Second Edition

Handbook of Biologically Active Peptides

Edited by
Abba J. Kastin

Handbook of Biologically Active Peptides

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Handbook of Biologically Active Peptides

Edited by Abba J. Kastin

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525 B Street, Suite 1900, San Diego, CA 92101-4495, USA
32 Jamestown Road, London NW1 7BY, UK
225 Wyman Street, Waltham, MA 02451, USA

First edition 2006
Second edition 2013

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British Library Cataloguing-in-Publication Data
A catalogue record for this book is available from the British Library

Library of Congress Cataloging-in-Publication Data
A catalog record for this book is available from the Library of Congress

ISBN: 978-0-12-385095-9

For information on all Academic Press publications visit our website at elsevierdirect.com

Typeset by TNQ Books and Journals Pvt Ltd.
www.tnq.co.in

Printed and bound in United States of America

13 14 15 16 17 10 9 8 7 6 5 4 3 2 1

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Preface to the 1st Edition

When Bernard Westerop, the skilled publishing editor of the journal *Peptides*, suggested that I edit a book on peptides, my initial reaction was the same as it had been when I was asked many years ago to be editor of the first peptide journal: I would better wait until I stop doing basic research.[1] However, it did not take me long to realize that if the book was properly organized, I could greatly accelerate my main goal as a journal editor: bringing together all aspects of biologically active peptides.

Investigators in selected areas of research, frequently, are oblivious to work in related areas. In the peptide field, for example, many investigators working with mammalian peptides are unaware of the large number of peptides from other forms of life, such as the hundreds of venom peptides from scorpions, snakes, sea anemones, spiders, insects, and worms. They also usually overlook the applicability of many pertinent observations made with different types of peptides, such as those in bacteria. This point is emphasized by a statement in the chapter by Nicolas and Amiche: "The discovery of the considerable extent of sequence identity between the neuropeptide and antimicrobial peptide precursors is unprecedented"; I suspect that it is also little known. and who would have thought that plants make peptides (the first being identified in 1991 by Clarence A. (Bud) Ryan, editor of the Plant Peptides Section) and that they can function as hormones.

Senior Publishing Editor, Tari Paschall, and Director of Development, Jasna Markovac, were very helpful and encouraged the publication of this amalgamation in a book large in length, width, and depth. One of the first decisions I had to make was whether the book should contain a few large chapters or many small ones. Since my goal was to assemble the disparate aspects of the field in one place for the first time, I chose to try to include as many types of peptides as possible, discussed by experts for each category. After careful consideration, I decided to organize this vast field into 20 sections, with some peptides intentionally discussed in more than one section. I then carefully chose the editors for these 20 sections. Thereafter, I was largely guided by their choices of chapters and authors. Although we tried to include articles for as many important peptides as possible, there were obviously some omissions, including at least one of my own favorite peptides, but we were more interested in showing the breadth of the peptide field.

The next big problem arose in enforcing the page restriction for each chapter. Understandably, there was the tendency for the authors to include everything. Although we tried to impose uniformity in design of the chapters, we were flexible in allowing authors to choose a balance between the amount of text and number of references within the space limitation. Reviews were cited wherever possible to save space. Even so, most chapters were initially too long to allow publication of all the material in a single volume.

There was one figure, however, suggested by Hubert Vaudry, that was incorporated into all chapters of his Brain Peptides section. This was a template of a sagittal section of the brain depicting the distribution of the peptide (cell bodies and fibers) or the peptide precursor mRNA. It serves to unify his large section of the Handbook, and his outline provided homogeneity to several other sections, including those involving Plant, Bacteria, Invertebrate, and Amphibian peptides:

1. Discovery
2. Structure of the Precursor mRNA/Gene
3. Distribution of the mRNA
4. Processing of the Precursor
5. Receptors and Signaling Cascades
6. Information on Active and/or Solution Conformations (if available)
7. Biological Actions Specific to the Organ or System
8. Pathophysiological Implications

Since the first few of these items could be discussed in several sections of the book, it was decided that the general background sections common to several chapters dealing with mammalian peptides would be discussed primarily in the Brain Peptides Section. For other sections, such as GI Peptides, this same outline is followed except that the focus is specific for the GI tract.

Variations of this outline are used in other sections. For example, for the Ingestive Peptides Section, David York recommended this outline:

1. Description of the effects on feeding behavior,
2. Studies from genetic manipulations or mutations, for example, knockouts, transgenics, siRNA experiments,
3. Functional response of the peptide/gene to differing metabolic and feeding states,
4. Sites of action and neural networks affected,

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5. Interactions with other peptidergic/aminergic systems,
6. Physiological and pathophysiological implications.

Other section editors recommended further variations. The Vaccine Peptides Section included the disease target as one of the categories. Naoto Minamino, discoverer of several peptides, recommended use of the Brain Peptides Section format but also noted that cardiovascular peptides are known to participate in the pathogenesis of cardiovascular diseases, and their plasma concentrations are often used as markers for their diagnosis, prognosis, and therapy. Therefore, he instructed authors in his Cardiovascular Peptides Section to focus on the peptides' endogenous form and concentration in plasma as well as their alteration under normal (physiological) and diseased (pathological) conditions.

The book tries to keep the abbreviations consistent. However, for some—for example, LHRH/GnRH and orexin/hypocretin—we allowed differences to reflect the authors' previous work already in the literature, even though I was an author of the paper showing that LHRH released both gonadotropins[4] and even though my one paper[2] on orexin/hypocretin used only the term *orexin*. Therefore, while there are a large number of chapters written by different authors, there is more uniformity in organization than would be expected from so many contributors. Discussion of some of the same peptides in different sections emphasizes the system involved. Conversely, some peptides discussed in only one section have actions that could fit into several sections (e.g. IGF-l). Many of the chapters are written by the investigator who made the initial discovery of the peptide being discussed.

For those mammalian peptides included in more than one section of the book, the emphasis is different in each section. Some degree of overlap was necessary, but it was kept to a minimum by the early transmission of the chapters in the Brain Peptides Section to the authors of chapters dealing with the same peptides in other sections. This was accomplished by the imposition of a deadline for the Brain Peptides chapters that was 2 months sooner than that for the other chapters. Moreover, each author was provided with the e-mail addresses of all authors and encouraged to communicate directly, especially after receiving drafts of related chapters from me.

It is very satisfying for me to see the tremendous body of knowledge in the field of biologically active peptides brought together in a single volume. I am especially gratified that so many chapters inherently reflect some of the concepts with which I have been personally involved: Peripheral peptides can affect the brain, endogenous antiopiate peptides exist, peptides can exert multiple effects, peptides can cross the blood–brain barrier directly, and others.[3] It is now almost inconceivable that they were once so controversial.

Despite the restrictions inherent in this large undertaking, the section editors and contributors were extremely cooperative, and I thank all of them. Their enthusiastic encouragement should ensure that this book will not only serve as an important reference source but will also widen the scope of the important field of biologically active peptides.

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After the publisher received all manuscripts, including this Preface, they decided that the formidable task of restricting the length of each chapter had been so successful that all materials designated for the supplemental disk could now be incorporated into this book.

REFERENCES

1. Kastin AJ. Editorial: Twenty-five years of peptides. *Peptides* 2004; **25**:315–17.
2. Kastin AJ, Akerstrom V. Orexin A but not orexin B rapidly enters brain from blood by simple diffusion. *J Pharmacol Exp Ther* 1999;**289**: 219–23.
3. Kastin AJ, Zadina JE, Banks WA, Graf MV. Misleading concepts in the field of brain peptides. *Peptides* 1984;**5**:249–53.
4. Schally AV, Arimura A, Kastin AJ. Hypothalamic regulating hormones. *Science* 1973;**179**:341–50.

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Preface to the 2nd Edition

The preface for the first edition of the *Handbook of Biologically Active Peptides* includes discussion of the conception, purpose, and organization of the book. These remarks remain applicable to this second edition. Obviously, the publication of a second edition indicates that the first must have fulfilled the need we perceived of bringing together in one place information about biologically active peptides originating in disparate communities of investigators. This is extremely gratifying to all of us.

Janice Audet, the Senior Acquisitions Editor, and Mary Preap, Editorial Project Manager, have been very receptive to improvements for this edition. We have added chapters to accommodate newly discovered biologically active peptides, shortened the chapter titles, broadened the section of general topics, and most importantly, added a section on peptide biosynthesis and processing. This section adds details for many of the peptides discussed elsewhere in the book. This intentional overlap, like that among many sections, reflects the differing focus of each section, even when discussing the same peptide.

With a large number of authors, every effort has been made to ensure some degree of uniformity. Where appropriate, chapters follow this basic format:

- Discovery
- Structure of the mRNA/gene
- Processing of the precursor
- Distribution of the mRNA/peptide
- Receptors and signaling cascades
- Biological actions
- Pathophysiological implications

More uniformity was attempted by my own editing of each chapter, even after that by the Section Editors, with enforcement of a short abstract, maximal total length, and (most difficult for the authors) limitation of references to no more than 35. This means that the work of many investigators could not be cited in their original form but rather in reviews or only by name or finding. As promised the authors, I accept full blame for this. The authors, section editors, publishers, and I hope you find this second edition of the *Handbook of Biologically Active Peptides* interesting and useful.

Abba J. Kastin

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Chapter 1

CLAVATA3

Shinichiro Sawa

ABSTRACT

Plant meristem is a unique structure made up of a self-renewing population of undifferentiated pluripotent stem cells. Shoot apical meristem (SAM) produces all aerial parts of postembryonic organs. *clavata3* (*clv3*) mutant produces fascinated SAM and flowers with increased floral organs in *Arabidopsis*. CLV3 restricts the size of the central zone in SAM and floral meristem (FM), controlling plant architecture. The *CLV3* gene encodes a precursor peptide with 96 amino acids. The mature CLV3 peptide is secreted from the apical tip of the SAM and FM as a glycopeptide of 13 amino acids with two hydroxyproline residues. The CLV3 peptide moves to the central part of the meristematic tissue and is recognized by at least three receptor complexes, CLV1, CLV2-Suppressor of LLP1 2 (SOL2)/CORYNE (CRN), and RECEPTOR LIKE PROTEIN KINASE 2 (RPK2). Phosphatases and kinases transmit the CLV3 signal. Here, we review the CLV3 peptide signaling network regulating meristem maintenance in *Arabidopsis*.

DISCOVERY

Shoot apical meristem (SAM) is known to be a meristem that provides cells for postembryonic growth and development (Fig. 1). The SAM is a collection of cells that continuously renew themselves by cell division and provide cells to new organs. Although continuous cell division and differentiation of their daughters are observed in meristematic regions, meristem size and the number of stem cells are kept constant. These observations suggest that stem cell maintenance and new organ formation are well balanced.[10,14] Recently, it has been shown that intercellular communication controls the organization and maintenance of SAM, as well as cell fate specification. Thus, stem cell maintenance requires well-coordinated regulation of different signals for meristem homeostasis.

The term meristem was first defined by Carl Wilhelm von Nageli in his book.[12] Meristem is a formative plant tissue made up of cells capable of dividing and giving rise to new cells.[16] Because of its essential role in higher plants, meristems have received much attention from plant scientists for >150 years.

The organization of the SAM can be divided into two areas (Fig. 1): the central zone (CZ), and the peripheral zone (PZ). In the SAM of plants, a balance between the division of stem cells in CZ and differentiating cells in PZ must be maintained. Genetic analyses of *Arabidopsis thaliana* show that *clv1*, *clv2*, and *clv3* are the loss-of-function mutants that exhibit remarkable phenotypes of shoot fascination, enlarged floral meristems, and increased numbers of floral organs (Fig. 2).[2] *CLV1* encodes a membrane-associated receptor-like kinase (RLK) that has leucine rich repeat (LRR) and kinase domains in its predicted extracellular and intracellular domain, respectively. *CLV2* encodes an LRR-receptor-like protein lacking a kinase domain.[3,5] *CLV3* encodes a 96-amino-acid peptide with a secretory signal sequence and a 14-amino-acid CLV3-ESR related (CLE) domain in its N-terminal and C-terminal region, respectively.[4] Together with genetic and biochemical analysis, CLV3 has been suggested as an extracellular signaling peptide that is responsible for the regulation of meristematic activity in *Arabidopsis*.

STRUCTURE OF THE PEPTIDE

The structure of the CLV3 peptide was examined by LC-MS/MS analysis by the use of peptides purified from a medium of cultured *Arabidopsis* plants overexpressing the *CLV3* gene.[13] The CLV3 peptide consists of 13 amino acids with two hydroxyproline residues (Fig. 3). The second hydroxyproline residue is modified with a linear b-1,2-linked triarabinoside. Dissociation constants (K_d) for the binding of CLV3 peptide with or without arabinose modification to the CLV1 ectodomain are 1.0 and 280 nM, respectively. Accordingly, arabinose modifies CLV3 peptide function in *Arabidopsis* compared with a CLV3 peptide without glycosylation.

However, a 12-amino-acid CLV3 peptide with two hydroxyproline was also isolated from callus cells,[8] and the corresponding synthetic peptide was also functional *in vivo*. However, its efficiency is lower than that of 13-amino-acid CLV3 peptides with a sugar modification. These results indicate that the arabinose chain is important for the function of the CLV3 peptide.

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00001-4
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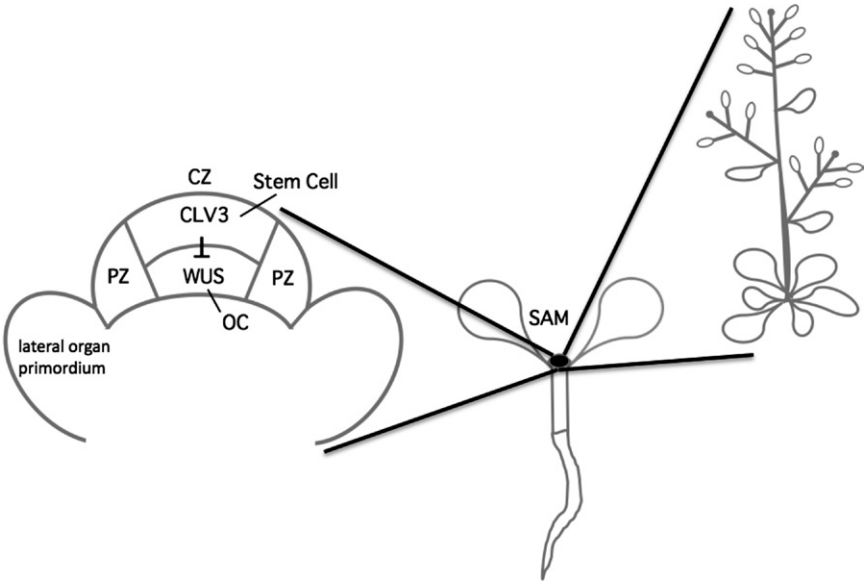

FIGURE 1 A model of the structures of SAM. SAM can be divided into two zones, the peripheral zone, PZ, and the central zone, CZ. CZ can be also divided into two regions, the stem cell region and the organizing center (OC). *CLV3* is expressed in the stem cell region, and *WUS* is expressed in the OC. All the upper ground tissues of the plant are produced by the activity of SAM.

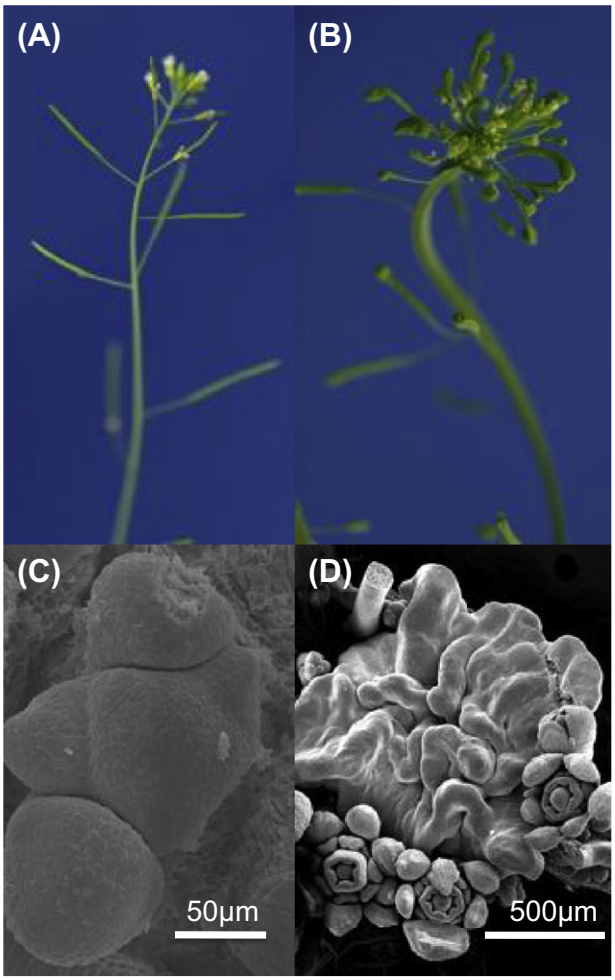

FIGURE 2 *clv3* mutant phenotypes. Compared with WT (A, C), *clv3* produces fasciated stem, increased number of floral organs, and larger shoot apical meristem (B, D).

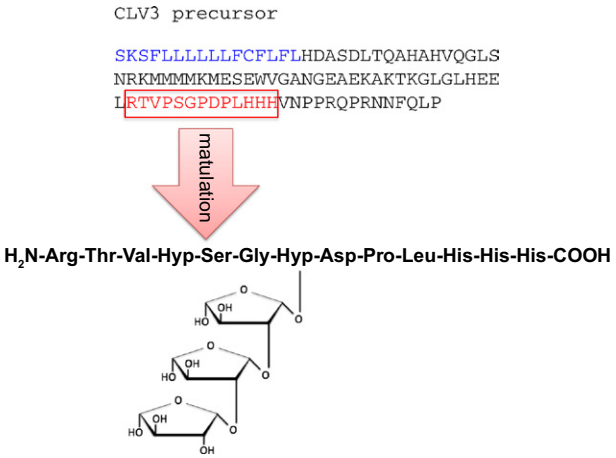

FIGURE 3 Structure of CLV3 preprotein and matured peptide. See color plate 1.

CLV3 is a member of the CLE family, and 26 kinds of CLE peptides are encoded in the *Arabidopsis* genome. The structure of CLE2 is similar to that of CLV3. However, CLE2 is a 12-amino-acid peptide.[13] So, CLE peptides seem to have various structural types, including different length and/or modifications.

DISTRIBUTION OF THE mRNA AND PEPTIDE

CLV3 gene expression is observed in the stem cell region at the apical tip of SAM (Fig. 1).[4] However, nobody knows the spatial distribution of mature CLV3 peptides.

Expression of *WUSCHEL* (*WUS*), a CLV3 downstream gene, is observed in the organizing center at the central part

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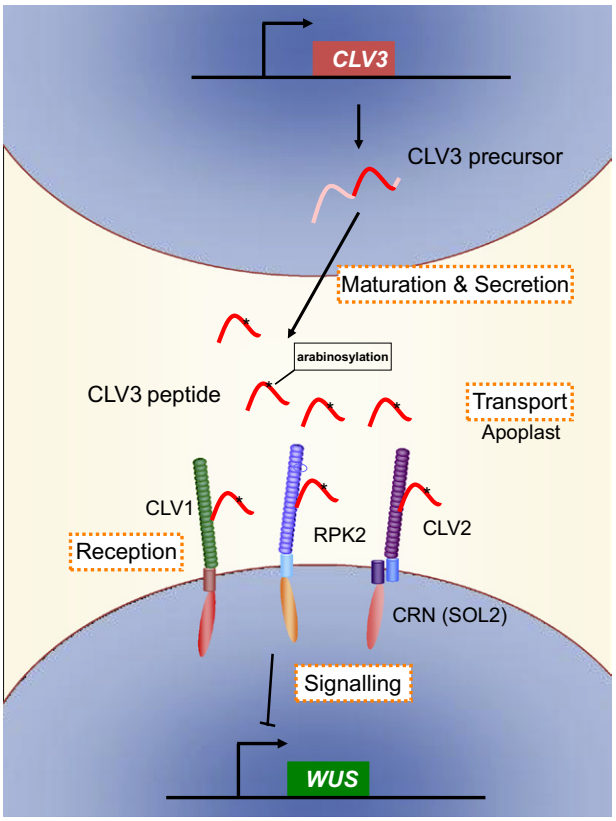

FIGURE 4 A schematic model of the CLV3 signaling pathway.

of SAM (Fig. 1), and the CLV3 peptide seems to move a short distance from the apical tip to the region around the central part of meristematic tissue.

BIOLOGICAL FUNCTIONS

clv mutants produce enlarged SAM and FM. Overexpression of the *CLV3* gene and application of the CLV3 peptide induce SAM and RAM consumption phenotypes.[8,6] In particular, *clv* mutants show enlarged CZ.[2] The CLV3 peptide seems to function to restrict the size of CZ, regulating meristematic tissue architecture.

RECEPTORS AND SIGNALING CASCADES

The CLV3 peptide is secreted from the apical tip of SAM to suppress a homeodomain transcription factor *WUS* gene expression noncell autonomously (Figs 1 and 4). However, *WUS* induces *CLV3* expression, giving rise to a negative feedback loop.[15]

CLV1, CLV2, SOL2/CRN, and RPK2 function as CLV3 receptors (Fig. 4). *RPK2* encodes an LRR-RLK, and *SOL2/CRN* encodes membrane bound kinase without an extracellular domain.[11,9,7] Genetic and biochemical analyses mainly agree that three major complexes perceive the CLV3 signal: CLV1, CLV2–SOL2/CRN hetero-oligomer, and RPK2 homooligomer.[7]

CLV3 ligand however induces CLV1 phosphorylation.[1] Type-2C kinase-associated protein phosphatase (KAPP) directly interacts with the CLV1 kinase domain, and functions as a negative regulator of CLV signaling transduction.[17] The *poltergeist* (*pol*) mutation suppresses the *clv1*, *clv2*, and *clv3* abnormalities.[18] *POL* and its homolog, *POLTERGEIST LIKE 1* (*PLL1*) encodes phosphatase type 2C (PP2C) protein, acting downstream of the CLV1 to maintain *WUS* expression. In addition to phosphatases, Rho GTPase-related protein (Rop), *MAP kinase kinase 4* (*MKK4*) and MAP kinase 6 (MPK6), components of the mitogen-activated protein (MAP) kinase cascade have been implicated in the mediation of phosphosignaling that regulates *WUS* expression downstream of the CLV3 peptide.[1,17]

REFERENCES

1. Betsuyaku S, Takahashi F, Kinoshita A, Miwa H, Shinozaki K, Fukuda H, et al. Mitogen activated protein kinase regulated by the CLAVATA receptors contributes to the shoot apical meristem homeostasis. *Plant Cell Physiol* 2011;**52**:14–29.
2. Clark SE, Running MP, Meyerowitz EM. CLAVATA3 is a specific regulator of shoot and floral meristem development affecting the same processes as CLAVATA1. *Development* 1995;**121**:2057–67.
3. Clark SE, Williams RW, Meyerowitz EM. The CLAVATA1 gene encodes a putative receptor kinase that controls shoot and floral meristem size in Arabidopsis. *Cell* 1997;**89**:575–85.
4. Fletcher JC, Brand U, Running MP, Simon R, Meyerowitz EM. Signaling of cell fate decisions by CLAVATA3 in Arabidopsis shoot meristems. *Science* 1999;**283**:1911–4.
5. Jeong S, Trotochaud AE, Clark SE. The Arabidopsis CLAVATA2 gene encodes a receptor like protein required for the stability of the CLAVATA1 receptor like kinase. *Plant Cell* 1999;**11**:1925–34.
6. Kinoshita A, Nakamura Y, Sasaki E, Kyozuka J, Fukuda H, Sawa S. Gain of fun ction phenotypes of chemically synthetic CLAVATA3/ESR related (CLE) peptides in *Arabidopsis thaliana* and *Oryza sativa*. *Plant Cell Physiol* 2007;**48**:1821–5.
7. Kinoshita A, Betsuyaku S, Osakabe Y, Mizuno S, Nagawa S, Stahl Y, et al. RPK2 is an essential receptor like kinase that transmits the CLV3 signal in Arabidopsis. *Development* 2010;**137**:3911–20.
8. Kondo T, Sawa S, Kinoshita A, Mizuno S, Kakimoto T, Fukuda H, et al. A plant peptide encoded by CLV3 identified by in situ MALDI TOF MS analysis. *Science* 2006;**313**:845–8.
9. Miwa H, Betsuyaku S, Iwamoto K, Kinoshita A, Fukuda H, Sawa S. The receptor like kinase SOL2 mediates CLE signaling in Arabidopsis. *Plant Cell Physiol* 2008;**49**:1752–7.
10. Miwa H, Kinoshita A, Fukuda H, Sawa S. Plant meristems: CLAVATA3/ESR-related signaling in the shoot apical meristem and the root apical meristem. *J Plant Res* 2009;**122**:31–9.
11. Muller R, Bleckmann A, Simon R. The receptor kinase CORYNE of Arabidopsis transmits the stem cell limiting signal CLAVATA3 independently of CLAVATA1. *Plant Cell* 2008;**20**:934–46.
12. Nageli C. *Beitrage zur Wissenschaftlichen Botanik. Erstes Heft*. Leipzig: Verlag von Wilhelm Engelmann; 1858.
13. Ohyama K, Shinohara H, Ogawa Ohnishi M, Matsubayashi Y. A glycopeptide regulating stem cell fate in *Arabidopsis thaliana*. *Nat Chem Biol* 2009;**5**:578–80.

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14. Sawa S, Kinoshita A, Nakanomyo I, Fukuda H. CLV3/ESR related (CLE) peptides as intercellular signaling molecules in plant. *Chem Rec* 2006;**6**:303–10.
15. Schoof H, Lenhard M, Haecker A, Mayer KF, Jurgens G, Laux T. The stem cell population of Arabidopsis shoot meristems in maintained by a regulatory loop between the CLAVATA and WUSCHEL genes. *Cell* 2000;**100**:635–44.
16. Scofield S, Murray JA. The evolving concept of meristem. *Plant Mol Biol* 2006;**60**:V–VII.
17. Trotochaud AE, Hao T, Wu G, Yang Z, Clark SE. The CLAVATA1 receptor like kinase requires CLAVATA3 for its assembly into a signaling complex that includes KAPP and a Rho-related protein. *Plant Cell* 1999;**11**:393–406.
18. Yu LP, Miller AK, Clark SE. POLTERGEIST encodes a protein phosphatase 2C that regulates CLAVATA pathways controlling stem cell identity at Arabidopsis shoot and flower meristems. *Curr Biol* 2003;**13**:179–88.

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Chapter 2

Defense Signal Peptides

Yube Yamaguchi, Gregory Pearce and Alisa Huffaker

ABSTRACT

Six genetically distinct families of plant defense signal peptides have been discovered. These include systemin, Plant elicitor peptides (Peps), and *Glycine max* (Pep914/890), all derived from larger precursors without traditional N-terminal secretion signals; hydroxyproline-rich systemins (HypSys), glycopeptides derived from precursors containing signal sequences for secretion through the ER and Golgi; and *Glycine max* Subtilisin Peptide (*Gm*SubPep) and inceptin, both embedded in proteins with nondefensive roles. These peptide signals may also be classified according to their regulatory functions: systemin, HypSys, and inceptin regulate antiherbivory responses, whereas Pep, *Gm*SubPep, and *Gm*Pep914/890 induce defenses against pathogens. With the exception of Peps, which are conserved across both monocots and dicots, each group of defense-signaling peptides is specific to particular plant families. This indicates that peptides evolved as signals through many distinct events during the arms race between plants and their biotic attackers, implying that more unique plant defense peptides are yet to be discovered.

DISCOVERY

Tomato and potato plants accumulate protease inhibitor proteins in leaves in response to herbivore attack or severe mechanical wounding. This accumulation occurs not only in wounded leaves but also systemically in undamaged leaves. After nearly two decades of intense efforts to identify the "protease inhibitor inducing factor," a microgram of the active factor was isolated from approximately 60 pounds of tomato leaves and identified as an 18 amino acid peptide called systemin (Sys).[19] When the peptide was chemically synthesized and supplied to young tomato plants, it induced protease inhibitor accumulation with a half-maximal activity of 40 fmol peptide per plant. Sys was the first peptide signal to be isolated and identified from a plant tissue.

Although a gene encoding systemin-like peptide is not found in the leaves of tobacco plants, leaves of young tobacco plants do exhibit systemic wound-inducible synthesis of trypsin inhibitor. Thus, a signaling pathway to activate protease inhibitor activity upon wounding as in tomato and potato must also exist in tobacco. These observations led to a search for defense peptide signals in the leaves of tobacco plants. This search was facilitated by the development of a biological assay using suspension-cultured cells that was much more rapid and simple than the whole-plant assay used for the isolation of Sys.[12,18] The new assay was based on the observation that addition of Sys to suspension-cultured tomato cells causes a rapid increase in the pH of the extracellular medium (up to 1 pH unit in 10min), heretofore referred to as the "alkalinization response." This pH change is among the earliest events induced by Sys and results from a calcium- and phosphorylation-dependent transient deactivation of a proton-pumping ATPase in the plasma membrane. The addition of microliter amounts of fractionated tobacco leaf extracts caused a strong alkalinization response in tobacco cells, suggesting that tobacco leaves may contain a signal analogous to Sys in tomato. With this assay, two 18 amino-acid glycopeptides that contain multiple hydroxyproline residues were identified and characterized.[16] Both peptides induce the synthesis of trypsin inhibitors when supplied through cut stems at nanomolar concentrations. Because of their similarities to tomato systemin in size, hydroxyproline content (derived from prolines), and their signaling characteristics, they were called Tobacco Systemin I and Tobacco Systemin II, designated here as *Nicotiana tabacum* Hydroxyproline-rich Systemins, or *Nt*HypSys I and *Nt*HypSys II.

Application of the alkalinization assay to nonsolanaceous plants has resulted in the isolation of several novel defense-related peptides, none of which bears homology to systemins or HypSys. Arabidopsis Plant Elicitor Peptide 1 (*At*Pep1, a 23 amino acid peptide) was isolated from *Arabidopsis thaliana* (mouse-ear cress),[6] and *Gm*Pep914 (an 8-amino acid peptide) and *Gm*SubPep (a 12-amino acid peptide) were isolated from soybean.[20,31] All these peptides seem to be primarily signals to activate pathogen defense responses.

Aside from systemin, inceptins are the only plant defense peptides identified without the use of the alkalinization assay.[28] Interestingly, inceptins, cyclic 11-amino acid peptides present in the Vigna and Phaseoulus species of the Fabaceae, were isolated from oral secretions of lepidopteran insect larvae as elicitors of plant ethylene production. Although inceptins are found in insect oral secretions, they are fragments

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00002-6
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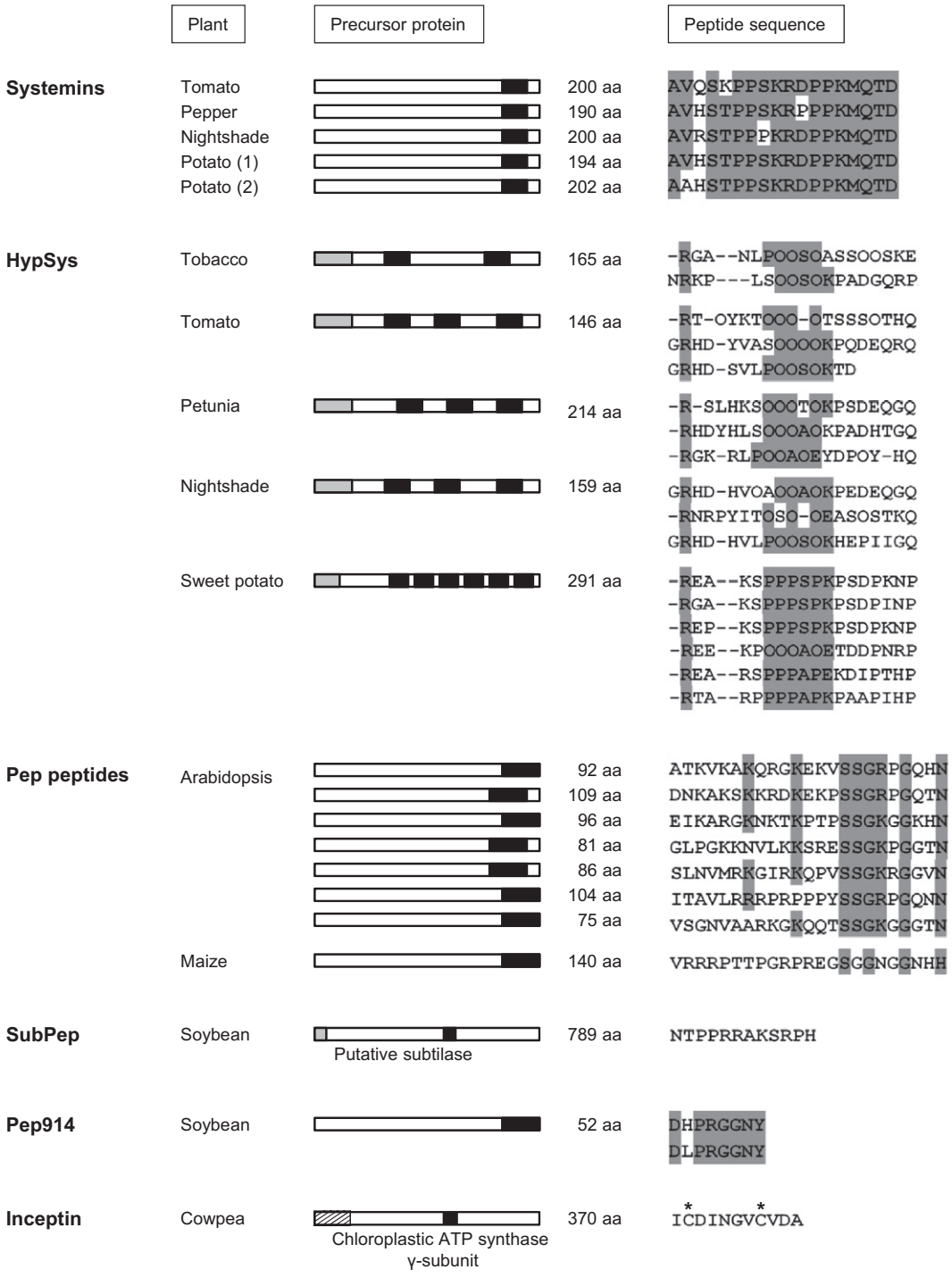

FIGURE 1 Structures of precursor proteins and peptide sequences. Black, gray and hatched boxes indicate positions of mature peptides, signal sequences for secretion, and signal sequences for chloroplast, respectively. Conserved amino acids are indicated with gray background and cysteines forming a disulfide bond are indicated with asterisks.

of the regulatory domain of plant chloroplastic ATPase subunit 3. As caterpillars feed on leaves, digestive enzymes in the larval midgut specifically process the cATPase protein to produce inceptins, which are then regurgitated back onto the leaf tissue during the course of feeding to induce plant defense. It is not yet known whether inceptins are ever produced in the absence of herbivory through endogenous proteolytic activities.

The structures of precursor proteins and mature peptide sequences characterized thus far are listed in Fig. 1.

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SYSTEMIN

Structure of the Peptide

Tomato systemin consists of the amino acid sequence: +AVQSKPPSKRDPPKMQTD– without any posttranslational modifications and is derived from the C-terminus of the 200 amino acid precursor protein called *Sl*proSys.[11,19] Systemin-like peptides are found in potato, nightshade, and pepper, all members of the Solanoideae subfamily of the Solanaceae family.[12] The gene has not been detected in tobacco or petunia, members of the Nicotianoideae and Petunioideae subfamilies, respectively, nor in species of any other plant families. The amino acid sequences of Sys peptides from tomato, potato, nightshade, and pepper differ by only a few residues.

Structural features of all systemin family members include charged amino acids flanking tandem proline motifs.[12,18] The tandem prolines are thought to cause the weak poly(L-proline) II, 3_1 helical conformation (PPII) found in *Sl*Sys. PPII conformations tend to form left-handed helices and are known to be involved in recognition events in animals. In each peptide, the tandem prolines are flanked by charged residues, and this charged PPII conformation may play an important role in receptor recognition. Substitution of the penultimate threonine residue at position #17 of *Sl*Sys with an alanine totally abolishes activity while generating a powerful competitive inhibitor.[26]

Structure of the Precursor Protein

The 200 amino acids composing *Sl*proSys are rich in charged residues, with 10% being Asp, 17% Glu, and 15% Lys. Regions rich in Lys and Glu residues resemble KEKE motifs that are hypothesized to mediate protein–protein interactions.[23] The processing enzymes that produce *Sl*Sys from *Sl*proSys have not been identified, nor is it known whether *Sl*Sys or *Sl*proSys is transported to the apoplast from the cytoplasm or by what mechanism this is accomplished. Wound-inducible proteases have been identified in leaves[18] and may play a role in processing *Sl*proSys to *Sl*Sys.

Prosystemin Gene Expression Patterns

The systemin precursor is located in the cytosol and nucleus of phloem parenchyma cells of minor veins and midribs of leaves and in the bicollateral phloem bundles of petioles and stems of tomato plants.[15] The gene encoding prosystemin is wound inducible in leaves; the transcript is detected within 30 min of wounding, and maximal accumulation occurs within 3h[26] When wound-responsive genes are temporally classified, *SlproSys* is among the "early genes" expressed along with several signaling genes. This is in contrast to genes encoding directly defensive proteins such as protease inhibitors that are "late genes," for which transcription begins 1–2h after wounding and maximizes after about 8 h.[26]

Biological Functions

*Sl*Sys was established as a primary wound signal for the systemic induction of defense genes in tomato plants through antisense technology, showing that the inhibition of the synthesis of *Sl*proSys also blocked systemic wound signaling and defense against herbivore attack.[12,18] Analyses using the systemin-signaling mutant, *spr2*, revealed that systemin induces JA synthesis in the companion cell-sieve element complex of the vascular bundles, leading to systemic protease inhibitor induction.[9,27] Systemin also increases the release of plant volatiles that attract natural enemies of insect herbivores.[12,18] Protease inhibitors induced by systemin are required for resistance against a fungal pathogen, *Botrytis cinerea*.[2]

Receptors and Signaling Cascades

By photoaffinity-labeling techniques, specific binding of systemin was detected in tomato suspension cells to SR-160, a 160kDa leucine-rich repeat receptor kinase (LRR-RK) identical to the tomato brassinosteroid receptor BRI1.[12,18] However, although SR160/BRI-1 binds systemin, it is probably not the physiological receptor that transduces the systemin signal.[10] The systemin receptor is hypothesized to be a BRI1-like receptor (BRL), and because systemin perception does not occur in leaf mesophyll cells, the receptor is thought to be localized to vascular tissues.[4] BRI1 interacts with BRI1-associated kinase 1 (BAK1), as do many other receptors, including PEPR1, FLS2, and EFR, all of which bind peptide signals that activate defense.[28] In addition to probably being highly homologous to BRI1, it is possible that the systemin receptor associates with BAK1, which could lead to colocalization with BRI1 on the plasma membrane.

Perception of systemin at the cell surface rapidly activates plasma membrane depolarization and causes ion channels to open resulting in an efflux of K^+ and influx of Ca^{2+} into the cell.[26] These changes are accompanied by the activation of both a MAP kinase and calmodulin and subsequent inactivation of a plasma membrane proton ATPase.[26] The main target of these events is stimulation of phospholipase activity, which results in the release of linolenic acid from thylakoid membranes in the chloroplast. Linolenic acid is the precursor to the production of the phytohormone jasmonic acid and other oxylipin signals through the octadecanoid pathway. The phytohormone ethylene is also produced in response to systemin and acts together with jasmonic acid to regulate the expression of defense genes and synthesis of defense metabolites.[26] In addition to ethylene and jasmonic acid, a number of other molecules have been identified as second messengers downstream of systemin signaling, including hydrogen peroxide, nitric oxide,

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and oligogalacturonic acid. Systemin amplifies wound signaling by rapidly inducing expression of its own gene and other signaling genes such as lipoxygenase and calmodulin in the vasculature.[26] This is hypothesized to allow signals to traverse the plant quickly through the phloem. Genes that encode defensive proteins such as protease inhibitors and polyphenol oxidase require additional response time and are expressed in the mesophyll cells rather than the vasculature.[26]

HypSys

Structure of the HypSys Peptides

Tobacco hydroxyproline-rich systemins, *Nt*HypSys I and II, originate from a single secreted prepropolypeptide of 165 amino acids that contains the *Nt*HypSys I sequence near the N-terminus and the *Nt*HypSys II sequence near the C-terminus.[16] Neither peptide exhibits homology with *Sl*Sys, but the –OOS– motifs are similar to the *Sl*Sys –PPS– motif and would result from posttranslational hydroxylation of proline during secretion. The two HypSys peptides have multiple regions that are contiguous hydroxyproline, serine, and threonine residues. These residues, flanked by charged amino acids, comprise >50% of each peptide. Mass spectroscopy of the two peptides revealed that the attached carbohydrate moieties consist of pentose residues: nine in *Nt*HypSys I and six in *Nt*HypSys II.[16] Multiple HypSys peptides have been isolated from petunia, nightshade, potato, and sweet potato and in each case are derived from single precursor proteins homologous to the tobacco precursor.[12,18] The native peptides are active at low nanomolar concentrations in the alkalinization assay, but the synthetic peptides without carbohydrate moieties have greatly reduced activity, indicating the importance of glycosylation for their biological activity. Because of the low binding affinity of synthetic HypSys peptides, receptors for HypSys peptides have not been isolated.

Structure of the Precursor Protein

The presence of multiple signaling peptides contained within a single precursor is a characteristic of many animal peptide hormones, but the tobacco HypSys precursor provides the first example in plants. Although the tobacco HypSys precursor protein contains two HypSys sequences, tomato, petunia, nightshade, and potato HypSys precursors, all contain three HypSys sequences, and the sweet potato precursor contains six HypSys peptides.[12,18]

All precursor proteins contain N-terminal secretion signals, and localization of *Sl*proHypSys in the cell wall matrix was confirmed by immunocytochemical staining.[14] *Sl*proHypSys processing must occur in the apoplast upon wounding, suggesting either that nonspecific cytoplasmic proteases are mixed with cell walls upon damage or that specific proteases are synthesized or activated in response to wounding.

Expression Patterns of *preproHypSys*

The transcripts of *preproHypSys* from tobacco, tomato, petunia, nightshade, and sweet potato accumulate upon treatment by wounding and MeJA,[12,18] while no changes in *N. attenuata* were observed.[1] Furthermore, the expression of *N. tabacum preproHypSys* was strongly induced by chewing and phloem-feeding insects, *Manduca sexta* larvae and *Bemisia tabaci* adult flies.[25] Observed transcript accumulation of these precursors in response to herbivores or wound-related stimuli supports the role of HypSys peptides in defense against herbivores.

Biological Functions

Supplying HypSys through the cut stem induced defense genes that are also induced by MeJA and by insects and/or pathogens: protease inhibitor genes in tomato, tobacco, nightshade, and sweet potato and a defensin gene in petunia.[12,18] In nightshade, genes encoding a lipoxygenase D and phospholipase A2, both involved in JA biosynthesis, are also rapidly induced by *Sn*HypSys. More direct evidence for the defensive role of HypSys peptides was obtained from experiments with *N. tabacum* and *N. attenuata*. Transgenic tobacco plants overexpressing *Nt*preproHypSys have increased levels of protease inhibitors and polyphenol oxidase and display enhanced resistance against *Helicoverpa armigera* larvae.[24] Experiments with transgenic *N. attenuata* silenced in the production of *Na*preproHypSys reveal that *Na*HypSys regulates a malonylation step in the synthesis of 17-hydroxygeranyllinallol diterpene glycoside, an antiherbivore compound.[3]

Tomato and nightshade have Sys in addition to HypSys. How the combined roles of Sys and HypSys are coordinated during wounding is not fully understood, but the production of multiple defense signaling peptides at wound sites may play an important role in stimulating rapid, robust synthesis of JA. If enzymes that process the peptide precursors were among the wound and jasmonate-induced proteases, then JA would promote production of both precursors and peptide signals as it traverses the vascular bundles. This would in turn serve to further amplify levels of JA. Both *Sl*proSys and *Sl*preproHypSys are localized in phloem parenchyma cells adjacent to companion cells[14,15] where JA biosynthesis occurs and a cooperative interaction between these cells may be important in tomato plants to achieve a vigorous defense response.[13] Tobacco, which has a much weaker systemic response than tomato, does not synthesize systemin but may amplify JA synthesis through release of HypSys

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peptides. Understanding the complex interactions among Sys, HypSys, and JA, as well as with other wound-induced signals, such as ET and H_2O_2, will be a key challenge for future research.

PLANT ELICITOR PEPTIDES (PEPS)

Structure of the Peptides

*At*Pep1 is derived from the 23 carboxyl-terminal residues of a 92 amino acid precursor polypeptide, *At*PROPEP1 (At5g64900).[6] Seven *At*PROPEP paralogs encoded in the Arabidopsis genome share a 10-amino acid motif, SSG(K/R)xGxxN, at the carboxyl-terminus of each deduced protein within the putative *At*Pep peptide region. Analyses of the biological activities of alanine-substituted analogs, or of *At*Pep1 with deletions at the N-terminus, reveal that the 10 C-terminal residues are necessary for activity, and modifications within the core motif, presented above, reduce the activity of the peptide several fold.[21] Substitution of Gly-17 in the conserved SSG sequence of the motif with Ala totally abolishes activity, but the analog is not a competitor of native *At*Pep1 in the alkalinization assay.[21] The amino-terminal regions of the *At*Pep peptides deduced from all the paralogs are cationic in nature. *At*Pep1 has a predicted p*I* of 11.2 with five lysines and two arginines concentrated at the amino-terminal half of the peptide.[21]

Structure of the Precursor Proteins

All the *At*PROPEP precursor proteins have negatively charged Glu/Asp repeats, interrupted by single Lys/Arg residues (KEKE motifs[23]). The precursors of all *At*Pep family members lack signal sequences that would target them through the classical secretory pathway. However, the *At*Peps interact with two cell surface receptors[32] to initiate defense signaling, indicating that AtPeps or their precursors are exported from the cell. The mechanisms of how *At*Peps arrive at the cell surface are not known, but the KEKE motifs along with an amphipathic helix motif conserved in all PROPEP precursor proteins have both been previously described as protein–protein interaction motifs.[23] Thus, these regions may interact with some yet unknown protein(s) such as chaperones or processing enzymes to facilitate processing and/or export.

*At*PROPEP orthologs have been predicted from the database of many plant species, including important crop plants such as canola, potato, rice, soybean, and maize.[6] All of these genes encode putative precursor proteins of 93–154 amino acids, and all lack signal sequences, contain KEKE motif regions, and have predicted amphipathic regions. The putative *At*Pep regions of these orthologs are Lys/Arg rich. The only absolutely conserved residue in the AtPep region is Gly that, when substituted with Ala in *At*Pep1, abolishes activity. The synthetic maize ortholog, ZmPep1, has biological activity.[5]

Expression Pattern of *At*PROPEPs

The genes encoding *At*PROPEPs are expressed at low basal levels in all Arabidopsis tissues from newly germinated seedlings to mature, seed-setting plants with basal levels in flowers and roots being somewhat higher than in other tissues.[6] The Arabidopsis *AtPROPEP* genes in leaves are differentially inducible by wounding, MeJA, ET, SA, microbe-associated molecular patterns (MAMPs), and pathogen infection.[6,7] Additionally, *AtPROPEP* genes are differentially inducible by supplying *At*Peps to young plants, indicating that feed-forward positive regulation exists within the signaling pathway of *At*Peps.[7] The gene encoding the maize ortholog, *Zm*PROPEP1 is transcribed in leaves in response to treatment with *Zm*Pep1 peptide or JA, further supporting a feed-forward amplification mechanism. Transcript corresponding to the *Zm*PROPEP1 gene also accumulates in leaves infected with the fungal pathogen *Cochliobolus heterostrophus*.[5]

Biological Functions

*At*Pep1 was the first peptide defense signal identified in the model plant *Arabidopsis thaliana* and has rapidly provided new insights into mechanisms regulating pathogen defense in this plant. Peps activate the accumulation of both transcripts encoding defense genes and defense-related metabolites. In Arabidopsis, the *PR-1* (pathogenesis-related protein) and *PDF1.2* (defensin) genes associated with the activation of SA- and JA-dependent defenses are transcribed in leaves supplied with *At*Pep peptides.[6,7] This transcriptional activation is compromised in mutants defective in biosynthesis or perception of the defensive hormones, JA, ET, and SA. Transgenic Arabidopsis plants with increased expression of *At*PROPEP1 also constitutively expressed defense genes including PDF1.2 and PR1. In maize, *Zm*Pep treatment stimulates the expression of genes encoding a number of defense proteins, including chitinases, a protease inhibitor and biosynthetic enzymes for the production of benzoxazinoid phytoalexins.[5] This activation of gene expression in maize is accompanied by *de novo* benzoxazinoid synthesis and accumulation of a particularly reactive phytoalexin precursor, HDMBOA-Glc.

Peps enhance disease resistance in both Arabidopsis and maize. Pretreatment of Arabidopsis leaves with *At*Peps before inoculation with the bacterial pathogen *Pseudomonas syringae* pv. tomato DC3000 results in a dramatically reduced bacterial propagation.[32] Arabidopsis plants constitutively expressing the *AtPROPEP1* gene are more resistant to root rot caused by the oomycyte root pathogen *Pythium irregulare*

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than are wild-type plants.[6] Pretreatment of maize leaves with *Zm*Pep1 reduces Southern leaf blight lesions and cell death caused by *Cochliobolus heterostrophus*.[5] Similarly, *Zm*Pep1 application to maize stems before inoculation with *Colletotrichum graminicola* reduces symptoms of anthracnose stalk rot as compared with water-treated stems. All these findings support the role of Peps as activators of pathogen defense responses.

Receptors and Signaling Cascades

Two *At*Pep receptors, *At*PEPR1 and *At*PEPR2, were identified by photoaffinity labeling and mutant analyses.[8,32] Both *At*PEPR1 and *At*PEPR2 are structurally similar to FLS2, EFR, and Xa21 receptors for peptide MAMPs and belong to the subfamily LRR XI. Kinetic analyses of *At*Pep1 binding to tobacco suspension-cultured cells expressing either *At*PEPR1 or *At*PEPR2 indicate that *At*Pep1 binds to the cell surface within a minute, with a K_d of 0.56 and 1.25nM, respectively.[32] In a double T-DNA insertional mutant of both *At*PEPR1 and *At*PEPR2, all effects caused by *At*Pep treatment are abolished, including reduced propagation of *P. syringae*.[32] In addition to an active kinase domain, the cytosolic portions of both *At*PEPR1 and *At*PEPR2 contain a guanylyl cyclase (GC) domain. This GC domain generates cGMP, and it is hypothesized that cGMP generated by this domain activates a cyclic nucleotide-gated Ca^{2+} conducting channel (CNGC2) that is required for the defense response.[22] In addition to Ca^{2+}, Peps induce the production of a number of downstream defense signals, including JA, ET, H_2O_2, and NO.[5–8] Perturbation of these downstream signals through mutation or application of inhibitors compromises AtPep-induced responses.[6,7]

Little is known about other downstream components of Pep signaling. Expression of genes encoding a number of defense-related kinases and WRKY transcription factors is activated by AtPep treatment.[32] This indicates that they may have a role in Pep signal transduction, but experimental evidence is required to confirm this hypothesis. *At*PEPR1, *At*PEPR2, EFR, and FLS2 all interact with the same coreceptor, BRI-associated kinase 1 (BAK1), which is required for both MAMPs and *At*Pep signaling.[30] These observations indicate that downstream pathways of Pep/PEPR overlap with MAMP signaling pathways and support the idea that *At*Peps amplify the defense responses initiated by MAMPs.[32]

*Gm*SubPep

Structure of the Peptide

The first identified peptide from soybean, *Gm*SubPep, is 12 amino acids in length with a highly basic net charge: NTPPRRAKSRPH.[20] *Gm*SubPep is active at low concentrations (<0.25nM) and produces a pH change of the suspension cell media within 10min. Alanine substitution at each amino acid position within the peptide results in almost a complete loss of alkalinization-inducing activity for [A^{10}]*Gm*SubPep (Arg-substituted) and [A^{12}]*Gm*SubPep (His-substituted), and both of these analogs successfully compete with *Gm*SubPep in the alkalinization assay.[17] Thus, *Gm*SubPep activity and possible association and transduction of signaling via its putative receptor are mediated through the carboxyl-terminal portion of the peptide. Truncated peptides produced by sequential removal of single amino acids from the amino terminus of *Gm*SubPep cause a 1000-fold decrease in activity after the removal of one amino acid [*Gm*SubPep(2–12)] and a complete loss of activity upon removal of three amino acids. However, these truncated analogs do not compete with *Gm*SubPep in the alkalinization assay.[17] The amino-terminal portion of the peptide may facilitate receptor binding but is not required for transduction of the signal, similar to systemin.[17] In agreement with these results, both [A^{10}]*Gm*SubPep and *Gm*SubPep(2–12) are incapable of activating expression of defense genes relative to *Gm*SubPep.

Structure of the Precursor Protein

The peptide sequence was derived from a gene designated Glyma18g48580 encoding a subtilisin-like protease (subtilase); thus, the peptide was named "*Glycine max* Subtilase Peptide" (*Gm*SubPep).[20] The full length coding region of Glyma18g48580 was sequenced, and reveals all the characteristics of a plant subtilase, including a signal sequence for secretion out of the cell. The *Gm*SubPep sequence is located just after the protease-associated domain within the peptidase S8 region, in a region unique to a small group of leguminous plants. In agreement with this, only soybean suspension cells are responsive to *Gm*SubPep in the alkalinization assay. Although more research is required, it would seem that *Gm*SubPep enters the apoplast embedded in the subtilase and a processing event releases *Gm*SubPep for association with its putative receptor. The processing events for release of *Gm*SubPep will be an exciting area of research along with the identification of a cell-surface receptor.

Biological Functions

*Gm*SubPep, in addition to its activity in the alkalinization assay, is also capable of inducing the expression of genes encoding defense proteins including a chitinase and a chalcone synthase involved in the biosynthesis of the phytoalexin glyceollin.[20] Interestingly, the expression of the gene encoding the subtilase (Glyma18g48680) is unresponsive to the application of hormones known to induce defense responses, such as JA, SA, and ET. This, coupled with high constitutive

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expression levels in all tissues of the plant, suggests a nondefensive role for the protease. The specific role of *Gm*SubPep will be revealed with insect/pathogen studies to elucidate mechanisms and temporal dynamics of *Gm*SubPep release from the subtilase.

*Gm*Pep914/890

Structure of the Peptides

A second peptide was isolated by the alkalinization assay from the crude soybean leaf extracts.[31] This eight amino acid peptide was named *Gm*Pep914 (DHPRGGNY), based on its monoisotopic mass. Synthetic *Gm*Pep914 peptide is capable of alkalinizing the cellular media at 0.25nM and requires only 5–10 min to achieve a maximal response. An alanine-substitution scan of *Gm*Pep914 reveals that substitution at positions 7 and 8 within the peptide ([A^7]*Gm*Pep914 and [A^8]*Gm*Pep914) causes a complete loss of alkalinizing activity. Neither analog is capable of competing with *Gm*Pep914 in the assay, suggesting that they are incapable of binding a putative membrane-bound receptor.[31] Additionally, the removal of either the amino-terminal or carboxyl-terminal amino acid abolishes peptide activity. This is in agreement with the strict conservation of the *Gm*Pep914 regions of the putative precursors found in the GenBank described below.

Structure of Precursor Proteins

A predicted coding sequence found in the soybean genome database (Glyma12g00990) reveals a 52-amino acid precursor (*Gm*proPep914) with *Gm*Pep914 located at the carboxyl-terminus of the putative protein. In addition to *Gm*proPep914, a second cDNA encoding a 52 amino acid precursor peptide was isolated with a very similar sequence, including an eight amino acid carboxy-terminal region that differs from *Gm*Pep914 by only one amino acid. This putative peptide was named *Gm*Pep890 and the precursor, Glyma09g36370, was named *Gm*proPep890. *Gm*Pep890 (DLPRGGNY) was synthesized and found to be as active as *Gm*Pep914 in the alkalinization assay. *Gm*proPep914 and *Gm*proPep890 share 84.6% sequence homology and, like the *At*proPeps and prosystemin (see above), have no discernible signal sequence for secretion.[31]

A search of homologs of *Gm*proPep914/890 reveals similar EST sequences in three other members of the Fabales: *Glycyrrhiza uralensis* (licorice), *Medicago truncatula*, and *Cajanus cajan*, a major legume crop of the tropics.[31] A search using only the *Gm*Pep914/890 peptide region reveals two additional homologs from the Cucurbitales family, a close relative to the Fabales. Thus, like systemin, it would seem that *Gm*Pep914/890 has evolved within a tight ecological niche.

Expression Pattern of *Gm*PROPEP914/890

*Gm*Pep914/890 induces the expression of the genes encoding their respective precursors *Gm*PROPEP914/890, when supplied to soybean suspension-cultured cells.[31] In experiments with soybean plants, *Gm*PROPEP914/890 exhibits high constitutive expression levels in roots with low basal levels in other tissues. However, upon treatment with jasmonate, salicylate, and ethylene, expression levels of both precursor genes increase in leaves. *Gm*proPep914 orthologs found in the EST database from other plant species are mainly contained in cDNA libraries prepared from underground tissues, which are surrounded by a plethora of potential microorganism invaders.[31]

Biological Functions

*Gm*Pep914/890 have no sequence homology to *Gm*SubPep; however, GmPep914/890 and GmSubPep induce the same defense genes in soybean suspension cells, including *CYP93A1*, *Chib1-1*, and *achs-1*.[31] Analysis of the biological functions of *Gm*Pep914/890 has just begun. However, similarities in activities of *Gm*Pep914/890 and the Plant Elicitor Peptides, such as transcriptional activation of genes encoding both peptide precursors and defense proteins, suggest that *Gm*Pep914/890 might act as amplifiers of defense responses initiated by biotic stresses, similar to AtPep peptides.

INCEPTINS

Peptide Structure

Inceptins are regulators of herbivore defense in *Vigna* and *Phaseoulus* species of legumes.[28] These disulfide-bridged peptides are released from a highly conserved regulatory domain in the γ-subunit of chloroplastic ATP synthase. Regulatory activity of the γ-subunit relies upon sequence fidelity at this position, with cATPase activation occurring through the reduction of a disulfide bridge between the regulatory domain and the inhibitory ε-subunit. Inceptin peptides contain two cysteines, including one that forms a bridge to the ε subunit and are classified as cryptides, peptide signals derived from proteins of unrelated function rather than from a standard propeptide precursor. The first cryptides discovered in plants, inceptins are proteolyzed from the cATPase by digestive enzymes in the midgut of the Fall armyworm (*Spodoptera frugiperda*) during herbivory.[28] While feeding, the insects regurgitate inceptins back onto the plant where it is recognized as an elicitor to activate antiherbivore defenses. Supplementation of insect diet with recombinant cATPase γ-subunit protein confirm that cATPase is the source of inceptins; insects supplied with cATPase-supplemented

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diet produce inceptin peptides, whereas those on nonsupplemented diet do not.[8] Furthermore, larvae feeding on roots, which do not contain chloroplastic ATPase, do not produce inceptins.

Inceptins are released by insect digestive enzymes through what is probably nonspecific proteolysis, and several forms can be detected in larval oral secretions.[29] Each inceptin is a short, acidic peptide with a core motif consisting of the disulfide-bridged cyclic domain. The bioactive peptides range in size from 11 to 13 amino acids because of amino-terminal extensions to the core domain of varying lengths. Eleven- and 12mer inceptins are slightly more active than the 13mer, with half-maximal active concentrations of 68 and 45fmol per leaf (as compared with 87fmol per leaf for the 13mer). Freshly collected insect oral secretions contain a blend of the three inceptins but with time the longer peptides break down to yield the very stable 11mer.[29]

Alanine substitution studies of the 11mer inceptin sequence, +ICDINGKCVDA–, reveals that Asp10 is absolutely required for elicitor activity and that substitutions at Asp3 and Cys8 substantially reduce bioactivity.[29] Interestingly, substitution at Cys2, which forms a disulfide bridge to Cys8, has little effect on elicitor activity. This indicates that although cyclization resulting from disulfide bonding confers stability to inceptins, it is not required for signaling function. As for many other plant peptide signals, inceptin activity requires conservation of the carboxyl-terminus but is tolerant of amino-terminal substitutions.

Distribution of the mRNA/peptide

The chloroplastic ATP synthase from which inceptin is derived is ubiquitous and constitutively present in foliar and other chloroplast-rich tissues. Because inceptins are the products of a domain particular to chloroplastic ATP synthases, they are not generated from other ATPase proteins. Because of the cleavage of inceptin from the γ-subunit by insect digestive enzymes, it seems probable that the production of bioactive peptide is limited to occasions of biotic interaction. It is not known whether endogenous plant proteolysis activities ever result in inceptin production in the absence of herbivory. Although inceptins act as defense elicitors only in *Vigna* and *Phaseoulus* species, the corresponding domain of the cATPase γ-subunit is widely conserved across plant species, and inceptin peptides are detected in oral secretions of armyworms that have fed on maize and tobacco as well as legumes.[28]

Biological Functions

Activation of plant defense against herbivores is associated with an ethylene burst, and *S. frugiperda* herbivory promotes ethylene synthesis in numerous plant species including *Vigna*. However, the fatty acid–amide conjugates from *S. frugiperda* oral secretions that elicit antiherbivore defense in some plant species are not active in *Vigna*. Inceptins were discovered through a search for the molecule responsible for elicitor activity in *Vigna* by the use of an induced-ethylene assay. In addition to promoting ethylene production, inceptins activate both direct and indirect herbivore defenses.[28]

Inceptin treatment results in the production of volatile organic compounds including a variety of terpenes as well as indole and methyl salicylate.[28] These volatiles mediate indirect plant defense by attracting natural enemies of *S. frugiperda*. In addition to promoting volatile emission, inceptins stimulate responses associated with direct defenses against herbivores. These include induced expression of a gene encoding a cystatin protease inhibitor and production of cinnamic acid, which is a precursor to phenylpropanoid defense metabolites.[28] The importance of inceptin-regulated defenses against *S. frugiperda* was confirmed in plants pretreated with the 11mer peptide. Larvae feeding on inceptin-pretreated leaves grow at a slower rate and have reduced endpoint larval biomass as compared with those allowed to feed upon leaves pretreated with a water control.[28]

Receptors and Signaling Cascades

The mechanism of inceptin perception is not yet known. The inceptin motif is present in cATP synthase proteins of most plants and *S. frugiperda* feeding on plants such as tobacco and maize produce inceptin peptides. However, the peptide activates downstream defenses only in *Vigna* and *Phaseolus* species. Because the proteolytic cleavage of inceptin seems to be fairly universal, it is not signal biogenesis that has specially evolved in these species but a mechanism for perception. As such, two potential mechanisms have been postulated for inceptin perception, R-gene guard protein mediated and receptor mediated.[28] According to the guard hypothesis, invading organisms are indirectly recognized through their effects on plant proteins: Proteases from these attackers cleave plant proteins (targets) into inappropriate nonnative fragments that are perceived by R protein guards. Inceptin functions as a classical guard hypothesis target in that proteolytic activity from caterpillars cleaves the cATPase γ-subunit into an inappropriate peptide fragment that is recognized by the plant. This peptide fragment might be recognized through the interaction with a guard R protein evolved in legume species. Alternatively, a pattern recognition receptor may have evolved in these species that interacts specifically with inceptin to activate signaling.

Although the initial perception of inceptin is not yet understood, downstream signal transduction is known to involve

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phytohormones. Inceptin activates concurrent synthesis of salicylic acid, jasmonic acid, and ethylene, indicating a cooperative role for the three phytohormones in coordinating defense against herbivores in *Vigna* and *Phaseolus*.[28]

CONCLUSIONS

Although systemin and HypSys were initially identified as elicitor peptides targeting herbivores, and Peps targeting pathogens, recent analyses suggest the involvement of these peptides in both herbivore and pathogen resistance. *Sl*Sys is required for resistance against the fungal pathogen, *Botrytis cinerea*,[2] *Ph*HypSys induces an antimicrobial protein, defensin,[12,18] and *Zm*Pep1 upregulates the production of benzoxazinoids, secondary metabolites associated with herbivore and pathogen resistance.[12,18] Because both herbivore and pathogen (especially fungal) resistances are mediated through the JA/ET signaling pathways, and most of the known elicitor peptides function through JA/ET signaling pathways, these results are logical. It would be interesting to investigate the possible involvement of the isolated pathogen defense peptides, *Gm*SubPep and *Gm*Pep914/890, for responses against herbivores.

Six families of endogenous elicitors have been identified thus far. Among these, Peps seem to be conserved throughout the angiosperms,[6] whereas the other elicitor peptides are specific to certain plant orders or families. Systemin has been found only in plants belonging to the Solanoidae subfamily of the Solanaceae family,[12,18] whereas HypSys is conserved within the Solanale order, which includes the sweet potato.[12,18] *Gm*Pep914 homologs are predicted from some legumes and from cucumber, both belonging to the nitrogen fixing clade of eurosids I.[31] *Gm*SubPep has been found only in soybean thus far.[20] These observations suggest that plant defense signaling peptides emerged multiple times during evolution, and Pep peptides probably emerged before the separation of dicots and monocots. For inceptin, only Vigna and Phaseoulus legume species respond to inceptin, although the peptide sequence is conserved in chloroplastic ATP synthase γ-subunits from various plants.[28] This suggests the recent emergence of receptors for inceptin in legumes.

It is evident that a diversity of peptide elicitors can exist even in a single plant species. Both systemin and HypSys are found in tomato, and systemin and HypSys regulate systemic wound signaling cooperatively through the octadecanoid pathway.[13] Soybean contains putative *At*Pep homologs, *Gm*SubPep and *Gm*Pep914/890, although their distinct roles in defense responses are yet to be investigated. The presence of multiple peptide elicitors and general elicitors such as oligogalacturonides in plants suggests that plants evolved multilayered systems to ensure a potent defense response against a wide array of invading organisms.

Studies of plant defense signaling peptides are still in their infancy, and the peptides identified thus far probably represent only a small fraction of elicitor peptides in the plant kingdom. A better understanding of the diversity and conservation of defense signaling peptides in higher plants will be facilitated by the isolation of peptides from as yet unstudied plant species. Continued study of signaling mechanisms and protective responses regulated by these peptides will facilitate their application to enhance plant resistance to both herbivores and disease.

REFERENCES

1. Berger B, Baldwin IT. The hydroxyproline-rich glycopeptide systemin precursor *Na*preproHypSys does not play a central role in *Nicotiana attenuata*'s anti-herbivore defense responses. *Plant Cell Environ* 2007;**30**:1450–64.
2. El Oirdi M, El Rahman TA, Rigano L, El Hadrami A, Rodriguez MC, Daayf F, et al. *Botrytis cinerea* manipulates the antagonistic effects between immune pathways to promote disease development in tomato. *Plant Cell* 2011;**23**:2405–21.
3. Heiling S, Schuman MC, Schoettner M, Mukerjee P, Berger B, Schneider B, et al. Jasmonate and ppHsystemin regulate key malonylation steps in the biosynthesis of 17-Hydroxygeranyllinalool Diterpene Glycosides, an abundant and effective direct defense against herbivores in *Nicotiana attenuata*. *Plant Cell* 2010;**22**:273–92.
4. Hind SR, Malinowski R, Yalamanchili R, Stratmann JW. Tissue-type specific systemin perception and the elusive systemin receptor. *Plant Signal Behav* 2010;**5**:41–4.
5. Huffaker A, Dafoe NJ, Schmelz EA. ZmPep1, an ortholog of Arabidopsis elicitor peptide 1, regulates maize innate immunity and enhances disease resistance. *Plant Physiol* 2011;**155**:1325–38.
6. Huffaker A, Pearce G, Ryan CA. An endogenous peptide signal in Arabidopsis activates components of the innate immune response. *Proc Natl Acad Sci USA* 2006;**103**:10098–103.
7. Huffaker A, Ryan CA. Endogenous peptide defense signals in Arabidopsis differentially amplify signaling for the innate immune response. *Proc Natl Acad Sci USA* 2007;**104**:10732–6.
8. Krol E, Mentzel T, Chinchilla D, Boller T, Felix G, Kemmerling B, et al. Perception of the Arabidopsis danger signal peptide 1 involves the pattern recognition receptor AtPEPR1 and its close homologue AtPEPR2. *J Biol Chem* 2010;**285**:13471–9.
9. Li C, Liu G, Xu C, Lee GI, Bauer P, Ling HQ, et al. The tomato suppressor of prosystemin-mediated responses2 gene encodes a fatty acid desaturase required for the biosynthesis of jasmonic acid and the production of a systemic wound signal for defense gene expression. *Plant Cell* 2003;**15**:1646–61.
10. Malinowski R, Higgins R, Luo Y, Piper L, Nazir A, Bajwa VS, et al. The tomato brassinosteroid receptor BRI1 increases binding of systemin to tobacco plasma membranes, but is not involved in systemin signaling. *Plant Mol Biol* 2009;**70**:603–16.
11. McGurl B, Ryan CA. The organization of the prosystemin gene. *Plant Mol Biol* 1995;**20**:405–9.
12. Narváez-Vásquez J, Orozco-Cárdenas ML, Pearce G. Plant peptide signals. In: Hughes A, editor. *Amino acids, peptides and proteins in organic chemistry*. : Wiley VCH; 2009. p. 599–631.
13. Narváez-Vásquez J, Orozco-Cárdenas ML, Ryan CA. Systemic wound signaling in tomato leaves is cooperatively regulated by systemin and hydroxyproline-rich glycopeptide signals. *Plant Mol Biol* 2007;**65**:711–8.

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14. Narváez-Vásquez J, Pearce G, Ryan CA. The plant cell wall matrix harbors a precursor of defense signaling peptides. *Proc Natl Acad Sci USA* 2005;**102**:12974–7.
15. Narvaez-Vasquez J, Ryan CA. The cellular localization of prosystemin: a functional role for phloem parenchyma in systemin signaling. *Planta* 2004;**218**:360–9.
16. Pearce G, Moura DS, Stratmann J, Ryan CA. Production of multiple plant hormones from a single polyprotein precursor. *Nature* 2001;**411**:817–20.
17. Pearce G, Munske G, Yamaguchi Y, Ryan CA. Structure–activity studies of GmSubPep, a soybean peptide defense signal derived from an extracellular protease. *Peptides* 2010;**31**:2159–64.
18. Pearce G. Systemin, hydroxyproline-rich systemin and the induction of protease inhibitors. *Curr Protein Pept Sci* 2011;**12**: 399–408.
19. Pearce G, Strydom D, Johnson S, Ryan CA. A polypeptide from tomato leaves activates the expression of proteinase inhibitor genes. *Science* 1991;**253**:895–7.
20. Pearce G, Yamaguchi Y, Barona G, Ryan CA. A subtilisin-like protein from soybean contains an embedded, cryptic signal that activates defense-related genes. *Proc Natl Acad Sci USA* 2010;**107**:14921–5.
21. Pearce G, Yamaguchi Y, Munske G, Ryan CA. Structure–activity studies of AtPep1, a plant peptide signal involved in the innate immune response. *Peptides* 2008;**29**:2083–9.
22. Qi Z, Verma R, Gehring C, Yamaguchi Y, Zhao Y, Ryan CA, et al. Ca^{2+} signaling by plant *Arabidopsis thaliana* Pep peptides depends on AtPepR1, a receptor with guanylyl cyclase activity, and cGMP-activated Ca^{2+} channels. *Proc Natl Acad Sci USA* 2010;**107**:21193–8.
23. Realini C, Rogers SW, Rechsteiner M. KEKE motifs: proposed roles in protein–protein association and presentation of peptides by MHC Class I receptors. *FEBS Lett* 1994;**348**:109–13.
24. Ren F, Lu YT. Overexpression of tobacco hydroxyproline-rich glycopeptides systemin precursor A gene in transgenic tobacco enhances resistance against *Helicoverpa armigera* larvae. *Plant Sci* 2006;**171**:286–92.
25. Rocha-Granados Cdel M, Sánchez-Hernández C, Sánchez-Hernández C, Martínez-Gallardo NA, Ochoa-Alejo N, Délano-Frier JP. The expression of the hydroxyproline-rich glycopeptide systemin precursor A in response to (a)biotic stress and elicitors is indicative of its role in the regulation of the wound response in tobacco (*Nicotiana tabacum* L.). *Planta* 2005;**222**:794–810.
26. Ryan CA. The systemin signaling pathway: differential activation of plant defensive genes. *Biochem Biophys Acta* 2000;**1477**: 112–21.
27. Schilmiller AL, Howe GA. Systemic signaling in the wound response. *Curr Opin Plant Biol* 2005;**8**:369–77.
28. Schmelz EA, Carroll MJ, LeClere S, Phipps SM, Meredith J, Chourey PS, et al. Fragments of ATP synthase mediate plant perception of insect attack. *Proc Natl Acad Sci USA* 2006;**103**:8894–9.
29. Schmelz EA, LeClere S, Carroll MJ, Alborn HT, Teal PE. Cowpea chloroplastic ATP synthase is the source of multiple plant defense elicitors during insect herbivory. *Plant Physiol* 2007;**144**:793–805
30. Schulze B, Mentzel T, Jehle AK, Mueller K, Beeler S, Boller T, et al. Rapid heteromerization and phosphorylation of ligand-activated plant transmembrane receptors and their associated kinase BAK1. *J Biol Chem* 2010;**285**:9444–51.
31. Yamaguchi Y, Barona G, Ryan CA, Pearce G. GmPep914, an eight-amino acid peptide isolated from soybean leaves, activates defense-related genes. *Plant Physiol* 2011;**156**:932–42.
32. Yamaguchi Y, Huffaker A, Bryan AC, Tax FE, Ryan CA. PEPR2 is a second receptor for the Pep1 and Pep2 peptides and contributes to defense responses in Arabidopsis. *Plant Cell* 2010;**22**:508–22.

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Chapter 3

DVL/RTFL

Elene R. Valdivia, Kate L. Hertweck, Sung Ki Cho and John C Walker

ABSTRACT

The DEVIL (DVL) or ROT-FOUR-LIKE (RTFL) family of small proteins was discovered during activation-tagging screens in *Arabidopsis*. Overexpression of several of these genes results in similar pleiotropic phenotypes: shortened rosette leaves and floral organs, altered silique development, and stalks at the base of pedicels and trichomes. Silenced lines or insertional mutants have no noticeable phenotype. More than 20 *DVL/RTFL* genes have been identified in Arabidopsis. The corresponding proteins (40–144 amino acids) share a highly conserved region of 30 amino acids located close to the C terminus, which is sufficient for their function. The overexpression of phenotypes might be explained by the alterations in polarized cellular proliferation. The mechanism of action of these small proteins is currently unknown. They seem to be localized to the plasma membrane where they might interact with a receptor. DVL/RTFL overexpression alters a large number of genes involved in signaling pathways, particularly in hormonal responses.

DISCOVERY

The first work identifying the DVL/RTFL gene family was published in 2004.[10] Because of their short open reading frames (~150 nt), these genes had not been detected during the initial annotation of the *Arabidopsis thaliana* genome. They were discovered when an activation-tag insertion that results in siliques with horned tips was mapped between genes At5g16020 and At5g16030. The insertion causes the overexpression of a 153-bp ORF, which was identified as an unannotated gene and named *DEVIL1* (*DVL1*). A search for homologous sequences in the Arabidopsis genome identified at least 20 additional members of the *DVL* family (Figure 1A). When overexpressed, several of them (*DVL2* to *DVL5*) showed phenotypes similar to those in the *DVL1* activation-tagged line (*dvl-1D*). In addition to altered siliques, other common phenotypes were rounder leaves and shorter petal and sepals.

Shortly after this first report, a second group published the characterization of an activation-tagged Arabidopsis line overexpressing another gene from this family.[8] The line was identified by its rounder leaves in a screen for mutations affecting leaf morphology. The altered gene was thus named *ROTUNDIFOLIA4* (*ROT4*), which corresponds to *DVL16*. The genes homologous to ROT4 were named ROT-FOUR-LIKE (RTFL).

STRUCTURE OF THE PEPTIDE

The Arabidopsis DVL/RTFL peptides/polypeptides range in size from 40 to 144 amino acids, but they all share a highly conserved region of about 30 amino acids located at or very close to the C-terminus[8,10] (Figure 1B). This region has been dubbed the RTF domain and is also conserved in DVL/RTFL proteins from other species. Overexpression of truncated or mutated versions of ROT4 has shown that this region, which is enriched in basic amino acids, is strictly required and sufficient for the peptides to induce phenotypic changes.[4,8,10] This observation could explain the similarity in the overexpression of phenotypes of different *DVL* genes. The remaining sequence of these short proteins is poorly conserved and frequently repetitive, with an abundance of polar residues. There is currently no information regarding possible function for the nonconserved regions.

It has been suggested that the one or two basic amino acid at the start of the conserved region could serve as a signal for proteolysis processing.[11] The DVL1 protein maintains its activity when fused to GFP at the N-terminus, but not if fused at the C-terminus, where the putative proteolytic processing would not remove this large addition. However, lines overexpressing ROT4 fused to GFP at both the N- and C-terminus showed phenotypic alterations, although these were milder in the second case.[9,11] That free GFP was not detected in these lines suggests that posttranslational proteolytic processing is not strictly required for DVL/RTFL activity, at least in this case.

DISTRIBUTION OF THE mRNA/PEPTIDE

More than 20 members of the DVL/RTFL family have been identified in Arabidopsis. Constitutive expression of 10 out of 11 *DVL* genes tested, including *DVL1* and *ROT4*/*DVL16*, results in similar phenotypes in rosettes, inflorescences, and siliques.[8,11] This could be interpreted as evidence of extensive

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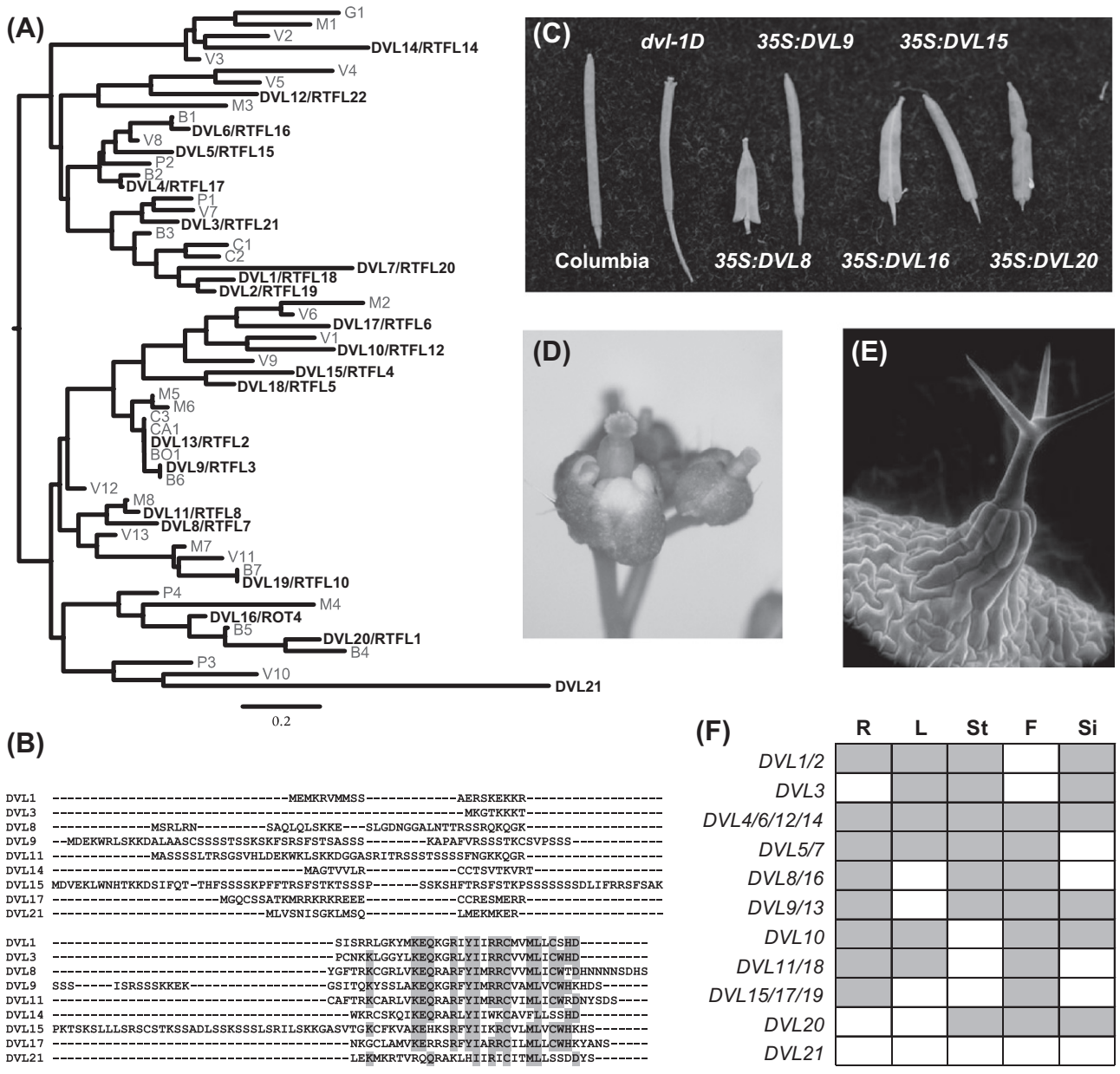

FIGURE 1 Diversification of the DVL/RTFL family. (A) Phenogram of DVL/RTFL proteins. *Arabidopsis* proteins are identified by their annotation. Proteins from other species were obtained from BAC sequences available on GenBank and are labeled as follows: *Boechera* (BO), *Brassica* (B), *Capsella* (CA), *Gossypium* (G), *Cleome* (C), *Medicago* (M), *Populus* (P), and *Vitis* (V). Sequences were aligned with ClustalW by a JTT subsitution matrix.[6] A neighbor-joining phenogram was constructed in MEGA.[5] The aligned sequences and tree are available on TreeBase (accession number 11894). The short length of the aligned matrix resulted in weak bootstrap support, but the general topological relationships appeared regardless of analysis parameters. (B) Alignment of selected DVL proteins. (C) Siliques from different *DVL* overexpressing lines. (D) Inflorescences from *dvl1-1D*. (E) Protrussion at the vase of tricomes in a *DVL9* overexpressing line. (F) Expression pattern of individual *DVL* genes determined by RT-PCR. Tested issues were root (R), leaf (L), stem (St), flower (F), and silique (Si). *DVL* genes with the same expression pattern are grouped together.

functional overlap. An alternative explanation is that these genes have specialized functions in different organs or cell types, but they work through a common mechanism and can substitute for each other.

To examine the expression profiles of the individual DVL genes, RT-PCR was performed on tissues from wildtype Col-0 (Figure 1F). All the DVL genes, with the exception of *DVL21* which was never amplified despite repeated attempts, are expressed in at least two of the tissues tested (root, seedling, stem, flower bud, and silique), although with different levels of expression. The lack of detectable expression of DVL21 was confirmed by the Arabidopsis Tiling Array Transcriptome Express Tool.[12] The highly divergent nature of this sequence suggests that it could be a pseudogene (Figure 1A).

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The phylogenetic analysis of the family suggests that diversification was already advanced by the time of divergence of the *Vitis* and Arabidopsis lineages (Figure 1A). The two most recently duplicated pairs of Arabidopsis genes are *DVL1*/*DVL2* and *DVL9*/*DVL13*. They also share the same expression profiles, and it would not be surprising if they have overlapping functions (Figure 1F). In older gene duplications, such as *DVL8*/*DVL11* or *DVL16*/*DVL20*, differences in the expression pattern can be detected, suggesting a higher degree of functional specialization. The widespread expression of the *DVL*/*RTFL* genes at the organ level suggests that the functional specialization in this family could be at the level of cell type or in the responses to internal or external stimuli.

BIOLOGICAL FUNCTION

Our understanding of the biological function of the *DVL*/*RTFL* peptides has been hampered by the lack of noticeable loss-of-function phenotypes in either mutants or silencing lines. Because of the small size of the *DVL/RTFL* genes insertional mutants are not very abundant. An Arabidopsis line with an insertion in *RTFL4*/*DVL15* and two rice lines with insertions in other *DVL/RTFL* genes have been described as lacking a morphological phenotype.[8] Similarly, RNAi constructs targeting *DVL1*, *DVL3* or both genes simultaneously failed to produce any visible alterations despite strong reductions in gene expression.[10] Our laboratory has analyzed many insertional mutants individually and in crosses, as well as additional silencing lines without observing any noticeable phenotypes. These results could be caused by genetic redundancy or by the involvement of these genes in responses to very specific conditions.

Currently, the only evidence for the biological function of the DVL/RTFL family is the phenotypes observed in overexpression lines, which involve severe alteration in plant development. It is difficult to draw firm conclusions about the function of these genes from the results of their expression at nonphysiological levels, but they seem to be able to alter the developmental program of many different organs. Because a 32-amino-acid fragment is sufficient for this effect, the most probable explanation is that the DVL/RTFL peptides play some sort of signaling role.

One of the characteristic phenotypes of DVL/RTFL overexpression is the alteration in silique development, giving rise to valves that are shorter and wider. Their precise shape is somewhat variable as overexpression of different DVL genes result in remarkably different shapes, with wider regions at the tip or the base (Figure 1C). Sepals and petals also tend to be shorter resulting in protruding pistils in young flowers (Figure 1D). It has been determined that the rounder leaves caused by overexpression of *ROT4*/*DVL16* have a reduced number of cells specifically in the longitudinal axis.[8] The cell size on the other hand does not appear to be altered. This phenotype suggests that *ROT4* supresses polarized cell proliferation. Because sepals, petals, and silique valves are leaf-derived organs, it is possible that a similar mechanism explains their shortening in *DVL*/*RTFL* overexpression lines. A more recent analysis revealed that ROT4 overexpression slows down the rate of cell production instead of prematurely ending the phase of cell division.[4] More precisely, it appears that, in this line, the size of the meristematic zone is somewhat reduced in the proximodistal axis. Additionally, overexpression of *ROT4* results in shorter petioles.

Another distinct phenotype caused by *ROT4* overexpression is a protrusion of the main inflorescence stem at the base of pedicels.[4] This protrusion does not look much different from the pedicel, from which it is separated by a sharp bend and a vestigial abscission zone. This same phenotype was observed in plants overexpressing DVL9,[11] and plants with inducible overexpression of DVL4. Another phenotype that is possibly related is a stalk-like protrusion at the base of the trichomes, which is filled with small mesophyll cells (Figure 1E). This phenotype is present in leaves, stems, and sepals and has been observed in lines overexpressing different *DVL* genes.[4,11] The pedicel and trichome phenotypes might involve a localized increase in polarized cell proliferation, opposite to what happens in leaves.

In *Medicago truncatula MtDVL1* is induced during nodule formation, and its overexpression results in reduced nodulation.[1] It is possible that this phenotype is also linked to the regulation of cell proliferation because cell-cycle reactivation in response to infection is a key step in the nodulation process.

To understand the pathways affected by *DVL* overexpression, a genomewide expression analysis was performed on a line where DVL4 expression is activated by dexamethasone application.[8] In this experiment, 310 genes were upregulated two-fold or more 9–10 h after induction. These genes were grouped by their biological functions using the functional categorization of the Munich Information Center for Protein Sequences (MIPS, http://www.mips.biochem.mpg.de).[2] The percentage of upregulated genes in each category was compared with the percentage of Arabidopsis genes classified in the same category (Figure 2A).

Among the genes upregulated in response to *DVL4* overexpression, there is a large percentage associated with metabolism (28.9%). Other functional categories that are overrepresented among the upregulated genes are 34 (interaction with environment), 36 (systemic interaction with the environment), and 40 (cell fate). Within category 34, the upregulated set of genes is enriched in the subcategory involved in sensing and reacting to environmental stimuli, such as chemoperception, gravitropism, and circadian rhythm (Figure 2B). Among the genes grouped in category 36, many of the upregulated genes are involved in response

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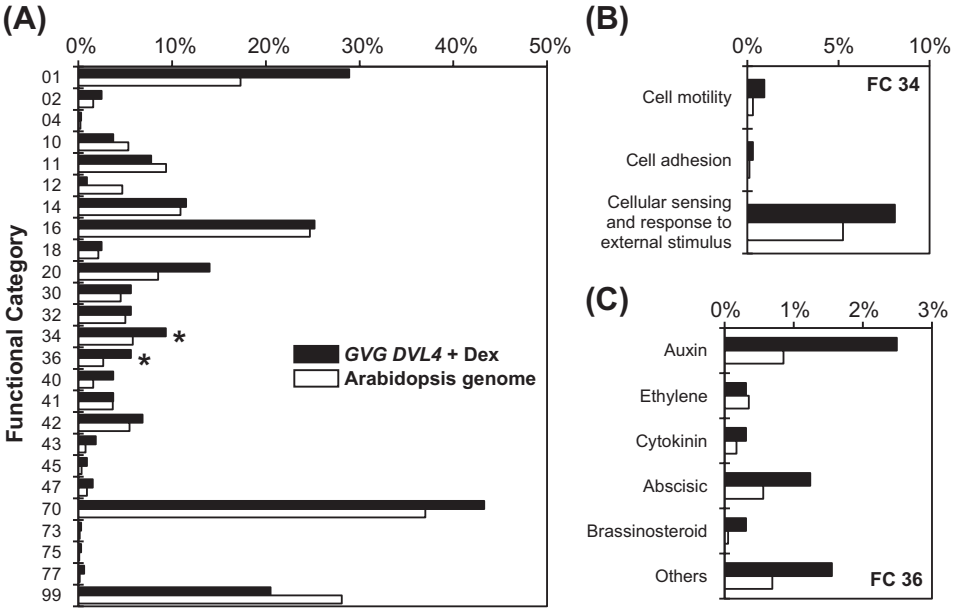

FIGURE 2 Comparative functional analysis of genes upregulated by inducible *DVL4* overexpression. (A) Distribution of genes upregulated by *DVL4* overexpression (black) and Arabidopsis genes (white) in the functional categories defined by the Munich Information Center for Protein Sequences. Asterisks indicate categories analyzed in B and C. Categories are labeled as follows: 01 metabolism, 02 energy, 04 storage protein, 10 cell cycle and DNA processing, 11 transcription, 12 protein synthesis, 14 protein fate (folding, modification, destination), 16 protein with binding function or cofactor requirement (structural or catalytic), 18 regulation of metabolism and protein function, 20 cellular transport, transport facility and transport routes, 30 cellular communication/signal transduction mechanism, 32 cell rescue, defense and virulence, 34 interaction with the environment, 36 systemic interaction with the environment, 40 cell fate, 41 development (systemic), 42 biogenesis of cellular components, 43 cell type differentiation, 45 tissue differentiation, 47 organ differentiation, 70 subcellular localization, 73 cell type localization, 75 tissue localization, 77 organ localization, 99 unclassified proteins. (B) Percentage of genes upregulated by DVL4 overexpression in subcategories within functional category 34 (Interaction with environment) compared to total Arabidopsis genes. (C) Percentage of genes upregulated by DVL4 overexpression in subcategories within functional category 36 (Systemic interaction with the environment) compared with total Arabidopsis genes.

to different hormones (Figure 2C). One of the genes involved in auxin responses (NAC1) is actually the gene showing the highest upregulation in response to *DVL4*. It is therefore possible that the alterations in cell proliferation observed in overexpression lines are the results of changes in hormonal responses. What seems clear is that *DVL/RTFL* overexpression is capable of causing profound changes in the developmental program.

RECEPTORS AND SIGNALING CASCADES

The similarity in the overexpression phenotypes of different *DVL* genes points to the possibility of a common receptor or several receptors with similar specificity. In addition, the fact that the conserved domain is required and sufficient for the activity of the peptides indicates that this is the region that interacts with the putative receptor. Even if the receptor is common or very similar for different members of the family, it is probable that different signaling cascades are triggered in different organs or cell types leading to the diversity of observed phenotypes.

One of the open questions about the DVL/RTFL family is whether these small proteins are secreted to the apoplastic space and interact with extracellular receptors or they exercise their effect in the cytoplasm interacting with intracellular partners. That these proteins lack a signal peptide points toward the second possibility, but there is significant evidence for the existence of non-classical or leaderless secretion pathways in plants.[9] Interestingly, most DVL/RTFL proteins are predicted as extracellular by SecretomeP, a program designed to identify nonclassically secreted proteins, although the prediction program was trained with mammalian sequences.[3] Weighting against an extracellular receptor is the observation that overexpression of GFP-DVL1 fusions with an added signal peptide did not produce phenotypic effects, whereas GFP-DVL1 overexpression in the cytoplasm resulted in a similar phenotype to overexpression of unmodified DVL1.[11] However, this result can also be interpreted as suggesting the need of some form of activation though posttranslational processing in the cytoplasm before secretion to the apoplast.

Both ROT4-GFP and GFP-ROT4 fusions are localized to the plasma membrane.[4,8] The overexpression of these constructs results in a similar phenotype to ROT4 overexpression, suggesting that the plasma membrane could be the native localization of DVL/RTFL proteins. Because ROT4 does not include a transmembrane domain, this could reflect the localization of a putative receptor or interacting

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partner. The question that remains to be solved is whether the interaction happens on the extracellular or intracellular side of the plasma membrane.

The localization of the receptor is related to the issue of transport. An intracellular receptor would suggest a local effect for the DVL/RTFL proteins limited to the same cell where they are produced or to the neighboring cells that they could reach through transport via plasmodesmata. Local overexpression of a GFP-ROT4 fusion in developing leaves was achieved through a construct in which Cre/Lox recombination was activated randomly by heat treatment.[4] Activation in young leaf primordia leads to large sectors of overexpressing cells. When these sectors are located in one of the leaf margins, the petiole on that side is shortened while the other side of the petiole is unaffected. This result points to a localized action of the DVL proteins and excludes a fast transport of GFP-ROT4 capable of establishing a uniform level of protein throughout the leaf. However, it is compatible with local cell-to-cell transport or even long distance transport at a slow rate.

Overexpression of DVL/RTFL genes results in the upregulation and downregulation of large numbers of genes, some of which are probably involved in signaling cascades. When DVL1, DVL2, DVL3, DVL4, or DVL5 are overexpressed, the mRNA level of *AGL/FUL8* decreases two- to five-fold.[10] This MADS-box gene participates in fruit–valve differentiation and could be involved in the alteration of silique development observed in these lines. A microarray analysis of Arabidopsis plants with constitutively activated ROT4 expression detected 17 genes that were upregulated three times or more, but none of these genes has a clear connection to signaling cascades or the regulation of cell division.[8] However, the list includes several genes that could be involved in pathogen responses.

An Arabidopsis line with expression of DVL4 controlled by dexamethasone application has permitted the identification of 422 genes whose expression is strongly upregulated or downregulated after 9–10 h of treatment.[7] Among these early responding genes transcription factors are overrepresented. The gene showing the strongest response in this experiment is *NAC1* (At1g56010), a transcription factor involved in auxin responses. When two Arabidopsis lines with independent insertions in NAC1 are crossed to the constitutively activated line *dvl1-1D*, the presence of either insertion in hemizygosis is sufficient to block the phenotype caused by *DVL1* overexpression. This blockage persists in the descendants of these crosses even when they did not receive the actual insertions in *NAC1*, suggesting some kind of epigenetic effect. The insertions in *NAC1* do not seem to affect the expression levels of *DVL1*. It is possible that the blockage of *DVL1* activity in these plants takes place at the translational or posttranslational level. An alternative explanation is that insertions in *NAC1* cause epigenetic modifications in genes involved in the signaling cascades activated by DVL1 of which NAC1 itself could be a member.

REFERENCES

1. Combier JP, Kuster H, Journet EP, Hohnjec N, Gamas P, Niebel A. Evidence for the involvement in nodulation of the two small putative regulatory peptide-encoding genes MtRALFL1 and MtDVL1. *Mol Plant–Microbe Interact* 2008;**21**:1118–27.
2. Frishman D, Albermann K, Hani J, Heumann K, Metanomski A, Zollner A, et al. Functional and structural genomics using PEDANT. *Bioinformatics* 2001;**17**:44–57.
3. Germain H, Chevalier E, Matton DP. Plant bioactive peptides: an expanding class of signaling molecules. *Can J Bot* 2006;**84**:1–9.
4. Ikeuchi M, Yamaguchi T, Kazama T, Ito T, Horiguchi G, Tsukaya H. ROTUNDIFOLIA4 regulates cell proliferation along the body axis in Arabidopsis shoot. *Plant Cell Physiol* 2011;**52**:59–69.
5. Kumar S, Nei M, Dudley J, Tamura K. MEGA: a biologist-centric software for evolutionary analysis of DNA and protein sequences. *Brief Bioinform* 2008;**9**:299–306.
6. Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, et al. Clustal W and Clustal X version 2.0. *Bioinformatics* 2007;**23**:2947–8.
7. Larue C, Wen J, Walker J. Interactions between a NAC-domain transcription factor and the putative small protein encoding DVL/ROT gene family. *Plant Mol Biol Report* 2010;**28**:162–8.
8. Narita NN, Moore S, Horiguchi G, Kubo M, Demura T, Fukuda H, et al. Overexpression of a novel small peptide ROTUNDIFOLIA4 decreases cell proliferation and alters leaf shape in *Arabidopsis thaliana*. *Plant J* 2004;**38**:699–713.
9. Rose JKC, Lee SJ. Straying off the highway: trafficking of secreted plant proteins and complexity in the plant cell wall proteome. *Plant Physiol* 2010;**153**:433–6.
10. Wen J, Lease KA, Walker JC. DVL, a novel class of small polypeptides: overexpression alters Arabidopsis development. *Plant J* 2004;**37**:668–77.
11. Wen J, Walker J. DVL peptides are involved in plant development. In: Abba JK, editor. *Handbook of biologically active peptides*. Burlington: Academic Press; 2006. p. 17–22.
12. Yamada K, Lim J, Dale JM, Chen H, Shinn P, Palm CJ, et al. Empirical analysis of transcriptional activity in the Arabidopsis genome. *Science* 2003;**302**:842–6.

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Chapter 4

EPF

Tatsuo Kakimoto

ABSTRACT

The *EPIDERMAL PATTERNING FACTOR* (EPF) family of *Arabidopsis thaliana* comprises nine genes that encode small cysteine-rich secretory peptides. *EPF2* is expressed in early-stage stomatal lineage cells, which contribute to the production of both stomatal and non-stomatal cells, and functions as a negative-feedback regulator that limits the number of epidermal cells. *EPF1* is also expressed in stomatal lineage cells and prevents contact formation of stomata by regulating the formative asymmetric cell division plane. Another family member, *EPF-LIKE9(EPFL9)/STOMAGEN*, is expressed in the inner cells of developing leaves and positively regulates stomatal density. Although the functions of these peptides are different, their genes require the same putative receptor components, ERECTA-family (ERf) transmembrane kinases and the TOO MANY MOUTHS (TMM) receptor-like protein. Their peptide signals are transmitted through an MAP kinase cascade, which destabilizes SPEECHLESS (SPCH) protein, a master regulator of stomatal lineage identity.

DISCOVERY

To identify new peptide mediators, we performed a screen involving the overexpression of many *Arabidopsis* genes that encode peptides/polypeptides (<150 amino acids) with a secretory signal sequence.[9] We identified genes that affect epidermal cell patterning, including those later designated *STOMAGEN/EPFL9*, *EPF1* (EPIDERMAL PATTERNING FACTOR 1), and *EPF2*, among which *EPF1* was the first to be reported.[9] Overexpression of the *STOMAGEN* gene increases the number of stomata, whereas overexpression of *EPF1* and *EPF2* genes decreases the number of stomata. Because overexpression possibly causes phenotypes unrelated to the gene's true role, we examined the expression patterns and phenotypes of loss-of-function mutants, the results of which let us focus on EPF1, EPF2,[10] and Stomagen.[14] EPF2[12] and Stomagen[11,22] were also identified as genes co-expressed with EPF1 and other genes that are expressed in the stomatal lineage cells. *CHALLAH (CHL)/EPFL6* was identified as the gene responsible for the *challah* mutant, which suppressed the lack-of-stomata phenotype of *too many mouths* mutants in hypocotyls and stems.[1] *CHAL* and two other genes with redundant functions, *CHL-LIKE* (*CHL)1* and *CHL2*, form the *CHAL* subfamily of the *EPF* family.[2]

STRUCTURE OF THE PEPTIDE

EPF-family genes (*EPF1*, *EPF2*, and [*EPF-LIKE EPFL*]*1–9*) encode 102–172 amino acid polypeptides with a secretory signal sequence at their N-terminus. Of these small proteins, the structure of the mature form has been experimentally determined for only Stomagen/EPFL9.[14,22] The mature Stomagen peptide is derived from the carboxy-terminal 41 amino acid residues of the gene-coded product, which share sequence similarity with all the other EPF-family members (see also the Stomagen section of this book). Stomagen has six cysteine residues that form three disulfide bonds.[14] These six cysteines are perfectly conserved in all EPF-family members. EPF1, EPF2, and EPFL7 have two additional cysteines (Fig. 1). This protein family is present in diverse plant species.

DISTRIBUTION OF THE mRNA/PEPTIDE

In *Arabidopsis*, a fraction of the leaf protodermal cells (undifferentiated epidermal cells) take the stomatal lineage and become meristemoid mother cells (MMCs), which each asymmetrically divide to form a small triangular cell (meristemoid) and its sister cell (termed a stomatal lineage ground cell, SLGC).[21] Cells that do not take the stomatal lineage differentiate into general epidermal cells called pavement cells. The meristemoid and the SLGC may also undergo asymmetric cell division forming a meristemoid and an SLGC. The meristemoid finally becomes a guard mother cell (GMC), which symmetrically divides to form a pair of guard cells that constitute a stoma. SLGCs finally differentiate into pavement cells. Thus, the number of MMCs and the number of asymmetric cell divisions determine the number of stomatal and non-stomatal epidermal cells (Fig. 2). These developmental steps have been anatomically examined in detail,[7] as have the underlying molecular mechanisms.[4,6,19,20]

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00004-X
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EPF1(At)        AGSRLP-DCSH-ACGSCSP-CRLVMVSFVCAS----------------VEEAETCPMAY--------------KCMCNNKSYPVP
EPF2(At)        TGSSLP-DCSY-ACGACSP-CKRVMISFECS-------------------VAESCSVIY--------------RCTCRGRYYHVPSRA
EPFL1(At)       LGSTPP-SCHN-RCNNCHP-CMAIQVPTLPTRSRFTRVNPFSGGFVRPPSSLTTVLDQYS------NYKPMGWKCHCNGHFYNP
EPFL2(At)       IGSRPP-RCERVRCRSCGH-CEAIQVPTNPQTKLHSPLTTSS------SSSSETIHLDYTRGDDSTNYKPMSWKCKCGNSIYNP
EPFL3(At)       IGSKPP-SCEK-KCYGCEP-CEAIQFPTI--------------------SSIPHLSPHYA------NYQPEGWRCHCP----PP
EPFL4(At)       PGSSPP-TCRS-KCGKCQP-CKPVHVPIQPGL----------------SMPLEYYPEAW--------------RCKCGNKLFMP
EPFL5(At)       PGSVPP-MCRL-KCGKCEP-CKAVHVPIQPGL----------------IMPLEYYPEAW--------------RCKCGNKLFM
EPFL6(At)       LGSSPP-RCSS-KCGRCTP-CKPVHVPVPPGT----------------PVTAEYYPEAW--------------RCKCGNKLYMP
EPFL7(At)       SGSSIP-DCSN-ACGPCKP-CKLVVISSTCS-------------------ASEACPLVY--------------KCLCKGKYYHVPSLT
EPFL8(At)       MGSEPP-VCAT-KCRNCKP-CLPYLFDIRGAHD-----------------DDDDSEPYYP-----------VKWICRCRDRVFEP
Stomagen(At)    IGSTAP-TCTYNECRGCRYKCRAEQVPVE---------------------GNDPINSAY------------HYRCVCHR
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FIGURE 1 Alignment of the C-terminal halves of the EPF-family proteins of *Arabidopsis*. EPFL6 = CHAL, EPFL5 = CLL1, EPFL4 = CLL2, Stomagen = EPFL9. This region represents the mature Stomagen/EPFL9.

The *EPF2* promoter–*GFP* (*GREEN FLUORESCENT PROTEIN*) signal is detected in MMCs, meristemoids, and SLGCs. The *EPF1* promoter–*GFP* signal is detected in meristemoids, GMCs, and young guard cells. *STOMAGEN* is expressed in the inner cells of developing leaves, carpels, stamens, and sepals, which have stomata. *CHAL/EPFL6* is preferentially expressed in the inner cells of hypocotyls and inflorescence stems, in midveins of leaves, and in the ovule chalazal region; however, it is not expressed in stomatal lineage cells.[1]

Biological Functions

Stomata consist of a pair of guard cells with a pore in-between. They change pore size, depending on the environmental status, through quick modulation of the turgor pressure of guard cells. Stomatal density and placing patterns are species-specific, but also change depending on the environment.[5]

In *Arabidopsis*, stomata are more evenly distributed than would occur randomly. In most plant species, stomata are not formed adjacent to one another, but are always separated by at least one pavement cell (one-cell-spacing rule[8]). This pattern is ensured by correct placement during the asymmetric cell division that forms the meristemoid; meristemoids are not formed in contact with previously formed meristemoids, GMCs, or young guard cells. It had been hypothesized that these cells produce an intercellular signaling molecule that inhibits the contact formation of new meristemoids.[4,7] This hypothetical molecule has been proven to be EPF1 based on three findings: first, *EPF1* is expressed in these cells; second, in the loss-of-function *epf1* mutant, the asymmetric cell division plane forming the meristemoid is placed in the wrong position, causing contact formation of stomata; and third, overexpression of *EPF1* decreases the number of stomata without decreasing the total epidermal cell number. These results indicate that EPF1 regulates the position of the asymmetric cell division plane, so that new meristemoids are not formed in contact with previously formed meristemoids, GMCs, or guard cells. The overexpression phenotype suggests that EPF1 may also regulate cell fate independently of its regulation of the cell division plane[9] (Fig. 2).

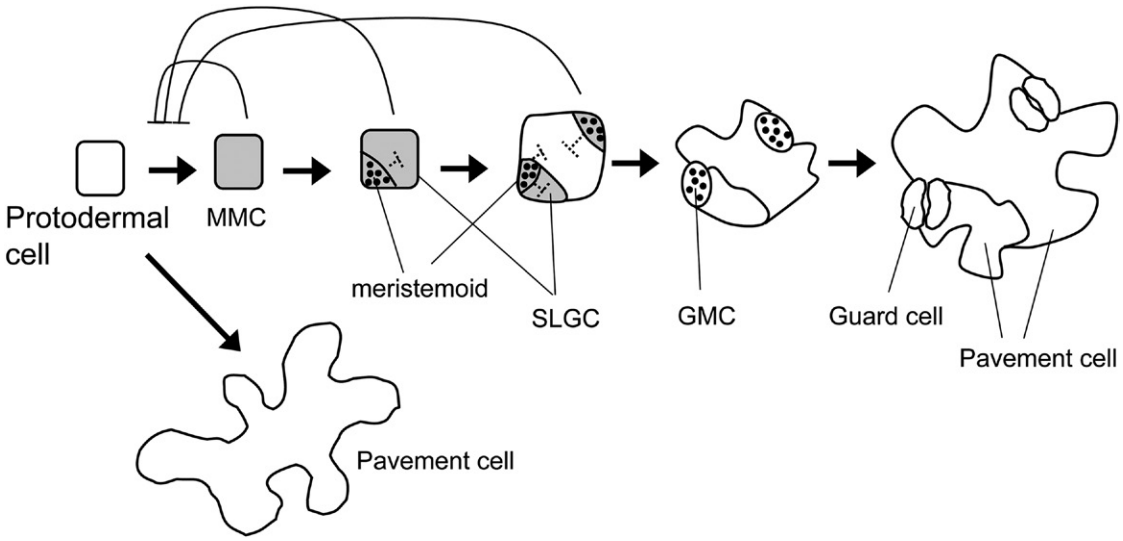

FIGURE 2 Epidermal lineages and regulatory mechanisms mediated by peptide signaling molecules. A fraction of the protodermal cells of the leaf primordium are selected to be meristemoid mother cells (MMCs), which asymmetrically divide to form small triangular cells (meristemoid) and sister cells (stomatal lineage ground cells, SLGCs). Meristemoids eventually give rise to pairs of guard cells that form the stoma, and the SLGCs eventually give rise to pavement cells. *EPF2*-expressing cells are shaded, and *EPF1*-expressing cells are dotted. EPF2 inhibits the formation of MMCs (solid line), limiting the number of epidermal cells. EPF1 regulates the positioning of the meristemoid (broken line). GMC, guard mother cell.

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Cell proliferation stops when the correct number of cells is formed. *EPF2* overexpression inhibits the formation of MMCs, thereby decreasing the total number of both stomatal and non-stomatal cells. By contrast, mutation in the *EPF2* gene results in increased number of both stomatal and non-stomatal cells, but the one-cell-spacing rule is maintained. Because *EPF2* is expressed in MMCs, meristemoids, and SLGCs, which are proliferative, extracellular EPF2 concentration should increase when the number of these proliferative cells increases. Thus, EPF2 acts as a negative-feedback molecule that limits the total number of epidermal cells.[10]

The *tmm* mutant has an increased number of stomata in its leaves and cotyledons, but lacks stomata in its hypocotyls and inflorescent stems. The *chal* mutation suppresses the stomata-less phenotype in the *tmm* hypocotyls and stems.[1] The *cll1* or *cll2* mutation enhances the *chal* phenotype in the *tmm* background. These results indicate that CHAL, CLL1, and CLL2 repress stomatal formation in the hypocotyls and in the stem, and that TMM represses the effects of CHAL family peptides. Because CHAL family genes are not highly expressed in stomatal precursor cells but are mainly expressed in the inner cells of the hypocotyl and of the stem, these peptides may act in a non-tissue autonomous manner. CHAL family peptides also control plant height, because *chal;cll1* and *chal;cll2* double mutants are short.[2] EPFL4/CLL2 and FPFL6/CHAL regulate stem elongation through the action of ER. EPF1 and EPF2 directly bind ERf receptor proteins.[23]

STOMAGEN/EPFL9 is expressed in the inner cells of developing leaves and is a positive regulator of stomatal formation. For the function of Stomagen, please refer to the chapter on Stomagen in this section of the book.

Receptors and Signaling Cascades

The candidate receptor components for EPF-family ligands are: ERECTA (ER), ERECTA-LIKE 1(ERL1), ERL2, and TMM. The ER-family proteins comprise an N-terminal leucine-rich repeat (LRR) followed by a transmembrane segment, and a C-terminal Ser/Thr kinase domain. TMM possesses only the LRR and the transmembrane segment. In the triple *er;erl1;erl2* mutant and the *tmm* mutant, the number of stomatal lineage cells is increased, which results in the production of more stomata and the one-cell-spacing rule is disrupted. This indicates that these putative receptor components control entry into the stomatal lineage and the asymmetric cell division that forms the meristemoid.[21]

Overexpression of *EPF1* or *EPF2* decreases the number of stomata in wildtype plants,[9,10,12] but not in the triple *er;erl1;erl2* mutant or in the *tmm* mutant.[9,10] This indicates that EPF1 and EPF2 require TMM and either ER, ERL1, or ERL2. Stomagen, on the other hand, decreases the stomatal number, when its gene is overexpressed or mature peptide is applied. Interestingly, Stomagen also requires TMM[14,22] (the requirement for the ER family has not been examined). EPF1 and EPF2 directly bind ERf receptor proteins.[16] Taken together, a plausible model would be that EPF2 and EPF1 compete with Stomagen for the same receptor complex that contains the ER family proteins and TMM. Supporting this model, Stomagen does not increase the stomatal number in the absence of *EPF1* and *EPF2*.[14]

An MAP kinase cascade is believed to be downstream of this receptor complex. In this MAP kinase cascade, MAP kinase kinase kinase, YODA (YDA)[3]; two MAP kinase kinases, MKK4 and MKK5; and two MAP kinases, MPK3 and MPK6 play important roles.[24] The MAP kinase system phosphorylates the beta helix-loop-helix transcription factor SPEECHLESS (SPCH) to destabilize it.[15] Because SPCH, which functions together with two other beta helix-loop-helix proteins (SCREAM and SCREAM2[13]), is required for the identity of early stomatal lineage cells,[17,18] destabilization of SPCH causes cells to become pavement cells.

To summarize the model, EPF1 and EPF2 are perceived by receptor complexes containing TMM and ER family receptor kinases, which in turn activate the MAP kinase cascade and the degradation of SPCH, inhibiting the acquisition or the maintenance of stomatal lineage identity. However, although the same set of receptor components is required for the functions of EPF1 and EPF2, these ligands are differentially recognized by the receptor(s). Overexpression of *EPF1* and *EPF2* leads to the loss of stomata, but through different pathways. The use of cell-type specific GFP marker lines showed that *EPF2*, but not *EPF1*, inhibits the formation of MMC. *EPF1*, however, inhibits the identity of the meristemoid.[10] The mechanism behind this recognition specificity is not yet known. EPF1 thus regulates both the asymmetric cell division plane and, independently, it may regulate cell identity. EPF1 most likely regulates cell identity through SPCH. EPF1 also controls the position of asymmetric cell division plane, perhaps independently of SPCH. The extracellular EPF1 gradient may cause subcellular local activation of the MAP kinase cascade, regulating the asymmetric cell division plane, via an as yet unknown mechanism.

The receptor complexes can also execute more complex tasks. *CHAL*, *CLL1*, and *CLL2* decreases leaf stomatal density when any one of them is overexpressed. These genes require ER family genes to function. Surprisingly, these three genes do not require TMM and also function more effectively in *tmm* mutants.[2] This suggests that TMM represses the signal transduction events initiated by these three peptides. CHAL, CLL1, and CLL2 may also regulate inflorescence elongation, since *chal;cll1;cll2* is a semidwarf mutant like *er*.[2]

The biological functions of the EPF-family peptides have been well characterized by genetic methods. However, information regarding the biochemical basis for the actions of these proteins is limited. How these peptide family members, together with the same set of receptor components, exert different functions is a challenging problem to solve.

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REFERENCES

1. Abrash EB, Bergmann DC. Regional specification of stomatal production by the putative ligand CHALLAH. *Development* 2010;**137**:447–55.
2. Abrash EB, Davies KA, Bergmann DC. Generation of signaling specificity in *Arabidopsis* by spatially restricted buffering of ligand–receptor interactions. *Plant Cell* 2011;**23**:2864–79.
3. Bergmann DC, Lukowitz W, Somerville CR. Stomatal development and pattern controlled by a MAPKK kinase. *Science* 2004;**304**:1494–7.
4. Bergmann DC, Sack FD. Stomatal development. *Annu Rev Plant Biol* 2007;**58**:163–81.
5. Casson SA, Hetherington AM. Environmental regulation of stomatal development. *Curr Opin Plant Biol* 2010;**13**:90–5.
6. Dong J, Bergmann DC. Stomatal patterning and development. *Curr Top Dev Biol* 2010;**91**:267–97.
7. Geisler MJ, Deppong DO, Nadeau JA, Sack FD. Stomatal neighbor cell polarity and division in *Arabidopsis*. *Planta* 2003;**216**:571–9.
8. Geisler MJ, Nadeau JA, Sack FD. Oriented asymmetric divisions that generate the stomatal spacing pattern in *Arabidopsis* are disrupted by the too many mouths mutation. *Plant Cell* 2000;**12**:2075–86.
9. Hara K, Kajita R, Torii KU, Bergmann DC, Kakimoto T. The secretory peptide gene EPF1 enforces the stomatal one-cell-spacing rule. *Genes Dev* 2007;**21**:1720–5.
10. Hara K, Yokoo T, Kajita R, Onishi T, Yahata S, Peterson KM, et al. Epidermal cell density is autoregulated via a secretory peptide, EPIDERMAL PATTERNING FACTOR 2 in *Arabidopsis* leaves. *Plant Cell Physiol* 2009;**50**:1019–31.
11. Hunt L, Bailey KJ, Gray JE. The signalling peptide EPFL9 is a positive regulator of stomatal development. *New Phytol* 2010;**186**:609–14.
12. Hunt L, Gray JE. The signaling peptide EPF2 controls asymmetric cell divisions during stomatal development. *Curr Biol* 2009;**19**:864–9.
13. Kanaoka MM, Pillitteri LJ, Fujii H, Yoshida Y, Bogenschutz NL, Takabayashi J, et al. SCREAM/ICE1 and SCREAM2 specify three cell-state transitional steps leading to *Arabidopsis* stomatal differentiation. *Plant Cell* 2008;**20**:1775–85.
14. Kondo T, Kajita R, Miyazaki A, Hokoyama M, Nakamura-Miura T, Mizuno S, et al. Stomatal density is controlled by a mesophyll-derived signaling molecule. *Plant Cell Physiol* 2010;**51**:1–8.
15. Lampard GR, Macalister CA, Bergmann DC. *Arabidopsis* stomatal initiation is controlled by MAPK-mediated regulation of the bHLH SPEECHLESS. *Science* 2008;**322**:1113–6.
16. Lee JS, Kuroha T, Hnilova M, Khatayevich D, Kanaoka MM, McAbee JM, et al. Direct interaction of ligand–receptor pairs specifying stomatal patterning. *Genes Dev* 2012;**26**:126–36.
17. MacAlister CA, Ohashi-Ito K, Bergmann DC. Transcription factor control of asymmetric cell divisions that establish the stomatal lineage. *Nature* 2007;**445**:537–40.
18. Pillitteri LJ, Sloan DB, Bogenschutz NL, Torii KU. Termination of asymmetric cell division and differentiation of stomata. *Nature* 2007;**445**:501–5.
19. Pillitteri LJ, Torii KU. Breaking the silence: three bHLH proteins direct cell-fate decisions during stomatal development. *Bioessays* 2007;**29**:861–70.
20. Shimada T, Sugano SS, Hara-Nishimura I. Positive and negative peptide signals control stomatal density. *Cell Mol Life Sci* 2011;**68**:2081–8.
21. Shpak ED, McAbee JM, Pillitteri LJ, Torii KU. Stomatal patterning and differentiation by synergistic interactions of receptor kinases. *Science* 2005;**309**:290–3.
22. Sugano SS, Shimada T, Imai Y, Okawa K, Tamai A, Mori M, et al. Stomagen positively regulates stomatal density in *Arabidopsis*. *Nature* 2010;**463**:241–4.
23. Uchida N, Lee JS, Horst RJ, Lai HH, Kajita R, Kakimoto T, et al. Regulation of inflorescence architecture by intertissue layer ligand–receptor communication between endodermis and phloem. *Proc Natl Acad Sci U S A* 2012;**109**:6337–42.
24. Wang H, Ngwenyama N, Liu Y, Walker JC, Zhang S. Stomatal development and patterning are regulated by environmentally responsive mitogen-activated protein kinases in *Arabidopsis*. *Plant Cell* 2007;**19**:63–73.

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Chapter 5

IDA/IDL

Melinka A. Butenko, Robert Kumpf and Reidunn B. Aalen

ABSTRACT

Floral organ abscission, the shedding of petals, sepals, and filaments, from *Arabidopsis thaliana* is a developmentally controlled process that requires the INFLORESCENCE DEFICIENT IN ABSCISSION (IDA) peptide ligand and the two leucine-rich repeat (LRR) receptor-like kinases, (RLK), HAESA (HAE) and HAESA-LIKE 2 (HSL2). IDA belongs to a larger protein family that together comprises the IDA-LIKE (IDL) proteins. These proteins are all extracellular localized proteins that share a common C-terminal domain—the EPIP domain—which contains the functional peptides. Although the exact functions of the IDL peptides are not known, the expression pattern of the *IDL* genes indicates that they may be involved in regulating cell separation processes important during plant development. Here we give an overview of the discovery of the *IDA* and *IDL* genes, their expression pattern, biological function, and genetic and biochemical studies that have been performed to reveal the IDA signaling pathway.

DISCOVERY

Since the first discovery of a plant peptide capable of having signaling activity, small molecules identified as plant peptide ligands have been identified by biochemical or genetic approaches. The INFLORESCENCE DEFICIENT IN ABCSISSION (IDA) peptide ligand was identified by traditional genetic screening based on random mutagenesis followed by cloning of the mutated gene. With the aim of identifying genetic components regulating floral organ abscission in *Arabidopsis thaliana*, a population of mutants with random T-DNA insertions was screened for deficiency in floral organ abscission.

In wildtype (WT) *Arabidopsis* flowers, the shedding of flower petals, sepals, and filaments supervenes shortly after pollination or anthesis (Figure 1A). By contrast, one of the mutant lines identified retain its perianth and stamens indeterminately (Figure 1B). This mutant, caused by a homozygous recessive monogenic mutation, was named *ida*.[5] PCR-based cloning techniques determined the T-DNA location of the *ida* mutation to be upstream of a small open reading frame, which later was annotated as *At1g68765*, and corresponds to the *IDA* gene.

IDA encodes a small protein of 77 amino acids (aa) with an N-terminal hydrophobic region, which was predicted by SignalP software to act as a signal peptide (SP), and this was proved by genetic and subcellular localization studies.[2,5,27] An *in silico* approach was undertaken to determine whether the *Arabidopsis* and other plant genomes encode proteins with similarities to *IDA*. Several *IDA-LIKE* (*IDL*) transcripts from both dicots and monocots were identified, including one cDNA (*IDL1*) and four unannotated putative *IDL* genes (*IDL2*, *IDL3*, *IDL4*, and *IDL5*) from *Arabidopsis*. RT-PCR was performed to verify that the unannotated *IDL* genes were expressed. The *IDL* transcripts encode proteins with predicted N-terminal SPs and a conserved C-terminal motif common to IDA, which we have named EPIP[5,27] (Figure 2A). The EPIP domain of IDA and the IDL proteins share similarities to that of the CLE domain[27] (Figure 2B) (see the chapter on CLV3 and CLE peptides elsewhere in

FIGURE 1 Phenotypes of the ida mutant. (A) In wildtype Arabidopsis plants, the floral organs (petals, sepals, and filaments) abscise shortly after the flower is pollinated (arrow head). (B) In the inflorescence deficient in abscission (ida) mutant, the floral organs remain attached indefinitely to the receptacle of the growing fertilized silique (arrow). See color plate 2.

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00005-1
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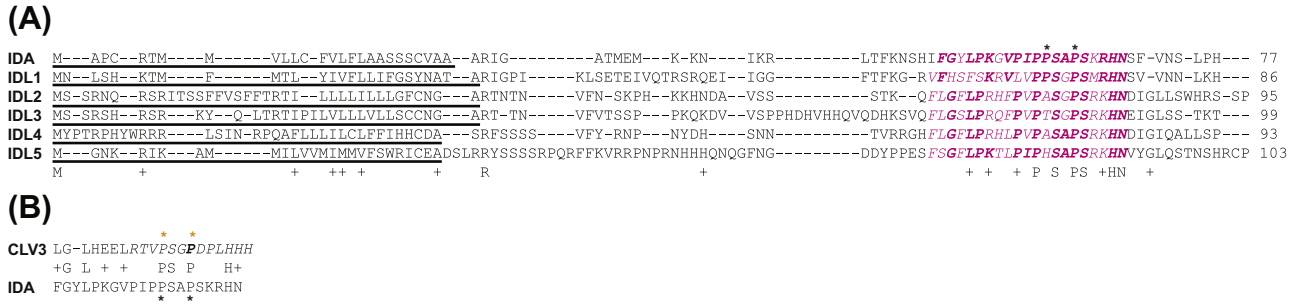

FIGURE 2 Alignment of IDA and IDL proteins and similarities to the mature CLV3 Peptide. (A) The IDA and IDL proteins have predicted N-terminal signal peptides (underlined) and a conserved C-terminal EPIP domain (italics in magenta). Amino acids (aa) in the EPIP domains of the IDL proteins identical to the IDA-EPIP domain are in bold italics, asterisks indicate prolines, which are probably hydroxyprolinated in IDA. Identical aa are indicated by letters and conserved aa by (+). (B) The mature CLV3 (MCLV3) peptide, indicated in italics, and some of the upstream aa have similarities to the EPIP domain of IDA. Identical aa are indicated by letters and conserved aa by (+). The MCLV3 peptide has two hydroxyprolines, indicated by orange asterisks, where one is posttranslationally modified with triarabinoside (bold italics). The predicted hydroxyprolines of the IDA-EPIP domain are indicated with black asterisks. See color plate 3.

this Plant Peptides Section of the book), and as no other hydrophobic regions, apart from the predicted SP, are found in these proteins, we infer that they were soluble and probably act as a new family of peptide ligands.[5] Further indication of their function as a new family of peptide ligands in *Arabidopsis* comes from *in planta* domain swap studies.[27]

STRUCTURE OF THE IDA AND IDL PEPTIDES

The IDA and IDL proteins can be divided into three regions: the N-terminal SP, a variable region with very little aa similarity between the proteins, and a conserved 20 aa EPIP domain (Figure 2A). A set of *in vivo* experiments elucidated the mature structure of the active peptide ligands.

When *Arabidopsis* plants overexpress (OX) IDA under the control of the cauliflower mosaic virus 35S promoter (35S), a series of phenotypes related to organ abscission are observed.[26] The plants portray precocious floral organ abscission, where all floral organs abscise at an earlier floral developmental stage than in WT plants; abscission of organs, such as cauline leaves and pedicels, not normally shed in *Arabidopsis*; and lastly an enlargement of the specialized abscission zone (AZ) cells at the point where the organs detach from the plant.[26] A certain amount of genetic redundancy exists between *IDA* and the *IDL* genes because plants which OX the *IDL* genes have phenotypes mimicking those of IDA OX lines to varying extents.[27] Interestingly, based on the full-length protein sequence, IDL1 is most closely related to IDA (Figure 2A), and OX of IDL1 gives the most similar phenotypes to plants OX IDA. *IDL1* has the ability to fully rescue *ida* when expressed under the regulatory elements of *IDA*, while plants expressing the other *IDL* genes show partial recovery of the abscission defect. Because the only conserved part between IDA and the IDL proteins is the EPIP domain (Figure 2A), domain swap experiments, in which the EPIP and C-terminal end (EPIP-C) of the IDL proteins are exchanged for that of IDA, were performed to test for possible rescue of the *ida* phenotype. The EPIP-C of IDL1 has the highest rescue ability; however, the EPIP-C of IDL2, IDL3, and IDL4 has a greater tendency to revert the abscission phenotype of *ida* than the IDL2, IDL3 and IDL4 gene swap constructs, indicating that the variable region of the IDA protein gives functional support to the EPIP domain.[27] For CLV3, the processing of the mature CLE peptide requires a few residues upstream from the CLE domain,[19] and it is therefore possible that residues in the variable region of the IDA protein are required to facilitate correct processing of the EPIP, or part of the EPIP, domain in floral AZ cells.

Both the gene swap and domain swap experiments between *IDA* and the *IDL* genes indicate that as for the CLE proteins,[18] the C-terminally located EPIP domain is sufficient for function. In accordance with this, *in vivo* results where deletion constructs of the IDA protein were used to complement the *ida* mutant phenotype, show that the EPIP domain is essential and sufficient for the function of the IDA protein.[27] Additionally, synthetic IDA-EPIP and IDL1-EPIP-C peptides have been used in a bioassay in which the percentage of flowers undergoing abscission at a given time point is recorded. There were a significant number of flowers undergoing abscission at earlier time points when treated with IDA-EPIP or IDL1-EPIP-C peptides than the untreated ones. Furthermore, the IDA-EPIP synthetic peptide could partially rescue the floral abscission defect of *ida* flowers.[27] However, relative high amounts (10 μM) of peptide needed to be used in the bioassay before a response was detected, and a full rescue of the abscission defect of *ida* flowers was not observed, indicating that the mature active peptide *in vivo* could be shorter than the EPIP domain, modified, or both. In higher plants, many of the peptide ligands identified are posttranslationally modified,[16] and the mature CLV3 peptide has hydroxyproline arabinosylation, which is important for its biological activity and high-affinity binding to CLV1.[20]

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Preliminary bioassay experiments with different synthetic IDA peptides indicate that the mature active peptide of IDA is shorter than the EPIP domain and also requires hydroxyprolination of key prolines (Figure 2A), which possibly may be further modified *in vivo*.

DISTRIBUTION OF THE mRNA AND BIOLOGICAL FUNCTION OF THE IDA AND IDL PEPTIDES

Floral organ abscission in *Arabidopsis* is divided into four distinct developmental stages.[21] The first stage, which normally occurs early during floral development, is the differentiation of specialized AZ cells where the organ will later separate from the plant. If the AZ cells are not differentiated, abscission will not take place, as with the double mutant of *BLADE-ON-PETIOLE 1* and *2* (*BOP1 BOP2*), which does not portray anatomically distinct AZ cells.[17] The second phase starts as the organs prepare to separate from the plant, often preceded by environmental or hormonal stimulus, and the AZ cells become competent to respond to abscission signals. Subsequently, the pectin in the cell walls between AZs start to degrade followed by an expansion in the size of the AZ cells.[24] Ultimately, the actual separation of the organ supervenes and a lignified protective layer develops on the distal side of the AZ to protect the plant from pathogen attack.

Mutations in *IDA* block floral abscission at a late stage in the floral abscission process which is consistent with the expression of *IDA* in the floral organ AZs during the course of the abscission process.[5,26] Both a *promoter:GUS* reporter of *IDA* and *in situ* experiments have shown that *IDA* is expressed shortly after the AZ cells become competent to respond to abscission signals and the initial loosening of the cell walls takes place.[5,6] The expression level is highest before organ separation and the specific AZ signal is maintained throughout the floral abscission process. The GUS expression is restricted to the AZ at the bases of all floral organs (filaments, petals, and sepals) in addition to the nectaries (Figure 3A). *GUS* expression is also observed after the floral organs are abscised but is then restricted to the nectaries.[5]

Evidence from many different abscission model systems supports the promoting effect of ethylene in the regulation of abscission in dicotyledonous plants. In *Arabidopsis*, exposure to exogenous ethylene accelerates the abscission process,[3,5] although the developmentally regulated pathway of floral abscission acts independent of ethylene.[3,5,12] Consistent with this, the *ida* mutant is sensitive to ethylene and exposure of exogenous ethylene does not rescue the abscission defect of *ida*. However, *GUS* expression driven by the *IDA* promoter in the ethylene insensitive *etr-1* mutant background[4] has a different spatial distribution compared with that of WT. The GUS signal is detected in the nectaries but is absent from the AZ region.[6] This makes it possible that the delay in floral abscission observed in the *etr-1* mutant[3] may in part result from the restricted expression pattern of *IDA*.

35S:IDA plants display ectopic abscission of pedicels and leaves, but OX of IDA does not induce middle lamella breakdown between all cells even if *IDA* is abundantly expressed in all organs.[26] This indicates that for cells to respond to IDA signaling some indispensable components must be present for IDA-induced abscission to occur. Although, WT *Arabidopsis* does not have fruit or leaf abscission, detailed morphological analysis shows the presence of vestigial AZs at the proximal end of the pedicels and cauline leaves, suggesting that the components necessary to respond to IDA signaling are present in these cells and that it is the restricted expression pattern of *IDA* that hinders cell separation from normally taking place in these tissues.[26] Consistent with this idea, if *IDA* is OX in the double mutant *bop1 bop2* background that does not differentiate floral or vestigial AZs, none of the *IDA* OX phenotypes are observed.[17]

The exact function of the *IDL* genes is not known. Because of the small size of the coding region of the genes (all under 350 bp), it is difficult to obtain insertion or point mutants, and where these exist, single mutants do not show distinct phenotypes, probably resulting from redundancy between genes with overlapping expression masking the effect of the mutation. We can therefore only speculate on the biological function of these genes based on their expression profiles which suggests that the encoded proteins may share a common role in regulating other cell to cell separation events during plant development.[27] The expression pattern of the different *IDL* genes was investigated with *promoter:GUS* reporter constructs. *IDL5* is expressed in the vascular tissue early during development, shortly after the seedling has opened the seed coat, and this expression persists until the seedling has opened its cotyledons (Figure 3B). After germination *IDL1*, *IDL2*, *IDL3*, and *IDL4* are all expressed in the vascular tissue of the expanding cotyledons and primary leaves (Figure 3C), and except for *IDL1* and *IDL5*, expression is also observed in the guard cells. In the leaf, during the maturation of continuous tubes of tracheary elements, the middle lamella between two neighboring tracheary elements is digested completely by cell-wall-degrading enzymes.[11] It is possible that the *IDL* genes may play a role in regulating this process.[27]

Consistent with the expression in the leaves, *IDL2*, *IDL3*, and *IDL4* are also expressed in the vascular tissue of the root where the expression accumulates around the emerging lateral roots (Figure 3D) and they may possibly be involved in connecting the xylem and phloem of the new meristem to the main root. Interestingly, in the mature plant these three genes are also expressed in the floral AZ; however, their expression is apparently not sufficient to induce cell separation of floral organs when *ida* is mutated, indicating that the active part of these IDL proteins, probably

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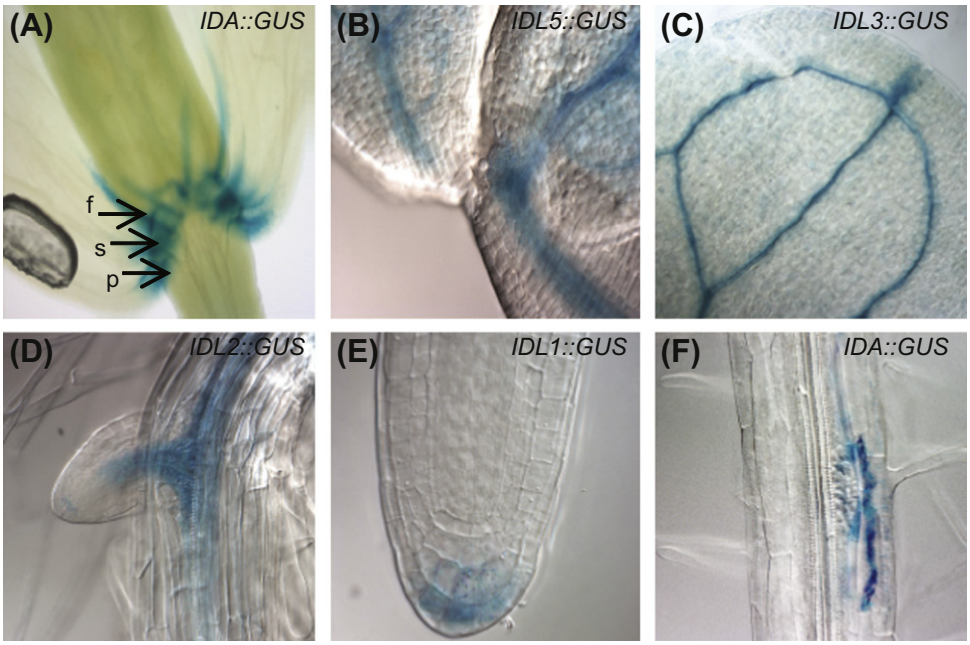

FIGURE 3 *Promoter:GUS* expression of IDA and IDL genes. (A) IDA is expressed in the filament (f), sepal (s), and petal (p) abscission zones during the abscission process. (B) IDL5 expression in the vascular tissue shortly after germination. (C) IDL1, IDL2, IDL3, and IDL4 are all expressed in the vascular tissue of the expanding cotyledon, here IDL3 is shown as a representative example. (D) IDL2, IDL3, and IDL4 are all expressed in the vascular tissue of the main root, and the expression accumulates around the lateral root; here, IDL2 is shown as a representative example. (E) IDL1 expression in the columella cells at tip of the main root. (F) IDA is also expressed in the cortex and epidermal cells of the main root during lateral root emergence. See color plate 4.

has a low affinity for the native receptors recognizing the IDA peptide ligand.

IDL1 has a specific pattern of expression in the columella cells of the tip of the primary root (Figure 3E). Interestingly, these cells undergo a cell separation event during root cap sloughing to allow the primary root to penetrate the soil.[8] Initial experiments with RNA interference lines indicates that this peptide ligand is involved in cell separation during columella cell loosening, which is important for the growth of the primary root. This, together with the genetic complementarity between IDL1 and IDA, opens the possibility that IDL1 and IDA may have similar signaling abilities.[7] Detailed studies of *IDA* expression during root development using a *promoter:GUS* construct show specific GUS expression in the cortex and epidermal cells overlaying the lateral root primordia (Figure 3F). As these cells need to undergo cell separation to facilitate the emergence of the outcoming primordia it appears that IDA plays a more general function in regulating cell separation outside the AZ cells. Initial experiments indicate that key hydrolytic enzymes necessary to degrade the cell wall during lateral root emergence are affected in the *ida* mutant. It is therefore possible that IDA induces similar signaling pathways as well as similar cell wall remodeling enzymes during different cell separation processes in *Arabidopsis*. Concurrent with this idea, *in situ* results have shown that *IDA* is also expressed in the dehiscence zone of the silique, where cell separation takes place when the valves open to allow for seed dispersal.[6]

RECEPTORS AND SIGNALING CASCADE OF IDA

Many mutants have been identified with changes in floral organ abscission.[1,14] Although some mutations lead to mutants with a delay in the abscission process, such as the *delayed in abscission* (*dab*) mutants,[22] others, such as *ida*, have a complete lack of abscission. These include the *bop1 bop2* double mutant,[17] the recently described mutant *never-shed* (*nev*) with a mutation in a gene encoding an ADP-ribosylation factor-GTPase-activating protein,[15] and a reported antisense line of the leucine-rich repeat (LRR) receptor-like kinase (RLK) encoding gene *HAESA* (*HAE*).[12] Because of the similarity in phenotypes between the *HAE* antisense line and the *ida* mutant it was early speculated whether HAE could function as a receptor for the IDA peptide ligand.[5]

Single knockout mutants of *HAE* are indistinguishable from WT plants and have normal floral organ abscission.[9,27] However, *HAE* belongs to a gene family that includes two paralogs,[25] *HAESA-LIKE 1* (*HSL1*) and *HAESA-LIKE 2* (*HSL2*) and a promoter driven *HSL2* GUS expression is restricted to floral organ AZ cells identical to that of *HAE*.[9] The overlapping expression pattern of *HAE* and *HSL2* suggests that these two genes are functionally redundant. Consistent with this,

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a knockout T-DNA line of *HSL2* shows normal abscission while a double mutant of *HAE* and *HSL2* is completely deficient in floral organ abscission.[9,27] As for *ida*, the morphology of the AZ in the *hae hsl2* plants appears normal, and the mutations affect the abscission process during the separation stage.[9,27] None of the OX phenotypes of *IDA* are present in *hae hsl2,* showing that *hae hsl2* is epistatic to *35S:IDA*.[9,27] This, in addition to IDA-EPIP peptides having no effect on the floral deficiency of *hae hsl2*,[27] substantiates the idea that HAE, HSL2, or both, function as receptors for IDA. It is still however, not clear which of the receptors, or if both receptors, have the predominant role in relaying the IDA signal. However, preliminary binding studies indicate that HSL2 may have a higher affinity for IDA than HAE.

Interestingly, both *HAE* and *HSL2* are expressed in the vestigial AZs where OX of *IDA* causes ectopic abscission. The similarity between the OX phenotypes of the *IDL* genes to those of *IDA* indicates that when these proteins are abundantly present they too can relay a signal via HAE, HSL2 or both. This opens the possibility that the native receptors to the IDL peptides may be other closely related HSL receptors.[7]

Genetic and biochemical studies have shown that the *MAP KINASE KINASE 4* (*MKK4*) and *MKK5* and the *MAP KINASE* 3 (*MPK3*) and *MPK6* act downstream of *IDA* and *HAE HSL2*.[9] Consequently, there seems to be a core signaling pathway regulating cell separation in floral abscission where IDA activates either one or both of the RLKs HAE and/or HSL2 causing autophosphorylation of the kinase receptor domains and the transduction of a phosphorelay signal through a MAPK cascade including MKK4, MKK5, MPK3, and MPK6 (Figure 4).

To identify additional components of the IDA signaling pathway we screened progeny of mutagenized *ida* seeds for suppressor mutants that showed normal floral organ abscission. Of the 13 revertant lines identified, two of them had, in addition to normal organ shedding, the characteristic downward pointing silique phenotype conferred by mutations in the *KNOTTED-LIKE HOMEOBOX* (*KNOX*) gene *BREVIPEDICELLUS* (*BP*)/*KNOTTED-LIKE FROM ARABIDOPSIS THALIANA1* (*KNAT1*).[10,28] Sequencing of the *BP*/*KNAT1* gene identified point mutations in each of the two revertant lines.[24] In addition, a null-allele deletion mutant of *BP*/*KNAT1*, *bp-3*, is capable of reverting the floral organ abscission defect of both *ida* and *hae hsl2*.[24]

Physiological and morphological analyses show that *bp-3* had a precocious dissolution of the middle lamella, indicating that *BP*/*KNAT1* functions as an inhibitor of abscission and is important for regulating the proper timing of cell separation.[24] This has also been proposed for *NEV*, and interestingly a suppressor of *nev*, the LRR-RLK *EVERSHED* (*EVR*)[13] is downregulated in the *bp* mutant.[29] However, unlike *bp-3*, *evr* is not capable of rescuing the abscission defect of *ida* or *hae hsl2* so EVR inhibits abscission independently of IDA, but BP/KNAT1 may mediate crosstalk between the IDA-HAE/HSL2 and the EVR pathways (Figure 4).

During inflorescence development, *BP*/*KNAT1* restricts the expression of the two KNOX domain genes *KNAT2* and *KNAT6* to promote correct inflorescence growth.[23] Expression studies from AZ cells with *promoter:GUS* constructs of *KNAT2* and *KNAT6* show that this is also the case in AZ cells. Moreover, a double mutant of *knat2 knat6* rescues the premature abscission observed in *bp-3* mutants.[24] This

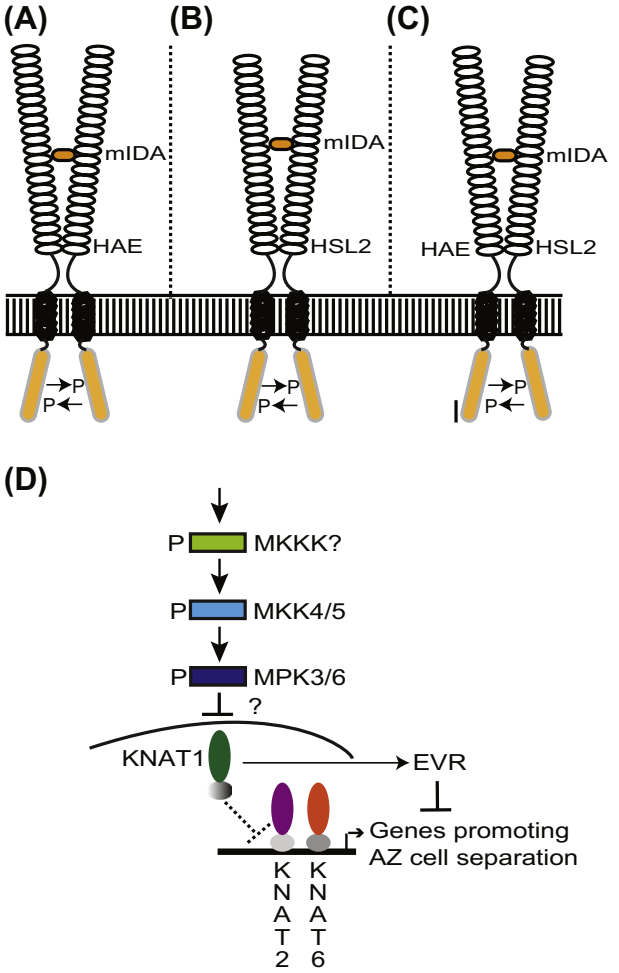

FIGURE 4 (A)–(C) A mature posttranslational modified IDA peptide consisting of the EPIP, or part of the EPIP, domain may bind the extracellular LRRs of an HAE homodimer (A), an HSL2 homodimer (B), or an HAE HSL2 heterodimer (C) and cause autophosphorylation (P) of the kinase receptor domains. (D) The signal from IDA-HAE/HSL2 is suggested to be transduced via a phosphorelay involving MKK4, MKK5, MPK3, and MPK6. Genetic evidence suggests that BP/KNAT1 acts as a negative downstream component of this signaling pathway by inhibiting cell separation and the expression of KNAT2 and KNAT6. On activation of the IDA signaling pathway, the BP/KNAT1 restriction of KNAT2 and KNAT6 is elevated, and these transcription factors can act as positive regulators of floral organ separation by positively regulating genes promoting AZ cell separation. In addition, BP/KNAT1 is a positive regulator of EVR, which influences cell separation by a separate parallel pathway.

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indicates that *KNAT2* and *KNAT6* act as positive regulators of cell separation during floral abscission downstream of *BP/KNAT1*. When *IDA* is OX in the *knat2 knat6* mutant background, the early abscission phenotype is lost. Conversely, when *KNAT2* or *KNAT6* are OX in the *ida* mutant background, rescue of the *ida* abscission defect is observed. This is consistent with the hypothesis that IDA signaling positively regulates *KNAT2* and *KNAT6*, and indeed promoter driven *GUS* expresssion of *KNAT2* and *KNAT6* is absent from the AZ of both the *ida* and *hae hsl2* mutants.[24]

The collected experiments performed by several scientists in the recent years have allowed us to propose the following signaling cascade for the IDA peptide ligand (Figure 4): A posttranslationally modified IDA peptide binds the LRR of either a homo- or heterodimer including HAE and/or HSL2 and induces a MAPK signaling cascade that acts to regulate *BP/KNAT1*. Because the transcriptional level of *BP/KNAT1* is unaltered in *ida* and *hae hsl2*, the regulation is probably at the protein level and may include dimerization with another homeodomain protein or posttranscriptional modifications of BP/KNAT1. The activation of the IDA pathway elevates the *BP/KNAT1* restriction of *KNAT2* and *KNAT6,* and these transcription factors may in turn positively regulate the transcription of genes involved in cell separation, such as cell wall remodeling (CWR) enzyme.

Future experiments investigating the cellular and subcellular localization of BP/KNAT1 during WT abscission compared with *ida* will hopefully provide an insight into how the IDA signaling pathway regulates the action of BP/KNAT1. Genetic identification of the remaining revertant lines will surely provide more detailed information on the IDA signaling system and certainly aid us in drawing parallels between the regulation of cell separation in the floral AZ and in lateral root emergence.

REFERENCES

1. Aalen RB. Flower and floral organ abscission—control, gene expression and hormone interaction. In: Yaish M, editor. *The flowering process and its control in plants: gene expression and hormone interaction*. Kerala: Research Signpost; 2011. p. 307–27.
2. Aalen RB, Butenko MA, Stenvik G-E, Tandstad NM, Patterson SE. Genetic control of floral abscission. In: Silva JT, editor. *Floriculture, ornamnetal and plant biotechnology: advances and topical issues*. London: Global Science Books; 2006. p. 101–8.
3. Bleecker A, Patterson SE. Last exit: senescence, abscission, and meristem arrest in *Arabidopsis*. *Plant Cell.* 1997;**9**:1169–79.
4. Bleecker AB, Estelle MA, Somerville C, Kende H. Insensitivity to ethylene conferred by a dominant mutation in *Arabidopsis thaliana*. *Science* 1988;**241**:1086–9.
5. Butenko MA, Patterson SE, Grini PE, Stenvik G-E, Amundsen SS, Mandal A, et al. *INFLORESCENCE DEFICIENT IN ABSCISSION* controls floral organ abscission in *arabidopsis* and identifies a novel family of putative ligands in plants. *Plant Cell.* 2003;**15**:2296–307.
6. Butenko MA, Stenvik GE, Alm V, Saether B, Patterson SE, Aalen RB. Ethylene-dependent and -independent pathways controlling floral abscission are revealed to converge using promoter::reporter gene constructs in the *ida* abscission mutant. *J Exp Bot* 2006; **57**:3627–37.
7. Butenko MA, Vie AK, Brembu T, Aalen RB, Bones AM. Plant peptides in signalling: looking for new partners. *Trends Plant Sci* 2009;**14**:255–63.
8. Campillo Ed, Abdel-Aziz A, Crawford D, Patterson SE. Root cap specific expression of an endo-beta-1,4-D-glucanase (cellulase): a new marker to study root development in Arabidopsis. *Plant Mol Biol* 2004;**56**:309–23.
9. Cho SK, Larue CT, Chevalier D, Wang H, Jinn TL, Zhang S, et al. Regulation of floral organ abscission in *Arabidopsis thaliana*. *Proc Natl Acad Sci USA* 2008;**105**:15629–34.
10. Douglas SJ, Chuck G, Dengler RE, Pelecanda L, Riggs CD. KNAT1 and ERECTA regulate inflorescence architecture in Arabidopsis. *Plant Cell* 2002;**14**:547–58.
11. Fukuda H. Signals that control plant vascular cell differentiation. *Nat Rev Mol Cell Biol* 2004;**5**:379–91.
12. Jinn T-L, Stone JM, Walker JC. HAESA, an Arabidopsis leucine-rich repeat receptor kinase, controls floral organ abscission. *Gene Dev* 2000;**14**:108–17.
13. Leslie ME, Lewis MW, Youn JY, Daniels MJ, Liljegren SJ. The EVERSHED receptor-like kinase modulates floral organ shedding in Arabidopsis. *Development* 2010;**137**:467–76.
14. Lewis MW, Leslie ME, Liljegren SJ. Plant separation: 50 ways to leave your mother. *Curr Opin Plant Biol* 2006;**9**:59–65.
15. Liljegren SJ, Leslie ME, Darnielle L, Lewis MW, Taylor SM, Luo R, et al. Regulation of membrane trafficking and organ separation by the NEVERSHED ARF-GAP protein. *Development* 2009;**136**:1909–18.
16. Matsubayashi Y. Post-translational modifications in secreted peptide hormones in plants. *Plant Cell Physiol* 2011;**52**:5–13.
17. McKim SM, Stenvik GE, Butenko MA, Kristiansen W, Cho SK, Hepworth SR, et al. The BLADE-ON-PETIOLE genes are essential for abscission zone formation in Arabidopsis. *Development* 2008;**135**:1537–46.
18. Ni J, Clark SE. Evidence for functional conservation, sufficiency and proteolytic processing of the CLAVATA3 CLE domain. *Plant Physiol* 2006;**140**:726–33.
19. Ni J, Guo Y, Jin H, Hartsell J, Clark S. Characterization of a CLE processing activity. *Plant Mol Biol* 2011;**75**:67–75.
20. Ohyama K, Shinohara H, Ogawa-Ohnishi M, Matsubayashi Y. A glycopeptide regulating stem cell fate in *Arabidopsis thaliana*. *Nat Chem Biol* 2009;**5**:578–80.
21. Patterson SE. Cutting loose. Abscission and dehisence in Arabidopsis. *Plant Physiol* 2001;**126**:494–500.
22. Patterson SE, Bleecker AB. Ethylene-dependent and -independent processes associated with floral organ abscission in Arabidopsis. *Plant Physiol* 2004;**134**:194–203.
23. Ragni L, Belles-Boix E, Gunl M, Pautot V. Interaction of KNAT6 and KNAT2 with BREVIPEDICELLUS and PENNYWISE in Arabidopsis inflorescences. *Plant Cell* 2008;**20**:888–900.
24. Shi C-L, Stenvik G-E, Vie AK, Bones AM, Pautot V, Proveniers M, et al. Arabidopsis class I KNOTTED-like homeobox proteins act downstream in the IDA-HAE/HSL2 floral abscission signaling pathway. *Plant Cell* 2011;**23**:2553–67.

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25. Shiu SH, Karlowski WM, Pan R, Tzeng YH, Mayer KF, Li WH. Comparative analysis of the receptor-like kinase family in Arabidopsis and rice. *Plant Cell* 2004;**16**:1220–34.
26. Stenvik GE, Butenko MA, Urbanowicz BR, Rose JK, Aalen RB. Overexpression of INFLORESCENCE DEFICIENT IN ABSCISSION activates cell separation in vestigial abscission zones in Arabidopsis. *Plant Cell* 2006;**18**:1467–76.
27. Stenvik GE, Tandstad NM, Guo Y, Shi CL, Kristiansen W, Holmgren A, et al. The EPIP peptide of INFLORESCENCE DEFICIENT IN ABSCISSION is sufficient to induce abscission in Arabidopsis through the receptor-like kinases HAESA and HAESA-LIKE2. *Plant Cell* 2008;**20**:1805–17.
28. Venglat SP, Dumonceaux T, Rozwadowski K, Parnell L, Babic V, Keller W, et al. The homeobox gene BREVIPEDICELLUS is a key regulator of inflorescence architecture in Arabidopsis. *Proc Natl Acad Sci USA* 2002;**99**:4730–5.
29. Wang X-Q, Xu W-H, Ma L-G, Fu Z-M, Deng X-W, Li J-Y, et al. Requirement of KNAT1/BP for the development of abscission zones in *Arabidopsis thaliana*. *J Int Plant Biol* 2006;**48**:15–26.

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Chapter 6

LUREs

Tetsuya Higashiyama

ABSTRACT

The pollen tube is a tip-growing cell that emerges from pollen grains. It contains two immotile sperm cells and transports them to the ovule, which contains the egg cell. Pollen-tube guidance to the ovule is critical for the successful fertilization of flowering plants. The existence of chemotropic factors derived from the ovule was postulated >140 years ago in the late 1860s. Ovular attractants, called LURE peptides, were first identified in 2009 in the model plant *Torenia fournieri*. LUREs are defensin-like peptides that are specifically and abundantly expressed in two synergid cells adjacent to the egg cell. LUREs show severe species preference and attraction specificity and might be involved in reproductive isolation. Unlike SCR/SP11, another defensin-like peptide for self-incompatibility in flowering plants, many LUREs are probably involved in generating the attraction signal for the pollen tube.

DISCOVERY

The study of pollen-tube guidance and associated attractants has a long history.[4,7] Chemotropic attractant factors derived from the ovule of flowering plants were first postulated as early as the 1860s[22] when researchers observed that pollen tubes grow toward the ovule *in vitro*. Since then, many plant biologists have attempted but failed to identify attractants derived from the ovule. These classical biochemical experiments were based on various bioassay systems and led to the identification of external calcium ions as a possible attractant in snapdragons.[18] However, external calcium ions are necessary for the growth of the tip of the pollen tube.[8] Attraction and growth stimulation were not clearly distinguished in these systems.[7,8]

One difficulty associated with bioassays of pollen-tube attraction is that pollen tubes still grow, but in random directions, without specific attractants if the medium contains the requisite compounds to sustain tube growth, such as water, calcium, borate, and sucrose.[8] In animals, the chemoattractant protein for the neuronal growth cone, netrin, was identified in 1994 using an *in vitro* assay.[14] This study showed that neural cells emerge from tissues only when netrin proteins are present in the medium.

In 1998, an *in vitro* system was developed in *Torenia fournieri*, a unique plant with a protruding female gametophyte.[10] The haploid female gametophyte, known as the embryo sac, usually has one egg cell and two synergid cells at the micropylar end, three antipodal cells at the opposite chalazal end, and one huge central cell in the middle.[5] Pollen tubes were directly attracted to the protruding micropylar end of this female gametophyte (Figure 1; for movies[7]). This was consistent with genetic analyses in *Arabidopsis thaliana* showing that the female gametophyte governed pollen-tube guidance to the ovule.[11] Laser ablation experiments showed that the attraction signal in *T. fournieri* was derived from the two synergid cells on the side of the egg.[12] This attraction activity has severe species preference and specificity even in closely related species,[9] suggesting that it is probably a rapidly evolving molecule(s). Thus, genes expressed in synergid cells of *Torenia* were examined using manually collected synergid cells to identify attractant candidates among the peptides and proteins.[20] The synergid cell showed a unique gene expression profile: various cysteine-rich peptides (CRPs) are abundantly expressed. Three major CRPs, TfCRP1, TfCRP2, and TfCRP3, were analyzed, and recombinant peptides of TfCRP1 and TfCRP3 showed strong activity to attract pollen tubes. These peptides were named LUREs (TfCRP1 as LURE1 and TfCRP3 as LURE2). Knockdown of *LURE1* or *LURE2* genes by injecting morpholino antisense oligomers impaired pollen-tube attraction. These results indicate that LURE1 and LURE2 are diffusible attractant molecules derived from the synergid cell of the ovule (Figure 2).[20] Genomic PCR analysis of TcCRP1 from a closely related species, *Torenia concolor*, identified a LURE1 homolog that also showed species-preferential attraction activity (Figure 2).[13]

STRUCTURE OF THE PEPTIDE

The apparent molecular masses of mature LURE1 and LURE2 are 8.6 and 9.8 kD, respectively, based on Western blotting of endogenous LUREs from the pistil.[20] Cleavage sites of the signal peptide of LURE1 and LURE2 have been precisely predicted using the SignalP program.

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00006-3
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The molecular weights of these endogenous LUREs are similar to those of recombinant LURE peptides expressed in *Escherichia coli* (predicted mature peptides lacking N-terminal signal sequences). These recombinant peptides of LURE1 and LURE2 show pollen-tube attraction activity at very low concentrations, as described below. Thus, it has been proposed that LURE1 and LURE2 are cleaved as predicted but are not substantially processed further. Secretion of endogenous LURE1 and LURE2 was confirmed by immunostaining, as described below.[20] The predicted mature peptides of LURE1 and LURE2 contain six cysteines. The molecular structures of the LUREs have not been resolved, but correct intramolecular disulfide bonds between the six cysteines have been suggested to be critical for their activity. Refolding is critical for pollen-tube attraction by recombinant LURE peptides, and refolded LUREs are mainly monomers.[20]

DISTRIBUTION OF THE mRNA/PEPTIDE

LURE1 and *LURE2* genes are specifically expressed in the two synergid cells on the side of the egg cell, independent of pollination, as shown by reverse transcription polymerase chain reaction (RT-PCR) of isolated female cells and immunostaining of ovules.[20] LURE1 and LURE2 peptides are secreted directionally toward the micropylar end of the egg and accumulate in the filiform apparatus. The filiform apparatus is a structure formed by the ingrown and/or thickened cell walls of the two synergid cells, and might be involved in active secretion and/or protection of the female gametophyte from pollen-tube thrust.[5] Accumulation of LURE peptides in the filiform apparatus is consistent with the behavior of pollen tubes; pollen tubes enter the female gametophyte through the region of the filiform apparatus.

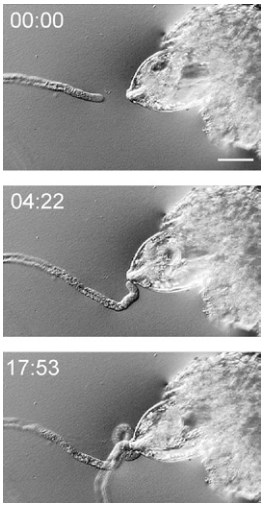

FIGURE 1 Pollen-tube attraction in the *in vitro Torenia* system. Two pollen tubes are attracted to the protruding embryo sac of Torenia fournieri. Pollen tubes are touching the filiform apparatus of two synergid cells in the embryo sac. Sequential images are shown and time after the start of observation is indicated as mm:ss. Modified after Ref. 7. Scale bar = 30 μm.

BIOLOGICAL FUNCTIONS

Pollen-tube guidance involves multistep controls exerted by various female tissues along the path of the pollen tubes.[7] LURE peptides govern the last guidance step over short distances, possibly at 100–200 μm from the female gametophyte.[21] The last guidance step is precise and is known as micropylar guidance.[21] The existence of attractants in other guidance steps remains unclear. Chemocyanin, a basic cell wall protein expressed in the lily stigma, is a good guidance molecule candidate.[15] Chemocyanin has ability to attract (reorient) pollen tubes *in vitro*; however, knockdown analyses have not been successful. It remains unknown whether LURE peptides are common in other flowering plants. Because CRPs generally show rapid molecular evolution, conventional homology searches are difficult for identifying LURE homologs in other plants. However, in *Arabidopsis thaliana*, CRPs are likely to be involved in synergid cell functions, including micropylar pollen-tube guidance.[21] ZmEA1, a nondefensin-like peptide, has been suggested as a possible pollen-tube attractant candidate for micropylar guidance in *Zea mays*.[2,17]

An *in vitro* attraction assay using recombinant LURE peptides has been used for characterization (Figure 3).[20] For quantitative analysis, recombinant peptides are embedded in gelatin beads approximately 40 μm in diameter and placed in front of each pollen tube using a micromanipulator to examine whether they attract pollen tubes. Pollen-tube attraction is a concentration-dependent process. At optimum concentrations, >60% of pollen tubes are attracted, which is similar to the frequency of attraction in manipulated ovules.[20] Surprisingly, the minimum concentration is 40 pM for both LURE1 and LURE2,[3,20] which means that only 1000 LURE molecules in the bead are sufficient to attract pollen tubes. Attraction of the pollen tube by LUREs might not simply

```
TfCRP1(LURE1)   22 GEIP--------PEQLRYVEFCD-LWSADFSG---SCGDLCKKKWGPNFVGDCDWYASTLWTSGDCVCSEKKKK 83
TcCRP1          22 GQIP--------PEPLRYVEFCD-LFSGDFSG---SCDELCKKKRGPNFVGDCDWYASTLWTRGDCVCSEKKKK 83
TfCRP3(LURE2)   24 SWIPFSKPKRGSYRLESDQERCAYLFPEDEAYAIESCNTRCKRTHGETAFGYCDF-TFPYWTAGECQCWSK    93

                                          C            CxxxC        GxC          CxC
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FIGURE 2 Alignments of LURE amino acid sequences. Alignments of LURE1, LURE2, and TcCRP1 (LURE1 homolog of Torenia concolor) are shown.[13,20] TcCRP1 preferentially attracted pollen tubes of its own species.[20] Conserved amino acids in more than two peptides are shaded gray. Conserved cysteines and glycines of the γ-core motif are shaded black and displayed in open boxes below the alignment.

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result from a LURE concentration gradient, as implied by visualization of LURE2 with Alexa labeling.[3]

The attraction activity of LUREs is similar to that of the synergid cell.[20] *T. fournieri* pollen tubes are preferentially and specifically attracted to ovules of their own species in the presence of ovules of *Torenia concolor* or *Lindernia micrantha*.[9] Consistently, pollen tubes of *T. concolor* and *L. micrantha* are less attracted and scarcely attracted, respectively, by LURE peptides from *T. fournieri*.[13,20] In the *in vitro Torenia* system, pollen tubes are grown through a cut style, as tubes become competent by growing through the pistil. The pollen tubes cultivated in semi-*in vitro* conditions, but not tubes germinated *in vitro*, are attracted to the synergid cell. Consistently, only competent pollen tubes growing through a cut style are attracted by LURE peptides.[20]

When LUREs were initially identified, only three major CRPs expressed in the synergid cell were examined. However, there are even more CRPs expressed in synergid cells, and some of them are likely to be candidate LUREs.[6] A mixture of plural or multiple LURE peptides might generate the attraction signal for micropylar guidance. It has also been proposed that LURE1 and LURE2 might have independent functions.[6]

RECEPTORS AND SIGNALING CASCADES

Receptors for LUREs have not been identified. The severe species preferentiality and specificity of LURE activity imply that some partner molecules on the plasma membrane of the pollen tube, possibly with highly variable domains, bind to the LUREs. Because the direction of tip growth is controlled at the tip by changing the exit site of secretory vesicles, it is plausible that the receptor is localized at the tip of the pollen tube.[21] Endocytosis actively occurs in the subapical region of the pollen tube for recycling of molecules at the tip.[1] Thus, the receptor might also be recycled at the tip. Pollen-tube competence, as described above, might involve some modifications of the receptor and/or signaling cascades.

Amino acid sequences are significantly divergent between LURE1 and LURE2, except for the conserved cysteines (Fig. 2).[20] The difference in amino acid sequence between LURE1 of *T. fournieri* and its homolog, *T. concolor* (TcCRP1), is only eight amino acids; however, this difference leads to significant species specificity.[13] Thus, because LURE1 and LURE2 are more highly diverged, they may bind to different receptors. Receptors for many plant peptide ligands, including defensin-like SCR/SP11, are serine–threonine receptor kinases with one transmembrane domain. However, the response of the pollen tube to LURE peptides is rapid and spatially precise. Involvement of other types of receptors has also been discussed.[21]

LURE-specific signaling cascades are unknown, but the directional signals from expected receptors are likely to modify signaling cascades related to tip growth of the pollen tube.[21] Alterations in the distribution of tip-focused concentration gradients of internal calcium ions and cAMP change the direction of growth, as shown using caged compounds and inhibitors.[16,19]

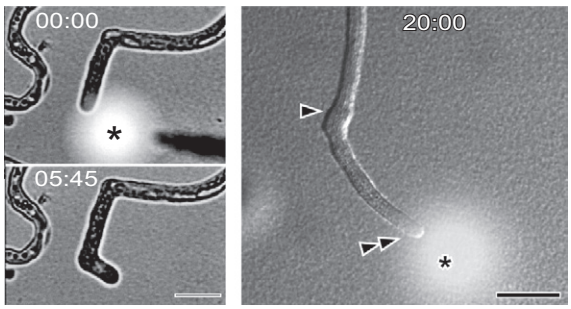

FIGURE 3 Pollen-tube attraction by recombinant LUREs. Pollen tubes were attracted using a micropipette (left) or a gelatin bead (right). Recombinant LURE2 (left) and LURE1 (right) were used. Note that the pollen tubes changed their direction to orient toward the injected LURE2 (left top, an asterisk) and a gelatin bead containing LURE1 (right, an asterisk). In the right panel, an arrowhead marks the position of the tip of the pollen tube when a gelatin bead was placed, and double arrowheads indicate the tip of the pollen tube growing toward the bead. Sequential images are shown in the left panels, and time after applying peptides is indicated as mm:ss. Modified after Ref. 20. Scale bar = 20 μm.

REFERENCES

1. Cheung AY, Hm Wu. Structural and signaling networks for the polar cell growth machinery in pollen tubes. *Annu Rev Plant Biol* 2008;**59**:547–72.
2. Dresselhaus T, Márton ML. Micropylar pollen tube guidance and burst: adapted from defense mechanisms? *Curr Opin Plant Biol* 2009;**12**:773–80.
3. Goto H, Okuda S, Mizukami A, Mori H, Sasaki N, Kurihara D, et al. Chemical visualization of an attractant peptide, LURE. *Plant Cell Physiol* 2011;**52**:49–58.
4. Heslop-Harrison J. Pollen germination and pollen-tube growth. *Int Rev Cytol* 1987;**107**:1–78.
5. Higashiyama T. The synergid cell: attractor and acceptor of the pollen tube for double fertilization. *J Plant Res* 2002;**115**:149–60.
6. Higashiyama T. Peptide signaling in pollen–pistil interactions. *Plant Cell Physiol* 2010;**51**:177–89.
7. Higashiyama T, Hamamura Y. Gametophytic pollen tube guidance. *Sex Plant Reprod* 2008;**21**:17–26.
8. Higashiyama T, Inatsugi R. Comparative analysis of biological models used in the study of pollen tube growth. *Plant Cell Monogr* 2006;**3**:265–86.
9. Higashiyama T, Inatsugi R, Sakamoto S, Sasaki N, Mori T, Kuroiwa H, et al. Species preferentiality of the pollen tube attractant derived from the synergid cell of *Torenia fournieri*. *Plant Physiol* 2006;**142**:481–91.
10. Higashiyama T, Kuroiwa H, Kawano S, Kuroiwa T. Guidance in vitro of the pollen tube to the naked embryo sac of *Torenia fournieri*. *Plant Cell* 1998;**10**:2019–31.

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11. Higashiyama T, Kuroiwa H, Kuroiwa T. Pollen-tube guidance: beacons from the female gametophyte. *Curr Opin Plant Biol* 2003;**6**:36–41.
12. Higashiyama T, Yabe S, Sasaki N, Nishimura Y, Miyagishima S, Kuroiwa H, et al. Pollen tube attraction by the synergid cell. *Science* 2001;**293**:1480–3.
13. Kanaoka MM, Kawano N, Matsubara Y, Susaki D, Okuda S, Sasaki N, et al. Identification and characterization of TcCRP1, a pollen tube attractant from *Torenia concolor*. *Ann Bot* 2011;**108**:739–47.
14. Kennedy TE, Serafini T, de la Torre JR, Tessier-Lavigne M. Netrins are diffusible chemotropic factors for commissural axons in the embryonic spinal cord. *Cell* 1994;**78**:425–35.
15. Kim S, Mollet J-C, Dong J, Zhang K, Park S-Y, Lord EM. Chemocyanin, a small basic protein from the lily stigma, induces pollen tube chemotropism. *Proc Natl Acad Sci USA* 2003;**100**:16,125–30.
16. Malho R, Trewavas AJ. Localized apical increases of cytosolic free calcium control pollen tube orientation. *Plant Cell* 1996;**8**:1935–49.
17. Márton ML, Cordts S, Broadhvest J, Dresselhaus T. Micropylar pollen tube guidance by EGG APPARATUS 1 of maize. *Science* 2005;**307**:573–6.
18. Mascarenhas JP, Machlis L. Chemotropic response of *Antirrhinum majus* pollen to calcium. *Nature* 1962;**196**:292–3.
19. Moutinho A, Hussey PJ, Trewavas AJ, Malhó R. cAMP acts as a second messenger in pollen tube growth and reorientation. *Proc Natl Acad Sci USA* 2001;**98**:10481–6.
20. Okuda S, Tsutsui H, Shiina K, Sprunck S, Takeuchi H, Yui R, et al. Defensin-like polypeptide LUREs are pollen tube attractants secreted from synergid cells. *Nature* 2009;**458**:357–61.
21. Takeuchi H, Higashiyama T. Attraction of tip-growing pollen tubes by the female gametophyte. *Curr Opin Plant Biol* 2011;**14**:614–21.
22. Van Tieghem P. Végétation libre du pollen et de l' ovule. *Ann Sci Nat Bot* 1869;**12**:312–28.

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Chapter 7

Phytosulfokine

Yoshikatsu Matsubayashi

ABSTRACT

Phytosulfokine (PSK) is a 5-amino-acid secreted peptide that contains two sulfated tyrosines. PSK was initially identified as a growth-promoting signal involved in the "density effect" in plant cell cultures. Addition of PSK to culture medium significantly improves the efficiency of cellular proliferation, even when the initial cell population is below the critical density. It was later shown that PSK also promotes *in vitro* tracheary element differentiation, somatic embryogenesis, and pollen germination. PSK is produced from approximately 80-amino-acid precursor peptides via post-translational sulfation and proteolytic processing. Genes encoding PSK precursors are widely expressed in a variety of tissues and upregulated by wounding. PSK is recognized by a membrane-localized leucine-rich repeat receptor kinase, PSKR1. Disruption of PSKR1 and its homologs in *Arabidopsis* causes growth defects such as short roots, smaller leaves, and early senescence. PSK signaling affects potential for growth and cellular longevity, thereby exerting a pleiotropic effect on individual cells in response to environmental conditions.

DISCOVERY AND STRUCTURE OF PSK

Almost all plant cells, even when fully differentiated, can dedifferentiate and proliferate *in vitro* to form a callus. Lines of evidence suggest that population of living cells are required to support callus formation and successive proliferation *in vitro*. This requirement is greater for more mature cells. Dilution of dispersed mature mesophyll cells in excess culture medium significantly prevents callus formation, even if auxins, cytokinins and nutrients are supplied.[15] A similar phenomenon has also been observed for transdifferentiation of mesophyll cells into tracheary elements (TEs).[16] If dispersed *Zinnia* mesophyll cells are cultured below a critical cell density, TE formation is greatly suppressed, indicating that cell-to-cell communication is also involved in transdifferentiation. Similarly, somatic embryo formation from a suspension of carrot cells is dependent on cell density.[7] Interestingly, this population dependence is lessened by the addition of conditioned medium in which cells have previously been grown, indicating that such cell-to-cell communication is mediated by secreted signal(s).[7,15,16]

Phytosulfokine (PSK), a 5-amino-acid-sulfated peptide is the primary signal molecule responsible for the above examples of cell-to-cell communication (Figure 1A).[4,7,15,16] PSK was first purified from conditioned medium derived from an asparagus (*Asparagus officinalis* L.) suspension culture, based on its ability to promote cell division of asparagus mesophyll cells cultured at low cell density.[15] PSK has been detected at nanomolar levels in culture media of various plant cell lines including asparagus,[15] rice,[17] maize,[17] *Zinnia*,[12] carrots,[4] and *Arabidopsis*.[25] This suggests that among higher plants, PSK is widely distributed with strict sequence conservation. Structure–activity analysis indicates that the N-terminal tripeptide fragment Tyr(SO_3H)–Ile–Tyr(SO_3H) is the active core of PSK.[10]

PSK is generated by tyrosine sulfation followed by enzymatic processing of approximately 80-amino-acid precursor peptides that have a secretion signal at its N-terminal (Figure 2).[11,24–26] In *Arabidopsis*, five paralogous genes encoding PSK precursors have been identified. Each predicted precursor peptides has a secretion signal at the N-terminus and a PSK sequence close to the C-terminus. However, these precursors exhibit extreme diversity, for example, only a small number of residues close to the C-terminus including PSK domain are conserved. Many amino acid substitutions are likely to be evolutionally allowed to accumulate in the outside region of the mature peptide domains because these regions are digested by proteolytic processing.

In vitro studies have suggested that proteolytic processing of PSK is mediated, at least in part, by a subtilisin-like serine protease, AtSBT1.1.[21] Its recognition site is, however, between Leu and His located several residues upstream of the mature PSK domain. This observation indicates that substrate specificity of plant subtilases is distinct from those of animal subtilisin/kexin-like prohormone convertases that exactly recognize the C-terminal side of paired basic amino acids. Plant subtilases have greater structural similarity with prokaryotic degrading-type subtilases than with animal processing-type subtilases, resulting from a lack of the P-domain characteristic of animal enzymes.[2] Proteolytic processing of plant peptide signals may involve several complex steps, such as initial cutting and further trimming of the peptides.

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00007-5
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BIOLOGICAL FUNCTION OF PSK *IN VITRO*

Biological function of PSK *in vitro* is pleiotropic. Addition of chemically synthesized PSK to culture medium significantly improves the efficiency of cellular proliferation (Figure 1B),[15,17] even when the initial cell population is below the critical density. PSK also stimulates *in vitro* tracheary element differentiation of *Zinnia* mesophyll cells (Figure 1C),[16] somatic embryogenesis (Figure 1D),[4,5,7] adventitious root formation[23] and pollen germination.[3] PSK also promotes production of taxol, a potent antitumor drug.[6] Microarray analysis of gene expression in mechanically dispersed *Zinnia* mesophyll cells under PSK treatment have shown that PSK downregulates stress-related genes such as chitinases, phenylpropanoid biosynthesis enzymes and 1-aminocyclopropane-1-carboxylic acid.[19] Supporting this, when PSK is applied to plant seedlings at high concentrations, it retards senescence under stress conditions.[22]

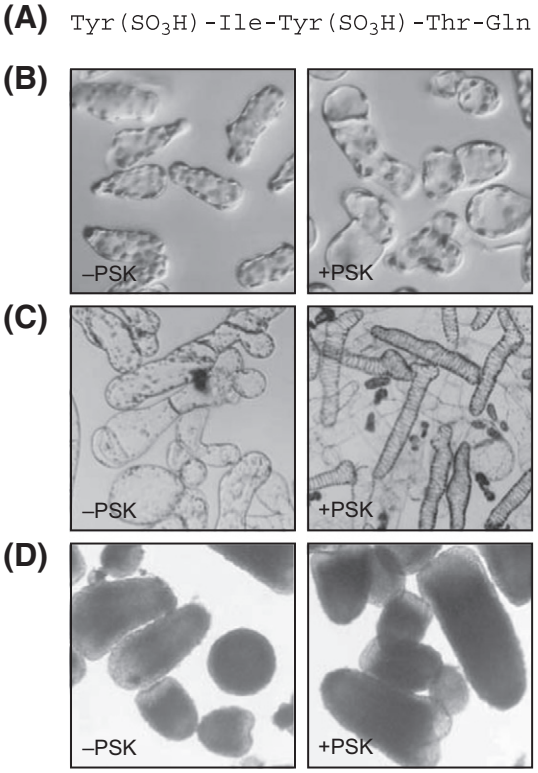

FIGURE 1 Structure and biological activities of PSK *in vitro*. (A) PSK is a 5-amino-acid peptide containing two sulfated tyrosines. (B–D) PSK promotes cell division of asparagus mesophyll cells (B), tracheary element differentiation of *Zinnia* mesophyll cells (C), and somatic embryogenesis of carrot suspension cells (D). PSK was added to each culture at final concentration of 10 nM.

EXPRESSION PATTERN AND *IN VIVO* FUNCTION OF PSK

PSK precursor genes are redundantly distributed in the genome (5 genes in *Arabidopsis*), and are found in a variety of angiosperm[9] and gymnosperm[5] plant species. In *Arabidopsis*, *AtPSKs* are expressed in a variety of tissues including roots, leaves, stems, flowers, siliques, and calluses.[11] Among the plant parts that are above the ground, *AtPSK2*, *AtPSK3*, *AtPSK4*, and *AtPSK5* were expressed in cotyledons and leaves and were most abundantly expressed in vascular bundles. In roots, *AtPSK3* was primarily expressed in root tips, whereas expression of *AtPSK2*, *AtPSK4*, and *AtPSK5* was mainly detected in the more mature regions of the root. Expression of *AtPSK4* and *AtPSK5* is highly upregulated on mechanical wounding.

PSK precursor genes are so small and redundant that the loss-of-function analysis is not easily applicable. Instead, knockout mutants of the genes for PSK receptors provide informative phenotypes (described below). Identification of tyrosylprotein sulfotransferase (TPST), an enzyme that catalyzes the tyrosine sulfation of the peptides, also provides a hint about *in planta* function of PSK.[8]

Arabidopsis TPST (AtTPST) is a Golgi-localized 62-kDa transmembrane protein.[8] AtTPST is expressed throughout the plant body, and the highest levels of expression are observed in the root apical meristem. A loss-of-function mutant of *AtTPST* (*tpst-1*) displays a semidwarf phenotype accompanied by stunted roots, loss of root stem cells, pale green leaves and early senescence.[8] Severe short root phenotype is characterized by considerable reduction in root meristem size and loss of coordination between cell elongation and expansion in the elongation-differentiation zone. Because TPST is an enzyme indispensable for biosynthesis of sulfated peptides, these phenotypes should reflect deficiencies in all the functional tyrosine-sulfated peptides. Interestingly, a novel sulfated peptide, root meristem growth factor (RGF), plays a central role in root meristem development, but RGF alone is not sufficient for full restoration of *tpst-1* root phenotype.[18] PSK is required

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PSK1   ------MKTKSEVLIFFFTLVLLLSMASSVILREDGFAPPKPSPTTHEKASTKGDRDGVECKNSDSEEEC-LVKKTVAA-HTDYIYTQDLNLSP   86
PSK2   MANVSALL-TIALLLCSTLMCT-ARPEPAISISITTAADPCNMEKKIEGKLDDMHMVDENC-GAD-DEDC-LMRRTLVA-HTDYIYTQKKKHP-   87
PSK3   --------MKQSLCLAVLFLILSTSSSAIRRGKEDQEINPLVSATSVEEDSVNKLMGMEYG-GEG-DEEC-LRRRMMTESHLDYIYTQHHKH--   81
PSK4   MGKFTTIF-IMALLLCSTLTYA-ARLTPTTTTALSR--EN-SV-KEIEG--DKVE--EESCNGIG-EEEC-LIRRSLVL-HTDYIYTQNHK-P-   79
PSK5   MVKFTTFLCIIALLLCSTLTHASARLNPTSV--YPE--EN-SF-KKLEQ--GEV-----ICEGVG-EEECFLIRRTLVA-HTDYIYTQNHN-P-   77
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FIGURE 2 Alignment of deduced amino acid sequences of PSK precursor proteins in *Arabidopsis*. The 5-amino-acid PSK domain is underlined. Identical amino acid residues are highlighted in black, and similar amino acid residues are highlighted in gray.

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for cell elongation in the elongation zone and enhances cell division in the meristematic zone.[18]

RECEPTOR FOR PSK AND *IN PLANTA* FUNCTION OF PSK

Specific binding sites for PSK were detected on the surface of suspension-cultured cells and in plasma-membrane-enriched fractions of various plants.[14,17] Scatchard analysis using [^{3}H]PSK has revealed the existence of a high-affinity PSK-binding site in microsomal fractions derived from carrot cells with a dissociation constant (K_d) of 4.2 nM.[12] This PSK receptor protein has also been visualized by photoaffinity labeling of carrot-plasma-membrane fractions using the photoactivatable ^{125}I-labeled PSK analog.[12,13]

The PSK-binding protein was purified from microsomal fractions of carrot (*Daucus carota* L.) cells by detergent solubilization and specific ligand-based affinity chromatography using a PSK-Sepharose column.[12] Molecular cloning of this protein revealed that PSK receptor (DcPSKR1) is a 1021 amino acid protein containing an N-terminal hydrophobic signal sequence, extracellular leucine-rich repeats (LRRs), a 36-amino-acid island domain, a transmembrane domain, and a cytoplasmic kinase domain. Photoaffinity labeling followed by fragmentation analysis revealed that the 15 amino acid region Glu^{503}–Lys^{517} located on the N-terminal side of the 36 amino acid island domain is the ligand binding domain of DcPSKR1.[20]

Arabidopsis PSK receptor (AtPSKR1) was further identified based on overall amino acid similarity to DcPSKR1.[11] AtPSKR1 was a 1008 amino acid LRR-RK that has 60% amino acid sequence identity to DcPSKR1 and contains 21 tandem copies of LRR, a 36-amino-acid island domain between the 17th and 18th LRRs, a single transmembrane domain, and a cytoplasmic kinase domain. In addition, amino acid sequences in the island domain are highly conserved between DcPSKR1 and AtPSKR1. PSK binds AtPSKR1 with a dissociation constant (K_d) of 7.7 nM, which is similar to the K_d of DcPSKR1.

Disruption of AtPSKR1 and its two homologs in *Arabidopsis* causes pleiotropic growth defects such as reduced root length, smaller leaves, and early senescence (Figure 3).[1,11] The short root phenotype is characterized by a significant decrease in cell size and a reduced meristem size, indicating that PSK regulates both meristem activity and cellular expansion (elongation). Adult plants of this triple mutant show a semidwarf phenotype with smaller leaves than that of wildtype plants, because of the decreases in cell number and cell size. At the bolting stage, mutant leaves exhibit premature senescence phenotypes. By contrast, seedlings overexpressing *AtPSKR1* develope larger leaves than the wildtype and exhibit delayed senescence. These results strongly suggest that PSK represents a novel class of hormones that affect the potential for cellular growth and longevity, thereby exerting a pleiotropic effect on individual cells in response to environmental conditions.

PSY1, A SULFATED GLYCOPEPTIDE FUNCTIONALLY SIMILAR TO PSK

PSY1 is an 18-amino-acid secreted glycopeptide containing one sulfated tyrosine residue and an L-arabinose sugar chain (Figure 4A). This glycopeptide was identified by exhaustive analysis of tyrosine-sulfated peptides in *Arabidopsis* cell culture media.[1] This peptide is derived from near the C-terminal region of the 75-amino-acid precursor peptide (Figure 4B). The *Arabidopsis* genome also contains two genes encoding PSY1 homologs with significant similarity within the mature peptide domain (Figure 4B). Interestingly, the physiological activity of PSY1 is closely similar to that of PSK. When PSY1 is directly added to the culture

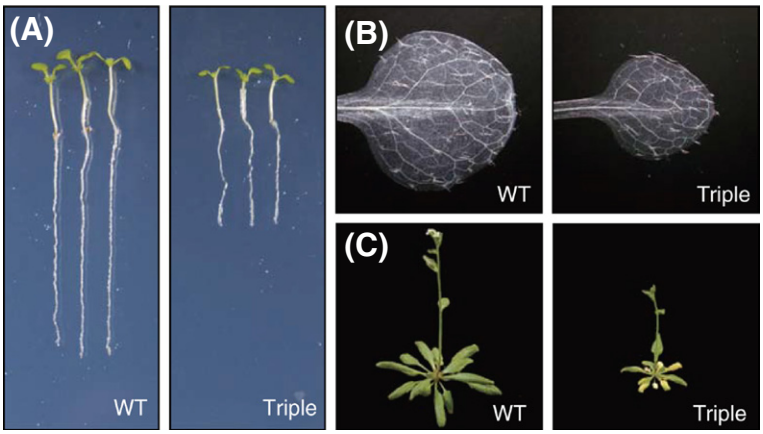

FIGURE 3 Phenotypes of the loss-of-function mutant of AtPSKR1 and its two homologs. (A) Photographs of WT and triple mutant seedlings grown on vertical agar plates for 10 days. (B) Photograph of the first true leaves of 2-week-old WT and triple mutant. (C) Photographs of WT and triple mutant 4 weeks after germination.

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(A) Asp-Tyr(SO3H)-Gly-Asp-Pro-Ser-Ala-Asn-Pro-Lys-His-Asp-Pro-Gly-Val-[(L-Ara)3]Hyp-Hyp-Ser

(B)

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PSY1        MTFVV--RL-L-VCLLTLTITSSLARNPVSVSGGFENSGFQRSLMVNVEDYGDPSANPKHDPGVPPSATGQRVVGRG    75
At3g47295   MSFGT--RL-L-LFLILTLPLVTSSSPNTLHVSG-IVKTGTTSRFLMMTIEDYDDPSANTRHDPSVPTNAKADTTP---  71
At2g29995   MGYSSSSRIGLCLFLFFTFALLSS-ARISLSFSEN-EMTVVPERSLMVSTNDYSDPTANGRHDP--PRGGRGRRR----  71
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FIGURE 4 Structure of PSY1 and alignment of PSY1 precursor homologs in *Arabidopsis*. (A) PSY1 is an 18-amino-acid glycopeptide containing one sulfated tyrosine. Identical amino acid residues are highlighted in black, and similar amino acid residues are highlighted in gray. Mature PSY1 domain is underlined.

medium of *Arabidopsis* suspension cells, it significantly promotes cellular proliferation in a dose-dependent manner. PSY1 also promotes proliferation of dispersed asparagus mesophyll cells at nanomolar concentrations. *PSY1* is widely expressed in various *Arabidopsis* tissues and is highly upregulated by wounding. Transgenic seedlings overexpressing PSY1 develop longer roots and larger cotyledons than wildtype do mainly because of an increase in cell size. These results indicate that PSY1 is involved in both cellular proliferation and expansion. Two structurally distinct sulfated peptides, PSY1 and PSK, act as physiologically similar growth-promoting signals.

REFERENCES

1. Amano Y, Tsubouchi H, Shinohara H, Ogawa M, Matsubayashi Y. Tyrosine-sulfated glycopeptide involved in cellular proliferation and expansion in *Arabidopsis*. *Proc Natl Acad Sci USA* 2007;**104**: 18,333–8.
2. Berger D, Altmann T. A subtilisin-like serine protease involved in the regulation of stomatal density and distribution in *Arabidopsis thaliana*. *Genes Dev* 2000;**14**:1119–31.
3. Chen YF, Matsubayashi Y, Sakagami Y. Peptide growth factor phytosulfokine-α contributes to the pollen population effect. *Planta* 2000;**211**:752–5.
4. Hanai H, Matsuno T, Yamamoto M, Matsubayashi Y, Kobayashi T, Kamada H, et al. A secreted peptide growth factor, phytosulfokine, acting as a stimulatory factor of carrot somatic embryo formation. *Plant Cell Physiol* 2000;**41**:27–32.
5. Igasaki T, Akashi N, Ujino-Ihara T, Matsubayashi Y, Sakagami Y, Shinohara K. Phytosulfokine stimulates somatic embryogenesis in *Cryptomeria japonica*. *Plant Cell Physiol* 2003;**44**:1412–6.
6. Kim BJ, Gibson DM, Shuler ML. Effect of the plant peptide regulator, phytosulfokine-α, on the growth and Taxol production from *Taxus* sp. suspension cultures. *Biotechnol Bioeng* 2006.
7. Kobayashi T, Eun C, Hanai H, Matsubayashi Y, Sakagami Y, Kamada H. Phytosulphokine-α, a peptidyl plant growth factor, stimulates somatic embryogenesis in carrot. *J Exp Bot* 1999;**50**:1123–8.
8. Komori R, Amano Y, Ogawa-Ohnishi M, Matsubayashi Y. Identification of tyrosylprotein sulfotransferase in *Arabidopsis*. *Proc Natl Acad Sci USA* 2009;**106**:15,067–72.
9. Lorbiecke R, Sauter M. Comparative analysis of PSK peptide growth factor precursor homologs. *Plant Sci* 2002;**163**:321–32.
10. Matsubayashi Y, Hanai H, Hara O, Sakagami Y. Active fragments and analogs of the plant growth factor, phytosulfokine: structure–activity relationships. *Biochem Biophys Res Commun* 1996;**225**: 209–14.
11. Matsubayashi Y, Ogawa M, Kihara H, Niwa M, Sakagami Y. Disruption and overexpression of *Arabidopsis* phytosulfokine receptor gene affects cellular longevity and potential for growth. *Plant Physiol* 2006;**142**:45–53.
12. Matsubayashi Y, Ogawa M, Morita A, Sakagami Y. An LRR receptor kinase involved in perception of a peptide plant hormone, phytosulfokine. *Science* 2002;**296**:1470–2.
13. Matsubayashi Y, Sakagami Y. 120- and 160-kDa receptors for endogenous mitogenic peptide, phytosulfokine-α, in rice plasma membranes. *J Biol Chem* 2000;**275**:15,520–5.
14. Matsubayashi Y, Sakagami Y. Characterization of specific binding sites for a mitogenic sulfated peptide, phytosulfokine-α, in the plasma-membrane fraction derived from *Oryza sativa* L. *Eur J Biochem* 1999;**262**:666–71.
15. Matsubayashi Y, Sakagami Y. Phytosulfokine, sulfated peptides that induce the proliferation of single mesophyll cells of *Asparagus officinalis* L. *Proc Natl Acad Sci USA* 1996;**93**:7623–7.
16. Matsubayashi Y, Takagi L, Omura N, Morita A, Sakagami Y. The endogenous sulfated pentapeptide phytosulfokine-α stimulates tracheary element differentiation of isolated mesophyll cells of zinnia. *Plant Physiol* 1999;**120**:1043–8.
17. Matsubayashi Y, Takagi L, Sakagami Y. Phytosulfokine-α, a sulfated pentapeptide, stimulates the proliferation of rice cells by means of specific high- and low-affinity binding sites. *Proc Natl Acad Sci USA* 1997;**94**:13,357–62.
18. Matsuzaki Y, Ogawa-Ohnishi M, Mori A, Matsubayashi Y. Secreted peptide signals required for maintenance of root stem cell niche in *Arabidopsis*. *Science* 2010;**329**:1065–7.
19. Motose H, Iwamoto K, Endo S, Demura T, Sakagami Y, Matsubayashi Y, et al. Involvement of phytosulfokine in the attenuation of stress response during the transdifferentiation of zinnia mesophyll cells into tracheary elements. *Plant Physiol* 2009;**150**:437–47.
20. Shinohara H, Ogawa M, Sakagami Y, Matsubayashi Y. Identification of ligand binding site of phytosulfokine receptor by on-column photoaffinity labeling. *J Biol Chem* 2006;**282**:124–31.
21. Srivastava R, Liu JX, Howell SH. Proteolytic processing of a precursor protein for a growth-promoting peptide by a subtilisin serine protease in *Arabidopsis*. *Plant J* 2008;**56**:219–27.
22. Yamakawa S, Matsubayashi Y, Sakagami Y, Kamada H, Satoh S. Promotive effects of the peptidyl plant growth factor, phytosulfokine-α, on the growth and chlorophyll content of *Arabidopsis* seedlings under high night-time temperature conditions. *Biosci Biotechnol Biochem* 1999;**63**:2240–3.
23. Yamakawa S, Sakurai C, Matsubayashi Y, Sakagami Y, Kamada H, Satoh S. The promotive effects of a peptidyl plant growth factor, phytosulfokine, on the formation of adventitious roots and expression of a gene for a root-specific cystatin in cucumber hypocotyls. *J Plant Res* 1998;**111**:453–8.

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24. Yang H, Matsubayashi Y, Hanai H, Nakamura K, Sakagami Y. Molecular cloning and characterization of *OsPSK*, a gene encoding a precursor for phytosulfokine-α, required for rice cell proliferation. *Plant Mol Biol* 2000;**44**:635–47.
25. Yang H, Matsubayashi Y, Nakamura K, Sakagami Y. Diversity of *Arabidopsis* genes encoding precursors for phytosulfokine, a peptide growth factor. *Plant Physiol* 2001;**127**:842–51.
26. Yang H, Matsubayashi Y, Nakamura K, Sakagami Y. *Oryza sativa PSK* gene encodes a precursor of phytosulfokine-α, a sulfated peptide growth factor found in plants. *Proc Natl Acad Sci USA* 1999;**96**:13,560–5.

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Chapter 8

POLARIS

Keith Lindsey, Saher Mehdi, Stuart A. Casson, Anna J. Mudge, Jennifer F. Topping and Junli Liu

ABSTRACT

The POLARIS (PLS) peptide was identified in the plant species *Arabidopsis thaliana* by promoter trapping. The gene encodes an mRNA of ca. 600 bases, at the 3′-end of which is a 36 amino acid open reading frame (ORF). Translation of the ORF is required for biological activity. The transcriptional start of the gene overlaps with the 3′-UTR of an upstream gene. Expression of the *POLARIS* gene is strongest in root tips, but is also detectable in young leaves, and is induced by auxin. Mutation of *POLARIS* leads to several developmental defects, including a short root phenotype and reduced vascular complexity in leaves. Recent results show a role for PLS in repressing ethylene responses, and in promoting ethylene-mediated auxin biosynthesis.

DISCOVERY

Arabidopsis thaliana has proved to be an enormously valuable model organism for the study of plant growth and development, not least because of its ease of transformation and small diploid genome, which has allowed the use of insertional mutagenesis for gene tagging and functional analysis. Several years ago we adopted a promoter trapping strategy to allow gene identification, based on the transcriptional activation of a promoterless *gusA* gene after random insertion into the genomes of plants, including *Arabidopsis*.

A major advantage of this approach over conventional mutagenic strategies is that it not only allows gene detection by the identification of mutant phenotypes, but also simultaneously can provide information on the expression pattern of the tagged gene. It also facilitates gene isolation because the insertion element provides a molecular tag on the genomic sequence in which the gene of interest is located. This can subsequently be cloned by one of a number of polymerase chain reaction (PCR)-based techniques.[9]

The promoter trap strategy we developed involved transforming populations of plants with a promoterless *gusA* gene engineered into a T-DNA vector, allowing agrobacterium-mediated gene transfer. This vector, designated as pΔGUSBin19,[24] is illustrated in Fig. 1. It is activated at high frequency in transgenic populations of several species, and GUS activity can be localized histochemically to allow the screening for insertion into genes that are expressed in tissue-specific patterns.[10]

An area of interest to us has been the identification and functional analysis of genes expressed in embryos and roots. The basal region of the embryo represents the cellular origin of the primary root.[4] We exploited the promoter trapping approach to screen transgenic *Arabidopsis* containing the vector pΔGUSBin19 for lines exhibiting GUS activity in embryos.[22] One line of interest, designated as AtEM101, showed a pattern of GUS activity that was polar in the developing embryo, and hence the tagged gene was named *POLARIS* (*PLS*). GUS activity is detectable from the early heart stage of embryogenesis, a stage at which apical–basal polarity is clearly visible in morphological terms, with cotyledonary primordia flanking the incipient shoot apical meristem at one end, and the developing root apical meristem adjacent to the suspensor at the other end, which is the region of attachment to the maternal tissue (Fig. 2). AtEM101 plants homozygous for the *PLS* promoter trap insertion event exhibited a short root phenotype.[2]

Using the promoter trap T-DNA as a molecular anchor point, we used RACE PCR to clone the *PLS* cDNA. Northern blot analysis using the *PLS* cDNA as a probe showed that wildtype (WT) seedlings accumulate a single low abundance transcript of ca. 500 nucleotides (Fig. 3A), and Southern analysis indicated a single gene copy per haploid genome.[2] Subsequent analysis of the *Arabidopsis* genome sequence has supported the view that *PLS* is a single copy gene.

STRUCTURE OF THE PRECURSOR mRNA/GENE

The genomic sequence at the *PLS* locus, which is on chromosome 4, reveals that the promoter trap T-DNA had inserted into a small ORF of 108bp, located in a 755bp region between two larger genes. One gene, which is not annotated and which we designated as *Gene X*, is 450bp upstream of the promoter trap T-DNA, encodes a predicted

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00008-7
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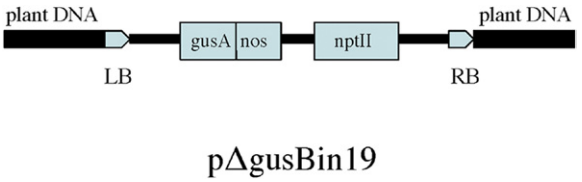

FIGURE 1 Diagrammatic representation of the promoter trap binary vector pΔGUSBin19, used to identify the *POLARIS* gene. At the T-DNA left border (LB) is a promoterless *gusA* gene, which can be activated after insertion into a transcription unit. At the T-DNA right border (RB) is a selectable marker, *npt-II*, conferring kanamycin resistance to transgenic plants. N = *nos* terminator.

92 amino acid peptide of unknown function. The second gene encodes the brassinosteroid receptor BRI1.[8] The identification of the ca. 500 nucleotide *PLS* transcript suggested that *PLS* encodes a distinct gene, and the ORF is not an exon of either of the flanking genes.

This was confirmed by both 5′-RACE PCR and RNAse protection assays, which showed that the transcription of the PLS ORF initiates within the 3′-untranslated region of *Gene X*. Specifically, we identified two transcript initiation sites, 95bp apart and one of which is used more frequently than the other (Fig. 3). The more distal start site (Start Site 1) occurs ca. 117bp upstream of the *Gene X* polyadenylation sequence, and has the sequence ATCCGTAT, with the underlined G representing the initiation site. The more proximal start site (Start Site 2), which is the more frequently used, is ca. 23bp upstream of the polyadenylation site of *Gene X*, with the sequence CCACTTAATA; it was not possible to resolve which of the three underlined bases is the start point for transcription.

Start Site 2 has a predicted TATA sequence (TATATAA) at positions −32 to −26, and Start Site 1 has a poorer TATA-like sequence at −35 to −29 (AATAATA). Sequence analysis of *PLS* 3′-RACE products revealed a variable length of polyadenylation, such that the length of the *PLS* transcript is between 427 and 606 nucleotides, depending upon which transcriptional start site is used and the length of the poly(A) tail.

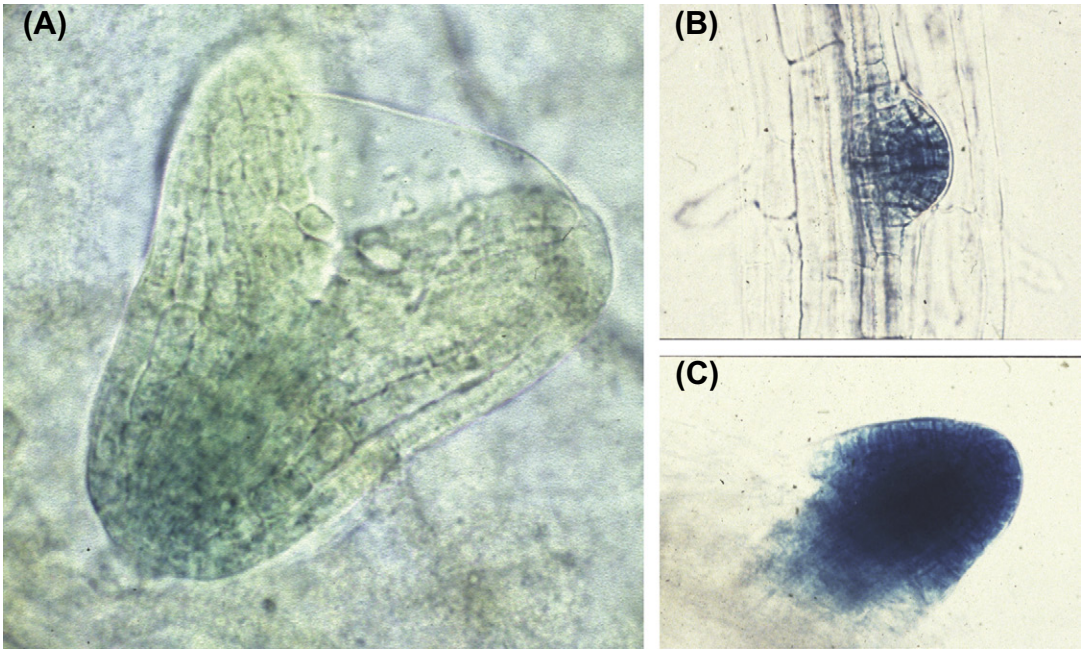

FIGURE 2 *POLARIS-GUS* expression in the basal region of a heart-stage embryo (A) and in the tips of emerging lateral roots of seedlings (B, C).

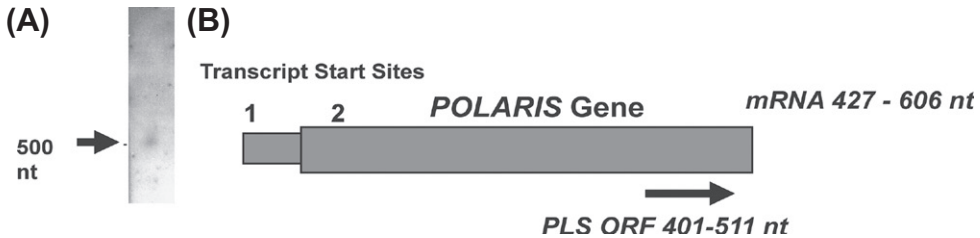

FIGURE 3 The *PLS* gene encodes a low abundance ca. 500 nt mRNA revealed in northern blots (A). Transcription initiated has been mapped by RNAse protection assays and 5′-RACE PCR to either of two start sites (1, 2), with the proximal site (2) used more frequently. The PLS ORF is located toward the 3′-end of the gene, at positions 401–511bp (including the translational stop codon) downstream of transcription Start Site 1.

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Sequences 5′ to the Start 2 transcription initiation site were cloned upstream of the *gusA* reporter gene, and transformed *Arabidopsis* plants were generated for functional analysis. A relatively short (−370bp) sequence was sufficient to drive GUS activity in the root tip of transgenics. A longer 5′-flanking sequence (−1190bp upstream of Start Site 2) promoted stronger GUS expression than the shorter sequence.[2] These results provided further support for transcription of the *PLS* gene initiating within the upstream *Gene X* sequence. While apparently unusual in eukaryotes, an overlapping organization has similarly been described for the *EhMCM3* and *EhPAK* genes of *Entamoeba histolytica*,[5] and for the *OTC* and *AUL1* genes of *Arabidopsis*.[17] Mouse and human examples have also been reported.[6,20]

The *PLS* gene was sequenced from two ecotypes of *Arabidopsis*, namely C24 (the original promoter trap background) and Columbia (Col-0), and was found to be identical. The PLS ORF, disrupted by the promoter trap T-DNA, encodes a 36 amino acid peptide of predicted 4.6kDa. The PLS ORF is located toward the 3′-end of the *PLS* transcript (Fig. 3B) and is preceded by an in-frame 27bp ORF (encoding a predicted 9 amino acid peptide) with a stop codon immediately preceding the PLS ATG, and an 8 amino acid peptide that overlaps with the PLS ORF. These upstream ORFs may play a role in regulating the *PLS* transcript stability and/or PLS translation, e.g. see Refs. 12 and 26.

Proof for a functional role for the PLS ORF comes from transgenic complementation experiments in which the short root phenotype of the mutant is complemented by transformation with a partial cDNA containing the WT PLS ORF sequence. Mutation of the PLS ORF ATG prevented complementation, showing that translation of the PLS peptide is required for biological activity.[2]

DISTRIBUTION OF THE mRNA

Northern blot, RT-PCR, and cloned promoter analyses have confirmed the distribution of *PLS* expression throughout different organs, as indicated by the histochemical localization of GUS activity in the original promoter trap line. The *PLS* mRNA is a very low abundance transcript, but is detectable most readily in the distal (apical) regions of roots, and also in young leaves as well as embryos. This is reflected also in promoter–GUS activity in transgenics. In the root, short (5min) histochemical staining times reveal GUS activity in the root columella initials and the lateral root cap, similar to the auxin-responsive DR5-GUS reporter.[18]

Indeed, *PLS* transcription is strongly auxin-inducible, as determined by promoter–GUS histochemistry, northern analysis, or competitor RT-PCR.[2] This induction, which occurs within 30min of auxin application, is only seen with functional auxins, and not with nonfunctional structural analogs, such as 2-naphthylacetic acid or 2,3-dichlorophenoxyacetic acid. The *PLS* promoter contains potential TGTCTC-like auxin response elements.[25] Further support for a dependence of *PLS* expression on correct auxin signaling comes from studies in the *gnom/emb30* mutant background. The GNOM protein is an ARF-GEF required for vesicle trafficking and the correct polar localization of PIN proteins, themselves components of the auxin efflux transport system.[21] In a severe *gn* genetic background, *PLS* expression in embryos and seedlings is depolarized, consistent with the depolarized auxin distribution.[23] The expression of *PLS* in young leaves is also consistent with their being a site of auxin biosynthesis and relatively high concentration, as for roots.[13,14] *PLS* expression is, therefore, a good marker of auxin responses and auxin localization.

PROCESSING

The N-terminal 24 amino acids of the PLS peptide are predicted to form two β-sheets, and the C-terminal 12 amino acids are predicted to form an α-helical domain. There is no obvious targeting sequence in the PLS sequence, suggesting that it is not secreted but remains cytosolic. There is a possibility that the N-terminal region may be processed, as the presence of three basic arginine residues at positions 10–12 may form a turn region and represent a cleavage site. The second β-sheet contains a repeated SIS sequence that, because of the proximity to the arginine residues, may represent a cAMP-/cGMP-dependent protein kinase phosphorylation site, although this has not been determined experimentally. The C-terminal α-helical domain contains the motif KLFKLFK, which has potential to act as a leucine zipper for protein–protein interactions.

BIOLOGICAL ACTIONS

Our effort to investigate the function of the PLS peptide has focused on an analysis of the *pls* mutant and transgenic overexpression phenotypes. Although *PLS* expression is detectable in the developing embryo, there is no obvious mutant phenotype at this stage of the life cycle. However, the first evidence of a role in development is seen at the seedling stage, with defects apparent in root architecture and leaf venation.[2]

Seedlings homozygous for the *pls* mutation show a reduced length of the primary root, to about 50% that of WT over the first 14 days post-germination (Fig. 4A). Cells of the root are shorter and wider (more isodiametric) than WT, and divide less frequently to account for the reduced root length. *pls* also produces fewer lateral roots.[3] Seedlings hemizygous for the *pls* mutation show a primary root length that is intermediate between WT and homozygous mutants, indicating that the mutation is semidominant.

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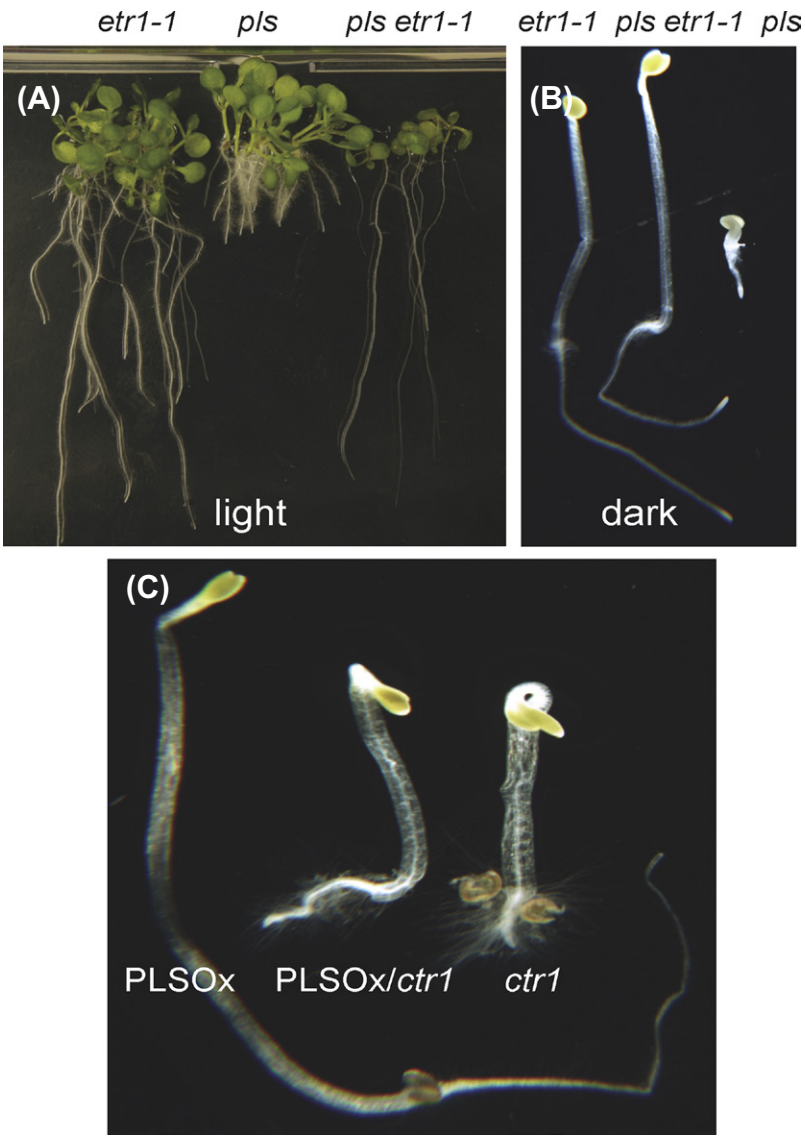

FIGURE 4 Phenotypes of seedlings of the *pls* mutant, the *etr1-1* mutant, and the *pls etr1-1* double mutant grown in the light (A) or in the dark (B). The PLS overexpressor (PLSOx) phenotype is repressed by the *ctr1* mutation (C), indicating that CTR1 functions downstream of PLS.

The overall stature of *pls* flowering plants is reduced compared to WT and more bushy, indicative of a reduced apical dominance. Microscopic analysis of leaves of the rosette at 12 days post-germination revealed a reduced venation compared with WT, and in particular a reduced number of higher order veins branching from the primary strands.[2]

Taken together, these results suggest a requirement for PLS in regulating cell shape and division in the root, vascular patterning in the leaf, and shoot architecture. One hypothesis we can consider is that PLS is required for correct auxin, cytokinin, or ethylene signaling, each of which is known to interact with the other hormone systems, and affect the development of the root and the shoot, including vascular patterning,[15] lateral root development,[1] and the control of apical dominance.[7]

To investigate auxin and cytokinin responses of the *pls* mutant, seedlings were grown *in vitro* in the presence of exogenous auxin or cytokinin over a range of concentrations. *pls* seedlings show reduced responses to auxins, seen as a reduced inhibition of primary root growth in the presence of exogenously supplied 2,4-D or 1-NAA. For example, in the presence of 10nm 1-NAA, WT primary roots are inhibited to 84% the length of untreated roots, whereas *pls* roots are 106% the length of untreated, i.e. showing a slight increase rather than inhibition in growth.[2] This suggests that *pls* seedlings may be defective in auxin transport, responses, or accumulation. Experimental analysis revealed a reduced

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rate of auxin transport in the *pls* mutant inflorescence stem to the root tip, which contains less free indoleacetic acid (IAA) than WT.[3] The expression of at least some auxin-regulated genes is correspondingly reduced in *pls*, shown as the reduced steady-state level of the *IAA1* gene transcript, supporting the view that *pls* has reduced auxin responses.

In the presence of exogenous cytokinins, *pls* primary roots exhibit an enhanced response compared with the WT. For example, in the presence of 10nm BA, the WT root is not significantly shorter than untreated. However, at the same concentration, the *pls* root growth is significantly inhibited to 71% the length of the untreated root.[2] Transgenic plants overexpressing the PLS transcript show reduced responses to cytokinins, and increased leaf vascularization, an auxin-regulated process, compared with the mutant. This antagonistic relationship between auxin and cytokinin responses in *pls* may reflect the antagonistic interplay of auxin and cytokinin biosynthesis[16] and suggests the requirement for PLS in regulating this interaction, either directly or indirectly.

Auxin and cytokinin also interact with ethylene signaling.[11] Evidence in support of a key role for PLS in modulating ethylene responses come from both mutant and transgenic overexpression analyses. The *pls* loss-of-function mutant exhibits a triple response phenotype (short and wide hypocotyl, exaggerated apical hook) when grown in the dark in air (Fig. 4B), suggestive of a defect in ethylene synthesis or response, but the mutant does not overproduce ethylene gas.[3] This implicates PLS as having a role in ethylene signal transduction. Consistent with this, the short root and triple response phenotypes of the mutant are rescued by the ethylene resistant *etr1* mutation; ETR1 is one of the ethylene receptor proteins.[19] Genetic analysis places PLS action upstream of CTR1, a negative regulator, or ethylene signal transduction (the PLS transgenic overexpression phenotype is blocked by the *ctr1* mutation; Fig. 4C), but downstream of the ethylene biosynthetic pathway. This suggests that PLS may interact directly with the ethylene receptor itself, a model that is supported by our unpublished experimental data but requires further work to confirm.

We have used mathematical modeling in combination with experimentation to analyze the role of PLS in hormone network interactions.[11] This work shows that the level of *PLS* expression influences ethylene, auxin, and cytokinin responses simultaneously. One prediction from the modeling was that the *pls* mutant would accumulate increased levels of biologically active cytokinins and a second prediction was that PLS is required for ethylene-mediated auxin biosynthesis in the root tip. Both predictions have been confirmed.[11]

In summary, the POLARIS peptide represents a new component required for cross-talk between more conventional plant signaling pathways, and modulation of its expression plays a significant role in the modulation of growth. Evidence suggests that the PLS peptide interacts with more than one protein partner—one may be the ethylene receptor complex, while a second appears to be an as yet unknown but distinct protein required for ethylene-mediated auxin biosynthesis.

REFERENCES

1. Casimiro I, Beeckman T, Graham N, Bhalerao R, Zhang H, Casero PJ, et al. Dissecting *Arabidopsis* lateral root development. *Trends Plant Sci* 2003;**8**:165–71.
2. Casson SA, Chilley PM, Topping JF, Evans IM, Souter MA, Lindsey K. The *POLARIS* gene of *Arabidopsis* encodes a predicted peptide required for correct root growth and leaf vascular patterning. *Plant Cell* 2002;**14**:1705–21.
3. Chilley PM, Casson SA, Tarkowski P, Wang KLC, Hawkins N, Hussey PJ, et al. The POLARIS peptide of *Arabidopsis* regulates auxin transport and root growth via effects on ethylene signaling. *Plant Cell* 2006;**18**:3058–72.
4. Dolan L, Janmaat K, Willemsen V, Linstead P, Poethig S, Roberts K, et al. Cellular organisation of the *Arabidopsis thaliana* root. *Development* 1993;**119**:71–84.
5. Gangopadhyay SS, Ray SS, Sinha P, Lohia A. Unusual genome organisation in *Entamoeba histolytica* leads to two overlapping transcripts. *Mol Biochem Parasitol* 1997;**89**:73–83.
6. Koskimies P, Spiess A-N, Lahti P, Huhtaniemi I, Ivell R. The mouse relaxin-like factor gene and its promoter are located within the 3′ region of the JAK3 genomic sequence. *FEBS Lett* 1997;**419**:186–90.
7. Leyser O. Regulation of shoot branching by auxin. *Trends Plant Sci* 2003;**8**:541–5.
8. Li J, Chory J. A putative leucine-rich repeat receptor kinase involved in brassinosteroid signal transduction. *Cell* 1997;**90**:929–38.
9. Lindsey K, Topping JF, Foster GD. Insertional mutagenesis by T-DNA. In: Twell D, editor. *Plant gene isolation*. Chichester: John Wiley & Sons; 1996. p. 275–305.
10. Lindsey K, Wei W, Clarke MC, McArdle HF, Rooke LM, Topping JF. Tagging genomic sequences that direct transgene expression by activation of a promoter trap in plants. *Transgenic Res* 1993;**2**:33–47.
11. Liu JL, Mehdi S, Topping J, Tarkowski P, Lindsey K. Modelling and experimental analysis of hormonal crosstalk in *Arabidopsis*. *Mol Syst Biol* 2010;**6**:373.
12. Linz B, Koloteva N, Vasilescu S, McCarthy JEG. Disruption of ribosomal scanning on the 5′-untranslated region, and not restriction of translational initiation *per se*, modulates the stability of nonaberrant mRNAs in the yeast *Saccharomyces cerevisiae*. *J Biol Chem* 1997;**272**:9131–40.
13. Ljung K, Bhalerao RP, Sandberg G. Sites and homeostatic control of auxin biosynthesis in *Arabidopsis* during vegetative development. *Plant J* 2001;**28**:465–74.
14. Ljung K, Hull AK, Celenza J, Yamada M, Estelle M, Normanly J, et al. Sites and regulation of auxin biosynthesis in *Arabidopsis* roots. *Plant Cell* 2005;**17**:1090–104.
15. Mattsson J, Sung ZR, Berleth T. Responses of plant vascular systems to auxin transport inhibition. *Development* 1999;**126**:2979–91.
16. Nordström A, Tarkowski P, Tarkowska D, Norbaek R, Astot C, Dolezal K, et al. Auxin regulation of cytokinin biosynthesis in *Arabidopsis thaliana*: a factor of potential importance for auxin–cytokinin-regulated development. *Proc Natl Acad Sci USA* 2004;**101**:8039–44.

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17. Quesada V, Ponce MR, Micol JL. OTC and AUL1, two convergent and overlapping genes in the nuclear genome of *Arabidopsis thaliana*. *FEBS Lett* 1999;**461**:101–6.
18. Sabatini S, Beis D, Wolkenfelt H, Murfett J, Guilfoyle T, Malamy J, et al. An auxin-dependent distal organizer of pattern and polarity in the *Arabidopsis* root. *Cell* 1999;**99**:463–72.
19. Schaller GE, Bleecker AB. Ethylene binding sites generated in yeast expressing the *Arabidopsis ETR1* gene. *Science* 1995;**270**:1809–11.
20. Speek M, Barry F, Miller WL. Alternate promoters and alternate splicing of human tenascin-X, a gene with 5′ and 3′ ends buried in other genes. *Hum Mol Gen* 1996;**5**:1749–59.
21. Steinmann T, Geldner N, Grebe M, Mangold S, Jackson CL, Paris S, et al. Coordinated polar localization of auxin efflux carrier PIN1 by GNOM ARF GEF. *Science* 1999;**286**:316–8.
22. Topping JF, Agyeman F, Henricot B, Lindsey K. Identification of molecular markers of embryogenesis in *Arabidopsis thaliana* by promoter trapping. *Plant J* 1994;**5**:895–903.
23. Topping JF, Lindsey K. Promoter trap markers differentiate structural and positional components of polar development in *Arabidopsis*. *Plant Cell* 1997;**9**:1713–25.
24. Topping JF, Wei W, Lindsey K. Functional tagging of regulatory elements in the plant genome. *Development* 1991;**112**:1009–19.
25. Ulmasov T, Murfett J, Hagen G, Guilfoyle TJ. Aux/IAA proteins repress expression of reporter genes containing natural and highly active synthetic auxin response elements. *Plant Cell* 1997;**9**:1963–71.
26. Wang L, Wessler SR. Inefficient reinitiation is responsible for upstream open reading frame-mediated translational repression of the maize *R* gene. *Plant Cell* 1998;**10**:1733–45.

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Chapter 9

RALF Peptides

Juliano R. Guerreiro, Gregory Pearce, Marcio C. Silva-Filho and Daniel S. Moura

ABSTRACT

RALF is a 5kDa peptide signal ubiquitous in the plant kingdom that has been associated with cell elongation. RALF precursors are found as single genes or members of multigene families. When added to the extracellular medium of suspension cells, RALF causes a pH increase followed by the activation of an MAP kinase. Plants overexpressing AtRALF1, one of the 34 isoforms present in *Arabidopsis*, have a semidwarf phenotype. Another RALF from *Arabidopsis*, AtRALF23, is down regulated by brassinolide and when overexpressed produces a shorter and bushier plant. *Nicotiana attenuata*, as opposed to *Arabidopsis*, has only one RALF that, when silenced, produces plants with longer roots. RALF is secreted and two membrane proteins from tomato cell suspensions were reported to bind specifically a ^{125}I-azido-labeled tomato RALF. AtRALF1 has also been found to induce a fast increase in cytoplasm Ca^{2+}, as a result of Ca^{2+} being mobilized from extracellular and intracellular stores.

DISCOVERY

The 49 amino acid peptide called RALF was discovered in leaf extracts of tobacco plants during a search for peptides with activity in the alkalinization assay.[19] The name RALF, short for Rapid Alkalinization Factor, is derived from the fast alkalinization response that the peptide elicits in the assay when compared with other plant bioactive peptides, such as systemin. When added to the media of suspension-cultured cells, RALF causes an alkalinization of the cell medium of approximately one pH unit, a characteristic response of other plant peptide hormones.[14,18,20] Crude protein extracts from tobacco leaves obtained in acidic conditions are rich in bioactive peptides.[19] As peptides from the extracts elute from HPLC columns, small aliquots from each fraction (1–10μL) are added to 1mL of suspension-cultured cells that are growing at a pH near 5. The fractions containing bioactive peptides are identified by the increase in pH of the cell medium that takes place within a few minutes after their addition to the cell cultures.[18,19] A peptide identified in the assay that caused an exceptionally rapid alkalinization response was called RALF and was isolated and characterized.[19] Homologous RALF peptides from tomato and alfalfa leaf extracts have been similarly identified and purified. The peptides have four cysteine residues that form two disulfide bridges, no other posttranslational modification has been found in the peptides and all have equivalent activities when assayed with tomato or tobacco cells. Chemically synthesized tomato RALF is as active as the native tomato RALF in the alkalinization assay.[19] RALF peptides from the model forest tree poplar and from the monocot sugarcane have also been biochemically isolated and proved to elicit similar responses in the alkalinization assay.[9,15]

STRUCTURE OF RALF

The tobacco *RALF* gene encodes a preproRALF precursor protein of 115 amino acids with the RALF sequence of 49 amino acids harbored within the C-terminal region of the protein.[19] This precursor contains six cysteines, with four in the active peptide region as disulfide bonds at Cys84–Cys94 and Cys107–Cys113 essential for its activity (Fig. 1A). The N-terminus contains a hydrophobic signal peptide typical of secreted proteins. A partial *Nicotiana benthamiana* RALF fused to the green fluorescent protein (GFP) confirms the extracellular location illuminating the ERECTA (ER) and the apoplast.[6] The primary structure is well conserved among different RALF isoforms.[19]

RALF precursor has also a dibasic site (Arg–Arg) just upstream of the active peptide, which is characterized by recognition sites for processing. *Arabidopsis* plants overexpressing the *AtRALF1* gene with a mutation at the second Arg of the dibasic site (R69A) do not show the characteristic semidwarf phenotype of AtRALF1 overexpressors.[12] Plants harboring the mutated *AtRALF1* gene show normal root and leaf growth with accumulation of the mutated proprotein and nearly undetectable levels of the processed peptide.[12] Similar results are obtained when the dibasic site of the AtRALF23 isoform is mutated. A subtilisin-like serine protease AtS1P is responsible for the AtRALF23 processing and release of the active peptide.[24]

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00009-9
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(A)

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MGVPSGLILCVLIGAFFISMAAAGDSGAYDWVMPARSGGGCKGSIGECIAEEEEFELDS  59
  **
ESRRILATKKYISYGALQKNSVPCSRRGASYYNCKPGAQANPYSRGCSAITRCRS     114
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(B)

PEPTIDES	PRIMARY STRUCTURE	ACTIVITY
SlRALF	ATTKYISYGALQKNSVPCSRRGASYYNCKPGAQANPYTRGCSAITRCRS	+++++
SlRALF (1-16)	ATTKYISYGALQKNSV	-
SlRALF(17-49)	PCSRRGASYYNCKPGAQANPYTRGCSAITRCRS	-
SlRALF(13-49)	KNSVPCSRRGASYYNCKPGAQANPYTRGCSAITRCRS	-
SlRALF (9-49)	GALQKNSVPCSRRGASYYNCKPGAQANPYTRGCSAITRCRS	-
SlRALF (5-49)	YISYGALQKNSVPCSRRGASYYNCKPGAQANPYTRGCSAITRCRS	+++++
ΔL^{11}SlRALF(10-49)	A-QKNSVPCSRRGASYYNCKPGAQANPYTRGCSAITRCRS	-
ΔL^{11}SlRALF (5-49)	YISYGA-QKNSVPCSRRGASYYNCKPGAQANPYTRGCSAITRCRS	+
ΔL^{11}SlRALF (1-49)	ATTKYISYGA-QKNSVPCSRRGASYYNCKPGAQANPYTRGCSAITRCRS	++
A^{5}SlRALF(5-49)	**A**ISYGALQKNSVPCSRRGASYYNCKPGAQANPYTRGCSAITRCRS	+++++
A^{6}SlRALF(6-49)	Y**A**SYGALQKNSVPCSRRGASYYNCKPGAQANPYTRGCSAITRCRS	+
A^{7}SlRALF(7-49)	YI**A**YGALQKNSVPCSRRGASYYNCKPGAQANPYTRGCSAITRCRS	+++++
A^{8}SlRALF(8-49)	YIS**A**GALQKNSVPCSRRGASYYNCKPGAQANPYTRGCSAITRCRS	+++++

FIGURE 1 RALF amino acid sequences. (A) Tobacco preproRALF. Signal peptide (underlined) and active peptide (double underlined) are indicated. Asterisks are above the two Arg implicated in processing and connected arrows point the Cys involved in disulfide bonds. (B) Amino acid sequences of tomato RALF-derived peptides used to determine activity in the alkalinization assay. Full alkalinization activity (+++++) was measured after 15min of the addition of 250nM of each peptide.[21]

The molecular weight of RALF is larger than defense-related peptides, such as systemins, and this only compounds the difficulty of identifying critical amino acids for their physiological effects. A structure–activity study revealed that the carboxy-terminal 33 amino acids, RALF(17–49), and the amino-terminal 16 amino acids, RALF(1–16), whether added alone or together exhibit no activity.[21] Sequential additions of four amino acid segments to the RALF(17–49) show that the activity is only recovered when the conserved motif YISY is added to the peptide. Sequential addition of four and eight amino acids, RALF(13–49) and RALF(9–49) respectively, does not restore the alkalinizing activity. Ala substitutions in the YISY motif further show that a substitution of the Ile at position six abolishes the activity. A deletion of the Leu at position 11, ΔL^{11}RALF(5–49), also causes a severe reduction in activity, showing that the amino and the carboxy terminus of RALF must be in the proper spatial arrangement (Fig. 1B).[2,21]

DISTRIBUTION OF THE mRNA/PEPTIDE

RALF, along with other peptide hormones, such as phytosulfokine and CLAVATA3, has highly conserved homologs throughout the plant kingdom.[3,19,26] RALF genes have now been identified and isolated in different tissues and organs from several species including poplar leaves, *Arabidopsis* pollen, *Solanum chacoense* ovule and ovary, *Primula vulgaris* pollen, *Nicotiana attenuata* plant, *Medicago truncatula* roots, soybean pollen, tomato pollen, broccoli flowers, and *Brassica campestris* flower buds.[1,4,5,7–9,11,13,25,27] RALF genes are found as a single copy, as is the case for *N. attenuata*, or as multigene families. *Arabidopsis* has a large family of 34 preproRALF homologs (AtRALFs) with C-terminal coding regions of various degrees of sequence identity with tobacco leaf preproRALF.[17,25] A subgroup of nine RALF homologs within this multigene family shows the highest primary structure identity to the original RALF isolated from tobacco leaves. *In silico* expression data of these nine RALF homologs show tissue specificity for some homologs, AtRALF1 and AtRALF4 for instance, and an almost ubiquitous expression for others, like AtRALF22, AtRALF23, and AtRALF33 (Fig. 2).[19]

In ovarian tissues of *S. chacoense*, five preproRALF cDNAs are present and expressed during fruit maturation.[7] Besides reproductive tissues, the five genes are differentially expressed in other parts of the plants, including roots, stems, and leaves. RALF peptides were purified from hybrid poplar leaves and two unique cDNA clones that code for RALF genes were identified in a poplar cDNA library.[9] Both cDNAs are differentially expressed in tissues of poplar saplings. Several RALF cDNAs have been isolated from pollen. A tomato RALF found in pollen (SlPRALF) has been implicated in the regulation of pollen tube elongation.[5] In *P. vulgaris*, a RALF-like cDNA was identified and its expression suggests an involvement in pollen maturation.[13]

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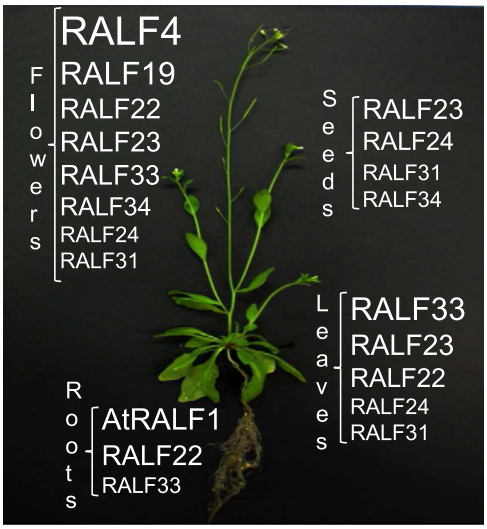

FIGURE 2 Tissue-specific expression of nine RALFs from *Arabidopsis* (AtRALFs) with high sequence identity to tobacco RALF. Font sizes used for AtRALFs indicate the level of expression of each isoform. Expression levels were obtained from eFP Browser at the Bio-Array Resource for Plant Biology.

A soybean pollen transcriptome survey identified a RALF cDNA as one of the top 30 most highly upregulated transcripts.[8]

Two RALF peptides were purified from the monocot sugarcane and gene expression analysis of four RALF genes (*SacRALFs*) showed low or almost undetectable gene expression levels in expanded leaves.[15] In expanding tissues, such as the base of half-emerged leaf blades, SacRALF transcripts are at high levels. The same transcripts are found at low levels at the apical portion, where cell expansion has stopped.[15] RALF gene expression has been associated with nodule formation in legumes and expression analysis of a RALF-like gene from *M. truncatula* shows upregulation upon treatment with nodulation factors.[4,23]

BIOLOGICAL FUNCTIONS

When added to the extracellular medium of suspension cells, RALF peptide causes an increase in the pH followed by the activation of an MAP kinase.[19] RALF also inhibits root growth and development of *Arabidopsis* and tomato seedlings when exogenously applied.[19] RALF inhibition of root growth is not a consequence of injury in the cells or a change in its morphology (Fig. 3). Gene expression profiles of RALF genes from several species also suggest a developmental role.[5,7,9,13,17] RALF peptide regulates the extracellular pH at the root hair tip during root hair development and also mobilizes extracellular and intracellular Ca^{+2}.[10,25] The pollen-specific RALF from tomato does not affect pollen viability but inhibits pollen tube elongation in a specific developmental window.[5,27] RALF peptides isolated from sugarcane (*Saccharum* spp.) leaves when added to culture media inhibit growth of microcalli derived from cell suspension cultures. Microcalli exposed to exogenous SacRALF also show a reduced number of elongated cells. Two RALF isoforms from *Arabidopsis*, AtRALF1 and ATRALF23, when overexpressed, produce semidwarf plants.[12,16] AtRALF23 gene is down regulated in response to treatment with brassinolide (BL) and it appears that AtRALF23 counteracts BL growth-promoting effects, since plants overexpressing AtRALF23 show a diminished response to BL treatments.[24] A clear picture of the function of the RALF peptide in plants is still lacking. However, all evidence points toward a role in plant development, more specifically cell elongation.[5,15,25]

RECEPTORS AND SIGNALING CASCADES

Two membrane proteins of 25 and 120kDa were identified on the external surface of suspension-cultured tomato cells by use of a photoaffinity analog of RALF.[22] Kinetic analyses indicate that the binding of RALF involves high affinity sites that are saturated at 2nM RALF. Unlabeled RALF strongly competes with labeling with the fully active

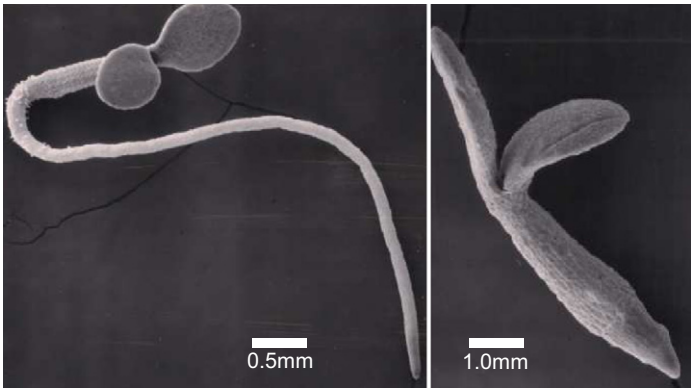

FIGURE 3 Scanning electron microscopy images of *Arabidopsis* seedlings treated with water (left panel) or with 10μM RALF (right panel).

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photoaffinity analog of RALF at a concentration as low as 2nM, and is completely blocked at 200nM, while two biologically inactive RALF analogs do not compete.[22] Suramin, an inhibitor of peptide ligand receptor interactions in animals and plants, is also a potent competitor of labeling. The properties of the two labeled proteins suggest that both are integral membrane components of the RALF receptor. While RALF–receptor interactions result in the alkalinization response and the activation of an MAP kinase, the mechanism of signal transduction remains obscure. A screening for cell signaling peptides able to induce changes in cytoplasmic Ca^{2+} of *Arabidopsis* seedlings identified AtRALF1 peptide.[10] AtRALF1 induces an increase in Ca^{2+} levels in approximately 40s and is able to reach its maximum at nanomolar concentrations. The increase in cytoplasmic Ca^{2+} was inhibited by pretreating seedlings with EGTA or inhibitors of IP_3-dependent signaling, LiCl and neomycin, indicating that Ca^{2+} is mobilized from the extracellular and intracellular compartments.

REFERENCES

1. Becker JD, Boavida LC, Carneiro J, Haury M, Feijó JA. Transcriptional profiling of *Arabidopsis* tissues reveals the unique characteristics of the pollen transcriptome. *Plant Physiol* 2003;**133**:713–25.
2. Bedinger PA, Pearce G, Covey PA. RALFs: peptide regulators of plant growth. *Plant Signal Behav* 2010;**5**:1342–6.
3. Cock JM, McCormick S. A large family of genes that share homology with CLAVATA3. *Plant Physiol* 2001;**126**:939–42.
4. Combier JP, Küster H, Journet EP, Hohnjec N, Gamas P, Niebel A. Evidence for the involvement in nodulation of the two small putative regulatory peptide-encoding genes MtRALFL1 and MtDVL1. *Mol Plant Microbe Interact* 2008;**21**:1118–27.
5. Covey PA, Subbaiah CC, Parsons RL, Pearce G, Lay FT, Anderson MA, et al. A pollen-specific RALF from tomato that regulates pollen tube elongation. *Plant Physiol* 2010;**153**:703–15.
6. Escobar NM, Haupt S, Thow G, Boevink P, Chapman S, Oparka K. High throughput viral expression of cDNA–green fluorescent protein fusions reveals novel subcellular addresses and identifies unique proteins that interact with plasmodesmata. *Plant Cell* 2003;**15**:1507–10.
7. Germain H, Chevalier E, Caron S, Matton DP. Characterization of five RALF like genes from *Solanum chacoense* provides support for a developmental role in plants. *Planta* 2005;**220**:447–54.
8. Haerizadeh F, Wong CE, Bhalla PL, Gresshoff PM, Singh MB. Genomic expression profiling of mature soybean (*Glycine max*) pollen. *BMC Plant Biol* 2009;**9**:25.
9. Haruta M, Constabel CP. Rapid alkalinization factors in poplar cell cultures. Peptide isolation, cDNA cloning, and differential expression in leaves and methyl jasmonate-treated cells. *Plant Physiol* 2003;**131**:814–23.
10. Haruta M, Monshausen G, Gilroy S, Sussman MR. A cytoplasmic Ca^{2+} functional assay for identifying and purifying endogenous cell signaling peptides in *Arabidopsis* seedlings: identification of AtRALF1 peptide. *Biochemistry* 2008;**47**:6311–21.
11. Li Y, Nie C, Cao J. Isolation and characterization of a novel BcMF14 gene from *Brassica campestris ssp. chinensis*. *Mol Biol Rep* 2011;**38**:1821–9.
12. Matos JL, Fiori CS, Silva-Filho MC, Moura DS. A conserved dibasic site is essential for correct processing of the peptide hormone AtRALF1 in *Arabidopsis thaliana*. *FEBS Lett* 2008;**582**:3343–7.
13. McCubbin AG, Lee C, Hetrick A. Identification of genes showing differential expression between morphs in developing flowers of *Primula vulgaris*. *Sex Plant Reprod* 2006;**19**:63–72.
14. Meindl T, Boller T, Felix G. The plant wound hormone systemin binds with the N-terminal part to its receptor but needs the C-terminal part to activate it. *Plant Cell* 1998;**10**:1561–70.
15. Mingossi FB, Matos JL, Rizzato AP, Medeiros AH, Falco MC, Silva-Filho MC, et al. SacRALF1, a peptide signal from the grass sugarcane (*Saccharum* spp.), is potentially involved in the regulation of tissue expansion. *Plant Mol Biol* 2010;**73**:271–81.
16. Nemhauser JL, Chory J. Bring it on: new insights into the mechanism of brassinosteroid action. *J Exp Bot* 2004;**55**:265–70.
17. Olsen AN, Mundy J, Skriver K. Peptomics, identification of novel cationic *Arabidopsis* peptides with conserved sequence motifs. *In Silico Biol* 2002;**2**:441–51.
18. Pearce G, Moura DS, Stratmann J, Ryan CA. Production of multiple plant hormones from a single polyprotein precursor. *Nature* 2001;**411**:817–20.
19. Pearce G, Moura DS, Stratmann J, Ryan CA. RALF, a 5-kDa ubiquitous polypeptide in plants, arrests root growth and development. *Proc Natl Acad Sci USA* 2001;**98**:12843–7.
20. Pearce G, Ryan CA. Systemic signaling in tomato plants for defense against herbivores. Isolation and characterization of three novel defense-signaling glycopeptide hormones coded in a single precursor gene. *J Biol Chem* 2003;**278**:30044–50.
21. Pearce G, Yamaguchi Y, Munske G, Ryan CA. Structure–activity studies of RALF, rapid alkalinization factor, reveal an essential–YISY–motif. *Peptides* 2010;**31**:1973–7.
22. Scheer JM, Pearce G, Ryan CA. *Le*RALF, a plant peptide that regulates root growth and development, specifically binds to 25 and 120 kDa cell surface membrane proteins of *Lycopersicon peruvianum*. *Planta* 2005;**221**:667–74.
23. Silverstein KA, Graham MA, VandenBosch KA. Novel paralogous gene families with potential function in legume nodules and seeds. *Curr Opin Plant Biol* 2006;**9**:142–6.
24. Srivastava R, Liu J, Guo H, Yin Y, Howell SH. Regulation and processing of a plant peptide hormone, AtRALF23, in *Arabidopsis*. *Plant J* 2009;**59**:930–9.
25. Wu J, Kurten EL, Monshausen G, Hummel GM, Gilroy S, Baldwin IT. NaRALF, a peptide signal essential for the regulation of root hair tip apoplastic pH in *Nicotiana attenuata*, is required of root hair development and plant growth in native soils. *Plant J* 2007;**52**:877–90.
26. Yang H, Matsubayashi Y, Hanai H, Sakagami Y. Phytosulfokine-α, a peptide growth factor found in higher plants: its structure, functions, precursor and receptors. *Plant Cell Physiol* 2000; **41**:825–30.
27. Zhang GY, Wu J, Wang XW. Cloning and expression analysis of a pollen preferential rapid alkalinization factor gene, BoRALF1, from broccoli flowers. *Mol Biol Rep* 2010;**37**:3273–81.

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Chapter 10

Root Meristem Growth Factor

Yoshikatsu Matsubayashi

ABSTRACT

Root meristem growth factor (RGF) is a 13-amino acid tyrosine-sulfated peptide involved in the maintenance of the root stem cell niche and regulation of meristematic activity in *Arabidopsis*. RGF1 was identified in a search for sulfated peptides that recover root meristem defects of the loss-of-function mutant of tyrosylprotein sulfotransferase. This approach is based on the assumption that phenotypes of loss-of-function mutants of posttranslational modification enzymes reflect deficiencies in the biosynthesis of all the functionally modified peptides. RGFs are produced from ~100-amino acid precursor peptides via posttranslational sulfation and proteolytic processing. Peptides of the RGF family are expressed mainly in the stem cell area and the innermost layer of central columella cells, and diffuse into the meristematic region. RGF peptides regulate root development by regulating expression levels and patterns of PLETHORA transcription factors, which are essential for the maintenance of the root stem cell niche and transit-amplifying cell proliferation.

DISCOVERY OF RGF

Secreted peptide hormones in plants often undergo complex posttranslational modifications that are mediated by specific enzymes, which recognize particular sequences of multiple target peptides. Because such modifications are generally critical for the functions of individual peptide hormones, the presence of novel peptide hormones should be revealed through phenotypic analysis of the mutants of posttranslational modification enzymes.

Tyrosine sulfation is a posttranslational modification that has been found in peptide hormones in both animals and plants.[9] This modification is mediated by tyrosylprotein sulfotransferase (TPST), which catalyzes the transfer of sulfate to the phenolic group of tyrosine. *Arabidopsis* TPST (AtTPST) is a type I transmembrane protein localized in *cis*-Golgi.[5] The loss-of-function mutant of *AtTPST* (*tpst-1*) shows stunted root phenotype accompanied by loss of maintenance of stem cells and a considerable decrease in meristematic activity (Fig. 1). Because *AtTPST* is a single copy gene, phenotypes of *tpst-1* mutant should reflect the deficiency in the biosynthesis of all the functional tyrosine-sulfated peptides found in *Arabidopsis*. Interestingly, two sulfated peptide hormones, PSK[7] and PSY1,[2] promoted cell elongation activity of *tpst-1* roots but did not restore the meristematic activity, indicating that tyrosine-sulfated peptide(s) other than PSK and PSY1 is involved in the maintenance of root stem cells and regulation of meristematic activity.

To identify this peptide signal, genes encoding possible sulfated peptides in the *Arabidopsis* genome were screened by an *in silico* approach. Known sulfated peptide hormones, PSK and PSY1, are encoded by multiple paralogous genes whose primary translated products are about 70- to 110-amino acid cysteine-poor (Cys < 6) peptides/polypeptides that contain secretion signal and Asp-Tyr sequences as a minimal tyrosine sulfation motif.[2,6] Based on these criteria, 34 candidate peptides/polypeptides were selected from 31,835 open reading frames in *Arabidopsis*. The RGF family was identified by determining their mature peptide structures followed by testing their activities to recover root meristem defects of *tpst-1* mutant.[8]

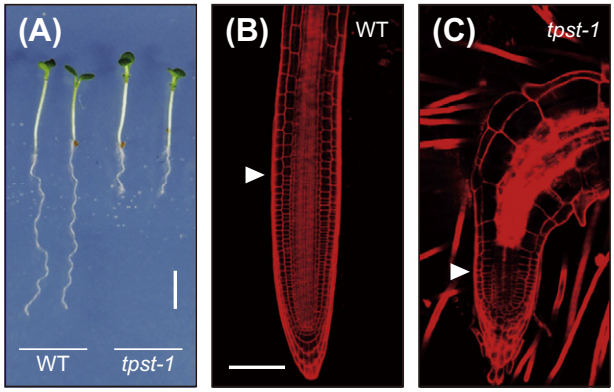

FIGURE 1 Root meristem phenotype of *tpst-1*. (A) Wild-type and *tpst-1* seedlings 7 days after germination. (B, C) Confocal images of root meristem of wild-type and *tpst-1* seedlings 3 days after germination. White arrows indicate the root meristem boundary. Scale bar: (A) 5 mm; (B, C) 100 μm.

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STRUCTURE, EXPRESSION, AND BIOLOGICAL FUNCTIONS OF RGF

The RGF family consists of nine gene members that encode peptides/polypeptides with a C-terminal conserved domain containing Asp-Tyr sequences.[8] Mature RGF1 is a 13-amino acid in length, carries a tyrosine sulfation, and derives from the C-terminal conserved domain of the precursor peptide through proteolytic processing (Fig. 2). Chemically synthesized mature RGF1 peptide restores stem cells and meristematic activity of *tpst-1* to a level comparable to that of the wild type when added in the culture medium (Fig. 3). Root meristem activity of *tpst-1* was also recovered, albeit to varying degrees, by the application of the synthetic sulfated peptides corresponding to the 13-amino acid conserved domain of the other members of this RGF peptide family.

RGF1 is specifically expressed in quiescent center cells and columella stem cells in the root tip (Fig. 4A). RGF2 and RGF3 are mainly expressed in the innermost layer of central columella cells. RGF4 shows a diffused expression pattern in the stem cell region. Secreted RGFs diffuse into the meristematic region (Fig. 4B). Although single loss-of-function mutants of RGFs do not exhibit any obvious phenotypes in roots, the *rgf1 rgf2 rgf3* triple mutant shows a short root phenotype characterized by the decrease in meristematic cell number, indicating that RGFs function redundantly.

MOLECULAR TARGET OF RGF SIGNALING

Although RGF receptors remain to be identified, one of the molecular targets of RGF signaling is PLETHORA (PLT) transcription factors. Involvement of PLTs in RGF signaling was identified by analysis of the response of several root-specific transcription factor mutants to RGF1 by exogenous application of RGF1 peptide.[8] The loss-of-function mutant of *PLETHORA* (*PLT*) transcription factors is less sensitive to RGF1. *PLT* genes, specifically expressed in root meristem, encode AP2-domain transcription factors that mediate patterning of the root stem cell niche.[1] PLT proteins display gradient distributions with

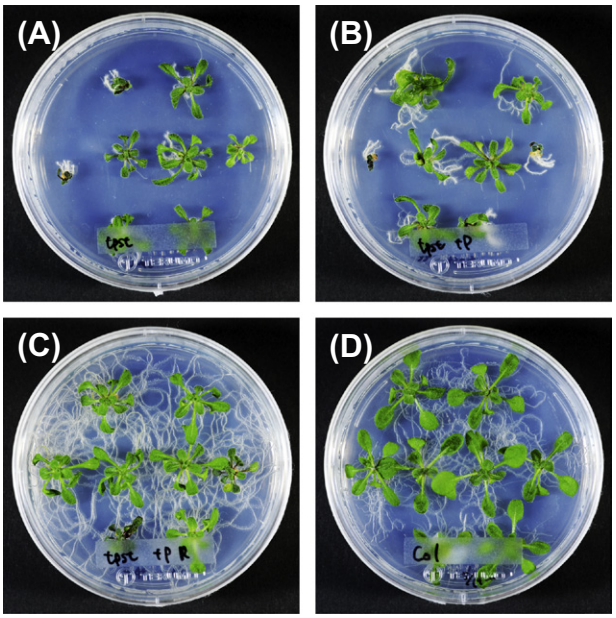

FIGURE 3 Effect of RGF1 on root phenotypes of *tpst-1*. Photographs of *tpst-1* seedlings grown in the absence (A) or the presence of PSK (100 nM) (B) or both PSK (100 nM) and RGF1 (100 nM) (C) 16 days after germination. PSK is required for full activity of RGF1. (D) Wild-type seedlings as a control. See color plate 5.

```
At5g60810 (RGF1)      1 ------------------------MVSIRVICYLLVFSVLQVHAKVSNANFNSQAPQMKNSEGLGASNGTQIAKKHAEDVIENRKTLKHV     66
At1g13620 (RGF2)      1 -------------------MTNITSSFLCLLILLLFCLSFGYSLHGDKDEVLSVDVGSNAKVMKHLDGDDAMKKAQVRGR----SGQEFS     67
At2g04025 (RGF3)      1 -------------------MTTL-SKILCVLIILLLCFSFRYSLHEDGNQQSSRDFVSTAKAIKY--GD-VMKKM-IRGRKLMMASGEKE     66
At3g30350 (RGF4)      1 MRFTIIVIAFLLIIQSLEEEQILVYARKGREACHKSLDYQGDQDSSTLHPKELYDAPRKVRFGRATRAEKEQVTAMNNDSWSFKISGASK     90
At5g51451 (RGF5)      1 ---------------------------------------------MSSIHVASMILLLF-LFLH-HSDSRHLDNVHI-TASRFSLVKDQ     41
At4g16515 (RGF6)      1 -------------------------------------------------------MSCSLRSGLVIVFCFIL-LLLSSNVGCASAARRL     33
At3g02240 (RGF7)      1 -------------------------------------MEMKKWSYANLITLALLFLFFIILLLAFQGGSRDDDHQHVHVAIRTKDISMG     52
At2g03830 (RGF8)      1 ------------------MKLIRVTLFLCALAILLLVTPTS-SLQL-KHPYSSPSQGLSKKIVTKM----ATRKLMIISSEYVMTSTSHE     66
At5g64770 (RGF9)      1 ------------------------------------------------------------MAIRVSHKSFLVALLLILFISSPTQARSL     29

At5g60810 (RGF1)     67 NVKVEANEKNGLEIESKEMVKKRKNKKRLTKTESLTADYSNPGHHPPRHN-------                                    116
At1g13620 (RGF2)     68 KETTKMMM--KKTTKKETN--VEEEDD---LVAY-TADYWKPRHHPPKNN-------                                    109
At2g04025 (RGF3)     67 EAETKMKRGNRETERNSSK--SVEEDG---LVAY-TADYWRAKHHPPKNN-------                                    110
At3g30350 (RGF4)     91 HLIVERKLGFHKRSKSSSFKWKPKKKKSSGPFVAFYDDYRGPARHPPRHNL------                                    141
At5g51451 (RGF5)     42 NVVSSSTSKEPVKVSRFV-PGPLKHH-HRRPPLL-FADYPKPSTRPPRHN-------                                     88
At4g16515 (RGF6)     34 RSHKHHHHKVASLDVFNGGERRRALGGVETGEEVVVMDYPQPHRKPPIHNEKS----                                     86
At3g02240 (RGF7)     53 RKLKSLKPINPTKKNGFEYPDQGSHDVQEREVYVELRDYGQRKYKPPVHN-------                                    102
At2g03830 (RGF8)     67 GSSEQLRVTSSGKSKDEEKKLSEEEEEKKALAKYLSMDYRTFRRRRPVHNKALPLDP                                    123
At5g64770 (RGF9)     30 R-EVVRNRTLLVVEKSQESRKIRHEGGGSDVDGLMDMDYNSANKKRPIHNR------                                     79
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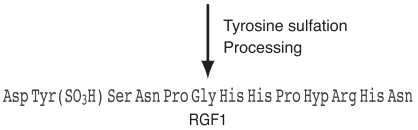

FIGURE 2 Multiple sequence alignments of the precursor polypeptides of the RGF family. Identical amino acids are shaded in black, and similar amino acids are shaded in gray.

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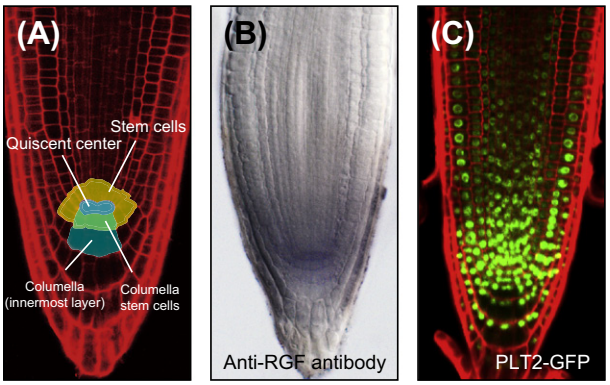

FIGURE 4 RGF peptides define expression levels and patterns of PLTs. (A) Schematic representation of the representative cell types present in the *Arabidopsis* root meristem. (B) Distribution of RGF peptides in the root meristem visualized by whole-mount immunostaining using anti-RGF antibody. (C) Expression pattern of PLT2-GFP protein showing gradient distribution with maxima in the stem cell area. See color plate 6.

maxima in the stem cell area, and this gradient is essential for maintenance of the root stem cell niche and transit-amplifying cell proliferation.[4] High levels of PLT activity promote stem cell identity and maintenance; lower levels promote mitotic activity of stem cell daughters; and further reduction in levels is required for cell differentiation.

In wild-type seedlings, PLT proteins show gradients that extend into the region of transit-amplifying cells or the elongation zone. This expression pattern of PLTs correlates well with the localization of RGF peptides (Fig. 4C). By contrast, PLT protein expression regions were considerably reduced, with weak expression in the meristematic zone in *tpst-1* or *rgf1 rgf2 rgf3* triple mutant seedlings.[8] Importantly, externally added RGF1 restores PLT protein expression levels and patterns in *tpst-1* without affecting *PLT* gene expression levels. Thus, RGF defines PLT expression levels and patterns mainly at posttranscriptional levels, possibly through stabilization of PLT proteins.[4]

Longitudinal auxin concentration gradients generated by the PIN auxin efflux facilitator network have been believed to underlie the gradient distribution of PLTs.[3] Discovery of RGF peptide signals, however, indicates that the regulatory system for PLTs is more complex than was previously thought. It will be very important to unravel how individual cells perceive and integrate the concentration-dependent information provided by RGFs and auxin to generate precise root meristem patterning during root development.

REFERENCES

1. Aida M, Beis D, Heidstra R, Willemsen V, Blilou I, Galinha C, et al. The *PLETHORA* genes mediate patterning of the *Arabidopsis* root stem cell niche. *Cell* 2004;**119**:109–20.
2. Amano Y, Tsubouchi H, Shinohara H, Ogawa M, Matsubayashi Y. Tyrosine-sulfated glycopeptide involved in cellular proliferation and expansion in *Arabidopsis*. *Proc Natl Acad Sci USA* 2007;**104**:18333–8.
3. Blilou I, Xu J, Wildwater M, Willemsen V, Paponov I, Friml J, et al. The PIN auxin efflux facilitator network controls growth and patterning in *Arabidopsis* roots. *Nature* 2005;**433**:39–44.
4. Galinha C, Hofhuis H, Luijten M, Willemsen V, Blilou I, Heidstra R, et al. PLETHORA proteins as dose-dependent master regulators of *Arabidopsis* root development. *Nature* 2007;**449**:1053–7.
5. Komori R, Amano Y, Ogawa-Ohnishi M, Matsubayashi Y. Identification of tyrosylprotein sulfotransferase in *Arabidopsis*. *Proc Natl Acad Sci USA* 2009;**106**:15067–72.
6. Matsubayashi Y, Ogawa M, Kihara H, Niwa M, Sakagami Y. Disruption and overexpression of *Arabidopsis* phytosulfokine receptor gene affects cellular longevity and potential for growth. *Plant Physiol* 2006;**142**:45–53.
7. Matsubayashi Y, Sakagami Y. Phytosulfokine, sulfated peptides that induce the proliferation of single mesophyll cells of *Asparagus officinalis* L. *Proc Natl Acad Sci USA* 1996;**93**:7623–7.
8. Matsuzaki Y, Ogawa-Ohnishi M, Mori A, Matsubayashi Y. Secreted peptide signals required for maintenance of root stem cell niche in *Arabidopsis*. *Science* 2010;**329**:1065–7.
9. Moore KL. The biology and enzymology of protein tyrosine *O*-sulfation. *J Biol Chem* 2003;**278**:24243–6.

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Chapter 11

ROTUNDIFOLIA4

Takahiro Yamaguchi, Momoko Ikeuchi and Hirokazu Tsukaya

ABSTRACT

In *Arabidopsis thaliana* (Arabidopsis), a dominant mutation in the ROTUNDIFOLIA4 (ROT4) gene results in a short-leaf phenotype with decreased cell proliferation, specifically along the proximodistal axis. *ROT4* encodes a peptide of 53 amino acids, which belongs to a plant-specific ROT-FOUR-LIKE/DEVIL (RTFL/DVL) family with 23 members in Arabidopsis. These members share a highly conserved RTF domain in the C-terminus, which serves as the core functional region. We have shown that ROT4 is not subjected to proteolytic processing and acts locally. We also propose that ROT4 is involved in the regulation of positional cues and polarized cell proliferation for multiple aspects of Arabidopsis development.

DISCOVERY

There are more than a dozen plant peptides which function as key players in diverse signaling pathways in plant development.[3] *ROT4* was identified by a gain-of-function genetic screen for genes affecting leaf development in *Arabidopsis thaliana* (Arabidopsis).[11] In an activation-tagging pool, one family displaying short, rounded leaves, and floral organs, and relatively short stems was isolated. Segregation analysis in the T_2 generation indicated a single dominant mutation, which was linked to the enhancer-containing T-DNA insertion. Thus, the mutation was named *rot4-1D*.[11]

Genomic sequence analysis around the T-DNA insertion shows a small potential open reading frame (ORF) of 53 amino acids, which was overlooked by the gene annotation program. Reverse transcription-polymerase chain reaction (RT-PCR) analysis shows that the mRNA corresponding to this small ORF accumulates at a higher level in the *rot4-1D* mutant than in the wildtype (WT). Moreover, overexpression of the full coding region of the ORF under the constitutive *35S* promoter results in the *rot4-1D* phenotype in Arabidopsis. These results indicate that the ORF comprises the *ROT4* gene.[11]

In Arabidopsis, several genes have been identified in the polar control of cell expansion and cell proliferation along the two planer leaf axes, the proximodistal (leaf-length) and the central-lateral (leaf-width) axes. For example, *ROTUNDIFOLIA3* and *ANGUSTIFOLIA* regulate leaf cell expansion along the proximodistal and mediolateral axes, respectively.[7,8,13] In *rot4-1D* mutants, the length of the leaf blades and petioles is severely shortened, while their width is only slightly affected (Fig. 1A, B). Closer observation shows that cell number, but not cell size, is greatly reduced specifically along the proximodistal axis in *rot4-1D* leaves.[11] These observations suggest that *ROT4* negatively regulates cell proliferation along the proximodistal axis when overexpressed.

STRUCTURE OF THE PEPTIDE

The longest *ROT4* cDNA sequence is 430bp and contains a 159-nt ORF encoding a putative 53 amino acid peptide.[11] There is no intron in the *ROT4* gene, and the deduced ROT4 peptide has a molecular mass of 6.2kDa. Database analysis has revealed 23 other putative Arabidopsis proteins homologous to ROT4; they have been designated the ROT-FOUR-LIKE/DEVIL (RTFL/DVL) family.[11,14] *RTFL/DVL* genes are found widely in angiosperm genomes and form a multigene family. For example, 26 *RTFL/DVL* genes (*OsRTFL1-26*) have been identified in the *Oryza sativa* genome. *RTFL/DVL* genes are also present in *Physcomitrella patens* (a moss) and *Selaginella moellendorffii* (a lycophyte) genomes.[2] No homologs were discovered from green algae.

Overexpression of at least six members of the RTFL/DVL family in Arabidopsis produces phenotypes similar to the *rot4-1D* mutant.[15] Overexpression of any of several *O. sativa RTFL/DVL* genes in Arabidopsis also results in the short-leaf phenotype, indicating that RTFL/DVL family members share similar protein function.[16] In *Medicago truncatula*, *MtDVL1* is expressed at the early stages of nodule formation and its overexpression abolishes nodulation.[1] Because the cell cycle is reactivated during nodule formation, *MtDVL1* could also be involved in the regulation of cell proliferation. Thus, the *RTFL/DVL* genes are widely conserved in land plants and may play an essential role in plant life.

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00011-7
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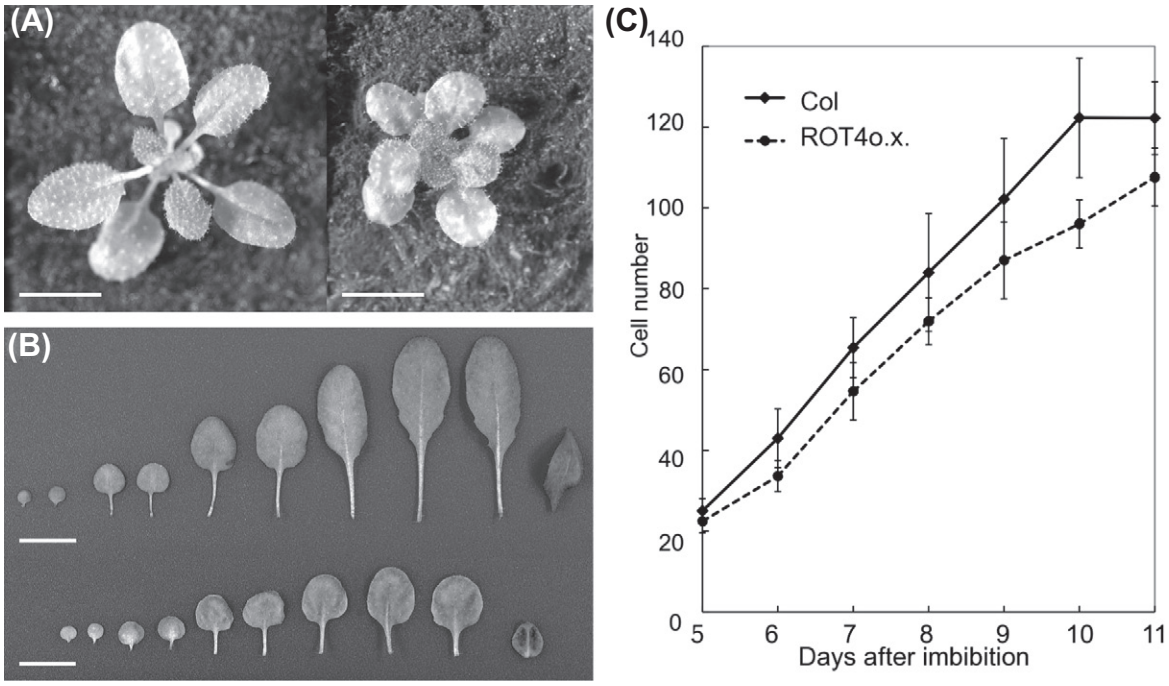

FIGURE 1 Overexpression of *ROT4* specifically reduces cell proliferation along the leaf proximodistal axis in *Arabidopsis*. (A) Seedlings of the WT (left) and *rot4-1D* (right) at 25 days. Scale bars = 10mm. (B) Leaves of the WT (top) and *rot4-1D* (bottom) at 33 days. Left to right: a cotyledon pair, the first through the seventh foliage leaves, and a cauline leaf. Scale bars = 10mm. (C) Time-course analysis of cell proliferation in the first leaf. Vertical axis indicates the cell number of the palisade layer along the proximodistal leaf axis. Reproduced from Refs. 6 and 11 with permission.

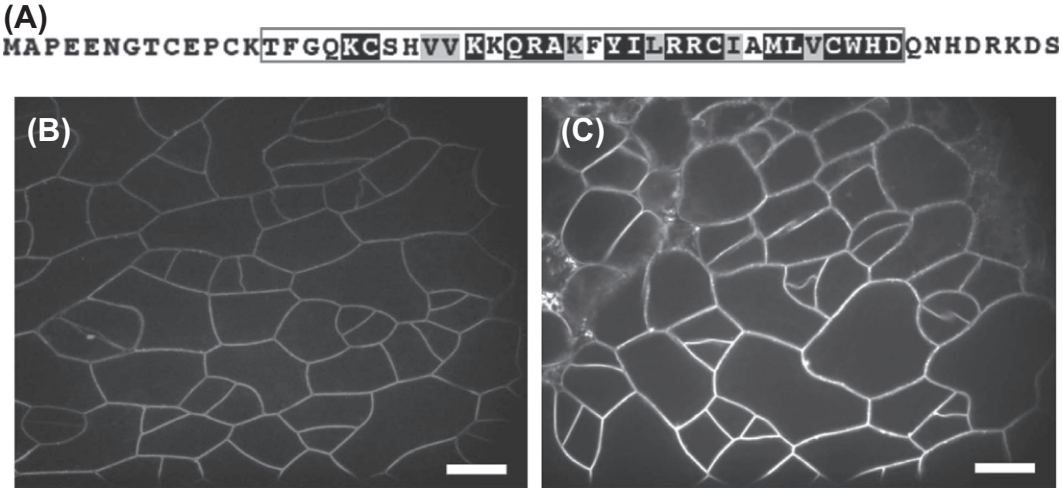

FIGURE 2 Structure of RTFL/DVL protein and intracellular localization of ROT4. (A) Amino acid sequence of ROT4. Black background shows highly conserved residues in the RTFL/DVL family, while gray background shows less-conserved residues. Box indicates the estimated core functional region. (B, C) Subcellular localization of ROT4-GFP (B) and of GFP-ROT4 (C) in Arabidopsis leaves. Scale bars = 10μm. Reproduced from Ref. 6 with permission.

RTFL/DVL proteins share a highly conserved region of approximately 30 amino acids in the C-terminus called the RTF domain, while the N-terminal region is less conserved among RTFL/DVL members and no predictable signal peptide has been identified (Fig. 2A).[11] The RTF domain is rich in basic amino acids, and does not show similarities to other proteins of known function. Overexpression of the conserved domain is sufficient and necessary to reproduce the *rot4-1D* phenotype.[11,15] We identified the precise functional region of ROT4 by overexpressing a series of truncated versions of *ROT4*. The results indicate that the core functional region of ROT4 consists of the 32 amino acid residues surrounding the RTF domain (from T14 to D45), with one or two additional adjacent amino acids required for full ROT4 activity (Fig. 2A).[6]

Plant peptides often undergo posttranslational proteolytic processing, which is essential for their function.[9] The

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highly conserved single or dibasic residues at the start of the conserved RTF domains have been hypothesized to serve as part of the recognition site for processing RTFL/DVL peptides from the precursor protein.[4,15,16] However, our study suggests that ROT4 is not subjected to proteolytic processing *in planta*. Overexpression of both GFP-ROT4 and ROT4-GFP fusion proteins produces a short-leaf phenotype similar to that of *rot4-1D*. By immunoblotting analysis with an anti-GFP (anti-green fluorescent protein) antibody, we detected the fusion protein (GFP-ROT4 or ROT4-GFP), but not free GFP, in each overexpression line.[6] Thus, ROT4 may be a unique peptide that can function without proteolytic processing.

DISTRIBUTION OF THE mRNA/PEPTIDE

Expression studies using RT-PCR show that *ROT4* is weakly expressed in flowers, shoots, and roots.[11] *ROT4* is expressed in leaves during the early stages of development, but it is not expressed in mature leaves. More precise localization of *ROT4* mRNA remains to be performed because clear signals cannot be obtained by in situ hybridization analysis in WT Arabidopsis plants. Preliminary analysis of a reporter β-glucuronidase (GUS) expression, driven by the 1.2-kb 5′-region of the *ROT4* translation start site, shows strong GUS localization in the petioles of young leaf primordia.[16] GUS expression becomes weaker as a leaf develops, indicating that the *ROT4* gene functions in young leaf primordia, the site of active cell proliferation.

BIOLOGICAL FUNCTIONS

Loss-of-function phenotypes of RTFL/DVL members are unknown. A single loss-of-function mutant did not produce any detectable phenotype in either Arabidopsis or *O. sativa*,[11] probably because of extensive functional redundancy among the more than 20 RTFL/DVL members. Generation of multiple loss-of-function mutants in *RTFL/DVL* genes is necessary to better understand their roles in WT development.

On the other hand, overexpression of *ROT4* inhibits cell proliferation specifically along the proximodistal axis in leaves, which results in the short-leaf phenotype. The effect of *ROT4* overexpression is also observed in other lateral organs, such as petals and carpels. In contrast, the effect is relatively mild in stems.[11] Thus, *ROT4* is more strongly associated with lateral organs that exhibit the development of determinate organ.

Decreases in the number of cells in the leaf could be caused by shortening of the cell proliferation period or by reduction in the cell proliferation rate. To distinguish these possibilities, we performed a time-course analysis of cell proliferation activity during leaf development. Results revealed that the rate of cell proliferation is lower in *rot4-1D* compared with the WT, while the duration of cell proliferation is not shortened in *rot4-1D* (Fig. 1C).[6] To further clarify ROT4 function, we also visualized proliferating cells in leaf primordia using the reporter construct *pCYCB1;1:CYB1;1-GUS*. We found that the size of the meristematic zone is reduced proximally in *rot4-1D* leaf primordia.[6] Therefore, ROT4 negatively regulates the rate of cell proliferation by reducing the size of the meristematic zone along the proximodistal axis during leaf development.

We also examined the effect of local overexpression of *ROT4* in developing leaves using an inducible chimera system.[5] When *GFP-ROT4* is overexpressed on one side of the leaf, the leaf blade–petiole boundary on that side is shifted proximally (Fig. 3A, B).[6] This result suggests that *ROT4* could be involved in the determination of organ boundary and positional cues along the proximodistal axis in leaf primordia.

In addition to the short-leaf phenotype, overexpression of *ROT4* shows an abnormal turning phenotype in pedicels or secondary inflorescence stems (Fig. 3C, D). Closer histological observation revealed that the boundary between pedicels or secondary inflorescence stem and the main inflorescence stem forms at this sharp turning point.[6] This observation has been confirmed using morphogenetic and molecular markers for the boundary. Thus, at the base of pedicels, the main inflorescence stem abnormally protrudes in overexpression of *ROT4*, which results in the turning phenotype seen in pedicels or secondary inflorescence stems. A similar protrusion phenotype is also observed at the base of trichomes during overexpression of some *RTFL/DVL* genes (Fig. 3E, F).[6,15] These phenotypes could be explained by a shift in the boundary or positional cues in inflorescences and at the base of trichomes, similar to the disturbance in positional cues observed upon local overexpression in leaves. Thus, it is possible that *ROT4* is involved in the determination of positional cues during various aspects of shoot development.

RECEPTORS AND SIGNALING CASCADES

Most small peptides are thought to function as peptide hormones, which are secreted into extracellular spaces and interact with specific receptors.[12] The lack of predicted signal sequences in ROT4 protein suggests that ROT4 does not enter the secretory pathway, although some plant peptides with no signal sequences are known to be secreted by non-canonical pathways.[9,12] When GFP-ROT4 is overexpressed on one side of the leaf, the petiole on the induced side is significantly shortened, while the petiole on the opposite side is not (Fig. 3A, B).[6] This result excludes the possibility of the long-distance transport of ROT4, although it is still possible that ROT4 moves locally to the neighboring cells.

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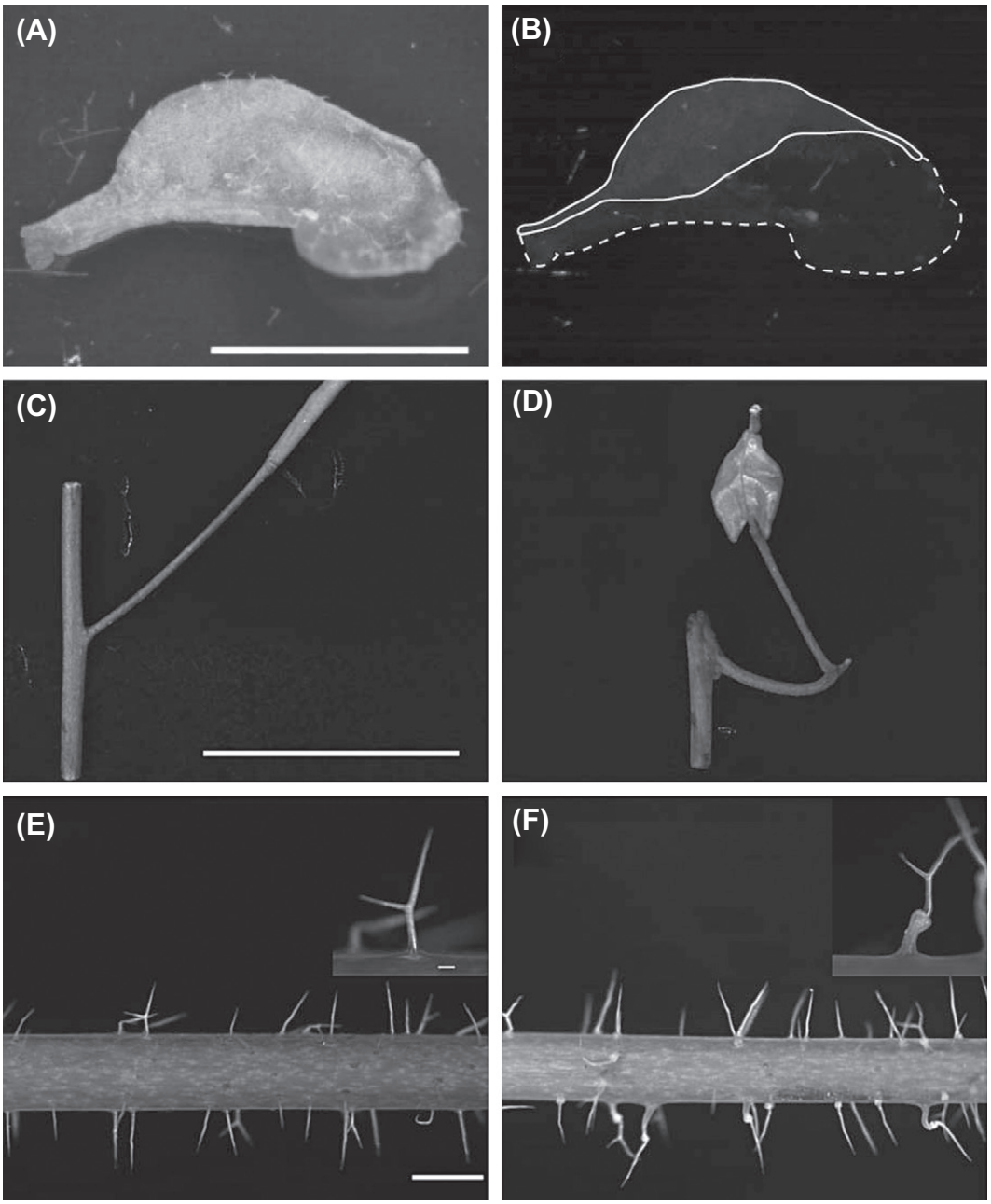

FIGURE 3 Leaf phenotype of localized overexpression of ROT4 and pleiotropic phenotypes of overexpression of ROT4. (A, B) A leaf with a sector overexpressing GFP-ROT4 in the marginal part. (A) A bright field image. (B) A fluorescent image. The sector with GFP fluorescence is outlined by a white line, and the remaining leaf part is outlined by a dashed line. (C, D) A pedicel in the WT (C) and in the overexpression of ROT4 showing a turning phenotype as a result of a protrusion of the inflorescence stem (D). (E, F) Trichome on the stem in the WT (E) and in the overexpression of ROT4 showing a protrusion of the trichome base (F). Bars = 5mm (A), 1mm (C), and 0.1mm (E). Reproduced from Ref. 6 with permission.

On a subcellular level, both the ROT4-GFP and GFP-ROT4 fusion proteins are localized to the plasma membrane and also to a limited number of small foci in the cytoplasm upon overexpression (Fig. 2B, C).[6,11] The appearance of a short-leaf phenotype in both overexpression lines suggests that the observed localization of ROT4 in the plasma membrane and in small cytoplasmic foci reproduces native intracellular localization. Because a transmembrane domain does not exist in ROT4, the protein could be a part of a protein complex localizing to the plasma membrane. Identification of protein partners of ROT4 and their loss-of-function mutants would greatly reveal insights into the role of ROT4 in WT development.

The downstream signaling cascade of RTFL/DVL is also of interest. RT-PCR analysis revealed that overexpression of

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the *DEVIL1 (DVL1/RTFL18)* gene results in down regulation of the MADS-box gene *FUL/AGL8*.[14] Because *FUL/AGL8* is involved in the regulation of fruit development, down regulation of this MADS-box gene may be related to the abnormal fruit morphology seen in overexpression of *RTFL/DVL* genes. Microarray analysis with inducible overexpression of *DVL4* identified 422 genes whose expression is strongly up or down regulated.[10] Laure et al. reported that, among them, *NAC1* expression is strongly upregulated by the induction of *DVL4*. *NAC1* encodes an NAC-domain transcription factor and is involved in the development of auxin-mediated lateral root in Arabidopsis. When a loss-of-function mutant of *NAC1* is crossed with *dvl1-1D*, all the phenotype characteristics of *dvl1-1D* are largely suppressed in the F1 plants, while the *DVL1* mRNA is still overexpressed. The effect of suppression persists in the F2 and F3 descendants, even in siblings homozygous for the WT *NAC1* allele, suggesting an epigenetic interaction between them.

In summary, the ROT4 peptide represents a unique component required for polarized cell proliferation and determination of positional cues in plant development. Key questions to be addressed include the clarification of the loss-of-function phenotypes, the identification of protein partners, and the elucidation of the signaling cascade downstream and upstream of the RTFL/DVL family. Further studies would greatly improve our understanding of the regulation of polarized growth in plants as well as the diverse roles of plant peptides.

REFERENCES

1. Combier JP, Kuster H, Journet EP, Hohnjec N, Gamas P, Niebel A. Evidence for the involvement in nodulation of the two small putative regulatory peptide-encoding genes *MtRALF1* and *MtDVL1*. *Mol Plant-Microbe Interact* 2008;**21**:1119–27.
2. Floyd S, Bowman J. The ancestral developmental tool kit of land plants. *Int J Plant Sci* 2007;**168**:1–35.
3. Fukuda H, Higashiyama T. Diverse functions of plant peptides: entering a new phase. *Plant Cell Physiol* 2011;**52**:1–4.
4. Harris RB. Processing of pro-hormone precursor proteins. *Arch Biochem Biophys* 1989;**275**:315–33.
5. Ichihashi Y, Kawade K, Usami T, Horiguchi G, Takahashi T, Tsukaya H. Key proliferative activity in the junction between the leaf blade and leaf petiole of *Arabidopsis thaliana*. *Plant Phys* 2011;**157**:1151–62.
6. Ikeuchi M, Yamaguchi T, Kazama T, Ito T, Horiguchi G, Tsukaya H. ROTUNDIFOLIA4 regulates cell proliferation along the body axis in Arabidopsis shoot. *Plant Cell Physiol* 2011;**52**:59–69.
7. Kim GT, Shoda K, Tsuge T, Cho KH, Uchimiya H, Yokoyama R, et al. The *ANGUSTIFOLIA* gene of *Arabidopsis*, a plant CtBP gene, regulates leaf-cell expansion, the arrangement of cortical microtubules in leaf cells and expression of a gene involved in cell-wall formation. *EMBO J* 2002;**21**:1267–79.
8. Kim GT, Tsukaya H, Uchimiya H. The *ROTUNDIFOLIA3* gene of *Arabidopsis thaliana* encodes a new member of the cytochrome P-450 family that is required for the regulated polar elongation of leaf cells. *Genes Dev* 1998;**12**:2381–91.
9. Matsubayashi Y. Post-translational modifications in secreted peptide hormones in plants. *Plant Cell Physiol* 2011;**52**:5–13.
10. Laure C, Wen J, Walker JC. Interactions between a NAC-Domain transcription factor and the putative small protein encoding DVL/ROT gene family. *Plant Mol Biol Rep* 2010;**28**:162–8.
11. Narita NN, Moore S, Horiguchi G, Kubo M, Demura T, Fukuda H, et al. Overexpression of a novel small peptide ROTUNDIFOLIA4 decreases cell proliferation and alters leaf shape in *Arabidopsis thaliana*. *Plant J* 2004;**38**:669–713.
12. Ryan CA, Pearce G, Scheer J, Moura DS. Polypeptide hormones. *Plant Cell* 2002:(Suppl.): S251–S264.
13. Tsuge T, Tsukaya H, Uchimiya H. Two independent and polarized processes of cell elongation regulate leaf blade expansion in *Arabidopsis thaliana* (L.) Heynh. *Development* 1996;**122**:1589–600.
14. Wen J, Lease KA, Walker JC. DVL, a novel class of small polypeptides: overexpression alters *Arabidopsis* development. *Plant J* 2004;**37**:668–77.
15. Wen J, Walker JC. DVL peptides are involved in plant development. In: Kastin AJ, editor. *Handbook of Biologically Active Peptides*. San Diego, CA, USA: Elsevier; 2006. p. 17–22.
16. Yamaguchi T, Tsukaya H. ROTUNDIFOLIA4: a plant-specific small peptide. In: Kastin AJ, editor. *Handbook of Biologically Active Peptides*. San Diego, CA, USA: Elsevier; 2006. p. 37–40.

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Chapter 12

SCR

Sushma Naithani, Palitha Dharmawardhana and June B. Nasrallah

ABSTRACT

The S-locus cysteine-rich protein (SCR) is a basic protein of about 6kDa that functions in the self-incompatibility (SI) response of the Brassicaceae. SI is a genetic system that prevents self-pollination by disrupting the interaction between pollen grains and the epidermal cells of the stigma, a specialized structure that caps the female reproductive apparatus. *SCR* is genetically and physically tightly linked to the gene for its receptor, the stigma-expressed single-pass transmembrane serine/threonine kinase S-locus receptor kinase (SRK). Like SRK, SCR is highly polymorphic, yet its variants adopt a conserved three-dimensional structure consisting of an α-helix and a triple-stranded antiparallel β-sheet stabilized by four intramolecular disulfide bonds. The allele-specific interactions of SCR with SRK, which underlie specificity in the SI response, trigger a signal transduction cascade within the stigma epidermal cell that leads to the inhibition of pollen tube growth. SCR is the prototypic member of the *SCR*-like (*SCRL*) family of genes, several of which might also function in plant reproduction.

DISCOVERY OF SCR

The *S*-locus cysteine-rich protein SCR, also known as SP11, was discovered in a search for the pollen determinant of self-incompatibility (SI) in the Brassicaceae (crucifer) family. SI is a generic term that encompasses several evolutionarily and mechanistically divergent systems that share one common feature, namely, the inhibition of self-pollination. The information provided here relates specifically to the SI system that operates in the Brassicaceae family, and *SCR* homologs are not expected to be present in families that use a different SI system.

The SI system of Brassicaceae is controlled by a single locus, the *S* (Sterility) locus [for recent reviews, see Refs 17, 32]. In self-incompatible members of the Brassicaceae [such as cultivated *Brassica oleracea* (includes cabbage, broccoli, kale, etc.), *B. rapa* (syn. *campestris*; includes Chinese cabbage, oil seed crops), *B. napus* (includes oil seed crops), as well as *Raphanus sativus* and *Arabidopsis lyrata*], pollinations between plants that express the same *S*-locus variant are prevented by the arrest of pollen tube development at the surface of the epidermal cells of the stigma, which are located at the tip of the female reproductive apparatus. Previous analysis of SI had indicated that the SI specificity-determining *S* locus, although behaving genetically as a single Mendelian locus, was a complex locus consisting of at least two genes, one encoding the stigma specificity determinant and the other encoding the pollen specificity determinant. Molecular analysis of the *S* haplotype had already identified the highly polymorphic *S*-locus receptor kinase (*SRK*) gene as the determinant of SI specificity in the stigma,[27,29] and detailed analyses of DNA flanking the *SRK* gene in various *S* haplotypes were initiated through long-range physical mapping coupled with transcriptional and sequence analyses.[6,21,28] In 1999, Schopfer et al. reported the isolation of *SCR* and provided the first experimental proof through loss- and gain-of-function experiments that *SCR* is required for SI and that it represents the pollen determinant of SI specificity.[21] A self-compatible γ-ray-induced induced mutant of *B. oleracea* that exhibits a pollen-specific defect in the SI response lacks *SCR* transcripts.[21] In addition, transformation of an S_2S_2 plant with an SCR_6 allele confers S_6 specificity on the pollen of transgenic plants.[21] Subsequent verification of these results was provided by additional transgenic experiments[25] and pollination bioassays in which SCR protein, either chemically synthesized or expressed in *E. coli*, was applied to stigmas and found to activate the SI response in an allele-specific manner.[10,31] Additionally, transformation of the self-fertile *Arabidopsis thaliana* with *A. lyrata* SRK_b and SCR_b genes showed that these two genes are sufficient to confer self-incompatibility in a self-compatible species.[15,16]

SCR Sequence Polymorphism

SCR alleles were first cloned from *Brassica* species (*B. rapa* and *B. oleracea*). The DNA sequence is now available for 20 *B. oleracea*, 23 *B. rapa,* 2 *B. napus*, and 10 *Raphanus sativus SCR* variants (http://www.ncbi.nlm.nih.gov/unigene). Additionally, *SCR* variants have been isolated from two other crucifer species [2]: one from *Capsella gradiflora* and a few from *Arabidopsis lyrata*, a self-incompatible close relative of the self-fertile model plant

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00012-9
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A. thaliana, which diverged from *Brassica* approximately 15–20 million years ago.[35] The total number of *SCR* alleles within a particular species is predicted to be much higher, because each *S* haplotype must contain an *SCR* variant and *S* haplotypes can number as many as 100 in one species. Consistent with their SI recognition functions, both *SCR* and its receptor *SRK* are highly polymorphic genes that must coevolve to maintain their allele-specific interaction. The diversification of *Brassica S* haplotypes is believed to have taken place over 20–40 million years ago, before speciation in the crucifer family. *S* haplotypes have been grouped into two classes on the basis of sequence similarity of their *SRK* alleles. *SCR* alleles exhibit a particularly high level of intraspecific sequence divergence, as illustrated in Fig. 1 for a representative sample of SCR variants from *Brassica* species and *A. lyrata*. In *Brassica* class-I *S* haplotypes, the mature SCR proteins share between 20 and 76% amino acid similarity while their predicted signal sequences are more conserved.[21] By contrast, *Brassica* class-II SCR proteins exhibit a relatively high degree of similarity (63–94%) in the sequence of both their predicted signal peptide and mature forms.[22] When all available SCR sequences are aligned, it is clear that only eight cysteine residues (designated C1–C8, the glycine in G x C2, and an aromatic residue in C3xxxY/F are conserved in most SCR variants (Fig. 1).

Criteria for Identification of SCR Alleles in Various Species of the Brassicaceae

Because of the extensive polymorphism of *SCR* variants and the existence of *SCR*-like genes in the genomes of Brassicaceae,[34] identification of a cloned sequence as an *SCR* allele is not straightforward. Linkage to the *S* locus and *SRK* is the major and essential criterion for identifying an *SCR*-like sequence as a *bona fide* new *SCR* allele. Accordingly, the isolation of a candidate *SCR* sequence must be verified by genetic analysis of plant populations that segregate for different *S* haplotypes or by physical linkage to *SRK*. When *SCR* alleles are sought from a new Brassicaceae species, the isolation of *SCR* alleles requires the cloning of the *S* locus from large-insert genomic libraries with subsequent "chromosome walking" from *SRK* to the *SCR* gene as was performed for *Arabidopsis lyrata*.[11] This already laborious process is made even more complicated by the fact that *S* haplotypes within one species exhibit extensive structural heteromorphism, a feature that is thought to contribute to the apparent reduced recombination in the *S*-locus region and the maintenance of the tight linkage of the *SRK–SCR* gene pair.[4] Both the orientation of *SCR* relative to *SRK* and its physical distance from *SRK* can vary among *S* haplotypes.[4,11] In *Brassica*, *S* haplotypes range from approximately 10 to >200kb in size, and they

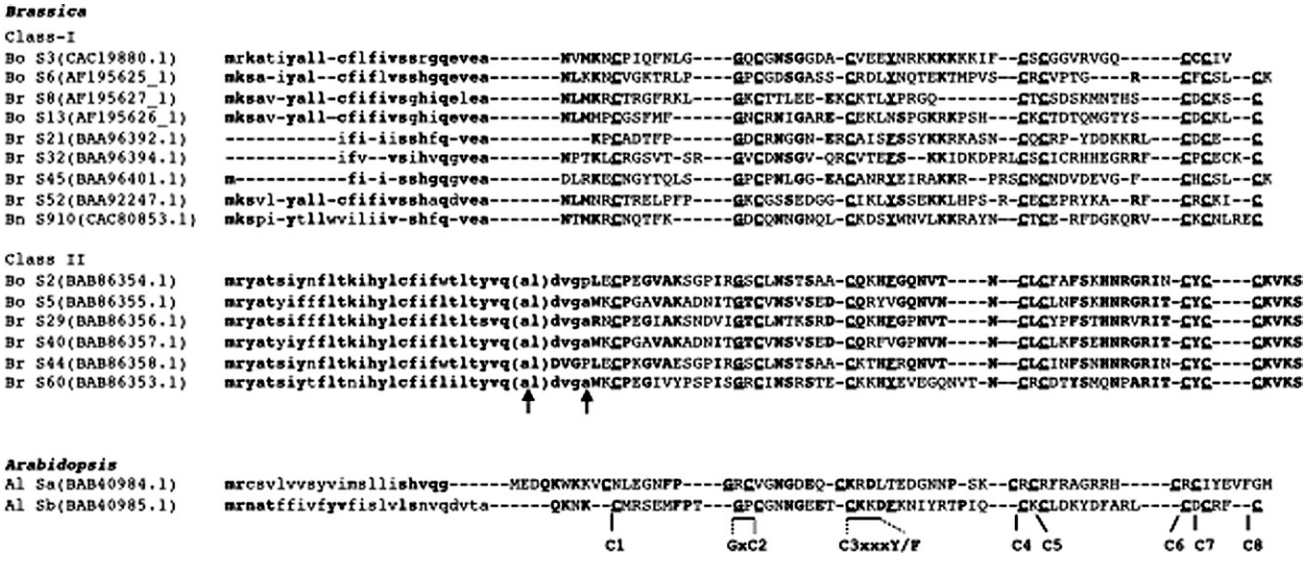

FIGURE 1 Multiple sequence alignment of the predicted amino acid sequences of SCR variants from various *S* haplotypes of different Brassicaceae species. The signal peptide sequence for each *Brassica* SCR variant is shown in lowercase letters. The SCRs from *Brassica oleracea* (Bo), *B. rapa* (Br), and *B. napus* (Bn) are arranged into class-I and class-II alleles, and two variants from *Arabidopsis lyrata* are grouped together. Residues that are conserved in most SCR variants within a group are shown in bold. Residues that are conserved among SCRs in the various groupings are underlined, and the consensus sequence motifs associated with some of these residues are shown below the alignment. In the class-II variants, arrows point to the predicted signal peptide cleavage sites in the proteins predicted from two alternative *Brassica* class-II *SCR* transcripts.[22] One form encodes an open reading frame of 95 amino acids that is predicted to produce a mature SCR protein of 66 amino acids after removal of the signal peptide (left arrow). The second form encodes a 93 amino acid protein that lacks two amino acids ("al" shown in parentheses), which would produce a mature SCR protein of 61 amino acids after cleavage of the signal peptide at an alternative cleavage site (right arrow).

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also differ in their complement of retroelements and haplotype-specific intergenic sequences.[4,11] Additionally, some *S* haplotypes contain partial or complete duplications of the SI-specificity genes. For example, two identical copies of *SCR* are found in the S_b haplotype of *A. lyrata*[11] and three nonidentical copies were reported in the S_{15} haplotype of *B. rapa.*[24]

SCR Pseudogenes in Self-fertile Brassicaceae

Not all strains within a predominantly self-incompatible species and not all species in the Brassicaceae are expected to have functional *SCR* genes, because the loss of SI that underlies the switch to self-fertility in this family is often accompanied by inactivation of *S*-locus genes. Therefore, the *S* loci of self-fertile species may contain only nonfunctional remnants of these genes. For example, the reference Col-0 accession of the self-fertile model plant *A. thaliana* contains only three truncated *SCR* sequences and an *SRK* pseudogene.[11] Other accessions contain *SRK*-linked *SCR* sequences that are highly diverged from the Col-0 *SCR* sequences and some of these potentially encode full-length open reading frames.[3] However, it is currently not known if their SCR products have retained functionality.

STRUCTURE OF THE PRECURSOR mRNA GENE

Comparison of *SCR* genomic and cDNA sequences indicates that the *SCR* gene consists of two exons separated by an intron. The first exon contains a predicted signal sequence of 29–32 amino acids, and the second exon contains an open reading frame of 50–66 amino acids that corresponds to the mature SCR protein.[21,30] The size of the intron varies among *SCR* alleles from 0.3kb in *B. rapa* SCR_{12} to 4.1 kb in SCR_8.[21,30] A single approximately 450-bp processed *SCR* transcript is observed for some *S* haplotypes that contain either one copy of the *SCR* gene[21,28] or two identical *SCR* genes (e.g., the *A. lyrata* S_b haplotype[11]). However, >1 *SCR* transcript has been reported for the S_{15} haplotype of *B. oleracea*, which contains three nonidentical tandem copies of *SCR* (designated S_{15}-*SP11a*, S_{15}-*SP11b*, and S_{15}-*SP11b'*).[24] Only the first two of these genes are transcribed to produce transcripts of two different sizes, a 0.65-kb transcript resulting from cotranscription of S_{15}-*SP11a* and exon 1 of S_{15}-*SP11b* and a less abundant 1.4-kb transcript containing the full-length S_{15}-*SP11a* and S_{15}-*SP11b* sequences and exon 1 of S_{15}-*SP11b'*.[24] Additionally, two SCR transcripts generated by alternative splicing have been detected for some class-II *SCR* alleles (e.g., S_{29}, S_{40}, S_{60} of *B. rapa*),[22] and these transcripts are predicted to encode two mature protein forms differing by five amino acids (Fig. 1). The biological significance, if any, of these alternative transcripts is not known.

DISTRIBUTION OF THE *SCR* mRNA AND THE BASIS OF ALLELIC DOMINANCE IN HETEROZYGOTES

All *SCR* alleles exhibit anther-specific expression, although the details of this expression vary with the allele being studied. In situ hybridization and reporter gene analyses have shown that all *SCR* alleles examined to date are expressed in the tapetum[11,30] and a subset of *SCR* alleles, such as the *Brassica* class-I *SCRs* and the *A. lyrata SCRb* allele, are also expressed in microspores. *SCR* expression in the tapetum is probably the biologically relevant site of expression: It is clearly sufficient for SI, and it is consistent with the localization of the SCR protein in the pollen coat and with the genetic control of pollen SI phenotype in the Brassicaceae.[11,22,30] The tapetum is a layer of nurse cells that lines the anther cavities in which pollen grains develop, and it serves as a source of many components of the pollen coat. The tapetum degenerates at late stages of pollen maturation, and as a result, tapetum-expressed *SCR* transcripts are lost at late stages of anther development. Furthermore, the tapetum is derived from sporophytic (i.e., 2N) cells of the anther. Expression of *SCR* in the tapetum thus explains the sporophytic control of SI in the Brassicaceae, in which the SI phenotype of a pollen grain is controlled by the diploid genotype of its parental plant rather than by its own haploid genotype. Thus, the pollen grains produced by an S_1S_2 plant will be phenotypically S_1S_2 (assuming codominance of S_1 and S_2) because SCR_1 and SCR_2 are expressed in the tapetum and the SCR_1 and SCR_2 proteins will become incorporated in the pollen coat of all pollen grains, whether they carry the SCR_1 or SCR_2 allele.

A peculiarity of the *SCR* gene is that its expression can be influenced by other *SCR* alleles in heterozygous anthers.[12,22] Some *SCR* alleles exhibit genetic interactions of dominance recessiveness. For example, in *Brassica*, class-II *SCR* alleles are generally recessive to class-I *SCR* alleles, and in *A. lyrata*, SCR_a is recessive to SCR_b. In situ hybridization has shown that the recessive *SCR* allele is silenced in the presence of the dominant *SCR* allele, leading to the absence of recessive *SCR* transcripts and protein in heterozygous anthers.[12,22] The silencing of the recessive SCR allele in heterozygotes results from specific methylation of its promoter region.[23] This methylation is associated with the accumulation of a 24 nucleotide sRNA, termed *Smi* (SP11(SCR)-methylation inducer), which is derived from a noncoding transcript originating within the dominant *S* haplotype.[33] The sequence of *Smi* transcripts derived from the dominant S_9 haplotype (Smi_D) is almost identical to the targeted regions in the promoters of the recessive *SCR* alleles (18 out of 19 nucleotide for SCR_{29}, SCR_{60} and SCR_{44}) but not to the same region in the dominant SCR_9 allele. This observation explains the specific silencing of recessive *SCR* alleles in the presence of the

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dominant allele. Interestingly, methylation of the promoter of the recessive *SCR* allele extends over approximately 300bp although the region that shares sequence similarity with the Smi_D transcript is only 18 bp long. How this spread of methylation occurs remains to be determined.

PROCESSING OF THE SCR PROTEIN

SCR variants differ in both length and amino acid composition (Fig. 1). Different *SCR* alleles predict open reading frames that range from 71 to 95 amino acids with a predicted signal peptide at their N terminus. For example, the mRNA sequence of the *B. rapa* SCR_8 allele predicts a 74 amino acid protein of approximately 8kDa, but the mature SCR protein extracted from the pollen coat was determined by mass spectrometric analysis to be 5.716kDa, which corresponds to 50 amino acids.[31] The primary SCR translational product is therefore processed by removal of the signal sequence that targets the protein for secretion. Although little biochemical characterization has been performed, the SCR protein is not apparently further processed or modified because SCR variants expressed in *E. coli*[10] or synthetic SCR[31] exhibit biological activity in pollination bioassays.

THE SCR RECEPTOR: THE *S*-LOCUS RECEPTOR KINASE (SRK)

The receptor for SCR, SRK, is encoded by a gene located within the *S* locus in tight genetic and physical linkage to *SCR*. *SRK* mRNA levels attain their highest levels in stigmas just before flower opening, which correlates with the onset of SI in the developing stigma.[27] The full-length *SRK* transcript encodes a protein that is expressed in the stigma epidermal cells and is integral plasma membrane protein.[26] SRK comprises an extracellular region (eSRK) of approximately 430 amino acids, followed by a single transmembrane domain, and a cytoplasmic serine/threonine kinase.[14] *SRK* alleles produce alternative mRNA transcripts[27] that produce a truncated protein equivalent to the eSRK in addition to the full-length SRK.[9] Interestingly, in *Brassica*, most *S* haplotypes contain a third gene, the *S*-locus glycoprotein (*SLG*) gene, which shares a high degree of sequence similarity with the *eSRK* sequence and is coordinately regulated with *SRK*.[7,9] Although SRK is clearly the sole determinant of SI specificity in the stigma, SLG is required for the accumulation of some SRKs to physiologically relevant levels,[7] and it can enhance the SI response in *SRK* transformants.[29] The eSRKs might have a role similar to that of SLG. Homology modeling of the eSRK suggests that this region consists of four structural modules: two N-terminal lectin-like domains (LLD1 and LLD2), followed by an EGF-like domain and a PAN_APPLE domain.[14] *In vitro* studies have suggested that the PAN_APPLE domain function in receptor dimerization.[14]

A major issue in understanding the SRK–SCR interaction is identification of the residues within eSRK that determine allele-specific interaction with the SCR ligand. Statistical analysis of multiple sequence alignments of eSRKs derived from several *Brassica* SRK variants identified three hypervariable regions and a less variable C-terminal region,[18] which were inferred to be targets for positive selection and potential candidates for residues involved in ligand recognition. However, experimental evidence was difficult to obtain because of the lack of efficient transformation methods in *Brassica*. This difficulty was recently overcome by the availability of a transgenic self-incompatible *A. thaliana* model system, which was generated by transformation with *SRK–SCR* gene pairs isolated from the self-incompatible species, *A. lyrata* and *C. grandiflora*.[2,15] Structure–function analysis of the eSRK in this *A. thaliana* model showed that only six residues occurring in two nonoverlapping clusters within the hypervariable regions are essential for allele-specific interaction between SRK and SCR and may form a ligand-binding pocket.[1] High-resolution three-dimensional structural analysis of the eSRK alone or in complex with its SCR ligand is required to confirm this hypothesis.

STRUCTURE OF SCR

The NMR structure of a synthetic SCR variant[13] and modeling of the 3-dimensional structures of several SCR variants are consistent with the conclusion that all SCR variants adopt a similar overall structure despite their extensive sequence divergence (Fig. 2). SCR is thought to fold into a compact α/β barrel structure made of a twisted three-stranded β-sheet backed by another layer formed by an α-helix with flanking loop regions. Four intramolecular disulfide bonds stabilize the structure. The disulfide bridges that link C2 to C5, C3 to C6, and C4 to C7 form the hydrophobic core of the molecule, whereas the C1–C8 bridge tethers the N and C termini. This structure is similar to that of plant defensins, with the difference being that the edges of the loop region between the α-helix and ß2 strand are extensively stabilized by disulfide bonds and hydrophobic packing in SCR. The aromatic (Y/F) residue that is conserved in many, but not all, SCRs (Fig. 1) appears to have a key role in stabilizing this loop, in part through its hydrophobic contact with C7. The conserved glycine in the G x C2 motif apparently facilitates the formation of a stable type-II β-turn in the flanking segment, connecting the β1 strand to the α-helix.

Structure–function Studies of SCRs

Structure–function studies of SCR variants have two aims: (i) to determine which residues, especially those conserved in most SCRs, are important for SCR function and (ii) to identify sequences that determine recognition specificity. While working with proteins encoded by the S_6 and S_{13} haplotypes

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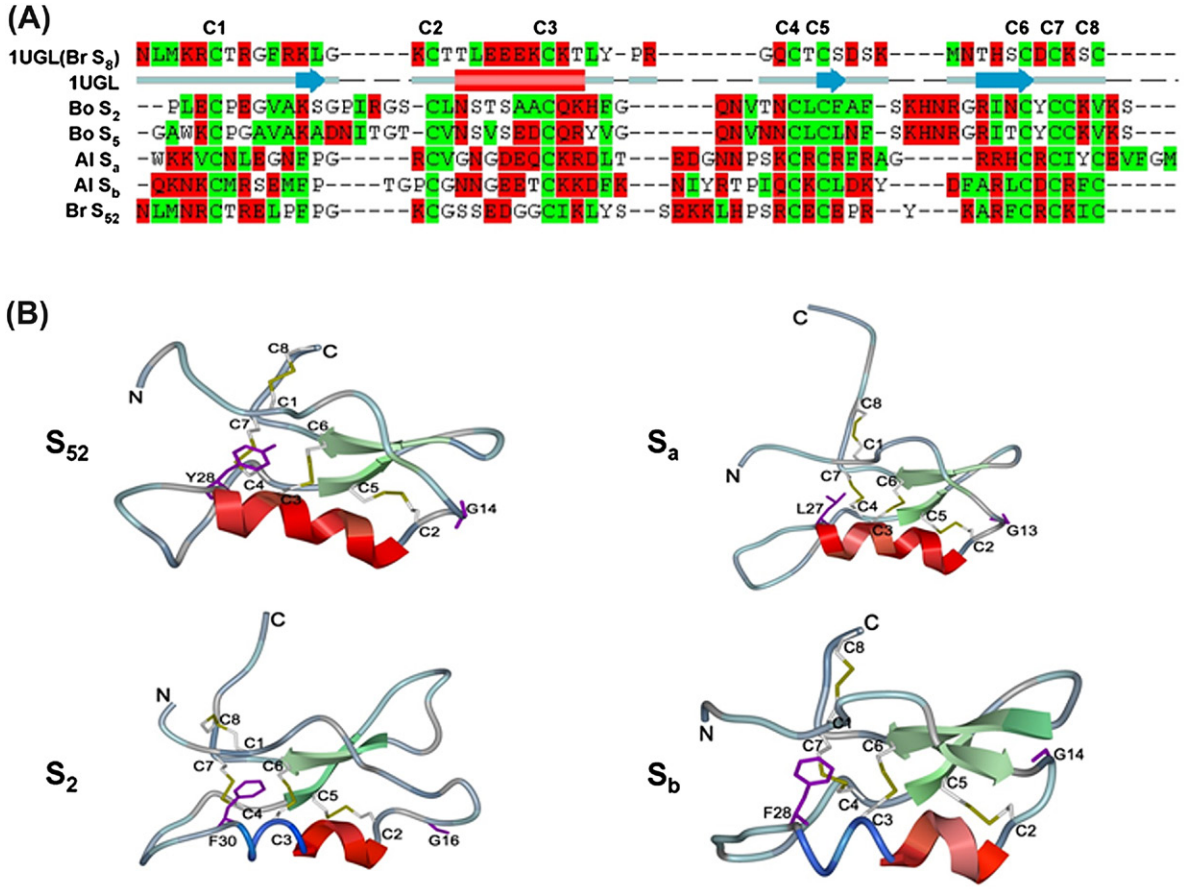

FIGURE 2 Predicted conservation of secondary structure in SCR variants. (**A**) Structure-based sequence alignment of SCR variants from *B. rapa* (Br) S_{52}, *B. oleracea* (Bo) S_2 and *S5*, and *A. lyrata* (Al) S_a and S_b. The NMR structure (model 1) of the *B. rapa* S_8 variant[13] (PDB code: 1UGL) was used as template. The secondary structure of the experimental model was computed by the method of Kabasch and Sanders.[5] The rectangle in the "1UGL" line depicts the α-helix and the arrows show the β-strands. Hydrophilic residues are shaded in dark gray and hydrophobic residues are shaded in light gray. The conserved C1–C8 residues are shown above the alignment. (**B**) Three-dimensional (3D) models of the SCR variants encoded by the *Brassica* S_{52} and S_2 and the *A. lyrata* S_a, and S_b haplotypes. These predicted models were generated with the program MODELLER[5] based on the S_8 SCR NMR structure (PDB code: 1UGL) determined by Mishima et al.[13] and the alignments given in A. The 3D models show the N/C termini and the location of the conserved cysteine residues (C1–C8) and glycine (G) in the G x C2 motif. Lines connecting pairs of cysteines depict the disulfide bridges. The α-helix is shown as a dark gray coiled ribbon and β-sheets are depicted by light gray arrows. The aromatic residue in the C3xxxY/F motif found in most SCRs is shown in stick representation for SCR variants of S_{52} (Y28), S_2 (F30), and S_b (F28). SCR_a contains a lysine (L27) at the corresponding position. See color plate 7.

of *B. oleracea*, SCR sequence variants were generated by site-directed mutagenesis of individual residues in SCR_6 or by swapping one or more regions between SCR_6 and SCR_{13}.[5] The mutated or chimeric SCRs were expressed in *E. coli* and tested *in vitro* for binding to the $eSRK_6$ and $eSRK_{13}$ and *in vivo* for activation of the SI response on stigmas.[5] These studies showed that the SCR function was abrogated by mutations of the conserved cysteine residues and the tyrosine residue in the C3xxxY/F motif, probably because these mutations disrupt the SCR structure. Surprisingly, mutation of the glycine residue in the G x C2 motif was tolerated. Analysis of SCR_6–SCR_{13} chimeras showed that SCR_{13} specificity resides in four contiguous amino acids within the C5–C6 loop.[5] However, the specificity-determining residues of SCR_6 could not be identified by this approach, possibly because noncontiguous residues in this variant determine specificity. These results indicate that residues imparting specificity to SCR can be arranged differently in different SCR variants. This conclusion is supported by *in vivo* studies using transgenic plants, which suggested that the C2–C3 and C5–C6 regions harbor the specificity determinants of the SCR variant encoded by the S_{52} haplotype of *B. rapa*, with some contribution from other regions.[20] Taken together, these studies point to the C5–C6 loop region as an important region for recognition specificity, in at least two SCR variants.

BIOLOGICAL ACTIONS OF SCR AND SI SIGNALING

SCR is the pollen determinant of specificity in the SI response, and it functions as a ligand for the stigma SRK receptor. Although the SCR protein is located in the pollen

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coat, the outermost surface of the pollen grain that makes initial contact with the stigma, the site of SCR activity is at the plasma membrane of the stigma epidermis, where its specific interaction with its SRK receptor determines the fate of a pollen grain. In self-pollination, the binding of SCR to its cognate "self" SRK results in autophosphorylation of the receptor[31] and activation of the SI-signal transduction pathway within the stigma epidermal cell, resulting in inhibition of pollen hydration, germination, and penetration of pollen tube into the papilla cell wall. In crosspollination, SCR cannot bind nor activate "nonself" SRK (i.e., an SRK encoded in an *S* haplotype different from that which encodes SCR), the signal transduction pathway is not activated, and pollen tube growth proceeds. Thus, SCR may be thought of as the molecular tag on pollen that allows the stigma epidermal cell to perceive, through its SRK, a pollen grain as being a "self" grain that must be arrested and prevented from hydrating, elaborating a pollen tube, and effecting fertilization.

The details of the SI-signal transduction pathway are not fully understood. Three proteins have been proposed to function as downstream effectors of SRK signaling in *Brassica*: the membrane-associated kinase MLPK, the ARM-repeat containing ARC1, which has E3 ubiquitin ligase activity and interacts with the SRK kinase [reviewed in Ref. 32], and the ARC1 interactor, Exo70A1, a component of the exocyst, which might be involved in the secretion of the factors required for pollen tube growth.[19] A model of SI signaling was proposed[19] in which interaction of the SRK ectodomain with its cognate SCR ligand causes autophosphorylation of the receptor, and the phosphorylated SRK, together with MLPK, then interacts with and phosphorylates ARC1. Activated ARC1 would subsequently target Exo70A1 for degradation, thus inhibiting the secretion of "compatibility" factors and preventing pollen tube formation. However, analysis of the *A. thaliana* orthologs of *MLPK*, *ARC1*, and *Exo70A1* failed to show a role for these genes in the SI response of *A. thaliana SRK–SCR* transformants, indicating that if these genes do function as reported in *Brassica*, their requirement for self-incompatibility is not universal.[17]

THE *SCR*-LIKE (*SCRL*) GENE FAMILY IN PLANTS

In most genome annotations of sequenced plants, genes encoding small peptides are routinely ignored. The difficulty in identifying these genes, including genes that encode SCR-like (SCRL) secreted peptides, stems primarily from the fact that gene-finding algorithms often ignore small ORFs (encoding < 50 amino acids) for which empirical evidence of expression is lacking. The identification of *SCRLs* in particular presents two additional difficulties. The first relates to the gene structure of *SCRL* genes, in which the signal sequence with its initiating ATG codon, is separated from the rest of the coding region by an intron. The second difficulty results from the fact that *SCR* alleles exhibit a high degree of sequence polymorphism (Fig. 1) and standard searches based on sequence homology are not suitable for identifying related sequences. Nevertheless, 28 *SCRL* genes that fall into 7 groups on the basis of sequence similarity (Fig. 3), were identified in the *A. thaliana* genome by iterative searches with the tBLASTN program using sequences of the seven most diverse SCR alleles and three pollen coat proteins from *Brassica* species.[34] Most *A. thaliana SCRLs* are predicted to encode ORFs containing a signal peptide, the conserved cysteine residues, the glycine in the G x C2 motif, and the aromatic amino acid in the C3xxxY/F motif found in most SCRs (Fig. 1). Three *SCRLs*, however, lack some of these conserved residues and are inferred to be nonfunctional. A substantial fraction of the *SCRL* genes are arranged in tandem in the genome, and these closely linked genes share relatively high sequence similarity, suggesting that they may have redundant functions.

Of 25 potentially functional SCRLs, none have been assigned a biological function. Furthermore, not much information is currently available regarding their expression patterns and the cells in which they are expressed. A search of ESTs, cDNAs, and microarray datasets available in the public domain identifies cDNAs and ESTs for six *SCRL* genes (At1g60986, At1g60987, At1g60985, At1g60989, At4g15735, At3g27503), most of which exhibit flower-specific expression patterns. Among these genes, only At1g60985 is represented on the ATH1 whole genome array, and expression data again indicate that this gene is expressed preferentially in floral organs, with the highest expression in carpels. Another *SCRL* gene, At1g65113, is represented only by ESTs in the NCBI database (http://www.ncbi.nlm.nih.gov/). A search of the MPSS database (http://mpss.udel.edu/at/), which harbors sequence information for small RNAs generated using Massively Parallel Signature Sequencing (MPSS), confirmed the expression of several *SCRL* genes (At1g65113, At1g60987, At1g60989, At1g60986) and additionally identified four *SCRL* genes (At4g10115, At4g32717, At4g22105, and At2g25685) for which no expression data were previously known. As shown in Fig. 4, these results show that 11 of the 25 apparently functional *SCRL* genes exhibit differential expression in various tissues, with the majority being predominantly expressed in flowers and thus possibly functioning in some aspect of reproduction. Furthermore, several *SCRL* genes have overlapping expression profiles, suggesting possible functional redundancy. Additional information related to gene expression, subcellular localization of the gene product, and mutant phenotypes is required to elucidate the biological function of the SCRL peptides.

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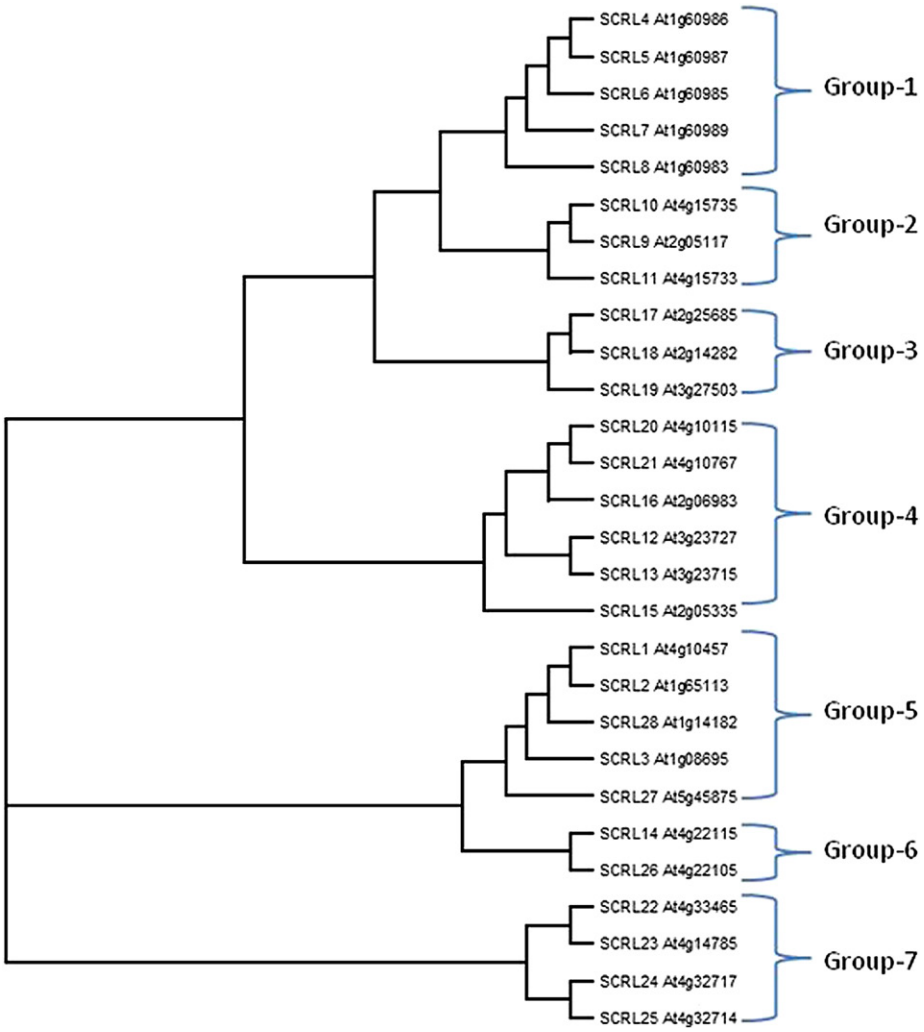

FIGURE 3 Grouping of *A. thaliana SCRL* genes based on phylogenetic relationships. The multiple sequence alignment of the predicted full-length SCRL proteins was generated using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/), and the tree was plotted using TreeView (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html). SCRL sequences were retrieved from the TAIR database (http://www.arabidopsis.org/).

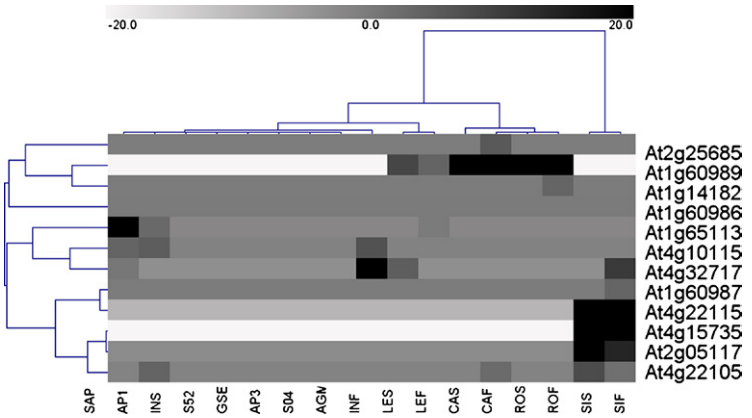

FIGURE 4 Analysis of *SCRL* gene expression using MPSS data.[8] Hierarchical clustering of gene expression pattern is based on Pearson correlation. The highest levels of upregulation and downregulation are indicated in black and white shading respectively. **SAP** – sup/ap1 inflorescence; **AP1** – ap1-10 inflorescence; **INS** – Inflorescence, signature MPSS, **S52** – Leaves, 52h after salicylic acid treatment**; GSE** – Germinating seedlings; **AP3** – inflorescence; **S04** – Leaves, 4h after salicylic acid treatment; **AGM** – agamous inflorescence; **INF** – Inflorescence - buds, classic MPSS; **LES** – Leaves – 21 day, untreated; **CAS** – Callus – actively growing, signature MPSS; **CAF** – Callus - actively growing, classic MPSS; **ROS** – Root – 21 day, untreated; **ROF** – root – 21 day, untreated, classic MPSS; **SIS** – Silique –24–48h postfertilization, signature MPSS; **SIF** – Silique –24–48h postfertilization, classic MPSS.

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ACKNOWLEDGMENTS

S. Naithani and P. Dharmawardhana are supported partially by the Gramene database project (NSF-IOS #0703908) and the plant ontology project (NSF-IOS #0822201). Work in the Nasrallah laboratory is supported by NSF grant IOS #0744579.

REFERENCES

1. Boggs NA, Dwyer KG, Nasrallah ME, Nasrallah JB. In vivo detection of residues required for ligand-selective activation of the S-locus receptor in Arabidopsis. *Curr Biol* 2009;**19**:786–91.
2. Boggs NA, Dwyer KG, Shah P, McCulloch AA, Bechsgaard J, Schierup MH, et al. Expression of distinct self-incompatibility specificities in *Arabidopsis thaliana*. *Genetics* 2009;**182**:1313–21.
3. Boggs NA, Nasrallah JB, Nasrallah ME. Independent S-locus mutations caused self-fertility in *Arabidopsis thaliana*. *PLoS Genet* 2009;**5**:e1000426.
4. Boyes DC, Nasrallah ME, Vrebalov J, Nasrallah JB. The self-incompatibility (S) haplotypes of *Brassica* contain highly divergent and rearranged sequences of ancient origin. *Plant Cell* 1997;**9**:237–47.
5. Chookajorn T, Kachroo A, Ripoll DR, Clark AG, Nasrallah JB. Specificity determinants and diversification of the *Brassica* self-incompatibility pollen ligand. *Proc Natl Acad Sci U S A* 2004;**101**:911–7.
6. Cui Y, Brugiere N, Jackman L, Bi YM, Rothstein SJ. Structural and transcriptional comparative analysis of the S locus regions in two self-incompatible *Brassica napus* lines. *Plant Cell* 1999;**11**:2217–31.
7. Dixit R, Nasrallah ME, Nasrallah JB. Post-transcriptional maturation of the S receptor kinase of *Brassica* correlates with co-expression of the S-locus glycoprotein in the stigmas of two *Brassica* strains and in transgenic tobacco plants. *Plant Physiol* 2000;**124**:297–311.
8. German MA, Pillay M, Jeong D-H, Hetawal A, Luo S, Janardhanan P, et al. Global identification of microRNA–target RNA pairs by parallel analysis of RNA ends. *Nat Biotech* 2008;**26**:941–6.
9. Giranton JL, Ariza MJ, Dumas C, Cock JM, Gaude T. The S locus receptor kinase gene encodes a soluble glycoprotein corresponding to the SRK extracellular domain in *Brassica oleracea*. *Plant J* 1995;**8**:827–34.
10. Kachroo A, Schopfer CR, Nasrallah ME, Nasrallah JB. Allele-specific receptor–ligand interactions in *Brassica* self-incompatibility. *Science* 2001;**293**:1824–6.
11. Kusaba M, Dwyer K, Hendershot J, Vrebalov J, Nasrallah JB, Nasrallah ME. Self-incompatibility in the genus Arabidopsis: characterization of the S locus in the outcrossing *A. lyrata* and its autogamous relative *A. thaliana*. *Plant Cell* 2001;**13**:627–43.
12. Kusaba M, Tung CW, Nasrallah ME, Nasrallah JB. Monoallelic expression and dominance interactions in anthers of self-incompatible *Arabidopsis lyrata*. *Plant Physiol* 2002;**128**:17–20.
13. Mishima M, Takayama S, Sasaki K, Jee JG, Kojima C, Isogai A, et al. Structure of the male determinant factor for *Brassica* self-incompatibility. *J Biol Chem* 2003;**278**:36389–95.
14. Naithani S, Chookajorn T, Ripoll DR, Nasrallah JB. Structural modules for receptor dimerization in the S-locus receptor kinase extracellular domain. *Proc Natl Acad Sci U S A* 2007;**104**:12211–6.
15. Nasrallah ME, Liu P, Nasrallah JB. Generation of self-incompatible *Arabidopsis thaliana* by transfer of two S locus genes from *A. lyrata*. *Science* 2002;**297**:247–9.
16. Nasrallah ME, Liu P, Sherman-Broyles S, Boggs NA, Nasrallah JB. Natural variation in expression of self-incompatibility in *Arabidopsis thaliana*: implications for the evolution of selfing. *Proc Natl Acad Sci U S A* 2004;**101**:16070–4.
17. Rea AC, Liu P, Nasrallah JB. A transgenic self-incompatible *Arabidopsis thaliana* model for evolutionary and mechanistic studies of crucifer self-incompatibility. *J Exp Bot* 2010;**61**:1897–906.
18. Sainudiin R, Wong WS, Yogeeswaran K, Nasrallah JB, Yang Z, Nielsen R. Detecting site-specific physicochemical selective pressures: applications to the Class I HLA of the human major histocompatibility complex and the SRK of the plant sporophytic self-incompatibility system. *J Mol Evol* 2005;**60**:315–26.
19. Samuel MA, Chong YT, Haasen KE, Aldea-Brydges MG, Stone SL, Goring DR. Cellular pathways regulating responses to compatible and self-incompatible pollen in Brassica and Arabidopsis Stigmas Intersect at Exo70A1, a putative component of the exocyst complex. *Plant Cell* 2009;**21**:2655–71.
20. Sato Y, Okamoto S, Nishio T. Diversification and alteration of recognition specificity of the pollen ligand SP11/SCR in self-incompatibility of Brassica and Raphanus. *Plant Cell* 2004;**16**:3230–41.
21. Schopfer CR, Nasrallah ME, Nasrallah JB. The male determinant of self-incompatibility in Brassica. *Science* 1999;**286**:1697–700.
22. Shiba H, Iwano M, Entani T, Ishimoto K, Shimosato H, Che FS, et al. The dominance of alleles controlling self-incompatibility in *Brassica* pollen is regulated at the RNA level. *Plant Cell* 2002;**14**:491–504.
23. Shiba H, Kakizaki T, Iwano M, Tarutani Y, Watanabe M, Isogai A, et al. Dominance relationships between self-incompatibility alleles controlled by DNA methylation. *Nat Genet* 2006;**38**:297–9.
24. Shiba H, Park JI, Suzuki G, Matsushita M, Nou IS, Isogai A, et al. Duplicated SP11 genes produce alternative transcripts in the S15 haplotype of *Brassica oleracea*. *Genes Genet Syst* 2004;**79**:87–93.
25. Shiba H, Takayama S, Iwano M, Shimosato H, Funato M, Nakagawa T, et al. A pollen coat protein, SP11/SCR, determines the pollen S-specificity in the self-incompatibility of *Brassica* species. *Plant Physiol* 2001;**125**:2095–103.
26. Stein JC, Dixit R, Nasrallah ME, Nasrallah JB. SRK, the stigma-specific S locus receptor kinase of *Brassica*, is targeted to the plasma membrane in transgenic tobacco. *Plant Cell* 1996;**8**:429–45.
27. Stein JC, Howlett B, Boyes DC, Nasrallah ME, Nasrallah JB. Molecular cloning of a putative receptor protein kinase gene encoded at the self-incompatibility locus of *Brassica oleracea*. *Proc Natl Acad Sci U S A* 1991;**88**:8816–20.
28. Suzuki G, Kai N, Hirose T, Fukui K, Nishio T, Takayama S, et al. Genomic organization of the S locus: identification and characterization of genes in SLG/SRK region of S(9) haplotype of *Brassica campestris* (syn. *rapa*). *Genetics* 1999;**153**:391–400.
29. Takasaki T, Hatakeyama K, Suzuki G, Watanabe M, Isogai A, Hinata K. The S receptor kinase determines self-incompatibility in *Brassica* stigma. *Nature* 2000;**403**:913–6.
30. Takayama S, Shiba H, Iwano M, Shimosato H, Che FS, Kai N, et al. The pollen determinant of self-incompatibility in *Brassica campestris*. *Proc Natl Acad Sci U S A* 2000;**97**:1920–5.
31. Takayama S, Shimosato H, Shiba H, Funato M, Che FS, Watanabe M, et al. Direct ligand–receptor complex interaction controls *Brassica* self-incompatibility. *Nature* 2001;**413**:534–8.

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32. Tantikanjana T, Nasrallah ME, Nasrallah JB. Complex networks of self-incompatibility signaling in the Brassicaceae. *Curr Opin Plant Biol* 2010;**13**:520–6.
33. Tarutani Y, Shiba H, Iwano M, Kakizaki T, Suzuki G, Watanabe M, et al. Trans-acting small RNA determines dominance relationships in Brassica self-incompatibility. *Nature* 2010;**466**:983–6.
34. Vanoosthuyse V, Miege C, Dumas C, Cock JM. Two large *Arabidopsis thaliana* gene families are homologous to the *Brassica* gene superfamily that encodes pollen coat proteins and the male component of the self-incompatibility response. *Plant Mol Biol* 2001;**46**:17–34.
35. Yang Y-W, Lai K-N, Tai P-Y, Ma D-P, Li W-H. Molecular phylogenetic studies of *Brassica, Rorippa, Arabidopsis* and allied genera based on the internal transcribed spacer region of 18S-25S rDNA. *Mol Phylogenet Evol* 1999;**13**:455–62.

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Chapter 13

Stomagen/EPFL9

Tomoo Shimada, Shigeo S. Sugano and Ikuko Hara-Nishimura

ABSTRACT

The stomata are valves found on aerial plant organs that are responsible for gas exchange between the atmosphere and the body of the plant. The two-dimensional pattern and the density of stomata are controlled by cysteine-rich peptides of the EPIDERMAL PATTERNING FACTOR (EPF) family. Stomagen/EPFL9 functions as a positive regulator, while EPF1, EPF2, and CHALLAH/EPFL6 function as negative regulators of stomatal development. Stomagen is a 45 amino acid peptide with three intramolecular disulfide bonds. The *STOMAGEN* gene is predominantly expressed in immature organs, including leaves, stems, and flower buds, where stomatal development occurs. Stomagen is produced in internal tissues and migrates to the epidermis, where it acts as an intercellular positive signal. Possible receptors for stomagen are the cell surface TMM and ERf receptors, both of which are also required for response to the negative signaling peptides, EPF1 and EPF2. These findings suggest that stomatal development is finely regulated by competitive binding of positive and negative peptides to the same receptor.

DISCOVERY

Stomagen/EPFL9 is a positive signaling factor for stomatal development. In 2010, the *STOMAGEN/EPFL9* gene was independently discovered by three research groups. Sugano et al.[24] and Hunt et al.[7] identified this gene among genes co-expressed with known stomatal regulators in *Arabidopsis*, while Kondo et al.[11] identified the gene among *Arabidopsis* genes encoding novel secreted peptides that influence plant development. *STOMAGEN*-like genes are found in various plants (Fig. 1A), including poplar, rice, and *Selaginella moellendorffii*, a primitive vascular plant, suggesting that stomagen is a conserved signaling molecule, which regulates stomatal density in vascular plants.

The *STOMAGEN* gene encodes a cysteine-rich secreted peptide that belongs to the EPIDERMAL PATTERNING FACTOR (EPF) peptide family.[6,10,20] In *Arabidopsis*, 11 EPF family peptides have been identified. Three of these family members, EPF1,[5] EPF2,[6,8] and CHALLAH/EPFL6,[1] have been shown to negatively regulate stomatal development. Overexpression experiments indicate that EPFL4 and EPFL5 are also capable of inhibiting stomatal development[6] but their endogenous functions remain to be determined. So far, stomagen is the only known positive signaling factor of stomatal development among EPF family peptides.

STRUCTURE OF THE PEPTIDE

The *STOMAGEN* gene encodes a small polypeptide of 102 amino acids with a putative signal peptide (SP) at the N-terminus (Fig. 1B). The mature form of the *STOMAGEN* gene product, stomagen, is a peptide consisting of the C-terminal 45 amino acids of the precursor protein.[11,24] In the mature peptide, there are six conserved cysteine residues that form three intramolecular disulfide bonds, Cys8–Cys41, Cys13–Cys20, and Cys16–Cys43 (Fig. 1B).[11] Just upstream of the cleavage site of stomagen, a putative dibasic signal (Arg–Arg or Lys–Arg) is well conserved among various plant species (Fig. 1A). Mature stomagen appears to lack posttranslational modifications, such as sulfation or hydroxylation, both of which are often found in other plant signaling peptides, as revealed by mass spectrometry analysis of endogenous stomagen from the apoplastic fluid of *STOMAGEN* overexpressors.[11] Indeed, a chemically synthesized 45 amino acid stomagen polypeptide exhibits stomata-inducing activity.[11,24] It is probable that other EPF family peptides are also subject to similar posttranslational processing, although the mature forms have not been determined. In summary, the *EPF* family genes, including *STOMAGEN*, are predicted to encode a preproprotein that consists of three domains, an SP, a proprotein region, and the mature peptide.

DISTRIBUTION OF THE mRNA/PEPTIDE

The *STOMAGEN* gene is predominantly expressed in immature organs, including leaves, stems, and flower buds,[7,11,24] which enable the gene to be identified by a co-expression analysis. Other stomatal regulators exhibit similar expression patterns. The expression patterns of the *STOMAGEN* gene are consistent with the fact that stomatal development occurs in these immature organs. Relatively low expression of the *STOMAGEN* gene was also observed in the root;[24] however, the physiological function of stomagen in the root is unknown.

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00013-0
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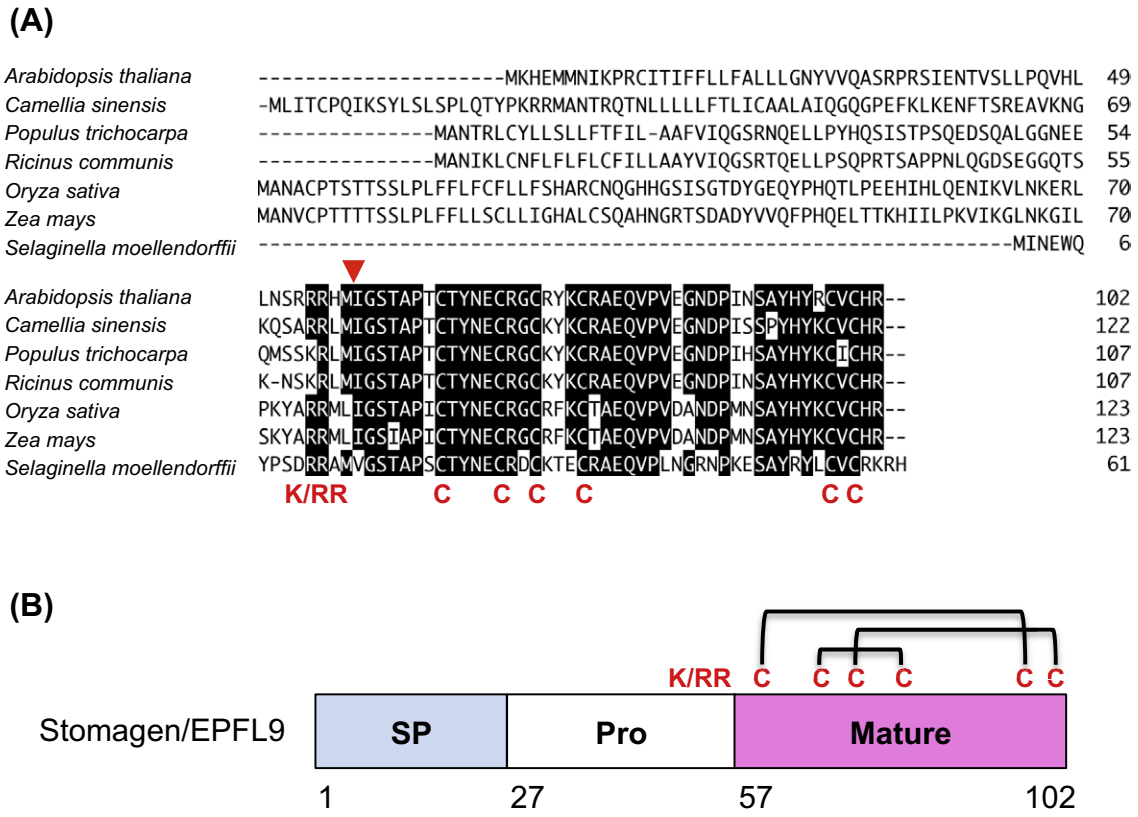

FIGURE 1 Stomagen/EPFL9 is a conserved cysteine-rich peptide. (A) A multiple alignment of stomagen/EPFL9 precursors from various plants, *Arabidopsis thaliana* (GI:15235542), *Camellia sinensis* (GI:330318758), *Populus trichocarpa* (GI:224065439), *Oryza sativa* (GI:115441801), *Zea mays* (GI:195649933), and *S. moellendorffii* (GI:302764960). The N-terminus of *Arabidopsis* mature stomagen is shown by an arrowhead. A putative cleavage signal (K/RR) and six conserved cysteine residues (C) are shown. Sequences are aligned by Clustal W and conserved amino acids among the sequences are shown in reverse. (B) Schematic representation of the domain structure of stomagen/EPFL9 precursor. The precursor is composed of the SP, the propeptide (Pro), and the mature domain. Three disulfide bridges of stomagen are indicated by solid lines. Amino acid positions are indicated below the structure.

Interestingly, the *STOMAGEN* gene is expressed in the internal tissues rather than the epidermis, where the stomata develop.[11,24] This *STOMAGEN* expression pattern is in sharp contrast to the expression of other stomatal regulators, which are expressed primarily in the epidermis or in the stomatal lineage cells. This finding indicates the existence of inter-tissue signaling that regulates stomatal development. Stomagen is predicted to be secreted from cells into the apoplast. In transgenic *Arabidopsis* expressing a STOMAGEN–Venus fusion protein, fluorescence was indeed observed outside the cells.[24] These findings indicate that stomagen is produced in internal tissues and migrates to the epidermis, where it acts as a positive signal for stomatal development.

BIOLOGICAL FUNCTIONS

The biological function of stomagen is to control the stomatal density or the stomatal index (defined as the ratio of the number of stomata to the total number of epidermal cells).[7,11,24] Overexpression of *STOMAGEN* increases the number of stomata, and suppression of *STOMAGEN* decreases the number of stomata in *Arabidopsis* (Fig. 2). A quantitative analysis revealed a positive correlation between the level of *STOMAGEN* messenger RNA and stomatal density.[24] Treatment of *Arabidopsis* seedlings with recombinant or chemically synthesized stomagen can induce the formation of stomata in a dose-dependent manner.[11,24] These findings indicate that stomagen is a positive regulator of stomatal development.

Stomata are formed from a protodermal cell, which is an undifferentiated epidermal cell, through a strict cell lineage in the very early stages of leaf development.[3,16] Stomagen appears to positively regulate several steps in the cell lineage during stomatal development.[21] Stomata in most dicotyledonous plants are separated by at least one non-stomatal cell,[21] which is thought to improve the efficiency of gas exchange. *STOMAGEN* overexpression lines exhibited many clustered stomata in mature leaves, indicating that the orientation of cell division in the stomatal lineage is defective in these lines.[7,11,24] The abnormal clustering, however, may be because of the ectopic expression of the *STOMAGEN* gene in the epidermis.

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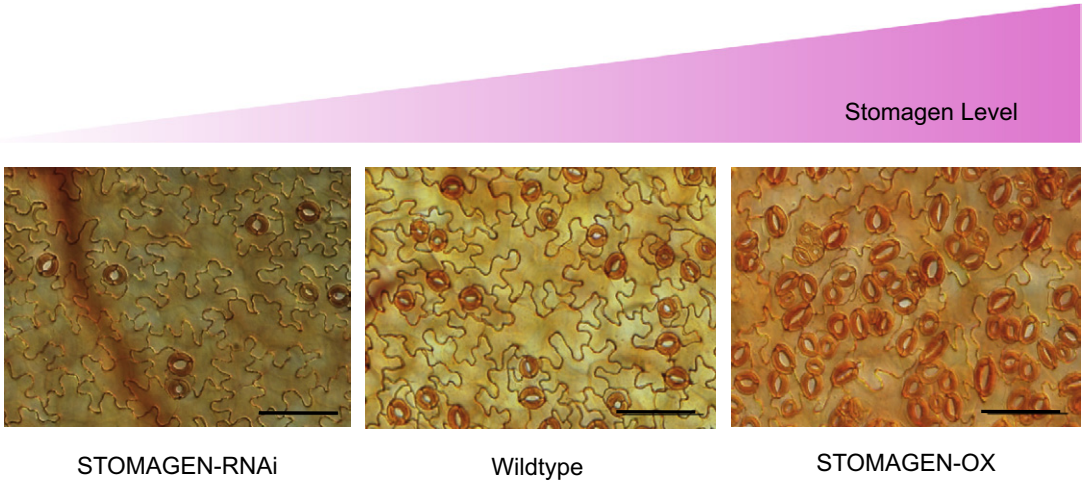

FIGURE 2 Stomagen/EPFL9 positively regulates stomatal density. Differential interference contrast (DIC) images of the abaxial epidermis of mature leaves of wild-type *Arabidopsis*, a *STOMAGEN*-silenced line (STOMAGEN-RNAi), and a *STOMAGEN*-overexpressing line (STOMAGEN-OX). The expected accumulation level of stomagen is illustrated above panels. Stomata were stained with Safranin-O. Scale bars = 100μm. See color plate 8.

RECEPTORS AND SIGNALING CASCADES

Putative receptors for stomagen are predicted to be two classes of cell surface receptors, the receptor-like membrane protein TOO MANY MOUTHS (TMM)[15,26] and three ERECTA family (ERf) receptor-like kinases (ER, ERL1, and ERL2)[23] (Fig. 3). Overexpression or silencing of the *STOMAGEN* gene in a *tmm* mutant did not affect the stomatal density.[24] The application of synthetic stomagen to *tmm* leaves also failed to increase the stomatal density.[11] These results indicate that stomagen requires TMM for the positive regulation of stomatal development. The TMM, ER, ERL1, and ERL2 receptors contain leucine-rich repeat motifs in their extracellular domains. TMM and the ERf proteins are thought to associate with each other in the plasma membrane and, because TMM lacks a cytosolic kinase domain, extracellular peptide signaling is transmitted into the cell by the kinase domain of the ERf.

TMM and ERf receptors have been characterized as negative regulators of stomatal development in leaves, since the loss-of-function of these receptors results in the overproliferation of stomata.[23,26] The possible ligands for these negative receptors are negative signaling peptides, such as EPF1[5] and EPF2.[6,8] EPF1 and EPF2 belong to the same EPF peptide family as stomagen. These observations support an "agonist–antagonist" hypothesis in which the positive peptide, stomagen, and negative peptides, EPF1 and EPF2, act antagonistically on the same receptor(s) during the process of stomatal development (Fig. 3). The *STOMAGEN* gene may have evolved from a negative peptide gene through gene duplication and the alteration of function.[22]

Once extracellular signaling is recognized by cell surface receptors, a mitogen-activated protein kinase (MAPK) cascade within the cell becomes activated, thereby regulating transcription factors (TFs) that promote stomatal development[4] (Fig. 3). A signaling module consisting of YODA (a MAPK kinase), MKK4/5/7/9 (MAPK kinases), and MPK3/6 (MAPKs) has been shown to mediate this signal transduction cascade.[2,12,25] Downstream of the MAPK cascade, two classes of basic helix-loop-helix (bHLH) TFs are involved in the developmental progression of the stomatal cell lineage. One class of bHLH factor contains three paralogous proteins,

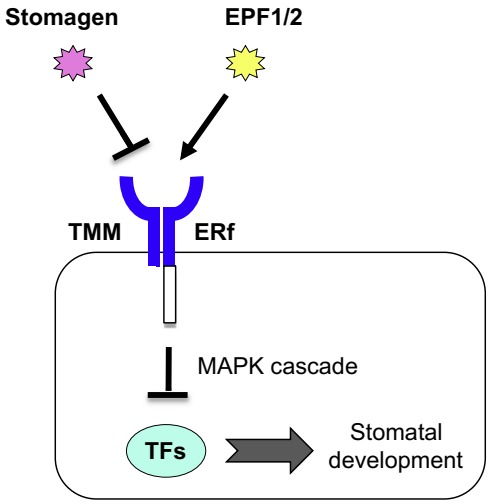

FIGURE 3 A model of peptide signaling for stomatal development in leaves. Stomatal development is promoted byTFs in the absence of receptor signaling and MAPK cascade. The negative peptides, EPF1 and EPF2 (EPF1/2), interact with negative receptors, ERf and TMM, which form a receptor complex at the plasma membrane. The activated ERf receptor transmits negative signal through its kinase domain to the MAPK cascade, which represses the function of TFs. The positive peptide stomagen competes with EPF1/2 for binding the receptor complex to attenuate the negative signal.

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SPEECHLESS (SPCH), MUTE, and FAMA, each of which regulates a distinct step in stomatal development.[14,17,19] The MAPK cascade regulates the phosphorylation status of SPCH, which triggers commitment to the stomatal lineage.[13] Both the overexpression of *STOMAGEN* in an *spch* mutant and the application of purified stomagen to an *spch* mutant failed to induce stomatal formation.[24] These results indicate that the stomata-inducing activity of stomagen is dependent on the SPCH pathway. The second class of bHLH proteins contains SCREAM/ICE1 (SCRM) and SCRM2.[9] The *SCRM* and *SCRM2* genes are expressed throughout the stomatal lineage and their gene products influence stomatal development by forming heterodimers with SPCH, MUTE, or FAMA. Recently, the NMR structure of stomatogen was reported.[18]

REFERENCES

1. Abrash EB, Bergmann DC. Regional specification of stomatal production by the putative ligand CHALLAH. *Development* 2010;**137**: 447–55.
2. Bergmann DC, Lukowitz W, Somerville CR. Stomatal development and pattern controlled by a MAPKK kinase. *Science* 2004;**304**: 1494–7.
3. Bergmann DC, Sack FD. Stomatal development. *Annu Rev Plant Biol* 2007;**58**:163–81.
4. Dong J, Bergmann DC. Stomatal patterning and development. *Curr Top Dev Biol* 2010;**91**:267–97.
5. Hara K, Kajita R, Torii KU, Bergmann DC, Kakimoto T. The secretory peptide gene *EPF1* enforces the stomatal one-cell-spacing rule. *Genes Dev* 2007;**21**:1720–5.
6. Hara K, Yokoo T, Kajita R, Onishi T, Yahata S, Peterson KM, et al. Epidermal cell density is autoregulated via a secretory peptide, EPIDERMAL PATTERNING FACTOR 2 in *Arabidopsis* leaves. *Plant Cell Physiol* 2009;**50**:1019–31.
7. Hunt L, Bailey KJ, Gray JE. The signalling peptide EPFL9 is a positive regulator of stomatal development. *New Phytol* 2010;**186**:609–14.
8. Hunt L, Gray JE. The signaling peptide EPF2 controls asymmetric cell divisions during stomatal development. *Curr Biol* 2009;**19**: 864–9.
9. Kanaoka MM, Pillitteri LJ, Fujii H, Yoshida Y, Bogenschutz NL, Takabayashi J, et al. *SCREAM/ICE1* and *SCREAM2* specify three cell-state transitional steps leading to *Arabidopsis* stomatal differentiation. *Plant Cell* 2008;**20**:1775–85.
10. Katsir L, Davies KA, Bergmann DC, Laux T. Peptide signaling in plant development. *Curr Biol* 2011;**21**:R356–64.
11. Kondo T, Kajita R, Miyazaki A, Hokoyama M, Nakamura-Miura T, Mizuno S, et al. Stomatal density is controlled by a mesophyll-derived signaling molecule. *Plant Cell Physiol* 2010;**51**:1–8.
12. Lampard GR, Lukowitz W, Ellis BE, Bergmann DC. Novel and expanded roles for MAPK signaling in *Arabidopsis* stomatal cell fate revealed by cell type-specific manipulations. *Plant Cell* 2009;**21**: 3506–17.
13. Lampard GR, MacAlister CA, Bergmann DC. *Arabidopsis* stomatal initiation is controlled by MAPK-mediated regulation of the bHLH SPEECHLESS. *Science* 2008;**322**:1113–6.
14. MacAlister CA, Ohashi-Ito K, Bergmann DC. Transcription factor control of asymmetric cell divisions that establish the stomatal lineage. *Nature* 2007;**445**:537–40.
15. Nadeau JA, Sack FD. Control of stomatal distribution on the *Arabidopsis* leaf surface. *Science* 2002;**296**:1697–700.
16. Nadeau JA, Sack FD. Stomatal development in *Arabidopsis*. In: Meyerowitz EM, Somerville CR, editors. *The Arabidopsis book*. Rockville, MD: American Society of Plant Biologists; 2002, doi: 101199/tab0066, http://wwwaspborg/publications/arabidopsis.
17. Ohashi-Ito K, Bergmann DC. *Arabidopsis* FAMA controls the final proliferation/differentiation switch during stomatal development. *Plant Cell* 2006;**18**:2493–505.
18. Ohki S, Takeuchi M, Mori M. The NMR structure of stomagen reveals the basis of stomatal density regulation by plant peptide hormones. *Nature Commun* 2011;**2**:512.
19. Pillitteri LJ, Sloan DB, Bogenschutz NL, Torii KU. Termination of asymmetric cell division and differentiation of stomata. *Nature* 2007;**445**:501–5.
20. Rychel AL, Peterson KM, Torii KU. Plant twitter: ligands under 140 amino acids enforcing stomatal patterning. *J Plant Res* 2010;**123**: 275–80.
21. Sachs T. *Pattern formation in plant tissues*. New York: Cambridge Univ. Press; 1991.
22. Shimada T, Sugano SS, Hara-Nishimura I. Positive and negative peptide signals control stomatal density. *Cell Mol Life Sci* 2011;**68**: 2081–8.
23. Shpak ED, McAbee JM, Pillitteri LJ, Torii KU. Stomatal patterning and differentiation by synergistic interactions of receptor kinases. *Science* 2005;**309**:290–3.
24. Sugano SS, Shimada T, Imai Y, Okawa K, Tamai A, Mori M, et al. Stomagen positively regulates stomatal density in *Arabidopsis*. *Nature* 2010;**463**:241–4.
25. Wang H, Ngwenyama N, Liu Y, Walker JC, Zhang S. Stomatal development and patterning are regulated by environmentally responsive mitogen-activated protein kinases in *Arabidopsis*. *Plant Cell* 2007;**19**:63–73.
26. Yang M, Sack FD. The *too many mouths* and *four lips* mutations affect stomatal production in *Arabidopsis*. *Plant Cell* 1995;**7**:2227–39.

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Chapter 14

TDIF

Yuki Kondo, Yuki Hirakawa and Hiroo Fukuda

ABSTRACT

The plant vascular system is a crucial network for the transport of water, nutrients, and signaling molecules. Small secretory molecules and plant hormones, play roles in vascular network formation. Here, we summarize the biological functions and signaling cascade of TDIF (tracheary element differentiation inhibitory factor) in vascular development. TDIF, a 12-amino acid peptide containing two hydroxyproline residues, was isolated originally as an inhibitory factor of tracheary element differentiation in a *Zinnia* xylogenic cell culture. Further analysis with *Arabidopsis* revealed non-cell-autonomous action of TDIF. TDIF is produced via processing and hydroxylation in phloem cells and is then perceived by procambial cells, including vascular stem cells, through a TDIF receptor (TDR/PXY). The signal both promotes proliferation of the procambial cells and suppresses their differentiation into tracheary elements. Analysis of downstream events clarified that a putative transcription factor, WUSCHEL-related HOMEOBOX 4 (WOX4), mediates TDIF signaling in the promotion of procambial cell proliferation.

DISCOVERY

Cell culture systems have been widely used to isolate key regulators governing plant cell differentiation and proliferation. Fukuda and Komamine (1980) established a xylogenic cell culture in which single mesophyll cells isolated from *Zinnia* leaves transdifferentiate synchronously and at high frequency into tracheary elements (TEs) without intervening cell division.[8] Detailed analysis of the *in vitro* differentiation process has revealed that not only TEs but also other vascular cells such as procambium-like cells and xylem parenchyma cells differentiate from the mesophyll cells.[9] A possible mechanism that allows differentiation into various cell types involves the interaction between cultured cells via signaling molecules secreted into the culture medium. Xylogen, an arabinogalactan protein, was discovered as such a secretory factor promoting TE differentiation in the *Zinnia* xylogenic culture.[22] Further analysis identified an inhibitor of TE differentiation, named TDIF (Fig. 1A).[14] TDIF was extracted with 20% methanol and purified by a poly Sulfoethyl A ion exchange column, tandem Super SW 3000 columns, and an Inertsil ODS3 reversed-phase column. Mass spectrometry and peptide sequencing clarified that TDIF is a dodeca amino acid peptide containing two hydroxylated proline residues.[14] Indeed, chemically synthesized TDIF peptide strongly inhibits TE differentiation at concentrations as low as 30 pM in the *Zinnia* xylogenic culture.[14] The 12 amino acid sequence was found near the C-terminus of a protein encoded by a *CLAVATA3/EMBRYO SURROUNDING REGION-related (CLE)* family gene in *Zinnia*. TDIF is also encoded at the C-termini of two *Arabidopsis CLE* genes, *CLE41* and *CLE44* (Fig. 1B).[14]

STRUCTURE OF THE PEPTIDE

The CLE family of small secretory peptides is conserved among land plants. In the *Arabidopsis* genome, there are 32 genes encoding CLE peptides.[16] CLE proteins contain an N-terminal signal peptide and a conserved C-terminal 14 amino acid CLE domain(s) from which a mature CLE peptide is produced through proteolytic cleavage (Fig. 1B).[14,17] The first identified natural CLE peptide, TDIF, consists of 12 amino acid residues containing two hydroxylated prolines at fourth and seventh residues.[14] The translated TDIF precursor protein is composed of 132 amino acids. TDIF sequence corresponds to H120 to N131. Addition of the C-terminal arginine residue (R132) to TDIF reduces its activity by 1/7th.[14] Therefore, proteolytic cleavage at both the N and C ends of the peptide is required to produce the mature peptide. In *Arabidopsis*, TDIF was identified from apoplast fluid of *CLE44*-overexpressing plants,[25] confirming that TDIF is a product of *CLE44* (Fig. 1B). Secretion of TDIF is shown *in vivo* by an immunohistochemical study using an anti-TDIF antibody.[11]

To deduce the chemical basis of TDIF function, TDIF derivatives were synthesized, and their activities were examined in the *Zinnia* xylogenic culture. Alanine substitution for H_1, V_3, G_6, N_8, P_9, and N_{12} within the 12 amino acid sequence all caused a severe loss of TDIF activity in the *Zinnia* xylogenic culture.[14] By contrast, replacement of the amino acids at positions E_2, S_5, Hyp_7, I_{10}, and S_{11} do not affect TDIF activity.[14] These alanine scanning experiments indicate that H_1, V_3, G_6, N_8, P_9, and N_{12} are the essential

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00014-2
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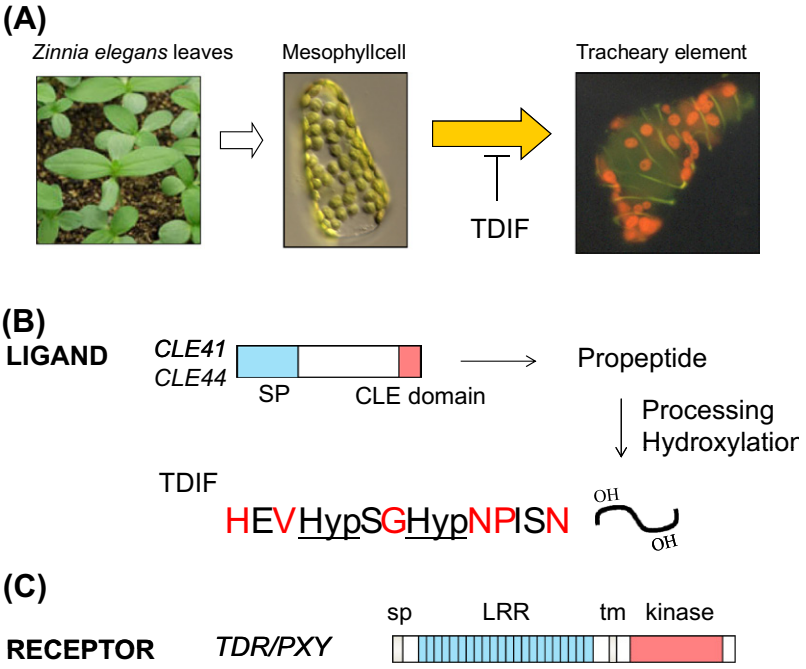

FIGURE 1 Identification and characterization of TDIF. (A) TDIF was isolated as a factor inhibiting TE differentiation from the *Zinnia* xylogenic culture. (B) Maturation of TDIF peptide through translation as a propeptide, processing, and hydroxylation. TDIF is produced as a 12-amino acid peptide with two hydroxyproline residues. Bold characters indicate indispensable amino acids for TDIF activity. (C) Structure of *TDR/PXY* encoding a TDIF receptor, which consists of a signal peptide (sp), leucine-rich-repeat domains (LRR), a transmembrane domain (tm), and a kinase domain. See color plate 9.

residues for TDIF activity (Fig. 1B).[14] Consistently, CLE42 peptide, which differs from TDIF at only one amino acid at the second position, also has an inhibitory effect on TE differentiation.[14]

All CLE peptides isolated so far have hydroxylation of proline residues at the 4th and 7th positions. In addition, CLV3 and CLE2 peptides are further modified with three arabinose residues on Hyp_7.[26] The glycosylation greatly increases the efficiency of binding of the CLE peptides to their receptors, indicating the importance of glycosylation for their biological activities.[26] By contrast, TDIF does not need glycosylation for its full activity. Indeed, alanine scanning analyses of TDIF synthetic peptide indicate the hydroxylation is dispensable for TDIF activity.[14]

DISTRIBUTION OF THE mRNA/PEPTIDE

The vascular system consists of two types of tissue, xylem and phloem, which are spatially separated by the intervening procambium/cambium/vascular meristem. Procambial/cambial cells, which give rise to xylem and phloem cells, behave as stem cells in the vascular meristem (Fig. 2). To ascertain where TDIF is produced *in vivo*, promoter activities of *CLE41* and *CLE44* were examined by use of the β-glucuronidase (GUS) reporter gene in *Arabidopsis*. In roots and hypocotyls, *pCLE41::GUS* activity is restricted to the phloem and the neighboring pericycle cells. *pCLE44::GUS* is expressed in endodermal cells as well as cells in the phloem and the adjacent pericycle.[11] Immunohistochemical analysis using an anti-TDIF antibody showed that TDIF accumulates in the apoplast of phloem cells and their surrounding cells.[11] By contrast, transcripts for *TDR,* which encodes a TDIF receptor, accumulate specifically in procambial cells.[11] These

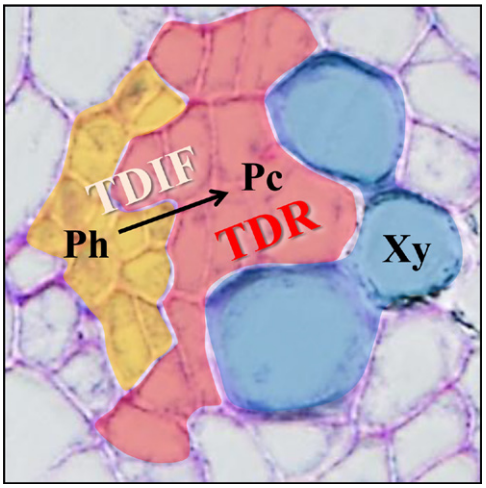

FIGURE 2 Non-cell-autonomous function of the TDIF signal TDIF is secreted from phloem cells and functions in procambial cells in a non-cell-autonomous fashion. Ph, Pc, and Xy indicate phloem, procambium, and xylem, respectively. See color plate 10.

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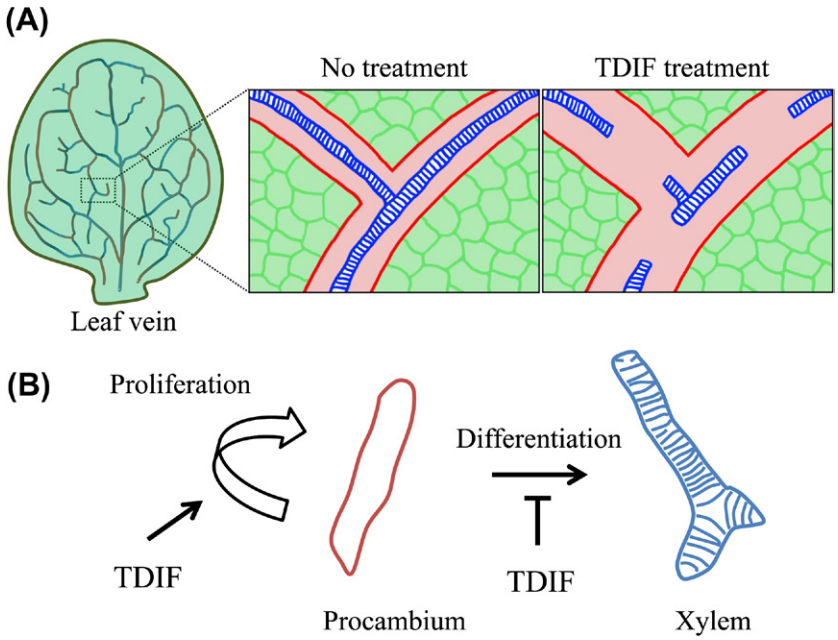

FIGURE 3 Biological functions of TDIF *in vivo*. (A) TDIF inhibits xylem strand formation but not phloem or procambium strand formation in leaf veins. (B) In a cellular level, TDIF promotes procambial cell proliferation and inhibits xylem cell differentiation from procambial cells.

results indicate that TDIF is produced in and secreted from the phloem cells, and then functions in the procambial region in a non-cell-autonomous fashion via the receptor, TDR (Fig. 2).

Interestingly, ectopic expression of *CLE41* under a xylem-specific promoter or the cauliflower mosaic virus 35S promoter leads to disorder of vascular tissues although *CLE41* overexpression under phloem-specific SUC2 promoter did not.[5] This result indicates that the location of TDIF production is essential for correct vascular patterning. Thus, TDIF diffusing from the phloem conveys positional information within the vasculature.

BIOLOGICAL FUNCTIONS

In *Zinnia* xylogenic culture, TDIF strongly inhibits TE differentiation at very low concentrations.[14] Similarly, TDIF application inhibits xylem vessel formation in leaves and hypocotyls in *Arabidopsis*, but it does not affect phloem strand formation.[11] In the region where xylem vessel formation is inhibited, procambial cells are formed normally (Fig. 3A).[11] These results indicate that TDIF specifically inhibits xylem differentiation of procambial cells. TDIF also promotes the proliferation of cells in the *Zinnia* culture[14] and of procambial cells in *Arabidopsis* hypocotyls.[11] These findings strongly suggest that functions in the maintenance of procambial cells by two different mechanisms: inhibiting differentiation of procambial cells into xylem cells and promoting proliferation of procambial cells (Fig. 3B). Indeed, *cle41* mutants often cannot maintain vascular stem cell activity in hypocotyls, resulting in the formation of xylem cells directly adjacent to phloem cells, an arrangement that does not occur in wild type.[12] This TDIF activity for procambial proliferation is greatly enhanced by the simultaneous application of TDIF with some CLE peptides belonging to groups A, B, and D, according to the previous classification.[13,30] CLE42 peptide, containing only one amino acid difference compared with TDIF also has TDIF activity. However, *CLE42* is not expressed in vascular tissues, but rather is expressed strongly in the shoot apical meristem (SAM) and axillary meristems.[31] Overexpression of *CLE42* and *CLE41* (encoding TDIF) enhanced axillary bud formation in leaf and cotyledon axils, in addition to causing xylem vessel discontinuity.[11,29–31]

RECEPTORS AND SIGNALING CASCADES

CLV1 and *CLV3* were identified genetically as key regulators of the SAM organization.[2,7] CLV3 peptide directly binds to the CLV1 receptor, which belongs to class XI of the leucine-rich repeat receptor-like kinase (LRR–RLK) family.[24] In *Arabidopsis*, there are >200 LRR–RLK members.[28] Based on the structural similarity between CLV3 peptide and TDIF, Hirakawa et al. (2008) predicted that TDIF may be recognized by an LRR–RLK.[11] To identify the receptor(s) for TDIF, they first selected about 60 LRR–RLK genes that are predicted to be expressed in procambium/cambium according to the expression data from the public database AtGenExpress and from the *Zinnia* xylogeninc culture and *Arabidopsis* xylogenic culture.[4,19] Screening T-DNA insertion lines for the 60

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LRR–RLK genes resulted in three lines being identified as mutants resistant to TDIF when assayed for xylem differentiation.[11] Each of these lines has a T-DNA insertion in the same gene, which was named *TDR*. TDR, previously designated as PHLOEM INTERCALATED WITH XYLEM (PXY), is a member of the class XI of the LRR–RLK family.[6] TDR protein comprises extracellular LRR domains, a transmembrane domain, and an intracellular kinase domain (Fig. 1C).[11] TDR localizes at the plasma membrane when overexpressed transiently in *Arabidopsis* cultured cells.[11] TDIF but not CLV3 binds to the extracellular LRR domain of TDR produced in BY-2 cells.[11] Loss-of-function mutants for *TDR* show abnormal polarity of vascular tissues and exhaustion of vascular stem cells, similar to the phenotype of *cle41*.[6,11,12]

The CLV3–CLV1–WUS (WUSCHEL) module is a well-known pathway in maintenance of the SAM.[1,21,27] *WOX4*, a member of the *WUSCHEL-related HOMEOBOX* (*WOX*) gene family, is upregulated within an hour upon TDIF application, suggesting that *WOX4* acts downstream of the TDIF–TDR signaling pathway.[12] *wox4* mutants exhibit resistance against TDIF treatment in terms of the promotion of vascular stem cell proliferation but not of inhibition of xylem differentiation.[12] Together with the observation that *WOX4* is expressed specifically in procambial cells,[12,15] it is concluded that *WOX4* is required for TDIF-dependent enhancement of procambial cell proliferation but not for TDIF-dependent suppression of their xylem differentiation (Fig. 4). This idea is supported by the observation that the vascular phenotype of *wox4* mutants differs from that of *tdr* mutants: in *wox4* mutants, procambial cell proliferation is reduced, whereas vascular stem cells still exist at least one cell layer between xylem and phloem cells.[12] These findings indicate that maintenance of vascular stem cells requires not only promotion of procambial cell proliferation but also suppression of xylem cell differentiation, both of which are regulated by TDIF–TDR. Further analysis is required to identify a putative transcription factor that functions in xylem cell differentiation downstream of the TDIF–TDR signaling pathway.

Plant hormones play crucial roles in vascular formation.[9] Cytokinin promotes the proliferation of procambium/cambium,[10,20,23] whereas brassinosteroids are capable of enhancing xylem differentiation.[3,32] A recent study on CLE peptides revealed that group-A CLE peptides including CLE9/10 inhibit protoxylem vessel formation in roots by modulating intracellular cytokinin signaling,[13,18] indicating crosstalk between CLE peptides and plant hormone signaling. Promising future work will help in understanding the crosstalk between the TDIF/CLE signaling pathways and plant hormones in the control of vascular cell differentiation and proliferation (Fig. 4).

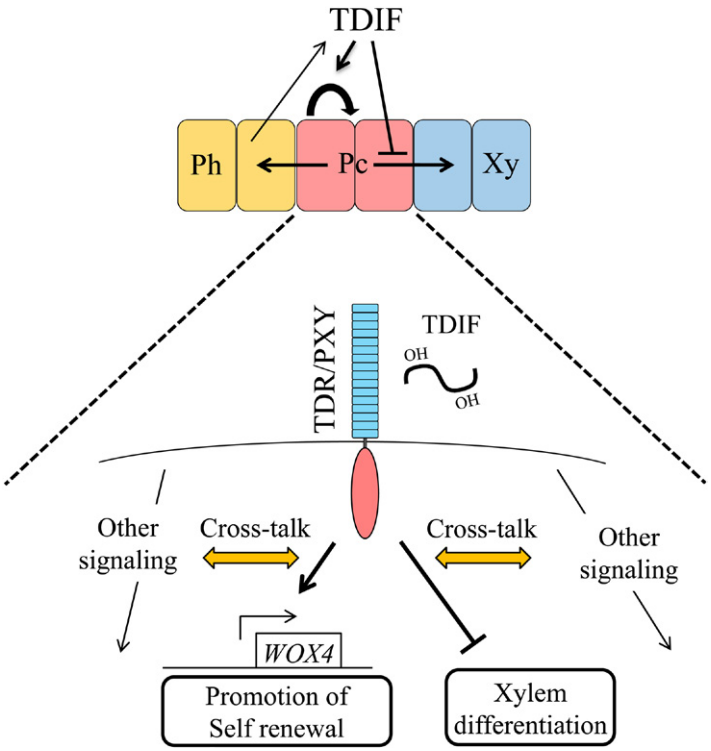

FIGURE 4 Intercellular and Intracellular TDIF–TDR signaling. TDIF is secreted from phloem cells and functions in procambial cells to maintain stem cell activity by both promoting cell division and suppressing differentiation into xylem cells. The former is regulated by a putative transcription factor WOX4.

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ACKNOWLEDGMENTS

The authors thank Dr. John L. Bowman for the critical reading of the manuscript. The authors acknowledge Yuuki Kobayashi for providing an illustration. This work was supported partly by Grants-in-Aid from the Ministry of Education, Science, Sports, and Culture of Japan (19060009) to H. Fukuda, from the Japan Society for the Promotion of Science (23227001 to H. Fukuda, JSPS Research Fellowships for Research Abroad to Y. Hirakawa, JSPS Research Fellowships for Young Scientists to Y. Kondo), and from Bio-oriented Technology Research Advancement Institution (BRAIN) to H. Fukuda.

REFERENCES

1. Brand U, Fletcher JC, Hobe M, Meyerowitz EM, Simon R. Dependence of stem cell fate in Arabidopsis on a feedback loop regulated by CLV3 activity. *Science* 2000;**289**:617–9.
2. Clark SE, Williams RW, Meyerowitz EM. The CLAVATA1 gene encodes a putative receptor kinase that controls shoot and floral meristem size in Arabidopsis. *Cell* 1997;**89**:575–85.
3. Caño-Delgado A, Yin Y, Yu C, Vafeados D, Mora-García S, Cheng JC, et al. BRL1 and BRL3 are novel brassinosteroid receptors that function in vascular differentiation in Arabidopsis. *Development* 2004;**131**:5341–51.
4. Demura T, Tashiro G, Horiguchi G, Kishimoto N, Kubo M, Matsuoka N, et al. Visualization by comprehensive microarray analysis of gene expression programs during transdifferentiation of mesophyll cells into xylem cells. *Proc Natl Acad Sci U S A* 2002;**99**:15794–9.
5. Etchells JP, Turner SR. The PXY-CLE41 receptor ligand pair defines a multifunctional pathway that controls the rate and orientation of vascular cell division. *Development* 2010;**137**:767–74.
6. Fisher K, Turner S. PXY, a receptor-like kinase essential for maintaining polarity during plant vascular-tissue development. *Curr Biol* 2007;**17**:1061–6.
7. Fletcher JC, Brand U, Running MP, Simon R, Meyerowitz EM. Signaling of cell fate decisions by CLAVATA3 in Arabidopsis shoot meristems. *Science* 1999;**283**:1911–4.
8. Fukuda H, Komamine A. Establishment of an experimental system for the study of tracheary element differentiation from single cells isolated from the mesophyll of *Zinnia elegans*. *Plant Physiol* 1980;**65**:57–60.
9. Fukuda H. Signals that control plant vascular cell differentiation. *Nat Rev Mol Cell Biol* 2004;**5**:379–91.
10. Hejátko J, Ryu H, Kim GT, Dobesová R, Choi S, Choi SM, et al. The histidine kinases CYTOKININ-INDEPENDENT1 and ARABIDOPSIS HISTIDINE KINASE2 and 3 regulate vascular tissue development in Arabidopsis shoots. *Plant Cell* 2009;**21**:2008–21.
11. Hirakawa Y, Shinohara H, Kondo Y, Inoue A, Nakanomyo I, Ogawa M, et al. Non-cell-autonomous control of vascular stem cell fate by a CLE peptide/receptor system. *Proc Natl Acad Sci U S A* 2008;**105**:15208–13.
12. Hirakawa Y, Kondo Y, Fukuda H. TDIF peptide signaling regulates vascular stem cell proliferation via the WOX4 homeobox gene in Arabidopsis. *Plant Cell* 2010;**22**:2618–29.
13. Hirakawa Y, Kondo Y, Fukuda H. Establishment and maintenance of vascular cell communities through local signaling. *Curr Opin Plant Biol* 2011;**14**:17–23.
14. Ito Y, Nakanomyo I, Motose H, Iwamoto K, Sawa S, Dohmae N, et al. Dodeca-CLE peptides as suppressors of plant stem cell differentiation. *Science* 2006;**313**:842–5.
15. Ji J, Strable J, Shimizu R, Koenig D, Sinha N, Scanlon MJ. WOX4 promotes procambial development. *Plant Physiol* 2010;**152**:1346–56.
16. Jun JH, Fiume E, Fletcher JC. The CLE family of plant polypeptide signaling molecules. *Cell Mol Life Sci* 2008;**65**:743–55.
17. Kondo T, Sawa S, Kinoshita A, Mizuno S, Kakimoto T, Fukuda H, et al. A plant peptide encoded by CLV3 identified by in situ MALDI-TOF MS analysis. *Science* 2006;**313**:845–8.
18. Kondo Y, Hirakawa Y, Kieber JJ, Fukuda H. CLE peptides can negatively regulate protoxylem vessel formation. *Plant Cell Physiol* 2011;**52**:37–48.
19. Kubo M, Udagawa M, Nishikubo N, Horiguchi G, Yamaguchi M, Ito J, et al. Transcription switches for protoxylem and metaxylem vessel formation. *Genes Dev* 2005;**19**:1855–60.
20. Matsumoto-Kitano M, Kusumoto T, Tarkowski P, Kinoshita-Tsujimura K, Václavíková K, Miyawaki K, et al. Cytokinins are central regulators of cambial activity. *Proc Natl Acad Sci U S A* 2008;**105**:20027–31.
21. Mayer KF, Schoof H, Haecker A, Lenhard M, Jürgens G, Laux T. Role of WUSCHEL in regulating stem cell fate in the Arabidopsis shoot meristem. *Cell* 1998;**95**:805–15.
22. Motose H, Sugiyama M, Fukuda H. A proteoglycan mediates inductive interaction during plant vascular development. *Nature* 2004;**429**:873–8.
23. Nieminen K, Immanen J, Laxell M, Kauppinen L, Tarkowski P, Dolezal K, et al. Cytokinin signaling regulates cambial development in poplar. *Proc Natl Acad Sci U S A* 2008;**105**:20032–7.
24. Ogawa M, Shinohara H, Sakagami Y, Matsubayashi Y. Arabidopsis CLV3 peptide directly binds CLV1 ectodomain. *Science* 2008;**319**:294.
25. Ohyama K, Ogawa M, Matsubayashi Y. Identification of a biologically active, small, secreted peptide in Arabidopsis by in silico gene screening, followed by LC–MS-based structure analysis. *Plant J* 2008;**55**:152–60.
26. Ohyama K, Shinohara H, Ogawa-Ohnishi M, Matsubayashi Y. A glycopeptide regulating stem cell fate in Arabidopsis thaliana. *Nat Chem Biol* 2009;**5**:578–80.
27. Schoof H, Lenhard M, Haecker A, Mayer KF, Jürgens G, Laux T. The stem cell population of Arabidopsis shoot meristems in maintained by a regulatory loop between the CLAVATA and WUSCHEL genes. *Cell* 2000;**100**:635–44.
28. Shiu SH, Karlowski WM, Pan R, Tzeng YH, Mayer KF, Li WH. Comparative analysis of the receptor-like kinase family in Arabidopsis and rice. *Plant Cell* 2004;**16**:1220–34.
29. Strabala TJ, O'Donnell PJ, Smit AM, Ampomah-Dwamena C, Martin EJ, Netzler N, et al. Gain-of-function phenotypes of many CLAVATA3/ESR genes, including four new family members, correlate with tandem variations in the conserved CLAVATA3/ESR domain. *Plant Physiol* 2006;**140**:1331–44.
30. Whitford R, Fernandez A, De Groodt R, Ortega E, Hilson P. Plant CLE peptides from two distinct functional classes synergistically induce division of vascular cells. *Proc Natl Acad Sci U S A* 2008;**105**:18625–30.
31. Yaginuma H, Hirakawa Y, Kondo Y, Ohashi-Ito K, Fukuda H. A novel function of TDIF-related peptides: promotion of axillary bud formation. *Plant Cell Physiol* 2011;**52**:1354–64.
32. Yamamoto R, Fujioka S, Demura T, Takatsuto S, Yoshida S, Fukuda H. Brassinosteroid levels increase drastically prior to morphogenesis of tracheary elements. *Plant Physiol* 2001;**125**:556–63.

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Chapter 15

Cathelicidins

Neeloffer Mookherjee, Kelly L. Brown and Robert E.W. Hancock

ABSTRACT

Cathelicidins are members of a family of protective, anti-infective peptides, also known as host defense or antimicrobial peptides, and are defined by the high degree of conservation of their "pre–pro" precursor sequences and exceptional diversity of sequence and structure in the mature peptide. These endogenous cationic peptides play a key role in innate immune responses and are widely distributed in mammalian species and being recently observed in nonmammalian vertebrates including hagfish, trout, and chicken. They exhibit a variety of functions ranging from direct antimicrobial activity against bacteria, fungi, eukaryotic parasites, and viruses, through to immunomodulatory and anti-inflammatory activities.

DISCOVERY

Cathelicidins are small cationic, amphiphilic peptides consisting of 12–97 amino acids. This family of peptides was first discovered in bovine neutrophils by Zanetti and collaborators in the early 1990s.[35] The term cathelicidin was coined in 1995 to describe this family of cationic, bipartite peptides sharing a conserved DNA and pre–pro protein sequence at the N-terminus that is highly homologous to that upstream of the coding sequence for the protein cathelin, a 96-amino-acid, cathepsin L protease inhibitor from porcine neutrophils. Thus, cathelicidins are paradoxically named based on this homology in the part of the precursor protein involved in secretion and processing, despite the fact that this family has become better known for the anti-infective properties of the mature (processed) protein, and potential protease inhibitory roles of cathelicidins are rarely investigated. Thus, all cathelicidins have a prosequence at their N-terminus as a part of the precursor protein, that is highly homologous, followed by a heterogeneous C-terminal mature peptide (Figure 1).[35]

Cathelicidins were originally described as antimicrobial peptides and include proteins such as pig protegrins and cattle indolicidins that served as templates for some of the most clinically advanced antimicrobial peptide drugs.[22,29] Cathelicidins have some direct antimicrobial activity in dilute media, but there is a broad range of potencies and in some cases antimicrobial activity is almost completely antagonized at the concentrations and salt conditions found physiologically.[1] Cathelicidins also exhibit a wide range of immunomodulatory and anti-inflammatory activities.[3,15,22] Therefore, the term "host defense peptides" can be used for cathelicidins to encompass their diverse biological functionality. Ongoing research to further understand the complex myriad of biological functions of cathelicidins has captured the interest of researchers, particularly in the health sciences.

STRUCTURE OF THE PRECURSOR mRNA/ STRUCTURAL GENE

The cathelicidin gene family can be traced back 300–500 million years,[30] showing the early evolutionary origin of these peptides. Like the defensin family, these peptides are encoded by a single gene that belongs to a gene family with three highly conserved exons. Cathelicidin encoding genes have a homologous 5′ region that encodes for the characteristic conserved cathelin prodomain, enabling the discovery of new cathelicidins by homology. Cathelicidin encoding genes contain four exons.[29] Exons 1–3 encode the conserved signal peptide (29–30 amino acids) and the cathelin-like domain (94–114 amino acids). By contrast, exon 4 is hypervariable, encoding the heterogenous mature peptide (12–100 amino acids) with very limited sequence similarity between species, in addition to the processing site and 3′ UTR (Figure 1). The 5′ flanking sequences upstream of the coding sequence and the promoter region have several consensus sequences for potential binding of transcription factors involved in hematopoiesis and inflammation.[9,29] Therefore, cathelicidin mRNA expression is, where studied, coregulated with (or by) inflammatory responses and other entities of innate immunity. Recent studies have demonstrated several mechanisms for the regulation of cathelicidin gene expression including the presence of splice variants,[9] possible interactions with

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00015-4
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the histone deacetylase complex,[27] and mediation via the vitamin D receptor complex.[31]

DISTRIBUTION OF CATHELICIDINS

Cathelicidins are extensively found in nature in both invertebrates such as hagfish and vertebrates such as fish, birds, reptiles and mammals.[15,29,30] Cathelicidin mRNAs and precursor or mature proteins are widely distributed in different mammalian species (Table 1) and in a variety of tissue types and cellular lineages. In mammals, cathelicidins are widely distributed among various tissue types, including the skin, intestine, oral cavity, cervix, lungs, mucosa, etc., and are found largely in their unprocessed (pre–pro) form in the granules of neutrophils as well as in myeloid precursors, epithelial cells, mast cells, keratinocytes, and lymphocytes. They are found in their processed forms in several bodily fluids including gastric juice, saliva, semen, sweat, plasma, airway surface liquid, and breast milk.[8,22]

The expression of cathelicidin mRNA can be constitutive or inducible and seems to be differentially regulated depending on the lineage and/or differentiation state of particular cell types. For example, the levels of expression of the genes encoding the precursors of human LL-37 and mouse CRAMP peptides are affected by infection (or the inflammation accompanying infection) and/or

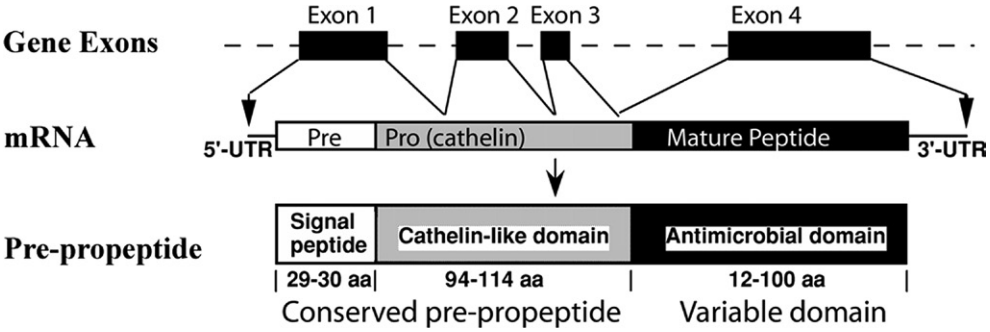

FIGURE 1 Gene structure and processing of cathelicidins (modified from *Circulation* 2004;**109**:1660).

TABLE 1 Distribution of Cathelicidins in Vertebrates

Peptide	Structure	Sequence	Origin
LL-37	α-Helical	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES	Human
RL-37	α-Helical	RLGNFFRKVKEKIGGGLKKVGQKIKDFLGNLVPRTAS	Rhesus monkey
mCRAMP	α-Helical	GLLRKGGEKIGEKLKKIGQKIKNFFQKLVPQPE	Mouse
rCRAMP	α-Helical	GLVRKGGEKFGEKLRKIGQKIKEFFQKLALEIEQ	Rat
CAP18	α-Helical	GLRKRLRKFRNKIKEKLKKIGQKIQGLLPKLAPRTDY	Rabbit
CAP11	α-Helical	GLRKKFRKTRKRIQKLGRKIGKTGRKVWKAWREYGQIPYPCRI	Guinea pig
BMAP-27	α-Helical	GRFKRFRKKFKKLFKKLSPVIPLLHL	Cow
BMAP-28	α-Helical	GGLRSLGRKILRAWKKYGPIIVPIIRI	Cow
BMAP-34	α-Helical	GLFRRLRDSIRRGQQKILEKARRIGERIKDIFR	Cow
SMAP-29	α-Helical	RGLRRLGRKIAHGVKKYGPTVLRIIRIA	Sheep
SMAP-34	α-Helical	GLFGRLRDSLQRGGQKILEKAERIWCKIKDIFR	Sheep
PMAP-23	α-Helical	RIIDLLWRVRRPQKPKFVTVWVR	Pig
PMAP-36	α-Helical	GRFRRLRKKTRKRLKKIGKVLKWIPPIVGSIPLGC	Pig
PMAP-37	α-Helical	GLLSRLRDFLSDRGRRLGEKIERIGQKIKDLSEFFQS	Pig
eCATH-1	α-Helical	KRFGRLAKSFLRMRILLPRRKILLAS	Horse

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TABLE 1 Distribution of cathelicidins in vertebrates—*Cont'd*

Peptide	Structure	Sequence	Origin
eCATH-2	α-Helical	KRRHWFPLSFQEFLEQLRRFRDQLPFP	Horse
eCATH-3	α-Helical	KRFHSVGSLIQRHQQMIRDKSEATRHGIRIITRPKLLLAS	Horse
Dog CATH	α-Helical	KKIDRLKELITTGGQKIGEKIRRIGQRIKDFFKNLQPREEKS	Dog
feCATH	α-Helical	QLGELIQQGGQKIVEKIQKIGQRIRDFFSNLRPRQEA	Cat
HFIAP-1	α-Helical	GFKKAWRKVKHAGRRVLDTAKGVGRHYVNNWLNRYR	Hagfish
HFIAP-3	α-Helical	GWFKKAWRKVKNAGRRVLKGVGIHYGVGLI	Hagfish
Bac4	Extended	RRLHPQHQRFPRERPWPKPLSLPLPRPGPRPWPKPL	Cow
Bac5	Extended	RFRPPIRRPPIRPPFYPPFRPPIRPPIFPPIRPPFRPPLGPFP	Cow
Bac7	Extended	RRIRPRPPRLPRPRPRPLPFPRPGPRPIPRPLPFPRPGPRPIPRPLPFPRP-GPRPIPRPL	Cow
OaBac5	Extended	RFRPPIRRPPIRPPFRPPFRPPVRPPIRPPFRPPFRPPIGPFP	Sheep
OaBac6	Extended	RRLRPRHQHFPSERPWPKPLPLPLPRPGPRPWPKPLPLPLPRP-GLRPWPKPL	Sheep
OaBac7.5	Extended	RRLRPRRPRLPRPRPRPRPRPRSLPLPRPQPRRIPRPILLPWRPPRP-IPRPQIQPIPRWL	Sheep
OaBac11	Extended	RRLRPRRPRLPRPRPRPRPRPRSLPLPRPKPRPIPRPLPLPRPRPKPIPRPL-PLPRPRPRRIPRPLPLPRPRPRPIPRPLPLPQPQPSPIPRPL	Sheep
Trout CATH	Extended	unknown	Rainbow trout
ChBac5	Extended	RFRPPIRRPPIRPPFNPPFRPPVRPPFRPPFRPPFRPPIGPFP	Goat
PR-39	Extended	RRRPRPPYLPRPRPPPFFPPRLPPRIPPGFPPRFPPRFP	Pig
Prophenin-1	Extended	AFPPPNVPGPRFPPPNFPGPRFPPPNFPGPRFPPPNFPGPRFPPPN-FPGPPFPPPIFPGPWFPPPPPFRP PPFGPPRFP	Pig
Prophenin-2	Extended	AFPPPNVPGPRFPPPNVPGPRFPPPNFPGPRFPPPNFPGPRFPPPNFP-GPPFPPPIFPGPWFPPPPPFRPPPFGPPRFP	Pig
Indolicidin	Extended	ILPWKWPWWPWWRR	Cow
Dodecapeptide	Cyclic β-turn	RLCRIVVIRVCR	Cow
OaDode	Cyclic β-turn	RYCRIIFLRVCR	Sheep
Protegrin-1	β-Hairpin	RGGRLCYCRRRFCVCVGR	Pig
Protegrin-2	β-Hairpin	RGGRLCYCRRRFCICV	Pig
Protegrin-3	β-Hairpin	RGGGLCYCRRRFCVCVGR	Pig
Protegrin-4	β-Hairpin	RGGRLCYCRGWICFCVGR	Pig
Protegrin-5	β-Hairpin	RGGRLCYCRPRFCVCVGR	Pig
CHICATH	Unknown	RVKRVWPLVIRTVIAGYNLYRAIKKK	Chicken
MaeuCath1	Unknown	KRGFGKKLRKRLKKFRNSIKKRLKNFNVVIPIPLPG	Tammar Wallaby

injury in skin keratinocytes.[15] Conversely, infection of the intestinal epithelium with particular pathogens, such as *Shigella*, leads to the downregulation of the expression of cathelicidin.[12] Expression of human cathelicidin LL-37 can also be upregulated in inflammatory diseases but is apparently not directly induced by microbial products. For example, LL-37 is found to be elevated in chronic inflammatory diseases such as rheumatoid arthritis,[25] Crohn's disease,[14] and eczema.[17] Similarly, the precursor protein of human LL-37 has recently been shown to

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be induced by vitamin D[31] and in endoplasmic reticulum stress via NF-κB activation.[24]

PROCESSING

Cathelicidins need to be proteolytically processed to be biologically active. Initially, they are synthesized as precursor peptides containing both a leader sequence (for secretion) and the pro (cathelin) domain (Figure 1). In neutrophils, these inactive pre-propeptides are stored at high concentrations in granules and upon neutrophil activation undergo processing by appropriate proteases to release the mature peptide that is found extracellularly. Various studies, including crystallographic analysis, have shown that mammalian cathelicidins are processed by serine proteases such as elastase, with the protease-sensitive sequence in a β-hairpin loop, and proteinase 3.[28] Another serine protease, kallikrein, was also recently shown to control the processing of cathelicidin precursor protein.[21] The exact function of the cathelin domain is still uncertain. It has been suggested that the cathelin-like domain exhibits antimicrobial and protease inhibitor activities, although these have not been proven to be relevant *in vivo*. Once released, the mature peptide often demonstrates resistance to proteolytic degradation by ubiquitous proteases resulting from mechanisms such as high proline content or posttranslational modifications such as bromination or C-terminal amidation.[28] The mature cationic peptide domain shows a broad range of an antimicrobial and immunomodulatory activities (discussed below).

RECEPTORS

The ligand–receptor interactions of cathelicidins are not well understood, with human cathelicidin LL-37 representing the most studied peptide in this regard. Overall LL-37 has been shown to have a variety of receptors according to the function being studied and the cell type. For example, LL-37 directly mediates chemotaxis of human peripheral blood neutrophils, monocytes, and T cells, and induces production of IL-8 in epithelial cells, through formyl peptide-receptor-like 1 (FPRL1),[6,16] whereas two alternative (as yet uncharacterized) receptors are involved in chemotaxis of mast cells by LL-37.[3] By contrast, it has been demonstrated that LL-37-mediated phosphorylation of mitogen-activated protein kinases (MAP kinases) p38 and Erk1/2 in human peripheral blood-derived monocytes is independent of FPRL1[2] and LL-37 activates MAP kinases in epithelial cells via the transactivation of epidermal growth factor receptors.[3] LL-37 has been shown to mediate IL-1β secretion from human monocytes by activating the P2X7 nucleotide scavenger receptor, but conversely porcine protegrins mediate IL-1β secretion independently of P2X7 receptors.[10] In addition, intracellular GAPDH has also been described as a functional receptor for LL-37 induction of chemokines (see Figure 2 for model).[19] One hypothesis to explain these results is that there are multiple

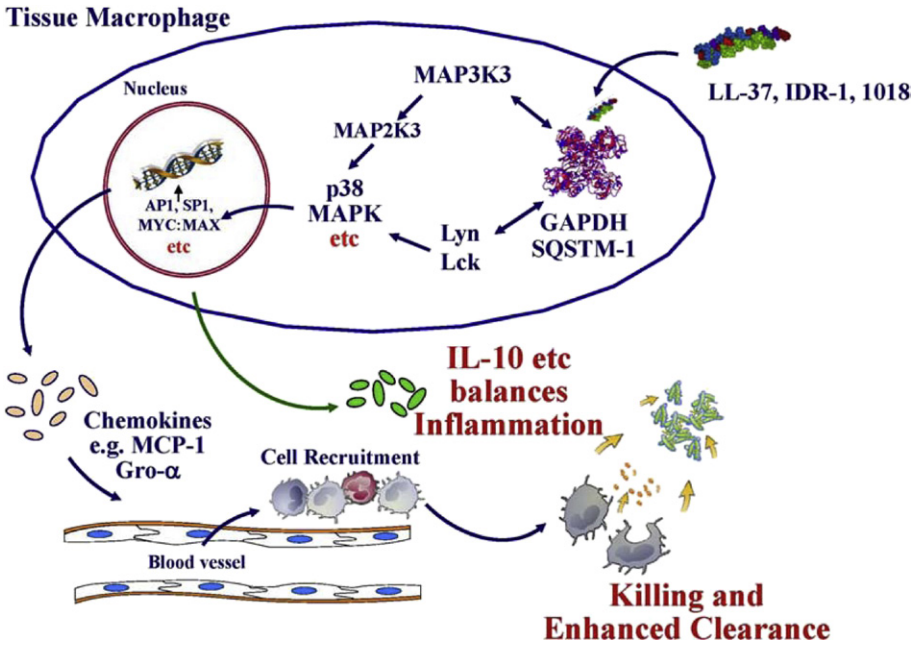

FIGURE 2 Proposed mechanism for cellular activation by LL-37: internalization of the peptide is facilitated by an atypical endocytic uptake, followed by interaction with the intracellular receptors. These interactions appear to facilitate modulation of various immune signaling pathways, mediates various immunomodulatory responses and overall results in the "selective" modulation of inflammatory responses (modified from *J Immunol* 2009;**183**:2688, and *Mol Biosystems* 2009;**5**:483).

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receptors for LL-37 that mediate different events in particular cells, but the issue of cathelicidin receptor interactions and their function(s) in mediating biological activity is not yet completely resolved.

INFORMATION ON ACTIVE AND/OR SOLUTION CONFORMATION

Peptides of the cathelicidin family include representatives of all known secondary structure groups into which antimicrobial peptides are classified. Indeed they exhibit a remarkable variety of sizes, sequences, and structures. They typically have a net positive charge at neutral pH of +2 to +7 resulting from excess lysine and arginine basic residues, and contain around 50% hydrophobic amino acids. This permits them to fold into amphipathic or amphiphilic structures (often upon membrane contact), which is important in their mechanisms of action.[4,29] Mature (processed, biologically active) cathelicidins fold into one of four structural classes: (i) amphipathic α-helices (that form after interaction with membranes), (ii) β-hairpin molecules with two β-strands interconnected by intramolecular disulfide bonds, (iii) extended structures (upon membrane interaction) including peptides enriched in one or two amino acids such as proline and arginine, or tryptophan, and (iv) peptides such as bactenecin (dodecapeptide) that are cyclized by a single disulfide bond and contain a β-turn structure (Figure 3, Table 1). Their distinct antimicrobial and immunomodulatory functions have been localized to different regions of the peptide, and it is probable that these functions have different (but perhaps somewhat overlapping) structural determinants. Several studies have indicated that the amino acid sequence of natural cathelicidins can be modified to obtain much more potent synthetic antimicrobial peptides with reduced undesirable properties, such as mast cell degranulation, hemolysis and cytotoxicity. The quantitative structure–activity relationships (QSAR) approach, based on sequences of natural cathelicidins, together with computational structural modeling has yielded biologically active peptides with potential therapeutic use.[11] Further structure–function studies will fuel the synthesis of peptides with potent and perhaps selective activities.

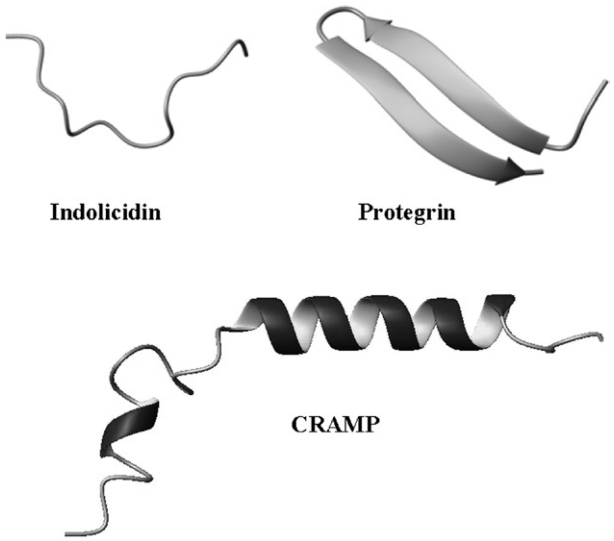

FIGURE 3 Ribbon structures of cathelicidin mature peptides bovine indolicidin (extended structure), porcine protegrin 1 (β-hairpin structure) and murine CRAMP (amphipathic α-helical structure).

BIOLOGICAL FUNCTIONS

Cathelicidins can function as immunomodulators and/or microbicidal agents in the innate immune system. The biological effects exerted by cathelicidins at a given time and place are probably determined by the specific activities of the peptide and the physiological setting, including the concentration of the peptide, cellular environment, and soluble components of the extracellular milieu. Although many publications refer to LL-37 as an antimicrobial peptide, in the presence of physiological salt, heparin, and serum conditions, LL-37 is not antimicrobial at the concentrations (generally 2 μg/ml) normally found in adults at mucosal surfaces, although it does exhibit immunomodulatory functions under these conditions. It is possible that the release of LL-37 during degranulation from neutrophils can considerably elevate local concentrations.

The most studied cathelicidin peptides, human LL-37, porcine PR-39, and bovine BMAP-28, have been shown, when added exogenously, to control bacterial load and prevent sepsis and mortality in rats and mice after bacterial challenge (Table 2). Mice deficient in the murine cathelicidin CRAMP also exhibit significant but moderate defects in defense against bacterial infections of the skin and respiratory tract as well as decreased angiogenesis and delayed wound closure. In humans with atopic dermatitis, low expression of LL-37 (and possibly other peptides) in skin lesions from patients coincides with their enhanced susceptibility to skin infections, while individuals with Morbus Kostmann, suffer from frequent oral bacterial infections and severe periodontal disease that correlates most with a deficiency in LL-37. Conversely, LL-37 is highly expressed in psoriatic skin and rosacea. Together, these observations demonstrate, *in vivo*, a significant role for cathelicidins in mucosal host defenses and wound healing.[3,15,22]

Anti-infective

The protective effects of cathelicidin peptides against different microorganisms (bacteria, viruses, yeast, fungi) have often been attributed to their direct antimicrobial killing properties, which can be mediated by bacterial cytoplasmic membrane disruption.[3,15] Indeed, some cathelicidin peptides have exceptionally good antimicrobial

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TABLE 2 Functions of Selected Cathelicidins

Cathelicidin functions	Human LL-37[1,3,8,22,34]	Murine CRAMP [15,29,34]	Bovine BMAP-28, Bac2A or indolicidin [29]	Porcine, PR-39[26]
Anti-infective effects observed in vivo				
Protects against bacterial challenge and sepsis	✓	✓	✓	
Controls bacterial load	✓	✓		
Selectively chemotactic	✓	✓		
Reduces amounts of proinflammatory mediators	✓		✓	
Enhances adaptive immune response	✓	✓		
Immunomodulatory effects observed in vitro				
Induces and influences signal transduction	✓	✓		✓
Induces gene expression and enhances protein secretion	✓		✓	✓
Suppresses inflammatory stimuli-induced gene expression and protein secretion	✓		✓	
Influences cell proliferation, differentiation, and migration	✓	✓	✓	✓
Promotes angiogenesis and/or wound healing	✓			✓
Cooperates with immune mediators	✓			
Influences adaptive immune response	✓			

activity (e.g., pig protegrin 1). Most cathelicidins, however, are not nearly as potent as protegrin yet may exert microbicidal action by other mechanisms, for example, LL-37 inhibits microbial attachment capabilities and biofilm formation.[5] LL-37 also exhibits antiviral effects by attacking viral membranes,[7] or targeting the reverse transcriptase of HIV.[32] Mouse cathelicidin CRAMP maintains colonic integrity and prevents inflammatory disease and infection in part by suppressing apoptosis and stimulating mucin gene expression and mucous production in the colon.[34]

Immunomodulatory

In addition to being antimicrobial, host defense peptides stimulate a broad range of biological effects in a innate immune cells (including neutrophils and epithelial cells), adaptive immune cells (T- and B-lymphocytes), and in cells that are involved in and bridge the innate and adaptive immune systems such as monocytes/macrophages and dendritic cells[3,8,15,22] (Table 2). They promote effector mechanisms that initiate and support innate and adaptive immune responses, yet they also protect the host against the detrimental effects of excessive inflammatory responses and support the subsequent resolution of inflammation.

Immune Activation

Cathelicidins promote chemotaxis of effector cells, induce the transcription and secretion of chemokines and cytokines, and induce mast cell activation and degranulation. Together these effects enhance vascular permeabilization, promote leukocyte extravasation, and regulate cellular responses at the site of infection/injury. Human cathelicidin LL-37 activates effector monocytes through many signaling pathways (IκB-α/NF-κB, PI3 K and the MAP kinases p38, ERK1/2 and JNK) and transcription factors (including NF-κB, AP-1, AP-2, SP-1, E2F1, CREB, and EGR) that induce the expression of nearly 500 immune-related genes.[18] LL-37 can also activate effector cells synergistically with proinflammatory stimuli (IL-1β, CpG, LTB_4), cellular differentiation factors (GM-CSF) and some TLR agonists (e.g., flagellin, polyI:C, and viral and microbial self-DNA/RNA); the latter synergy is in line with the observed adjuventicity of cathelicidins LL-37 and CRAMP.[1,3,22]

Inflammatory Control

In a diverse range of cells exposed to proinflammatory bacterial products, cathelicidins suppress gene transcription and the release of proinflammatory mediators. The antiendotoxin (LPS) effect of cathelicidins has been well described in numerous immune cells and correlates with protection in

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several animal models of sepsis.[20] Recently, it was shown that LL-37 inhibited both innate and adaptive cellular responses to IFNγ, the key cytokine of Th1-polarized immunity.[23] Cathelicidins exert these effects through intracellaular and/or surface receptors that trigger intracellular signaling pathways that influence MAP kinases, Ca^{2+} moblilization and NF-κB.[18] Cathelicidins can also directly bind to negatively charged microbial components (e.g., LPS) and can also control inflammation by preventing the release of toxic cellular components that cause excess tissue damage and inflammation. For example, PR-39 inhibits the production of reactive oxygen species and BMAP-28 induces cell death via apoptosis of activated (infected) lymphocytes, which, unlike necrosis, occurs without releasing toxic cytoplasmic molecules. LL-37 also modulates apoptosis, necrosis and secondary necrosis with differing effects that are cell type specific; affected cells include neutrophils, keratinocytes, epithelial cells, endothelial cells, $CD4^+$ $CD25^+$ $FoxP3^+$ regulatory T cells, and cytotoxic, but not helper, T cells.[3]

Immune Resolution

Cathelicidins are strongly expressed during wound healing and have been shown to promote reepithelialization of human skin wounds and rat gastric ulcers. These effects may be mediated by cathelicidin-induced angiogenic effects, induction of epithelial cell migration and proliferation, and inflammatory modulation. LL-37-induced migration of keratinocytes and proliferation of epithelial cells is mediated by pathways involving EGFR/STAT3 and GPCR/EGFR/ERK.[34]

The neutralizing and resolving effects of cathelicidins provide a balance between the protective and destructive components of inflammation, protecting against lethal conditions, while promoting responses to eradicate the infectious agent. These neutralizing activities also implicate cathelicidins in maintaining homeostasis, particularly in regions of the gut rich in commensals that contain the same bacterial molecules that activate the innate immune response through TLRs.

THERAPEUTIC POTENTIAL

Recent advances in understanding the bioactivity of cathelicidins suggest a broad use of optimized derivatives of these peptides as therapeutic interventions. The most prominent therapeutic potential of cathelicidins is established in the protection against infections.[3] There has been considerable interest in developing synthetic peptide derivatives with enhanced therapeutic potential, for example, against drug-resistant pathogens, while eliminating undesirable cytotoxic properties associated with natural cathelicidins. For example, a bovine cathelicidin derivative, peptide Omiganan, has been shown to be effective against infections and in inflammatory conditions in clinical trials.[22] Recent studies have also explored the use of cathelicidin peptides in wound healing by promoting reepithelialization and angiogenesis, and in controlling inflammation.[34] Similarly, the immunomodulatory functions of these peptides have lead to their potential use as vaccine adjuvants.[22] The human cathelicidin LL-37 is now known to be a modulator of tumor growth, which seems to be tissue-specific. For example, LL-37 can promote breast and lung cancers and in contrast suppress tumor growth in gastric cancers.[33] Thus fragments derived from LL-37 are being developed as anticancer agents.[33] Another area for the use of these peptides is as biomarkers for disease diagnosis. Recent studies have demonstrated that human LL-37 may serve as a biomarker in inflammatory disorders such as eczema and chronic obstructive pulmonary disease.[13,17] Despite extensive proof-of-principle studies and some clinical trials, development of these peptides as therapeutics has several challenges,[11] primarily pharmacokinetics, peptide stability, possible side effects of systemic administration and associated production costs.

In summary, cathelicidins actively participate in the regulation of host defenses, exerting antimicrobial activity through the stimulation of biological functions in immune effector cells during the inflammatory and immune response and/or direct killing. The functional redundancy between species, the antiseptic activities, and adjuvant properties of cathelicidins make them attractive anti-infective therapeutic agents. The ability of cathelicidins to reduce proinflammatory mediators, induce chemotaxis and enhance the adaptive immune response *in vivo* validates these as key functions of cathelicidins in protection against bacterial challenge. However, the vast number of reports under varying conditions illuminates the need for experiments to confirm that *in vitro* activity is physiologically relevant *in vivo*.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the support of the Canadian Institutes of Health research to REW Hancock for our Peptide research. REW Hancock was supported by a Canada Research Chair Award. N Mookherjee is supported by Manitoba Health Research Council, and Health Sciences Centre Foundation, Manitoba, Canada. KL Brown is supported by the European Community's Seventh Framework Programme (FP7/2007–2013) under grant agreement number 221094.

REFERENCES

1. Bowdish DM, Davidson DJ, Lau YE, Lee K, Scott MG, Hancock REW. Impact of LL-37 on anti-infective immunity. *J Leukoc Biol* 2005;**77**:451–9.
2. Bowdish DM, Davidson DJ, Speert DP, Hancock REW. The human cationic peptide LL-37 induces activation of the extracellular signal-regulated kinase and p38 kinase pathways in primary human monocytes. *J Immunol* 2004;**172**:3758–65.

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3. Bucki R, Leszczynska K, Namiot A, Sokolowski W. Cathelicidin LL-37: a multitask antimicrobial peptide. *Arch Immunol Ther Exp (Warsz)* 2010;**58**:15–25.
4. Burton MF, Steel PG. The chemistry and biology of LL-37. *Nat Prod Rep* 2009;**26**:1572–84.
5. Chennupati SK, Chiu AG, Tamashiro E, Banks CA, Cohen MB, Bleier BS, et al. Effects of an LL-37-derived antimicrobial peptide in an animal model of biofilm Pseudomonas sinusitis. *Am J Rhinol Allergy* 2009;**23**:46–51.
6. De Y, Chen Q, Schmidt AP, Anderson GM, Wang JM, Wooters J, et al. LL-37, the neutrophil granule- and epithelial cell-derived cathelicidin, utilizes formyl peptide receptor-like 1 (FPRL1) as a receptor to chemoattract human peripheral blood neutrophils, monocytes, and T cells. *J Exp Med* 2000;**192**:1069–74.
7. Dean RE, O'Brien LM, Thwaite JE, Fox MA, Atkins H, Ulaeto DO. A carpet-based mechanism for direct antimicrobial peptide activity against vaccinia virus membranes. *Peptides* 2010;**31**:1966–72.
8. Doss M, White MR, Tecle T, Hartshorn KL. Human defensins and LL-37 in mucosal immunity. *J Leukoc Biol* 2010;**87**:79–92.
9. Elloumi HZ, Holland SM. Complex regulation of human cathelicidin gene expression: novel splice variants and 5'UTR negative regulatory element. *Mol Immunol* 2008;**45**:204–17.
10. Elssner A, Duncan M, Gavrilin M, Wewers MD. A novel P2X7 receptor activator, the human cathelicidin-derived peptide LL37, induces IL-1 beta processing and release. *J Immunol* 2004;**172**:4987–94.
11. Hadley EB, Hancock REW. Strategies for the discovery and advancement of novel cationic antimicrobial peptides. *Curr Top Med Chem* 2010;**10**:1872–81.
12. Islam D, Bandholtz L, Nilsson J, Wigzell H, Christensson B, Agerberth B, et al. Downregulation of bactericidal peptides in enteric infections: a novel immune escape mechanism with bacterial DNA as a potential regulator. *Nat Med* 2001;**7**:180–5.
13. Jiang Y, Xiao W, Zhang Y, Xing Y. Urokinase-type plasminogen activator system and human cationic antimicrobial protein 18 in serum and induced sputum of patients with chronic obstructive pulmonary disease. *Respirology* 2010;**15**:939–46.
14. Kubler I, Koslowski MJ, Gersemann M, Fellermann K, Beisner J, Becker S, et al. Influence of standard treatment on ileal and colonic antimicrobial defensin expression in active Crohn's disease. *Aliment Pharmacol Ther* 2009;**30**:621–33.
15. Lai Y, Gallo RL. AMPed up immunity: how antimicrobial peptides have multiple roles in immune defense. *Trends Immunol* 2009;**30**:131–41.
16. Lau YE, Rozek A, Scott MG, Goosney DL, Davidson DJ, Hancock RE. Interaction and cellular localization of the human host defense peptide LL-37 with lung epithelial cells. *Infect Immun* 2005;**73**:583–91.
17. Leung T, Ching K, Kong A, Wong G, Chan J, Hon K. Circulating LL-37 is a biomarker for eczema severity in children. *J Eur Acad Dermatol Venereol* 2012;**26**:518–22.
18. Mookherjee N, Hamill P, Gardy J, Blimkie D, Falsafi R, Chikatamarla A, et al. Systems biology evaluation of immune responses induced by human host defence peptide LL-37 in mononuclear cells. *Mol Biosyst* 2009;**5**:483–96.
19. Mookherjee N, Lippert DN, Hamill P, Falsafi R, Nijnik A, Kindrachuk J, et al. Intracellular receptor for human host defense peptide LL-37 in monocytes. *J Immunol* 2009;**183**:2688–96.
20. Mookherjee N, Rehaume LM, Hancock REW. Cathelicidins and functional analogues as antisepsis molecules. *Expert Opin Ther Targets* 2007;**11**:993–1004.
21. Morizane S, Yamasaki K, Kabigting FD, Gallo RL. Kallikrein expression and cathelicidin processing are independently controlled in keratinocytes by calcium, vitamin D(3), and retinoic acid. *J Invest Dermatol* 2010;**130**:1297–306.
22. Nijnik A, Hancock REW. The roles of cathelicidin LL-37 in immune defences and novel clinical applications. *Curr Opin Hematol* 2009;**16**:41–7.
23. Nijnik A, Pistolic J, Wyatt A, Tam S, Hancock REW. Human cathelicidin peptide LL-37 modulates the effects of IFN-gamma on APCs. *J Immunol* 2009;**183**:5788–98.
24. Park K, Elias PM, Oda Y, Mackenzie D, Mauro T, Holleran WM, et al. Regulation of cathelicidin expression by an ER stress-signaling, vitamin D receptor-independent pathway. *J Biol Chem* 2011;**286**:34121–30.
25. Paulsen F, Pufe T, Conradi L, Varoga D, Tsokos M, Papendieck J, et al. Antimicrobial peptides are expressed and produced in healthy and inflamed human synovial membranes. *J Pathol* 2002;**198**:369–77.
26. Sang Y, Blecha F. Porcine host defense peptides: expanding repertoire and functions. *Dev Comp Immunol* 2009;**33**:334–43.
27. Schauber J, Iffland K, Frisch S, Kudlich T, Schmausser B, Eck M, et al. Histone-deacetylase inhibitors induce the cathelicidin LL-37 in gastrointestinal cells. *Mol Immunol* 2004;**41**:847–54.
28. Shinnar AE, Butler KL, Park HJ. Cathelicidin family of antimicrobial peptides: proteolytic processing and protease resistance. *Bioorg Chem* 2003;**31**:425–36.
29. Tomasinsig L, Zanetti M. The cathelicidins—structure, function and evolution. *Curr Protein Pept Sci* 2005;**6**:23–34.
30. Uzzell T, Stolzenberg ED, Shinnar AE, Zasloff M. Hagfish intestinal antimicrobial peptides are ancient cathelicidins. *Peptides* 2003;**24**:1655–67.
31. White JH. Vitamin D as an inducer of cathelicidin antimicrobial peptide expression: past, present and future. *J Steroid Biochem Mol Biol* 2010;**121**:234–8.
32. Wong JH, Legowska A, Rolka K, Ng TB, Hui M, Cho CH, et al. Effects of cathelicidin and its fragments on three key enzymes of HIV-1. *Peptides* 2011;**32**:1117–22.
33. Wu WK, Wang G, Coffelt SB, Betancourt AM, Lee CW, Fan D, et al. Emerging roles of the host defense peptide LL-37 in human cancer and its potential therapeutic applications. *Int J Cancer* 2010;**127**:1741–7.
34. Wu WK, Wong CC, Li ZJ, Zhang L, Ren SX, Cho CH. Cathelicidins in inflammation and tissue repair: potential therapeutic applications for gastrointestinal disorders. *Acta Pharmacol Sin* 2010;**31**:1118–22.
35. Zanetti M. Cathelicidins, multifunctional peptides of the innate immunity. *J Leukoc Biol* 2004;**75**:39–48.

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Chapter 16

Class II Non-Lantibiotic Bacteriocins

Ingolf F. Nes, Dag A. Brede and Dzung B. Diep

ABSTRACT

The Class II bacteriocins, which often are termed non-lantibiotic and heat stable bacteriocins, encompass a larger group with high diversity with respect to structure and antimicrobial activity. Modifications have been identified in some new bacteriocins, and there are formylated methionines in the leaderless bacteriocins. It is becoming increasingly evident that Class IIa bacteriocins need a receptor for targeting bacteria. So far, only the Class IIa bacteriocins and a few non-related one-peptide bacteriocins have been experimentally proven to need a receptor. Despite there being no sequence homology between lactococcin A and the Class IIa bacteriocins, they are recognizing the same receptor, the membrane embedded part of the mannose PTS transport system. The two membrane proteins ManC and ManD that constitute the receptor and are needed for exerting antimicrobial activity are differently recognized by lactococcin A and the Class IIa bacteriocins. It seems probable that the target specificity of most Class II bacteriocins rely on a receptor.

BACKGROUND

Bacteriocins are defined as ribosomally synthesized antibacterial peptides/proteins that either kill or inhibit the growth of closely related bacteria. These bacteriocins are divided into two major classes: the Class I lantibiotics (see the chapter by Dischinger et al. in this section of the book) and the Class II non-modified bacteriocins, with the latter also being called the non-lantibiotics. The Class II bacteriocins, which are the focus of this chapter, are presently divided into: (a) the anti-listeria, pediocin-like bacteriocins that have very similar amino acid sequences at their N-terminus, (b) the two-peptide bacteriocins whose activity depends on two different peptides, (c) the cyclic bacteriocins, and (d) the linear nonpediocin-like one-peptide (LINPLOP) bacteriocins (Table 1). In addition, there is a group named leaderless bacteriocins because they are synthesized without an N-terminal leader peptide. Most bacteriocins are cationic and contain 30–70 amino acid residues, and their mode of killing is through membrane permeabilization of the target cells. Class II bacteriocins were originally defined as non-modified bacteriocins. However, during the last few years, it has been shown that some bacteriocins that are included in the Class II bacteriocins may have modifications such as formylation of the N-terminal residue[21] and glycosylation.[34] The classification of bacteriocins is still a matter of controversy, though there is a general agreement as to how certain subclasses are defined.

New Class II bacteriocins are frequently identified, and they are found among bacteria, mostly lactic acid bacteria (LAB), in a great variety of ecological niches such as cereal, milk, and meat-based foods and feeds, different kinds of silage and plants, and the intestinal tract of animals including humans. Class II bacteriocins have not been found in many applications to date. However, starter cultures for food fermentation (dairy and meat products) use bacteriocin-producing strains, and several commercial starter cultures are known to produce bacteriocins.

In recent years, probiotic bacteria have attracted an increased awareness among consumers. Such "health-promoting" bacteria are used in numerous food products and dietary supplements. Several probiotic cultures produce bacteriocins, and there is good circumstantial evidence that they contribute to a healthy gut microflora. Among the many ecological niches studied for the presence of bacteriocin-producing LAB, the fecal flora of babies, seem to represent an environment enriched with bacteriocin-producing LAB. In particular, bacteriocin-producing enterococci, the dominant LAB in the colon, are found at a high frequency. The role of bacteriocin-producing probiotic LAB was convincingly shown when a bacteriocin producer efficiently prevented the development of pathogenic *Listeria* in infected mice.[6]

NON-LANTIBIOTIC BACTERIOCINS: PROPERTIES AND STRUCTURAL FEATURES

Class IIa: Pediocin-like Bacteriocins

Class II bacteriocins are generally synthesized as a prebacteriocin with an N-terminal leader, which is removed during the process of secretion.[19] The leader is almost exclusively

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00016-6
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TABLE 1 The Different Subclasses of Class II Bacteriocins

Class II subgroups	Example peptides	PFAM*	Transport mechanism	Inhibition spectrum	PMID†
Pediocin family	Pediocin PA1, Pediocin AcH	PF01721	Double Gly	A**	1575516
	Enterocin P	PF01722	Sec-dependent	A**	11073927
Two-Peptide Bacteriocins	Lactococcin G (LcnGα and LcnGß)		Double Gly	*Lactococcus*	1512201
Cyclic Bacteriocins	Gassericin A	PF12173	Dedicated ABC-transporter	*Lactobacillus, Listeria, Bacillus cereus, Staphylococcus aureus*	9648219
	Uberolysin	PF09221	Dedicated ABC-transporter	*Gram-positive bacteria*	17464077
Leaderless Bacteriocins	Single peptide: Aureocin A53	PF11758	Dedicated ABC-transporter	*Staphylococcus, Enterococcus, Micrococcus, Corynebacterium fimi, Listeria innocua*	12054867
	Two synergistic peptides: Enterocin L50 (A and B)		Dedicated ABC-transporter	LAB,‡ *Listeria, Bacillus, Clostridium, Staphylococcus*	11073927
	Four synergistic peptides: Aureocin A70 (AurA, AurB, AurC, AurD)			LAB,‡ *Listeria*	11531330
S-Glycosylated bacteriocins	Sublancin		Double Gly	*Bacillus, S. aureus, S. pyogenes*	21196935
	Glycocin F		Double Gly	*Lactobacillus, Enterococcus, Leuconostoc*	21251913
Cysteine sulfur to α-carbon crosslinked bacteriocin peptides (sactibiotic)	One-peptide: Thuricin H		Dedicated ABC-transporter	*Listeria, Bacillus, C. piscicola*	21786372
	Circular: Subtilosin	PF11420	Dedicated ABC-transporter	*Listeria*	15035610
	Two peptides: Thuricin CD		Dedicated ABC-transporter	*Clostridium, Bacillus, Lactobacillus,*	21526839
Antimicrobial Peptides Produced From Larger Proteins	PAMP		Sec-dependent	*Propionibacterium, Lactobacillus*	12057960
	Closticin 574		Sec-dependent	*Lactobacillus, Clostridium*	12620847
	Propionicin F		Dedicated ABC-transporter	*Propionibacterium freudenreichii*	15574930
	AP48-MapA		Sec-dependent	LAB,‡ *S. aureus, Listeria, Bacillus*	20833791
Subclass II LINPLOPS$ Bacteriocins	Lactococcin A		Double gly	*Lactococcus*	1904860
	Lactococcin 972	PF09683	Dedicated ABC-transporter	*Lactococcus*	10589723
	Enterocin B		Double gly	*Enterococcus, Lactobacillus*	9245817
	Piscicolin 61		Double gly	*Carnobacterium Enterococcus, Listeria*	7764997
	Divergicin A		Sec-dependent	*Carnobacterium*	7768812
	Propionicin T1		Sec-dependent	*Propionibacterium*	11010864

*PFAM: The Protein Family Database.
†PMID: Reference Pubmed Identifier.
**A: Selected members within Enterococcus, Lactobacillus, Leuconostoc, Pediococcus, Camobacterium, Listeria.
‡LAB: lactic acid bacteria.
$Linear nonpediocin-like one-peptide bacteriocins.

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a so-called double-glycine type with exceptions for some bacteriocins that use a common signal sequence for secretion (Sec leader).[4] To understand the antimicrobial activity of bacteriocins, it is important to understand their structure. Structural studies have provided important information and new ideas as to how such peptides kill target bacteria. Class IIa bacteriocins are the most thoroughly studied Class II bacteriocins.

The present model of how such bacteriocins interact with the target membrane is partly based on investigation of their three-dimensional structures in dodecylphosphocholine (DPC) micelles and in trifluoroethanol (TFE) by the use of nuclear magnetic resonance spectroscopy (NMR) and circular dichroism (CD), mutation analysis, and construction of hybrid molecules that have been studied in the groups of Vederas (Canada), Nissen-Meyer, (Norway), and others.[32] A common structural feature of Class IIa bacteriocins is their N-terminal consensus sequence (KYYGNGL/VXCXKXX-CXVDW) that is organized into an S-shaped antiparallel β-sheet structure stabilized by a disulfide bridge. In the absence of this bridge, the antimicrobial activity is drastically reduced.

The C-terminus of Class IIa is less conserved and comprises a more hydrophobic or amphiphilic α-helical-structure that has been proposed to interact with the hydrophobic core of target-cell membranes. The N-terminal and C-terminal regions seem to be linked together by a flexible hinge. Figure 1 shows the structures of some Class IIa bacteriocins. The C-terminus seems to be folded back, and in enterocin A and pediocin PA1, this structure is forced by a second disulfide bridge. This folded back structure seems to enhance the antimicrobial activity because introducing a similar disulfide bridge in sakacin P significantly improves the activity with respect to both potency and broadening the inhibitory spectrum.[11] Also temperature studies show that the bacteriocins without the second C-terminal disulfide bond are 30–50 times less active at 37 °C compared with the activity at 25 °C. Such heat instability is not observed for the bacteriocins with a second N-terminal disulfide bond.[16]

Class IIa bacteriocins are structured in membrane-mimicking solvents, and for a long time, the killing mode of such bacteriocins was based on the formation of a barrel-stave bacteriocin-based pore that provided a channel in the target-cell membrane for free diffusion of ions and/or small molecules. Even early experiments suggested that this was a simplified model and that more specific targets (receptors) were probably involved in the killing.

A detailed picture of target-specific interaction was first obtained for the lantibiotic nisin, which uses lipid II as a specific docking site to interact with target cells.[3] Years later, it was conclusively shown that many one-peptide bacteriocins including Class IIa bacteriocins target the membrane-located part (MptCD proteins) of the phosphoenolpyruvate:mannose phosphotransferase system (Man-PTS), which is the major transport system for glucose in bacteria.[8] Based on this and other studies, it was conclusively shown that such bacteriocins lead to permeabilization of targeted bacteria that encode a phylogenetically defined subgroup of Man-PTS. Particularly, a defined region of 40 amino acids that encompasses an extracellular loop of the membrane-located protein MptC is responsible for the specific target recognition by the Class IIa bacteriocins.[20]

The present model on how Class IIa and other one-peptide bacteriocins use ManCD (homologs of MptCD) as a receptor is depicted in Fig. 2. The mechanism of killing includes specific binding to or interaction with MptC (ManC) in the presence of MptD (ManD) by triggering an irreversible opening of the transporter which in effect permits a free diffusion of ions across the membrane that eventually kills the target cell.

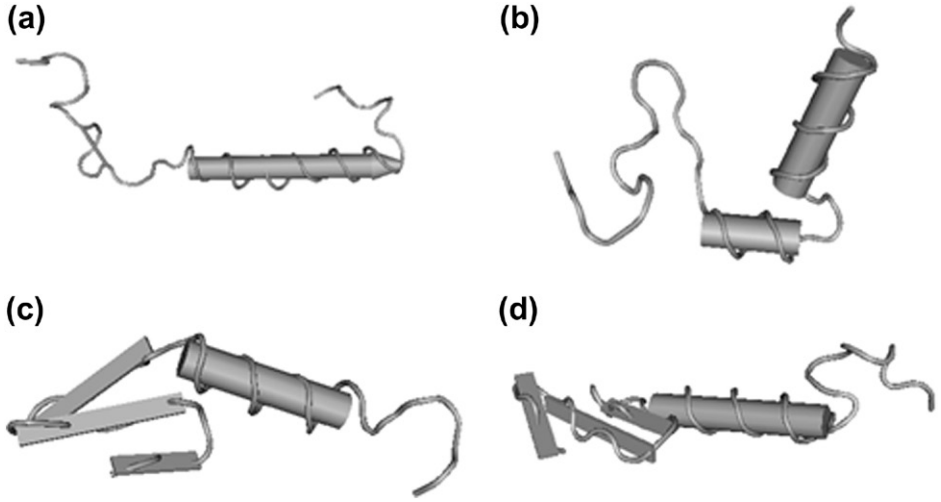

FIGURE 1 Structure of Class IIa bacteriocins. a, Carnobacteriocin B2; b, Curvacin A; c, Leucocin; d, Sakacin P. In A the conserved N-terminal disulphide bridge is shown. In c and d the N-terminal conserved S-shaped reminiscence of a three-stranded antiparallel β-sheet structure is shown.

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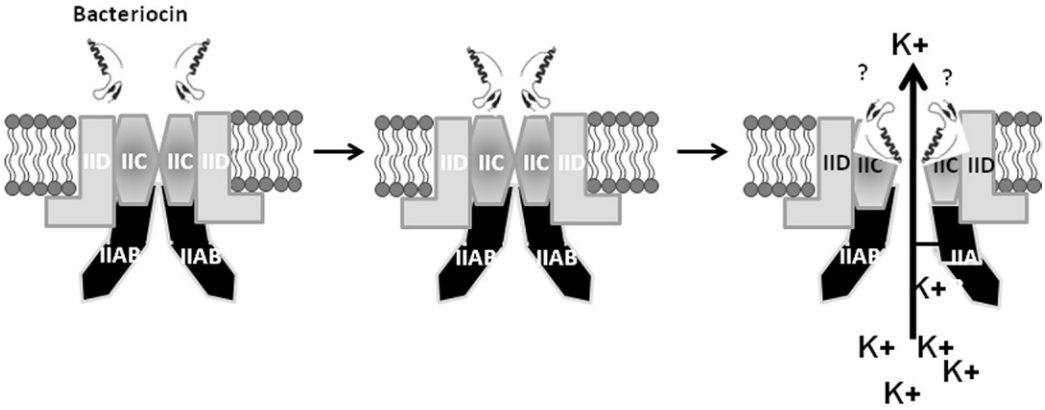

FIGURE 2 The model of killing by Man-PTS targeting bacteriocins. Shown here is the binding of a Class IIa bacteriocin to its receptor and the membrane embedded component ManCD (IIC and IID) of the Man-PTS transporter. This binding irreversibly opens the pore of the transporter and allows free diffusion of ions (such as potassium ions) from the targeted bacterium. Subunits IIA and IIB, which are domains reversibly associated with their membrane-located partners at the cytosolic side, are required for the sugar transport function but not for the bacteriocin receptor function.

Despite the observation that these bacteriocins target the membrane-located constituents of the Man-PTS transporter, it is not possible to exclude direct interaction between the bacteriocin and the hydrophobic membrane of the targeted bacterium as contributing to bacterial killing.

Class IIb: The Two-Peptide Bacteriocins

The two peptides that constitute a two-peptide bacteriocin are normally synthesized as precursor peptides each with a 15- to 30-residue double-glycine-type N-terminal leader sequence. The leader is cleaved at the C-terminal side of the two glycine residues by a dedicated ABC-transporter that concomitantly transfers the mature peptides across the cell membrane, as observed for most Class II bacteriocins and some lantibiotic bacteriocins. The features that identify a two-peptide bacteriocin are: (i) the bacteriocin comprises two non-identical peptides that are encoded by two separate genes located adjacent to one another, (ii) the individual peptides pose little or no antibacterial activity, (iii) the two peptides act together in a one to one molar ratio, and (iv) only one immunity protein is required to protect the producer.

Like other bacteriocins, two-peptide bacteriocins also attack the integrity of the target membrane thereby causing leakage of intracellular compounds such as potassium ions. But the mechanism behind the permeabilization is still poorly understood.

The individual peptides of lactococcin G bind independently to the membrane/target cells, but the antimicrobial activity is manifested only when both peptides are present concurrently.[26] It also seems that there is no diffusion of peptides between cells, and that the peptides seem to be immediately and irreversibly bound by the membrane/cell surface of the target cells.

Ion selective efflux is also observed among two-peptide bacteriocins. In the plantaricin system where 2 two-peptide bacteriocins are colocalized in the same gene cluster, plantaricin EF causes leakage of monovalent cations while plantaricin JK preferentially permits efflux of anions.[22]

The three-dimensional structures of some two-peptide bacteriocins have been elucidated by both CD and NMR spectroscopy in DPC micelles and in TFE.[27] These peptides contain long amphiphilic α-helical-structures, and the two peptides interact in a way that enhances their structuring in membrane-mimicking solvents. Experiments also suggest that the beta-peptide of lactococcin G or enterocin 1071 is important for the target specificity.[27] Structure–function studies and sequence analysis suggest that the two peptides form a membrane-penetrating stacked helix structure involving helix–helix-interacting GxxxG motifs that are present in virtually all characterized two-peptide bacteriocins.

In a study addressing the importance of the ends of the alpha and beta peptides of lactococcin G, selected N- and C-terminal L-amino acid residues were replaced with the D-isomeric counterparts.[29] Activity studies show that when the D-amino acid containing peptides are combined with their wildtype peptide counterparts, antimicrobial activities are partly retained. Studies combined with structural analysis of peptides modified at the C-terminal region of the alpha-LcnG peptide and at the C- and N-terminal regions of beta-LcnG peptide suggest that these terminal regions are not crucial in maintaining antibacterial activity.[29] The results indicate that these regions are not structurally essential for interpeptide interactions or in their interactions with cellular components. In addition, the immunity protein of lactococcin G also retains its function when the D-amino acid containing bacteriocin peptides are tested.[29]

Based on structural information, genetic, mutagenic, and complementary studies, a model as to how a two-peptide bacteriocin can form a pore in a membrane has

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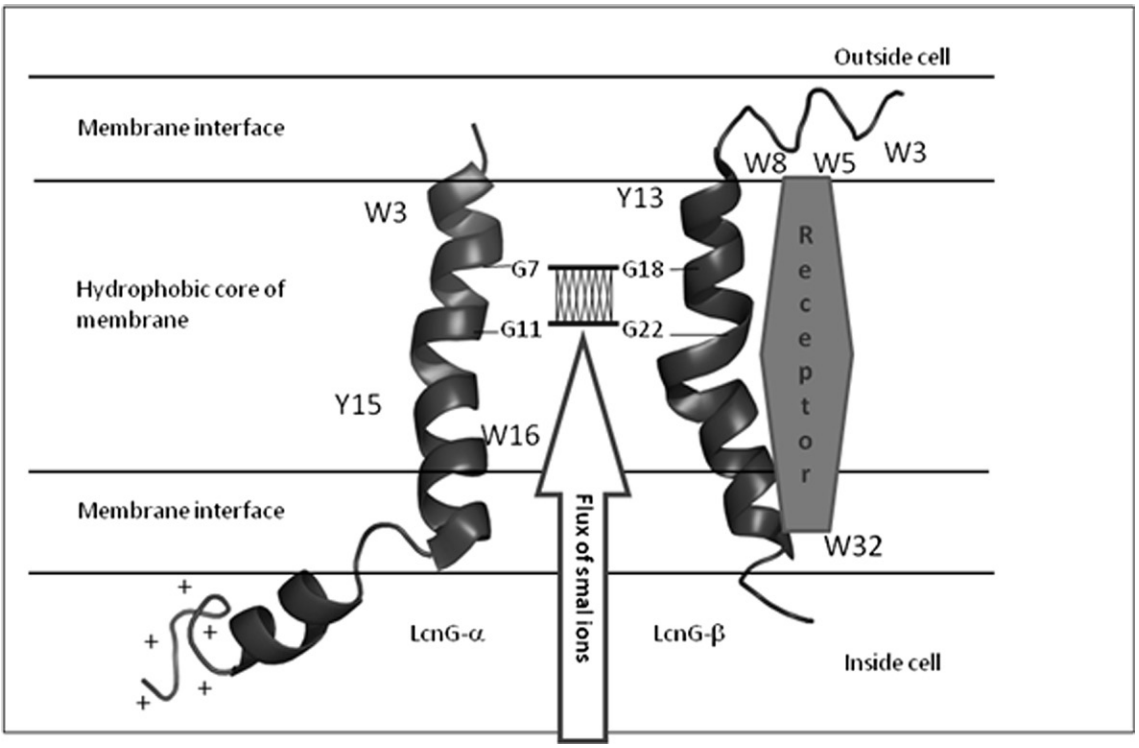

FIGURE 3 The model of how lactococcin G causes permeabilization in a bacterium. The two lactococcin G peptides become structured when they contact and insert into the target membrane. The two peptides interact with each other presumably when they become structured within the membrane and form a stacked helix structure involving the two GxxxG motifs (G7–G11 and G18–G22). A putative receptor molecule is included in the model.

been proposed by Nissen-Meyer and collaborators (Fig. 3). This model proposes that the two individual peptides in a two-peptide bacteriocin form a helix–helix structure when embedded in the membrane creating an open pore for free migration of ions between the cell and its environment. This model does not include any specific receptor, though such a target has been suggested. If one considers the narrow target specificity of the two-peptide bacteriocins, it seems probable that a receptor is involved in the killing as seen with many one-peptide bacteriocins, including the Class IIa ones, but such a target is yet to be identified.

It is proposed that many membrane-active peptide bacteriocins kill target cells through basically the same mechanism; such a common theme includes the concept that a membrane-penetrating part of the bacteriocin binds to a membrane embedded region of an integrated membrane protein (receptor) thereby causing conformational alterations in the structure of the target protein and/or membrane that in turn leads to membrane-leakage and eventually cell death.[27]

Class IIc: Circular Bacteriocins

Circular bacteriocins are antimicrobial peptides produced by a variety of Gram-positive bacteria. They are part of a growing family of ribosomally synthesized peptides that are circularized by an amide bond between the N and C termini of the linear propeptide. Circular bacteriocins adopt a four- or five-α-helix structure encompassing a hydrophobic core.[33] They are exceptionally stable and show increased resistance to proteases.

A cyclic bacteriocin is synthesized as a prebacteriocin with a defined leader, but the length of leaders and their sequences vary between the different bacteriocins. The cyclic bacteriocins are presently divided into two groups: (i) those with long (>20 amino acid residues) and (ii) those with very short (2–6 amino acid residues) leader sequences. Although the mechanisms behind their biosynthesis, including the cyclization process have not been disclosed, the minimum number of genes required for production has been determined for several cyclic bacteriocins.[33]

Cyclic bacteriocins permeabilize the membrane of sensitive bacteria, causing loss of ions and dissipation of the membrane potential. Carnocyclin A forms anion selective pores as seen with the two-peptide plantaricin JF.[12] Still most of the studies concerning mechanisms of action have been performed on *in vitro* systems where non-physiological concentrations of the bacteriocin are used. Because of the physicochemical properties of bacteriocins (e.g. cationic, hydrophobic, skewed composition of amino acids), such studies do not give a valid picture of the mechanism behind killing as seen with early studies of other bacteriocins such as nisin, where additional complexity is involved in receptors

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(lipid II) and cell wall inhibition. Because of their stability and inertness to protease activity, the circular bacteriocins should have great potential in various applications from food to medical treatment.

Classification of bacteriocins can be a problem as seen with the head-to-tail cyclic bacteriocin subtilosin A that posttranslationally links cysteine sulfurs to alpha-carbons of certain amino acids as seen for the sactibiotics (see below).[17]

Class IId: LINPLOP Bacteriocins

This group of bacteriocins has sometimes been referred to as a miscellaneous group because it comprises a diverse group of bacteriocins with characteristics that do not fit them into any of the other subclasses. Some members of the LINPLOP subclass are lactococcins A and B, and enterocin B. Although the LINPLOP bacteriocins share no sequence similarity to the pediocin-like bacteriocins, some members of this group (lactococcins A and B) seem, surprisingly, to share some common mechanistic features with them in cell targeting and immunity.[8] Like the pediocin-like bacteriocins (Class IIa), lactococcin A uses the membrane-located components (ManCD) of the Man-PTS as receptor (Fig. 2). In both types of bacteriocins, their immunity proteins seems to exist in two different settings: one setting involves a soluble and/or loosely membrane-associated form in the absence of the cognate bacteriocins, and the second, which occurs when they confer immunity, involves a protein complex tightly associated with the Man-PTS (receptor) and the cognate bacteriocin. However, they differ greatly from each other in their inhibitory spectra. The lactococcal bacteriocins target basically only cells of lactococcal species while the pediocin-like bacteriocins, as a group, target cells of diverse bacterial genera but not cells of the *Lactococcus* genus. This substantial difference in target specificity relies mainly on small differences in the primary sequences of the membrane-located components of Man-PTS of the lactococcal cells and the non-lactococcal cells.[19,20]

Leaderless Bacteriocins

Leaderless bacteriocins have been found in the lactococci, enterococci, staphylococci, and others.[5,7,14,15,25] Historically, these bacteriocins have been grouped in the same subclass as the LINPLOP bacteriocins, but recent findings regarding their distinct genetics and biosynthesis suggest a separate subclass for these bacteriocins. The leaderless bacteriocins are synthesized without N-terminal signal peptide extensions, and consequently need no activation during excretion. Thus, they have several inherent traits distinct from other types of bacteriocins. They are not subject to posttranslational modifications or processing, although in 2002 the presence of an *N*-formyl-methionine residue (normally removed in bacteria during protein synthesis) was reported in aureocin A53.[24] Since then, another study confirmed formylation of the N-terminal methionine in such a leaderless bacteriocin,[21] and now it seems quite probable that such leaderless bacteriocins all have formylated N-terminal methionine residues. However, whether the formyl group is required for the antimicrobial activity or not has not been disclosed.

How the producer avoids being harmed by a leaderless bacteriocin before secretion is not understood, but it seems plausible that bactericidal activity is only possible from the outside of the cell. Evidence for a dedicated immunity gene/protein has been found in certain systems such as the enteriocin Q and aureocin 70.[7]

There are two different mechanisms by which leaderless bacteriocins are secreted. Some leaderless bacteriocins are exported by dedicated ABC-transporters, which lack domains typical for the recognition of Sec- or double-glycine leaders. Notably, the primary sequences of the four (A–D) aureocin 70 peptides are recognized by a single transporter. For other peptides, the transport mechanisms remain to be confirmed, although there is some evidence for members of *Staphylococcus* hemolytic protein family peptides (PF05480) that the transport is facilitated by the general secretion pathway. The primary structure of some leaderless bacteriocins shows homology to *Staphylococcus* hemolytic protein family peptides, which include phenol-soluble modulins (PSMs). PSMs are amphipathic, α-helical peptides found in *Staphylococcus aureus*, *Staphylococcus epidermidis* and other staphylococci. A subclass of these peptides also has bacteriocin activity.

Genes of leaderless bacteriocins can be found as clusters in the genomes, and up to four such genes have been found located next to each other, although single genes have also been identified. When more than two bacteriocins are found, they share strong sequence homology, suggesting that gene duplications have taken place. It is also surprising that they act synergistically, and the potency is increased and the spectra widened when these homologous peptides act together.[5,25]

In vitro experiments revealed that the four peptides of aureocin 70 oligomerize to enable pore formation. It has been shown that the leaderless aureocin A53 permeabilizes the bacterial membrane; its activity leads to cell death within minutes, and it was suggested that the bactericidal activity of aureocin A53 develops through a generalized membrane destruction rather than by formation of discrete pores within the membrane.

The most challenging aspect of the leaderless bacteriocins is their apparent structural similarity to hemolytic peptides and toxins found among certain pathogens.[9] Some of these toxins also possess antibacterial activity; consequently, leaderless bacteriocins should always be thoroughly scrutinized for toxic properties before being considered as antimicrobial remedies.

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Antimicrobial Peptides Derived from Larger Proteins by Proteolytic Processing

An increasing number of bacteriocins derived from large precursor proteins are being characterized. *Propionibacterium jensenii* and *P. thoenii* secrete large amounts of the pro-PAMP protein, which on encountering proteases in its resident milieu is specifically processed into the protease-activated antimicrobial peptide.[10] It seems that propeptide is a microbial booby trap that targets proteolytic bacteria. Propionicin F is a 4.4-kDa bacteriocin derived from a 29-kDa precursor, which undergoes both N- and C-terminal proteolytic processing. In contrast to most bacteriocins, this peptide is negatively charged but displays bactericidal activity at nanomolar concentrations. In the intestinal isolates of *Lactobacillus reuteri*, the mucus adhesion protein MapA is degraded by the producer into specific peptides with antimicrobial activity.[2] This pleiotropic function of the MapA protein offers a new perspective on how bacteria gain a competitive edge in the gut epithelium. Presently, the mechanism behind the production of MapA-derived antimicrobial peptide, and whether it plays a role in defenses against pathogenic bacteria in the gastrointestinal tract is unknown.

OTHER NON-LANTIBIOTICS BUT MODIFIED BACTERIOCINS

In 1996, a new bacteriocin was found to be produced by a *Lactobacillus plantarum* strain.[18] Subsequently, it was shown that this bacteriocin (named glycocin F) was posttranslationally glycosylated in two amino acid residues.[34] An N-acetylglucosamine was linked to the oxygen atom in Ser18, and an *N*-acetylhexosamine was S-linked to the cysteine in position 43. Glycocin F is synthesized with a double-glycine leader as found in many bacteriocins. Some bacteriocins such as plantaricin ASM[13] were previously classified as lantibiotics, but now there is a good reason to believe that such bacteriocins are not lantibiotics but belong to the group of glycosylated bacteriocins. The glycosylated bacteriocins may include, in addition to glycocin F and sublancin 168[28] (originally classified as a lantibiotic), bacteriocins such as plantaricin ASM, and enterocin 96 though the last two have not been experimentally proven to contain such modifications.

Thuricin CD, produced by *Bacillus thuringiensis*, is another posttranslationally modified bacteriocin that does not fit into the class of lantibiotics and has been allocated to a new class of bacteriocins named sactibiotics.[23] The sactibiotics have a unique modification wherein sulfur atoms are covalently linked to the α-carbon backbone of amino acids. Thuricin CD is a bacteriocin that consists of two peptides, each having three sulfur to α-carbon bridge modifications and these peptides share 45.3% sequence similarity.[31] The individual peptides exhibit some antimicrobial activity but when combined they exhibit much stronger activity. Optimal activity is achieved when the two peptides are mixed in a one to one molar ratio. In addition, the two peptides are synthesized in a prepeptide form with typical double-glycine leaders, as found for many other bacteriocins within Classes I and II. The circular subtilosin A can also be classified as a sactibiotic because it covalently links sulfur to certain α-carbon-amino acids.

A bioinformatic search in bacterial genome databases identified 15 novel thuricin CD-like gene clusters, based on the presence of homologs of thuricin-like bacteriocin genes.[23] Thus, sactibiotic-like bacteriocins seem to be abundant in Gram-positive bacteria. Thuricin CD seems to have a great potential for use in the treatment of *Clostridium difficile* infections resulting from its unique anticlostridial activity with little or no impact on the commensal gut flora. In contrast, other antimicrobial agents greatly affect the composition of the human microflora of the gut.[30]

CONCLUDING REMARKS

Over the years, we have seen an increasing number of new bacteriocins with different properties. New classes of bacteriocins are found with new and interesting structures and antimicrobial activities, which will make them useful for combating pathogens. Bacteriocins are among the most potent antimicrobial compounds known, but they are limited by their narrow specific activity. Many bacteriocins do not target all bacteria within a species, whereas others are able to kill selected isolates from different genera. Although several reports claim that Gram-positive bacteriocins are able to kill Gram-negative bacteria, this is largely by a combined action with other antimicrobial or chemical entities. On the other hand, it is interesting to note that not just the Class IIa bacteriocins need the Man-PTS system as a receptor as certain Gram-negative bacteriocins also require membrane components of the Man-PTS to exert their antimicrobial effects.[1] The Man-PTS system is ubiquitous in bacteria but not found in eukaryotes, and therefore, it should be possible to develop a strategy to fight all bacteria with peptide drugs targeting at the Man-PTS.

The greatest genetic diversity is found among bacteria, and we will certainly, in the future, discover a large number of new and chemically diverse bacteriocins with specificities against different bacterial targets. This will give us a great opportunity to develop new and potent antimicrobial compounds to fight pathogens. Such an effort is a human necessity because the antibiotic era is slowly but surely coming to a halt.

REFERENCES

1. Bieler S, Silva F, Belin D. The polypeptide core of microcin E492 stably associates with the mannose permease and interferes with mannose metabolism. *Res Microbiol* 2010;**161**:706–10.
2. Bohle LA, Brede DA, Diep DB, Holo H, Nes IF. Specific degradation of the mucus adhesion-promoting protein (MapA) of *Lactobacillus reuteri* to an antimicrobial peptide. *Appl Environ Microbiol* 2010;**76**:7306–9.

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3. Brotz H, Josten M, Wiedemann I, Schneider U, Gotz F, Bierbaum G, et al. Role of lipid-bound peptidoglycan precursors in the formation of pores by nisin, epidermin and other lantibiotics. *Mol Microbiol* 1998;**30**:317–27.
4. Cintas LM, Casaus P, Havarstein LS, Hernandez PE, Nes IF. Biochemical and genetic characterization of enterocin P, a novel sec-dependent bacteriocin from *Enterococcus faecium* P13 with a broad antimicrobial spectrum. *Appl Environ Microbiol* 1997;**63**:4321–30.
5. Cintas LM, Casaus P, Holo H, Hernandez PE, Nes IF, Havarstein LS. Enterocins L50A and L50B, two novel bacteriocins from *Enterococcus faecium* L50, are related to staphylococcal hemolysins. *J Bacteriol* 1998;**180**:1988–94.
6. Corr SC, Li Y, Riedel CU, O'Toole PW, Hill C, Gahan CG. Bacteriocin production as a mechanism for the antiinfective activity of *Lactobacillus salivarius* UCC118. *Proc Natl Acad Sci USA* 2007;**104**:7617–21.
7. Criado R, Diep DB, Aakra A, Gutierrez J, Nes IF, Hernandez PE, et al. Complete sequence of the Enterocin Q-encoding plasmid pCIZ2 from the multiple bacteriocin producer *Enterococcus faecium* L50 and genetic characterization of Enterocin Q production and immunity. *Appl Environ Microbiol* 2006;**72**:6653–66.
8. Diep DB, Skaugen M, Salehian Z, Holo H, Nes IF. Common mechanisms of target cell recognition and immunity for class II bacteriocins. *Proc Natl Acad Sci USA* 2007;**104**:2384–9.
9. Donvito B, Etienne J, Denoroy L, Greenland T, Benito Y, Vandenesch F. Synergistic hemolytic activity of *Staphylococcus lugdunensis* is mediated by three peptides encoded by a nonagr genetic locus. *Infect Immun* 1997;**65**:95–100.
10. Faye T, Holo H, Langsrud T, Nes IF, Brede DA. The unconventional antimicrobial peptides of the classical propionibacteria. *Appl Microbiol Biotechnol* 2011;**89**:549–54.
11. Fimland G, Johnsen L, Axelsson L, Brurberg MB, Nes IF, Eijsink VG, et al. A C-terminal disulfide bridge in pediocin-like bacteriocins renders bacteriocin activity less temperature dependent and is a major determinant of the antimicrobial spectrum. *J Bacteriol* 2000;**182**:2643–8.
12. Gong X, Martin-Visscher LA, Nahirney D, Vederas JC, Duszyk M. The circular bacteriocin, carnocyclin A, forms anion-selective channels in lipid bilayers. *Biochim Biophys Acta Biomembr* 2009;**1788**:1797–803.
13. Hata T, Tanaka R, Ohmomo S. Isolation and characterization of plantaricin ASM1: a new bacteriocin produced by *Lactobacillus plantarum* A-1. *Int J Food Microbiol* 2010;**137**:94–9.
14. Hyink O, Balakrishnan M, Tagg JR. *Streptococcus rattus* strain BHT produces both a class I two-component lantibiotic and a class II bacteriocin. *FEMS Microbiol Lett* 2005;**252**:235–41.
15. Iwatani S, Zendo T, Yoneyama F, Nakayama J, Sonomoto K. Characterization and structure analysis of a novel bacteriocin, lacticin Z, produced by *Lactococcus lactis* QU 14. *Biosci Biotechnol Biochem* 2007;**71**:1984–92.
16. Kaur K, Andrew LC, Wishart DS, Vederas JC. Dynamic relationships among type IIa bacteriocins: temperature effects on antimicrobial activity and on structure of the C-terminal amphipathic alpha helix as a receptor-binding region. *Biochemistry* 2004;**43**:9009–20.
17. Kawulka K, Sprules T, McKay RT, Mercier P, Diaper CM, Zuber P, et al. Structure of subtilosin A, an antimicrobial peptide from *Bacillus subtilis* with unusual posttranslational modifications linking cysteine sulfurs to alpha-carbons of phenylalanine and threonine. *J Am Chem Soc* 2003;**125**:4726–7.
18. Kelly WJ, Asmundson RV, Huang CM. Characterization of plantaricin KW30, a bacteriocin produced by *Lactobacillus plantarum*. *J Appl Microbiol* 1996;**81**:657–62.
19. Kjos M, Borrero J, Opsata M, Birri DJ, Holo H, Cintas LM, et al. Target recognition, resistance, immunity and genome mining of class II bacteriocins from Gram-positive bacteria. *Microbiology* 2011;**157**:3256–67.
20. Kjos M, Salehian Z, Nes IF, Diep DB. An extracellular loop of the mannose phosphotransferase system component IIC is responsible for specific targeting by class IIa bacteriocins. *J Bacteriol* 2010;**192**:5906–13.
21. Liu X, Vederas JC, Whittal RM, Zheng J, Stiles ME, Carlson D, et al. Identification of an N-terminal formylated, two-peptide bacteriocin from *Enterococcus faecalis* 710C. *J Agric Food Chem* 2011;**59**:5602–8.
22. Moll GN, van den Akker E, Hauge HH, Nissen-Meyer J, Nes IF, Konings WN, et al. Complementary and overlapping selectivity of the two-peptide bacteriocins plantaricin EF and JK. *J Bacteriol* 1999;**181**:4848–52.
23. Murphy K, O'Sullivan O, Rea MC, Cotter PD, Ross RP, Hill C. Genome mining for radical SAM protein determinants reveals multiple sactibiotic-like gene clusters. *PLoS One* 2011;**6**.
24. Netz DJ, Pohl R, Beck-Sickinger AG, Selmer T, Pierik AJ, Bastos Mdo C, et al. Biochemical characterisation and genetic analysis of aureocin A53, a new, atypical bacteriocin from *Staphylococcus aureus*. *J Mol Biol* 2002;**319**:745–56.
25. Netz DJ, Sahl HG, Marcelino R, dos Santos Nascimento J, de Oliveira SS, Soares MB, et al. Molecular characterisation of aureocin A70, a multi-peptide bacteriocin isolated from *Staphylococcus aureus*. *J Mol Biol* 2001;**311**:939–49.
26. Nissen-Meyer J, Holo H, Havarstein LS, Sletten K, Nes IF. A novel lactococcal bacteriocin whose activity depends on the complementary action of two peptides. *J Bacteriol* 1992;**174**:5686–92.
27. Nissen-Meyer J, Oppegard C, Rogne P, Haugen HS, Kristiansen PE. Structure and mode-of-action of the two-peptide (class-IIb) bacteriocins. *Probiotics Antimicrob Proteins* 2010;**2**:52–60.
28. Oman TJ, Boettcher JM, Wang H, Okalibe XN, van der Donk WA. Sublancin is not a lantibiotic but an S-linked glycopeptide. *Nat Chem Biol* 2011;**7**:78–80.
29. Oppegard C, Rogne P, Kristiansen PE, Nissen-Meyer J. Structure analysis of the two-peptide bacteriocin lactococcin G by introducing D-amino acid residues. *Microbiology* 2010;**156**:1883–9.
30. Rea MC, Dobson A, O'Sullivan O, Crispie F, Fouhy F, Cotter PD, et al. Effect of broad- and narrow-spectrum antimicrobials on *Clostridium difficile* and microbial diversity in a model of the distal colon. *Proc Natl Acad Sci USA* 2011;**108**(Suppl. 1):4639–44.
31. Rea MC, Sit CS, Clayton E, O'Connor PM, Whittal RM, Zheng J, et al. Thuricin CD, a posttranslationally modified bacteriocin with a narrow spectrum of activity against *Clostridium difficile*. *Proc Natl Acad Sci USA* 2010;**107**:9352–7.
32. Uteng M, Hauge HH, Markwick PR, Fimland G, Mantzilas D, Nissen-Meyer J, et al. Three-dimensional structure in lipid micelles of the pediocin-like antimicrobial peptide sakacin P and a sakacin P variant that is structurally stabilized by an inserted C-terminal disulfide bridge. *Biochemistry* 2003;**42**:11417–26.
33. van Belkum MJ, Martin-Visscher LA, Vederas JC. Structure and genetics of circular bacteriocins. *Trends Microbiol* 2011;**19**:411–8.
34. Venugopal H, Edwards PJ, Schwalbe M, Claridge JK, Libich DS, Stepper J, et al. Structural, dynamic, and chemical characterization of a novel S-glycosylated bacteriocin. *Biochemistry* 2011;**50**:2748–55.

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Chapter 17

Colicins

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ABSTRACT

The functions of receptor binding, cytotoxicity, and translocation across the cell envelope, which are necessary for colicins to enter the cell to exert their lethal effect, are encoded in defined domains. Import into the cell is guided by a transenvelope network of "tol" or "ton" proteins. The crystal structures of several colicins or one of their domains are known. Structures have also been obtained for the outer membrane vitamin B_{12} (BtuB) and CirA receptors, in complex with the receptor (R)-domain of the nuclease E-colicins and the channel-forming colicin Ia, respectively. Both extended (>100 Å) colicin receptor–binding domains protrude obliquely from the membrane plane at a 45° angle, resembling a "fishing pole." The disordered colicin E3 N-terminal peptide inserts into an OmpF pore, as shown by crystal structures. Occlusion of OmpF channels by the unfolded colicin E3 C-terminal cytotoxic nuclease domain supports the premise that the colicin C–terminal domain is cotranslocated through OmpF.

DISCOVERY AND CLASSIFICATION

Colicins are plasmid-encoded bacteriocins, produced by *Escherichia coli* under stress conditions, which are cytotoxic to closely related strains that contain the required outer membrane receptor(s) but do not produce the cognate immunity protein. Colicins were first discovered in 1925 by Gratia who showed that one strain of *E. coli* ("V" for virulent) released a substance found in the filtrate that was toxic to a sensitive strain.[3] Gratia also showed that colicin-resistant mutants could be derived from sensitive strains. Fredericq later suggested that resistance to colicins involved the loss of a surface receptor specific for that type of colicin.[3] Indeed, as was confirmed later, colicins enter cells by parasitizing outer membrane receptors that have the physiological purpose of binding and import of metabolites (e.g., vitamins, sugars, metals such as iron). Subsequently, several colicins differing in their mode of action and the receptors they parasitize to gain entry into target cells have been identified. The domain organization of colicins, with each domain performing a distinct function in colicin uptake,[1] is shown (Fig. 1A). Ribbon diagrams of those colicins (Ia, N, B, which are pore formers, and E3, an rRNase) for which the 3-D structure has been solved, are shown in Fig. 1 B–F. Colicins have also been classified according to the translocation system that they appropriate to enter the bacteria. Group A colicins use the bacterial Tol-dependent translocation system consisting of the proteins TolA, TolB, TolQ, TolR, and Pal (Table 1). The "Tol" nomenclature is derived from the observation that mutants of such gene products are "tolerant" to colicin action. Group B colicins use the ton-dependent system, consisting of TonB and ExbB–ExbD (Table 2). "Ton" mutants are so named because they are resistant to the phage T1. As can be seen from the tables, the lethal action of most known colicins is exerted either by cellular deenergization[8,19] that results from the formation of a highly conducting pore in the cytoplasmic membrane or by an enzymatic nuclease digestion mechanism (reviewed in Ref. 3).

COLICIN, IMMUNITY, AND LYSIS PROTEINS

Cells are themselves protected from the action of the colicins they produce by coordinate synthesis of an immunity protein that acts specifically against that colicin. Immunity proteins are small (MW ~10kDa) polypeptides that protect the colicin-producing cells from both endogenous and exogenous colicins. Immunity proteins bind tightly to the enzymatic colicins and prevent them from acting on the colicin-producing cell. In the case of the pore-forming colicins, a small number of immunity molecules (~10^2–10^3) in the cytoplasmic membrane are able to provide immunity against the formation of a single functional and lethal channel in that membrane. Regions of homology between the immunity proteins of pore-forming colicins A, B, and N have suggested that extramembrane loops L1 and L3 of the 3-4 TM helix immunity protein interact with the respective colicins. A "lysis protein," localized in the periplasmic space, functions to release colicin from producer cells to the extracellular medium, both for the colicin-immunity protein complex of enzymatically active colicins and for pore-forming colicins. The gene encoding the colicin, its cognate immunity protein, and the lysis protein form an operon that

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00017-8
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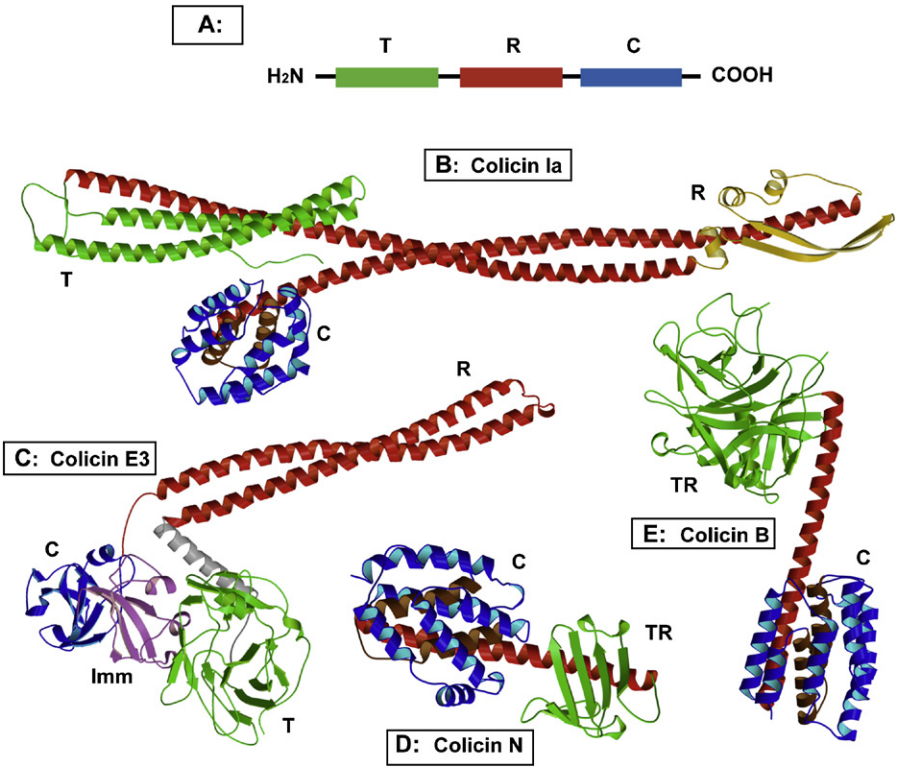

FIGURE 1 (**A**) Generic domain structure of colicin polypeptides (top): T, translocation; R, receptor binding; C, catalytic or activity containing domain (B–E). Ribbon diagrams of structures of pore-forming colicins, Ia (**B**), B (**E**), and N (**D**), and of rRNase colicin E3 (**C**). Note the prominent long (210 and 100 Å in colicins Ia and E3) coiled-coil structure of the R-domain. Structures, predominantly a-helical, have also been obtained of the C-domains of (i) colicins A and E1 by X-ray diffraction and (ii) of E7 and E9 by solution NMR. The structures of E3 and E7 contain bound immunity protein, as is characteristic of the nuclease colicins. See color plate 11.

TABLE 1 Colicins that are Imported via the Tol System. Although these Colicins have a Common Theme of Import into Target Cells, the Receptors and Translocators Used by these Colicins are Different. They also Differ in their Cytotoxic Activities

Colicin	Activity protein[1]	Immunity protein[1]	Receptor	Translocation	Cytotoxic activity
A[2]	592	178	BtuB	OmpF, TolQRAB	Pore forming
E1	522	113	BtuB	TolC, TolQRAB	Pore forming
N	387	131	OmpF	TolQRA	Pore forming
E2	581	86	BtuB	OmpF, TolQRAB	DNase
E7	566	87	BtuB	OmpF, TolQRAB	DNase
E9	582	86	BtuB	OmpF, TolQRAB	DNase
E3	551	85	BtuB	OmpF, TolQRAB	16SrRNase
E6	551	85	BtuB	OmpF, TolQRAB	16SrRNase

Tol-dependent colicins E4, E5, E8, K, U, 28b, and DF13 have not been included in the table.
[1]*Length in amino acids.*
[2]*Sequence of Colicin A from* Citrobacter freundii.

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TABLE 2 Colicins that are Imported via the Ton System. Although the Mechanism of Import of the Various Colicins into the Target Cells is Similar, the Cytotoxic Activity Differs. A Common Feature of these Colicins is the Presence of a TonB Box that Interacts with the TonB Protein, which Transduces Energy from the Inner Membrane to these Import Processes[3]

Colicin	Activity protein[1]	Immunity protein[1]	Receptor protein	Activity
B	510	175	FepA	Channel
D	697	87	FepA	Protein synthesis[2]
Ia	626	111	Cir	Channel
Ib	626	115	Cir	Channel

Ton-dependent colicins Js, M, 5, and 10 have not been included in the table.
[1]Length in amino acids.
[2]Inhibits protein synthesis by cleavage of arginine tRNAs.

is regulated by an SOS (DNA-damage–induced) promoter. In the absence of induction, the immunity gene is expressed by a constitutive promoter that provides basal levels of immunity protein for protection against exogenous colicins (reviewed in Ref. 3).

THE DOMAIN CONCEPT

There are three defined steps in the killing of sensitive *E. coli* by most colicins, each of which is carried out by a separate domain of the colicin that occupies approximately one-third of the polypeptide (Fig. 1A). The first step involves the central "R" (receptor) domain, which mediates the binding of colicin to its outer membrane receptor. The N-terminal "T" (translocation) domain mediates the second step of translocation from the outer membrane receptor to the colicin target in the cell. Finally, the carboxy-terminal "C" (catalytic) domain functions in cytotoxicity.

CRYSTAL STRUCTURES OF COLICINS (FIG. 1B–E)

(A) The four complete or incomplete structures of colicins that have been solved by X-ray diffraction of three-dimensional crystals and their major properties are summarized: (i) The structure of residues 23–39 and 83–624 of the 626 residue pore-forming colicin Ia ("B-type," translocated by the Ton system) has been resolved to 3.0 Å resolution (Fig. 1B[29]) and (ii) that encompassing residues 84–551 of the 551 residue endoribonuclease colicin E3 ("A-type," translocated by the Tol system) to 3.0 Å (Fig. 1C[24]). The crystal structure of a complex of the BtuB receptor and bound coiled-coil R-domain of colicins E2[21] and E3,[16] relevant to the mechanism of their cellular import through the outer membrane, is discussed below. (iii) A 3.1-Å structure of a 292 residue segment, of a 321 residue chymotryptic fragment of the 387 residue pore-forming colicin N, contains the globular channel domain (Fig. 1D) but is missing a substantial part (N-terminal 90 residues) of the T- (and possibly of the R-) domain.[27] (iv) The structure of most of the 511 residue pore-forming colicin B (ton dependent) (Fig. 1E) was solved at a 2.5-Å resolution.[11] The X-ray and NMR structures of the C-domains of pore-forming colicins A[18] and E1[7] show a bundle of ten α-helices with a hydrophobic helical hairpin anchor at the core. The structures of the endonuclease domain of colicins E3, E7, and E9 (tol dependent), and ton-dependent colicin D, in complex with Imm or nucleic acids, have also been solved.[9,15,26]

The T-, R-, and C-domains in colicins E3 (RNase; tol dependent) and Ia (pore forming; ton dependent) are structurally defined (Fig. 1B, C) and connected by long α-helices that form coiled coils, whereas in colicin B (pore forming; ton dependent; Fig. 1E), and probably in colicin N (pore-forming; tol-dependent; Fig. 1D), the T- and R-domain functions are located in a globular domain that is separated from the C-domain by a single long helix, forming the shape of a dumbbell. These two distinct shapes are not related to either the import pathway (tol or ton dependent) or to the mechanism of cytotoxicity (pore formation or endonuclease). It has been proposed that the extended coiled-coil structure ("fishing pole") in nuclease colicins such as E3 is necessary for delivery of T- and C-domains of a receptor-bound colicin to the outer membrane porin OmpF, through which it is translocated to the periplasm.[16] Outer membrane porins do not seem to be involved in the import of the ton-dependent colicin Ia.[13]

(B) *T-domains.* The N-terminal T-domain of colicin Ia contains a "TonB box" (E23–V27) that is necessary for translocation across the periplasm as part of an antiparallel helix bundle. This secondary structure of the T-domain of colicin Ia contrasts with the β-sheet "jelly roll" that forms most of the colicin E3 T-domain. The N-terminal region of the nuclease E colicin T-domain contains an extended "TolB box" needed for translocation,[10] and is glycine-rich

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(34 of 79 residues), accounting for the disorder in this region of the structure. A possible reason for the difference in the T-domain structures of colicins Ia and E3 is that the immunity protein is tightly bound to the T-domain in the latter. The N-terminal domain of colicin B is composed of 17 β-strands, 2 α-helices, and long loops. The central region (residues 130–291) of its sequence shares some similarity with the T-domain of colicin E3, but the folding pattern does not fit to a jelly roll motif. In both colicins Ia and B, the N-terminal segments, containing the TonB box (residues 23–27 and 17–21 in Ia and B, respectively) are folded back and interact with antiparallel helices of the coiled-coil and T-domain in colicin Ia and with the N-terminal lobe (TR-domains) of colicin B.

(C) *R-domains.* The 104 residue region at the tip of the 160-Å coiled coil, consisting of an amphipathic two-stranded β-hairpin folded around the tip-terminal helix of the coiled coil was proposed to bind to the colicin Ia Cir receptor.[29] The R-domain of colicin N was proposed to consist of a 65 residue (residues 97–161) six-stranded β-strand wrapped around the C-terminal end of a 45 residue extended helix (65 Å) that would be part of the pore-forming domain.[27] The receptor-binding function of colicin B was attributed to two antiparallel β-strands (residues 262–282) of the same N-terminal lobe that contains the T-domain.

Comparison of the structure of colicin Ia (Fig. 1B) with genetic and functional analysis of the pore-forming colicins implies that the entire coiled coil might be a good fit to the receptor-binding R-domain. Subsequent studies of the minimum receptor-binding domain in the A type colicins E9 and E3 that inhibit intracellular protein synthesis have indicated that a large part of the coiled-coil, but perhaps only its distal half, might be necessary for recognition and effective binding to the receptor. The coiled-coil nature of the receptor domain in the structure of intact colicins Ia and E3 implies that this is a necessary structural motif for interaction with the receptor that initially sequesters the colicin.

The markedly smaller length of the extended helices of colicins N and B is suggestive of a mechanism that is different from those used by colicins Ia and E3 for receptor binding and translocation across the outer membrane and periplasmic space.

(D) *Cytotoxicity domains.* The isolated soluble pore-forming 20-kDa domain of colicins A[18] and E1,[7] along with these domains in the intact colicins B, Ia, and N, have a similar 10 helix globular structure with a tightly packed apolar core formed by a hydrophobic helical hairpin consisting of helices VIII and IX. This hairpin is surrounded by 8 hydrophilic helices, some of which are amphipathic. The average length of the two hairpin helices is 15–16 residues, shorter than usually considered necessary (~20 residues) to span the membrane bilayer. The average length of the surrounding 8 helical segments is 12–14 residues. Beyond the implied role of the buried helical hydrophobic hairpin as a potential membrane anchor, the structure of the soluble channel domain provides few clues about the mechanism of pore formation and insertion into the membrane, particularly since the one-hit mechanism of cell killing implies that the channel is formed by a single colicin molecule. In addition, the channel-forming domain of the soluble colicin must undergo large voltage-dependent conformational changes to accomplish insertion of the channel.[17,33,34]

The function of the hydrophobic helical hairpin is to serve as an anchor during pore-forming domain insertion into membrane bilayer. This interaction is dependent on the anionic lipid content, that is, and is optimal at approximately 20–30% anionic lipid and a negative membrane surface potential of approximately −60 mV.[34] Electrostatic interactions with the negatively charged membrane surface (i) guides binding of the C-terminal pore-forming domain to the membrane surface and (ii) induces unfolding that results in the formation of a surface-bound prepore state.[17,33]

The ability of pore-forming colicins to form ion channels in planar bilayer membranes was demonstrated for the first time by Schein et al.[19] The transition of the colicin from a closed-to-open state requires the presence of a transmembrane potential, negative on the side of the membrane opposite (*trans*) to the side of protein binding and insertion. The electrical field across the cytoplasmic membrane (*in vivo*) created by the electrochemical proton gradient thus favors pore formation. The structure of the open channel state is not known. The one-hit killing first described by F. Jacob implies that the channel is formed by a single colicin molecule or unit. In the open state of the channel, a significant part of the C-domain (68 residues, helices II–V of colicin Ia) becomes accessible to ligand binding from the *trans* side of the membrane.[23] The dependence of channel formation by colicins on the lipid content of membrane points to the involvement of lipids in the formation and a structure of the ion channel that has been characterized as a "toroidal pore."[32]

The cytotoxic activity of endonuclease colicins also resides in the C-terminal domain. The size of the C-terminal cytotoxic domain of these colicins ranges from 96 to 135 residues. The immunity proteins bind to these domains with very high affinity ($K_d < 10^{-13}$M[28]; Zakharov et al., unpublished), efficiently blocking the interaction of colicins with their substrates, DNA or ribosomes. Extensive, highly complementary interaction surfaces in endonuclease–Imm complexes that involve hydrophobic and polar side chains of both proteins, account for the high-affinity nature of binding of Imm. Active sites in both DNase and rRNase colicins involve residues on the C-terminal side relative to the residues involved in Imm binding. This can allow Imm binding to an incompletely translated colicin to afford protection to host cells during the toxin synthesis.

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CRYSTAL STRUCTURES OF COLICIN–RECEPTOR COMPLEXES (FIG. 2)

Crystal structures of complexes formed by polypeptide fragments of (i) the receptor-binding domain of colicins E2, E3, and Ia with the outer membrane primary receptor, BtuB[16,21] or Cir[2] and (ii) the N-terminal T-domain of colicins E3 and E9 with OmpF[12,30] have provided an insight into the initial molecular events that concentrate the colicin from three-dimensional solution space to the two-dimensional cell surface. Here, we discuss these structures together with the information they provide regarding the mechanism of colicin translocation across the *E. coli* outer membrane.

Colicin R Domain and the Primary Receptor

(A) Comparison of E2R135–BtuB and E3R135–BtuB, and with apo-BtuB –Crystallization of colicin E2[21] (Fig. 2B) and E3[16] (Fig. 2A) R-domains (135 residues) in complex with their primary receptor, BtuB, yielded crystal structures at 3.5- and 2.75-Å resolution, respectively. The two crystal structures showed the colicin R domain bound peripherally and at an oblique angle of 45° to the membrane plane. There was no disorder seen in the plug domain of either crystal structures, implying that the colicins do not translocate through the BtuB central pore. Further evidence in this regard comes from planar bilayer studies that did not show an opening of the BtuB pore upon addition of colicin E3 R-domain.[16] The oblique angle of the R-domain is thought to be physiologically relevant and is hypothesized to position the T-domain of the colicin in close proximity to the outer membrane where it enables a search for the secondary receptor/translocator, OmpF, or OmpC. The ample abundance of OmpF and/or OmpC (~10^5/cell) ensures that at least one of these porins can be found within easy reach of the obliquely bound colicin. The natively unstructured N-terminal 83 residues of colicins E2 and E3 have been inferred to enable the colicin to bind to and traverse the outer membrane to reach the periplasmic TolB protein. The two structures were found to be remarkably similar with the main difference being that the R domain in E2R135–BtuB was two and five residues longer, respectively, on the N- and C-termini. Although the R-domain was bound peripherally, the interaction between the R-domain and BtuB is surprisingly strong. The K_d of the interaction was estimated to be approximately 10^{-9}M, implying a binding energy of approximately −12kcal/mol.[16] This strong interaction suggests that the C-domain of colicin reaches its cytosolic target not by dissociation of the colicin from its tightly bound receptor but by separation of the C-domain from the rest of the colicin. The cleaved C-domain then enters the cell cytosol.

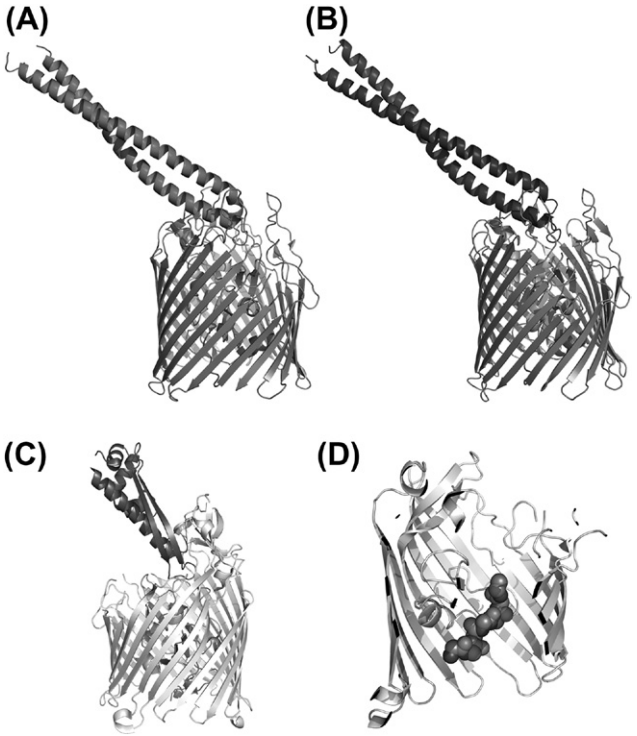

FIGURE 2 Crystal structures of receptor-binding R135 fragment of colicin (A) E3 and (B) E2 (residues 313–447) in complex with the high-affinity outer membrane vitamin B12 receptor at 2.75 and 3.5 Å, respectively. (C) A 2.5-Å resolution structure of colicin Ia R104 (aa 282–385) fragment bound to its Cir receptor showing the colicin Ia bound at the same 45° angle to membrane plane, as seen for colicin E2 and E3R135–BtuB structures above. (D) A 3.0-Å structure of OmpF porin with a small fragment of the translocating N-terminal peptide segment of colicin E3, corresponding to carbon backbone of a string of 7 residues, bound in the central pore.

At least some degree of colicin unfolding is required for its entry into the cell. The structure of E2R135–BtuB and E3R135–BtuB has revealed unwinding of intertwined coiled-coil R-domain and unfolding of the ends of the alpha-helices suggesting that the binding energy of BtuB and colicin R-domain can be used for colicin unfolding.[16] The binding of colicin E2 and E3 R-domain causes some changes in the BtuB structure, too. Most of the changes are in the extracellular loops of BtuB, some of which become ordered on binding of the R-domain. Two residues, Ser4 and Pro5 of the TonB box, become ordered up binding of the R-domain.

(B) Ia R104-Cir complex (Fig. 2C) – The structure of the R-domain (104 residues, aa 282–385) of the pore-forming Group B colicin Ia in complex with its Cir receptor has been solved to 2.5-Å resolution.[2] The structure revealed the Ia R-domain to be bound at a 45° angle to the membrane plane, an orientation very similar to that seen in the E2R135–BtuB and E3R135–BtuB structures. The Ia R-domain binds peripherally, yet tightly (dissociation constant, ~10^{-10}M) to Cir. As in the structures of nuclease colicin R-domains with BtuB, no significant disorder was seen in the plug domain

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of the Cir receptor, suggesting that Ia translocates across the outer membrane through a secondary receptor/translocator. This secondary receptor/translocator was inferred to be another Cir molecule.[13] Superimposition of the complete colicin Ia structure onto the structure of Ia–Cir complex reveals that because of the long 160-Å helices connecting R-domain to the C- and T-domain, the C- and T-domains are suspended approximately 100 Å above the membrane surface. It is unclear how these domains of the colicin would traverse this distance to reach the membrane surface.

(C) Binding of Colicin N to OmpF – Electron microscopic analysis of 2-D crystals of colicin N along with its receptor/translocator OmpF revealed the colicin to be bound at the periphery of OmpF trimer where it could displace LPS molecules bound to OmpF.[5] Neutron scattering indicates that the pore-forming domain of colicin N binds in the interstices of the OmpF trimer, inserting into an OmpF–lipid interface.[5] Interaction of the colicin with LPS is further corroborated by the results of genomic screening that identified LPS biosynthesis genes to have a function in the cytotoxicity of colicin N.[20] Genes *gmhA*, *gmhB*, gmhC, *gmhD* that function in synthesizing the ADP-L, D-Hep moiety, *waaC*, and *waaF* that use the ADP-L, D-Hep to form the LPS inner core and *waaG* and *galU* that, in concert, add the first glucose to this inner core, were all identified to be required for colicin cytotoxicity. These results imply that the LPS inner core together with the first added glucose forms the minimum binding site for colicin N.

It is unclear whether colicin N translocation across the outer membrane occurs through the central OmpF lumen or at the OmpF-lipid interface. There is evidence to suggest that the colicin T-domain interacts with the OmpF lumen.[14,31] One of the possible mechanisms of colicin N entry that can accommodate these findings is that the colicin T-domain interacts and traverses the outer membrane through OmpF to reach the periplasm. In the periplasm, the TolB box in the colicin T-domain interacts with the TolB protein that is a part of the Tol complex. This interaction together with the colicin N–OmpF binding energy causes partial unfolding of the colicin, enabling the C-domain to traverse the outer membrane at the membrane-OmpF interface. In this model, the colicin R-domain remains anchored to the outer membrane through its interactions with OmpF, whereas the C-domain reaches the inner membrane where it forms channels killing the cell. Support for this model also comes from planar bilayer studies that show that the T-domain of colicin N can occlude OmpF channels, whereas the RC domain does not.[31]

Colicin E3 T-domain and OmpF (Fig. 2D)

Subsequent steps in the import of the nuclease colicins E2, E3, and E9 across the outer membrane include facile lateral diffusion across the outer membrane[25] and formation of a transient complex with the OmpF porin to which the colicin N-terminal T-domain binds and inserts. The latter inference is based on structure data derived from diffraction data at 3.0-Å resolution obtained from cocrystals of OmpF with N-terminal peptides of colicins E3[30] or E9.[12] Electron density was consistent with a seven residue segment of the colicin E3 T-domain, the sequence of which was not determined in the original study but overlapped a 15 residue peptide T-domain peptide of colicin E9 that spans the aqueous channel running through OmpF. These structures define the anchor formed by these colicins with the OmpF pore protein through which their cytotoxic C-terminal segment must be inserted. Although a structure has not yet been obtained of the latter complex, it has been shown that the C-terminal peptide of colicin E3 will occlude OmpF channels in planar bilayer membranes.[35]

MECHANISM OF IMPORT ACROSS THE OUTER MEMBRANE (FIG. 3)

Several steps have been identified in the uptake and subsequent cytotoxicity of the colicins. These steps have been hypothesized in both Group A and B colicins. In the first step (Fig. 3, Step 1), colicins bind tightly to their primary receptor, through their R-domain. This binding is at an oblique angle to the plane of the membrane, as seen in the crystal structures of E2R135–BtuB, E3R135–BtuB, and Ia R104–Cir. This allows the T-domain of the colicin to potentially scour the neighboring area for the secondary receptor/translocator, OmpF/OmpC in the case of nuclease E colicins and Cir in the case of colicin Ia (Fig. 3, Step 2). The T-domain then interacts with either the Tol or the Ton network (Fig. 3, Step 3). This interaction leads to the release of the immunity protein[6] (Fig. 3, Step 4). Release of

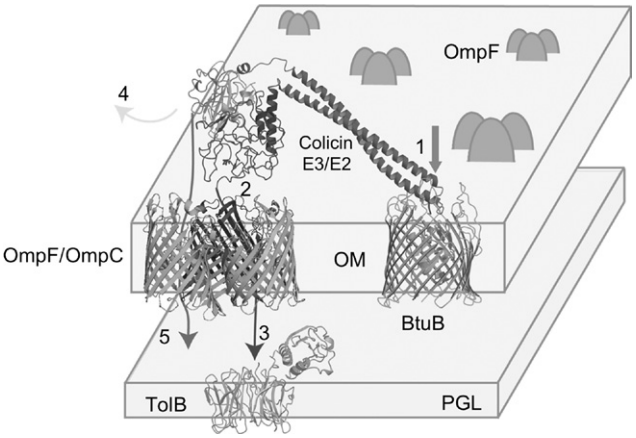

FIGURE 3 Events occurring during the import of colicin E3/E2: **(1)** High-affinity binding to the primary receptor BtuB; **(2)** interaction with and insertion through the secondary receptor/translocator OmpF/OmpC; **(3)** binding to TolB; **(4)** release of Immunity; **(5)** proteolytic cleavage of the cytotoxic C-domain followed by its insertion into the cell where it exerts its cytotoxic effects.

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immunity protein has been shown to cause a partial unfolding of the colicin E3 C-domain allowing it to interact with OmpF[35] (Fig. 3, Step 5). Concomitantly, a protease then functions to separate the C-domain from the rest of the colicin by cleaving at the junction between the C- and the R-domain[22] (Fig. 3, Step 5). In contradiction to an earlier work that found the protease to be periplasmic for colicin E7,[22] a recent work has identified the protease to be an inner membrane protein, FtsH,[4] in the case of colicins D and E3. The mechanism of translocation of C-domain into the cell across the inner membrane is yet to be elucidated but is widely believed to be mediated by the Tol or the TonB–ExbBD complexes.

AREAS OF RESEARCH ENCOMPASSED BY COLICIN STUDIES

As described above, colicin studies span a broad range of general interests that include mechanisms of protein secretion, protein import, protein–receptor interactions, high-affinity protein–protein interactions, ion channel structures and function, and intracellular nuclease activities.

ACKNOWLEDGMENTS

Studies of the authors reported here were supported by NIH GM-018457 and the Henry Koffler Professorship (W.A.C.).

REFERENCES

1. Benedetti H, Frenette M, Baty D, Knibiehler M, Pattus F, Lazdunski C. Individual domains of colicins confer specificity in colicin uptake, in pore-properties and in immunity requirement. *J Mol Biol* 1991;**217**:429–39.
2. Buchanan SK, Lukacik P, Grizot S, Ghirlando R,MM, Barnard TJ, et al. Structure of colicin I receptor bound to the R-domain of colicin Ia: implications for protein import. *EMBO J* 2007;**26**:2594–604.
3. Cascales E, Buchanan SK, Duche D, Kleanthous C, Lloubes R, Postle K, et al. Colicin biology. *Microbiol Mol Biol Rev* 2007;**71**:158–229.
4. Chauleau M, Mora L, Serba J, de Zamaroczy M. FtsH-dependent processing of RNase colicins D and E3 means that only the cytotoxic domains are imported into the cytoplasm. *J Biol Chem* 2011;**286**:29397–407.
5. Clifton LA, Johnson CL, Solovyova AS, Callow P, Weiss KL, Ridley H, et al. Low resolution structure and dynamics of a Colicin-Receptor complex determined by neutron scattering. *J Biol Chem* 2011;**287**:337–46.
6. Duche D, Frenkian A, Prima V, Lloubes R. Release of immunity protein requires functional endonuclease colicin import machinery. *J Bacteriol* 2006;**188**:8593–600.
7. Elkins P, Bunker A, Cramer WA, Stauffacher CV. A mechanism for toxin insertion into membranes is suggested by the crystal structure of the channel-forming domain of colicin E1. *Structure* 1997;**5**:443–58.
8. Gould JM, Cramer WA. Studies on the depolarization of the *Escherichia coli* cell membrane by colicin E1. *J Biol Chem* 1977;**252**:5491–7.
9. Graille M, Mora L, Buckingham RH, van Tilbeurgh H, de Zamaroczy M. Structural inhibition of the colicin D tRNase by the tRNA-mimicking immunity protein. *EMBO J* 2004;**23**:1474–82.
10. Hands SL, Holland LE, Vankemmelbeke M, Fraser L, Macdonald CJ, Moore GR, et al. Interactions of TolB with the translocation domain of colicin E9 require an extended TolB box. *J Bacteriol* 2005;**187**:6733–41.
11. Hilsenbeck JL, Park H, Chen G, Youn B, Postle K, Kang C. Crystal structure of the cytotoxic bacterial protein colicin B at 2.5 Å resolution. *Mol Microbiol* 2004;**51**:711–20.
12. Housden NG, Wojdyla JA, Korczynska J, Grishkovskaya I, Kirkpatrick N, Brzozowski AM, et al. Directed epitope delivery across the *Escherichia coli* outer membrane through the porin OmpF. *Proc Natl Acad Sci U S A* 2010;**107**:21412–7.
13. Jakes KS, Finkelstein A. The colicin Ia receptor, Cir, is also the translocator for colicin Ia. *Mol Microbiol* 2010;**75**:567–78.
14. Jeanteur D, Schirmer T, Fourel D, Simonet V, Rummel G, Widmer C, et al. Structural and functional alterations of a colicin-resistant mutant of OmpF porin from *Escherichia coli*. *Proc Natl Acad Sci U S A* 1994;**91**:10675–9.
15. Kühlmann UC, Pommer AJ, Moore GR, James R, Kleanthous C. Specificity in protein–protein interactions: the structural basis for dual recognition in endonuclease colicin-immunity protein complexes. *J Mol Biol* 2000;**301**:1163–78.
16. Kurisu G, Zakharov SD, Zhalnina MV, Bano S, Eroukova VY, Rokitskaya TI, et al. The structure of BtuB with bound colicin E3 R-domain implies a translocon. *Nat Struct Biol* 2003;**10**:948–54.
17. Lindeberg M, Zakharov SD, Cramer WA. Unfolding pathway of the colicin E1 channel protein on a membrane surface. *J Mol Biol* 2000;**295**:679–92.
18. Parker MW, Postma JP, Pattus F, Tucker AD, Tsernoglou D. Refined structure of the pore-forming domain of colicin A at 2.4 Å resolution. *J Mol Biol* 1992;**224**:639–57.
19. Schein SJ, Kagan BL, Finkelstein A. Colicin K acts by forming voltage-dependent channels in phospholipid bilayer membranes. *Nature* 1978;**276**:159–63.
20. Sharma O, Datsenko KA, Ess SC, Zhalnina MV, Wanner BL, Cramer WA. Genome-wide screens: novel mechanisms in colicin import and cytotoxicity. *Mol Microbiol* 2009;**73**:571–85.
21. Sharma O, Yamashita E, Zhalnina MV, Zakharov SD, Datsenko KA, Wanner BL, et al. Structure of the complex of the colicin E2 R-domain and its BtuB receptor. The outer membrane colicin translocon. *J Biol Chem* 2007;**282**:23163–70.
22. Shi Z, Chak KF, Yuan HS. Identification of an essential cleavage site in ColE7 required for import and killing of cells. *J Biol Chem* 2005;**280**:24663–8.
23. Slatin SL, Qiu XQ, Jakes KS, Finkelstein A. Identification of a translocated protein segment in a voltage-dependent channel. *Nature* 1994;**371**:158–61.
24. Soelaiman S, Jakes K, Wu N, Li C, Shoham M. Crystal structure of colicin E3: implications for cell entry and ribosome inactivation. *Mol Cell* 2001;**8**:1053–62.
25. Spector J, Zakharov S, Lill Y, Sharma O, Cramer WA, Ritchie K. Mobility of BtuB and OmpF in the *Escherichia coli* outer membrane: implications for dynamic formation of a translocon complex. *Biophys J* 2010;**99**:3880–6.
26. Sui MJ, Tsai LC, Hsia KC, Doudeva LG, Ku WY, Han GW, et al. Metal ions and phosphate binding in the H–N–H motif: crystal structures of the nuclease domain of ColE7/Im7 in complex with a phosphate ion and different divalent metal ions. *Protein Sci* 2002;**11**:2947–57.

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27. Vetter IR, Parker MW, Tucker AD, Lakey JH, Pattus F, Tsernoglou D. Crystal structure of a colicin N fragment suggests a model for toxicity. *Structure* 1998;**6**:863–74.
28. Walker D, Moore GR, James R, Kleanthous C. Thermodynamic consequences of bipartite immunity protein binding to the ribosomal ribonuclease colicin E3. *Biochemistry* 2003;**42**:4161–71.
29. Wiener M, Freymann D, Ghosh P, Stroud RM. Crystal structure of colicin Ia. *Nature* 1997;**385**:461–4.
30. Yamashita E, Zhalnina MV, Zakharov SD, Sharma O, Cramer WA. Crystal structures of the OmpF porin: function in a colicin translocon. *EMBO J* 2008;**27**:2171–80.
31. Zakharov SD, Eroukova VY, Rokitskaya TI, Zhalnina MV, Sharma O, Loll PJ, et al. Colicin occlusion of OmpF and TolC channels: outer membrane translocons for colicin import. *Biophys J* 2004;**87**:3901–11.
32. Zakharov SD, Kotova EA, Antonenko YN, Cramer WA. On the role of lipid in colicin pore formation. *Biochim Biophys Acta* 2004;**1666**:239–49.
33. Zakharov SD, Lindeberg M, Griko Y, Salamon Z, Tollin G, Prendergast FG, et al. Membrane-bound state of the colicin E1 channel domain as an extended two-dimensional helical array. *Proc Natl Acad Sci U S A* 1998;**95**:4282–7.
34. Zakharov SD, Rokitskaya TI, Shapovalov VL, Antonenko YN, Cramer WA. Tuning the membrane surface potential for efficient toxin import. *Proc Natl Acad Sci U S A* 2002;**99**:8654–9.
35. Zakharov SD, Zhalnina MV, Sharma O, Cramer WA. The colicin E3 outer membrane translocon: immunity protein release allows interaction of the cytotoxic domain with OmpF porin. *Biochemistry* 2006;**45**:10199–207.

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Chapter 18

Defensins

Nikolinka Antcheva, Filomena Guida and Alessandro Tossi

ABSTRACT

The term defensin relates to a set of host defense peptides (HDPs) in vertebrates, invertebrates, plants, and molds that display structural similarities based on a cystine stabilized antiparallel β-sheet core, with an α-helical stretch also present in some members. Defensins of different origins exhibit structural and functional similarities, with phylogenetic relationships between invertebrate and plant defensins and between different types of vertebrate defensins, and a possible common bacterial ancestor. Most defensins demonstrate a direct antimicrobial action *in vitro* with varying activity spectra. This requires interaction with the components of the microbial cell wall, and often involves membrane permeabilization, although the mode of action differs markedly for defensins both within and from different families. Defensins also play important regulatory roles in the immune system of animals and plants, acting as a bridge between innate and adaptive immunity in vertebrates. Roles outside of host defenses have also been reported.

DISCOVERY

Defensins were among the first HDPs to be discovered. α-Defensins were first identified in the early 1980s as cationic, cysteine-rich components of mammalian neutrophil phagocytes, by Lehrer et al., who coined the name "defensin."[14,15] Analogous molecules termed "cryptdins" were later found in specialized host defense cells of the intestinal crypts (see the chapter on Paneth cell defensins in the Gastrointestinal Peptides section of this book). The ability to inhibit adrenocortical steroidogenesis also led to the alternative definition "corticostatin."[32] In the early 1990s, "β-defensins," with a different cysteine-bridge connectivity, were identified in mammalian epithelia and neutrophils, as well as in avian leukocytes,[3,13–15,23,26] and more recently also in reptiles and teleost fish.[4,35] In the late 1980s, isolation of the inducible peptides from insect hemolymph[5] with a significant similarity to the mammalian peptides is what prompted them to be named "insect defensins." Cysteine-rich peptides with antimicrobial properties—the "thionins"—were known in plants since the early 1950s,[2] but it was only in the midnineties that the γ-thionins, based on their resemblance to the insect and mammalian defensins, were redefined as "plant defensins" (see also the chapter on Plant Antifungal Peptides in the Plant Peptides section of this book).[13] A backbone circularized peptide that is structurally quite different from that of defensins was isolated in Old World monkeys but was found to derive from the head-to-tail ligation of two truncated α-defensins, so it was assigned the name "θ-defensin" with reference to its cyclic structure.[8,14,23] Finally, the recent discovery of a several families of defensin-like peptides in a fungus underlines the antiquity of this peptide superfamily of innate immune effectors.[34]

The term "defensin" thus defines several peptide families based on functional (host defense) and structural (a compact cysteine-stabilized β-sheet structure) similarities[1,10,26,27] that seem to span a vast phylogenetic continuum. There is evidence that fungal, invertebrate animal and plant defensins form an evolutionarily related group,[5,7,19,34] and all the vertebrate defensins form another.[29] These two groups may be phylogenetically related through ancient prokaryotic ancestors as evidenced by the discovery of defensin-like peptides in some bacteria.[1,27,31,33]

STRUCTURE OF PRECURSOR mRNA AND GENE

Analysis of defensin gene organization in numerous species indicates that they occur in multigene families, as a result of numerous duplication and diversification events. They are synthesized as prepropeptides (Fig. 1) and processed to varying degrees depending on the context.

The mammalian α- and β-defensins are products of distinct gene families that probably derive from an ancestral β-defensin gene, as only this latter type are found in non-mammalian vertebrates.[25,26,28] Primate θ-defensin precursors are truncated α-defensin paralogs from which fragments are excised and spliced head to tail to give the mature, backbone cyclized, homodimeric or heterodimeric peptide (Fig. 2).[8,14,23] Their genes are active in Old World monkeys and Orangutans, not present in New World monkeys or prosimians, and present only as pseudogenes in chimpanzees and humans.

In humans, a gene cluster for both α- and β-defensins was mapped to a structurally variable region of chromosome

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00018-X
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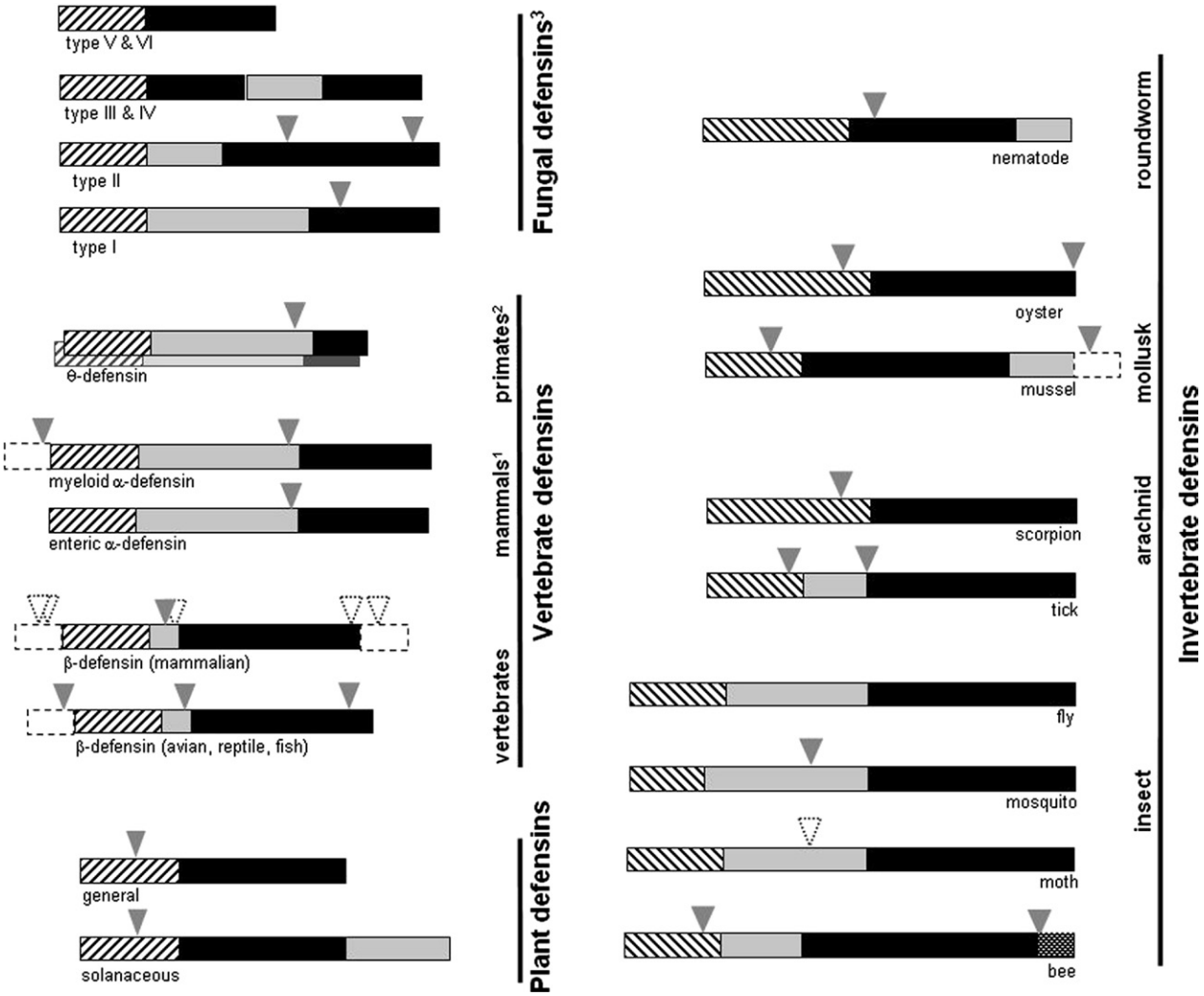

FIGURE 1 Gene organization of different defensin families. The coding region for the mature peptide is in black. The signal or leader sequences are hashed. The proregions are in gray. Broken rectangle indicates the 5′- or 3′-UTR regions, only when introns fall into them. Introns are indicated by triangles. Full gray triangles represent introns normally present, whereas dotted triangles represent additional introns present only in some members. Coding region relative sizes are only indicative. For θ-defensin, two different genes provide precursors as indicated in the circle that are subsequently trimmed and spliced head to tail into a single backbone-cyclized molecule. The dotted part of the bee defensin mature peptide region represents a C-terminal extension found only in hymenopterans defensins.[1,2] For vertebrate defensins, β-defensins are present in all vertebrates, whereas α-defensins are present in all tested mammals except in artiodactyls. θ-Defensins are present in Old but not in New world monkeys, and also in orangutans, but only as pseudogenes in humans and chimpanzees.[3] Fungal defensin gene families are as indicated by Zhu.[34] Adapted from Refs. 4, 7, 11, 13, 18, 20, 21, 23, 29, 34.

8 (8p21–p23[22]), and there is evidence for a similar arrangement in syntenic chromosomal locations in other mammals confirming the homology between these gene families. Clusters of β-defensin-like peptides of unknown function map to other chromosomal locations in humans, mice, rats, and dogs.[18,22] In nonmammalian vertebrate species, only β-defensin genes have been identified; 14 and 22 genes are clustered, respectively, on chicken and finch chromosome 3 (3q3.5–q3.7 in chicken).[11] Mammalian β-defensin genes have at least two exons, the first encoding for the signal and the propiece, and the second for the HDP (Fig. 1), separated by an intron of variable size, just before the mature peptide coding region. Myeloid α-defensins have a third exon in the 5′-UTR, as do avian, reptile, and fish but only a few mammalian β-defensins.[4,18,23,35]

Invertebrate defensins exhibit a more varied gene organization (Fig. 1). In addition to presenting diverse coding regions, the presence, number, and location of introns also vary.[7,20] Defensin genes from related species, or even the same species, can show significant homology in the mature HDP region but little in that coding for the leader and propeptides.

Plant defensins show two types of gene organization (Fig. 1),[13] the most common consisting of only signal peptide and the mature defensin domain, the other, found in

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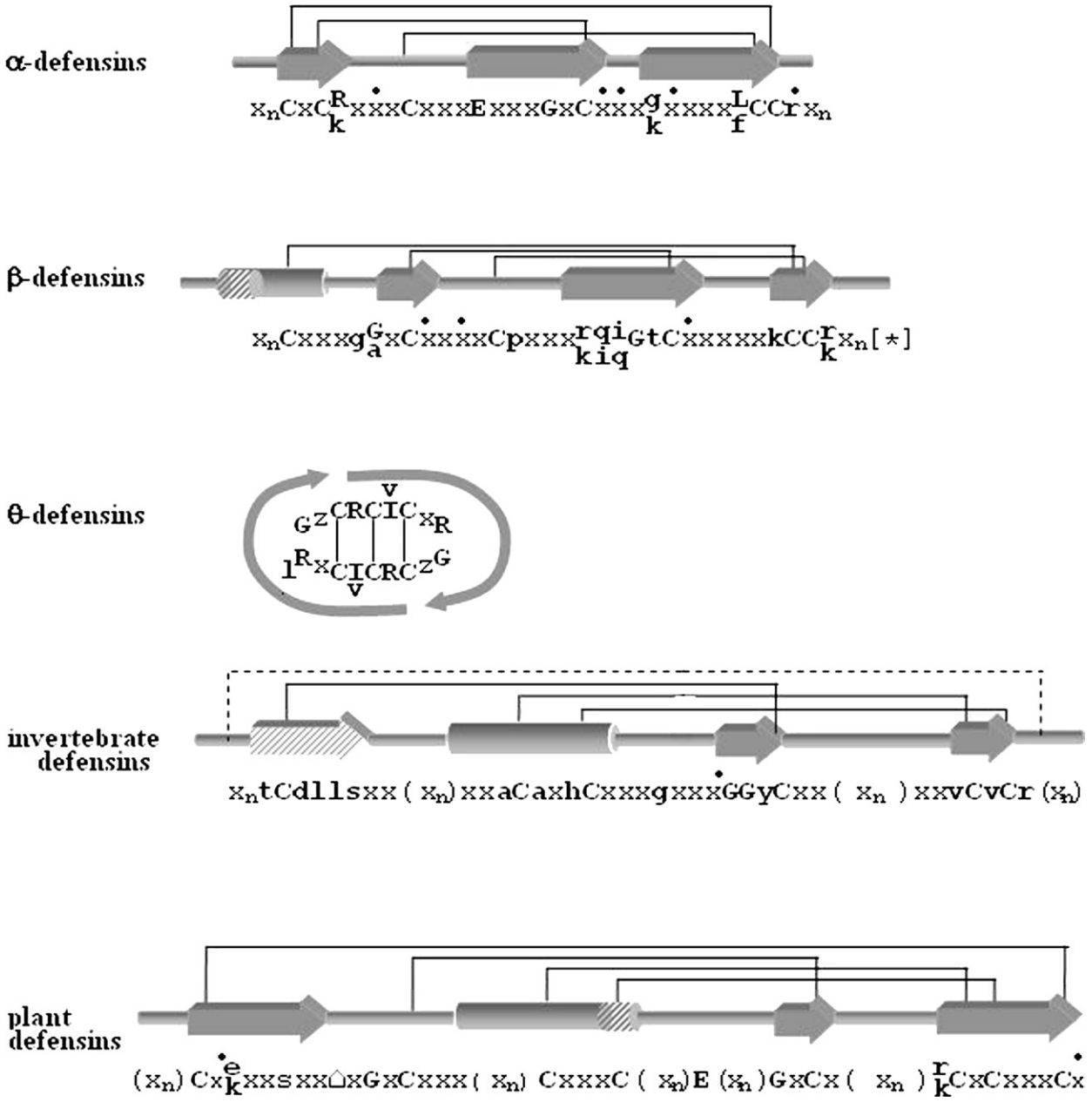

FIGURE 2 Schematic representation of the primary structure, topology, disulfide connectivities, and residue variation in defensin families. Highly conserved residues are in uppercase, conserved residues are in lowercase, x indicates variable residues, and stretches of variable size are indicated by x_n; dots above the sequence indicate positions of possible residue deletions in some family members with respect to others. For β-defensins, * indicates a long C-terminal extension present in some members (e.g. epididymis hBD25-29). For θ-defensins, z indicates variable but hydrophobic residues; the only known fungal defensin structure is very similar to the six cysteine mussel defensin structure. For plant defensins, ⌂ indicates aromatic residues. Gray arrows represent β-strands. Cylinders represent α-helical segments. Hashed segments are present in some family members but not in others.

solenaceous species, presenting a C-terminal propiece. Only one intron is present.

The propiece in plant, invertebrate, and vertebrate defensins is often anionic, in contrast to the cationic mature HDP. This may be required for folding, subcellular trafficking, or processing, or may serve to render the HDP nontoxic until needed, protecting the producer cell.

DISTRIBUTION AND EXPRESSION OF THE mRNA

Defensins are expressed at sites where the producer organism encounters pathogens or needs to control the natural microbial flora, namely, in phagocytic cells and at epithelial surfaces. Mammalian defensins are stored in the granules of

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neutrophils or paneth cells or are generated by monocytes, macrophage, keratinocytes, or mucosal epithelial cells of the respiratory, digestive, urogenital, or reproductive system. α-Defensins were originally isolated from the cytoplasmic granules of polymorphonuclear phagocytes, where they are present at high concentrations (>10 mg/ml).[15,23] Human and mouse Paneth cells also store α-defensins in secretory granules for release into intestinal crypts, where a concentration of 10 mg/ml can be reached. In both situations, expression seems to be constitutive. β-Defensins have a wider tissue distribution, involving barrier and secretory epithelial cells in particular and can also be produced at quite high concentrations. Expression is in some cases is constitutive and in others induced by bacteria or their components (e.g. LPS, LTA) or by proinflammatory cytokines (interferons-γ and -1β, TNFα), and is often mediated by Toll-like receptors, induction being NF-κB dependent.[10,15]

The distribution pattern in both granulocytes and epithelial cells varies considerably among vertebrate species, as well as within each species.[10,15,23] α-Defensins have been isolated from the leukocytes of primates and various rodents but not from mice. In humans, the expression of defensins has been reported in monocytes, NK, and T-cells, but only rabbits have appreciable amounts in alveolar macrophages. Only certain primate species express θ-defensins in monocytes and neutrophils.[8,23] Artiodactyls express numerous β-defensins, but apparently not α-defensins, and in avian species, only the more ancient β-defensins are expressed in a specific manner in the heterophils, epithelia, and eggs.[26] However, as α-defensin genes are present in monotremes and in basal mammals they developed early in mammalian evolution.

Invertebrate defensins have mainly been isolated from the hemolymph of insects, arachnids, and mollusks.[5,7,28] In insects, they are induced by bacterial components, in the fat body for release into the hemolymph, in surface epithelia, and in the midgut. In the latter case, they may serve both to prevent infection of blood-sucking insects by blood-borne pathogens and parasites, and to protect the stored blood meal from microbial attack. Curiously, a similar role has been proposed for the avian β-defensin spheniscin, isolated from the preserved stomach contents of the king penguin.[26]

The distribution of plant defensins also is consistent with their defensive role,[13,24] as they are mainly found in vulnerable tissues or at entry points for pathogens, where they are constitutively produced in a tissue- or organ-specific manner, or induced in peripheral cell layers, and in cells that line cavities such as stomata. Most of the plant defensins identified are seed derived and sufficiently abundant to provide seedlings with protection against soil fungi. They have also been identified in other tissues, such as leaves, tubers, roots, flowers, pods, and fruits with a wide distribution in species throughout the plant kingdom.[13]

A common feature of many defensins in animals and plants is their induction by pathogen-associated molecular patterns (PAMPs) interacting with Toll or Toll-like receptors.[6,10,13,15,23] The regulatory mechanisms show a remarkable analogy, although it is uncertain whether they represent an ancient evolutionary relationship or a case of convergent evolution. Invertebrate defensins are principally selective for Gram-positive bacteria and fungi, and activation occurs via NF-κB-like signaling pathways initiated by Toll receptors, which respond to cytokines produced after activation of pattern recognition receptors for different types of PAMPs.[6] Induction of mammalian defensins can be mediated by Toll-like receptors (TLRs), which directly recognize PAMPs, or by receptors for chemokines induced by bacterial components,[10,15,23] and is often under NF-κB control. Plants have also acquired the ability to recognize PAMPs via receptors that resemble animal TLRs, and several components of the resulting signaling cascades leading to the transcriptional activation of immune response genes are shared among the two kingdoms.[16]

PROCESSING

Defensins are synthesized as precursors with an N-terminal signal or leader sequence necessary for secretion or storage, and usually also with a prosequence that can be either N- or C-terminal to the mature peptide region (Fig. 1). The posttranslational processing steps are well understood for myeloid and enteric α-defensins, which are transferred into the ER by the signal peptide, where it is removed leaving a relatively long anionic propiece. This may serve as an intramolecular chaperone for folding, and/or to neutralize the activity of the mature peptide region preventing cell damage.[14,15,23] The myeloid defensins are stored as the mature, active form, on removal of the propiece during granulogenesis. In mice, enteric α-defensins are also stored in granules in the mature form, after processing by the matrix metalloproteinase 7 (matrylisin), whereas they are stored in the proform in humans, with processing by Paneth cell trypsin during or after release.

β-Defensin precursors are simpler, having a shorter propiece or lacking it altogether, and their biosynthesis and intracellular trafficking seem quite different from that of the α-defensins.[15,23] Human β-defensin 1 (hBD1) is constitutively produced in many epithelial cells and is present in plasma and urine, where several N-terminal truncated forms indicate continued processing. hBD2 lacks a propiece and is processed to the mature form by signal peptidases in the ER, ready for secretion. The biosynthesis of ovine neutrophil β-defensins is completed early in myelopoiesis, and the peptides are stored in the dense granules in the mature form.

θ-Defensins are derived from the processing of α-defensin isoforms in which a premature stop-codon interrupts

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translation of the mature peptide after 12 residues. Although the intermediate processing steps are unknown, in monkey PMN two of these truncated peptides are trimmed and spliced head-to-tail to give the mature, eighteen-residue cyclic peptide.[8,23]

Invertebrate defensins are also synthesized as precursors composed of a hydrophobic signal peptide and a relatively long prosequence, joined to the C-terminal mature HDP by a dibasic processing site.[5] This overall organization resembles that of vertebrate and plant defensins. The prodomain, similarly to the vertebrate α-defensins, shows a high content of anionic residues and likely has similar chaperone or protective functions. In contrast to animal systems, there is little information about proteolytic processing of defensins in plants.

RECEPTORS

The antimicrobial activity of mammalian defensins derives from both direct and indirect mechanisms. With respect to direct mechanisms, they act as immune effectors via a direct interaction with the microbial cell-surface and/or membrane components, leading to a breach of the membrane or a compromise of essential cell membrane enzymes involved in processes such as cell wall synthesis or cell division, as part of the killing mechanism, or to translocation into the cytoplasm where they can access intramolecular targets. Membrane lysis is not necessarily a receptor mediated process, although the ability to recognize specific membrane components can be important. In particular, the antifungal activity of insect and plant defensins requires either direct interaction with specific sphingolipids and related glucosylceramides in lipid rafts, or with phosphatidyl inositol anchored proteins associated with these rafts.[11,13,27] The antiviral activity of θ-defensins has been related to their capacity to act as lectins and bind viral glycoproteins or glycolipids.[15] More recently, it has been reported that different classes of defensins, including fungal plectasin, invertebrate defensins, and plant defensins can all interact specifically with and block lipid II, thereby inhibiting cell wall biosynthesis in susceptible bacteria.[27]

Indirect antimicrobial action involves an immune regulatory role as shown for some mammalian defensins that act as signaling molecules to recruit or activate cellular components of innate or adaptive immunity. They have variously been shown to chemoattract leukocytes, antigen presenting cells, and lymphocytes.[12,15,17,30] Human and mouse β-defensins are chemotactic for a broad spectrum of leukocytes via the CCR2 or CCR6 receptors. Certain α-defensins selectively chemoattract T-cells and iDC by G-protein-coupled receptors, as this activity is pertussis toxin sensitive. Mouse β-defensin 2 has the ability to stimulate iDC maturation, acting through TLR4. The capacity of certain α-defensins to interfere with the production of immunosuppressive glucocorticoids, possibly by blocking ACTH receptors, was also recognized very early.[32] They have a large variety of other immune regulatory functions, and many of these are probably receptor-mediated including cytokine/chemokine induction, regulation of IL-1β secretion and homeostasis through the P_2X_7 nucleotide scavenging receptor, regulation of cell death mechanisms, and effects on cellular growth and wound repair.[19]

ACTIVE CONFORMATION

Defensins have variable primary structures, not only by comparison of members of different families but also for members within each family (Figs 2 and 3). Effectively, only the cysteine residues involved in disulfide bonding, and few other residues are conserved, whereas the remainder show varying degrees of variability, as do the number of residues separating the cysteines (Fig. 2). The connectivities differ between families but are conserved within each family, being C1-C6, C2-C4, C3-C5 for α-defensins; C1-C5, C2-C4, C3-C6 for β-defensins; C1-C4, C2-C5, C3-C6 for invertebrate defensins; and C1-C8, C2-C5, C3-C6, C4-C7 for plant defensins. Some insect defensin-like peptides such as drosomycin also show an added C0-C7 disulfide bridge.[5,10,13,25,26,28,34]

Despite the differences in sequence and connectivities, molecules from the different families fold into quite similar tertiary structures (Fig. 4). This involves a core formed by a twisted antiparallel β-sheet, generally comprising two or three strands connected to each other and to the rest of the molecule by the disulfide bridges. In insect and plant defensins, the presence of a helix gives rise to the cysteine-stabilized α/β fold (CSαβ), which defines a superfamily in the SCOP classification that also includes scorpion and spider toxins and protease and amylase inhibitors. Vertebrate α- and β-defensins share an overall similar fold, although not all peptides have a C-terminal α-helical segment. This segment, when present, makes the fold similar to the CSαβ fold, but they have been placed in a separate "defensin-like" SCOP superfamily. Thus, defensins have in common a conserved scaffold that seems to be insensitive to sequence variations. The distribution of cationic and hydrophobic residues on this scaffold tends to be arranged so as to result in amphipathic structures that favor interaction with microbial membranes, which are rich in anionic components, and can also account for more specific receptor/based interactions. The lack of sequence constraints and the fact that these peptides are involved in host:pathogen interactions has ensured their rapid evolution and explains the heterogeneity of their sequences.

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ALPHA DEFENSINS

```
                                  ------c-c----c-------g-c----------fcc
                                                                    l
Cavia porcellus                   ----rrcicttrtcrfpyrrlgtcifqnrvyt--fcc---------
(guinea pig)                      ----rrcicttrtcrfpyrrlgtclfqnrvyt--fcc---------
Dasypus novemcinctus              …qqrmicfcrsdvcnlgeidfrtcilkgtah---lccs--------
(armadillo)                       …lqgttclcrtdvcklgeidfgtctlkgtphk--lccs--------
                                  …qertscyckttackfgefpsgtctlkgtpqk--lccs--------
                                  …qqratcyckttactlgeiskgncmlygtpqk--lcci--------
                                  …qqraacycrtkacpsgeislgtctlnskthk--fccr--------
                                  …qqraicscrcgfcrigqhlvgtctlngfhhr--fcch--------
                                  …qqraicycrtgscrfrehivgtcicnhiphr--fccc--------
                                  …hrkaacfcrtgrclfnerqlgicirngrryn--fccs--------
                                  …qwravcfcrilpcfflerqqgictvngrsyn--yccs--------
                                  …qrratclcrtgpclsnerqlgicisngrryn--lcclnlvn----
                                  …qrnmtctcrfgrcqskerlrgiciglrlglq---cch--------
                                  …qrrmactcrfgrcqsgerfrgvcvglrlglq---ccr--------
Echinops telfairi                 …partkctcrrksciypeesvsqcningvpfk--fccr--------
(tenrec)                          …mkrsicycrrsrcafgehrrgicshnsfrri--rccr--------
                                  …xxxxxcscrrksciypeqtvshcningiifk--fcc---------
                                  …lkkttcycrrglcklgerlsgmcrigslphn--lccnwf------
                                  …lkrktcycrrgrcqrgerlygmcskfflahn--lccs--------
Equus caballus                    …gtasctcrrawicrwgerhsgkcidqkgstyr-lccrr-------
(horse)                           …gtasctcrrawvckwgerhsgncidqegsayr-lccrr-------
                                  …gtatctcrhsnsclshergsgmciskkgkayk-lcclr-------
                                  …katlvchcsrtfcqnsyqelgtctgipsgtlyrlccrrg------
                                  …qgtptchcrpftcysgespsghcegkgfqrrr--ccrr-------
                                  …qgtptchcraftcnsgespsghcegkgfqhrr--ccrr-------
                                  …qsihfcicrygrchshersssgscpqissrhkr--cclr-------
                                  …qeastcrcrvf-crfrerrsgtcagrgiqnr--lccprrppnrf…
                                  …qeastcrcrvf-chfrerrsgtcagrgiqnr--lccprrppnrf…
                                  …rpglichcssf-cqrpykqegictt-gpfqr--lccrpdrrrgpr
                                  …rpglichcssf-cqrpyreegictr-glpir--lccrpdrrrgpr
                                  …rpglichcssf-cqrphkqegictr-gklkr--lccrpdgrrgpr
                                  …rpglicrcssf-cqrpyreegictt-glpkr--lccrpdrrrgpr
                                  …rpglichcssf-crrpyreegictr-gqpkr--fccrrgpr----
                                  …rsalichcsfs-crspykkigychlrrlh----fccpswlnfvd-
Homo sapiens                      -----acycripaciagerrygtciyqgrlwa--fcc---------
(human)                           ------cycripaciagerrygtciyqgrlwa--fcc---------
                                  -----dcycripaciagerrygtciyqgrlwa--fcc---------
                                  -----vcscrlvfcrrtelrvgncliggvsft--ycctrv------
                                  -qaratcycrtgrcatreslsgvceisgrlyr--lccr--------
                                  straftchcrr-scysteysygtctvmginhr--fccl--------
Loxodonta africana                …kknltcycrsvrcriservsgfcktkgiayl--fccr--------
(elephant)                        …kktttcycrsvrcfrferllgrctiqgvvsl--fccr--------
                                  …rsnltcycrfglclfperrygicirngirla--fccr--------
                                  …kktiichcrrllclssehlsgictikgvryp--fccr--------
Macaca mulatta                    -----acycripaclagerrygtcfylgrvwa--fcc---------
(rhesus macaque)                  -----acycripaclagerrygtcfymgrvwa--fcc---------
                                  -----acycripaclagerrygtcfyrrrvwa--fcc---------
                                  -----acycripaclagerrygtcfylrrvwa--fcc---------
                                  ---rrtcrcrfgrcfrresysgscningrifs--lccr--------
                                  ----rtcrcrfgrcfrresysgscningrifs--lccr--------
                                  ---rrtcrcrfgrcfrresysgscningriss--lccr--------
                                  ----rtcrcrfgrcfrresysgscningriss--lccr--------
Mammuthus primigenius(mammoth)    …kktvicycrrarclssellsgictisgvryp--fccr--------
Mesocricetus auratus              ----vtcfcrrrgcasrerhigycrf-gntiyr-lccrr-------
(hamster)                         ------cfckrpvcdsgetqigycrl-gntfyr-lccrq-------
                                  ----vtcfcrrrgcasrerligycrf-gntiyg-lccrr-------
                                  ----vtcfckrpvcdsgetqigycrl-gntfyr-lccrq-------
Mus musculus                      -lrdlvcycrsrgckgrermngtcrk-ghllyt-lccr--------
(Mouse)                           -lrdlvcycrtrgckrrermngtcrk-ghlmyt-lccr--------
                                  -lrdlvcycrkrgckrrermngtcrk-ghlmyt-lccr--------
                                  -lrgllcycrkghckrgervrgtc---g-irfl-yccprr------
                                  -lkklicycrirgckrrervfgtcrn-lfltfv-fccs--------
                                  -lrdlvcycrargckgrermngtcrk-ghllym-lccr--------
                                  -lrdlvcycrtrgckrrehmngtcrk-ghlmyt-lccr--------
                                  -lrdlvcycrkrgckrrehmngtcrk-ghlmyt-lccrmw------
                                  -lrdlvcycrkrgckrrehmngtcrk-ghllym-lccr--------
                                  -lrdlvcycrkrgckgrermngtcrk-ghllytm-ccr--------
                                  -lrdlvcycrsrgckgrermngtcrk-ghllym-lccr--------
                                  -lrdlvcycrkrgckrrehmngtcrr-ghlmyt-lccr--------
                                  -lrdlvcycrtrgckrrermngtcrk-ghlmht-lccr--------
```

FIGURE 3 Sequences of members of different defensin families. Sequences are aligned and gaps inserted so as to maintain as far as possible a constant cysteine residue spacing. Residues or types of residues that are significantly conserved are shaded. For θ-defensins, only a hemisequence is shown. The full peptide is formed by the head to tail homo- or heterodimeric ligation of two hemisequences in a circular structure (see Fig. 2). Defensin nomenclature has not yet been fixed, so that the peptide names are either those present in the literature or in the UniProt database. The beginning of the mature peptide region is often inferred or unknown, in which case it is indicated by ….

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```
                                        ------c-c----c-------g-c----------fcc
                                                                          l
                          Defa15        -lrdlvcycrkrgckrrehingtcrk-ghllym-lccr--------
                          Defa16        -lrdlvcycrsrgckgrermngtcrk-ghlmyt-lccr--------
                          Defa17        -lrdlvcycrkrgckrrehmngtcrk-ghllyt-lccr--------
                          Defa20        ---dlicycrkggcnrgeqvygtcs--grll---fccrrrhrh---
                          Defa21        -srdliclcrnrrcnrgelfygtca--gpfl---rccrrrr-----
                          Defa22        -srdliclcrkrrcnrgelfygtca--gpfl---rccrrrr-----
                          Defa23        -lrdlvcycrtrgckrrermngtcrk-ghliyt-lcc---------
                          Defa24        -lrdlvcycrargckgrermngtcsk-ghllym-lcc---------
                          Defa25        -cedlicycrtrgckrrerlngtcrk-ghlmymlwcc---------
                          Defa26        -lrdlgcycrkrgctrreringtcrk-ghlmyt-lccl--------
Oryctolagus cuniculus     CS-I          ----gicacrrrfcpnserfsgycrvngaryvr--ccsrr------
(rabbit)                  CS-II         ----grcvcrkqlcsyrerrigdckirgvrfp--fccpr-------
                          CS-III        ----vvcacrralclprerragfcrirgrihp--lccrr-------
                          CS-IV         ----vvcacrralclplerragfcrirgrihp--lccrr-------
                          CS-VI         ----gicacrrrfclnfeqfsgycrvngaryvr--ccsrr------
                          NP-4          ----vsctcrrfscgfgerasgsctvngvrht--lccrr-------
                          NP-5          ----vfctcrgflcgsgerasgsctingvrht--lccrr-------
                          RK1           ----mpcsckk-ycdpwevidgscglfnskyi---ccrek------
Ornithorhynchus anatinus  DefA1         --dttfcrcrv-scnilekysgkcelsgrtari--cc---------
(platypus)                DefA3         --riitcscrt-fcflgerisgrcyqsvfiyr--lccrg-------
                          DefA4         ----hscvcrr-icaarqvrkgrcsrrrri-----ccly-------
Papio anubis              DEFA1         --ariichcqigrcsrpesysricilhgqvar--lccrras-----
(baboon)                  DEFA2         --arrtcycrt-rcytpefhsgkclfkgltyk--lccr--------
Papio hamadryas           PhD1          ---rricrcrigrclglevyfgvcflhgrlarr--ccr--------
                          PhD2          ----ricrcrigrclglevyfgvcflhgrlarr--ccr--------
                          PhD3          ----rtcrcrlgrcsrresysgscningri-ys-lccr--------
Pan troglodytes           DEFA1         -----acycripaclagerrygtciyqgrlwa--fcc---------
(chimpanzee)              DEFA4         -----vcscrlvfcrrtelrvgncliggvsft--ycctrvd-----
                          DEFA5         -qaratcycrighctileslsgvceisgrlyr--lccr--------
                          DEFA6         straftchcrr-scysteysygtctvmginhr--fccl--------
                          DEFA7         ……kglichcrvlyclfgehlggtsfihgeryp--iccy
                          PhD4          -----acycripacfagerrygtcfylgrvwa--fcc---------
Rattus norvegicus         RatNP1        ----vtcycrrtrcgfrerlsgacgyrgri-yr-lccr--------
(rat)                     RatNP2        ----vtcycrstrcgfrerlsgacgyrgri-yr-lccr--------
                          RatNP3        ------cscrtsscrfgerlsgacrlngri-yr-lcc---------
                          RatNP4        -----acycrigacvsgerltgacglngri-yr-lccr--------
                          RD-5          -lrdlkcfcrrkscnwgegimgickkrygspi--lccr--------
                          Defa6         grpvrrcrcran-cgpkeyatafcaq-gpfkqfkfccta-------
                          Defa7         --sgvtcycrllscqfgerlagscrsggvt-yp-lcch--------
                          Defa8         gwplkqchcrkf-crpyekaegscrp-glfikrkicciqqwtpgrt
                          Defa9         rwpwkrchcrsf-crpyenatsfcaq-glfkqhkfccletwpprmk
                          Defa10        ---gstcscrigtcvsgewlswvcringri-yr-lccr--------
```

BETA DEFENSINS

```
                                        ----------c-----g-c----c-------g-c-------cc---------------
Anas platyrhynchos        Apl_AvBD2     --------flc---rigschfgrcpihlvrvgsc--fgfrsccksp wdv---------
(duck)                    Apl_AvBD5     -----glpqdc-errggfcshksccppgigriglc--skedfccrsrwys---------
                          Apl_AvBD6     -----spihac-ryqrgvcipgpcrwpyyrvgsc-gsglksccvrnrwa---------
                          Apl_AvBD7     -----rpidtc-rlrngicfpgicrrpyywigtc-nngigsccargwrs---------
                          Apl_AvBD9     -----adtlac-rqshgscsfvacrapsvdigtc-rggklkcckwapss---------
                          Apl_AvBD10    ----fadtaac-rsqgnfcragacpptfaasgsc-hggllnccak-------------
Anolis Carolinensis       AcBD1         …eakikdhqqc-ryahgncysffcprgtvtigkc--sftqkcce--------------
(lizard)                  AcBD2         -saiitnekqc-krekgycayilpkpctfpfsyagkcstwkacckk------------
                          AcBD3         -ladpkttdec-tkaggvckhllcgtsmtdigvc--ggknvcckkk------------
                          AcBD4         --tgpksgaec-vkaggecdlrtclsplkpigkc-erresvcckk-------------
                          AcBD5         -ltgpktaaqc-vkaggkcevgkclsptkrigtc-enkdsvccmk-------------
                          AcBD6         -lskidsakec-rsangkcykrkcpssyknigrc--ykkvyccr-------------
                          AcBD7         fstnihrakdc-kvangicrlstclhypwerigkc-gdkrvcci--------------
                          AcBD8         nteeitdtmac-fkaqgdcvkkdc-vllkqigtc--drqllcck-------------
                          AcBD9         …kpkiknasdc-vdaggtceekkc--ttkklgkc--dkkllcce--------------
                          AcBD10        …kprikstvqc-rhqggtc-gkkc-tmakkigkc--dkkfqcck-------------
                          AcBD11        …kpkikttaqc-leeggtcrkgkc-ssavkigtc--gkktlcck-------------
                          AcBD12        …gkkindsfec-ldaggrcekkec-flskkignc--skkgicck-------------
                          AcBD13        …aadmnseaec-keangfcrefycitgstkigkc---gktvcck-------------
                          AcBD14        …ekidekacekgggycrplnekscrfpfveegnc-gfswrlcck-------------
                          AcBD15        …qgphddtiac-grgggscqpvpcrglsveagtc-qggtmkccr-------------
                          AcBD16        nadpapdtmackindrgfckygecpgitqtggtc-qwgslvcck-------------
                          AcBD17        giayianhalc-isfdgscehkkcpptht avgyc--hnawtcclr------------
                          AcBD18        …vfysrtpeec-lflggvcktlfcplvynrsgrc--skhkpcctptlsaysylk----
                          AcBD19        …ppledtiqc-knvggfcktgpcpttfslgstc-hggalsccr--------------
                          AcBd20        aivqprsrifc-taaggvcrqnecrsfekkggyc--ltgvpcclsf------------
                          AcBD21        …kmhvngrilc-raiggncrr-scrnheisggkc--lsevhccvgydlv---------
                          AcBD22        vaiqprntlfc-naagarcrs-rclfrermigrc--nakkrcclqyg-----------
```

FIGURE 3 *Continued.*

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                                  ----------c-----g-c----c-------g-c-------cc----------------
                    AcBD23        …eakiktrgec-easwggcytffcprgsseigkc--sfakkccv--------------
                    AcBD24        …kakiktrgec-evalggcftffcprgsseigkc--sfakkccv--------------
                    AcBD25        …krrikshrdc-yrarsvcysffcprgtltmgkc--sftqkcck--------------
                    AcBD26        …twtddptvkc-rtsggycdsaicrwplknagyrc-ksgrpcck--------------
                    AcBD27        …akrptsdlec-qkakglclhqrcrrpwrsvgtc-nnlqhhccq--------------
                    AcBD28        ------gtgrc-rrlkgvcrhtlchpvevyvgrc-nngmgnccvddaedirkyrq---
                    AcBD29        …qrvprfvshc-lrrggicryddcsegeeqigtc-yhhtmiccrdevm----------
                    AcBD30        --ngdfvtlsc-vfrggtcetricrenevkigtc-skikklcckkpkpkrvmnltel…
                    AcBD31        atgdlydtlec-rnhqgrc-rrhcfyneehigtc-tggrqlcck--------------
                    AcBD32        …anrprtrrec-yqanggcryrrcsfpmmekgrc--nsrkrccv--------------
A. patagonicus      Apa_AvBD1     ------sfglc-rlrrgfcahgrcrfpsipigrc--srfvqccrrvw-----------
(penguin)           Apa_AvBD      ------sfglc-rlrrgfcargrcrfpsipigrc--srfvqccrrvw-----------
Bos Taurus          BNBD1         ------dfasc-htnggiclpnrcpghmiqigic-frprvkccrsw------------
(cow)               BNBD2         ----vrnhvtc-rinrgfcvpircpgrtrqigtc-fgprikccrsw------------
                    BNBD3         --qgvrnhvtc-rinrgfcvpircpgrtrqigtc-fgprikccrsw------------
                    BNBD4         --qrvrnpqsc-rwnmgvcipflcrvgmrqigtc-fgprvpccrr-------------
                    BNBD5         --qvvrnpqsc-rwnmgvcipiscpgnmrqigtc-fgprvpccrrw------------
                    BNBD6         --qgvrnhvtc-riyggfcvpircpgrtrqigtc-fgrpvkccrrw------------
                    BNBD7         --qgvrnfvtc-rinrgfcvpircpghrrqigtc-lgprikccr--------------
                    BNBD8         ----vrnfvtc-rinrgfcvpircpghrrqigtc-lgpqikccr--------------
                    BNBD9         --qgvrnfvtc-rinrgfcvpircpghrrqigtc-lapqikccr--------------
                    BNBD10        --qgvrsylsc-wgnrgiclnrcpgrmrqigtc-laprvkccr--------------
                    BNBD11        ------gplsc-rrnggvcipircpgpmrqigtc-fgrpvkccrsw------------
                    BNBD12        ------gplsc-grnggvcipircpvpmrqigtc-fgrpvkccrsw------------
                    BNBD13        --sgisgplsc-grnggvcipircpvpmrqigtc-fgrpvkccrsw------------
                    BD119         ----rrhmlrc-mgdlgic-rpacrqseepylyc--rnyqpcclpfyvridisgkeg…
                    BBD(C7)       ------gplsc-rrkggicilircpgpmrqigtc-fgrpvkccrsw------------
                    EAP           ------nplsc-rlnrgicvpircpgnlrqigtc-ftpsvkccrwr------------
                    LAP           ---gvrnsqsc-rrnkgicvpircpgsmrqigtc-lgaqvkccrrk------------
                    TAP           ------npvsc-vrnkgicvpircpgsmkqigtc-vgravkccrkk------------
Capra hircus        BD1           ------srrsc-hrnkgvcaltrc-rnmrqigtc-fgppvkccrkk------------
(goat)              BD2           --qgiinhrsc-yrnkgvcaparc-rnmrqigtc-hgppvkccrkk------------
Cercopithecus preussi BD1         ------dhync-vrsggqclysacpiytkiqgtc-yhgkakcck--------------
(monkey)            BD2           ---dirnpvtc-lrsgaichpgfcprrykhigvc-gvsaikcckkp------------
                    BD3           giintlqkyyc-rvrggrcavlsclpkeeqigkc-strgrkccrrkk-----------
C. erythrogaster    BD1           ---dhyi---c-vrsggqclysacpiytkiqgtc-yhgkakcck--------------
                    BD2           ---dirnpitc-lksgaichpgfcprrykhigvc-gvsaikcckkp------------
                    BD3           giintlqkyyc-rvrggrcavlsclpkeeqigkc-strgrkccrrkk-----------
Columba livia(pidgeon) Cli_AvBD4  -----ryhmqc-gyrgtfctpgkcphgnaylglc--rpkysccrwl------------
Coturnix coturnix   Cco_AvBD1     -----gkreq-ryrqkgfcaflkcpsltiisgkc--srfhfcckniwg----------
(quail)             Cco_AvBD5     -----aspqdc-errggfcphsscptgirriglc--seqvfccr--------------
                    Cco_AvBD6     -----spirac-rlrrgtcffgrcpqpyhhvgsc--glrsscwv-------------
                    Cco_AvBD9     -----pdtlac-rqghgscsfvacrapsvdigtc-rggklkcckwapss---------
                    Cco_AvBD10    ----fpdtaac-rsqgnfcrvgvcpptftisgsc-hgglhccakipaq---------
Danio rerio         zfBD1         ----asfpwsc-aslsgvcrqgvclpselyfgplgcgkgflccvshfl----------
(zebra fish)        zfBD2         -aevqiqnwtc--gygglc-rrfcfdqeyivahhgcprryrccavrf------------
                    zfBD3         -ndtdvqrwtc--gyrglc-rkhcyareymigyrgcprryrccalrf-----------
E. orbicularis (turtle) tBD01     ----ydlsknc-rlrggicyigkc--prrffrsgscsrgnvcclrfg------------
Equus caballus(Horse) BD103A      giinmlqksyc-kirkgrcallgclpkeeqigsc-svsgrkccrkkk-----------
Galagoides demidoff BD1           ------dhymcvkn-kgiclysacplytkiigtc-yggkakccg--------------
Gallus gallus       Gga_AvBD1     -----grksdc-frksgfcaflkcpsltlisgkc--srfylcckriwg----------
(chicken)           Gga_AvBD1A    -----grksdc-frkngfcaflkcpyltlisgkc--srfhlcckriw----------
                    Gga_AvBD2     --------lfc---kggschfggcpshlikvgsc--fgfrscckwpwna---------
                    Gga_AvBD3     ------tatqc-rirggfcrvgscrfphiaigkc--atfiscccgray----------
                    Gga_AvBD4     -----ryhmqc-gyrgtfctpgkcphgnaylglc--rpkysccrwl------------
                    Gga_AvBD5     -----glpqdc-errggfcshkscppgigriglc--skedfccrsrwys---------
                    Gga_AvBD5A    -----glpqdc-errggfcshrscppgigriglc--skedfccrsrwys---------
                    Gga_AvBD6     -----spihac-ryqrgvcipgpcrwpyyrvgsc-gsglkscccvrnrwa---------
                    Gga_AvBD7     -----rpidtc-rlrngicfpgicrrpyywigtc-nngigsccargwrs---------
                    Gga_AvBD8     -----nneaqc-eqaggicskdhcfhlhtrafghc-qrgvpccrtvyd----------
                    Gga_AvBD9     -----adtlac-rqshgscsfvacrapsvdigtc-rggklkcckwapss---------
                    Gga_AvBD10    ----fpdtvac-rtqgnfcragacpptftisgqc-hgglnccakipaq---------
                    Gga_AvBD11    ---lprdtsrcvgyhgycirskvcpkpfaafgtc-swrqktccvdttsdfhtcqdkg…
                    Gga_AvBD12    -----hgpdsc-nhdrglcrvgncnpgeylakyc-fepvilcckplsptptkt-----
                    Gga_AvBD13    ----fsdsqlc-rnnhghc-rrlcfhmeswagsc-mngrlrccrfstkqpfsnp----
                    Gga_AvBD14    -----sdtvtc-rkmkgkcsfllcpffkrssgtc-ynglakccrpfw-----------
Gorilla gorilla     BD1           ------dhync-vssggqclysacpiftkiqgtc-yggkakcck--------------
                    BD2           ---gigdpvtc-lksgaichpvfcprrykqigtc-glpgtkcckkp------------
                    BD3           giintlqkyyc-rvrggrcavlsclpkeeqigkc-strgrkccrrkk-----------
                    BD104A        ---efeldric-gygtarc-rkkcrsqeyrigrc--pntfacclrkwdesllnrtkp-
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FIGURE 3 *Continued.*

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                                  ----------c-----g-c----c-------g-c-------cc----------------
                BD105A            …sgefavcesc-klgrgkc-rkeclenekpdgnc--rlnflccrqri-----------
                BD106A            -----ffdekc-nklkgtc-knncgkneelialc--qkslkccrtiqpcgsiid----
                BD107A            ---aihralic-krmeghc-eaecltfeakiggcraelapfcckrkkh---------
                BD118             ---aysgekkc-wnrsghc-rkqckdgeavkdtc--knlraccvpsnedh--------
                BD119A            ----krhilrc-mgnsgic-rasckkneqpylyc--rnyqscclqsymrisisgkee…
                BD119B            --------vec--wmdghc-rllckdgedsiirc--rnrkrccvpshyltiqpvtih…
                BD123             ------gtqrc-wnlygkc-ryrcskkervyvyc--innkmccvkpkyqpkerwwpf-
                BD125             ----sfepqkcwknnighc-rrrcldtercillc--rnklscciyiiisheytqrpa…
                BD126             ----nwyvkkcl-ndvgic-kkkckpeemhvkngwamcgkqrdccvpad---------
                BD129             ---efiglrrcl-mglgrc-rdhcnvdekeiqkc----kkkccvgpkvv kliknyl…
                BD132             ------ggskcvsntpayc-rtychwgetalfmf--nasrkccisysflpkpdlpql-
Hylobates concolor  BD1           ------dhync-vrsggqclysacpiytkiqgtc-yqgkakcck--------------
(gibbon)        BD2               ---dirnpvtc-lksgaichpvfcprrykqigtc-glpgtkccrkp------------
                BD3               glmntlqkyyc-rvrggwcavlsclpkeeqigkc-strgrkccrrkk-----------
H. lar          BD1               ---dhyn---c-vrsggqclysacpiytkiqgtc-yqgkakcck--------------
                BD2               ---dirnpvtc-lksgaichpvfcprrykqigtc-glpgtkccrkp------------
                BD3               giintlqkyyc-rvrggrcavlsclpkeeqigkc-pmrgrkccrrkk-----------
                BD104A            ---efewdric-gygtarc-rnkcrsqeyrigrc--pntfacclrkwdesllnstkp-
                BD105A            …sgefavfesc-kfsrgkc-rkeclenekpdgnc--rlnflccrqsi-----------
                BD106A            -----ffddkc-dklrgtc-knscekneeltsfc--qkslkccrtiqtcgnttd----
                BD107A            ---aihralic-krmeghc-eaecltfevkiggcraelapfcckrkkh---------
                BD118             ---aysgekkc-wnrsghc-rkqckdgeavkdtc--knlraccvpsnedh--------
                BD119A            ---gkhhilrc-mgnsgic-rasckkneqpylyc--rnyqhcclqsymrisisgeee-
                BD119B            --------vec--wmdghc-rllckdgedsiirc--rnrkrccvpsryltiqpvtih…
                BD125             ----sfeppkcwknnighc-rrrcldteryilvc--rnklsccisiiisheytrrpa…
                BD126             ----nwyvkkc-lndvgic-kkkckpeelhvkngramcgkqrdccvpad---------
                BD128             -----arlkkcfnnitgyc-rkkckvgeryeigc--lsgklccvndeeekkhvsfkk…
                BD129             ---efiglrrc-lmgfgrc-rdhcnvdekeiqkc---kmkkccvgpkvvkliknylq…
                BD132             ------ggskcvsdtqgyc-rtychqgetalfmc--nasrkccasysflpkpdlpql…
H. moloch       BD1               ------dhync-vrsggqclysacpiytkiqgtc-yqgkakcck--------------
Homo sapiens    BD1               ------dhync-vssggqclysacpiftkiqgtc-yrgkakcck--------------
                BD2               ---gigdpvtc-lksgaichpvfcprrykqigtc-glpgtkcckkp------------
                BD3               giintlqkyyc-rvrggrcavlsclpkeeqigkc-strgrkccrrkk-----------
                BD4               ---efeldric-gygtarc-rkkcrsqeyrigrc--pntyacclrkwdesllnrtkp-
                BD5               …sgefavcesc-klgrgkc-rkeclenekpdgnc--rlnflccrqri-----------
                BD6               -----ffdekc-nklkgtc-knncgkneelialc--qkslkccrtiqpcgsiid----
                BD7               ---aihralis-krmeghc-eaecltfevkiggcraelapfcckrkkh---------
                BD8B              -----kfkeic-erpngsc-rdfcleteihvgrc--lnsqpcclplghqpriesttp…
                BD9               --glgpaeghc-lnlfgvcrtdvcnivedqigac--rrrmkccrawwilmpiptpli…
                BD10A             …ygsldlrrec-rignggc-knqcheneiriayc-irpgthcclqq------------
                BD10B             …fepkyrferc-ekvrgic-ktfcddveydygyc-ikwrsqccv--------------
                BD13              …revaerkrec-qlvrgac-kpecnsweyvyyyc---nvnpccavweyqkpiinkit…
                BD14              --------drc-tkrygrc-krdclesekqidic-slprkicckteklyeeddmf----
                BD15              qtapdgwirrc-yygtgrc-rkscckeierkkekc--gekhiccvpkekdklshihdq…
                BD116             …gksrepwnpc-elyqgmc-rnacreyeiqyltc--pndqkcclklsvkitssknvk…
                BD18              ---aysgekkc-wnrsghc-rkqckdgeavkdtc--knlraccipsnedh--------
                BD19A             ----krhilrc-mgnsgic-rasckkneqpylyc--rncqscclqsymrisisgkee…
                BD119B            --------vec--wmdghc-rllckdgedsiirc--rnrkrccvpsryltiqpvtih…
                BD121             ---qvtpvmkc-wgksgrc-rttckesevyyilc--kteakccvdpkyvpvkpkltd…
                BD23              ------gtqrc-wnlygkc-ryrcskkervyvyc--innkmccvkpkyqpkerwwpf-
                BD24              ------efkrc-wkgqgac-qtyctrqetymhlc--pdaslcclsyalkpppvpkhe…
                BD25              ----sfepqkcwknnvghc-rrrcldteryillc--rnklsccisiisheytrr----
                BD26              ----nwyvkkc-lndvgic-kkkckpeemhvkngwamcgkqrdccvpad---------
                BD27              -------lkkcwnnyvqghcrkicrvnevpealc--engrycclnikeleac------
                BD28              -----arlkkcfnkvtgyc-rkkckvgeryeigc--lsgklccandeeekkhvsfkk…
                BD29              ---efiglrrc-lmglgrc-rdhcnvdekeiqkc---kmkkccvgpkvvkliknylq…
                BD30              --gvipgqkqc-ialkgvcrdklcstlddtigic--negkkccrrwwilepyptpvp…
                BD31              ----fisndec-pseyyhc-rlkcnadehairyc--adfsicckkliiieidgqkkw--
                BD32              ------ggskcvsntpgyc-rtcchwgetalfmc--nasrkccisysflpkpdlpql…
                BD33              ---avkdtysc-fimrgkc-rhechdfekpigfc-tklnancym--------------
                BD34              …slssemhkkc-ykngi-c-rlecyesemlvayc--mfqleccvkgnpap--------
                BD35              …vyiqkifasc-wrlqgtc-rpkclkneqyrilc--dtihlccvnpkylpiltgk---
                BD36              …gndgvkvrtc-tsqkavc-ffgcppgyrwiafc--hnilsccknmtrfqppqakdp…
Macaca fascicularis BD1           ------dhync-vrsggqclysacpiytriqgtc-yhgkakcck--------------
(macaque)       BD2               ---dirnpvtc-vrsgaiclpgfcprrykhigvc-gvsaikcckkp------------
                BD3               giintlqkyyc-rvrggrcavlsclpkeeqigkc-strgrkccrrkk-----------
                BD105A            …sgrfavcesc-klgrgkc-rkeclenekpdgsc--rlnflccrprm-----------
                BD118             ---ayggekkc-wnrsghc-rkqckdgeavketc--knhraccvpsnedh--------
M. mulatta      BD1               ------dhync-vrsggqclysacpiytriqgtc-yhgkakcck--------------
                BD2               ---dirnpvtc-vrsgaiclpgfcprrykhigvc-gvsaikcckkp------------
                BD3               giintlqkyyc-rvrggrcavlsclpkeeqigkc-strgrkccrrkk-----------
                BD4A              ---gigdpvtc-lkngaichpvfcprrykqigtc-glpgtkcckkp------------
                BD18              ---ayggekkc-wnrsghc-rkqckdgeavketc--knhraccvpsnedh--------
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                                  ----------c-----g-c----c-------g-c-------cc---------------
                BD19A             ---gkrhilrc-mgnsgic-rasckkneqpylyc--rnyqacclqsymrisisgkee-
                BD19B             --------vec--wmdghc-rllckdgedsiirc--rnrkrccvpsryltiqpvtih…
                BD21              ---qvtpvtkc-wgksgrc-rttckksevyyilc--kteakccvdpkyvpvrskltd…
                BD22              ----vgsiekc-wnfrgsc-rdeclknekvyvfc--msgklcclkpkdqphlpqrtkn
                BD23              ------gtqrc-wnlygkc-rhrcskkervyvyc--lnnkmccvkpkyqpkekwwpf-
M. gallopavo    Mga_AvBD1         -----gkrekc-lrrngfcaflkcptlsvisgtc--srfqvcckttllg----------
(turkey)        Mga_AvBD2         --------lfc---krgtchfgrcpshlikvgsc--fgfrscckwpwda---------
                Mga_AvBD3         -----gtpiqc-rirggfcrfgscrfphiaiakc--atfipccgsiwg----------
Mus musculus    BD1               ------dqykc-lqhggfclrsscpsntklqgtc-kpdkpnccks-------------
(mouse)         BD1P              ------vrikc-mpkmtavfgdncsfyssmgdlc-nntksvccmvpvrmdni------
                BD2               ---e-aeldhc-htnggycvraicppsarrpgsc-fpeknpcckymk-----------
                BD3               --kkinnpvsc-lrkggrc-wnrcigntrqigsc-gvpflkcckrk------------
                BD4               --qiinnpitc-mtngaic-wgpcptafrqignc-ghfkvrcckir------------
                BD5               --ktinnpvsc-cmiggic-rylckgnilqngnc-gvtslncckrk------------
                BD6               --qlinspvtc-msyggsc-qrscnggfrlgghc-ghpkirccrrk------------
                BD7               --qdinskrac-yreggec-lqrciglfhkigtc--nfrfkcckfqipekktkil---
                BD8               --qkinepvsc-irnggic-qyrciglrhkigtc--gspfkcck--------------
                BD9               -iigvsemerc-hkkggyc-yfycfsshkkigsc-fpewprccknik-----------
                BD10              …lilkaqltrc-ykfggfchynicpgnsrfmsnc-hpenlrcckniqkf---------
                BD11              …lilkaqlarc-ykfggfcynsmcpphtkfignc-hpdhlhccinmkelegst-----
                BD12              …ggeiavcetc-rlgrgkc-rrtciesekiagwc--klnffccreri-----------
                BD13              ---tlyrrflc-kkmngqc-eaecftfeqkigtc--qanflccrkrkeh---------
                BD14              flpktlrkffc-rirggrcavlnclgkeeqigrc-snsgrkccrkkk-----------
                BD15              -----ffdekc-srvngrc-tasclkneelvalc--qknlkccvtvqpcgksksnqsd
                BD17              …ygsldlrkec-kmrrghc-klqcsekelrisfc-irpgthccm--------------
                BD18              …revaerthkc-slvrgtc-kseclnsweykynyc---htepccvvrykrmekllstp-
                BD19              ---gknpilqc-mgnrgfc-rssckkseqayfyc--rtfqmcclqsyvrisltgvddn
                BD20              --------krcfsnvegyc-rkkcrlveisemgc--lhgkyccvnelenkkhkkhsv…
                BD25              ------efkrc-wngqgac-rtfctrqetfmhlc--pdaslcclsysfkpsrpsrvg…
                BD29              …skrqepwiac-elyqglc-rnacqkyeiqylsc--pktrkcclkyprkit-------
                BD30              …myikriydtc-wklkgic-rntcqkeeiyhifc--giqslcclekkempvlfvk---
                BD33              -rkrnskfrpc-ekmggicksqkthgcsilpaec-ksrykhccrl-------------
                BD34              ---ffffdekc-sringrc-tasclkneelvalc--wknlkccvtvqscgrskgnqs…
                BD35              ---eiavcetc-rlgrgkc-rraciesekivgwc--klnffccreri-----------
                BD36              --------qkc-wnlhgkc-rhrcsrkesvyvyc--tngkmccvkpkyqpkpkpwmf-
                BD37              …pvamldtiac-ienkdtcrlkncprlhnvvgtc-yegkgkcch kn-----------
                BD38              ---igpdtkkc-vqrknachyfecpwlyysvgtc-ykgkgkccqkry-----------
                BD39              -----ddsiqc-fqknntchtnqcpyfqdeigtc-ydkrgkccqkrllhirvprkkkv
                BD40              -----ldtikc-lqgnnnchiqkcpwfllqvstc-ykgkgrccqkrrwfarshvyhv-
                BD41              …idiknqierc-ekvrgmc-ktvcdideydygyc-irwrnqcci--------------
                BD43              ----flenqdc--skhrhc-rmkckaneyavryc--edwticcrvkkkeskkkkmw--
                Bin-1             …ippgirntvc-lmqqghcrlfmcrsgerkgdic-sdpwnrccvpysvkdrr------
Ovis aries      BD1               --qgvrnrlsc-hrnkgvcvpsrc-rhmrqigtc-rgppvkccrkk-----------
(sheep)         BD2               --hgvtdslsc-rwkkgicvltrc-gtmrqigtc-fgppvkccrlk-----------
O.anatinus      DefB1             -----gmkekc-vtmggyc-rkqcrvqdalsgyc--rnenpccvrr-----------
(platypus)      DefB2             -----glnnkc-ayfrgqc-rrkcpqrdiffgfc--rnhdqcclsslhtrh-------
                DefB3             ---gisrvric-rekgghc-dadchleerhlggcraayltfccrk-------------
                DefB4             ------gprrc-kgklgyc-rskcqskqvelgkc--stkaiccgistgtsssqgshe…
                DefB5             --------kkc-rergqgchsgvcswnekfigfc--sfarpccrkrravp--------
                DefB6             …cdinkksfdc-yhrngrc-rfncrkreynngdc--sqyqsccclptrnl---------
                DefB-vL           …vrrrrrrppc-edvngqc-qprgnpclrlrgac--prgsrccmptvaah--------
Pan troglodytes BD1               ------dhync-vssggqclysacpiftkiqgtc-yggkakcck--------------
(chimpanzee)    BD2               ---gisdpvtc-lksgaichpvfcprrykqigtc-glpgtkcckkp------------
                BD3               giintlqkyyc-rvrggrcavlsclpkeeqigkc-strgrkccrrkk-----------
                BD4                  efeldric-gygtarc-rkkcrsqeyrigrc--pntyacclrkwdesllnrtkp-
                BD5               …sgefavcesc-klgrgkc-rkeclenekpdgnc--rlnflccrqri-----------
                BD6               -----ffdekc-nklkgtc-knncgkneelialc--qkslkccrtiqpcgsiid----
                BD7               ---aihralis-krmeghc-eaecltfevktggcraelapfccknrkkh---------
                BD8               -----kfkeic-erpdgsc-rdfcleteihvgrc--lnsrpcclplghqpriesttp…
                BD9               --glgpaeghc-lnlsgvcrrdvckvvedqigac--rrrmkccrawwilmpiptp---
                BD10              …ygsldlrrec-rmgngrc-knqcheneiriayc-irpgthcclqq------------
                BD13              …revaekkrec-qlvrgac-kpecnsweyvyyyc---nvnpccvvqeyqkpiinkit…
                BD16              …gksrepwnpc-elyqgmc-rnacreyeiqyltc--pndqkcclklsvkitssknvk…
                BD118             ---aysgekkc-wnrsghc-rkqckdgeavkdtc--knlraccvpsnedh--------
                BD19A             …lrrmgnsgic----rasc-----kkneqpylyc--rnyqscclqsymrisisgkee…
                BD19B             --vecwmdghc----rllc-----kdgedsiirc--rnrkrccvpsryltiqpvtih…
                BD21              ---qvtpvmkc-wgksgrc-rttckesevyyilc--kteakccvdpkyvpvkpkltd…
                BD123             ------gtqrc-wnlygkc-ryrcskkervyvyc--innkmccvkpkyqpkerwwpf-
                BD124             ------efkrc-wkgqgac-rtyctrqetymhlc--pdaslcclsyalkpppvpkhe…
                BD25              ----sfepqkcwknnighc-rrrcldteryillc--rnklsccisliisqeytrrpa…
                BD26              ----nwyvkkc-lndvgic-kkkckpgemhikngwatcgkqrdccvpad---------
                BD27              -------lkkcwnnyvqghcrkicrvnevpealc--engryccclnikeleac------
                BD29              ---efiglrrc-lmglgrc-rdhcnvdekeiqkc---kmkkccvgpkvvkliknylq…
```

FIGURE 3 *Continued.*

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```
                                          ----------c-----g-c----c-------g-c-------cc----------------
                                BD30      --gvipgqkqc-ialkgvcrdklcstlddtigic--negkkccrrwwilepyptpvp…
                                BD131     ----fisndecpseyyyhc-rlkcnadehairyc--adfsiccklkiieihgqkkw--
                                BD32      ------ggskcvsntpgyc-rtychwgetalfmc--nasrkccvsysflpkadlprl…
                                BD33      vkcavkdtysc-fivrgkc-rhechdfekpigfc-tklnancym--------------
                                BD34      -----emhkkc--ykngic-rlecyesemlvayc--mfqleccvkgnpap--------
                                BD35      …vyiqkifasc-wrlqgtc-rpkclknetisyfg--vilyicgcvnpkylpiltgk--
                                BD36      fgndgvkvrtc-tsqkavc-ffgcppgyrwiafc--hnilsccknmtrfqplqakdp…
Papio anubis                    BD1       ------dhync-vrsggqclysacpiytriqgtc-yhgkakcck--------------
(baboon)                        BD2       ---dirnpvtc-vrsgaiclpgfcprrykhigvc-gvsaikcckkp------------
                                BD3       giintlqkyyc-rvrggrcavlsclpkeeqigkc-strgrkccrrkk-----------
P. sinensis (turtle)            pelovaterin ---ddtpssrcgsggwgpc-lpivdllcivhvtvgcsggfgccrig
Pongo pygmaeus                  BD1       ------dhync-vssggqclysacpiftkiqgtc-yrgkakcck--------------
(orangutan)                     BD2       ---gigdpvtc-lksgaichpvfcprrykqigtc-glpgtkcckkp------------
                                BD3       giintlqkyyc-rvrggrcavlsclpkeeqigkc-strgrkccrrkk-----------
                                BD4       ---efeldric-gygtarc-rkkcrsqeyrigrc--pntyacclrkwdesllnrtkp-
                                BD5       …sdefavcesc-klgrgkc-rkeclenekpdgnc--rlnflccreri-----------
                                BD6       -----ffdekc-gklkgtc-knncgkneelialc--qkslkccrtiqpcgsiid----
                                BD7       ---aihralic-krmeghc-eaecltfevkiggcraelapfcckrkkh---------
                                BD18      ---aysgekkc-wnrsghc-rkqckdgeavkdtc--knlraccvpsnedh--------
                                BD19A     ----kryilrc-mgnsgic-rasckrneqpylyc--knyqscclqsymrisisgkee-
                                BD19B     --------vec--wmdghc-rllckdgedsiirc--rnrkrccvpshyltiqpvtih…
                                BD123     ------gtqrc-wnlygkc-rhrcskkervyvyc--vnnkmccvkpkyqpkerwwrf-
                                BD25      ----sfepqkcwknnighc-rrrcldteryillc--rnklsccisiiisheytrrpa-
                                BD28      -----arlkkcfnnvtgyc-rkkckvgeryeigc--lsgklccinyeeekehvsfkk…
                                BD29      ---efiglrrc-lmgfgrc-rdhcnvdekeiqkc---kmkkccvgpkvvkliknylq…
                                BD32      ------ggskcvsntpgyc-rtychqgetalfmc--nasrkccvsysflpkpdlpql…
Presbytis cristata              BD1       ------dhync-vrsggqclysacpiytkiqgtc-yhgkakcck--------------
(surili)                        BD2       ---dirnpitc-lksgaichpgfcpgrykhigvc-gvsaikcckkp------------
                                BD3       giintlqkyyc-rvrggrcallsclpkeeqigkc-strgrkccrrkk-----------
P. melalophos                   BD1       ------dhync-vrsggqclysacpiytkiqgtc-yhgkakcck--------------
                                BD2       ---dirnpvtc-lksgaichpvfcprrykqigtc-glpgtkccrkp------------
                                BD3       giintlqkyyc-rvrggrcallsclpkeeqigkc-strgrkccrrkk-----------
P. obscurus                     BD1       ------dhync-vrsggqclysacpiytkiqgtc-yhgkakcck--------------
                                BD2       ---dirnpitc-lksgaichpgfcprrykhigvc-gvsaikcckkp------------
                                BD3       giintlqkyyc-rvrggrcallsclpkeeqigkc-strgrkccrrkk-----------
Saguinus oedipus                BD1       ------dhync-vkggqclysacpiytkvqgtc-yggkakcck--------------
(tamarin)                       BD3       giintlqkyyc-rvrggrcavlsclpkeeqigkc-strgrkccrrkk-----------
Rattus norvegicus               BD1       ------dqyrc-lqnggfclrsscpshtklqgtc-kpdkpnccrs-------------
(rat)                           BD2       --qsinnpitc-ltkggvc-wgpctggfrqigtc-glprvrcckk------------
                                BD3       --kkvynavsc-mtnggic-wlkcsgtfreigsc-gtrqlkcckk------------
                                BD5       --qsinnpvsc-vthggic-wgrcpgsfrqigtc-glgkvrcckk------------
                                BD9       …lglktefeqc-qrirgyclntycrfptshvgsc-ypekryccknir-----------
                                BD10      …lgmtaetewc-rlfegfchdkncppptshvgsc-hpekrscckdrr-----------
                                BD11      …rrsvsgfqec-hskggycyryycprphrrlgsc-ypyaanccrrrr-----------
                                BD12      …ggefavcetc-rlgrgkc-rrtcldsekiagkc--klnffccreri-----------
                                BD13      ---tlyrrflc-kkmkgrc-etaclsfekkigtcradltplcckekkkh---------
                                BD14      fipkslrrffc-rvrggrcailnclgkeeqigrc-snrgqkccrkk-----------
                                BD15      ---ffdekekc-sringrc-tesclkneelialc--qknlkccvtvqpcgrdkgdel…
                                BD17      eygsldlrkec-rmskghc-klqcseneiriafc-irpgthcci-------------
                                BD18      …rdvlerthkc-flvggec-ksecssweyeyvfc---ytgpccvmreykrvekfsnt-
                                BD20      --------krcfsnvagyc-rkrcrlveisemgc--lhgkyccvnelenkrhkkdtv…
                                BD25      ------efkrc-wngqgac-rtyctrqekfihlc--pdaslcclsyslkasphsrag…
                                BD29      …gkrrepwvsc-elyqgsc-rnacqkyeiqyltc--pkkrkcclkfpmkitrv-----
                                BD30      …myirqiydtc-wklkghc-rnvcgkkeifhifc--gtqflccierkempvlfvk---
                                BD33      -rkrntkfrqc-ekmggickyqkthgcsilpaec-ksrykhccrl------------
                                BD38      ---tdqdtakc-vqkknvcyyfecpwlsisvstc-ykgkakccqkry----------
                                BD39      -----idsvkc-fqknntchtircpyfqdevgtc-yegrgkccqkrllsirvpkkkv-
                                BD43      ----flanqec-fseyrhc-rmkckaneyairyc--adwticcrvkkreakkkimw--
                                BD50      hpgtvhvrfkc-ipkiaavfgdncpfygnvdglc-ndkksvccmvpvrldni------
                                Bin-1a    ----girntvc-fmqrghcrlfmcrsgerkgdic-sdpwnrccvsssiknr-------
Sus scrofa                      BD1       ---nignsvsc-lrnkgvcmpgkcapkmkqigtc-gmpqvkcckrk----------
(pig)                           BD2       -------hyic-akkggtcnfspcplfnriegtc-ysgkakccir------------
                                BD3       -------ryyc-kirrgrcavlgclpkeeqigsc-svsgrkccrkrk----------
                                BD4       -------rrec-rrgngrc-klecregeiriayc-mrpatycclqk----------
                                BD104     -------dric-gygtsrc-rryckrqeirigrc--phtypcclkkwrdsslnp----
                                BD114     -------perc-skmygqc-rtrcykiekqidic-yspskicciqrafeedls-----
                                BD123     -------amkc-wsalgrc-rttcqesevfhilc--rdatmccvhpkyipikt-----
                                BD125     -------vercwrynighc-rrrclhlerykhlc--mnkracciplssddp-------
                                BD129     -------lgrc-vmglgrc-kdhcavnekeidkc---kkkkccigpkgiqliksyl--
                                EP2C      -------vinc-krsegrc-qnfcnymemqigyc-sknkdpcclpqn----------
                                EP2E      --------vic-lmqhgtcrlffchsgekkseic-sdpwnrccvpnteeekrs-----
```

FIGURE 3 *Continued.*

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```
                                          ----------c-----g-c----c-------g-c-------cc---------------
Struthio camelus        Sca_AvBD1         ---apgnkaec-erekgycgflkcsfpfvvsgkc--srfffcckniw-----------
(ostrich)               Sca_AvBD2         --------lfc---rkgtc-hggcpahlvkvgsc--fgfracckwpwdv---------
                        Sca_AvBD4         -----ryhmqc-gyrgtfctpgkcphgnaylglc--rpkysccrwl------------
                        Sca_AvBD7         ---iprpldpc-iaqngrcftgicrypyfwigtc--rngkscrrr------------
                        Sca_AvBD8         ---lpvneaqc-rqvggycglricnfpsrflglc--trnhpccsrvwv----------
Taeniopygia guttata     Tgu_AvBD2         -----remflc---rggschfggcpihlikvgrc--fgfrsccks-------------
(finch)                 Tgu_AvBD4         -----rpymqc-gyrgtfchkgkcprgnyylgpc--rsgysccrw-------------
                        Tgu_AvBD5         -----asaqdc-errggfcshrscppgigriglc--seqefccrm-------------
                        Tgu_AvBD7         -----rlnnpc-lmqnglcfrgicrrpyywigtc--sngyscckr-------------
                        Tgu_AvBD8         -----ntevqc-rqaggvcssdrcpppphtrpfgr-cqqgipccrt-------------
                        Tgu_AvBD9         -----adtlac-rqsrgscsfvpcsaplvdigtc-rggklrcckw-------------
                        Tgu_AvBD10        -----adtvec-rsqgrfcragacpptfaatgtc-hggllnccsk-------------
                        Tgu_AvBD11        -----rdtlrcleyhgycfhlkscpepfaafgtc-yrrrrtcclg-------------
                        Tgu_AvBD12        -----hgpdsc-nhggglcrmgscvsgeyvaqyc-fepiilcckn-------------
                        Tgu_AvBD13        -----sdtqqc-rssrghc-rrlcfhmerwegsc-sngrlrccr--------------
```

Ovodefensins (egg white -defensins)

```
                                          ----------c-----g-c----c-------g-c-------cc---------------
duck                    BPS1              -----qkkgfc----agycsyscaktdewtfhqtc-gkmyccipppkkg---------
                        BPS2              -----qvrkyc--pkvgycsskcskadvwslssdc--kfycclppgwk----------
chicken                 gallin-1          ------ilkyc--pkigycsntcsktqiwatshgc--kmycclpaswkwk--------
                        gallin-2          ------vlkyc--pkigycsntcsktqiwatshgc--kmycclpaswkwk--------
                        gallin-3          ------vlkyc--pkigycsntcsktqiwatshgc--kmycclpanwkwk--------
finch                   taeniopygin-1     -----kfrktc--apmgycspkcrvmdlkytsgdc--kyscciptawkgk--------
                        taeniopygin-2     -----qpkrsc----rghcsrtcgkgereehtedc-ggmhcclthrkr----------
turkey                  meleagrin         -----qvlkyc--pkigycsskcskaevwayspdc--kvhccvpanqk----------
swan                    cygnin            -----qvrkyc--pkvgycsskcskadvwslssdc--kfycclppgwk----------
marine turtle           TEWP              -------qkkc----pgrctlkcgkherptlpync-gkyiccvpvkvk----------
```

-Defensin-like venoms

```
                                          ----------c-----g-c----c-------g-c-------cc---------------
Crotalus durissus       DLP/crotamine[1]  -------ykqc-hkkgghcfpkekicippssdfgkmdcrwrwkcckkgsgk
O.anatinus              DLP1[1]           …afvqhrprdc-esingvcrhkdtvncreifladcyndeqkccrk-------------
                        DLP2              -------mqac-wshsgvcrdksernckpmawtycenrnqkccey-------------
                        DLP3              -----femqyc-wshsgvcrdksernnkpmawtycenrqkkcef--------------
                        DLPA              …piffygrqpc-syydgvcrdksdvnckyiaftycenpnqrccyy-------------
```

THETA DEFENSINS (*)

```
Hylobates syndactylus   DEFT-1                    rcicgrgvc
Kikuyu colob            DEFT-1                    rcvctrgfc
Macaca mulatta          DEFT-1                    rcictrgfc
                        DEFT-2                    rclcrrgvc
                        DEFT-3                    rcicvlgic
                        DEFT-4                    rcictrgvc
Macaca nemestrina       DEFT-1                    rcicrrgvc
Papio anubis            BTD-a                     rcvctrgfc
                        BTD-b                     rcvcrrgvc
                        BTD-c                     rciclgic
                        BTD-d                     rcfcrrgvc
P. hamadryas            PhTD-1                    rcvcrrgvc
                        PhTD-3                    rcvctrgfc
Pongo pygmaeus          DEFT-1,2,4                rcicrrgvc
                        DEFT-3                    rcicgrgvc
                        DEFA-1                    rcicrrgic
S.syndactylus           DEFT-1                    rcicgrgvc
```

INVERTEBRATE DEFENSINS

Insect

```
                                          --tcdlls-----------acaahc---g---ggyc------vcvcr-------------
Acalolepta luxuriosa    DEF1              rftcdvlsveakgvklnhaacgihclfrrrt-ggycnkk--rvcicr-------------
Aedes aegypti           DEFA              -atcdlls----gfgvgdsacaahciarrnr-ggycnak--kvcvcrn-------------
                        DEFB              -atcdlls----gfgvgdsacaahciargnr-ggycnsq--kvcvcrn-------------
                        DEFC              -atcdlls----gfgvgdsacaahciargnr-ggycnsk--kvcvcrn-------------
Aedes albopictus        DEF               -atcdlls----gfgvgdsacaahciarrnr-ggycnak--tvcvc--------------
                        DEFD              -atcdlls----gfgvgdsacaahciargnr-ggycnsk--kvcvcpi------------
Aeschna cyanea          DEFI              gfgcpl----------dqmqchrhcqtitgrsggycsgplkltctcyr------------
Allomyrina dichotoma    DEFI              -vtcdllsfeakgfaanhslcaahclaigrr-ggscer---gvcicrr------------
Anomala cuprea          DEFA              -vtcdllsfeakgfaanhsicaahclaigrk-ggscqn---gvcvcrn------------
                        DEFB              -vtcdllsfeakgfaanhsicaahclvigrk-ggacqn---gvcvcrn------------
Anopheles gambiae       DEF1              -atcdlas----gfgvgsslcaahciarryr-ggycnsk--avcvcrn------------
```

FIGURE 3 *Continued.*

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		--tcdlls----c------acaahc---gc--ggyc-----avcvcr----------
	DEF2	-atcdlas----gfgvgnnlcaahciarryr-ggycnsk--avcvcrn----------
	DEF3	…lacvtne----gpkwantycaavchmsgrg-agscnak--decvcsmt----------
Apis mellifera	**DEFI**	-vtcdllsf---kgqvndsacaanclslgka-gghcek---gvcicrktsfkdlwdkyf-
	DEF2	-vtcdvlswqskwlsinhsacairclaqrrk-ggscrn---gvcicrk----------
Archaeoprepona demophon	**DEFN**	igscvwgav------nytsncnaeckrrgyk-gghcgsfanvncwcet
Bombus pascuorum	**DEFI**	-vtcdllsi---kgvaehsacaanclsmgka-ggrcen---giclcrkttfkelwdkrf-
Bombus ignitus	**DEF1**	-vtcdllsi---kgvaehsacaanclsmgka-ggrcen---gvclcrktnfkdlwdkrfg
Drosophila melanogaster	**DEFI**	ratcdlls----kwnwnhtacaghciakgfk-ggycndk--avcvcrn----------
	DMYC*	-adclsgrykgpcavwdnetcrrvckeegrs-sghcsps--lkcwcegc----------
Eristalis tenax	**DEF**	-atcdlls----flnvnhaacaahclskgyr-ggycdgk--kvcncr----------
Formica aquilonia	**DEF**	-ftcdlls----gagvdhsacaahcilrgkt-ggrcnsd--rvcvcra----------
Galleria mellonella	**DEF1**	igscvw------gatnytsdcnaeckrrgyk-gghcgsflnvncwce----------
	DEF2	igscvw------gatnytsdcnaeckrrgyk-gghcgsfwnvncwcee----------
Glossina morsitans	**DEFA**	…vtcni----------gewvcvahcnskskk-sgycsr---gvcyctn----------
Heliothis virescens	**DEFN**	igscvw------gavnytsdcngeckrrgyk-gghcgsfanvncwcet----------
Holotrichia diomphalia	**DEF1**	-vtcdllslqikgiaindsacaahclamrrk-ggsckq---gvcvcrn----------
Lonomia oblique	**DFP6**	…grcnqk----------qcdadcvkkgyf-gglctlt---scfctgsrs---------
Lucilia sericata	**DEFI**	-atcdlls----gtgvkhsacaahcllrgnr-ggycngra--icvcrn----------
Mamestra brassicae	**DEFA**	pascylldgyaagrddgrahcia---prnr--rlycas--yqvcvcry----------
Oryctes rhinoceros	**DEFI**	rltcdllsfeakgfaanhslcaahclaigrk-ggacq---ngvcvcrr----------
Palomena prasina	**DEFI**	-atcdalsfsskwltvnhsacaihcltkgyk-ggrcvn---ticncrn----------
Pandinus imperator	**SCRP**	efqcmanm-------dmlgncekhcqtsgek--gych---gtkckcgtplsy--------
Phlebotomus duboscqi	**DEFI**	-atcdlls----afgvghaacaahcighgyr-ggycnsk--avctcrr----------
Protophormia terranovae	**DEFI**	ratcdlls----gtginhsacaahcllrgnr-ggycngk--gvcvcrn----------
Pyrrhocoris apterus	**DEFI**	-atcdilsfqsqwvtpnhagcalhcvikgyk-ggqcki---tvchcrr----------
Rhodnius prolixus	**DEFA**	-atcdlfsfrskwvtpnhaacaahcllrgnr-ggrck---gtichcrk----------
	DEFC	-atcdllsltskwftpnhagcaahciflgnr-ggrcv---gtvchcrk----------
Sarcophaga peregrine	**SAPB**	-ltcei----------drslcllhcrlkgyl-raycsqq--kvcrcvq----------
	SAPC	-atcdlls----gigvqhsacalhcvfrgnr-ggyctgk--gicvcrn----------
	SAPE	-atcdlls----gtginhsacaahcllrgnr-ggycngk--avcvcrn----------
Stomoxys calcitrans	**DEF1**	gitcdlls----lwkvghaacaahclvlgdv-ggyctke--glcvcke----------
	DEF1A	-itcdlls----lwkvghaacaahclvlgnv-ggyctke--glcvcke----------
	DEF2	ratcdlls----mwnvnhsacaahclllgks-ggrcndd--avcvcrk----------
	DEF2A	ratcdlls----mwnvnhsacaahclllgks-ggrcndd--avcvcrk----------
Tenebrio molitor	**DEFI**	-vtcdilsveakgvklndaacaahclfrgrs-ggycngk--rvcvcr----------
Zophobas atratus	**DEFA**	-ftcdvlgfeiagtklnsaacgahclalgrr-ggycnsk--svcvcr----------
Arachnid		--tcd-l----------ac--hc---g---ggyc-------c-c----------
Amblyomma americanum	**DEFI**	gfgcpf----------nqyqchshclsigrr-ggycggsfkttctcyn----------
Androctonus australis	**DEF4**	gfgcpf----------nqgachrhcrsirrr-ggycaglfkqtctcyr----------
Boophilus microplus	**DEFI**	gfgcpf----------nqgachrhcrsirrr-ggycaglikqtctcyrn----------
entruroides limpidus	**DEF1**	--acqf----------wscnsscisrgyr-qgycwgiqykycqcq----------
Dermacentor variabilis	**DEF1**	gfgcpl----------nqgachnhcrsirrr-ggycsgiikqtctcyrn----------
D. andersoni	**DEFI**	gfgcpl----------nqgachnhcrsirrr-ggycsgiikqtctcyrn----------
H. longicornis	**DEFL0**	dfgcgq---------gmifmcqrrcmrlypgstgfcrgfr---cmcdthiplrppfmvg-
Ixodes ricinus	**DEF**	gyycpf----------fqdkchrhcrsfgrk-agycggflkktcicv----------
Leiurus quinquestriatus	**DEF4**	gfgcpl----------nqgachrhcrsirrr-ggycagffkqtctcyrn----------
O. carinatus	**DEF1,3**	…fmcva-------nvdmtkscdthcqkasge-kgychgtk---ckcgvplsy--------
	DEF2	…fmcma-------nmdptgscethcqkasge-kgychgtk---ckcgvplsy--------
	DEF4	…fmcva-------nidmtkscdthcqkasge-kgychgtk---ckcgvplsy--------
Ornithodoros coriaceus	**DEF1**	rrgcgcpl--------nqgachrhcksigrr-ggycagflkqtctcyrn----------
Tityus discrepans	**DEF**	-rycprnp----------eacynyclrtgrp-ggycggrsritcfcfr----------
Mollusc		--tcd----------c--hc---g---ggyc-------c-c----------
Crassostrea virginica	**DEF1**	gfgcp----------wnryqchshcrsigrl-ggycagslrltctcyrs----------
C. gigas	**DEFh2***	gfgcp----------gdgyecnrhcrsigcr-agycdavtlwlrctctgcsgkk------
M. galloprovincialis	**DEFA**	gfgcp----------ndypchrhcksipgrxggycggxhrlrctcyr----------
	DEFB	gfgcp----------ndypchrhcksipgryggycggxhrlrctc----------
	DEFI/MGD-1*	gfgcp----------nnyqchrhcksipgrcggycggwhrlrctcyrcg----------

* 8-cysteine invertebrate defensins

FIGURE 3 *Continued.*

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FUNGAL DEFENSINS

```
                                        ---cc-------c-------c--hc---g---ggyc------vc-c-
Pseudoplectania nisella  plectasin      gfgcngpwd------eddmqchnhcksikgykggycakg-gfvckcy------------
Aspergillus clavatus     aclasin        gwscgffgg-------ndepchqhcksirgyrggyckfg--gickcy------------
                         aclasinC*      -siccsfpd-----pwggfccedhcsyig-kpggqcsdkk--vctcn------------
A. flavus                aflasin-2      -sscqlgdv--wdlnaadaacsascaiqhgdkhgghcdknk-vcvcn------------
                         aflasin-1C     -siscnfpd-----pfgglicenhcayig-kpggqcsdqk--vctcn------------
                         aflasin-1N     ---fchdsi--scmvggdnvcnnvcvrqgnpnggrclprdgcpgndicacypqs-----
A. fumicatus             afusinC*       -siccsfpd-----pwgglccedhcsyig-kpggqcsdkg--vctcn------------
                         afusinN        ---fchnsi--scmmggdstcnnvcvrqgnpnggrclprdgcpgydicacypnn-----
A. terreus               atesin-1       gygc-----------pndyscsnycssi-grnggycggflwqtckcnekk---------
                         atesin-2       gygc-----------pndyacssycssi-grnggycggflwqtckcnekk---------
                         atesin-3       -sscqlgdi----wgagdaacsasciaqgegyhgghcndd-svcvcny-----------
A. oryzae                aorsinC        -siscnfpd-----pfgglicenhcayig-kpggqcsdqk--vctcn------------
                         aorsinN        ---fchdsi--scmvggdnvcnnvcvrqgnpnggrclprdgcpgndicacypqs-----
A. nidulans              anisin-1       -sscqlggi----fgagdaacsasciragtyhggycndk--qvcicth-----------
                         anisin-2       -ascqigdi----wgagdaacsasclkdgtyhggycnee--svcvcty-----------
Chaetomium globusum      cglosin-1C*    -svccsfpd-----pvgglcceahcqqighleggqctaqn--vctcg------------
                         cglosin-1N     --qdcs-sv--vcvlg-dgacnrvcemeghteggkcvprdgcpagseicvcgakka---
Neosartrya fischeri      nefisin-2C*    -siccsfpd-----pwgglccedhcsyig-kpggqcsdks--vctcn------------
                         nefisin-1C*    -svccslpd-----pwkgfcceahcdkig-kpggqcsdkn--vctcn------------
                         nefisin-2N     ---lchnsi--scmmggdstcnnvcvrqgnpsggrclprdgcpgydicacypns-----
                         aclasinN       ---lchnsi--scalggdnvcnnvcvrqgndnggrclprdgcpgydicacyprs-----
                         nefisin-1N     ---fchnsi--scalggdstcnnvcvrqgnphggrclprdgcpgydicacypnn-----
```

* 8-cysteine fungal defensins

PLANT DEFENSINS

```
                                        ----------c-e--s--f-g-c---------c---c---e------g-c---f----kc-c-----
                                                            w
Aesculus hippocastanum   AhAMP1         ---------lcnerpsqtwsgncgntah---cdkqcqdwe--kashgachkrenhw-kcfcyfnc-
Arabidopsis thaliana     PDF1.1         -------qklc-erpsgtwsgvcgnsna---cknqcinle--karhgscnyvfpah-kcicyfpc-
                         PDF1.2         -------qklc-ekpsgtwsgvcgnsna---cknqcinle--gakhgscnyvfpah-kcicyvpc-
                         PDF1.3         -------qklc-ekpsgtwsgvcgnsna---cknqcinle--gakhgscnyvfpah-kcicyfpc-
                         PDF1.4         temmavegric-errsktwtgfcgntrg---cdsqckrwe--rashgachaqfpgf-acfcyfnc-
                         PDF1.5         --------elc-kresetwsgrcvndyq---crdhcinnd--rgndgycaggypwyrscfcffsc-
                         PDF2           -------aelc-kresetwsgrcvndyq---crdhcinnd--rgndgycaggyp-yrscfcffsc-
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                         PDF2.2         --------rtc-esqshrfkgtcvsasn---canvchn-e--gfvggncrg-frr--rcfctrhc-
                         PDF2.3         --------rtc-eskshrfkgpcvsthn---canvchn-e--gfgggkcrg-frr--rcyctrhc-
                         PDF2.4         --------rtc-etssnlfngpclsssn---canvchn-e--gfsdgdcrg-frr--rclctrpc-
                         PDF2.5         --------rtc-qskshhfkymctsnhn---caivcrn-e--gfsggrchg-fhr--rcyctrlc-
                         PDF2.6         --------rtc-espsnkfqgvclnsqs---cakacps-e--gfsggrcssl-----rcycskac-
                         PDF3           -------artc-esqshrfkgtcvsasn---canvchn-e--gfvggncrg-frr--rcfctrhc-
                         PDF4           -------artc-asqsqrfkgkcvsdtn---cenvchn-e--gfpggdcrg-frr--rcfctrnc-
Beta vulgaris            PDEF2          --------atc-rkpsmyfsgacfsdtn---cqkacnr-e--dwpngkclvgf----kcecqrpc-
                         PDEF11         --------aic-kkpskffkgacgrdadv--cekacdq-e--nwpggvc-vpfl---rcecqrsc-
Brassica campestris      BSD1           -------qrsc-krqpnsgskncmkdse---crevciyae--kamratcdytfprr-rcfchfpcq
Brassica napus           PDEF1          -------qklc-erpsgtwsgvcgnnna---cknqcinle--karhgscnyvfpah-k--------
                         PDEF3          ------eaklc-erssgtwsgvcgnnna---cknqcirle--gaqhgscnyvfpah-kcicyfpc-
Brassica rapa            PDEF3          ------aqklc-erssgtwsgvcgnnna---cknqrinle--garhgscnyvfpyh-rcicyfpc-
                         AFP1           -------qklc-erpsgtwsgvcgnnna---cknqcin----------------------------
                         AFP2           -------qklc-erpsgt-sgvcgnnna---cknqcir----------------------------
Cajanus cajan            PDEF1          -------aktc-enladkyrgpcfsg-----cdthcttke--havsgrcrddf----rcwctkrc-
Capsicum annuum          PDEF1          --------kic-ealsgnfkglclssrd---cgnvcrr-e--gftdgscig-frl--qcfctkpca
                         PDEF2          --------rtc-esqshrfkglcfsksn---cgsvcht-e--gfngghcrg-frr--rcfctrhc-
Capsicum chinense        CCD1           ------qnnic-kttskhfkglcfadsk---crkvciq-e-dkfedghcsklqr---kclctknc
                         PDEF           -ldmmaeakic-ealsgnfkglclssrd---cgnvcrr-e--gftsgvcrg-fpl--kcfcrkpca
Cassia fistula           CFD1           --------ktc-ekpskffsggcigttgnkqcdylcrrge--gllsgackgl-----kcvctkac-
                         CFD2           --------ktc-evlsgkfggacstiingpkcdktcknqe--hyisgtcksdf----kcwctknc-
Clitoria ternatea        PDEF1          --------nlc-erasltwtgncgntgh---cdtqcrnwe--sakhgachkrginw-kcfcyfdc-
                         CtAMP1         --------nlc-erasltwtgncgntgh---cdtqcrnwe--sakhgachkrg-nw-kcfcyfnc-
Dahlia merckii           PDEF1          --------elceekasktwsgncgntgh---cdnqckswe--gaahgachvrngkh-mcfcyfnc-
                         DmAMP1         --------elc-ekasktwsgncgntgh---cdnqckswe--gaahgachvrngkh-mcfcyfnc-
                         DmAMP2         --------evc-ekasktwsgncgntgh---c-------------------------------
Echinochloa crus         EcAMPD1        --------rec-qsqshrykgacvhdtn---casvcqt-e--gfsggkcvg-frg--rcfctkac-
                         EcAMPD2        --------rec-qsqshrykgacvhdtn---casvcqt-e--gfsggkcvg-frg--rcfctkhc-
```

FIGURE 3 *Continued.*

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```
                                  ----------c-e--s--f-g-c--------c---c---e------g-c---f----kc-c-----
                                                     w
Elaeis guineensis        EgAD1    --------rtc-esqshkfqgtclresn---canvcqt-e--gfqggvcrg-vrr--rcfctrlc-
Glycine max              SE60     --------rvc-esqshgfhglcnrdhn---calvcrn-e--gfsggrckg-frr--rcfctric-
Hardenbergia violacea    HvAMP1   --------ktc-eslantyrgpcftdgs---cddhcknke--lislgrcrndv----rcwctrnc-
Helianthus annuus        PDEFA    --------rtc-esqshkfkgtclsdtn---canvchs-e--rfsggkcrg-frr--rcfctthc-
Heliothis virescens      HvD      ----dkligsc-vwgavny--t--sd-----cngecllr---gykgghc-g-fanv-ncwcet---
Heuchera sanguinea       PDEF1    -----dgvklc-dvpsgtwsgvcgsssk---csqqckdreehfayggachyqfpsv-kcfckrqc-
                         Hs-AFP1  -----dgvklc-dvpsgtwsghcgsssk---csqqckdre-hfayggachyqfpsv-kcfckrqc-
Hordeum vulgare          PDEF     --------ric-rrrsagfkgpcvsnkn---caqvcmq-e--gwgggncdg--plr-rckcmrrc-
Lycopersicon esculentum  TPP      --------qic-kapsqtfpglcfmdss---crkycik-e--kftgghcsklqr---kclctkpc-
                         TGAS118  --------rtc-esqshrfkgpcvsekn---casvcet-e--gfsggdcrg-fff--fcfctrpc-
Medicago sativa          MsDef1   --------rtc-enladkyrgpcfsg-----cdthcttke--navsgrcrddf----rcwctkrc-
Medicago truncatula      MtDef2   --------ktc-enladkyrgpcfsg-----cdthcttkenavs--grcrddf----rcwctkrc-
                         MtDef4   --------rtc-esqshkfkgpcasdhn---casvcqt-e--rfsggrcrg-frr--rcfctthc-
                         PDEF3.1  -eivvteantc-enlagtfrgpcfgnsn---cdfhcrtke--hlvsgrcrddf----rcwctrnc-
Nicotiana alata          Nad1     --------rec-ktesntfpgicitkpp---crkacis-e--kftdghcskilr---rclctkpc-
Nicotiana tabacum        PDEF1    --------rec-ktesntfpgicitkpp---crkacis-e--kftdghcskllr---rclctkpc-
                         NTS13    --------rtc-esqshrfkgpcsrdsn---catvclt-e--gfsggrcpwipp---rcfctspc-
Nicotiana excelsior      NETHIO1  --------rec-are-i-ftglcitnpq---crkaci-ke--kftdghcskilr---rclctkpc-
                         NETHIO2  --------kdc-ktesntfpgicitkpp---crkaci-ke--kftdghcskilr---rclctkpc-
Nicotiana paniculata     PDEF1    -------kstc-kaesntfpglcitkpp---crkacls-e--kftdgkcskilr---rcicykpc-
Nigella sativa           NsD1     --------kfc-ekpsgtwsgvcgnsga---ckdqcirle--gakhgscnykppah-rcicyyec-
                         NsD2     --------kfc-ekpsgtwsgvcgnsga---ckdqcirle--gakhgscnyklpah-rcicyyec-
Oryza sativa             PDEF1    -------arhc-lsqshrfkgmcvssnn---canvcrt-e--sfpdgeckshgler-kcfckkvc-
Pachyrhizus erosus       SPE10    --------ktc-enladtfrgpcftdgs---cddhcknkehlik--grcrddf----rcwctrnc
Petunia inflate          PDEF1    --------rtc-esqshrfhgtcvresn---casvcqt-e--gfiggncra-frr--rcfctrnc-
[2]Petunia hybrida       Phd1     --------atc-kaecptwdsvcinkkp---cvacckka---kfsdghcskilr---rclctkecv
                         Phd2     --------gtc-kaecptwegicinkap---cvkcckaqpe-kftdghcskilr---rclctkpca
Picea abies              SPI1     --------rtc-ktpsgkfkgvcassnn---cknvcqt-e--gfpsgscdfhvanr-kcycskpcp
Picea glauca             PDEF1    -------artc-ktpsgkfkgvcassnn---cknvcqt-e--gfpsgscdhvanr--kcycskpcp
Pisum sativum            PDEF1    --------ntc-ehladtyrgvcftnas---cddhcknka--hlisgtchd-w----kcfctqnc-
                         PDEF2    --------ntc-enlagsykgvcfgg-----cdrhcrtqe--gaisgrcrddf----rcwctknc-
                         DRR230-C --------ntc-ehladtyrgvcftdas---cddhcknka--hlisgtchn-f----kcfctqnc-
                         Psd1     --------ktc-ehladtyrgvcftnas---cddhcknka--hlisgtchn-w----kcfctqnc-
                         PDEF4    --------ktc-enlsgtfkgpcipdgn---cnkhcrnne--hllsgrcrddf----rcwctnrc-
Prunus persica           PPDFN1   --------rtc-esqsnrfkgtcvstsn---casvcqt-e--gfpgghcrg-frr--rcfctkhc-
Pyrus pyrifolia          PDN-1    --------rtc-eaasgkfkgmcfssnn---cantcar-e--kfdggkckg-frr--rcmctkkc-
Raphanus sativus         RsAfp1   -------qklc-erpsgtwsgvcgnnna---cknqcinle--karhgscnyvfpah-kcicyfpc-
                         RsAfp2   -------qklc-qrpsgtwsgvcgnnna---cknqcirle--karhgscnyvfpah-kcicyfpc-
                         PDEF3    --------klc-erssgtwsgvcgnnna---cknqcirle--gaqhgscnyvfpah-kcicyfpc-
                         PDEF4    -------qklc-erpsgtwsgvcgnnna---cknqcinle--garhgscnyifpyh-rcicyfpc-
Sinapis alba             PDEF1    -------qklc-erpsgtwsgvcgnnna---cknqcinle--karhgscnyvfpah-kcicyfpc-
                         PDEF2A   -------qklc-qrpsgtwsgvcgnnna---crnqcinle--karhgscnyvfpah-kcicyfpc-
                         PDEF2B   -------qklcarpsgtwssgncrnnna---crnfcikle--ksrhgscnipfpsn-kcicyfpc-
Solanum tuberosum        PDEF1    --------rhc-eslshrfkgpctrdsn---casvcet-e--rfsggnchg-frr--rcfctkpc-
Sorghum bicolor          PDEF1    --------rvc-mgksqhhsfpcisdrl---csnecvk-eeggwtagych-l-r---ycrcqkac-
                         PDEF2    --------rvc-mgksagfkglcmrdqn---caqvclq-e--gwgggncdgvmr---qckcirqc-
                         PDEF3    --------rvc-rrrsagfkglcmsdhn---caqvclq-e--gwgggncdgvir---qckcirqc-
Spinacia oleracea        PDEF2    ---gifssrkc-ktpsktfkgictrdsn---cdtscry-e--gypagdckgirr---rcmcskpc-
Saccharum spp            PDEF1    --------ryc-lsqshrfkglcmsssn---canvcqt-e--nfpggeckadgatr-kcfckkic-
Triticum aestivum        PDEF1    --------kic-rrrsagfkgpcmsnkn---caqvcqq-e--gwgggncdgpfr---rckcirqc-
                         PDEF2    --------kvc-rqrsagfkgpcvsdkn---caqvclq-e--gwgggncdgpfr---rckcirqc-
                         TAD1     --------rtc-lsqshkfkgtclsnsn---caavcrt-e--nfpdgecnthlver-kcyckrtc-
Triticum kiharae         AMP-D1   --------rtc-qsqshkfkgacfsdtn---cdsvcrt-e--nfprgqcnqhhver-kcycerdc-
                         AMP-D1.1 --------rdc-esdshkfhgacfsdtn---canvcqt-e--gftagkcvgvqr---hchctkdc-
                         AMP-D2   --------rtc-esqshkfkgpcfsdsn---catvcrt-e--nfprgqcnqhhver-kcycersc-
                         AMP-D3   --------rdc-ksdshkfhgacfsdtn---canvcqt-e--gftrgkcdgi-----hchcikdc-
```

FIGURE 3 *Continued.*

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                                  ----------c-e--s--f-g-c--------c---c---e------g-c---f----kc-c-----
                                                   w
                      AMP-D4      --------rdc-tsqshkfvglclsdrn---casvclt-e--yftggkcdhr-----rcvctkgc-
                      AMP-D5      --------rec-rseskkfvglcvsdtn---casvclt-e--rfpggkcdg-yr---rcfctkdc-
                      AMP-D6      --------rdc-rsqsktfvglcvsdtn---casvclt-e--hfpggkcdg-yr---rcfctkdc-
                      AMP-D6.1    --------rec-rsqskqfvglcvsdtn---casvclt-e--hfpggkcdg-yr---rcfctkdc-
Triticum monococcum   AMP-D1.2    --------rtc-qsqshkfkgacfsdtn---casvcrt-e--nfprgqcnqhhver-kcycerdc-
Wasabia japonica      PDEF1       ------aqklc-ekssgtwsgvcgnnna---cknqcinle--garhgscnyifpyh-rcicyfpc-
Vicia faba            PDEF1,2     ------llgrc-kvksnrfhgpcltdth---cstvcrg-e--gykggdchglrr---rcmc-l-c-
Vigna radiata         VrD1        --------rtc-mikkegw-gkclidtt---cahscknr---gyiggnckgmtr---tcyclvnc-
                      VrD2        --------ktc-enlantyrgpcfttgs---cddhcknke--hlrsgrcrddf----rcwctrnc-
Vigna unguiculata     PDEF1       --------ktc-enlvdtyrgpcfttgs---cddhcknke--hllsgrcrddv----rcwctrnc-
                      THGC        --------rvc-esqshgfkgactgdhn---calvcrn-e--gfsggncrg-frr--rcfcltkc-
Zea mays              PDEF1       --------rvc-rrrsagfkgvcmsdhn---caqvclq-e--gygggncdgimr---qckcirqc-
                      THZ2        --------rvc-mgksqhhsfpcisdrl---csnecvk-edggwtagychlr-----ycrcqkac-
```

FIGURE 3 *Continued.*

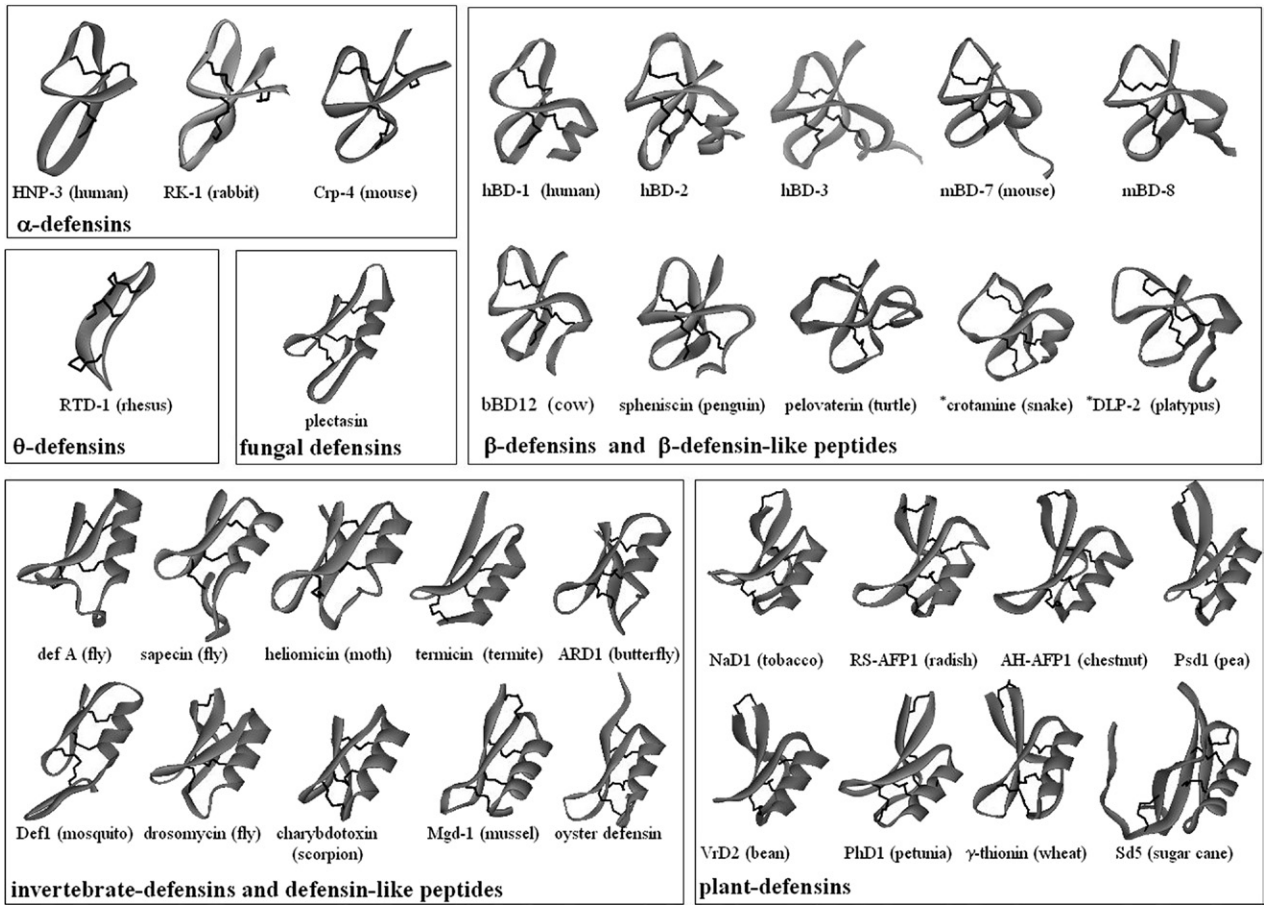

FIGURE 4 Comparison of structurally characterized defensins and defensin-like peptide from different families. Structures were prepared with DSViewer pro from Accelrys, using coordinates deposited in the PDB databank with the following PDB IDs: HNP3, 1DFN; RK-1, 1EWS; cryptdin-4, 1TV0; hBD-1, 1IJV; hBD-2, 1FD3; hBD-3, 1KJ6; mBD8, 1E4R; mBD-7, 1E4 T; bBD12, 1BNB; spheniscin, 1UT3; pelovaterin, 2JR3; crotamine, 1H5O; DLP-2, 1D6B; RTD-1, 1HVZ; plectasin, 1ZFU; fly def A, 1ICA; sapecin 1L4 V; heliomicin, 1I2U; termicin, 1MMO; ARD1, 1OZZ; Def1 (mosquito), 2NY8; drosomycin, 1MYN; charybdotoxin, 2CRD; Mgd-1 (mussel), 1FJN; oyster defensin, 2B68; NaD1, 1MR4; RS-AFP-1, 1AYJ; AH-AFP1, 1BK8; Psd1, 1JKZ; VrD2, 2GL1; PhD-1, 1N4 N; γ-thionin, 1GPS; Sd5, 2KSK. *Snake crotamine and platypus DLP2 are venom peptides.

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BIOLOGICAL ACTIONS

Defensins have an undeniably important role in host defense, as both effectors and regulators.[2,3,5,10,13–15,19,23–28] As effectors, most defensins show a direct activity, *in vitro*, against bacteria and fungi, which is however quite medium and salt sensitive. α- and θ-Defensins tend to have a broader spectrum of activity than β-defensins do, whereas the canonical insect defensins tend to be more active against Gram-positive bacteria and the four cystine insect defensin-like peptides and plant defensins tend to be principally active against fungi. The precise mechanisms of action of these peptides, regarding this direct microbicidal activity, are still undefined. For many of them, interaction with the microbial membrane seems to be a crucial step, although the mode of action can vary remarkably. Membrane disruption would lead to the loss of the transmembrane potential gradient and release of cytoplasmic contents and may contribute to the microbicidal activity.

For the vertebrate defensins and the canonical three cystine invertebrate defensins, bacterial membrane interaction was proposed to occur principally with anionic phospholipids such as phosphatidylglicerol or cardiolipin.[2,5,10,12,23,25] This could then lead to pore formation by barrel stave, toroidal or aggregate mechanisms or membrane disruption by a detergent-like "carpet mechanism." However, although the relevance of initial membrane interactions is undisputed, that of membrane disruption may be overestimated. It is now apparent that bacterial membranes demonstrate considerable functional organization of their protein machinery, whose assembly requires patches of anionic phospholipids, so that HDPs could preferentially target these regions and affect coordinated processes required for bacterial survival. Further, for fungal, mollusk, and mammalian defensins, it has been shown that the lipid II, which carries cell wall building blocks, is also an important target, especially for Gram-positive bacteria.[27] By binding to it, these peptides impede cell wall biosynthesis, analogously to some clinically used antibiotics. It is interesting to note that the affinity for mammalian α-defensin HNP1 and β-defensin hBD3 is an order of magnitude lower than for the fungal defensin, and in their case, pleiotropic effects on membrane localized processes may act in conjunction with cell wall inhibition. The lower affinity probably also correlates with the broader activity spectrum of these peptides, which also includes Gram-negative bacteria, and that requires affinity for additional targets.

For the four cystine antifungal insect and plant defensin that are principally antifungal, a common membrane target has been identified in fungal sphyngolipids or related glucosylceramides (see above).[1,13,27] These sphingolipids colocalize in fungal membranes with ergosterol in lipid-rafts rich in membrane proteins, and may represent high-affinity binding sites for the defensins. Different antifungal defensins bind in different manners with one or more different subsequent effects, including membrane disruption, inactivation of calcium channels, activation of signaling cascades, induction of ROS production, and induction of apoptosis.[1,27]

For mammalian defensins in particular, roles as regulators of innate and adaptive immune cells have been amply demonstrated.[10,12,15,17,19,30] These variously include the capacity to chemoattract monocytes, neutrophils, immature dendritic cells, mast cells, and T-cells; to stimulate the maturation of antigen presenting cells; to induce or suppress proinflammatory mediators; to activate phagocytes; to regulate the function of complement components, to inhibit the production of immunosuppressive adrenal glucocorticoids, and to act as adjuvants for antigen presenting cells. Some mammalian defensins also play other roles outside immunity, which include the stimulation of wound healing, reproduction, and curiously the determination of the fur color in dogs. Certain plant defensins act as inhibitors of proteinases, α-amylase, or protein translation. These are structurally and functionally distinct from antifungal defensins and serve to protect from predators.[26] Plant defensins are also implicated in plant growth and development.[24]

ACKNOWLEDGMENTS

N. Antchera and F. Guida, respectively, acknowledge postdoctoral and doctoral grants from the University of Trieste. The FVG regional R3A2 network and EU IAPP project NAM are acknowledged for support.

REFERENCES

1. Aerts AM, François IE, Cammue BP, Thevissen K. The mode of antifungal action of plant, insect and human defensins. *Cell Mol Life Sci* 2008;**65**:2069–79.
2. Castro MS, Fontes W. Plant defense and antimicrobial peptides. *Protein Pept Lett* 2005;**12**:13–8.
3. Crovella S, Antcheva N, Zelezetsky I, Boniotto M, Pacor S, Verga Falzacappa MV, et al. Primate beta-defensins—structure, function and evolution. *Curr Protein Pept Sci* 2005;**6**:7–21.
4. Dalla Valle L, Benato F, Maistro S, Quinzani S, Alibardi L. Bioinformatic and molecular characterization of beta-defensins-like peptides isolated from the green lizard *Anolis carolinensis*. *Dev Comp Immunol* 2012;**36**:222–9.
5. Dimarcq JL, Bulet P, Hetru C, Hoffmann J. Cysteine-rich antimicrobial peptides in invertebrates. *Biopolymers* 1998;**47**:465–77.
6. Ferrandon D, Imler JL, Hoffmann JA. Sensing infection in Drosophila: toll and beyond. *Semin Immunol* 2004;**16**:43–53.
7. Froy O, Gurevitz M. Arthropod and mollusk defensins—evolution by exon-shuffling. *Trends Genet* 2003;**19**:684–7.
8. Garcia AE, Osapay G, Tran PA, Yuan J, Selsted ME. Isolation, synthesis, and antimicrobial activities of naturally occurring theta-defensin isoforms from baboon leukocytes. *Infect Immun* 2008;**76**(12):5883–91.
9. Graham MA, Silversein KA, VandenBosch KA. Defensin-like genes: genomic perspectives on a diverse superfamily in plants. *Crop Sci* 2008;**48**:S3–11.

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10. Hazlett L, Wu M. Defensins in innate immunity. *Cell Tissue Res* 2011;**343**(1):175–88.
11. Hellgren O, Ekblom R. Evolution of a cluster of innate immune genes (beta-defensins) along the ancestral lines of chicken and zebra finch. *Immunome Res* 2010;**6**:3.
12. Lai Y, Gallo RL. AMPed up immunity: how antimicrobial peptides have multiple roles in immune defense. *Trends Immunol* 2009;**30**:131–41.
13. Lay FT, Anderson MA. Defensins—components of the innate immune system in plants. *Curr Protein Pept Sci* 2005;**6**:85–101.
14. Lehrer RI. Primate defensins. *Nat Rev Microbiol* 2004;**2**:727–38.
15. Lehrer RI. Multispecific myeloid defensins. *Curr Opin Hematol* 2007;**14**:16–21.
16. Nurnberger T, Brunner F, Kemmerling B, Piater L. Innate immunity in plants and animals: striking similarities and obvious differences. *Immunol Rev* 2004;**198**:249–66.
17. Oppenheim JJ, Yang D. Alarmins: chemotactic activators of immune responses. *Curr Opin Immunol* 2005;**17**:359–65.
18. Patil AA, Cai Y, Sang Y, Blecha F, Zhang G. Cross-species analysis of the mammalian beta-defensin gene family: presence of syntenic gene clusters and preferential expression in the male reproductive tract. *Physiol Genomics* 2005;**23**:5–17.
19. Rehaume L, Hancock RE. Neutrophil-derived defensins as modulators of innate immune function. *Crit Rev Immunol* 2008;**28**: 185–200.
20. Rodriguez de la Vega RC, Possani LD. On the evolution of invertebrate defensins. *Trends Genet* 2005;**21**:330–2.
21. Schmitt P, Gueguen Y, Desmarais E, Bachère E, de Lorgeril J. Molecular diversity of antimicrobial effectors in the oyster Crassostrea gigas. *BMC Evol Biol* 2010;**10**:23.
22. Schutte BC, Mitros JP, Bartlett JA, Walters JD, Jia HP, Welsh MJ, et al. Discovery of five conserved beta-defensin gene clusters using a computational search strategy. *Proc Natl Acad Sci U S A* 2002;**99**:2129–33.
23. Selsted ME, Ouellette AJ. Mammalian defensins in the antimicrobial immune response. *Nat Immunol* 2005;**6**:551–7.
24. Stotz HU, Thomson JG, Wang Y. Plant defensins: defense, development and application. *Plant Signal Behav* 2009;**4**:1010–2.
25. Tossi A, Sandri L. Molecular diversity in gene-encoded, cationic antimicrobial polypeptides. *Curr Pharm Des* 2002;**8**:743–61.
26. van Dijk A, Veldhuizen EJ, Haagsman HP. Avian defensins. *Vet Immunol Immunopathol* 2008;**124**:1–8.
27. Wilmes M, Cammue BP, Sahl HG, Thevissen K. Antibiotic activities of host defense peptides: more to it than lipid bilayer perturbation. *Nat Prod Rep* 2011;**28**:1350–8.
28. Wong JH, Xia L, Ng TB. A review of defensins of diverse origins. *Curr Protein Pept Sci* 2007;**8**(5):446–59.
29. Xiao Y, Hughes AL, Ando J, Matsuda Y, Cheng JF, Skinner-Noble D, et al. A genome-wide screen identifies a single beta-defensin gene cluster in the chicken: implications for the origin and evolution of mammalian defensins. *BMC Genomics* 2004;**5**:56.
30. Yang D, Liu ZH, Tewary P, Chen Q, de la Rosa G, Oppenheim JJ. Defensin participation in innate and adaptive immunity. *Curr Pharm Des* 2007;**13**(30):3131–9.
31. Yeaman MR, Yount NY. Unifying themes in host defence effector polypeptides. *Nat Rev Microbiol* 2007;**5**(9):727–40.
32. Zhu Q, Bateman A, Singh A, Solomon S. Isolation and biological activity of corticostatic peptides (anti-ACTH). *Endocr Res* 1989;**15**:129–49.
33. Zhu S. Evidence for myxobacterial origin of eukaryotic defensins. *Immunogenetics* 2007;**59**:949–54.
34. Zhu S. Discovery of six families of fungal defensin-like peptides provides insights into origin and evolution of CSαβ defensins. *Mol Immunol* 2008;**45**:828–38.
35. Zou J, Mercier C, Koussounadis A, Secombes C. Discovery of multiple beta-defensin like homologues in teleost fish. *Mol Immunol* 2007;**44**:638–47.

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Chapter 19

Lantibiotics

Jasmin Dischinger, Imke Wiedemann, Gabriele Bierbaum and Hans-Georg Sahl

ABSTRACT

Lanthionine-containing antibiotic peptides (lantibiotics) have been known for >60 years, and the prototype peptide nisin has been used safely in food preservation for half a century. Lantibiotics derive from ribosomally synthesized prepeptides through unique post-translational modifications involving serine, threonine, and cysteine residues. About 80 lantibiotics are known to date, and even more gene clusters, containing the determinants for prepeptides, modification, processing, export, and producer self-protection, have been sequenced or detected by genome mining. Recent progress in *in vitro* modification and insights into multiple antibiotic mechanisms combined in a single molecule make lantibiotics attractive model compounds for design of novel antiinfective drugs.

DISCOVERY

The most prominent lantibiotic nisin was first described as a group-**N** streptococci **i**nhibiting **s**ubstance in the 1920s and identified as a lanthionine-containing peptide by Berridge in 1949. Its structure was elucidated by Gross and Morrel. The designation "lantibiotics" was introduced to emphasize the occurrence of lanthionine and the antibiotic nature of these peptides (**lan**thionine-containing an**tibiotics**)[7] (Fig. 1). Since 1985, a significant number of new lantibiotics have been discovered,[3,7] among them numerous natural variants. "Prototype" peptides, such as nisin, mersacidin, lacticin 481, Pep 5, and lacticin 3147 were defined for various subgroups, which are shown in Table 1. Genomic mining shows that putative lantibiotic gene clusters are widespread among many genera and even present in phyla, for example, the cyanobacteria that had previously not been associated with lantibiotic production.[20,22]

STRUCTURAL *lanA* GENES

Lantibiotics are expressed as prepropeptides, with an N-terminal leader sequence and a C-terminal propeptide part, which is posttranslationally modified. Biosynthesis starts with enzymatic dehydration of serine and/or threonine residues in the propeptide part yielding the unusual amino acids 2,3-dehydroalanine (Dha) and 2,3-dehydrobutyrine (Dhb). To some of these dehydroamino acids, the thiol group of a neighboring cysteine residue is added resulting in the characteristic lanthionine (from Dha) and methyllanthionine (from Dhb) residues. The stable, thioether-based intramolecular rings determine the three-dimensional structure of the peptides and are essential for biological activity. The substrate recognition of the modification enzymes seems to reside in the leader peptide, for example, it is possible to synthesize pneumococcin using the nisin modification enzymes after a fusion to the nisin leader.[21]

BIOSYNTHETIC GENE CLUSTERS

For a long time, lantibiotic production was exclusively associated with Gram-positive bacteria; however, genetic determinants for lantibiotic synthesis are also present in other genera and phyla and can be located on either chromosomes or mobile elements such as plasmids and transposons. Nearly always, the prepeptide structural genes (*lanA*) are part of gene clusters that also contain the genes responsible for modification (*lanB*, *lanC*, *lanM*, *labKC*, *lanL*, and *lanD*), proteolytic processing (*lanP* and *lanT*), transport (*lanT*), immunity (*lanI*, *lanFEG*) and biosynthesis regulation (*lanK*, *lanR*, *lanQ*) (Fig. 2) (reviewed in Refs. 3, 7, 35). By contrast, in the marine cyanobacterium *Prochlorococcus*, one LanM enzyme is able to modify a plethora of LanA precursor peptides that are encoded all over the chromosome.[20] Currently, lantibiotics are classified according to their modification machinery and antibiotic activity: Type I and II lantibiotics show antibacterial activity and are distinguished by their modification enzymes as discussed below, whereas type III lantibiotics show only little antibiotic activity and, for example, may be involved in morphogenesis of the producer strain.[35]

BIOSYNTHESIS OF THE RING STRUCTURES

Several different biosynthetic machineries have been identified. The first requires two separate enzymes (LanB and LanC) to synthesize lanthionines and – by definition – is

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00019-1
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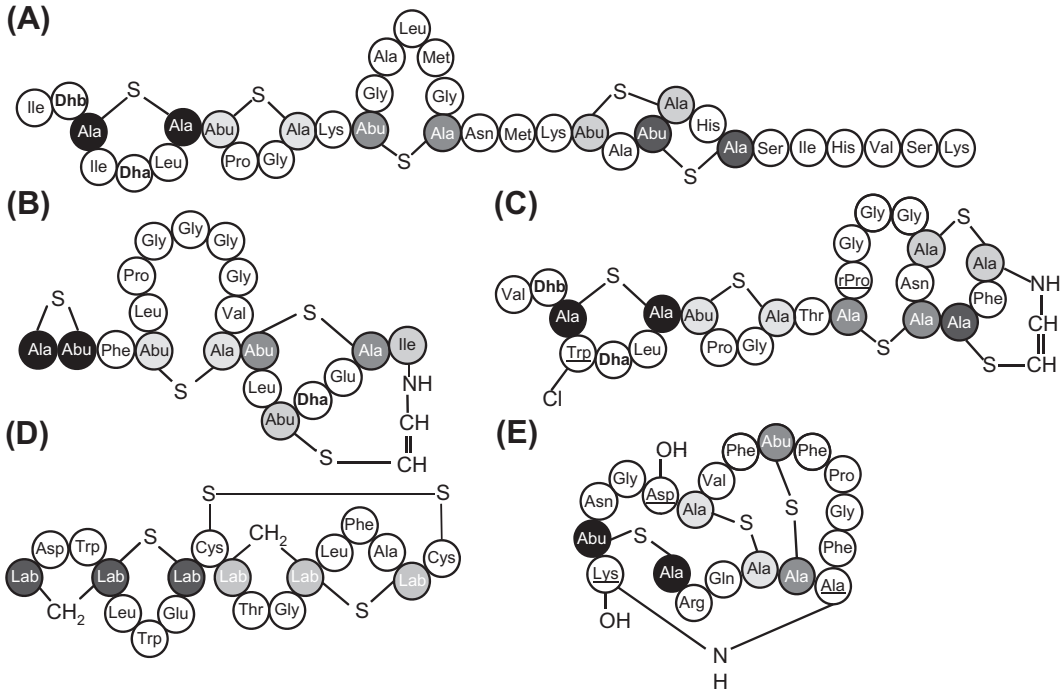

FIGURE 1 Structures of the lantibiotics nisin A (A), mersacidin (B), microbisporicin (C), labyrinthopeptin A2 (D), and cinnamycin (E). Residues involved in (Me)Lan or labionin formation are highlighted by shades of gray, and residues that contribute to one thioether are marked in the same shade of gray. Additionally, serine and threonine residues that are dehydrated to Dha and Dhb in the mature peptide are in bold. Additional modifications are underlined.

found in gene clusters of the type I lantibiotics (Table 1).[7] In this pathway, the LanB enzyme dehydrates the Ser and Thr residues of the C-terminal core propeptide or propeptide sequence. The LanC enzyme then catalyzes the cyclization reactions to form the thioether structures. The structure of NisC has been well studied; the enzyme coordinates a zinc ion that is involved in the activation of the thiolate.[3] In type II lantibiotics (Table 1), only one enzyme, LanM, is necessary to form the thioether bridge and catalyzes both reactions.[3,7] Several LanM enzymes are active in *in vitro* modification assays, and the dehydration of the hydroxy amino acids is mediated via an ATP-dependent phosphorylation.[3,20,23] The

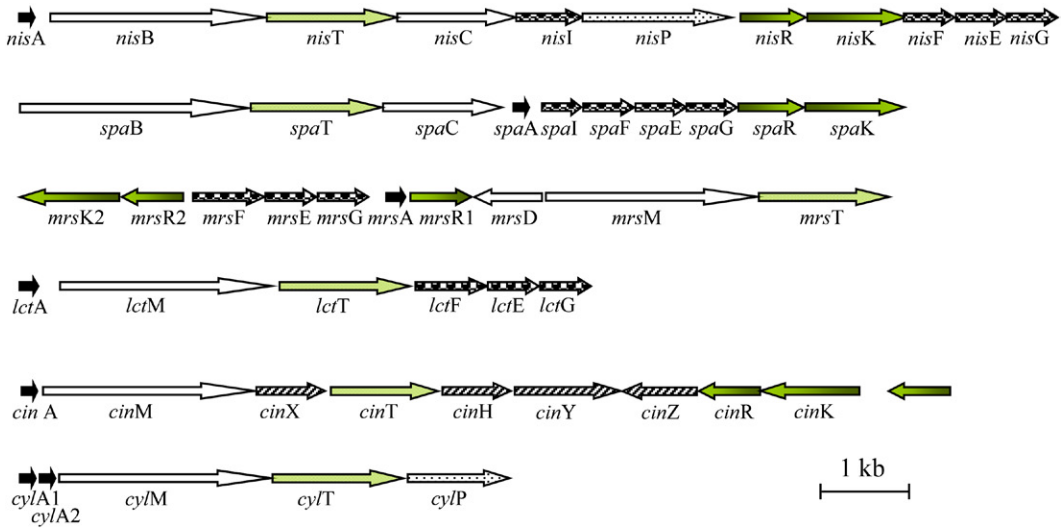

FIGURE 2 Examples for lantibiotic biosynthesis gene clusters. *lanA* genes encode the lantibiotic prepeptides; *lanB*, *lanC*, *lanM*, and *lanD*, modification enzymes; *lanP*, proteolytic enzymes; *lanT*, transporters; *lanI*, *lanE*, *lanF*, and *lanG*, immunity determinants; *lanR*, *lanK*, and *lanQ*, regulators of biosynthesis. Gene nomenclature has been unified for clarity and is not consistent with the original publications. From the top to the bottom, the gene clusters of nisin (*nis*), subtilin (*spa*), mersacidin (*mrs*), lacticin 481 (lc*t*), cinnamycin (*cin*), and cytolysin (*cyl*) are shown.

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TABLE 1 Overview of Lantibiotics

Name	Producer	MW	Propeptide
Class I lantibiotics (LanBC Modified, Elongated Peptides)[1]			
Nisin-like Lantibiotics			
Nisin A	*Lactococcus lactis* ATCC 11454	3353	ITSISLCTPGCKTGALMGCNMKTATCHCSIHVSK
Nisin Q	*Lactococcus lactis* 61-14	3327	ITSISLCTPGCKTGVLMGCNLKTATCNCSVHVSK
Nisin U	*Streptococcus uberis* 42	3029	ITSKSLCTPGCKTGILMTCPLKTATCGCHFG
Nisin Z	*Lactococcus lactis* N8, NIZO22186	3330	ITSISLCTPGCKTGALMGCNMKTATCNCSIHVSK
Nisin F	*Lactococcus lactis* F10	3457	ITSISLCTPGCKTGALMGCNMKTATCNCSVHVSK
Subtilin	*Bacillus subtilis* ATCC 6633	3317	WKSESLCTPGCVTGALQTCFLQTLTCNCKISK
Entianin[10]	*Bacillus subtilis* DSM 15029T	3348	WKSESVCTPGCVTGLLQTCFLQTITCNCKISK
Ericin S	*Bacillus subtilis* A1/3	3442	WKSESVCTPGCVTGVLQTCFLQTITCNCHISK
Ericin A	*Bacillus subtilis* A1/3	2986	VLSKSLCTPGCITGPLQTCYLCFPTFAKC
Planosporicin (LAB97518)	*Planomonospora* spec.	2194	ITSVSWCTPGCTSEGGGSGCSHCC
Streptin 1	*Streptococcus pyogenes* M25	2424	VGSRYLCTPGSCWKLVCFTTTVK VGSRYLCTPGSCWKLVCFTTTVK two putative structures predicted
Streptin 2		2821	TPYVGSRYLCTPGSCWKLVCFTTTVK TPYVGSRYLCTPGSCWKLVCFTTTVK two putative structures predicted (elongated streptin 1)
Epidermin-Like Lantibiotics			
Epidermin	*Staphylococcus epidermidis* Tü3298	2164	IASKFLCTPGCAKTGSFNSYCC AviCys (19+22)
[Val1,Leu6] Epidermin	*Staphylococcus epidermidis* BNV1/BNV301	2151	VASKFLCTPGCAKTGSFNSYCC AviCys (19+22)
Gallidermin	*Staphylococcus gallinarum* (F16/P57) Tü3928	2165	IASKFLCTPGCAKTGSFNSYCC AviCys (19+22)
Staphylococcin T	*Staphylococcus cohnii* StT		
Mutacin B-Ny266	*Steptococcus mutans*	2270	FKSWSFCTPGCAKTGSFNSYCC AviCys (19+22); structure predicted
Mutacin 1140 / Mutacin III	*Steptococcus mutans*	2263	FKSWSLCTPGCARTGSFNSYCC AviCys (19+22)
Clausin[5]	*Bacillus clausii* OC	?	FTSVSFCTPGCGETGSFNSFCC AviCys (19+22)
Mutacin I	*Steptococcus mutans*	2364	FSSLSLCSLGCTGVKNPSFNSYCC predicted structure; AviCys (19+22)
Microbisporicin (different variants)	*Microbiospora* species (*corallina*)	2246	VTSWSLCTPGCTSPGGGSNCSFCC; AviMeCys (21–24) **variant: 107891 A1:** chloro-tryptophan (4), dihydroxyproline (14)
		2230	**variant: 107891 A2:** chloro-tryptophan (4), hydroxyproline (14)

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TABLE 1 Overview of Lantibiotics—*Cont'd*

Name	Producer	MW	Propeptide
Pep5-Like Lantibiotics			
Pep5	*Staphyloccoccus epidermidis* 5	3488	TAGPAIRASVKQCQKTLKATRLFTVSCKGKNGCK 2-oxobutyryl (1)
Epilancin K7	*Staphyloccoccus epidermidis* K7	3032	SASVLKTSIKVSKKYCKGVTLTCGCNITGGK 2-hydroxypropionyl (1)
Epicidin 280	*Staphyloccoccus epidermidis* BN 280	3133	SLGPAIKATRQVCPKATRFVTVSCKKSDCQ predicted structure; 2-hydroxypropionyl (1)
Epilancin 15X	*Staphyloccoccus epidermidis* 15×154	3173	ASIVKTTIKASKKLCRGFTLTCGCHFTGKK lactate (1)
Class II lantibiotics (LanM Modified, Globular Peptides)			
Lacticin 481-Like Lantibiotics			
Lacticin 481	*Lactococcus lactis* CNRZ 481	2901	KGGSGVIHTISHECNMNSWQFVFTCCS
Mutacin II, JT-8, H-29B	*Streptococcus mutans* T8	3245	NRWWQGVVPTVSYECRMNSWQHVFTCC
Mutacin K8	*Streptococcus mutans* K8	2734	MGKGAVGTISHECRYNSWAFLATCCS predicted structure, corresponds to mass present in culture supernatant, 2 more putative *lanA* genes in the gene cluster
Nukacin ISK-1, 3299	*Staphylococcus warneri* ISK1	2960	KKKSGVIPTVSHDCHMNSFQFVFTCCS predicted structure
Nukacin KQU-131[34]	*Staphylococcus hominis* KQU-133	3004	KKKSGVIPTVSHDCHMNSFQFMFTCCS predicted structure
Streptococcin SA-FF22	*Streptococcus pyogenes* SA-FF22	2795	GKNGVFKTISHECHLNTWAFLATCCS structure not fully confirmed
Streptococcin AM49	*Streptococcus pyogenes*		
Macedocin	*Streptococcus macedonicus* ACA-DC198		
Macedocin A1	*Streptococcus macedonicus* ACA-DC198	?	GHGVNTISAECRWNSLQAIFSCC structure predicted, based on similarity to lacticin 481
Butyrivibriocin OR79A	*Butyrivibrio fibrisolvens*	2910	GNGVIKTISHECHMNTWQFIFTCCS structure not fully elucidated/predicted based on similarity to lacticin 481
Butyrivibriocin OR36		?	GDGVFRTISHECHMNTWMFIFTCCS putative *lanA*, structure predicted based on similarity to lacticin 481
Butyrivibriocin OB247		?	GDGVFRTISHECAMNTWMFIFTCCS putative *lanA*, structure predicted based on similarity to lacticin 481
Salivaricin A	*Streptococcus salivarius* 20P3	2315	KRGSGWIATITTDDCPNSVFVCC structure not fully elucidated
Salivaricin B	*Streptococcus salivarius* K12	2740	GGGVIQTISHECRMNSWQFLFTCCS putative *lanA;* structure predicted based on similarity to lacticin 481

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TABLE 1 Overview of Lantibiotics—*Cont'd*

Name	Producer	MW	Propeptide
Salivaricin A1	*Streptococcus agalactiae* 120 *Streptococcus pyogenes* MGAS315 *Streptococcus pyogenes* M1 GAS *Streptococcus pyogenes* MGAS10394	2321	KKGSGWFATITDDCPNSVFVCC unknown structure
Salivaricin A2	*Streptococcus salivarius*	?	KRGTGWFATITDDCPNSVFVCC unknown structure (*lanA* identified)
Salivaricin A3		?	KKGPGWIATITDDCPNSIFVCC unknown structure (*lanA* identified)
Salivaricin A4		?	KRGPGWIATITDDCPNSIFVCC unknown structure (*lanA* identified)
Salivaricin A5		?	KRGPGWIATITDDCPNSVFVCC unknown structure (*lanA* identified)
Salivaricin 9[33]	*Streptococcus salivarius* strain 9	2560	GNGVVLFLTHECNLATWTKKLKCC structure predicted (similarity to lacticin 481)
Variacin	*Kocuria varians* MCV8/12	2658	GSGVIPTISHECHMNSFQFVFTCCS structure predicted (similarity to lacticin 481)
Plantaricin C	*Lactobacillus plantarum* LL441	2880	KKTKKNSSGDICILLTSECDHLATWVCC structure partially elucidated, predicted
Bovicin HJ50 = Thermophilin 1277[19]	*Streptococcus bovis* HJ50 and *thermophilus* SBT1277	3428	ADRGWIKTLTKDCPNVISSI**C**AGTIITA**C**KNCA ADRGWIKTLTKDCPNVISSI**C**AGTIITA**C**KNCA structure predicted; disulfide bridge (21 + 29)
Ruminococcin A	*Ruminococcus gnavus* E1	2675	GNGVLKTISHECNMNTWQFLFTCC structure predicted
Mersacidin-Like Lantibiotics			
Mersacidin	*Bacillus amyloliquefaciens* HIL-Y-85, 54728	1825	CTFTLPGGGGVCTLTSECIC AviMeCys (15 + 20)
Actagardine	*Actinoplanes liguriae*	1890	SSGWVCTLTIECGTVICAC lanthionine sulfoxide (14 + 19)
Ala(O)-Actagardine	*Actinoplanes liguriae*	1961	ASSGWVCTLTIECGTVICAC lanthionine sulfoxide (14 + 19)
Deoxy-actagardine B (DAB)	*Actinoplanes liguriae* NCIMB41362		SSGWVCTLTIECGTLVCAC lanthionine sulfoxide (14 + 19)
Michiganin A	*Clavibacter michiganensis* subspec.	2145	SSSGWLCTLTIECGTIICACR structure predicted
Cinnamycin-Like Lantibiotics			
Cinnamycin/ lantibiotic Ro 09-198	*Streptomyces cinnamoneus*; *Streptomyces griseoverticillatus*	2042	CRQSCSFGPFTFVCDGNTK lysinoalanine (19 + 6); hydroxyaspartate (15)
Duramycin	*Streptomyces cinnamoneus* DSM 40646	2014	CKQSCSFGPFTFVCDGNTK lysinoalanine (19 + 6); hydroxyaspartate (15)

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TABLE 1 Overview of Lantibiotics—*Cont'd*

Name	Producer	MW	Propeptide
Duramycin B	*Streptoverticillium* spec.	1951	CRQSCSFGPLIFVCDGNIK lysinoalanine (19 + 6); hydroxyaspartate (15)
Duramycin C	*Streptomyces griseoluteus* R2017	2008	CANSCSYGPLIWSCDGNIK lysinoalanine (19 + 6); hydroxyaspartate (15)
Ancovenin	*Streptomyces* spec.	1959	CVQSCSFGPLIWSCDGNIK lysinoalanine (19 + 6)
Two-Peptide lantibiotics (synergistic action)			
Lacticin 3147	*Lactococcus lactis* subsp. *lactis* DPC3147	3322	CSTNTFSLSDYWGNNGAWCTLIHECMAWCK D-Ala (7)
		2847	TTPATPAISILSAYISTNTCPITKCTRAC 2-oxobuturyl (1); D-Ala (9; 12)
Staphylococcin C55	*Staphylococcus aureus* C55	3339	CSTNTFSLSDYWGNKGNWCTAIHECMSWCK structure predicted; D-Ala (7)
		2993	GTPLALLGGAATGVIGYISNQTCPTTACTRAC structure predicted
Plantaricin W	*Lactobacillus plantarum*	3223	KCKWWNISCDLGNNGHVCTLSHECQVSCN structure predicted; disulfide bridge (2 + 9)
		3099	SGIPCTIGAAVAASIAVCPTTKCSKRCGKRKK structure predicted
Cytolysin	*Enterococcus faecalis* V583	4164	TTPACFTIGLGVGALFSAKFC structure predicted (2 thioether bridges confirmed)
		2631	TTPVCAVAATAAASSAACGWVGGGIFTGVTVVVSLKHC unknown structure (1 thioether bridge confirmed)
Haloduracin	*Bacillus halodurans* C-125	3043	CAWYNISCRLGNKGAYCTLTVECMPSCN disulfide bridge (1–8)
		2330	TTWPCATVGVSVALCPITKCTSQC
Smb	*Streptococcus mutans* GS5	?	IGTTVVNSTFSIVLGNKGYICTVTVECMRNCSK predicted structure
		?	STPACAIGVVGITVAVTGISTACTSRCINK predicted structure
Pneumococcin[21]	*Streptococcus pneumoniae* R6	?	WTPTPIILKSAAASSKVCISAAVSGIGGLVSYNNDCLG structure not fully elucidated
		?	STIICSATLSFIASYLGSAQTRCGKDNKKK structure not fully elucidated
BHT	*Streptococcus ratti* BHT	3375	IGTTVVNSTFSIVLGNKGYICTVTVECMRNCQ predicted structure
		2802	STPACAIGVVGITVAVTGISTACTSRCINK predicted structure
Lichenicidin[2]	*Bacillus licheniformis* DSM13/ATCC14580/VK21	3250	TITLSTCAILSKPLGNNGYLCTVTKECMPSCN 2-oxobuturyl (1)
		3021	TTPATTSSWTCITAGVTVSASLCPTTKCTSRC 2-oxobuturyl (1)

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TABLE 1 Overview of Lantibiotics—*Cont'd*

Name	Producer	MW	Propeptide
Class III Lantibiotics / Lantipeptides with Morphogenetic Function			
SapB	*Streptomyces coelicolor*	2026	TGSRASLLLCGDSSLSITTCN predicted structure
SapT	*Streptomyces tendae*	2032	YTQGCSGLCTIVICATVVICG
AmfS	*Streptomyces griseus*	?	QVSLLVCEYSSLSVVLCTP unknown structure
Prochlorosin[20]	*Prochlorococcus* MIT9313	-	In total, 29 putative *lanA* genes with homologous leaders; modified by one LanM enzyme (experimentally shown for 6 peptides; different ring structures)
Labyrinthopeptins (Labionin Containing Peptides; LabKC Modified)			
Labyrinthopeptin A1[25]	*Actinomadura namibiensis* DSM6313	?	DSNASVWECCSTGSWVPFTCC labionin (2 + 5 + 9; 11 + 14 + 19); disulfide bridge (10 + 20)
Labyrinthopeptin A3[25]		?	SNASVWECCSTGSWVPFTCC labionin (1 + 4 + 8; 10 + 13 + 18); disulfide bridge (9 + 19)
Labyrinthopeptin A2[25]		1923	SDWSLWECCSTGSLFACC labionin (1 + 4 + 8; 10 + 13 + 17); disulfide bridge (9 + 18)
Lantibiotic Peptides Without Classification /Unknown Structure and Biosynthesis			
Lactocin S	*Lactobacillus sake* L-45	3764	STPVLASVAVSMELLPTASVLYSDVAGCFKYSAKHHC D-Ala (7; 11; 19)
Pediocin PD-1[1]	*Pediococcus damnosus* NCFB 1832	2866	KKIKKSXSGDIXXLXXEXDHLAXXXXX only partially identified, no structure
Carnocin UI49	*Carnobacterium pisciola*	4635	GSEIQPR... only partially identified (N-terminus), no structure
Paenibacillin[16]	*Paenibacillus* spec.	2983	ASIIKTTIKVSKAVCKTLTCICTGSCSNCK predicted structure; *N*-acetylalanine (1)
unnamed	*Bifidobacterium longum* DJO10A	?	only prepeptide sequence available, cleavage site not identified
Venezuelin[11]	*Streptomyces venezuelae* ATCC 10712	?	TCECVGLLTLLNTVCIGISCA structure partially elucidated, cleavage site unknown
Related Peptides (Formerly Falsely Classified as Lantibiotics)			
Cypemycin[8]	*Streptomyces* spec.	2094	ATPATPTVAQFVIQGSTICLVC AviCys (19 + 22); **Linaridin**
Sublancin 168[27]	*Bacillus subtilis* 168	3876	GLGKAQCAALWLQCASGGTIGCGGGAVACQNYRQFCR disulfide bridge (7 + 36; 14 + 29); glycosylation (22) **S-linked glycopeptide**

[1]*Classification according to Refs. 36 and 3. Residues involved in Lan and MeLan bridge formation are highlighted by different shades of gray, and residues that are involved in one thioether amino acid are marked in the same shade of gray (1. 2. 3. 4. 5.). The positions (x) of additional modifications are underlined. Serine and threonine residues that are dehydrated to Dha and Dhb are designated in* **bold**.

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LanD enzymes catalyze the oxidation of the C-terminal Cys residue present in epidermin and its analogs and in mersacidin.[7,31] The labyrinthopeptins contain a novel modified amino acid, labionin, a quaternary α,α-disubstituted amino acid and are modified in a yet different biosynthetic mechanism that involves a GTP-dependent phosphorylation step by the LabKC enzyme.[24,25] Because an antibiotic action has not yet been reported and the LabKC enzyme displays similarities to RamC, which modifies the type III lantibiotic SapB, these peptides are grouped with the type III lantibiotics. A further novel thioether biosynthesis pathway, catalyzed by the LanL enzyme, was described for venezuelin.[12]

PROCESSING

After posttranslational modification, lantibiotics become activated by removal of the leader peptide segment. Genetic determinants for dedicated proteases are present in most gene clusters.

LanP proteases are associated with the LanBC modification system of type I lantibiotics and show sequence similarity to subtilisin-like serine proteases (reviewed in Refs. 3, 7). Peptides that are processed by such LanP proteases have leader sequences with a number of charged residues and relatively conserved motifs at position −15 and −20 (FNLD) and at the cleavage site. NisP and EpiP are synthesized as preproproteins, that is, with a signal peptide for export and a prosequence, which possibly functions as an intracellular chaperone. NisP possesses, in addition, a C-terminal extension that might act as membrane anchor to attach the enzyme to the cytoplasmic membrane; EpiP lacks the C-terminal anchor. No details are known for MutP proteins (from mutacins I and III) that have strong similarity to NisP and EpiP. PepP, ElkP, and LasP seem to be located in the cytoplasm.[7]

Type II lantibiotic peptides resulting from LanM biosynthesis machineries, such as mersacidin, lacticin 481 (and analogs), and many two-peptide lantibiotics such as lacticin 3147 A1/A2 are processed concomitantly with export by bifunctional ABC transporters LanT.[7] Such LanT transporters possess an N-terminal extension that contains a cysteine protease domain. The cleavage site of these peptides is usually preceded by a "double-Gly" motif, referring to conserved GG (GA or GS) residues in positions −1 and −2 (Fig. 1); bifunctional transporters are also found with many nonlantibiotic bacteriocins.[15]

RECEPTORS

The antimicrobial activity of lantibiotics does not involve receptors *sensu strictu*, although specific targets have been identified (see Biological Activities). However, lantibiotic biosynthesis may be autoregulated through a quorum-sensing process, in which the respective lantibiotic acts as an upregulating signal. In this case, the lantibiotic binds to a receptor that is the histidine kinase of a dedicated two-component regulatory system (LanRK).[3]

PRODUCER SELF-PROTECTION

Lantibiotic producers always contain peptides or proteins that confer producer self-protection or "immunity." Usually, ABC transporters (LanEFG), single proteins or peptides (LanI), or accessory proteins are involved (LanH). For a recent review, see Ref. 26.

INFORMATION ON ACTIVE AND/OR SOLUTION CONFORMATION

The antibiotic activity is strictly associated with the fully modified lantibiotic, that is, lanthionine ring structures and dehydroresidues provide structural features that are essential for activity. The distinction between the two groups of antibacterial peptides, type I and II lantibiotics, is also reflected in their overall structures.

Typical type I lantibiotics such as nisin, subtilin, gallidermin, and Pep5 are elongated and flexible in solution. Defined conformations can only be found within or close to the rings. In membrane mimicking solvents, these peptides adopt rod-shaped structures with pronounced amphiphilicity.[7]

The type II lantibiotics adopt a more globular conformation than type I lantibiotics, and they possess much less conformational freedom than the type I peptides. Cinnamycin, duramycins, and ancovenin have an intertwined bridging pattern including a head-to-tail thioether. Studies on the conformation of cinnamycin and duramycins in water and water:acetonitrile (1:1) have shown that these peptides present a U-shaped topology, with the hydrophobic residues clustered in the U-bend area of the molecule and the hydrophilic side chains located in the terminal segments.[7]

The solution conformations of the type II lantibiotics mersacidin and actagardine were determined in methanol and acetonitrile:water (7:3), respectively. Both peptides adopt an overall globular structure with neutral side chains pointing outwards. The hydrophilic residue Glu, which belongs to a highly conserved motif in mersacidin, actagardine and many of the α-peptides of the two-peptide lantibiotics, is directed to the center of the molecule. Mersacidin is the only lantibiotic for which a crystal structure is available.[30]

As shown for the solution structure of plantaricin C, the type II lantibiotics of the lacticin 481 group[9] share features from both groups. The first six N-terminal residues, four of which are lysines, are structurally undefined; the remaining part of the molecule is globular because of a bridge connecting residues 7 and 27 and the presence of three internal rings.[32]

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BIOLOGICAL ACTIVITIES

Most lantibiotics are active against Gram-positive bacteria and exert multiple activities at the cytoplasmic membrane.[3,7] Resistance of Gram-negative bacteria results from the protective effect of the outer membrane that is impenetrable to these peptides. Microbisporicin is a particularly active agent against pathogenic Gram-positive bacteria (methicillin-resistant staphylococci and enterococci).[6]

Numerous studies performed with intact bacterial cells, membrane vesicles, and artificial liposomes have shown that nisin and other type-A lantibiotics in μM concentrations are able to destabilize membranes by forming nonselective, transient pores, which leads to dissipation of the membrane potential, rapid efflux of small metabolites, and cessation of cellular biosynthetic processes (reviewed in Ref. 3).

The membrane-bound peptidoglycan precursor lipid II was identified as a specific target for many lantibiotics. These substances belong to the nisin group (nisin and subtilin), the epidermin group (epidermin, gallidermin, mutacin 1140, and clausin[5]), or the lacticin 481-group (lacticin 481[4] and plantaricin C), the mersacidin group (mersacidin and actagardine) and the α-peptides of the two-peptide lantibiotics (lacticin 3147). Binding of lipid II blocks the precursor from incorporation into the bacterial cell wall. Although this seems to be the only bactericidal activity of mersacidin, the nisin-type lantibiotics and the two-component lantibiotic lacticin 3147 combine this activity with a unique, target-mediated pore formation.[3] Such a combination of two killing mechanisms may explain the potent *in vivo* activity (minimal inhibitory concentration in the nanometer range) of nisin and related lantibiotics.

The molecular basis of the interaction between nisin and lipid II was intensively investigated. Genetically engineered nisin variants and NMR spectroscopy identified structural features of nisin, which are important for its biological activities. The first two rings in the N-terminal part of the peptide are responsible for interaction with lipid II, whereas the C-terminal and the central flexible hinge region are essential for pore formation. Obviously, the pyrophosphate moiety of the lipid II molecule contributes significantly to binding nisin. The N-terminus of nisin was found to form a cage-like structure, which allows the formation of five intermolecular hydrogen bonds between the backbone amides of nisin and the pyrophosphate linkage group of lipid II.[18] These structural elements are also found in a complex of nisin and undecaprenylpyrophosphate; however, for target-mediated pore formation, nisin seems to need a second docking site on the Mur-NAc-pentapeptide moiety present in lipids I and II. Eight nisin molecules and four lipid II molecules are intrinsic parts of the pore.[13] Furthermore, nisin is able to sequester lipid II from the division septum and form large nisin–lipid II aggregates in the membranes.[14]

Mersacidin and actagardine also bind to lipid II and thus inhibit the cell wall biosynthesis at the level of transglycosylation. It was shown that the binding site of these peptides differs from that of the glycopeptide antibiotic vancomycin. NMR studies of the interaction of lipid II and mersacidin identified a hinge region between residues 12 and 13, which serves in opening and closing of the adjacent rings during lipid II binding. Moreover, Glu-17 is indispensable for antibiotic activity.[17] The two-peptide lantibiotic lacticin 3147 and its relatives like haloduracin seem to be interesting cases in which the two functions, lipid II binding and pore formation, are attributed to two individual peptides, rather than being combined in one molecule.[3,28]

In both cases, nisin and mersacidin, the structural elements that are predicted to be directly involved in the interaction with lipid II, that is, the first two rings in nisin and the intertwined rings in mersacidin with the conserved negative charge are also found in many other peptides. In Table 1, lantibiotics were grouped according to their ring patterns. It seems probable that peptides that possess these conserved structural features exert the same mechanism of action as the respective prototype lantibiotics, at least regarding their interactions with lipid II.

The cinnamycin-like lantibiotics display bactericidal activity against a few bacterial strains, in particular *Bacillus* strains. The basis for the antibiotic activity seems to be specific binding of phosphoethanolamine, which causes increased membrane permeability, impaired ATP-dependent protein translocation and calcium uptake, and phospholipase inhibition (reviewed in Ref. 3).

The structure–function relationships of several lantibiotics have been addressed by extensive engineering of site-directed mutants, and these investigations have recently been systematically reviewed.[29]

The lanthionine-containing peptides of the group III lantibiotics probably have non-antibiotic functions, and the designation lantipeptides has been proposed.[11] For example, SapB does not display antibacterial properties but functions as biological surfactant. It is important for aerial mycelium formation by the filamentous bacterium *Streptomyces coelicolor*. The functions of other lantipeptides, for example, from the cyanobacterium *Prochlorococcus*, are still unknown.[20] Cytolysin is another two-peptide lantibiotic produced by some enterococci, has cytotoxic activity, and has been shown to represent a significant virulence factor in clinical cases of enterococcal infections.

ACKNOWLEDGMENTS

The authors receive support from the German Research Foundation (DFG, various projects).

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REFERENCES

1. Bauer R, Chikindas ML, Dicks LM. Purification, partial amino acid sequence and mode of action of pediocin PD-1, a bacteriocin produced by *Pediococcus damnosus* NCFB 1832. *Int J Food Microbiol* 2005;**101**(1):17–27.
2. Begley M, Cotter PD, Hill C, Ross RP. Identification of a novel two-peptide lantibiotic, lichenicidin, following rational genome mining for LanM proteins. *Appl Environ Microbiol* 2009;**75**(17):5451–60.
3. Bierbaum G, Sahl HG. Lantibiotics: mode of action, biosynthesis and bioengineering. *Curr Pharm Biotechnol* 2009;**10**(1):2–18.
4. Böttiger T, Schneider T, Martinez B, Sahl HG, Wiedemann I. Influence of Ca(2+) ions on the activity of lantibiotics containing a mersacidin-like lipid II binding motif. *Appl Environ Microbiol* 2009;**75**(13):4427–34.
5. Bouhss A, Al Dabbagh B, Vincent M, Odaert B, Aumont-Nicaise M, Bressolier P, et al. Specific interactions of clausin, a new lantibiotic, with lipid precursors of the bacterial cell wall. *Biophys J* 2009;**97**(5):1390–7.
6. Castiglione F, Lazzarini A, Carrano L, Corti E, Ciciliato I, Gastaldo L, et al. Determining the structure and mode of action of microbisporicin, a potent lantibiotic active against multiresistant pathogens. *Chem Biol* 2008;**15**(1):22–31.
7. Chatterjee C, Paul M, Xie L, van der Donk WA. Biosynthesis and mode of action of lantibiotics. *Chem Rev* 2005;**105**(2):633–84.
8. Claesen J, Bibb M. Genome mining and genetic analysis of cypemycin biosynthesis reveal an unusual class of posttranslationally modified peptides. *Proc Natl Acad Sci U S A* 2010;**107**(37):16297–302.
9. Dufour A, Hindre T, Haras D, Le Pennec JP. The biology of lantibiotics from the lacticin 481 group is coming of age. *FEMS Microbiol Rev* 2007;**31**(2):134–67.
10. Fuchs SW, Jaskolla TW, Bochmann S, Kotter P, Wichelhaus T, Karas M, et al. Entianin, a novel subtilin-like lantibiotic from *Bacillus subtilis* subsp. *spizizenii* DSM 15029T with high antimicrobial activity. *Appl Environ Microbiol* 2011;**77**(5):1698–707.
11. Goto Y, Li B, Claesen J, Shi Y, Bibb MJ, van der Donk WA. Discovery of unique lanthionine synthetases reveals new mechanistic and evolutionary insights. *PLoS Biol* 2010;**8**(3):e1000339.
12. Goto Y, Okesli A, van der Donk WA. Mechanistic studies of Ser/Thr dehydration catalyzed by a member of the LanL lanthionine synthetase family. *Biochemistry* 2011;**50**(5):891–8.
13. Hasper HE, de Kruijff B, Breukink E. Assembly and stability of nisin-lipid II pores. *Biochemistry* 2004;**43**(36):11567–75.
14. Hasper HE, Kramer NE, Smith JL, Hillman JD, Zachariah C, Kuipers OP, et al. An alternative bactericidal mechanism of action for lantibiotic peptides that target lipid II. *Science* 2006;**313**(5793):1636–7.
15. Havarstein LS, Diep DB, Nes IF. A family of bacteriocin ABC transporters carry out proteolytic processing of their substrates concomitant with export. *Mol Microbiol* 1995;**16**(2):229–40.
16. He Z, Kisla D, Zhang L, Yuan C, Green-Church KB, Yousef AE. Isolation and identification of a *Paenibacillus polymyxa* strain that coproduces a novel lantibiotic and polymyxin. *Appl Environ Microbiol* 2007;**73**(1):168–78.
17. Hsu ST, Breukink E, Bierbaum G, Sahl HG, de Kruijff B, Kaptein R, et al. NMR study of mersacidin and lipid II interaction in dodecylphosphocholine micelles. Conformational changes are a key to antimicrobial activity. *J Biol Chem* 2003;**278**(15):13110–7.
18. Hsu ST, Breukink E, Tischenko E, Lutters MA, de Kruijff B, Kaptein R, et al. The nisin-lipid II complex reveals a pyrophosphate cage that provides a blueprint for novel antibiotics. *Nat Struct Mol Biol* 2004;**11**(10):963–7.
19. Kabuki T, Uenishi H, Seto Y, Yoshioka T, Nakajima H. A unique lantibiotic, thermophilin 1277, containing a disulfide bridge and two thioether bridges. *J Appl Microbiol* 2009;**106**(3):853–62.
20. Li B, Sher D, Kelly L, Shi Y, Huang K, Knerr PJ, et al. Catalytic promiscuity in the biosynthesis of cyclic peptide secondary metabolites in planktonic marine cyanobacteria. *Proc Natl Acad Sci U S A* 2010;**107**(23):10430–5.
21. Majchrzykiewicz JA, Lubelski J, Moll GN, Kuipers A, Bijlsma JJ, Kuipers OP, et al. Production of a class II two-component lantibiotic of *Streptococcus pneumoniae* using the class I nisin synthetic machinery and leader sequence. *Antimicrob Agents Chemother* 2010;**54**(4):1498–505.
22. Marsh AJ, O'Sullivan O, Ross RP, Cotter PD, Hill C. In silico analysis highlights the frequency and diversity of type 1 lantibiotic gene clusters in genome sequenced bacteria. *BMC Genomics* 2010;**11**:679.
23. McClerren AL, Cooper LE, Quan C, Thomas PM, Kelleher NL, van der Donk WA. Discovery and in vitro biosynthesis of haloduracin, a two-component lantibiotic. *Proc Natl Acad Sci U S A* 2006;**103**(46):17243–8.
24. Müller WM, Ensle P, Krawczyk B, Süssmuth RD. Leader peptide-directed processing of labyrinthopeptin A2 precursor peptide by the modifying enzyme LabKC. *Biochemistry* 2011:[epub ahead of print].
25. Müller WM, Schmiederer T, Ensle P, Süssmuth RD. In vitro biosynthesis of the prepeptide of type-III lantibiotic labyrinthopeptin A2 including formation of a C–C bond as a post-translational modification. *Angew Chem Int Ed Engl* 2010;**49**(13):2436–40.
26. Okuda K, Sonomoto K. Structural and functional diversity of lantibiotic immunity proteins. *Curr Pharm Biotechnol* 2011;**12**(8):1231–9.
27. Oman TJ, Boettcher JM, Wang H, Okalibe XN, van der Donk WA. Sublancin is not a lantibiotic but an S-linked glycopeptide. *Nat Chem Biol* 2011;**7**(2):78–80.
28. Oman TJ, van der Donk WA. Insights into the mode of action of the two-peptide lantibiotic haloduracin. *ACS Chem Biol* 2009;**4**(10):865–74.
29. Ross AC, Vederas JC. Fundamental functionality: recent developments in understanding the structure–activity relationships of lantibiotic peptides. *J Antibiot (Tokyo)* 2011;**64**(1):27–34.
30. Schneider TR, Kärcher J, Pohl E, Lubini P, Sheldrick GM. Ab initio structure determination of the lantibiotic mersacidin. *Acta Crystallogr D Biol Crystallogr* 2000;**56**(Pt 6):705–13.
31. Sit CS, Yoganathan S, Vederas JC. Biosynthesis of aminovinyl-cycteine-containing peptides and its application in the production of potential drug candidates. *Acc Chem Res* 2011;**44**(4):261–8.
32. Turner DL, Brennan L, Meyer HE, Lohaus C, Siethoff C, Costa HS, et al. Solution structure of plantaricin C, a novel lantibiotic. *Eur J Biochem* 1999;**264**(3):833–9.
33. Wescombe PA, Upton M, Renault P, Wirawan RE, Power D, Burton JP, et al. Salivaricin 9, a new lantibiotic produced by. *Streptococcus Salivarius Microbiol* 2011;**157**(Pt 5):1290–9.
34. Wilaipun P, Zendo T, Okuda K, Nakayama J, Sonomoto K. Identification of the nukacin KQU-131, a new type-A(II) lantibiotic produced by *Staphylococcus hominis* KQU-131 isolated from Thai fermented fish product (Pla-ra). *Biosci Biotechnol Biochem* 2008;**72**(8):2232–5.
35. Willey JM, van der Donk WA. Lantibiotics: peptides of diverse structure and function. *Annu Rev Microbiol* 2007;**61**:477–501.

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Chapter 20

Microcins

Sylvie Rebuffat

ABSTRACT

Microcins are gene-encoded antimicrobial peptides (molecular weights <10 kDa) produced by Gram-negative bacteria, essentially Enterobacteria. They belong to a large class of bacteriocins and are involved in microbial competition. Microcins are highly stable peptides that display heterogeneous structures arising from various and complex posttranslational modifications acquired through specific enzyme machineries. These modifications include heterocycles (thiazole, oxazole), and nucleotide, or siderophore entities that contribute to the potent activity exerted by microcins toward bacterial species generally closely related to the producing bacteria. Most microcins use subtle Trojan horse strategies to kill sensitive bacteria: they either hijack iron siderophore receptors to enter cells or require processing when inside target bacteria to generate the toxic species. Subsequently, some inhibit strategic intracellular enzymes and functions (DNA gyrase, RNA polymerase, ATP-synthase, mRNA translation); others act at the cytoplasmic membrane level. Therefore, microcins provide fascinating models for novel antimicrobial strategies that evade common resistance mechanisms.

DISCOVERY AND CLASSIFICATION

Most bacteria and archaea produce an arsenal of toxic peptides and proteins, which are major actors of microbial competition. They are called bacteriocins, a term first introduced by François Jacob in the 1950s and further used to define proteinaceous compounds of bacterial origin that are lethal to bacteria differing from the producing strain. This term is today currently applied to antimicrobial peptides, polypeptides, and proteins that are ribosomally synthesized by bacteria, have most often a narrow spectrum of antibacterial activity, and against which the producing strains are protected by a dedicated immunity system. In the literature, the term bacteriocin very often applies to antibacterial peptides from Gram-positive bacteria and particularly lactic acid bacteria, but the first bacteriocin to be discovered was produced by the Gram-negative bacterium *Escherichia coli* and was a microcin.[25] Microcins are different from colicins, which are large proteins (between 30 and 80 kDa), in their lower molecular masses (<10 kDa) and especial resistance to proteases, extreme pH, and temperature.[1] They are essentially hydrophobic and form a very restricted, but highly heterogeneous, class of bacteriocins, with only 14 representatives identified to date,[6] among which only 8 have been isolated and structurally characterized (Figure 1). Because of this restricted number of representatives, classifying microcins appears difficult in contrast with bacteriocins from Gram-positive bacteria and with colicins, where identification of a larger number of representatives sharing common structural features and mechanisms of action helps in identifying classes.[25] Most microcins are plasmid-encoded peptides. They arise from biosynthetic pathways that may include the acquisition of complex and diverse posttranslational modifications, exhibit a high diversity in structures and mechanisms of action, and display various mechanisms of uptake. Therefore, the classification adopted presently, which divides microcins into two classes (Figure 1), takes into account these criteria.[6,25] Class I encompasses three plasmid-encoded peptides with molecular mass <5 kDa that have extensive backbone posttranslational modification: microcins B17, C (also called C7, C51 or C7/C51), and J25.[6,30] More precisely, microcin B17 is a 43-amino-acid peptide that carries four thiazole and four oxazole heterocycles, which arise from the modification of six glycine, four serine, and four cysteine residues among the 69 amino acids that form the microcin precursor. Microcin C is a nucleotide-peptide. It is an N-formylated heptapeptide that is covalently linked to a modified adenosine monophosphate by its C-terminal aspartate through an N-acyl phosphoramidate linkage; a propylamine is attached to the phosphate group, thus modifying adenosine. Microcin J25 is a 21 amino acid lasso peptide. It consists of a macrolactam ring closed between the N-terminal amino group and the side-chain carboxylate of an aspartate at position 8 through which the tail is threaded and trapped by steric interactions, thus forming a lasso. Class II contains higher molecular mass peptides, in the 5- to 10-kDa range and is itself further subdivided into two subclasses, IIa and IIb. (Figure 1). Class IIa consists of three plasmid-encoded peptides,

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00020-8
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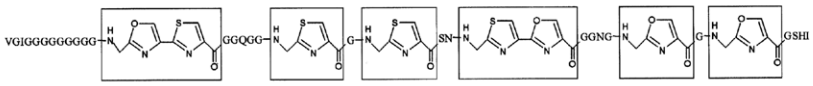
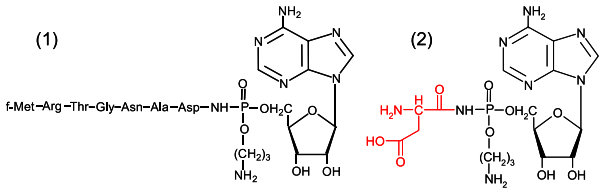
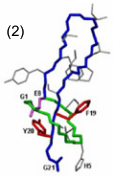
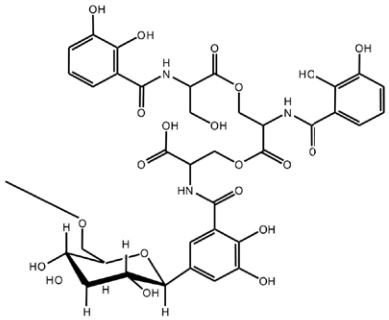
Microcin	Structure
Class I	
B17 (3093 Da)	
C (1176 Da) (C7-C51) (1) mature microcin (2) toxic entity generated by proteolytic cleavage in target cells	
J25 (2107 Da) (1) primary structure; (2) three-dimensional structure (PDB: 1Q71)	(1) G G A G H V P E Y F V G I G T P I S F Y G 
Class II	
Class IIa	
V (8734 Da)	ASGRDIAMAI GTLSGQFVAG GIGAAAGGVA GGAIYDYAST HKPNPAMSPS GLGGTIKQKP EGIPSEAWNY AAGRLCNWSP NNLSDVCL
L (8884 Da)	GDVNWVDVGK TVATNGAGVI GGAFGAGLGC PVCAGAFAVG SSAAVAALYD AAGNSNSAKQ KPEGLPPEAW NYAEGRMCNW SPNNLSDVCL
Class IIb	
E492 (7887 Da)	GETDPNTQLL NDLGNNMAWG AALGAPGGLG SAALGAAGGA LQTVGQGLID HGPVNVPIPV LIGPSWNGSG SGYNSATSSS GSGS
M (7283 Da)	DGNDGQAELI AIGSLAGTFI SPGFGSIAGA YIGDKVHSWA TTATVSPSMS PSGIGLSSQF GSGRGTSSAS SSAGSGS
H47 (4864 Da)	GGAPATSANA AGAAAIVGAL AGIPGGPLGV VVGAVSAGLT TAIGSTVGSG SASSSAGGGS
	 Post-translational modification linked to the C-terminal Ser of class IIb microcins

FIGURE 1 Structures of isolated and characterized microcins of classes I and II. Important residues are highlighted in gray; disulfide bonds are indicated by bars connecting the cysteines concerned.

microcins L, V,[6] and N (previously named microcin 24)[4] that are devoid of posttranslational modifications and have two, one, and no disulfide bond(s), respectively. Class IIb includes those chromosome-encoded linear microcins that carry a C-terminal siderophore posttranslational modification, thus forming a small family of related peptides: microcins E492, M, H47, and presumably I47 and G492, which have been hypothesized through genome analyses but have not been isolated and characterized to date.[21,34] The structure of microcin E492 has been

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examined in detail. It consists of an 84-amino-acid peptide that can be either unmodified as primarily described,[23] or modified as a siderophore peptide.[32] Indeed, the modified microcin E492 carries a C-glucosylated linear trimer of N-2,3-(dihydroxybenzoyl)-L-serine (DHBS) linked to the C-terminal serine carboxylate through an O-glycosidic bond. With enterobactin (a cyclic trimer of DHBS) and salmochelin (the C-glycosylated counterpart of enterobactin), DHBS is one of the major catechol siderophores used by Gram-negative bacteria to bind iron and import it into cells via high affinity receptors. The modified form of microcin E492 that carries a salmochelin-like siderophore was the first natural siderophore peptide to be isolated.[32] It is the mature form of the microcin that displays improved activity.[33,17,18]

Therefore, because of their diversity of structures and mechanisms of action, microcins appear as attractive models for the design of novel bioactive peptides with antiinfective or antitumor properties.[5,6,12,22,26]

GENETIC SYSTEMS AND BIOSYNTHESIS

Although they share with colicins a common gene cluster organization, microcins have more complex genetic systems that include several genes encoding auxiliary proteins or modification enzymes, and in this regard are more similar to bacteriocins from Gram-positive bacteria. All microcins are synthesized by ribosomal machinery and share a common maturation process. They are translated from their respective mRNAs as precursor peptides containing an N-terminal leader peptide extension and a core peptide, which eventually undergoes more or less complex posttranslational modifications before becoming the mature active microcin.

Microcins are encoded by gene clusters, generally carried by plasmids and in certain cases (microcins of class IIb) by the chromosome. These clusters include a variable number of genes, but show a conserved organization (Figure 2).[6,30] The minimal structure of the gene clusters consists of (i) a structural gene encoding the precursor of the microcin, (ii) the self-immunity gene, which is generally adjacent to the former and encodes the factors that protect the producing strain against its own toxic peptide (immunity protein or/and system connected to the export system), and (iii) genes encoding the microcin export system required for secretion of the antimicrobial peptide outside the cells. A variable number of genes coding for posttranslational modification enzymes are found additionally for modified microcins (Figure 2). Gene clusters encoding unmodified class IIa microcins only contain four genes among which two are involved in export. By contrast, the siderophore microcins (class IIb), which are chromosomally encoded, exhibit very complex gene clusters that contain at least ten genes, as exemplified by microcin E492 (*mceA* to *mceJ*), *mceC*, *D*, *I*, and *J* being involved in the acquisition of the siderophore posttranslational modification (Figure 2). For class I, production of, immunity to, and export of microcin B17 require seven genes forming an operon: *mcbA*, *mcbB*, *mcbC*, *mcbD*, *mcbE*, *mcbF*, and *mcbG*. The genes *mcbC*, *D*, *E* encode the three components of the microcin B17 synthetase that provide the posttranslational modifications, that is, the thiazole and oxazole rings. The main cluster of genes encoding microcin C (*mccABCDE*) allows acquisition of the nucleotide and propylamine parts linked to the heptapeptide via the phosporamidate bond, while the structural gene *mccA* that encodes the 7-amino-acid precursor, is one of the shortest genes known (24 bp).[9] By contrast, microcin J25 production only requires four genes *mcjA* (structural gene), *mcjB, mcjC* (modification enzymes), and *mcjD* (export), although the precursor must undergo cleavage of the leader and cyclization to the macrolactam ring, together with adequate folding to enable adoption of the unique threaded lasso structure.

All microcins are produced as inactive precursor peptides endowed with leader peptide extensions of various sizes and sequences that are located N-terminal to the mature peptide region. Leader peptides of class II microcins are approximately 15 amino acids in length, whereas posttranslationally processed microcins of class I have longer leaders of 19–37 amino acids (58 for microcin J25, 69 for microcin B17).[6,30] Microcin C does not possess a leader peptide and is the only one to be secreted without cleavage of a precursor. Similarly to bacteriocins from Gram-positive bacteria, cleavage of the microcin leaders most often occurs at a specific cleavage site. Class II microcin leader peptides contain a conserved sequence MRXI/LX(9)GG/A (X = any amino acid) that ends with the typical double-glycine motif or glycine-alanine motif that is found in proteins exported through ABC (ATP-binding cassette) transporters as an alternative to the GG motif.[6] Leader peptides of class I microcins do not display common features, either in length or in amino acid sequence. The roles of the leaders have not been clarified to date for microcins and bacteriocins, and differ for the different classes and subclasses of peptides considered. However, they probably contribute to the maturation, the secretion, the protection of the precursor against degradation, or to keeping the precursor inactive inside the host cells during biosynthesis.[6,19]

Export of the mature microcins generally occurs on cleavage of the leader peptides. Similar to bacteriocins from Gram-positive bacteria, secretion of class II microcins into the extracellular medium uses ABC transporters,[26] which cleave the typical GG-containing leader sequences of the precursors, concomitantly with translocation of the microcins across the membrane. The machineries in charge of the secretion of these microcins have a canonical organization consisting of three components, an ABC transporter and its

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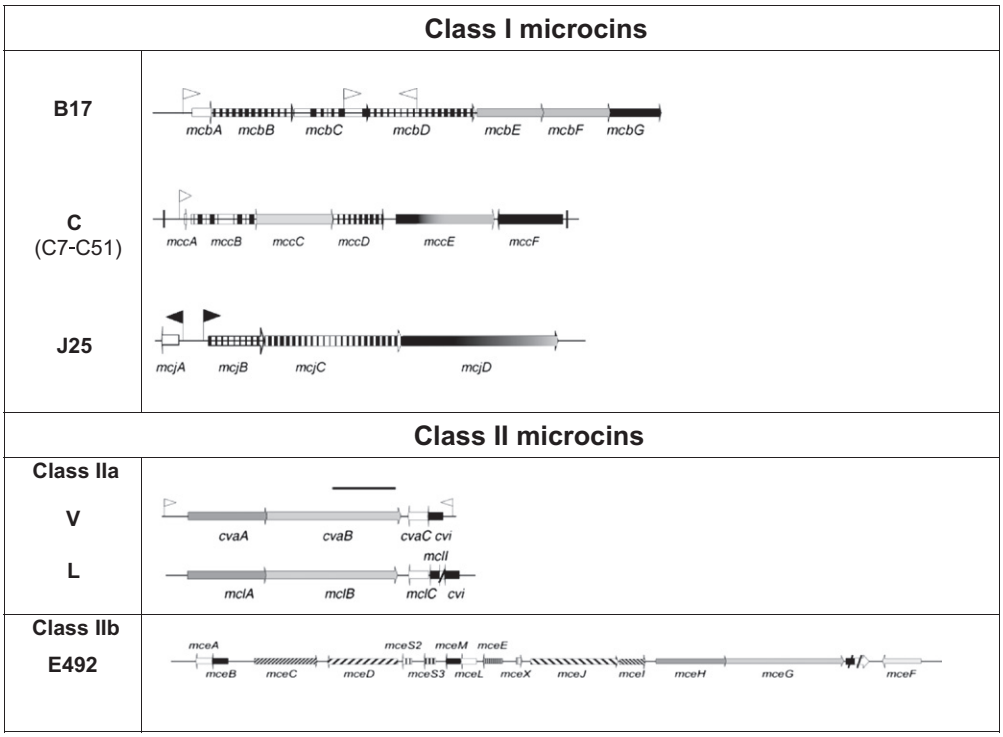

FIGURE 2 Typical gene clusters characterizing microcins of classes I and II. Genes encoding the microcin precursors are indicated in white; those required for self-immunity, export and for both immunity and export are labeled in black, gray and shaded tones, respectively; those required for post-translational modifications are labeled in vertical hatching and in right- and left-inclined hatching for class I and class II microcins, respectively.

accessory protein, both of which are encoded by the microcin gene cluster, and the protein TolC, which is located at the outer membrane and is encoded outside the microcin gene cluster.[34] There is no standard export machinery for class I microcins. They use either ABC transporter-like systems (microcins B17 and J25) or efflux pumps (microcin C), which are encoded by their genetic systems, in association with outer membrane components. Before proteolytic cleavage, some precursors are posttranslationally modified, particularly the class I and IIb microcins.

Most results on microcin B17 maturation were published in the 1990s, coincident with the isolation of the McbBCD enzyme complex[12] and reviewed in two articles published in 2007.[6,30] Microcin B17 was the first microcin to be reconstituted *in vitro* from its precursor McbA in the presence of the modification enzymes McbB, McbC, and McbD that form the microcin B17 synthetase complex.[12] The oxazole and thiazole heterocycles that take part in the final structure of the active microcin result from a series of cyclization, dehydration, and dehydrogenation reactions occurring from Gly–Ser (oxazoles) and Gly–Cys (thiazoles) dipeptides or Gly–Cys–Ser (thiazole-oxazole) and Gly–Ser–Cys (oxazole-thiazole) tripeptides displayed in the sequence of the 69 amino-acid precursor McbA. The McbBCD synthetase recognizes the leader peptide extension that has a helical structure. On removal of the N-terminal 26 amino acid leader peptide by the dedicated ABC transporter McbEF, mature microcin B17 is then exported outside the cells. Severinov's group showed that a major portion of the posttranslationally modified mature microcin contains an additional unusual modification, a backbone ester bond connecting residues Cys51 and Ser52.[8] The formation of the depsipeptide bond very probably results from an N–O acyl rearrangement that occurs during cyclization at Ser52 located immediately C-terminal to one of the thiazole rings. It is postulated that this novel modified microcin is therefore possibly an intermediate form in the biosynthesis pathway of microcin B17.

The maturation process of microcin C is particularly original in the sense that the last cleavage step leading to the toxic entity does not occur in producing cells, but in target cells.[14] Before this last processing step, three modifications of the precursor MccA are required to link the modified nucleotide to the heptapeptide: (i) conversion of Asn7 to a derivatized isoAsn7, (ii) formation of an N–P bond between the amide group of isoAsn7 and Pα of ATP, facilitating the peptidyl–AMP linkage via a phosphoramidate bond, which is ensured by a specific enzyme MccB encoded by the microcin gene cluster *mccABCDE*,[28] and (iii) aminopropylation of the phosphoramidate by two enzymes encoded by genes *mccD* and *mccE*. The aminopropyl chain increases activity several fold, indicating

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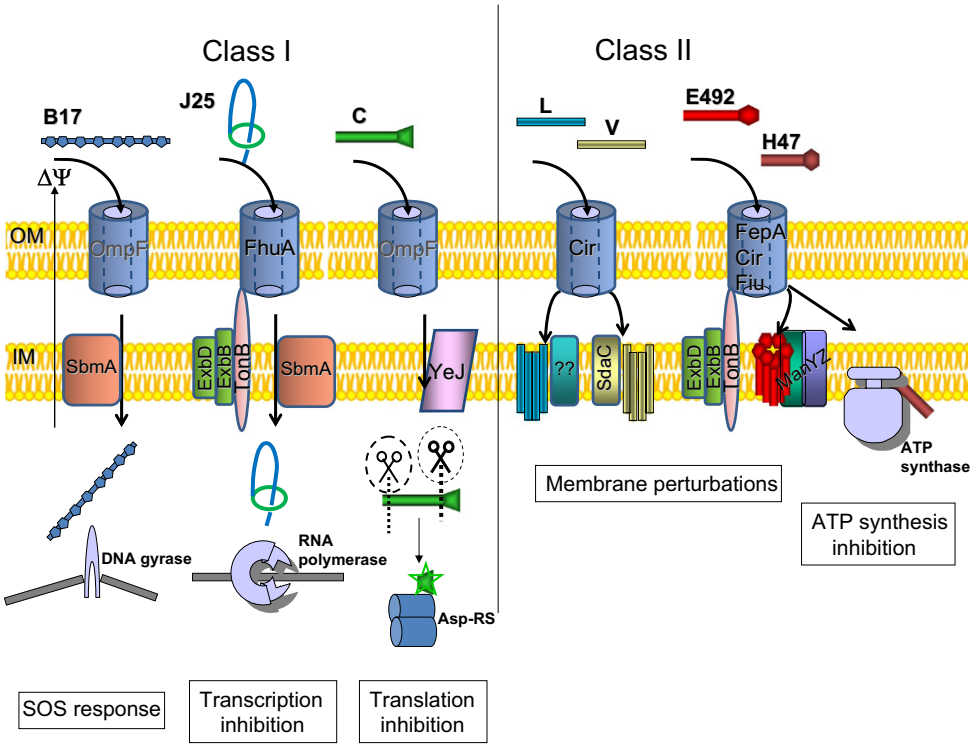

FIGURE 3 Synthetic scheme of the mechanisms of action of microcins of classes I and II. See color plate 12.

its importance for antibacterial activity.[31] The modified precursor itself (i.e., the N-formylated heptapeptide nucleotide) is exported out of the producing cells and enters into the target bacteria using OmpF and Yej. The final maturation steps required for MccC activity (i.e., deformylation and cleavage of the peptide part) happen in target cells[31] (see "Receptors and Biological Activity" and Figure 3).

Microcin J25 is the archetype of lasso peptides. Lasso peptides form a growing class of bioactive peptides synthesized by bacteria that differ most often from enterobacteria, and particularly by actinomyocetes.[10,24,29] They have attracted considerable interest based on the high stability of the fascinating lasso structure, which can be used as a template for peptide bioengineering,[11] as well as the biological activities of known lasso peptides that inhibit enzymes or antagonize receptors.[24] In microcin J25, the amino group of Gly1 is linked to the side-chain carboxylate of Glu8 by a lactam bond, forming an N-terminal ring that is threaded by the C-terminal tail, thus forming the lasso (Figure 1). This particular structure is maintained by the two bulky side chains of two aromatic residues at the end of the tail, Phe19 and Tyr20 that straddle the ring and prevent the tail escaping from the ring. With microcin B17,[12] microcin J25 is the sole microcin that can be produced by *in vitro* reconstitution of an enzyme complex capable of modifying an exogenously supplied precursor peptide.[7] This pioneering work in the connected fields of microcins and lasso peptides showed that the two proteins McjB and McjC encoded in the *mcjABCD* gene cluster are the two enzymes that control the acquisition of the lasso structure from the 58 amino acid precursor. Only the presence of ATP and Mg^{2+} is additionally required to produce the mature and active lasso microcin.[7] Following hypotheses arising from gene sequence comparisons,[7,30,31] recent evidence obtained in my laboratory (Yan K-P, et al. *ChemBioChem* 2012) indicates that McjB is a cysteine protease that cleaves off the leader peptide, whereas McjC is a lactam synthetase responsible for the formation of the amide bond between the glycine at position 1 generated upon proteolytic cleavage of the leader and the Glu8 side chain. McjB and McjC are two interdependent enzymes that mutually need the physical presence of their partner to fulfil their enzymatic function, probably acting as a protein complex. Moreover, the leader peptide with a basic character plays a role in the maturation process, being implicated in the recognition of the precursor McjA by the maturation machinery.

Microcin E492 is the most studied siderophore microcin. The modification anchored at its C-terminal serine is a C-glucosylated linear trimer of DHBS, which is a linear breakdown product of the cyclic catechol-type siderophore enterobactin. Biosynthetic pathways of siderophore microcins (E492, M, H47, and presumably I47 and G492) are dependent on the enterobactin and salmochelin synthesis pathways.[17,34] These pathways use nonribosomal peptide synthetases (NRPS), and their initial steps involve

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the shikimate pathway that starts from phosphoenol pyruvate and erythrose-4-phosphate. Four genes *mceCDIJ* in the siderophore-microcin gene clusters are required for modifying the peptide at its C-terminal serine. For microcin E492, proteins MceC and MceD ensure enterobactin glucosylation and linearization,[17] whereas MceI and MceJ are responsible for the formation of the O-glucosidic bond between the glucosylated enterobactin and the C-terminal serine of the microcin.[18]

Thus, it seems that the elucidation of microcin biosynthetic pathways points to specific, and in some cases novel, bacterial enzymes that could be either targets for antibiotics or tools for peptide bioengineering.

IMMUNITY OF PRODUCING BACTERIA TO MICROCINS

Contrary to Gram-positive bacteriocins for which several three-dimensional structures of immunity proteins have been determined, assisting in the elucidation of their immunity mechanisms, no immunity protein from a microcin-producing bacterium has been characterized. Although the mechanisms by which microcinogenic strains acquire self-immunity to their own microcin remain largely unexplained, it seems that at least one resistance-conferring gene is associated with the production of a given microcin. Immunity to class I microcins principally involves efflux pumps. Indeed, self-immunity to microcin B17 involves three genes in the microcin gene cluster. However, only strains harboring the three *mcbEFG* genes are fully resistant to the toxic peptide, while those having only two of these genes are only partly resistant to the microcin.[6] In addition to the McbEF export pump, the protein McbG, which is a member of the growing family of pentapeptide repeat proteins, contributes to the immunity of the producing cells to microcin B17.[31] Similarly, self-immunity of the producing strains to microcin C involves at least two proteins, MccC and E, the first one being an efflux pump. In addition, a third protein, MccF contributes to self-immunity of the producing strains.[30,31] The mechanism by which the producing strains are protected against microcin J25 also involves efflux pumps.[6,31] One, McjD, encoded by one of the genes of the microcin biosynthesis cluster, is also involved in the export of the microcin out of the producing bacteria. The other, YojI, is an ABC transporter. Both McjD and YojI require TolC to export microcin J25. By contrast and more similarly to Gram-positive bacteriocins, immunity to class II microcins involves dedicated peptides/polypeptides that range from 51 to 144 amino acids in length. Generally, these small proteins are membrane associated.[6] No three-dimensional structures of these microcin immunity proteins have been determined, and further studies are required to elucidate the mechanisms involved. They should be taken into account in the context of the elaboration of novel strategies against bacterial resistance.

RECEPTORS AND BIOLOGICAL ACTIVITIES

Similar to colicins, microcins have receptor-mediated mechanisms of antimicrobial activity. Such a mechanism is reflected in their low minimal inhibitory concentrations that are found in the nanomolar range, contrasting with antimicrobial peptides of eukaryotic origin that act through a direct interaction with phospholipid membrane bilayers and exert their activity in the micromolar range. It also explains the narrow spectrum of activity generally exhibited by microcins, which are essentially active against bacterial strains very similar to the producing Gram-negative bacteria. Thus, microcin J25 that is produced by *E. coli* mainly targets *Salmonella* sp., whereas microcin E492 produced by *Klebsiella pneumoniae* preferentially kills *Escherichia* sp. To improve uptake into sensitive bacteria, microcins hijack receptors involved in the uptake of essential nutrients, which therefore constitute an Achille's heel for the bacteria (Figure 3). Many microcins parasitize iron siderophore receptors, either of the catechol- (FepA, Cir, Fiu), in the case of microcins E492, H47, L, M, V, or the hydroxamate-type (FhuA) for microcin J25, whereas others (microcin B17 and C) use the porin OmpF. However, the affinity of the receptors for the microcins varies, as microcins E492, M, and H47 are recognized essentially by FepA and also to a lesser extent by Cir and Fiu, whereas microcin L and V on the one hand and J25 on the other hand are specifically and exclusively recognized by Cir and by FhuA, respectively.[6,15] Translocation of many microcins (E492, M, H47, J25) requires the TonB-ExbB-ExbD inner-membrane protein complex,[3,6,15] which uses the proton-motive force from the cytoplasmic membrane for energy transduction to the outer membrane and its receptors. After having crossed the outer membrane through porin OmpF, microcin B17 and C require the proteins SbmA and the ABC-transporter YejABEF, respectively, to be transported through the inner membrane.[26,31]

Microcins exhibit very heterogeneous, complex and subtle bacteria killing mechanisms (Figure 3). Class I microcins mainly inhibit vital bacterial enzymes.[31] Microcins B17 targets DNA gyrase (a topoisomerase essential for ATP-dependent negative supercoiling of DNA, similarly to clinically important quinolones but through a different mechanism). Microcin B17 can inhibit both the DNA supercoiling and relaxation reactions of gyrase.[20] It induces accumulation of covalent complexes of DNA gyrase with cleaved DNA, leading to the formation of double stranded DNA breaks, inhibition of DNA replication, and triggering of the SOS response.[20,31] The intracellular target of microcins J25 is RNA polymerase (RNAP). The lasso microcin binds in the secondary channel that directs NTP substrates

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toward the RNAP active site, provoking its obstruction in a cork-in-the-bottle fashion[16] and therefore blocking transcription. However, in addition to this enzyme inhibition, microcin J25 targets mitochondria and the respiratory chain at higher concentrations.[6,31] To become active, microcin C requires a cleavage step inside target bacteria to generate the toxic entity.[14] Once inside the cells, it is processed in two steps, first by peptide deformylase, which removes the N-terminal formyl group and second by several broad-specificity aminopeptidases that are able to target the ultimate peptide bond Ala6–Asp7 and cleave off the peptide part.[31] As a result of intracellular processing, the active compound, a modified nonhydrolyzable aspartyl adenylate, is released (Figures 1 and 3). The processed molecule, which is a mimic of aspartyl adenylate, is a potent inhibitor of aspartyl tRNA synthetase, which therefore blocks protein synthesis at the translation step.[13,14]

Class II microcins, however, target the inner membrane or their components.[6] Microcin H47 targets the F_0 proton channel of ATP synthase.[27] Microcins V and E492 both permeabilize the inner membrane. However, microcins also require proteins in the inner membrane for activity. Microcin E492 not only forms channels in the inner membrane, but it also requires the ManYZ inner-membrane components of the mannose permease,[2] which is involved in the active uptake of mannose and related hexoses, to exert its bactericidal activity. Similarly, microcin V needs SdaC, which is involved in serine uptake, and both microcins B17 and J25 need SbmA, a protein of unknown function,[6,30,31] whereas microcin L does not require the inner-membrane proteins ManYZ or SdaC.[15] These proteins presumably help microcins pass through the inner membrane.

STRUCTURE/ACTIVITY RELATIONSHIPS

Except for microcin J25, which has been the subject of extensive structural analysis leading to the clear description of its lasso structure,[24] the three-dimensional structures of other microcins remain poorly identified. Detailed structure–activity relationships of class I microcins, however, have been undertaken, while such studies are not described for class II microcins. A single study from M. Laviña's group, which constructed chimeric peptides with exchanged C-terminal sequences of microcins V and H47, has clearly shown that the C-terminus is involved in recognition by the receptors and therefore in the uptake of the microcin, whereas the N-terminus is responsible for toxicity and specificity.[6]

The unique mode of action of microcin C, for which final processing steps occur inside target bacteria, makes it an attractive model for elaborating analogs targeting other aminoacyl-tRNA synthetases than the aspartyl-tRNA synthetase naturally inhibited by the natural microcin. As saturating mutagenesis of the *mcjA* gene failed, a chemical strategy was developed to prepare a series of microcin C analogs, which retain the initial Trojan horse mechanism of antibacterial activity displayed by the natural microcin (i.e., similar import by YejABEF into target bacteria and processing by aminopeptidases inside cells), while targeting different aminoacyl-tRNA synthetases specified by the last amino acid located proximal to the adenosine part.[35] This synthetic approach is the first step to further create new compounds derived from microcin C and inhibiting different targets. There is no systematic structure–activity relationship analysis of microcin B17, but some point mutation studies indicate that the number and position of the thiazole and oxazole heterocycles in the microcin structure are important and that the C-terminal bis-heterocycle is critical, the activity being completely lost on complete destruction of these two cycles.[31] A systematic structure–activity relationship study of microcin J25 has been performed in Severinov's laboratory, leading to 380 singly substituted variants and paving the way for the future design of strategic variants.[31] The most remarkable conclusion is that the production and activity of the lasso microcin are both tolerant to amino acid substitutions at most of the positions in the 21-amino-acid sequence. Ongoing site-directed mutagenesis studies in my group aiming at deciphering the residues that are critical for both the lasso fold and the antibacterial activity are in progress and point out particularly the stringent roles of Tyr20, which traps the tail into the ring, and of Gly21. The stable lasso structure of microcin J25 is an efficient scaffold for epitope grafting,[11] affording proof of concept of the capacity to provide novel biological functions to the lasso fold and thus very promising tracks for future drug design.

CONCLUDING REMARKS

Bacteriocins exert their potent and selective antimicrobial activities through original, sometimes unique, mechanisms of action that show them to be excellent models for scientists to mimic their strategies. Many bacteriocins from Gram-positive bacteria target components or intermediates in the synthesis of peptidoglycan. Microcins from Gram-negative bacteria, however, use Trojan horse mechanisms to kill target bacteria. Either they use molecular mimicry to bind receptors involved in vital bacterial functions, such as iron import or sugar transport, and to cross the outer or inner membrane, or they enter freely into target bacteria as a harmless entity, which is then processed inside target bacteria to generate a toxic compound. It now becomes clear from structure–activity relationship studies that scientists can take advantage of these elegant and sophisticated strategies to generate potent and selective drugs. The rapid development of bioinformatics provides powerful tools to assist in the discovery of novel microcins or related peptides endowed with interesting biological properties, either inhibiting specific enzymes or perturbing essential bacterial functions. In this regard,

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posttranslationally modified microcins and particularly class I microcins, seem particularly suitable for drug design, because of their lower molecular masses and unusual posttranslational modifications. Moreover, the mechanisms of immunity developed by the microcin-producing bacteria to evade the toxic peptides they synthesize should provide powerful models for circumventing bacterial resistance. Taken together, this shows that microcins, and bacteriocins in general, could become important tools in the rational design and engineering of biologically active peptides.

REFERENCES

1. Baquero F, Moreno F. The microcins. *FEMS Microbiol Lett* 1984;**23**:117–24. Review.
2. Bieler S, Silva F, Soto C, Belin D. Bactericidal activity of both secreted and nonsecreted microcin E492 requires the mannose permease. *J Bacteriol* 2006;**188**:7049–61.
3. Braun V, Patzer SI, Hantke K. Ton-dependent colicins and microcins: modular design and evolution. *Biochimie* 2002;**84**:365–80. Review.
4. Corsini G, Karahanian E, Tello M, Fernandez K, Rivero D, Saavedra JM, et al. Purification and characterization of the antimicrobial peptide microcin N. *FEMS Microbiol Lett* 2010;**312**: 119–25.
5. Darst SA. New inhibitors targeting bacterial RNA polymerase. *Trends Biochem Sci* 2004;**29**:159–60:Review.
6. Duquesne S, Destoumieux-Garzón D, Peduzzi J, Rebuffat S. Microcins, gene-encoded antibacterial peptides from enterobacteria. *Nat Prod Rep* 2007;**24**:708–34. Review.
7. Duquesne S, Destoumieux-Garzón Zirah S, Goulard C, Peduzzi J, Rebuffat S. Two enzymes catalyze the maturation of a lasso peptide in *Escherichia coli*. *Chem Biol* 2007;**14**:793–803.
8. Ghilarov D, Serebryakova M, Shkundina I, Severinov K. A major portion of DNA gyrase inhibitor microcin B17 undergoes an N, O-peptidyl shift during synthesis. *J Biol Chem* 2011;**286**: 26,308–18.
9. González-Pastor JE, San Millán JL, Moreno F. The smallest known gene. *Nature* 1994;**369**:281.
10. Knappe TA, Linne U, Zirah S, Rebuffat S, Xie X, Marahiel MA. Isolation and structural characterization of capistruin, a lasso peptide predicted from the genome sequence of *Burkholderia thailandensis* E264. *J Am Chem Soc* 2008;**130**:11,446–54.
11. Knappe TA, Manzenrieder F, Mas-Moruno C, Linne U, Sasse F, Kessler H, et al. Introducing lasso peptides as molecular scaffolds for drug design: engineering of an integrin antagonist. *Angew Chem Int Ed Engl* 2011. http//dx.doi.org/10.1002/anie.201102190. [Epub ahead of print].
12. Li YM, Milne JC, Madison LL, Kolter R, Walsh CT. From peptide precursors to oxazole and thiazole-containing peptide antibiotics: microcin B17 synthase. *Science* 1996;**274**:1188–93.
13. Metlitskaya A, Kazakov T, Kommer A, Pavlova O, Praetorius-Ibba M, Ibba M, et al. Aspartyl-tRNA synthetase is the target of peptide nucleotide antibiotic microcin C. *J Biol Chem* 2006;**281**:18,033–42.
14. Metlitskaya A, Kazakov T, Vondenhoff GH, Novikova M, Shashkov A, Zatsepin T, et al. Maturation of the translation inhibitor microcin C. *J Bacteriol* 2009;**191**:2380–7.
15. Morin N, Lanneluc I, Connil N, Cottenceau M, Pons AM, Sablé S. Mechanism of bactericidal activity of microcin L in *Escherichia coli* and *Salmonella enterica*. *Antimicrob Agents Chemother* 2011;**55**: 997–1007.
16. Mukhopadhyay J, Sineva E, Knight J, Levy RM, Ebright RH. Antibacterial peptide microcin J25 inhibits transcription by binding within and obstructing the RNA polymerase secondary channel. *Mol Cell* 2004;**14**:739–51.
17. Nolan EM, Fischbach MA, Koglin A, Walsh CT. Biosynthetic tailoring of microcin E492m: posttranslational modification affords an antibacterial siderophore-peptide conjugate. *J Am Chem Soc* 2007;**129**:14,336–47.
18. Nolan EM, Walsh CT, Koglin A. Investigations of the MceIJ-catalyzed posttranslational modification of the microcin E492 C-terminus: linkage of ribosomal and nonribosomal peptides to form "Trojan horse" antibiotics. *Biochemistry* 2008;**47**:9289–99.
19. Oman TJ, van der Donk WA. Follow the leader: the use of leader peptides to guide natural product biosynthesis. *Nat Chem Biol* 2010;**6**:9–18. Review.
20. Pierrat OA, Maxwell A. Evidence for the role of DNA strand passage in the mechanism of action of microcin B17 on DNA gyrase. *Biochemistry* 2005;**44**:4204–15.
21. Poey ME, Azpiroz MF, Laviña M. Comparative analysis of chromosome-encoded microcins. *Antimicrob Agents Chemother* 2006;**50**: 1411–8.
22. Pons AM, Lanneluc I, Cottenceau G, Sable S. New developments in non-post translationally modified microcins. *Biochimie* 2002;**84**: 531–7. Review.
23. Pons AM, Zorn N, Vignon D, Delalande F, Van Dorsselaer A, Cottenceau G. Microcin E492 is an unmodified peptide related in structure to colicin V. *Antimicrob Agents Chemother* 2002;**46**: 229–30.
24. Rebuffat S, Blond A, Destoumieux-Garzón D, Goulard C, Peduzzi J. Microcin J25, from the macrocyclic to the lasso structure: implications for biosynthetic, evolutionary and biotechnological perspectives. *Curr Protein Pept Sci* 2004;**5**:383–91. Review.
25. Rebuffat S. Bacteriocins from Gram-negative bacteria: a classification? In: Drider D, Rebuffat S, editors. *Prokaryotic antimicrobial peptides: from genes to applications*. New York: Springer; 2011, Review. p. 55–75.
26. Rebuffat S. Microcins from Enterobacteria: on the edge between Gram-positive bacteriocins and colicins. In: Drider D, Rebuffat S, editors. *Prokaryotic antimicrobial peptides: from genes to applications*. New York: Springer; 2011, Review. p. 333–53.
27. Rodríguez E, Laviña M. The proton channel is the minimal structure of ATP synthase necessary and sufficient for microcin H47 antibiotic action. *Antimicrob Agents Chemother* 2003;**47**:181–7.
28. Roush RF, Nolan EM, Lohr F, Walsh CT. Maturation of an *Escherichia coli* ribosomal peptide antibiotic by ATP-consuming N–P bond formation in microcin C7. *J Am Chem Soc* 2008;**130**: 3603–9.
29. Rosengren KJ, Craik DJ. How bugs make lassos. *Chem Biol* 2009;**16**:1211–2.
30. Severinov K, Semenova E, Kazakov A, Kazakov T, Gelfand MS. Low-molecular-weight post-translationally modified microcins. *Mol Microbiol* 2007;**65**:1380–94. *Erratum in: Mol Microbiol* 2007;**66**:277. Review.

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31. Severinov K, Semenova E, Kazakov T. Class I microcins: their structures, activities, and mechanisms of resistance. In: Drider D, Rebuffat S, editors. *Prokaryotic antimicrobial peptides: from genes to applications*. New York: Springer; 2011, Review. p. 289–309.
32. Thomas X, Destoumieux-Garzón D, Peduzzi J, Afonso C, Blond A, Birlirakis N, et al. Siderophore peptide, a new type of post-translationally modified antibacterial peptide with potent activity. *J Biol Chem* 2004;**279**:28,233–42.
33. Vassiliadis G, Peduzzi J, Zirah S, Thomas X, Rebuffat S, Destoumieux-Garzón D. Insight into siderophore-peptide biosynthesis: enterobactin is a precursor for microcin E492 post-translational modification. *Antimicrob Agents Chemother* 2007;**51**:3546–53.
34. Vassiliadis G, Destoumieux-Garzón D, Lombard C, Rebuffat S, Peduzzi J. Siderophore microcins form the first family of structure-related antimicrobial peptides from Enterobacteriaceae: isolation and characterization of microcins M and H47. *Antimicrob Agents Chemother* 2010;**54**:288–97.
35. Vondenhoff GH, Dubiley S, Severinov K, Lescrinier E, Rozenski J, Van Aerschot A. Extended targeting potential and improved synthesis of Microcin C analogs as antibacterials. *Bioorg Med Chem* 2011 Aug 3. [Epub ahead of print].

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Chapter 21

Nonribosomal Peptide Synthesis

Jan Grünewald and Mohamed A. Marahiel

ABSTRACT

Nonribosomal peptides (NRPs) are highly diverse natural products of bacterial and fungal origin. In contrast to ribosomally produced peptides, which are diversified by posttranslational modification events (analogous to postsynthetic modifications of NRPs, which will be not discussed here), complexity-generating structural transformations of the NRP scaffold occur at several additional levels within the biosynthetic pathway. First, the biosynthetic enzymes of NRPs, the nonribosomal peptide synthetases (NRPSs), are able to process at least 25-fold more monomers than the ribosomal biosynthetic machinery. Second, NRPs are further diversified during chain initiation and elongation by a variety of NRPS condensation domain subtypes that integrate D-amino acids, heterocyclic elements, fatty acids, and other moieties into the peptide backbone. Third, complexity-generating chain release from the NRPS multienzyme complex often aims to rigidify the NRP scaffold. This step is mainly catalyzed by thioesterase and reductase domains and is discussed at the end of this chapter.

INTRODUCTION

Diversity is the key characteristic of nonribosomal peptides. The Norine database currently includes >1120 of these highly complex natural products (http://bioinfo.lifl.fr/norine/). NRPs are mainly derived from the secondary metabolism of microorganisms such as bacteria and fungi.[6,21] Their proteolytic stability and highly selective bioactivity have made the diverse scaffolds of NRPs an attractive source of novel drugs for the pharmaceutical industry. For instance, cyclosporin is an efficient immunosuppressive agent that down regulates T cells by binding to cyclophillin,[2] and daptomycin is an antibiotic against multiresistant Gram-positive bacteria that acts through rapid depolarization of the bacterial cell membrane.[1] These are just two examples of the numerous biological applications of NRPs. Hence, the chemical and structural diversity of these secondary metabolites correlates with a large set of highly diverse biological targets with which NRPs can interact. "What determines the high diversity of NRPs?" The answer to this question lies in the biosynthetic logic of NRP production. NRPs are synthesized by large multidomain enzymes, the NRPSs, which catalyze the formation of these natural products in a templated assembly-line fashion.[6] Unlike ribosomal translation of mRNA, NRPS assembly lines can process >520 monomers (http://bioinfo.lifl.fr/norine/, September 2011) for the biosynthesis of NRPs. In addition to high molecular diversity at the monomer level, additional diversification of NRPs occurs during chain assembly on the NRPS multienzyme complex. For instance, on-template heterocyclization significantly increases the rigidity of NRPs and protects them against proteolytic degradation. Chain termination is another diversification step during NRP biosynthesis. For example, full-length NRPs can be cleaved from NRPS enzymes as linear peptides, as peptide amides, as peptide alcohols, and as cyclic or branched-cyclic macrocycles, such as macrolactones, macrolactams, and cycloimines.[4] Finally, another diversification step is achieved by postassembly-line tailoring reactions[33] that are analogous to posttranslational modifications (PTMs) of ribosomally synthesized peptides such as the lantibiotics, microcins, cyanobactins, cyclotides, and linaridins. While PTMs are chemical modifications of peptides after the release from the ribosome, postassembly-line tailoring occurs after the cleavage of NRPs from the NRPS assembly line. In contrast to the PTM machinery, which is the only complexity-generating tool for ribosomally synthesized peptides, postassembly-line tailoring is only one of several steps to generate diversity in nonribosomal peptide synthesis. Further, genomic databases contain thousands of NRPS gene clusters that have not been biochemically characterized to date.[34] Thus, it is expected that many more NRP diversity-generating tools will be discovered in the future. The scope of this chapter is to provide a brief overview of the biosynthesis of NRPs, and to describe how the NRPS machinery is

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00021-X
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optimized to introduce a high degree of chemical diversity into peptides that rarely exceed 20 monomers in length.

MONOMER DIVERSITY

The biosynthetic genes for NRP assembly are usually physically clustered within the respective bacterial or fungal genome. This also applies to the genes that encode for the biosynthesis of the numerous monomers of NRPs. For example, the 82-kb gene cluster of the calcium-dependent antibiotic (CDA) from *Streptomyces coelicolor* contains at least 40 genes, 17 of which are predicted to encode enzymes for the biosynthesis and tailoring of monomers such as the 2,3-epoxyhexanoyl fatty acid and the nonproteinogenic amino acids 3-methylglutamic acid (3-MeGlu), 3-hydroxyasparagine, 3-phosphohydroxyasparagine, and 4-hydroxyphenylglycine (Hpg).[8] Further, the high flexibility in monomer selection combined with the NRPS machinery's lack of an efficient proofreading mechanism often leads to inhomogeneous mixtures of NRP natural products. One example is the depsipeptide daptomycin. This approved last-resort antibiotic was originally isolated as a minor component of the A21978C group of acidic lipopeptides derived from the fermentation of *Streptomyces roseosporus*.[1] A21978C is a complex mixture of depsipeptides that differ in the nature of the fatty acyl groups N-terminally attached to an identical peptide core. The major components A21978C_1, A21978C_2, and A21978C_3 contain branched chain C_{11}, C_{12}, and C_{13} fatty acyl groups, respectively. The ratio of these components can be easily manipulated by precursor-directed biosynthesis. Addition of valine to *S. roseosporus* fermentations leads to the production of mainly A21978C_2, whereas feeding with isoleucine dramatically shifted the production toward A21978C_1 and A21978C_3.[1] Analogously, the production of daptomycin containing a straight chain C_{10} fatty acyl group could be significantly optimized by adding decanoic acid to the *S. roseosporus* fermentation medium,[1] thereby illustrating how the diversity of NRP biosynthesis can be easily manipulated to produce a desired peptidic compound. Hence, the monomer diversity of NRPs is a great opportunity for natural-product-based drug discovery efforts.

According to a bioinformatic study based on the Norine database, proteinogenic L-amino acids form only 40% of the total number of NRP monomers.[3] Most of the NRP monomers are of nonproteinogenic origin, such as D-amino acids, noncanonical amino acids, lipids, sugars, and polyketide building blocks. Although both bacterial- and fungal-derived NRPs contain a similar degree of monomer complexity, bacteria and fungi show profound differences in monomer preference. Bacterial NRPs often contain D-serine, D-alanine, 2,4-diaminobutyric acid (Dab), and Hpg as nonproteinogenic building blocks, which are rarely found or even absent in fungal NRPs. By contrast, fungal NRPs are unique in preferring nonproteinogenic monomers such as 2-aminoisobutyric acid (Aib), phenylalaninol, leucinol, and isovaline.[3] Besides monomer-based differentiation between fungal and bacterial NPSs, bioinformatics may also prove useful to assign specific NRP monomer compositions to certain biological activities. By using the Norine database in combination with the known monomeric compositions of the NRPs orfamide, fuscachelin, and aibellin, Jacques and coworkers were able to correctly predict their biological activities as being surfactant, siderophoric, and antibiotic, respectively.[3] Together, bioinformatics in combination with NRP monomer manipulation, such as precursor-directed biosynthesis and mutasynthesis, offer an enormous potential to precisely engineer peptidic natural products with desired biological activities.

INITIATION OF NRP ASSEMBLY

The selection of a highly diverse set of monomers for peptide synthesis is only one aspect that contributes to the overall complexity of NRPs. Further diversification occurs during chain initiation, chain elongation, and chain termination of NRP biosynthesis. All these steps are catalyzed by the NRPS multienzyme complex that covalently binds both the monomers and the nascent peptide chain as thioesters to highly flexible 4′-phosphopantetheine (4′-PP) cofactors.[6] This covalent mode of peptide synthesis minimizes side reactions and eliminates diffusion limitations of single enzyme pathways. The 4′-PP cofactor is posttranslationally attached via a phosphodiester bond to a conserved serine residue of the peptidyl carrier protein (PCP) also referred to as thiolation (T) domain. During NRP biosynthesis, the α-amino group of a covalently attached monomer nucleophilically attacks the electrophilic thioester of a peptidyl or aminoacyl group that is tethered to the upstream PCP. Hence, peptide chain growth by one monomer unit per catalytic cycle is accompanied by the transfer of the nascent oligomer from an upstream to a downstream PCP, thereby also ensuring peptide chain elongation from N- to C-terminus. Besides unidirectionality of peptide synthesis, the prosthetic 4′-PP group of the PCP provides the basis of diversification during NRP biosynthesis. Substrates tethered to this flexible 18 Å long 4′-PP cofactor can reach various domains within the NRPS multienzyme complex that catalyze transformations such as the heterocyclization of serine and cysteine residues, N-methylation of amide bonds, and epimerization of L- to D-amino acids. The importance of the PCP for biosynthesis and diversification of NRPs has led to a "PCP-centered view" of NRPS multienzyme complexes.[12] Structural biology efforts have focused on elucidating the molecular interaction of PCPs with the various catalytic domains of the NRPS machinery. A major breakthrough was the solution

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structure of the entire 144-kDa termination module of the *Bacillus subtilis* surfactin synthetase.[31] The 2.6 Å X-ray structure shows the PCP in the context of a fully functional NRPS module containing the complete catalytic machinery necessary for chain elongation by one monomer.

N-Acylation is a common strategy in nonribosomal peptide biosynthesis to increase the hydrophobicity of intrinsically hydrophilic NRP natural products. This initiation step in NRP biosynthesis also significantly contributes to the diversification of the peptide scaffold. For example, most of the daptomycin-related acidic lipopeptide antibiotics are naturally produced as mixtures composed of various N-terminal fatty acid chains.[1] Because of its potential importance for the bioengineering of novel lipopeptide drugs, the mechanism of NRPS-mediated lipoinitiation has been the object of intense research. Initially, Baltz et al. proposed a three-step pathway for the N-terminal lipidation of NRPs.[1] First, fatty acyl-CoA ligase (FACL) catalyzes the formation of acyl-CoA from ATP and the respective fatty acid, which is either recruited from primary metabolism or is synthesized or derivatized by enzymes encoded by the respective biosynthetic gene cluster. Second, the activated acyl moiety of the CoA thioester intermediate is transferred to the holo form of a dedicated acyl carrier protein (ACP) in a transthioesterification reaction. Third, the interaction of this acyl-S-pantetheinyl-ACP intermediate with the NRPS multienzyme complex leads to the transfer of the lipid moiety to the tethered monomer of the NRPS initiation module. This final step of nonribosomal lipoinitiation is assumed to be catalyzed by the N-terminal condensation (C) domain of the NRPS megaenzyme, which is also referred to as "starter" C domain or as type C^{III} domain. The suggested mechanism is supported by the presence of both ACP and FACL in several biosynthetic gene clusters of the acidic lipopeptide antibiotics, namely, A54145, friulimicin, and daptomycin.[1] However, two studies on the glidobactin and the surfactin biosynthetic pathways shed light on an alternative mechanism of NRP lipoinitiation[10,16] (Fig. 1). Interestingly, although no ACPs could be located in either of the two biosynthetic gene clusters, *in vitro* studies show that the respective starter C domains catalyze amide bond formation between the respective PCP-tethered aminoacyl acceptor substrate and the acyl-CoA donor substrate. Further, knockouts of FACL genes in the genome of the surfactin producer *Bacillus subtilis* and enzymatic assays with recombinant FACLs indicate direct recruitment of the acyl-CoA donor substrate from primary metabolism, thereby also explaining the absence of a dedicated FACL gene in the surfactin biosynthetic gene cluster.[16] In glidobactin biosynthesis, the acyl-CoA donor is presumably recruited from the catabolism of fatty acyl-CoA precursors[10] Moreover, although the dissected starter C domain of the surfactin synthetase exhibits stringent substrate specificity for the 3-hydroxy tetradecanoic acyl-CoA donor substrate, the corresponding domain of the glidobactin synthetase tolerates various lipid donors for chain-initiating N-acylation that structurally significantly differ from the cognate 2(E),4(E)-diene fatty acids. Overall, the example of the fatty acid diversity in the acidic lipopeptide antibiotics indicates that many more of such starter C domains with relaxed acyl-CoA donor specificity may exist. Such promiscuous starter C domains would serve as ideal tools for NRPS reengineering efforts to generate novel NRPs with custom-made N-terminal fatty acid moieties, thereby allowing the fine tuning of the hydrophobicity of these natural products.

Besides catalyzing lipoinitiation of NRP synthesis, starter C domains are also responsible for the N-terminal incorporation of aromatic or heteroaromatic carboxylic acids into NRPs. In the biosynthesis of the siderophore enterobactin, the starter C domain catalyzes amide bond formation between serine-S-pantetheinyl EntF and upstream 2,3-dihydroxybenzoate (2,3-DHB), which is tethered to the 4′-PP group of an aryl carrier protein (ArCP) in the two-domain protein EntB.[23] Other examples include starter C domain-mediated chain initiation reactions with 4-methyl-3-hydroxyanthranilic acid (4-MHA) in actinomycin D biosynthesis and 3-hydroxypicolinic acid (3-HPA) in pristinamycin I biosynthesis.[23] A special case is observed in the biosynthesis of the siderophore vibriobactin. In this instance, the starter C domain VibH is a stand-alone domain that catalyzes amide bond formation between two nonproteinogenic building blocks: 2,3-DHB as the donor substrate and norspermidine as the acceptor substrate.[11] Hence, the examples discussed thus far show that starter C domains are versatile biocatalysts capable of processing highly diverse monomeric building blocks for the initiation of NRP assembly. However, one exception is the N-formylation of anabaenopeptilides and linear gramicidins. This NRP initiation reaction is suggested to be catalyzed by formylation domains. For the linear gramicidins, *in vitro* enzymatic assays reveal that the formylation domain of the first module LgrA1 uses both N^{10}-formyltetrahydrofolate and N^{5}-formyltetrahydrofolate as cofactors to catalyze N-formylation of PCP-tethered valine, and, to a lesser degree, leucine and isoleucine.[29]

ELONGATION DURING NRP ASSEMBLY

While the nascent peptide is covalently tethered to the NRPS assembly line, chain elongation is another opportunity to diversify the NRP scaffold. Common diversifying modifications during chain assembly include the epimerization of distinct monomers of the peptide chain, the introduction of heterocyclic structural elements, the N-methylation of peptide bonds, and the reduction of α-keto acids leading to ester bond formation.[34] These complexity-generating

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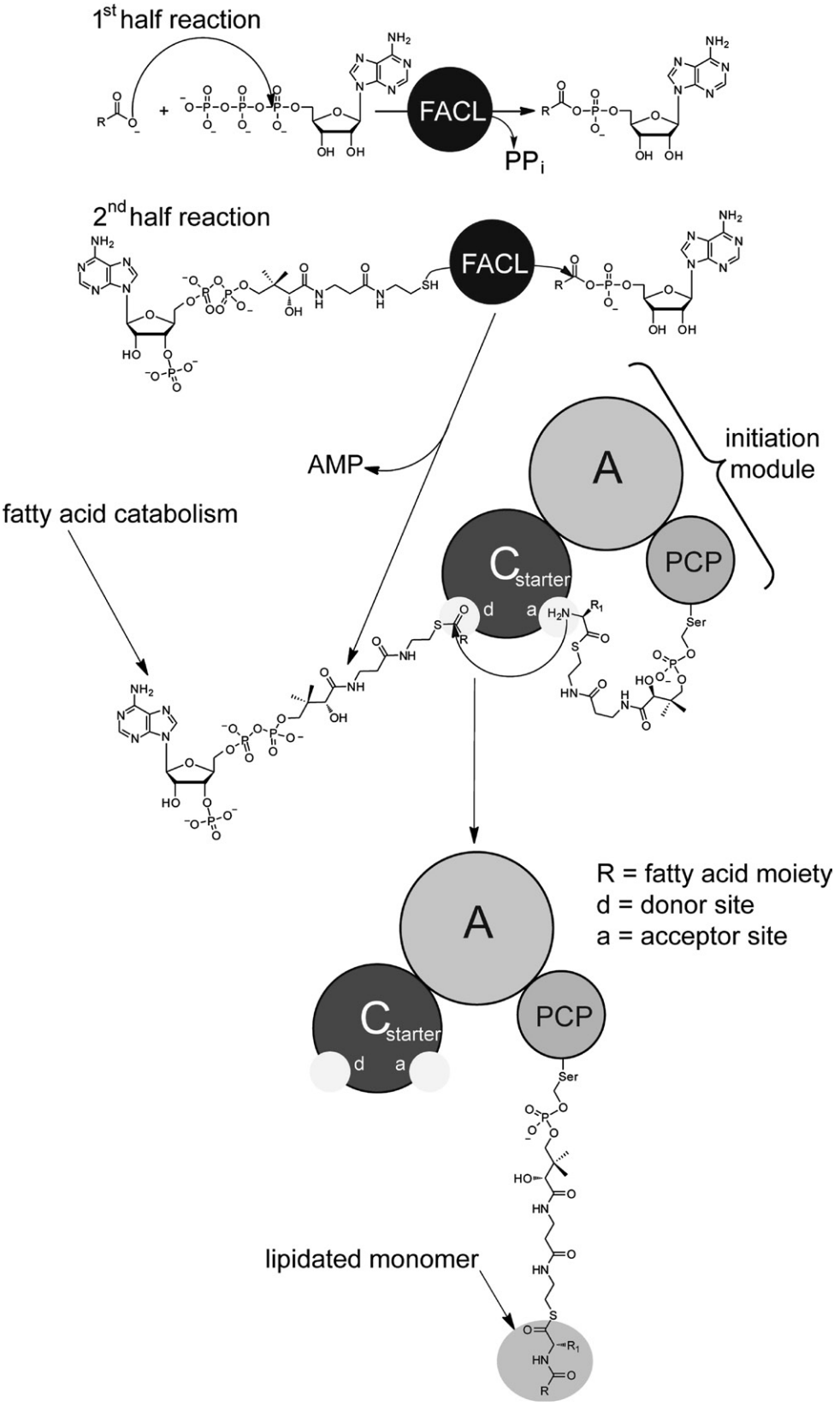

FIGURE 1 Proposed mechanism of NRP lipoinitiation. Acyl-CoA donor substrates are directly recruited from primary metabolism. In the surfactin biosynthetic pathway, formation of the lipid-CoA donor substrate is catalyzed by FACLs.[16] The respective fatty acid is first activated as an adenylate (first half reaction), which is then converted into an energy-rich CoA thioester (second half reaction). Alternatively, the acyl-CoA donor substrate can also be derived from the catabolism of acyl-CoA precursors,[10] as suggested for the glidobactin biosynthetic pathway. In either case, the dedicated acyl-CoA donor directly serves as a substrate for the starter C domain, which catalyzes the transfer of the activated fatty acyl moiety to the PCP-bound aminoacyl acceptor via amide bond formation.

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transformations during peptide assembly, combined with the high diversity of NRP monomers, pose a significant challenge to the enzymatic machinery of the NRPS. Besides lipoinitiation, the C domain is the key enzymatic element accounting for efficient peptide elongation with highly diverse NRP building blocks. Further, it even contributes to several "on-assembly-line" modifications that further increase the complexity of NRPs. To better accommodate the variety of enzymatic transformations, the C domain is split into several functional subtypes. Phylogenetic studies carried out by Rausch et al. reveal the existence of six C domain subtypes in NRPSs: the already mentioned starter C domain, the $^{L}C_{L}$ domain, the $^{D}C_{L}$ domain, the epimerization (E) domain, the dual E/C domain, and the heterocyclization (Cy) domain[23] (Fig. 2). A solid understanding of the catalytic functions and substrate specificities of this diverse set of C domain subtypes will be beneficial for the design of future bioactive NRP derivatives by NRPS reengineering efforts. The major functional and structural aspects of C domain subtypes in peptide elongation will be discussed in the following section.

In nonribosomal peptide elongation, the C domain catalyzes the nucleophilic attack of the α-amino group of the PCP-tethered monomer on the electrophilic thioester of the nascent peptidyl-S-pantetheinyl intermediate of the upstream PCP. X-ray crystallographic studies reveal that the

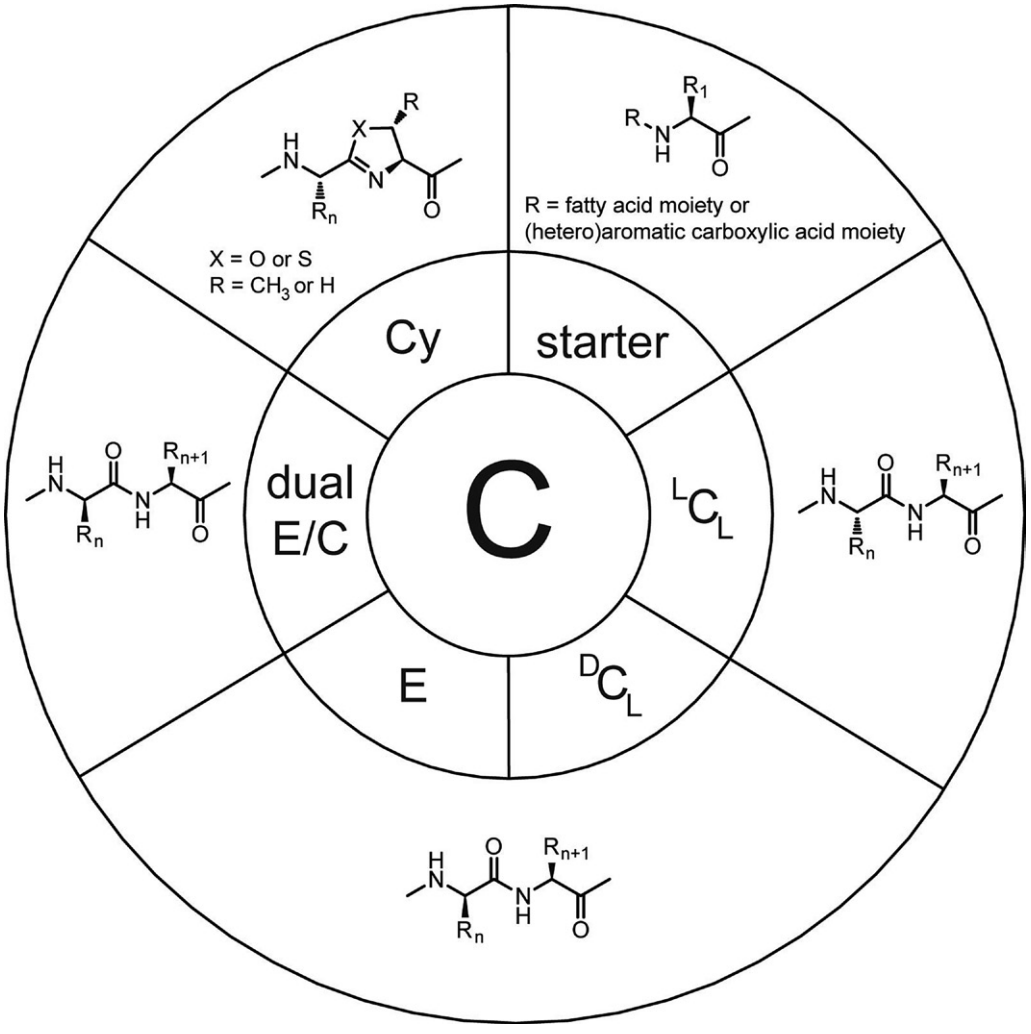

FIGURE 2 NRP diversification during peptide assembly by various C domain subtypes. According to Rausch et al.,[23] the C domain can be subdivided into six different functional subtypes: starter C domain, $^{L}C_{L}$ domain, $^{D}C_{L}$ domain, E domain, dual E/C domain, and Cy domain. The starter C domain is involved in the attachment of a diverse set of fatty acid moieties or (hetero)aromatic building blocks to the N-terminus of the nascent NRP natural product. The $^{L}C_{L}$ domain catalyzes amide bond formation between two L-configured amino acid monomers, whereas the two-domain E/$^{D}C_{L}$ system and the dual E/C domain stereospecifically catalyze amide bond formation between an upstream D-configured amino acid monomer and a downstream L-configured amino acid monomer. The major difference between the dual E/C domain and the two-domain E/$^{D}C_{L}$ system is that in the latter amino acid epimerization is catalyzed by an autonomous catalytic domain, the E domain. Finally, the Cy domain catalyzes both peptide bond formation and its subsequent cyclodehydration to generate thiazoline (X = S; R = H) or oxazoline heterocycles (X = O; R = CH_3 or H).

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C domain has a V-like structure, in which two sub-domains are separated by a groove containing the putative active site HHXXXDG of the C3 core motif (His-motif).[31] The groove provides enough space for the 4′-PP-tethered condensation substrates to reach the highly conserved motif from two opposing faces, the donor and the acceptor sites. Initially, the second histidine residue of the His-motif was postulated to function as a catalytic base in the deprotonation of the positively charged α-amino group of the acceptor monomer.[26] However, mutational studies on the VibH C domain show that the conserved histidine residue is not essential for the catalysis of amide bond formation.[11] Further, pK_a value calculations of the respective histidine residue in the C domain of the 6th module of the tyrocidine synthetase TycC indicated complete protonation of the histidine side chain under physiological conditions.[28] Essen and coworkers therefore suggested that the protonated residue is involved in the stabilization of the tetrahedral oxyanion intermediate during the nucleophilic attack on the thioester bond. Similar to the still elusive mechanism of amide bond formation, the interdomain dynamics involved in nonribosomal peptide elongation are currently far from being understood. Despite the availability of X-ray crystallographic information on a PCP-C didomain[28] and even an entire NRPS termination module,[31] X-ray crystallography can only provide static snapshots of distinct conformational states and crystal-packing effects may alter the NRPS interdomain interactions in a nonphysiological way. However, in contrast to this, nuclear magnetic resonance studies on the PCP indicated that NRPS assembly-line synthesis is a highly dynamic process that is driven by major conformational changes of this small protein domain.[12] Hence, a better understanding of nonribosomal peptide elongation would require alternative structure solution methodologies that better capture the native dynamic processes involved in amide bond formation. In addition, as suggested by Strieker et al., a yet to be determined 3-D structure of a multimodular NRPS fragment containing the C domain together with its interacting upstream and downstream partner PCP domains would give better insights into the underlying interdomain interactions.[31]

One striking feature of the C domain, which has not yet been rationalized on a structural basis, is its stereoselectivity for both condensation substrates. The $^{D}C_{L}$ domain catalyzes the stereospecific condensation of a D-configured donor substrate with an L-configured acceptor substrate, whereas the $^{L}C_{L}$ domain exhibits strict L-specificity on both donor and acceptor sites. Switching between both C domain subtypes by rational mutagenesis would allow complete control over the stereochemistry of the final NRP natural product. Although reprogramming of C domain subtypes would be highly desirable for the engineering of novel NRPs, stereospecificity-determining key residues have not been identified yet. Further, phylogenetic studies indicate no significant differences between the putative catalytically or structurally relevant residues, for example, both subtypes contain the highly conserved His-motif.[23] However, profound differences between the two subtypes could be detected within and downstream of core motif C4. Mutually nonconserved residues in these regions may serve as a promising starting point for future mutational studies aiming to alter the stereospecificity of the C domain donor site.

Besides its stereospecificity, the C domain also exhibits significant selectivity toward the side-chain structure of the acceptor monomer. Hence, the acceptor site of the C domain is a second selectivity filter that acts downstream of monomer selection and activation by the adenylation (A) domain. This tight functional interaction between both domains ensures the incorporation of the correct monomer into the nascent peptide chain. Both the C domain and its downstream A domain also tightly interact at a structural level. According to the X-ray crystal structure of the termination module of the surfactin NRPS, both domains share a large surface area to form a rigid catalytic platform with their active sites aligned on the same side of the platform.[31] This close functional and structural association of both domains explains the difficulty of reengineering NRPSs by domain swapping. Reprogramming of NRPSs by swapping, deletion, or insertion of entire modules is preferable as the structural integrity of this catalytic platform is maintained. Moreover, the low side chain specificity of the C domain donor site, which is part of the module–module interface, increases the chances of success of such reengineering efforts.

The $^{D}C_{L}$ domain is closely related to the dual E/C domain, which was first characterized by Walsh and Fischbach.[6] Both C domain subtypes act as stereoselective $^{D}C_{L}$ catalysts that share significant similarities in the C5 core motif.[23] However, analysis of the primary sequence of the dual E/C domain reveals the existence of a unique N-terminal extension that contains another elongated His-motif, HH[I/L]XXXDG. This second His-motif is believed to exhibit epimerization activity toward the C-terminal L-amino acid of the nascent peptide chain, which is tethered to the preceding module. Specifically, E/C domain-catalyzed epimerization results in the generation of a diastereomeric mixture of the donor peptidyl chain containing the C-terminal monomer in both D- and L-configurations. Interestingly, biochemical studies with recombinant NRPS components of the arthrofactin synthetase indicate that this epimerization activity is triggered by the presence of the downstream monomer-*S*-pantetheinyl-PCP intermediate.[6] Epimerization is then followed by stereoselective peptide bond formation, in which only the D-configured C-terminal monomer of the upstream peptidyl-S-pantetheinyl moiety is transferred to the downstream L-aminoacyl-S-pantetheinyl

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acceptor. The strict order of the dual catalytic activity of this C domain subtype ensures unidirectionality of peptide synthesis and the stereoselective incorporation of D-configured monomers.

D-Configured building blocks diversify NRPs and contribute to the exceptional stability of these natural products by conferring resistance to naturally L-specific proteases. Further, they allow the NRP scaffold to adopt unique structural conformations, which play an important role in their bioactivity. For the vast majority of characterized NRPSs, the incorporation of D-configured monomers is mediated by two distinct C domain subtypes, namely, E and $^{D}C_{L}$ domains. By analogy to the catalytic activity of the dual E/C domain subtype, E-domain-catalyzed epimerization is followed by $^{D}C_{L}$-domain-mediated condensation of the downstream L-aminoacyl-S-pantetheinyl acceptor with the thioester-bound D-configured monomer of the upstream donor substrate.[6] Mutational studies have indicated that epimerization is catalyzed by the second histidine residue of the E domain's HHXXXDG motif,[30] which is supposedly involved in the rapid deprotonation and reprotonation of the Cα atom of the aminoacyl- or peptidyl-S-pantetheinyl donor.

Heterocyclization is another tailoring step in nonribosomal peptide synthesis that occurs while the nascent peptide chain is tethered to the NRPS assembly line. In addition to further diversification of the NRP natural product, the introduction of heterocyclic structural elements increases resistance to proteolytic degradation and also significantly contributes to the rigidity of the peptide scaffold. Moreover, five-membered heterocyclic rings, such as thiazolines, oxazolines, and their oxidized derivatives, are important binding motifs that allow NRPs to chelate metal ions and to specifically interact with DNA, RNA, and proteins.[27] In NRP biosynthesis, the formation of oxazoline and thiazoline heterocyclic moieties is catalyzed by the heterocyclization (Cy) domain. Similar to the dual E/C domain, this C domain subtype catalyzes multiple catalytic transformations. The first catalytic step is mechanistically identical to C-domain-mediated peptide bond formation in which the nascent peptidyl-S-pantetheinyl donor is transferred to the downstream monomer-S-pantetheinyl-PCP intermediate. Specific to the Cy domain, the acceptor substrate is always a 4′-PP group-tethered serine, threonine, or cysteine moiety, because the nucleophilic side chains of these amino acids attack the carbonyl carbon atom of the newly formed amide bond in the second catalytic step. Dehydration of the cyclization product then leads to the formation of the final oxazoline (derived from serine or threonine) or thiazoline (derived from cysteine) ring systems.[5] Overall, the Cy domain is a dual-functioning C domain subtype that catalyzes the cyclodehydration of its own generated peptide bond. In common with the dual E/C domain, the Cy domain has a unique sequence motif. The His-motif HHXXXDG is replaced by the conserved signature motif, DXXXD, which is believed to be crucial for the catalysis of amide bond formation.[11] Further, mutational studies of the Cy domain of the bacitracin synthetase identified two conserved residues, Asn^{900} and Ser^{984}, which seem to be essential for cyclodehydration catalysis.[5] Interestingly, the corresponding residues are absent in the Cy2 domain of the VibF subunit of the vibriobactin synthetase, and the catalytic activity of Cy2 is restricted to amide bond formation.[18] By contrast, the adjacent Cy1 domain of the VibF subunit, which contains both key residues, is mainly involved in the catalysis of the cyclodehydration reaction. Hence, in analogy to the two-domain E/$^{D}C_{L}$ system, Cy1 and Cy2 are believed to act in tandem with Cy1 restricted to the catalysis of cyclodehydration and Cy2 restricted to the catalysis of amide bond formation. The cyclodehydration-specialized Cy domain may therefore be regarded as the evolutionary counterpart of the epimerization-specialized E domain.

In many cases, Cy-domain-catalyzed cyclodehydration of peptide bonds is followed by subsequent oxidation or reduction of the resulting oxazoline, β-methyl oxazoline, and thiazoline heterocyclic ring systems.[6] This further increases the number of possible heterocyclic elements in NRPs to a total of nine different species. Heterocycle diversification is either catalyzed by oxidation (Ox) or reduction (R) domains (Fig. 3). The Ox domain, which is integrated into the NRPS multienzyme complex, uses the cofactor flavin mononucleotide (FMN) to oxidize oxazoline and thiazoline to oxazole and thiazole moieties, respectively.[6] Conversely, the R domain acts as a free-standing enzyme by catalyzing the NADPH-cofactor-dependent reduction of oxazoline to oxazolidine rings or thiazoline to thiazolidine

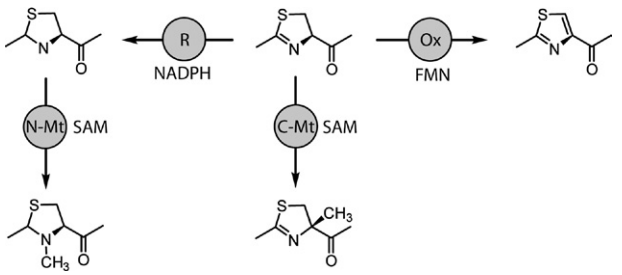

FIGURE 3 Heterocycle diversification in NRP biosynthesis as exemplified for the thiazoline moiety. Peptide bond formation and subsequent cyclodehydration to form a thiazoline heterocycle is the starting point of several complexity-generating catalytic transformations. The FMN-dependent Ox domain can carry out a two-electron oxidation resulting in the generation of an aromatic thiazole moiety. Conversely, two-electron reduction by the NADPH-dependent R domain yields a thiazolidine heterocycle. Because of the high basicity of the nitrogen atom in the thiazolidine ring, this moiety can undergo N-methylation by an SAM-dependent N-Mt domain as observed in pyochelin biosynthesis.[22] Alternatively, SAM-dependent C-methylation of the thiazoline heterocycle occurs in yersiniabactin biosynthesis.[19] This regiospecific methylation of the C2 atom is catalyzed by a C-Mt domain via a putative carbanion intermediate.

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heterocycles.[24] Because both Ox and R domain could be heterologously expressed in *Escherichia coli* as catalytically active enzymes, they may be used as versatile catalytic tools for the *in vivo* production of novel peptides using engineered NRPSs expressed in this genetically most-manipulable microorganism.

Besides oxidation and reduction, heterocyclic moieties can be C-methylated by an S-adenosylmethionine (SAM)-dependent C-methyltransferase (C-Mt) domain (Fig. 3). In the synthetase of the siderophore yersiniabactin, the C-Mt domain is located downstream of a Cy domain that catalyzes the cyclization of a cysteine side chain onto the preceding amide carbonyl group.[19] The resulting thiazoline moiety is then regiospecifically methylated at the C2 atom by the C-Mt domain. Mechanistically, C-methylation is believed to involve C-Mt-domain-mediated deprotonation of the C2 hydrogen atom, leading to a delocalized carbanion intermediate that nucleophilically attacks the electron-deficient methyl group of the enzyme-bound SAM cofactor. Similar to Ox and R domains, C-Mt domain catalysis requires the substrate to be covalently tethered to the 4′-PP cofactor of the PCP. Hence, all these complexity-generating tailoring steps on heterocyclic elements occur during chain elongation of the NRP nascent product. The same applies to the N-methylation of these rigidifying moieties (Fig. 3). For instance, *in vitro* reconstitution of the biosynthetic pathway of the siderophore pyochelin indicated that N-methylation of the C-terminal thiazolidine heterocycle occurs during NRPS assembly-line synthesis.[22] The enzymatic cascade reaction is initiated by a Cy domain, which catalyzes both peptide bond formation and cyclodehydration. An *in trans*-acting R domain then converts the resulting C-terminal thiazoline into a thiazolidine moiety. This imine reduction renders the heterocyclic amine nitrogen more basic, thereby facilitating the final nucleophilic attack onto the SAM electrophile. This final step in heterocycle tailoring is catalyzed by an *in cis*-acting N-Mt domain, which is an embedded catalytic entity of the pyochelin synthetase. N-methylation may prevent opening of the reduced thiazolidine ring, which is assumed to be involved in the siderophore chelation of ferric iron.[22] However, more common than N-methylation of heterocyclic moieties is the N-methylation of noncyclized peptide bonds in nonribosomal peptide biosynthesis. N-Methylation of peptide bonds is an important *in cis* tailoring step that renders NRPs more hydrophobic and inhibits proteolytic cleavage of the peptide backbone. For example, the cyclosporin synthetase contains six embedded N-Mt domains that contribute to the N-methylated peptide bonds of the undecapeptide.[32] In contrast to the N-methylation of heterocyclic elements, SAM-dependent N-methylation occurs before amide bond formation resulting from the higher basicity of the free amino group of the 4′-PP-bound aminoacyl acceptor substrate. Further, molecular modeling studies on the C domain of the second module of cyclosporin synthetase reveal specific recognition of the N-methyl group of the downstream N-MeLeu^3-S-PCP acceptor nucleophile by the acceptor site.[32] Specifically, the N-methyl group is involved in hydrophobic interactions with Phe^{1399}, which is part of a hydrophobic pocket in the C domain acceptor channel. This key residue is only conserved among C domains in the cyclosporin synthetase that catalyze the formation of N-methylated peptide bonds.

Diversification of NRPs during chain elongation is mainly generated by the C domain and its various subtypes. Interestingly, instead of catalyzing amide bond formation, several C domains of bacterial and fungal NRPSs supposedly act as chemoselective ester synthases. For example, the terminal C domain of the CesA module of the cereulide NRPS has been proposed to use the α-hydroxy moiety of the 4′-PP-bound L-α-hydroxy isovaleric acid (L-HIV) as the incoming nucleophile.[17] Ester bond formation is proposed to occur with $^{D}C_{L}$ stereospecificity, leading to the observed D-Ala-L-HIV connectivity of the cereulide molecule.

Diversification of NRPs by the various C domain subtypes usually occurs *in cis*. However, as already exemplified for heterocycle reduction in pyochelin biosynthesis, many additional tailoring reactions during chain elongation are catalyzed by *in trans*-acting enzymes that interact intermolecularly with the NRPS-tethered peptide substrate. According to Walsh and Fischbach, these enzymatic transformations can be classified into two categories: group transfer reactions and oxidative transformations.[34] Tailoring enzymes involved in group transfer reactions catalyze the conjugation of an electrophilic moiety of a cosubstrate to a nucleophilic center of the NRP scaffold. One example is the N-methylation of 4′-PP-bound 2,3-diaminobutyric acid (2,3-DABA) by the external SAM-dependent methyltransferase PacV in pacidamycin biosynthesis.[35] By contrast, oxidative transformations are catalyzed by tailoring enzymes that use molecular oxygen as a cosubstrate. For instance, nonheme Fe^{II} halogenases of various biosynthetic gene clusters were discovered that use molecular oxygen and α-ketoglutarate to oxidatively activate halogen atoms such as bromine and chlorine. As shown for the syringomycin E biosynthetic pathway, these enzymes regiospecifically halogenate unactivated carbon centers on 4′-PP-tethered aminoacyl moieties.[20] Another example of oxidative transformations is the crosslinking of aromatic side chains of vancomycin-type glycopeptide antibiotics by cytochrome P450 monooxygenases. In vancomycin biosynthesis, the three enzymes OxyA, OxyB, and OxyC catalyze these rigidifying oxidative transformations on the PCP-tethered aglycone in a highly regiospecific fashion.[34] However, most of the enzymatic group transfer reactions and oxidative transformations occur postsynthetically after the release of the peptide from the NRPS multienzyme complex. These postsynthetic tailoring reactions show parallels

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to the diversifying posttranslational modifications found in ribosomally produced peptides. A comprehensive review of these complexity-generating modifications has been provided by Walsh et al.[33]

TERMINATION OF NRP ASSEMBLY

Chain termination in NRP biosynthesis combines peptide release from the NRPS assembly line with further diversification of the peptidic scaffold. In most cases, diversification is directed toward the rigidification of the short peptide chain consisting of usually <20 monomers. Chain release by macrocyclization is the most common strategy in NRP biosynthesis to constrain the peptide backbone in a biologically active conformation. According to the Norine database, 64% of all nonribosomally produced peptides consist of at least one cyclized form.[3] Besides being important for bioactivity, these nonlinear structures significantly contribute to the proteolytic resistance of the NRP natural products, thereby increasing their half-lives. Interestingly, although the biologically active conformation of most ribosomally produced peptides is stabilized by cystine disulfide bonds or lanthionine thioether bridges, cysteine constitutes a rare monomer in NRPs. The role of cysteine in the rigidification of the NRP scaffold is mainly confined to thiazoline, thiazole, and thiazolidine heterocycle formation. Instead, covalent linkage between nonadjacent residues of the NRP chain occurs mostly via macrolactonization and macrolactamization. This final step in templated NRP synthesis is typically catalyzed by a thioesterase domain (TE domain), which usually constitutes the most downstream domain of the NRPS assembly line.[13] TE domains belong to the superfamily of α/β hydrolases, which use a catalytic triad of serine, histidine, and aspartate for catalysis. Specifically, the reactive hydroxyl moiety of the triad serine residue nucleophilically attacks the thioester of the 4′-PP cofactor-bound full-length peptidyl chain of the most downstream PCP domain. The resulting peptidyl-O-TE oxoester intermediate then undergoes regioselective attack by an amine or hydroxyl group of the tethered peptide moiety, thereby triggering the release of the macrocyclic product from the NRPS template.[13] For instance, daptomycin is released as a macrolactone, whereas bacitracin A is released as a macrolactam (Fig. 4). Valuable insights into the mechanistic aspects of TE-domain-catalyzed peptide cyclization have been gained from X-ray crystallography and NMR spectroscopy, as well as *in vitro* enzymatic assays with recombinant TE domains.[7,13] These studies conclude that substrate recognition by the TE domain is basically limited to the peptide's C-terminal residues and the residue containing the cyclization nucleophile. The active site of the TE domain allows hydrogen bond-assisted folding of the bound peptide into a precyclic conformation, which orients the cyclization nucleophile for the subsequent attack on the transient oxoester linkage. In a minority of NRPSs, this TE-domain-mediated chain termination step involves hydrolysis or aminolysis of the peptidyl-O-TE intermediate, leading to the release of a linear NRP product. For example, the glycopeptides of the vancomycin family are hydrolytically released from the NRPS multienzyme complex as linear peptide acids (Fig. 4). As mentioned earlier, scaffold rigidification of these NRPs by oxidative side-chain cross-linking takes place before chain termination. In a third variant of chain termination, the peptidyl-O-TE intermediate is nucleophilically attacked by several species of nucleophiles. As shown in the biosynthesis of gramicidin S, the TE-bound pentapeptidyl oxoester is cleaved by the N-terminal α-amino group of a second pentapeptidyl moiety as a pseudoexternal nucleophile, which is tethered to the most downstream PCP domain.[9] The resulting 4′-PP cofactor-bound head-to-tail dimerized decapeptidyl moiety is then transferred back to the TE domain, which catalyzes the release of the final ten-residue macrolactam ring via nucleophilic attack of the internal N-terminal α-amino group. Such cyclooligomerization reactions have been observed for both macrolactone- and macrolactam-forming TE domains.[13] In addition, as shown in Fig. 4, cyclooligomerization involving the generation of two thioester bonds has been described for the TE domain of the thiocoraline synthetase.[25] Even more remarkable, iterative TE domain-mediated diversification of NRP scaffolds also includes C–C bond formation (Fig. 4). Such a chain termination strategy was discovered during the reconstitution of the terrequinone A biosynthetic pathway *in vitro*. Specifically, Balibar et al. showed that TE domain-catalyzed release of a terrequinone A precursor is coupled to two tandem Claisen condensations. These two sequential C–C bond formations generate the quinone core which bridges the two indole moieties of terrequinone A.[4] Further, in some NRPSs, a chain releasing C domain can functionally substitute a TE domain. Examples are C-domain-catalyzed macrolactamization of cyclosporin A[13] and C domain-catalyzed aminolytic release in vibriobactin biosynthesis[11] (Fig. 4). Hence, the C domain is the most versatile catalytic entity of NRPSs, because it can contribute to all three biosynthetic phases of NRP biosynthesis, namely, initiation, elongation, and termination.

In NRP biosynthesis, chain release can also be triggered by an NAD(P)H-dependent reductase domain (R domain), which typically constitutes an integrated domain at the C-terminal end of the NRPS assembly line. Both two-electron and four-electron reductions of the PCP-bound peptidyl thioester have been observed. Two-electron reductive release via formation of a peptide aldehyde intermediate occurs in the biosynthesis of saframycin A, anthramycin, and linear gramicidin A.[4] For the latter NRP, the two-electron reductive release is followed by another two-electron reduction, which is catalyzed by an external R domain, leading to the observed C-terminal ethanolamine moiety of the final product (Fig. 4). By contrast, lyngbyatoxin A is reductively released from the biosynthetic template as a peptidyl

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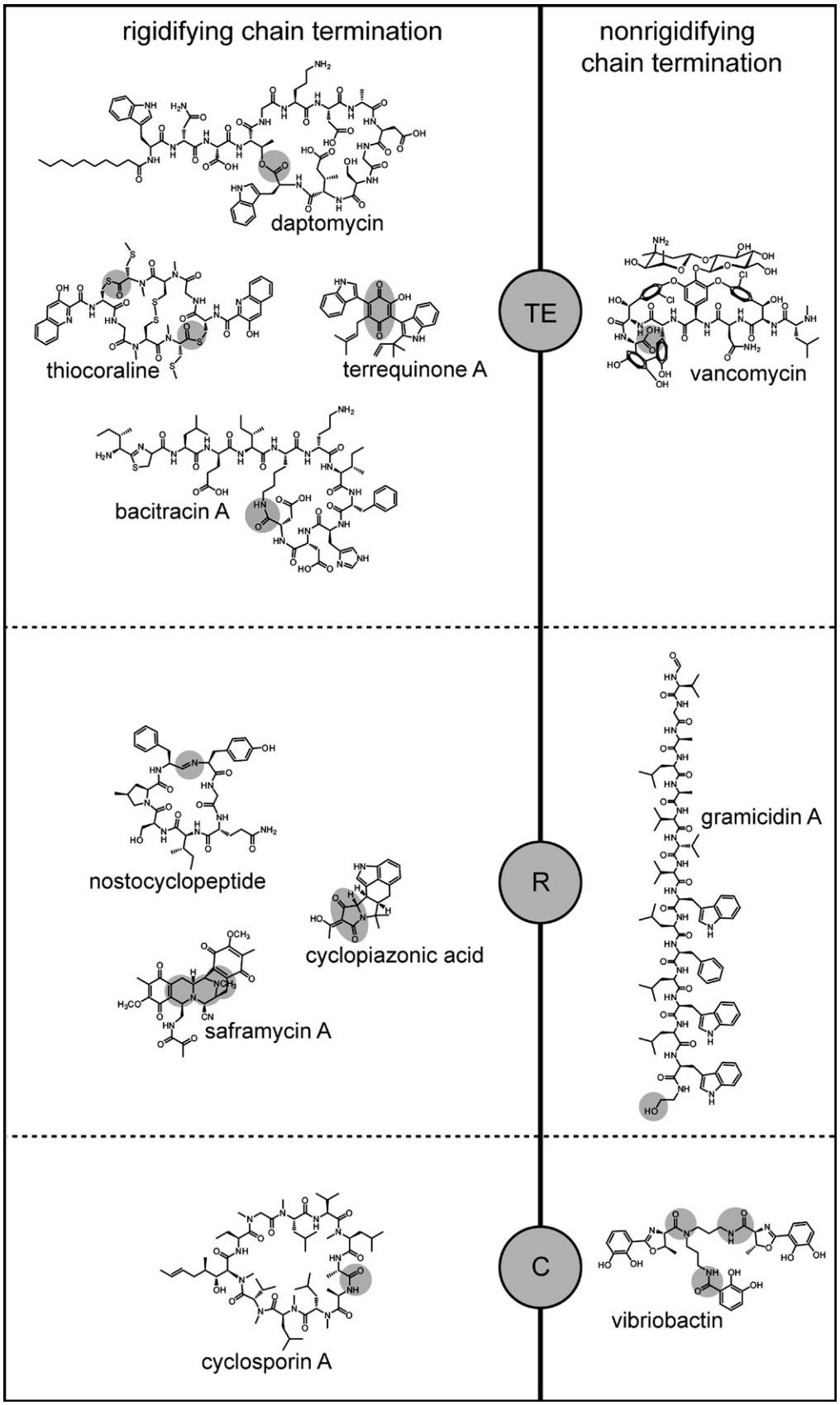

FIGURE 4 NRP diversification by chain termination. An overview of characteristic NRPs that represent specific chain termination strategies in NRP biosynthesis is shown. These chain termination strategies are subdivided into rigidifying (left) and nonrigidifying (right). Further, the NRPs are categorized by the termination domain that is involved in the chain release of the respective peptidic product. Finally, the structural motif that is associated with NRP chain release is highlighted by shading.

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alcohol by the catalytic action of a single integrated R domain that carries out a four-electron reduction.[4]

In many cases, R domain-catalyzed reductive release is followed by various crosslinking events that lead to a substantial rigidification of the initially released linear product. For example, in saframycin A biosynthesis, the reductively released aldehyde species form the substrates of the iterative Pictet–Spengler reactions that build up the rigid polycyclic tetrahydroisoquinoline core of the antibiotic (Fig. 4).[14] Even peptide macrocyclization can be triggered by an R domain as shown for the cytotoxin nostocyclopeptide.[15] After product release via a two-electron reduction of the PCP-bound peptidyl thioester, the linear heptapeptide aldehyde undergoes spontaneous formation of a macrocyclic imine (Fig. 4). Experiments with synthetic peptide aldehydes indicate that cyclic imine formation occurs in the absence of the R domain and is solely driven by the autonomous folding of the linear precursor into a precyclic conformation.[15] By contrast, direct involvement of the R domain in chain-terminating cyclization reactions occurs in the biosynthesis of equisetin, tenellin, and cyclopiazonic acid.[4] Here, chain release is mediated by a redox-inactive R domain that catalyzes the formation of a five-membered ring via Dieckmann condensation (Fig. 4). The nonrequirement of a reducing cofactor for this type of chemistry also rationalizes the existence of TE domains that act as Dieckmann cyclases as observed in the biosynthesis of the polyketide aflatoxin.[4] In addition to the aforementioned chain termination strategies, diversifying NRP off-loading mechanisms also include chemistries such as diketopiperazine formation and α-oxoamine formation, which have been reviewed by Du and Lou[4] as well as Walsh and Fischbach.[34]

The biotechnological potential of chain-terminating NRPS domains is enormous. Bypassing the selectivity restrictions imposed by A and C domains, researchers have used excised TE domains in combination with solid phase peptide synthesis to create large libraries of macrocyclic peptides.[13] This chemoenzymatic approach is ideal for the high throughput screening of NPSs for improved bioactivity. Lead peptides could then be translated back into reengineered NRPSs for large-scale production by fermentation.

In conclusion, the enormous structural complexity of NRPs originates from a total of five biosynthetic events: 1. Enzymatic pathways that contribute to monomer diversification; 2. Starter C-domain-mediated complexity generation during the initiation of NRP biosynthesis; 3. Diversification by various C-domain subtypes while the peptide is elongated on the NRPS assembly line; 4. Chain-terminating complexity generation by various enzymatic peptide release mechanisms; 5. Postsynthetic tailoring to complete the biosynthesis of the released peptide. These many levels of complexity generation make this biosynthetic system a unique tool for the generation of tailored drug scaffolds. By using methods such as precursor-directed biosynthesis, mutasynthesis, directed protein evolution, domain/module swapping, and chemoenzymatic NRP reengineering, researchers have already begun to rationally manipulate each of these biosynthetic steps with the eventual goal to improve the pharmacological properties of NRP natural products.

REFERENCES

1. Baltz RH, Miao V, Wrigley SK. Natural products to drugs: daptomycin and related lipopeptide antibiotics. *Nat Prod Rep* 2005;**22**:717–41.
2. Borel JF, Baumann G, Chapman I, Donatsch P, Fahr A, Mueller EA, et al. In vivo pharmacological effects of ciclosporin and some analogues. *Adv Pharmacol* 1996;**35**:115–246.
3. Caboche S, Leclere V, Pupin M, Kucherov G, Jacques P. Diversity of monomers in nonribosomal peptides: towards the prediction of origin and biological activity. *J Bacteriol* 2010;**192**:5143–50.
4. Du L, Lou L. PKS and NRPS release mechanisms. *Nat Prod Rep* 2010;**27**:255–78.
5. Duerfahrt T, Eppelmann K, Muller R, Marahiel MA. Rational design of a bimodular model system for the investigation of heterocyclization in nonribosomal peptide biosynthesis. *Chem Biol* 2004;**11**:261–71.
6. Fischbach MA, Walsh CT. Assembly-line enzymology for polyketide and nonribosomal Peptide antibiotics: logic, machinery, and mechanisms. *Chem Rev* 2006;**106**:3468–96.
7. Frueh DP, Arthanari H, Koglin A, Vosburg DA, Bennett AE, Walsh CT, et al. Dynamic thiolation-thioesterase structure of a non-ribosomal peptide synthetase. *Nature* 2008;**454**:903–6.
8. Hojati Z, Milne C, Harvey B, Gordon L, Borg M, Flett F, et al. Structure, biosynthetic origin, and engineered biosynthesis of calcium-dependent antibiotics from Streptomyces coelicolor. *Chem Biol* 2002;**9**:1175–87.
9. Hoyer KM, Mahlert C, Marahiel MA. The iterative gramicidin s thioesterase catalyzes peptide ligation and cyclization. *Chem Biol* 2007;**14**:13–22.
10. Imker HJ, Krahn D, Clerc J, Kaiser M, Walsh CT. N-acylation during glidobactin biosynthesis by the tridomain nonribosomal peptide synthetase module GlbF. *Chem Biol* 2010;**17**:1077–83.
11. Keating TA, Marshall CG, Walsh CT, Keating AE. The structure of VibH represents nonribosomal peptide synthetase condensation, cyclization and epimerization domains. *Nat Struct Biol* 2002;**9**:522–6.
12. Koglin A, Walsh CT. Structural insights into nonribosomal peptide enzymatic assembly lines. *Nat Prod Rep* 2009;**26**:987–1000.
13. Kohli RM, Walsh CT. Enzymology of acyl chain macrocyclization in natural product biosynthesis. *Chem Commun (Camb)* 2003:297–307.
14. Koketsu K, Watanabe K, Suda H, Oguri H, Oikawa H. Reconstruction of the saframycin core scaffold defines dual Pictet–Spengler mechanisms. *Nat Chem Biol* 2010;**6**:408–10.
15. Kopp F, Mahlert C, Grunewald J, Marahiel MA. Peptide macrocyclization: the reductase of the nostocyclopeptide synthetase triggers the self-assembly of a macrocyclic imine. *J Am Chem Soc* 2006;**128**:16478–9.
16. Kraas FI, Helmetag V, Wittmann M, Strieker M, Marahiel MA. Functional dissection of surfactin synthetase initiation module reveals insights into the mechanism of lipoinitiation. *Chem Biol* 2010;**17**:872–80.

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17. Magarvey NA, Ehling-Schulz M, Walsh CT. Characterization of the cereulide NRPS alpha-hydroxy acid specifying modules: activation of alpha-keto acids and chiral reduction on the assembly line. *J Am Chem Soc* 2006;**128**:10698–9.
18. Marshall CG, Hillson NJ, Walsh CT. Catalytic mapping of the vibriobactin biosynthetic enzyme VibF. *Biochemistry* 2002;**41**:244–50.
19. Miller DA, Walsh CT, Luo L. C-methyltransferase and cyclization domain activity at the intraprotein PK/NRP switch point of yersiniabactin synthetase. *J Am Chem Soc* 2001;**123**:8434–5.
20. Neumann CS, Fujimori DG, Walsh CT. Halogenation strategies in natural product biosynthesis. *Chem Biol* 2008;**15**:99–109.
21. Nolan EM, Walsh CT. How nature morphs peptide scaffolds into antibiotics. *ChemBioChem* 2009;**10**:34–53.
22. Patel HM, Walsh CT. In vitro reconstitution of the *Pseudomonas aeruginosa* nonribosomal peptide synthesis of pyochelin: characterization of backbone tailoring thiazoline reductase and N-methyltransferase activities. *Biochemistry* 2001;**40**:9023–31.
23. Rausch C, Hoof I, Weber T, Wohlleben W, Huson DH. Phylogenetic analysis of condensation domains in NRPS sheds light on their functional evolution. *BMC Evol Biol* 2007;**7**:78.
24. Reimmann C, Patel HM, Serino L, Barone M, Walsh CT, Haas D. Essential PchG-dependent reduction in pyochelin biosynthesis of Pseudomonas aeruginosa. *J Bacteriol* 2001;**183**:813–20.
25. Robbel L, Hoyer KM, Marahiel MA. TioS T-TE–a prototypical thioesterase responsible for cyclodimerization of the quinoline- and quinoxaline-type class of chromodepsipeptides. *FEBS J* 2009;**276**:1641–53.
26. Roche ED, Walsh CT. Dissection of the EntF condensation domain boundary and active site residues in nonribosomal peptide synthesis. *Biochemistry* 2003;**42**:1334–44.
27. Roy RS, Gehring AM, Milne JC, Belshaw PJ, Walsh CT. Thiazole and oxazole peptides: biosynthesis and molecular machinery. *Nat Prod Rep* 1999;**16**:249–63.
28. Samel SA, Schoenafinger G, Knappe TA, Marahiel MA, Essen LO. Structural and functional insights into a peptide bond-forming bidomain from a nonribosomal peptide synthetase. *Structure* 2007;**15**:781–92.
29. Schoenafinger G, Schracke N, Linne U, Marahiel MA. Formylation domain: an essential modifying enzyme for the nonribosomal biosynthesis of linear gramicidin. *J Am Chem Soc* 2006;**128**:7406–7.
30. Stachelhaus T, Walsh CT. Mutational analysis of the epimerization domain in the initiation module PheATE of gramicidin S synthetase. *Biochemistry* 2000;**39**:5775–87.
31. Strieker M, Tanovic A, Marahiel MA. Nonribosomal peptide synthetases: structures and dynamics. *Curr Opin Struct Biol* 2010;**20**:234–40.
32. Velkov T, Horne J, Scanlon MJ, Capuano B, Yuriev E, Lawen A. Characterization of the *N*-methyltransferase activities of the multifunctional polypeptide cyclosporin synthetase. *Chem Biol* 2011;**18**:464–75.
33. Walsh CT, Chen H, Keating TA, Hubbard BK, Losey HC, Luo L, et al. Tailoring enzymes that modify nonribosomal peptides during and after chain elongation on NRPS assembly lines. *Curr Opin Chem Biol* 2001;**5**:525–34.
34. Walsh CT, Fischbach MA. Natural products version 2.0: connecting genes to molecules. *J Am Chem Soc* 2010;**132**:2469–93.
35. Zhang W, Ntai I, Bolla ML, Malcolmson SJ, Kahne D, Kelleher NL, et al. Nine enzymes are required for assembly of the pacidamycin group of peptidyl nucleoside antibiotics. *J Am Chem Soc* 2011;**133**:5240–3.

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Chapter 22

Peptaibols

César de la Fuente-Núñez, L. Whitmore and B.A. Wallace

ABSTRACT

The peptaibols are a large family (>440 members identified to date) of homologous linear peptides isolated from soil fungi, ranging in length from 5 to 20 amino acids. They are characterized as having a high content of α-Aminoisobutyric acid (Aib) residues and generally have both N-terminal (mainly acetyl groups) and C-terminal modifications in the form of amino alcohol groups rather than amino acids. Peptaibols are nonribosomally synthesized on large protein complexes that permit the incorporation of nonstandard amino acids. During the process of peptide chain elongation, some of the synthetase modules are not absolutely specific for amino acid type, resulting in a diversity of isoforms being produced by a single organism. Most members of the family show antibacterial or antifungal activity, which has been attributed to their ability to induce membrane permeability by forming multimeric helical bundle ion channels. Some peptaibols possess antiviral and antitumor activities and promote wound healing.

INTRODUCTION

The naturally occurring peptaibols are a family of short peptides isolated from soil filamentous fungi, especially from members of the genus *Trichoderma*. This chapter represents an update of the previous version found in the first edition of this Handbook.[30] The name peptabiol derives from the chemical composition of these molecules: pept<u>ides</u> containing <u>Aib</u> (α-Aminoisobutyric acid) residues and ending in a C-terminal alcoh<u>ol</u>. They are mainly distinguished by compositions consisting of a large number of "nonstandard" amino acid residues, with Aib accounting for a high proportion of all residues in each of the sequences (for examples of some peptaibol sequences, see Fig. 1). Peptaibols are synthesized on large protein synthetase complexes rather than ribosomally, which permits the inclusion of nonstandard, noncoded residues. As would be expected for a family of peptides with a single dominant residue, there is considerable sequence identity between different peptaibols, especially between peptides isolated from the same biological source. It is possible to group the peptaibol sequences from a range of biological sources into subfamilies according to their sequences. At present, >440 peptaibol sequences have been identified,[8,28,30] although high-resolution structures are only available for a few of them.[1,29] All those structures are primarily helical, as would be expected because Aib is a strongly helix-promoting residue. The helical nature of these peptides plays a key role in their biological activity, which seems to be primarily based on their ability to form helical bundles that facilitate ion transport/leakage across membranes. This activity can be harmful to organisms and can cause cell death. Some authors argue that fungi produce peptaibols as a passive defense mechanism. As a result, many peptaibols are effective antibiotics, with varying degrees of activity against different target microbes. An online searchable database of natural peptaibol sequences, structures, nomenclature, and literature references[28] may be found at http://peptaibol.cryst.bbk.ac.uk although these have not been updated recently. The numbers presented in this chapter have been updated based on recent literature searches.

CHARACTERISTIC FEATURES OF THE PEPTAIBOLS

An important feature of peptaibol sequences is the presence of a high proportion of α,α-di-substituted amino acids, primarily Aib. The Aib residue, also known as α-methylalanine, has two methyl groups attached to its α-carbon atom, making it more hydrophobic and conformationally constrained than the standard (ribosomally encoded) L-alanine and thus promoting the formation of helical structures (Fig. 2). Another disubstituted residue found in peptaibols is isovaline (Iva), which is also known as methylethylalanine. Percentages and locations of Aib residues in peptaibol sequences vary among different members of the same family.[29,30] However, no peptaibols have as yet been discovered with a C-terminal hydroxylated Aib residue. Similarly, in the longer sequences, Aib has not yet been found at positions 18, 19, or 20. On average, almost 40% of peptaibol residues in the currently known peptaibols are Aib, and >3% are isovaline (Iva) or α-ethylalanine; the upper and

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00022-1
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lower ranges for Aib composition of individual sequences are 56% and 14%, respectively. Some trends regarding Aib content have been identified. Thus, shorter peptaibols tend to contain greater proportions of Aib than do longer ones. The distribution of residues Aib-neighboring residues is not random, as certain pairs of residues occur more frequently than expected statistically. A prime example of this is that virtually all proline residues in peptaibols (>98%) have Aib as their N-terminal neighbor; the Aib–Pro peptide bond forms the characteristic kink that is often seen in the peptaibol structures. Furthermore, 100% of nonproteinogenic hydroxyproline (Hyp) residues are preceded by disubstituted amino acids (75% Aib and 25% isovaline).

Although Aib is the most common noncoded residue to occur in peptaibols, it is not the only one. The imino acid hydroxyproline comprises >2% of all residues, and etnorvaline

```
a)     Ac Phe Aib Aib Aib Iva Gly Leu Aib Aib Hyp Gln Iva Pro Aib Pro Phe OH
b)     Ac Aib Gly Aib Leu Aib Glu Aib Aib Aib Ala Aib Aib Pro Leu Aib Iva Gln Val OH
c) Ac Aib Pro Aib Ala Aib Ala Gln Aib Val Aib Gly Leu Aib Pro Val Aib Aib Gln Gln Phe OH
d)                                Ac Aib Asn Ile Ile Aib Pro Leu Leu Aib Pro Ile OH
e)     Ac Leu Aib Leu Aib Phe OH
```

FIGURE 1 Examples of sequences of peptaibols of different lengths (a) antiamoebin II, (b) trichotoxin A_40, (c) alamethicin F_50, (d) trichorovin TV_XIIa, and (e) peptaibolin. α-Aminoisobutyric acid (Aib) residues are shaded in light gray, and proline (Pro) residues are shaded in darker gray.

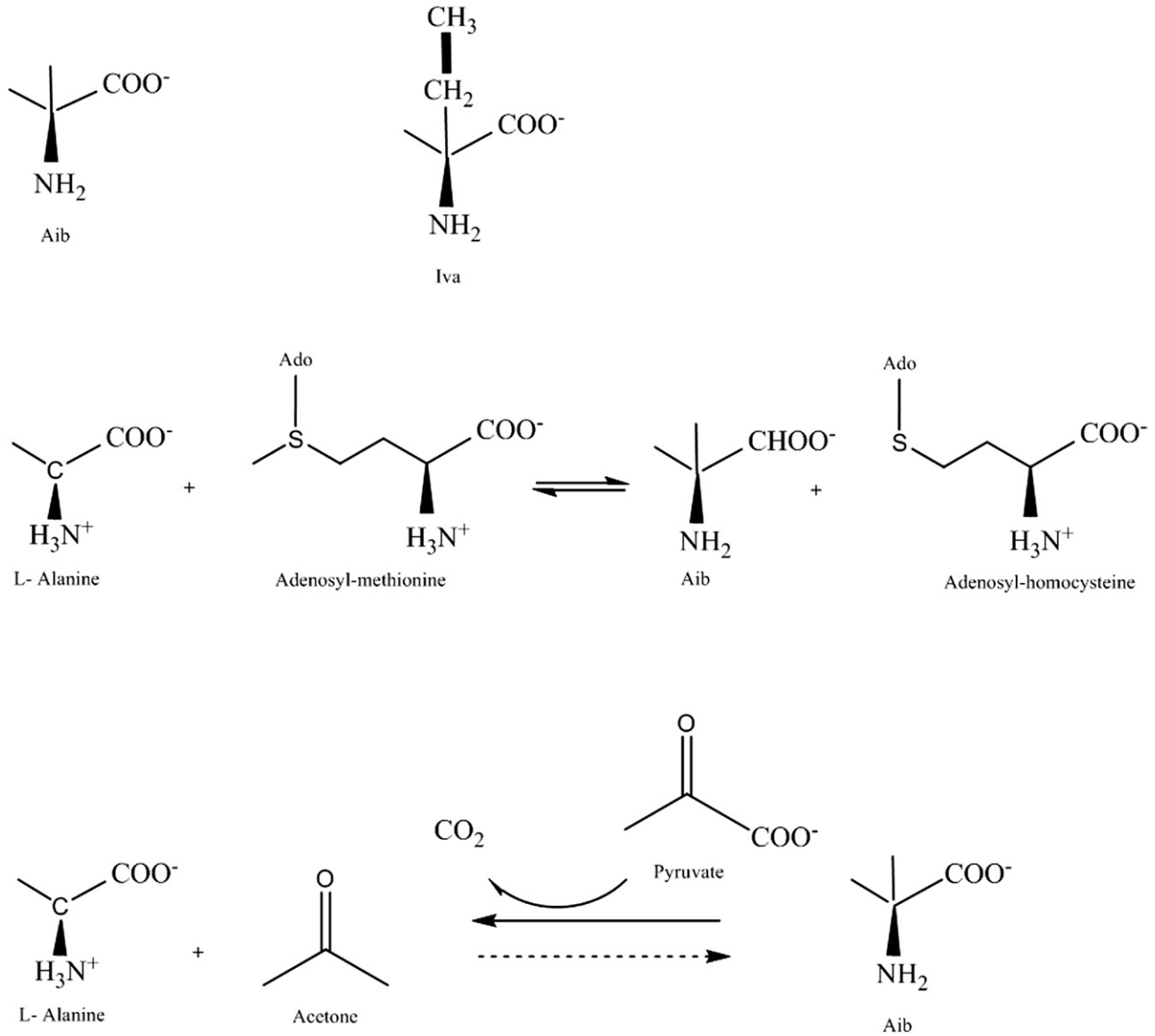

FIGURE 2 (a) Chemical structures of Aib and Iva residues and (b) possible enzymatic reactions leading to the formation of Aib (Adapted from Ref. 14).

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(α-amino-α-ethyl-n-pentanoic acid) and 2-amino-6-hydroxy-4-methyl-8-oxodecanoic acid (AHMO) are also rarely found (in <10 examples across all known peptaibols).

In addition to containing these nonstandard amino acids, peptaibol sequences also have a distinctive lack of many charged and polar amino acids.[29] Although the functionally important glutamine is abundant in peptaibol sequences, constituting around 6% of the residues and featuring in >65% of the sequences, there are as yet no known peptaibols containing arginine, asparagine, cysteine, histidine, lysine, or methionine residues, and there is only one example of a tyrosine-containing peptaibol. Glutamines (or occasionally glutamic acids) are found at specific locations, primarily at position 6 or near the C-terminus; the position and number of these polar residues seem to be related to their functional properties as ion channels.

Prolines are also frequently found, accounting for >7% of all peptaibol residues and are often located near the middle of the sequences. These residues tend to break helical structures because of the absence of the amino protons needed to form intramolecular hydrogen bonds. It has been proposed that they may be responsible for producing kinks or bends in helices or β-turns, which may be related to their ion channel gating properties.[5]

Aromatic residues are found infrequently in peptaibols, comprising approximately 4% of the total amino acid residue content. They tend to cluster at the termini, where they are thought to contribute to stabilize the transmembrane orientations of the peptide chains within the bilayer, in a manner similar to the mechanism proposed for the transmembrane segments of membrane proteins.[27]

A further sequence feature of peptaibols is that both their N- and C-termini tend to be modified. The N-terminal amino acids are typically attached to an acyl group, although other aliphatic groups, such as octyl groups in the trikoningins,[29,30] have also been reported. The C-terminal residues, however, generally have their carbonyl groups reduced to hydroxyl groups, explaining why they are amino alcohols rather than amino acids. Interestingly, clonostachin is a peptaibol that has an ester-linked sugar alcohol terminal group.[4]

Sequence length is often considered to be a defining feature of peptaibols. The shortest known natural peptaibol, peptaibolin, is five residues long.[13] The longest ones are 20 residues long. Of all known peptaibols, a substantial proportion of sequences (>30%) have 20 residues, and the distribution of sequence size shows a decreasing trend with decreasing peptide size (Fig. 3). The predominance of longer sequences probably results from the role of these peptides as transmembrane permeabilizers. Remarkably, even-numbered members predominate over odd-numbered ones, with the exception of the highly populated 11-residue group (which might be found more frequently than expected because its size corresponds approximately to a half-bilayer spanning helix).[29]

BIOLOGICAL ORIGINS AND BIOSYNTHESIS OF PEPTAIBOLS

All known peptaibols have been isolated from soil fungi or molds. The majority (>50%) have been isolated from various *Trichoderma* species.[6,16,18–20,24] Others come from other fungal species, including *Hypocrea peltata*,[28]

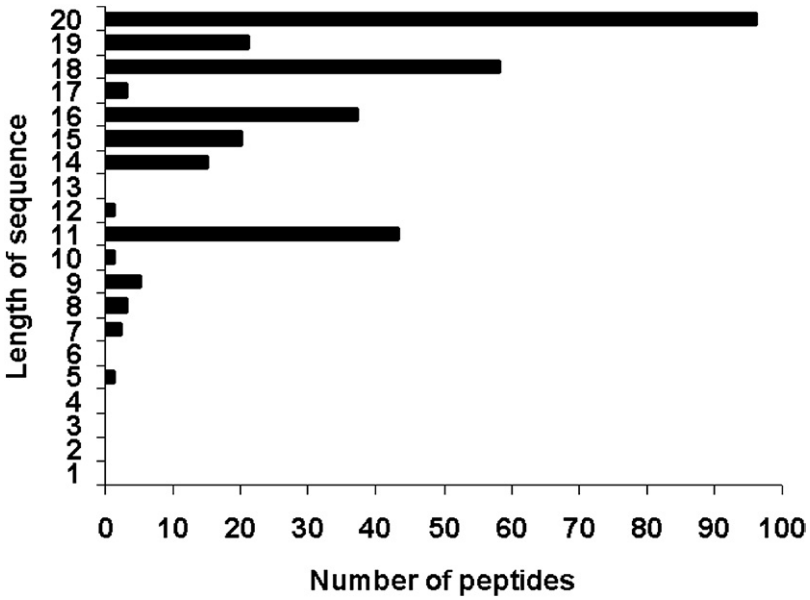

FIGURE 3 Distribution of peptaibol sequence lengths as of 2004 (after Ref. 29).

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Clonostachys,[4] *Apiocrea chrysosperm*,[9] *Sepedonium ampullosporum*,[21,30] *Acremonium tubakii*,[25] *Mycogone cervina*,[32] *Mycogone rosea*,[30] and *Verticimonosporium ellipticum.*[28] Some particular sequences are known to be produced by different organisms. Antiamoebins, for example, have been isolated from at least three different species: sequences I–V are from *Emericellopsis poonensis*, *Emericellopsis synnematicola*, and *Cephalosporium pimprina*,[30] whereas sequences VI–XVI are obtained from *Stilbella erythrocephal*, *Stilbella fimetaria*, and *Gliocladium catenulatum.*[15]

Peptaibols are not formed ribosomally. Instead, they are synthesized by multidomain enzymes on large nonribosomal peptide synthetase (NRPS) complexes[30] (Fig. 4). The peptaibols are assembled by these NRPSs from various precursor units, responsible for adding both standard and nonstandard amino acids to the peptide sequence. The synthetases have a modular structure, with different modules independently responsible for recognizing, activating, and modifying specific consecutive residues. Each module can be further organized into different adenylation, thiolation, and condensation domains depending on the specific reaction they catalyze. The number of modules usually correlates with the number of amino acid residues present in the peptide sequence. Synthetase complexes have been cloned and expressed, and depending on the modules present, used to produce defined peptaibol molecules.[31] Some modules seem to be specific for particular residues, whereas others have two or three specificities and will attach residues according to their availability within the growth medium.[21] This variable specificity leads to the microheterogeneity of the peptaibols extracted from a given organism. Also, there are indications that other factors may modify the peptaibol sequence. Raap et al.[23] showed that zervamicins produced in a medium with labeled isovaline contained both labeled isovaline and labeled Aib residues. The tendency of these multidomain complexes to synthesize peptaibols with an even number of residues, pairwise distribution of amino acids and longer sequences, indicates that the biological role of these peptides is dictated by these large nonribosomal complexes.

Postsynthesis modifications of peptaibols may also contribute to define specific characteristics. Sharman et al.[28] have shown that XR586, a peptaibol-like peptide from *Acremonium persicinum*, is sequentially very similar to the zervamicins, although it terminates in the sequence Phe–Gly (i.e., with an extra Gly), rather than the Phe residue common to the zervamicins. This might suggest that the final stage of synthesis of some peptaibols could include a cleavage enzyme that is not present in all organisms.

PEPTAIBOL STRUCTURES

Various methods have been employed to group peptaibols according to their sequences. The original classification method was based on sequence length and terminal groups.

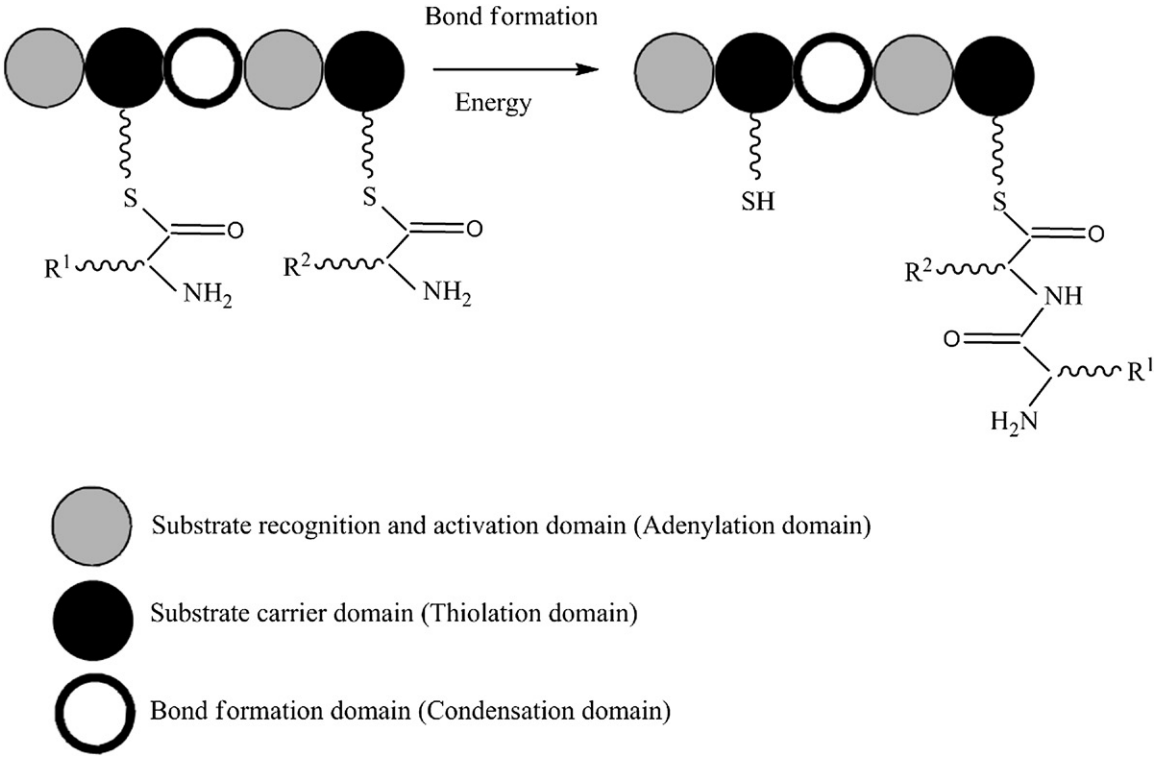

FIGURE 4 *In vivo* posttranslational synthesis of peptaibols by nonribosomal peptide synthetases (NRPS), after Ref. 17.

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Accordingly, groupings were established as "long," "short," and "lipopeptaibol." The long peptaibols are characterized by a sequence length of 18–20 residues (e.g., saturnisporins). Short peptaibols have sequence lengths in the range of 11–16 residues and include the trichorovins[28] (11 residues) and heptaibin[14] (15 residues). It should be noted that this classification was made before the identification of 17 residue peptaibols such as cephaibols P and Q.[25] Lipopeptaibols are 11 residues or shorter and have fatty acids such as octanoic acid at their N-termini, as exemplified by trichogin A IV.[2]

A classification method based on sequence homology was devised by Chugh and Wallace in 2001.[5] Homology grouping into subfamilies, with special attention to functionally important residues, had the advantage of being relatively sequence length-independent, and tended to cluster family members with similar functional properties. A total of nine distinct subfamilies with sequence identities of >50% were identified. This classification makes it clear that closely related sequences reflect a naturally occurring pool of "mutants" with slightly modified functional properties, which can be used in structure/function studies.

In contrast to the wealth of information available about peptaibol sequences, there are relatively few published peptaibol structures with complete coordinates. This is probably related to the fact that peptaibols fall in the intermediate size category, smaller than the macromolecular structures traditionally found in the Protein Data Bank, and larger than the small molecule structures deposited in the Cambridge Structural Data Bank. There are only a limited number of X-ray structures and even fewer deposited NMR structures (http://peptaibol.cryst.bbk.ac.uk/structure.htm). For one of the peptaibols, antiamoebin I, two crystal structures (in different solvents) and an NMR structure have been identified. For zervamicin IIb, there are two NMR structures in different environments (one from an isotropic solvent and one in micelles) and a crystal structure for a modified version of this peptaibol. The observation that different peptaibol structures occur in different environments suggests flexibility and adaptability. The dearth of structures may reflect difficulties for crystallizing membrane-active peptides and solving intermediate-sized crystal structures,[28] as well as for determining structures of peptides with high redundancy of residue types from NMR data.

Less detailed structural information under various solvent conditions and/or in lipid bilayers or micelles has been frequently obtained using circular dichroism (CD) spectroscopy. CD information is available for hypelcins, trichorzianines, alamethicin,[3] paracelsin, trichogins, trikoningins, trichotoxin A-40, chrysospermins, trichosporins, saturnisporins, harzianins, trichologins, antiamoebin, and XR586, a peptaibol-like antibiotic molecule [http://peptaibol.cryst.bbk.ac.uk/singlerecords/XR586.html]. They all have common spectral shapes typical of helical structures, featuring negative peaks at approximately 205–209 and 221–226 nm and positive peaks at approximately 192–198 nm. These broad ranges reflect the total span of peak positions quoted in the literature. As the structures are highly constrained by extensive hydrogen-bonded networks, they tend to show little solvent dependence, although their spectra (especially the peak positions) do exhibit a significant dependence on solvent permanent dipole (dielectric constant).

All the peptaibol structures determined to date include helical conformations, although they exhibit considerable structural diversity in the type of helix present (3_{10} or α) and in whether they are straight or bent. The bending tends to be the result of the number and position of the proline or hydroxyproline residues present. Nonetheless, not all imino acid-containing peptaibols are bent. For example, the structure of trichotoxin, which has a central proline, is essentially a straight helix [http://peptaibol.cryst.bbk.ac.uk/trichotoxin1.htm].

FUNCTIONS AND ACTIVITIES OF PEPTAIBOLS

Functionally, most peptaibols exhibit varying antibacterial and antifungal properties against different target organisms. Thus, for example, although chrysospermins display activity against a range of bacteria, including Gram-negative *Escherichia coli*,[9] trikoningins inhibit the Gram-positive bacterium *Staphylococcus aureus*.[30] Antifungal behavior includes that of hypelcin A, which prevents the growth of *Lentinus edodes*, a Japanese edible mushroom and other fungi.[30]

The physical basis of peptaibol antibiotic properties seems to lie in their ability to permeabilize lipid bilayers. They form transmembrane pores and carriers and can also modify the membrane surface in a surfactant-like mode. When pores are formed in membranes, they generally take the form of voltage-gated ion channels.[10,11,22] Typically, voltage-dependent activity is usually associated with the longer peptaibols (that can directly span a bilayer) and has also been observed in peptaibols as short as 14 residues. The channels themselves are thought to be formed by several peptaibol molecules coming together to form a bundle,[10] which is then inserted into the membrane, creating a hydrophilic channel through its center[3] (Fig. 5). The longer peptaibols (16–20 residues), when in a helical conformation, have the appropriate length to span a lipid bilayer.[5,26] The number of peptide chains in the bundle differs depending on the peptaibol type, ranging from 6 to >12. The basis for the antimicrobial activity of these compounds can be explained by the leakage of cytoplasmic material through the peptaibol-induced channels.

Somewhat surprisingly, the membrane activity of peptaibols is as pronounced in some short peptides as it is in longer ones. Auvin-Guette et al.[2] reported that among the trikoningins, the 11-residue lipopeptaibols KB I and

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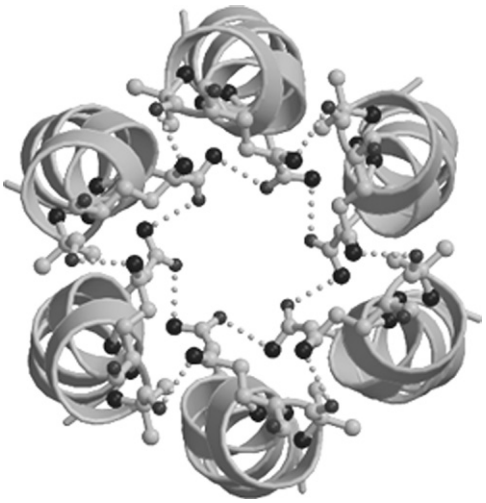

FIGURE 5 End-on view of the hexameric bundle pore structure of trichotoxin, after Ref.10.

KB II show similar antibacterial activity against *Staphylococcus aureus* as does the 19- residue kA V. Similarly, the 11-residue trichogin A IV was shown to have significant membrane-perturbing properties in experiments measuring carboxyfluorescein leakage from egg phosphatidylcholine unilamellar vesicles.[2] Clearly, the shorter peptaibols could not span a membrane in the same way as the longer ones. There are a variety of potential mechanisms for these short peptides as discussed previously.[1,12] Two possible mechanisms of action could occur in these cases: either the shorter peptaibols form end-to-end bundles within the bilayer, thus effectively doubling up their length perpendicular to the bilayer, or, as suggested for the antimicrobial peptides, form membrane-associated aggregates, surface-associated "carpets," membrane spanning toroidal channels or act via a detergent-like mechanism.

Finally, although membrane permeabilization is clearly a feature common to all peptaibols, it remains uncertain whether this is their true biological function within the native organism. Hypothetically, they could act as an *in vivo* defense mechanism against other organisms with different membrane characteristics to their own. In fact, many of them exhibit differential activity when targeting different membrane lipid types.[10,11]

In summary, the peptaibols are a naturally occurring family of hydrophobic nonribosomally produced peptides that seem to have the capability of altering membrane permeability. They show considerable sequence diversity because of their multiple specificities displayed by nonribosomal peptide synthetases[5,7,31] and represent an excellent natural resource of "mutant" peptides, allowing the study of structure–activity relationships. The antibiotic properties of peptaibols seem to be related to ion channel formation.[5,22]

ACKNOWLEDGMENTS

This work was supported, in part, by grants from the UK Biotechnology and Biological Sciences Research Council to B.A. Wallace. C de la Fuente-Núñez received a scholarship from the Fundación "la Caixa" and Fundación Canadá (Spain). The original idea of creating a Peptaibol Database was suggested by Dr. Chris Snook, when he was a student in the Wallace Laboratory. The database was designed and is curated by Dr. Whitmore. Some updates of the database and curation were the work of Dr. Jasveen Chugh. We thank both Drs. Chugh and Snook for helpful discussions and Dr. Chugh for Fig. 4.

REFERENCES

1. Aravinda S, Shamala N, Balaram P. Aib residues in peptaibiotics and synthetic sequences: analysis of nonhelical conformations. *Chem Biodivers* 2008;**5**:1238–62.
2. Auvin-Guette C, Rebuffat S, Prigent Y, Bodo B. Trichogin A IV, an 11-Residue Lipopeptabiol from *Trichoderma longibrachiatum*. *J Am Chem Soc* 1992;**114**:2170–4.
3. Cascio M, Wallace BA. Conformation of alamethicin in phospholipid vesicles: implications for insertion models. *Proteins* 1988;**4**:89–98.
4. Chikanishi T, Hasumi K, Harada T, Kawasaki N, Endo A. Clonostachin, a novel peptaibol that inhibits platelet aggregation. *J Antibiot* 1997;**50**:105–10.
5. Chugh JK, Wallace BA. Peptaibols: models for ion channels. *Biochem Soc Trans* 2001;**29**:565–70.
6. Chutrakul C, Alcocer M, Bailey K, Peberdy JF. The production and characterisation of trichotoxin peptaibols, by *Trichoderma asperellum*. *Chem Biodivers* 2008;**5**:1694–706.
7. Daniel JF, Filho ER. Peptaibols of *Trichoderma*. *Nat Prod Rep* 2007;**24**:1128–41.
8. Degenkolb T, Brückner H. Peptaibiomics: towards a myriad of bioactive peptides containing C(alpha)-dialkylamino acids? *Chem Biodivers* 2008;**5**:1817–43.
9. Dornberger K, Ihn W, Ritzau M, Gräfe U, Schlegel B, Fleck WF, Metzger JW. Chrysospermins, new peptaibol antibiotics from *Apiocrea chrysosperma* Ap101. *Antibiot* 1995;**48**:977–89.
10. Duclohier H, Alder GM, Bashford CL, Brückner H, Chugh JK, Wallace BA. Conductance studies on trichotoxin_A50E and Implications for Channel Structure. *Biophys J* 2004;**87**:1705–10.
11. Duclohier H, Snook CF, Wallace BA. Antiamoebin can function as a carrier or as a pore-forming peptaibol. *Biochim Biophys Acta* 1998;**1415**:255–60.
12. Duclohier H. Antimicrobial peptides and peptaibols, substitutes for conventional antibiotics. *Curr Pharm Des* 2010;**16**:3212–23.
13. Hülsmann H, Heinze S, Ritzau M, Schlegel B, Gräfe U. Isolation and structure of peptaibolin, a new peptaibol from *Sepedonium* strains. *J Antibiot* 1998;**51**:1055–8.
14. Ishiyama D, Satou T, Senda H, Fujimaki T, Honda R, Kanazawa S. Heptaibin, a novel antifungal peptaibol antibiotic from *Emericellopsis sp.* BAUA8289. *J Antibiot* 2000;**53**:728–32.
15. Jaworski A, Brückner H. New Sequences and New Fungal Producers of Peptabiol Antibiotics Antimoebins. *J Pept Sci* 2000;**6**:149–67.
16. Kubicek CP, Komoń-Zelazowska M, Sándor E, Druzhinina IS. Facts and challenges in the understanding of the biosynthesis of peptaibols by *Trichoderma*. *Chem Biodivers* 2007;**4**:1068–82.
17. Llewellyn NM, Spencer JB. Biological chemistry: Enzymes line up for assembly. *Nature* 2007;**448**:755–6.

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18. Maddau L, Cabras A, Franceschini A, Linaldeddu BT, Crobu S, Roggio T, et al. Occurrence and characterization of peptaibols from *Trichoderma citrinoviride*, an endophytic fungus of cork oak, using electrospray ionization quadrupole time-of-flight mass spectrometry. *Microbiology* 2009;**155**:3371–81.
19. Mohamed-Benkada M, Montagu M, Biard JF, Mondeguer F, Verite P, Dalgalarrondo M, et al. New short peptaibols from a marine *Trichoderma* strain. *Rapid Commun Mass Spectrom* 2006;**20**:1176–80.
20. Mukherjee PK, Wiest A, Ruiz N, Keightley A, Moran-Diez ME, McCluskey K, et al. Two classes of new peptaibols are synthesized by a single non-ribosomal peptide synthetase of *Trichoderma virens*. *J Biol Chem* 2011;**286**:4544–54.
21. Neuhof T, Berg A, Besl H, Schwecke T, Dieckmann R, von Döhren H. Peptaibol production by *Sepedonium* strains parasitizing boletales. *Chem Biodivers* 2007;**4**:1103–15.
22. O'Reilly AO, Wallace BA. The peptaibol antiamoebin as a model ion channel: similarities to bacterial potassium channels. *J Pept Sci* 2003;**9**:769–75.
23. Raap J, Erkelens K, Ogrel A, Skladnev DA, Brückner H. Fungal biosynthesis of non-ribosomal peptide antibiotics and α, α-dialkylated amino acid constituents. *J Pept Sci* 2005;**11**:331–8.
24. Ruiz N, Wielgosz-Collin G, Poirier L, Grovel O, Petit KE, Mohamed-Benkada M, et al. New Trichobrachins, 11-residue peptaibols from a marine strain of *Trichoderma longibrachiatum*. *Peptides* 2007;**28**:1351–8.
25. Schiell M, Hofmann J, Kurz M, Schmidt FR, Vértesy L, Vogel M, et al. Cephaibols, new peptaibol antibiotics with anthelmintic properties from *Acremonium tubakii* DSM 12774. *J Antibiot* 2001;**54**:220–33.
26. Wallace BA. Common structural features in gramicidin and other ion channels. *Bioessays* 2000;**22**:227–34.
27. Wallace BA, Janes RW. Tryptophans in membrane proteins. X-ray crystallographic analyses. *Adv Exp Med Biol* 1999;**467**:789–99.
28. Whitmore L, Wallace BA. The Peptabiol Database: a database for sequences and structures of naturally occurring peptaibols. *Nucleic Acids Res* 2004;**32**:D593–4.
29. Whitmore L, Wallace BA. Analysis of peptaibol sequence composition: implications for in vivo synthesis and channel formation. *Eur Biophys J* 2004;**33**:233–7.
30. Whitmore L, Wallace BA. Peptabiols. In: Kastin AJ, editor. Handbook of biologically active peptides. Academic Press: 2006. p. 83–8.
31. Wiest A, Grzegorski D, Xu BW, Goulard C, Rebuffat S, Ebbole DJ, et al. Identification of peptaibols from *Trichoderma virens* and cloning of a peptaibol synthetase. *J Biol Chem* 2002;**277**:20862–8.
32. Wilhelm C, Anke H, Flores Y, Sterner O. New peptaibols from *Mycogone cervina*. *J Nat Prod* 2004;**67**:466–8.

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Chapter 23

Antifungal and Antibacterial Peptides of Fungal Origin

Tzi Bun Ng

ABSTRACT

Many diseases in humans, livestock, and agricultural crops are caused by fungi. Nonproteinaceous and proteinaceous/peptidic molecules are produced by humans and other living organisms to combat fungal assault. Much effort has been directed at isolating antifungal proteins and peptides from living organisms and making use of them in antifungal strategies such as producing transgenic crops overexpressing the antifungal proteins and peptides and thus acquiring enhanced resistance against pathogenic fungi. Antifungal peptides produced by fungi comprise peptaibols, which also display antibacterial activity, cyclic peptides, and linear peptides. Proteins with antifungal and antibacterial activities are also produced by fungi.

INTRODUCTION

Antifungal proteins are produced by humans, animals, plants, and microbes for protection against pathogenic fungi. A spectacular array of antifungal proteins with a diversity of molecular masses, N-terminal or complete amino acid sequences, and specificity, and mechanism of antifungal action exist. Some of the antifungal proteins also possess antibacterial activity. The intent of this chapter is to review antifungal peptides and antibacterial peptides of fungal origin (Table 1).

Peptaibols with Antifungal and Antibacterial Activities

Nine membrane-active peptaibols, designated as trichorzianines A, were isolated from *Trichoderma harzianum* cultures. They are characterized by an acetylated N-terminal amino acid, and L-tryptophanol or L-phenylalaninol at the C-terminal, and possession of seven to eight alpha-aminoisobutyric acid residues. The isovaline residue present has the D-configuration.[10] Two nonadecapeptide peptaibols, tricholongins BI and BII, expressing antifungal and antibacterial activities, have been isolated from *Trichoderma longibrachiatum* cultures. They possess mostly hydrophobic L-amino acids, eight to nine 2-aminoisobutyric acid residues, an acetylated N-terminal residue, and a C-terminal leucinol. Both peptides adopt a similar alpha-helical conformation with a hinge around Pro13 resulting from two 3(10) bonds. The results suggest that the N-terminus contains mixed alpha/3(10) bonds.[20] Four nonadecapeptide peptaibols with antibacterial and antifungal activities, designated as chrysospermins A, B, C, and D, were isolated from *Apiocrea chrysosperma* mycelia.[9] Ampullosporin was isolated from *Sepedonium ampullosporum* mycelia as a peptaibol with narrow-spectrum antibacterial and antifungal activities. It induced pigment formation by *Phoma destructiva*, elicited hypothermia and inhibited spontaneous locomotor activity in mice.[21]

Two linear 19-amino-acid peptaibols with antifungal and antibacterial activities, septocylindrin A and septocylindrin B, which are peptides with a modified phenylalanine C-terminus related to the membrane-channel-forming peptaibol alamethicin, were isolated from an isolate of the fungus *Septocylindrium* sp. The 18th residue, was Glu in septocylindrin A and Gln in septocylindrin B.[25] From liquid culture of a cork oak endophytic strain of *Trichoderma citrinoviride*, a mixture of peptaibols, 20-residue antifungal peptides with N-terminal acetylation and C-terminal phenylalaninol, was isolated. The peptide mixture shows remarkable toxicity in a brine shrimp bioassay.[18]

Trichogin GA IV from the fungus *Trichoderma longibrachiatum* is a protease-stable lipopeptaibol with attenuated hemolytic activity. It exhibits folding in a mixed 3(10)-/α-helical conformation with an amphiphilic character, possibly accounting for its ability to perturb bacterial membranes and to induce cell death. It displays antibacterial activity toward Gram-positive bacteria, especially methicillin-resistant *S. aureus*.[31]

Peptaibols trichorzianines A1 and B1, chitobiohydrolase, endochitinase, and beta-1,3-glucanase activities were found in culture supernatants of *Trichoderma harzianum*

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00023-3
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TABLE 1 Amino Acid Sequences of Peptides With Antifungal, Antibacterial and HIV-Reverse Transcriptase Inhibitory Activities

Peptide	Amino acid sequence
Ampullosporin[21]	Ac-Trp-Ala-Aib-Aib-Leu-Aib-Gln-Aib-Aib-Aib-Gln-Leu-Aib-Gln-Leuol
Russula paludosa peptide[27]	Lys-Arg-Glu-His-Gly-Gln-His-Cys-Glu-Phe
Cicadapeptin I[16]	Hyp-Hyp-Val-Aib-Gln-Aib-Leu
Cicadapeptin II[16]	Hyp-Hyp-Val-Aib-Gln-Aib-Ile
Penicillium canescens antifungal peptide	D-Phe-L-Val-D-Val-L-Tyr
Ulocladium atrum cyclopeptolide[29]	HPA-homoPro-NMeVal(1)-Val-NMeAsp-NMeIle(1)-NMeIle(2)-Gly-NMeVal(1)-OMeTyr
Isarfelin A[11]	Pro-Leu-Phe-Gly-Ala
Isarfelin B[11]	Pro-Val-Phe-Gly-Ala
Aspergillus clavatus antimicrobial peptide[3]	D-Phe-L-Val-D-Val-L-Tyr
Trichogin GA IV[31]	n-Oct-Aib-Gly-Leu-Aib-Gly-Gly-Leu-Aib-Gly-Ile-Lol
Cordycommunin[13]	Thr-Ala-Ala-Gln-Tyr-Val
Alternaramide[14]	Pro-Phe-Hiv-Pro-Phe
Septocylindrin A[25]	Ac-Aib-Pro-Aib-Ala-Gln-Aib-Val-Aib-Gly-Leu-Aib-Pro-Val-Aib-Aib-Glu-Gln-Phaol
Septocylindrin B[25]	Ac-Aib-Pro-Aib-Ala-Gln-Aib-Val-Aib-Gly-Leu-Aib-Pro-Val-Aib-Aib-Gln-Glu-Phaol
Aspergillus giganteus antifungal peptide[17]	Asp-Glu-Ser-Ala-Val-Leu-Ala-Thr-….

mycelia cultivated on glucose as the only carbon source and transferred to fresh medium with *Botrytis cinerea* cell walls. The culture supernatants impede spore germination and hyphal elongation of *B. cinerea*. Culture supernatants from mycelia cultured in fresh medium in the absence of *B. cinerea* cell walls do not show hydrolase activities, synthesis of peptaibol-like compounds, or suppression of fungal growth. Purified trichorzianines A1 and B1, chitobiohydrolase, endochitinase, or beta-1,3-glucanase inhibited spore germination and hyphal elongation. There is a synergism between the antifungal actions of the enzymes and peptaibols.[23] Also see the peptaibol chapter in the Bacterial/Antibacterial Peptides section of the book.

Cyclic Antifungal Peptides

Isarfelin, a cyclic peptide with inhibitory activity toward the fungi *Rhizoctonia solani* and *Sclerotinia sclerotiorum* and insecticidal activity toward *Leucania separata* but devoid of antibacterial activity, was isolated from *Isaria felina* mycelia. The isolation procedure involved ethanol extraction, adsorption on YPR II macropore adsorption resin, ethyl acetate extraction, petroleum ether precipitation, and recrystallization from ethyl acetate.[11]

The saprophytic fungus *Ulocladium atrum Preuss*, promising for biocontrol of *Botrytis cinerea* on crops cultivated in the greenhouse and the field, produces a cyclopeptolide characterized by an abundance of N-methylated amino acids and potent antifungal activity against *Botrytis cinerea* and moderate activity against *Alternaria alternata* and *Magnaporthe grisea.*[29]

A cyclic depsipeptide, eujavanicin A, which suppresses growth in the human pathogenic filamentous fungus *Aspergillus fumigatus*, was isolated from *Eupenicillium javanicum.*[19]

The filamentous soil fungus *Coleophoma empetri* F-11899 produces a water-soluble echinocandin type 1 antifungal lipopeptide FR901379, the original source of micafungin used in therapy of deep-seated mycoses. FR901379 consists of a cyclic hexapeptide nucleus with a sulfonated and fatty acid acylated N-terminus. FR901379 exerts its activity against *Aspergillus* and *Candida*

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species by inhibiting 1,3-b-glucan and hence fungal cell wall synthesis.[28]

A novel cyclodepsipeptide, cordycommunin, with growth retarding activity toward *Mycobacterium tuberculosis* H37Ra and weak cytotoxicity toward KB cells, was isolated from the entomopathogenic fungus *Ophiocordyceps communis.*[13]

Other Antifungal Peptides

A thermostable 5773-Da antifungal peptide was purified from an indigenous fungal strain (VR) of *Aspergillus clavatus* by ultrafiltration and reverse phase-HPLC on a C18 column. It inhibits mycelial growth of *Alternaria solani*, *Aspergillus niger*, *Botrytis cinerea*, *Fusarium oxysporum*, and *F. solani*, but it has no effect on growth in yeasts and bacteria.[24]

A new tetrapeptide D-Phe-L-Val-D-Val-L-Tyr, with antifungal activity similar to that of the commercial product benomyl against the soybean pathogen *Fusarium virguliforme*, was isolated from a *Penicillium canescens* culture collected from beehive pollen.[3]

Penicillium antifungal protein (PAF) from *Penicillium chrysogenum* is a small cationic protein devoid of toxicity on mammalian cells and is thus a drug candidate for treating the usually fatal Aspergillus infections in humans. Its mechanism of antifungal action entails G-protein-coupled signaling followed by apoptosis of sensitive fungi. Its solution structure has been determined by nuclear magnetic resonance. PAF comprises five beta-strands forming two orthogonally packed beta-sheets that share a common interface. Its highly conserved and positively charged lysine-rich surface region contributes to an augmented toxicity.[2]

A killer toxin with a molecular mass <3 kDa and antifungal activity against *Penicillium expansum* strain #2 was partially purified by normal phase HPLC, using TSK-gel Amide-80 analytical and preparative columns from cell-free culture supernatants of *Pichia ohmeri 158* and *Candida guilliermondii P3*.[8] The basic, low-molecular-weight antifungal protein from *Aspergillus giganteus* is secreted as an inactive precursor with a six amino-acid extension at the NH_2-terminal and shows antifungal activity against phytopathogens *in vitro* and *in vivo*.[17]

The (2–10) kDa protein fraction of the culture supernatant of *Saccharomyces cerevisiae* CCMI 885 contained antimicrobial peptides with fungistatic effect against *Hanseniaspora guilliermondii, Kluyveromyces thermotolerans,* and *Torulaspora delbrueckii,* and fungicidal activity against *Kluyveromyces marxianus.*[1]

A 6.0-kDa thermostable, protease-sensitive, cysteine-rich, basic antimicrobial peptide from *Aspergillus clavatus* ES1 with antifungal and antibacterial activities, designated as AcAMP, shows high sequence homology to other ascomycete peptides. It might find application in biocontrol of plant diseases and food preservation.[12]

A 9.5-kDa antifungal protein, with N-terminal amino acid sequence homology to *Clostridium thermocellum* ribonuclease H and antifungal activity against *Flammulina velutipes*, was purified from the mushroom *Hypsizygus marmoreus*.[26] A 6.6-kDa basic and cysteine-rich antifungal peptide, with homology to similar proteins from *Aspergillus clavatus*, *A. giganteus*, *A. niger*, and *Penicillium chrysogenum*, was isolated from the culture supernatant of the mold, *Neosartorya fischeri* (anamorf: *Aspergillus fischerianus*). Its in silico presumed tertiary structure including the presence of five antiparallel β-sheet connected with filaments and stabilized by three disulfide bridges, closely resembles those of defensin-like peptides. It is protease resistant and stable within broad pH and temperature ranges.[15]

Aspergillus giganteus produces a cationic cysteine-rich defensin-like antifungal protein AFPNN5353, which suppresses the germination and growth of filamentous ascomycetes, such as human and plant pathogens and the model organisms *Aspergillus nidulans* and *A. niger*. Binder et al.[4] determined an AFPNN5353 hypersensitive phenotype of nonfunctional *A. nidulans* mutants in the protein kinase C (Pkc)/mitogen-activated protein kinase signaling pathway and the induction of α-glucan synthase A promoter in a transgenic *A. niger* strain, which point at the stimulation of the cell wall integrity pathway and cell wall remodeling in response to the antifungal protein. Stimulation of the cell wall integrity pathway by the antifungal protein operates independently from RhoA, which regulates signal transduction of the fungal cell wall integrity pathway. Calcium signaling has an essential role in the mechanism of action of this antifungal protein, which elevates the cytosolic free calcium ion concentration of a transgenic *A. niger* strain expressing codon optimized aequorin. Calcium chloride supplementation of the growth medium antagonizes toxicity of the antifungal protein, reduces perturbation of the cytosolic free calcium ion concentration, and inhibits protein uptake into *Aspergillus* cells.[4]

Hybrid Peptide Polyketides

Five hybrid peptide polyketides, curvularides A–E, were isolated from the endophytic fungus *Curvularia geniculata* obtained from the limbs of *Catunaregam tomentosa*. Curvularide B shows antifungal activity against *Candida albicans* and synergistic activity with a fluconazole drug.[6]

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Antibacterial Peptides

A cyclic pentadepsipeptide designated as alternaramide, with slight antibacterial activity toward *Bacillus subtilis* and *Staphylococcus aureus*, has been isolated from an ethyl acetate extract of the fungus *Alternaria* sp. SF-5016.[14] Fermentation extracts of *Cordyceps heteropoda* (ARSEF #1880) isolated from an Australian cicada yielded two major peptides, cicadapeptins I and I, both with N-terminal acylation by *n*-decanoic acid and C-terminal amidation by 1,2-diamino-4-methylpentane. The cicadapeptins display antibacterial activity and slight antifungal activity.[16]

HIV-1 RT Inhibitory Peptide

From the hot water extract of *Russula paludosa*, a 4.5-kDa peptide with the N-terminal amino acid sequence KREHGQHCEF and HIV-1 RT inhibitory activity (IC50 = 11 μM) was isolated by anion exchange chromatography on DEAE cellulose and gel filtration on Superdex 75. The peptide is devoid of antifungal activity.[27]

Antifungal and Antibacterial Proteins

Two forms of the 31-kDa chitosanase PgChP produced by *Penicillium chrysogenum* AS51D, which differ in the extent of glycosylation, exhibit antifungal activity against toxigenic molds. PgChP manifests pronounced sequence homology to fungal chitosanases and high catalytic activity on chitosan. Thus, the possible applications of PgChP include the use as a food preservative against toxigenic molds and a tool for obtaining chitosan oligomers for food additives and neutraceuticals.[22]

A 44-kDa dimeric antibacterial protein from the wild mushroom *Clitocybe sinopica* exhibits antibacterial activity against a variety of bacteria including *Agrobacterium rhizogenes*, *A. tumefaciens*, *A.vitis*, *Xanthomonas malvacearum*, and *X. oryzae* but not against *Erwinia herbicola*, *Escherichia coli*, *Pseudomonas batatae*, and *Staphylococcus aureus.* It has no antifungal activity.[30]

The yeast *Kluyveromyces siamensis* HN12-1 isolated from mangrove ecosystem produces a 66.4-kDa killer toxin against the pathogenic yeast (*Metschnikowia bicuspidata* WCY) in crabs.[5]

Mushroom Antifungal Peptides and Proteins

Antifungal peptides and proteins designated as agrocybin, alveolarin, eryngin, ganodermin, hypsin, lyophylin, Lyophyllum antifungal protein, *Hypsizygus marmoreus* antifungal protein, lentin, and pleurostrin have been isolated from mushrooms. They inhibit mycelial growth with an IC50 value at micromolar concentrations. They manifest a range of molecular masses (9–28 kDa) and a variety of N-terminal sequences.[7,26]

REFERENCES

1. Albergaria H, Francisco D, Gori K, Arneborg N, Gírio F. *Saccharomyces cerevisiae* CCMI 885 secretes peptides that inhibit the growth of some non-Saccharomyces wine-related strains. *Appl Microbiol Biotechnol* 2010;**86**:965–72.
2. Batta G, Barna T, Gáspári Z, Sándor S, Kövér KE, Binder U, et al. Functional aspects of the solution structure and dynamics of PAF—a highly-stable antifungal protein from *Penicillium chrysogenum*. *FEBS J* 2009;**276**:2875–90.
3. Bertinetti BV, Peña NI, Cabrera GM. An antifungal tetrapeptide from the culture of *Penicillium canescens*. *Chem Biodivers* 2009;**6**:1178–84.
4. Binder U, Bencina M, Eigentler A, Meyer V, Marx F. The *Aspergillus giganteus* antifungal protein AFPNN5353 activates the cell wall integrity pathway and perturbs calcium homeostasis. *BMC Microbiol* 2011;**11**:209.
5. Buzdar MA, Chi Z, Wang Q, Hua MX, Chi ZM. Production, purification, and characterization of a novel killer toxin from *Kluyveromyces siamensis* against a pathogenic yeast in crab. *Appl Microbiol Biotechnol* 2011;**91**:1571–9.
6. Chomcheon P, Wiyakrutta S, Aree T, Sriubolmas N, Ngamrojanavanich N, Mahidol C, et al. Curvularides A-E: antifungal hybrid peptide-polyketides from the endophytic fungus *Curvularia geniculata*. *Chemistry* 2010;**16**:11178–85.
7. Chu KT, Wang HX, Ng TB. Fungal peptides with antifungal activity. In: Kastin AJ, editor. *Handbook of Biologically Active Peptides*. Academic Press, 2006; San Diego, USA. p. 125–129 [Chapter 19].
8. Coelho AR, Tachi M, Pagnocca FC, Nobrega GM, Hoffmann FL, Harada K, et al. Purification of *Candida guilliermondii* and *Pichia ohmeri* killer toxin as an active agent against *Penicillium expansum*. *Food Addit Contam Part A Chem Anal Control Expo Risk Assess* 2009;**26**:73–81.
9. Dornberger K, Ihn W, Ritzau M, Gräfe U, Schlegel B, Fleck WF, et al. Chrysospermins, new peptaibol antibiotics from *Apiocrea chrysosperma* Ap101. *J Antibiot (Tokyo)* 1995;**48**:977–89.
10. el Hajji M, Rebuffat S, Lecommandeur D, Bodo B. Isolation and sequence determination of trichorzianines A antifungal peptides from *Trichoderma harzianum*. *Int J Pept Protein Res* 1987;**29**:207–15.
11. Guo YX, Liu QH, Ng TB, Wang HX. Isarfelin, a peptide with antifungal and insecticidal activities from *Isaria felina*. *Peptides* 2005;**26**:2384–91.
12. Hajji M, Jellouli K, Hmidet N, Balti R, Sellami-Kamoun A, Nasri M. A highly thermostable antimicrobial peptide from *Aspergillus clavatus* ES1: biochemical and molecular characterization. *J Ind Microbiol Biotechnol* 2010;**37**(8):805–13.
13. Haritakun R, Sappan M, Suvannakad R, Tasanathai K, Isaka M. An antimycobacterial cyclodepsipeptide from the entomopathogenic fungus *Ophiocordyceps communis* BCC 16475. *J Nat Prod* 2010;**73**:75–8.
14. Kim MY, Sohn JH, Ahn JS, Oh H. Alternaramide, a cyclic depsipeptide from the marine-derived fungus *Alternaria* sp. SF-5016. *J Nat Prod* 2009;**72**:2065–8.
15. Kovács L, Virágh M, Takó M, Papp T, Vágvölgyi C, Galgóczy L. Isolation and characterization of *Neosartorya fischeri* antifungal protein (NFAP). *Peptides* 2011;**32**:1724–31.
16. Krasnoff SB, Reátegui RF, Wagenaar MM, Gloer JB, Gibson DM. Cicadapeptins I and II: new Aib-containing peptides from the entomopathogenic fungus *Cordyceps heteropoda*. *J Nat Prod* 2005;**68**:50–5.

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17. López-García B, Moreno AB, San Segundo B, De los Ríos V, Manning JM, Gavilanes JG, et al. Production of the biotechnologically relevant AFP from *Aspergillus giganteus* in the yeast *Pichia pastoris*. *Protein Expr Purif* 2010;**70**:206–10.
18. Maddau L, Cabras A, Franceschini A, Linaldeddu BT, Crobu S, Roggio T, et al. Occurrence and characterization of peptaibols from *Trichoderma citrinoviride*, an endophytic fungus of cork oak, using electrospray ionization quadrupole time-of-flight mass spectrometry. *Microbiology* 2009;**155**(Pt 10):3371–81.
19. Nakadate S, Nozawa K, Sato H, Horie H, Fujii Y, Nagai M, et al. Antifungal cyclic depsipeptide, eujavanicin A, isolated from *Eupenicillium javanicum*. *J Nat Prod* 2008;**71**:1640–2.
20. Rebuffat S, Prigent Y, Auvin-Guette C, Bodo B. Tricholongins BI and BII, 19-residue peptaibols from *Trichoderma longibrachiatum*. Solution structure from two-dimensional NMR spectroscopy. *Eur J Biochem* 1991;**201**:661–74.
21. Ritzau M, Heinze S, Dornberger K, Berg A, Fleck W, Schlegel B, et al. Ampullosporin, a new peptaibol-type antibiotic from *Sepedonium ampullosporum* HKI-0053 with neuroleptic activity in mice. *J Antibiot (Tokyo)* 1997;**50**(9):722–8.
22. Rodríguez-Martín A, Acosta R, Liddell S, Núñez F, Benito MJ, Asensio MA. Characterization of the novel antifungal chitosanase PgChP and the encoding gene from *Penicillium chrysogenum*. *Appl Microbiol Biotechnol* 2010;**88**:519–28.
23. Schirmböck M, Lorito M, Wang YL, Hayes CK, Arisan-Atac I, Scala F, et al. Parallel formation and synergism of hydrolytic enzymes and peptaibol antibiotics, molecular mechanisms involved in the antagonistic action of *Trichoderma harzianum* against phytopathogenic fungi. *Appl Environ Microbiol* 1994;**60**:4364–70.
24. Skouri-Gargouri H, Gargouri A. First isolation of a novel thermostable antifungal peptide secreted by *Aspergillus clavatus*. *Peptides* 2008;**29**:1871–7.
25. Summers MY, Kong F, Feng X, Siegel MM, Janso JE, Graziani EI, et al. Septocylindrins A and B: peptaibols produced by the terrestrial fungus *Septocylindrium* sp. LL-Z1518. *J Nat Prod* 2007;**70**:391–6.
26. Suzuki T, Umehara K, Tashiro A, Kobayashi Y, Dohra H, Hirai H, et al. An antifungal protein from the culinary-medicinal beech mushroom, *Hypsizygus marmoreus* (Peck) Bigel. (Agaricomycetideae). *Int J Med Mushrooms* 2011;**13**:27–31.
27. Wang J, Wang HX, Ng TB. A peptide with HIV-1 reverse transcriptase inhibitory activity from the medicinal mushroom *Russula paludosa*. *Peptides* 2007;**28**:560–5.
28. Yamada M, Yawata K, Orino Y, Ueda S, Isogai Y, Taguchi G, et al. *Agrobacterium-tumefaciens*-mediated transformation of antifungal-lipopeptide-producing fungus *Coleophoma empetri* F-11899. *Curr Genet* 2009;**55**:623–30.
29. Yun BS, Kwon EM, Kim JC, Yu SH. Antifungal cyclopeptolide from fungal saprophytic antagonist *Ulocladium atrum*. *J Microbiol Biotechnol* 2007;**17**:1217–20.
30. Zheng S, Liu Q, Zhang G, Wang H, Ng TB. Purification and characterization of an antibacterial protein from dried fruiting bodies of the wild mushroom *Clitocybe sinopica*. *Acta Biochim Pol* 2010;**57**:43–8.
31. Zotti MD, Biondi B, Peggion C, Formaggio F, Park Y, Hahm KS, et al. A versatile template for the synthesis of novel peptaibiotics. *Org Biomol Chem* 2012;**10**:1285–99.

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Chapter 24

Fungal Peptides with Ribonuclease and Ribosome Inactivating Activities

Tzi Bun Ng

ABSTRACT

Fungi including molds and mushrooms produce a variety of proteins and peptides with ribonuclease- and ribosome-inactivating activities. In addition to their ability to inhibit protein synthesis, they may have other effects such as antiproliferative activity toward tumor cells and inhibitory activity toward viral enzymes. They manifest a diversity of structures and molecular sizes.

MUSHROOM UBIQUITIN-LIKE PEPTIDES

Ubiquitin-like peptides with RNase activity have been isolated from *Agaricus cylindracea*, *Catharellus cibarius*, *Calvatia caelata*, *Pleurotus ostreatus*, *P. sajor-caju*, and *Termitomyces globulus*. Most of them have a molecular mass of 8–9 kDa and are unadsorbed on DEAE-cellulose but adsorbed on Affi-gel blue gel, Mono S and CM-Sepharose/CM-cellulose. However, they differ in polyhomoribonucleotide specificity, optimum pH, and optimum temperature. They exhibit marked sequence similarity to human ubiquitin.[16]

MUSHROOM RNases

RNases were isolated from mushroom fruiting bodies by a combination of ion exchange chromatography on cationic and anionic exchangers and gel filtration. Sometimes, affinity chromatography on Affi-gel blue gel was used. They are unadsorbed on DEAE-cellulose, adsorbed on Affi-gel blue gel and CM-Sepharose and Mono S.[7,16–19,21,24,25] RNases have been reported from different Pleurotus species including *P. eryngii*, *P. pulmonarius*, *P. sajor-caju*, *P. tuber-regium*, and *P. ostreatus*. They exhibit a range of molecular masses running from 9 to 29 kDa, with most of them in the range of 9–14 kDa. They have an optimum pH of approximately 7. They differ in their N-terminal sequence, optimum temperature, and polyhomoribonucleotide specificity. RNases have also been isolated from non-Pleurotus species including *Clitocybe maxima*, *Dictyophora indusiata*, *Ganoderma lucidum*, *Irpex lacteus*, *Lentinus edodes*, *Russula virescens*, and *Volvariella volvaceae*. They display a molecular mass in the range of 17.5–43 kDa. They differ from one another in their optimum temperature, optimum pH, and polyhomoribonucleotide specificity. *C. maxima* RNase displays some sequence homology to *P. ostreatus* RNase. *I. lacteus* and *L.edodes* RNases are very similar, whereas *P. pulmonarius* RNase and black oyster mushroom RNases are somewhat similar.[16]

Ganoderma lucidum RNase and *Volvariella volvac*ea RNases have the largest molecular mass (42.5–43 kDa), followed by RNases from *Boletus griseus* (29 kDa)[16] and *Dictyophora indusiata* (28 kDa).[16] *Clitocybe maxima*[16] and *Agaricus bisporus*[19] RNases have an intermediate molecular mass (17.5 and 14 kDa, respectively).

B. griseus RNase expresses maximal activity at pH 3.5.[16] RNase from *G. lucidum*, *R. virescens*, *D. indusiata*, and *P. djamor* demonstrate an optimum pH at 4–4.6. RNase from *R. delica*, *A. bisporus*, and *H. marmoreus* display maximal activity at pH 5. RNase from *V. volvaceae*, *P. tuber-regium*, *C. maxima*, *T. ganbajun*, and *L. shimeiji* have an optimum pH between pH 6 and pH 7. The following RNases have an optimum pH between pH 7 and pH 8: *P. eryngii, P. ostreatus,* and *P. pulmonarius.*[16,21,25] All the following RNases express maximal activity at 60 °C: *D. indusiata*, *G. lucidum*, *R. virescens*, *R. delica*, *P. djamor*, and *A. bisporus.* RNases from *P. eryngii* and *B. griseus* have an optimum temperature between 60 °C and 70 °C. RNases from *C. maxima*, *H. marmoreus*, and *L. shimeiji* have maximal activity at 70 °C. *T. ganbajun* RNase has an optimum temperature at 40 °C.[7,16,18,19,21,24,25] *P. pulmonarius* RNase has optimum activity at 55 °C.[16]

Different RNases from different Pleurotus species display different specificities for polyhomoribonucleotides.[16] The

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00024-5
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same phenomenon is observed when the pairs *R. virescens* and *R. delica* RNases and *L. shimeiji* and *H. marmoreus* are compared. *P. djamor* RNase[21] and *L. shimeiji* RNase[24] resemble in their polyhomoribonucleotide specificity to some extent. *P. pulmonarius* RNase[16] and *H. shimeji* RNase is another pair with similar polyhomoribonucleotide specificity. *C. maxima* RNase[16] and *P. eryngii* RNase[16] is yet another pair with similarity. The others exhibit different specificities.

RNases from *H. marmoreus*, *L. shimeiji*, *R. delica*, and *P. djamor* exert antiproliferative activity toward tumor cells. RNases from *L. shimeiji* and *T. ganbajun* but not that from *R. delica* inhibit HIV-1 reverse transcriptase.[7,21,24,25]

Saccharomyces cerevisiae RNases

In *Saccharomyces cerevisiae*, RNase MRP is composed of an RNA subunit and ten proteins. Thirty-one direct protein–protein interactions are found. Each protein interacts with three or more others. Seven proteins exhibit self-interaction. Six protein subunits interact directly with MRP RNA and four protein subunits interact with pre-rRNA.[1]

Saccharomyces cerevisiae RNase H2 is a complex of three proteins [Rnh2Ap (Rnh201p), Ydr279p (Rnh202p), and Ylr154p (Rnh203p)] all of which are required for RNase H2 activity. Deletion of the gene encoding any protein or mutations in the catalytic site in Rnh2A abolishes RNase H2 activity. The multisubunit nature of the RNase may have structural importance and play a role in interaction with other proteins involved in DNA replication/repair and transcription.[10]

Aspergillus niger RNase

A 40.1-kDa extracellular RNase, with the N-terminal amino acid sequence TIDTYSSDSP, designated as *A. niger* SA-13-20 RNase, was isolated from a mutant of *Aspergillus niger.* It differs in molecular weight and pI value from RNase produced by the wildtype.[22]

FUNGAL RIBONUCLEOLYTIC TOXINS

Fungal ribotoxins, such as mitogillin and the related *Aspergillus* toxins restrictocin and alpha-sarcin, are ribonucleases that bring about enzymatic inactivation of the ribosome by cleaving 28S RNA of the large ribosomal subunit, at a site between G4325 and A4326, present in a 14-base sequence (the alpha-sarcin loop) conserved among the large subunit rRNAs of all organisms. Consequently, elongation factor binding is disrupted, and protein synthesis is suppressed. Because the His-49Tyr, Glu-95Lys, Arg-120Lys, and His-136Tyr mutations adversely affect the ribonucleolytic activity of mitogillin, these residues seem to be the active site residues.[11]

Restrictocin has two disulfide bonds, one between Cys 5 and Cys 147 and another between Cys 75 and Cys 131. The presence of the two disulfide bonds is essential for ribonucleolytic and protein synthesis inhibitory activities.[12]

The putative catalytic site of restrictocin includes Tyr47, His49, Glu95, Arg120, and His136. Intact N- and C-termini are required for restrictocin to express full activity.[13]

In restrictocin, P112 is crucial for maintaining the functionally active conformation. Prolines at positions 112, 118, and 126 are not directly involved in the membrane interaction activity of restrictocin.[5]

A total of 93 out of 149 (62%) of the amino acid residues in restrictocin are not essential for the action of the toxin. Of the five proposed active site residues, three can be deleted without loss of activity, possibly because the loss of these three residues is compensated for by a residue in the vicinity that fills the structural space produced by the larger amino acid deletions.[6]

The loop L4 in restrictocin spanning residues 99–117 is more important than loop L2 spanning residues 36–48 for interaction with the specific substrate.[4] To elucidate the molecular basis of toxin sarcin action, Correll et al.[3] determined three cocrystal structures of the sarcin homolog restrictocin bound to analogs that mimic the target sarcin/ricin loop (SRL) structure of the rat 28S rRNA. In these structures, restrictocin contacts the bulged G motif and an unfolded form of the tetraloop of the SRL RNA. In one structure, toxin loops guide selection of the target site by contacting the base critical for recognition (G4319) and the surrounding S-shaped backbone. In another structure, base flipping of the tetraloop enables cleavage by placing the target nucleotide in the active site with the nucleophile nearly inline for attack on the scissile bond. These structures provide the views of how a site-specific protein endonuclease recognizes and cleaves a folded RNA substrate.[23]

Sarcin targets a universal RNA structure in the ribosome designated as the sarcin/ricin loop. A resolution structure of a minimal sarcin/ricin loop RNA substrate reveals that the loop portion of the substrate folds into two common building blocks of RNA structure: a bulged-G motif (recognition site) and a GAGA tetraloop (cleavage site). Site selection by the toxin depends on direct base and shape recognition of the sarcin/ricin loop RNA, and cleavage by the toxin depends on a base flipping mechanism that positions the nucleophile for in-line attack on the scissile bond.[3]

All species assigned to *Aspergillus* section Clavati carry ribotoxin genes classified into different groups including the alpha-sarcin, gigantin, c-sarcin, and mitogillin/restrictocin families. *A. longivesica* and *N. acanthosporus* produce ribotoxins only slightly related to gigantins and c-sarcins, respectively. All of them possess the cysteine residues, and the two Trp residues of alpha-sarcin conserved in ribotoxins.[17]

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TABLE 1 N-Terminal Amino Acid Sequences of Fungal Ribonucleases and Ribosome Inactivating Proteins

Ribonuclease from *Pleurotus djamor*	DTACNCRKQV
Ribonuclease from *Lyophyllum shimeiji*	AATCW KTSTA
Ribonuclease from *Agaricus bisporus*	ACAANNAARRYYASSVGNNN
Ribonuclease from *Russula delica*	GCGATACKQV
Ribonuclease from *Thelephora ganbajun*	DADIAVWAPPVNAQN
Insecticidal protein hirsutellin A from *Hirsutella thompsonii*	APIVTCRPKLDGRE
Ribonuclease from *Hypsizigus marmoreus*	TFPDFANPTQNAVITQAFKDAHQLVTLAVSYITLKGARN
Ribonuclease from *Boletus griseus*	DGTSTSG
Ribosome inactivating protein (hypsin) from *Hypsizigus marmoreus*	ITFQGDLDARQDVITNADTRRKRDVRAAVR
Ribosome inactivating protein (velutin) from *Flammulina velutipes*	XHPDLFXXRPDNTASPKFEDPRLNP
Ribosome inactivating protein (lyophyllin) from *Lyophyllum shimeji*	ITFQGASPARQTVITNAITRARADVRAAVS
Ribosome inactivating protein (marmorin) from *Hypsizigus marmoreus*	AEGTLLGSRATCESGNSMY
Robotoxin from *Aspergillus clavatus*	EARAVTWTCLNDQKNPKTNK

N-terminal sequences from Refs. 2–8, 21–25.

MUSHROOM RIBOSOME INACTIVATING PROTEINS (RIPs)

Mushroom RIPs that have been isolated from mushrooms comprise flammin, velin, flammulin, velutin, hypsin, lyophyllin, pluturegin, calcaelin, and volvarin. They are unadsorbed on DEAE-cellulose, and adsorbed on Affi-gel blue gel, and CM-Sepharose/CM-cellulose. Hypsin and lyophyllin closely resemble each other in amino acid sequence. They display a molecular mass in the range of 13.8–40 kDa. They inhibit translation in a cell-free rabbit reticulocyte lysate system with an IC50 in the range 0.24–7 nM[14]

The teratogenicity of three fungal ribosome-inactivating proteins, hypsin from *Hypsizigus mamoreus*, lyophyllin from *Lyophyllum shimeji*, and velutin from *Flammulina velutipes*, has been studied using microinjection and post-implantation whole embryo culture. The results show that hypsin and lyophyllin induce abnormal embryonic development at 2.5 μM during the organogenesis period from E8.5 to E9.5. The total number of abnormal embryos showed a dose-dependent increase, accompanied by a reduction in the final somite number, and an increase of abnormal structures including open cranial neural tube, abnormal branchial arches, absence of forelimb buds, and twisted body axis. The otic and optic placodes, however, are affected to a lesser extent. Histological study disclosed a correlation of increased cell death with abnormal structures, indicating that induction of cell death by hypsin and lyophyllin may explain its teratogenicity. By contrast, velutin does not have any adverse effect on mouse development.[2,15]

A 9567-Da RIP with a novel N-terminal sequence from fresh fruiting bodies of the mushroom *Hypsizigus marmoreus*, designated as marmorin, inhibits proliferation of hepatoma Hep G2 cells and breast cancer MCF-7 cells, HIV-1 reverse transcriptase activity, and translation in the rabbit reticulocyte lysate system with an IC50 of 0.15, 5, 30 μM, and 0.7 nM, respectively. Compared with RIPs from hairy gourd, bitter gourd, ridge gourd, garden pea, and the mushroom *Flammulina velutipes*, marmorin is more potent in its antiproliferative activity toward hepatoma (HepG2) and breast cancer (MCF-7) cells, similar in inhibitory potency toward HIV-1 reverse transcriptase (with the exception that it was more potent than ridge gourd RIP and bitter gourd RIP), and less potent in translation-inhibitory potency. Marmorin is devoid of antifungal, protease, RNase, mitogenic, antimitogenic, nitric-oxide-inducing, hemagglutinating, and trypsin inhibitory activities.[20]

The N-terminal amino acid sequences of some fungal ribonucleases and ribosome inactivating proteins are presented in Table 1.

REFERENCES

1. Aspinall TV, Gordon JM, Bennett HJ, Karahalios P, Bukowski JP, Walker SC, et al. Interactions between subunits of *Saccharomyces cerevisiae* RNase MRP support a conserved eukaryotic RNase P/MRP architecture. *Nucleic Acids Res* 2007;**35**:6439–50.
2. Chan WY, Ng TB, Lam JS, Wong JH, Chu KT, Ngai PH, et al. The mushroom ribosome-inactivating protein lyophyllin exerts deleterious effects on mouse embryonic development in vitro. *Appl Microbiol Biotechnol* 2010;**85**:985–93.

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3. Correll CC, Yang X, Gerczei T, Beneken J, Plantinga MJ. RNA recognition and base flipping by the toxin sarcin. *J Synchrotron Radiat* 2004;**11**(Pt 1):93–6.
4. Dey P, Tripathi M, Batra JK. Involvement of loops L2 and L4 of ribonucleolytic toxin restrictocin in its functional activity. *Protein Pept Lett* 2007;**14**:125–9.
5. Goyal A, Seth D, Batra JK. Role of cis prolines 112 and 126 in the functional activity of ribonucleolytic toxin restrictocin. *Biochem Biophys Res Commun* 2002;**295**:812–7.
6. Glück A, Wool IG. Analysis by systematic deletion of amino acids of the action of the ribotoxin restrictocin. *Biochim Biophys Acta* 2002;**1594**:115–26.
7. Guan GP, Wang HX, Ng TB, Zhang RY. A novel ribonuclease with antiproliferative activity from fresh fruiting bodies of the edible mushroom *Hypsizigus marmoreus*. *Biochim Biophys Acta* 2007;**1770**:1593–7.
8. Herrero-Galán E, Lacadena J, Martínez del Pozo A, Boucias DG, Olmo N, Oñaderra M., et al. The insecticidal protein hursutellin A from the mite fungal pathogen *Hirsutella thompsonii* is a ribotoxin. *Proteins* 2008;**72**:217–28.
9. Gavilanes JG. The insecticidal protein hirsutellin A from the mite fungal pathogen *Hirsutella thompsonii* is a ribotoxin. *Proteins* 2008;**72**:217–28.
10. Jeong HS, Backlund PS, Chen HC, Karavanov AA, Crouch RJ. RNase H2 of *Saccharomyces cerevisiae* is a complex of three proteins. *Nucleic Acids Res* 2004;**32**:407–14.
11. Kao R, Shea JE, Davies J, Holden DW. Probing the active site of mitogillin, a fungal ribotoxin. *Mol Microbiol* 1998;**29**:1019–27.
12. Nayak SK, Rathore D, Batra JK. Role of individual cysteine residues and disulfide bonds in the structure and function of Aspergillus ribonucleolytic toxin restrictocin. *Biochemistry* 1999;**38**:10052–8.
13. Nayak SK, Batra JK. Localization of the catalytic activity in restrictocin molecule by deletion mutagenesis. *Eur J Biochem* 2000;**267**:1777–83.
14. Ng TB. Fungal ribosome-inactivating proteins. In: Kastin AJ, editor. *Handbook of Biologically Active Peptides*. Academic Press; 2006; San Diego, USA. p. 145–149 [Chapter 22].
15. Ng TB, Lam JS, Wong JH, Lam SK, Ngai PH, Wang HX, et al. Differential abilities of the mushroom ribosome-inactivating proteins hypsin and velutin to perturb normal development of cultured mouse embryos. *Toxicol In Vitro* 2010;**24**:1250–7.
16. Ng TB, Wang HX. Fungal peptides with ribonucleolytic activity. In: Kastin AJ, editor. *Handbook of Biologically Active Peptides*. Academic Press; 2006. p. 137–143 [Chapter 22].
17. Varga J, Samson RA. Ribotoxin genes in isolates of Aspergillus section Clavati. *Antonie Van Leeuwenhoek* 2008;**94**:481–5.
18. Wang HX, Ng TB. Purification of a novel ribonuclease from dried fruiting bodies of the edible wild mushroom *Thelephora ganbajun*. *Biochem Biophys Res Commun* 2004;**324**:855–9.
19. Wang HX, Ng TB. A novel ribonuclease from fresh fruiting bodies of the portabella mushroom *Agaricus bisporus*. *Biochem Cell Biol* 2006;**84**:178–83.
20. Wong JH, Wang HX, Ng TB. Marmorin, a new ribosome inactivating protein with antiproliferative and HIV-1 reverse transcriptase inhibitory activities from the mushroom *Hypsizigus marmoreus*. *Appl Microbiol Biotechnol* 2008;**81**:669–74.
21. Wu X, Zheng S, Cui L, Wang H, Ng TB. Isolation and characterization of a novel ribonuclease from the pink oyster mushroom *Pleurotus djamor*. *J Gen Appl Microbiol* 2010;**56**:231–9.
22. Xiong YH, Liu JZ, Song HY. Purification and partial characterization of an extracellular ribonuclease from a mutant of *Aspergillus niger*. *Appl Biochem Biotechnol* 2005;**125**:201–10.
23. Yang X, Gérczei T, Glover LT, Correll CC. Crystal structures of restrictocin-inhibitor complexes with implications for RNA recognition and base flipping. *Nat Struct Biol* 2001;**8**:968–73.
24. Zhang GQ, Hu DD, Wang HX, Ng TB. A novel ribonuclease with antiproliferative activity from fresh fruiting bodies of the edible mushroom *Lyophyllum shimeiji*. *Biochem Genet* 2010;**48**:658–68.
25. Zhao S, Zhao Y, Li S, Zhang G, Wang H, Ng TB. An antiproliferative ribonuclease from fruiting bodies of the wild mushroom *Russula delica*. *J Microbiol Biotechnol* 2010;**20**:693–9.

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Chapter 25

Fungal Toxins

Jack Ho Wong

ABSTRACT

Fungi exist in a diversity of habitats, encompassing oceans, rocks, deserts, very salty environments, and areas of extraordinarily high or low temperatures. They acquire their nutrition by absorbing nutrients from the surroundings. Many fungi play a crucial role in decomposition and returning nutrients to the ecosystem. They are also exploited in medicine, a well-known example being the antibiotic penicillin, as well as in industry and cuisine preparation. There are two sides of a coin. Many fungi produce toxic materials that threaten the health and safety of human beings and livestock. In this chapter, toxic peptides such as amatoxins and phallotoxins produced by higher fungi (mushrooms) and depsipeptides from phytopathogenic molds are reviewed.

INTRODUCTION

Fungi have a wide geographical distribution. A countless number of fungal species have been identified. Fungi produce a spectacular array of toxins for counteracting predators or minimizing competition from other organisms. Based on the phylogenetics, fungal toxins can be categorized into (1) mycotoxins formed by hyphae of common molds; and (2) mushroom toxins formed in the fruiting bodies of higher fungi. Based on their molecular structure, fungal toxins can be divided into two groups: nonpeptidic toxins and toxic peptidic toxins. Examples of nonpeptidic toxins include orellanine, gyromitrin, and coprine from mushrooms[1,4,7] and other toxins from molds comprising aflaxonin produced by *Aspergillus niger*,[5] ergot alkaloids from *Clarviceps* spp,[6] zearalenone from *Fusarium* species,[13] and trichothecenes from a variety of molds.[16] In this chapter, toxic peptides such as amatoxins and phallotoixns from higher fungi (mushroom) and some depsipeptides from phytopathogenic fungi (molds) are discussed.

STRUCTURAL FEATURES OF THE PEPTIDIC FUNGAL TOXINS

Amatoxins and phallotoxins constitute the two major groups of peptidic mushroom toxins. Different amatoxins including alpha-, beta-, and gamma-amanitins have been isolated. They are octapeptides with a molecular weight in the vicinity of 900 Da. In addition, a D-amino acid is present in phallotoxins. Amatoxins and the related phallotoxins are bicyclic peptides that possess a distinctive Trp–Cys crossbridge (having the name tryptathionine) and several hydroxylated amino acids.[10] The detailed structures of amatoxins and phallotoxins were presented in the first edition of Handbook of Biologically Active Peptides in Chapter 20. Depsipeptides are nonribosomal peptides. Their biosynthesis is facilitated by the biosynthetic programming of the nonribosomal peptide synthetases, the deployment of non-proteinogenic amino acid precursors, and the synthesis of straight-chain, macrocyclic or branched macrocyclic structures with amide, ester or even thioester or imino ring closures.[11,2] In the nonribosomal depsipeptides, at least one bond of the peptide backbone is substituted by an ester bond. These link carboxy groups of amino acids with a 2-hydroxycarboxylic acid, or furnish another routing of the chain through side chain hydroxy groups of amino acids and the C terminal region of the peptide.

Different from depsipeptides, the synthesis of amatoxins and phallotoxins takes place on ribosomes.[3] The gene coding for alpha-amanitin yields a proprotein with 35-amino-acid residues, and then the mature toxin (IWGIGCNP). Genes coding for beta-amanitin (IWGIGCDP), phallacidin (AWLVDCP), and phalloidin (AWLATCP) have also been detected in *Amanita* species. It is believed that *Amanita* species synthesize a much greater number of cyclic peptides than those reported in the literature, because their genomes harbor a minimum of 30 sequences.[3]

BIOSYNTHESIS OF PEPTIDIC FUNGAL TOXINS

The biosynthesis of amatoxins and phallotoxins occurs on ribosomes. The genome of *Amanita bisporigera* encompasses a sizable family of related genes, which is designated as the MSDIN family. The genes were revealed by genomic survey sequencing using 454 pyrosequencing of *A. bisporigera*, AMA1 (for alpha-amanitin) and PHA1 (for phallacidin). The proproteins of the family members consist of 34–39 amino

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00025-7
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acids and are devoid of signal peptides. In the mature toxins, 6–10 amino acids are present, and the amino acid sequences are hypervariable in comparison with that of adjacent sequences. These compounds have the smallest size of all known cyclic peptides that are synthesized on ribosomes. A proline (Pro) residue is the immediate upstream amino acid and also the last amino acid in the predicted hypervariable regions. All reported amatoxins and phallotoxins and all predicted MSDIN peptides possess a minimum of one Pro residue. Only amatoxins and phallotoxins have Trp and Cys residues and thus tryptathionine can be formed only by these toxins. Several monocyclic hexapeptides and decapeptides have been isolated from *A. phalloides*. All 20 proteinogenic amino acids are present at least once in one of the MSDIN family members of *A. bisporigera*. PCR has been used to amplify members of the MSDIN family from *A. phalloides* and *A. ocreata*.[17]

The steps in *Amanita* toxin biosynthesis after translation comprise proteolytic excision, cyclization, hydroxylation, Trp–Cys (tryptathionine) crosslinking, and epimerization of one amino acid in the case of phallotoxins. These steps do not always take place in the above sequence. *Amanita* toxin biosynthesis might also necessitate genes coding for export and regulatory proteins. Cyclization might be effected before or after tryptathionine biosynthesis.

Together with the complete amino acid sequence deduced from a cDNA, a protein was identified as a member of the prolyl oligopeptidase (POP) family of serine proteases[15] involved in the synthesis of mushroom toxins. The enzyme excises at both Pro residues in the synthetic phalloidin peptide to liberate the mature linear peptide.

Most probably, a protease or protease-like enzyme catalyzes the cyclization of the *Amanita* toxins. However, no evidence from chromatographic or mass spectrometric experiments has been reported for the formation of cyclized product during incubation of toxin precursors with POP and thus catalysis of the cyclization by POP is very probable. With respect to the sequence of processing steps, cyclization might take place subsequent to the formation of the Trp–Cys crossbridge (tryptathionine), because this would promote cyclization by bringing the N and C termini of the nascent peptide into proximity of each other.

The biosynthesis of fungal depsipeptides is accomplished with the catalytic help of cyclooligomer depsipeptide synthetases (CODS), which have modules with a role in the programmed iterative synthesis of the peptide/peptidol monomer units. Their cyclooligomerization mechanisms entail the participation of various domains

In comparison with other fungal CODSs, the 10 known full-length CODS have approximately 3150 amino acid residues and display a molecular mass of 350 kDa. They exhibit 55–74% amino acid sequence identity. The two *Fusarium oxysporum* CODSs, FolCODS and FoCODS, show 68% identity. All identified CODS, notwithstanding their involvement in the formation of hexa- or octadepsipeptides, manifest identical extended bimodular architecture with the same domain arrangement: C1A1T1–C2A2M2T2aT2b–C3.[14]

PROPOSED USAGE OF FUNGAL TOXINS

Riede showed that amanitin suppresses the activity of leukemia cells and advocated a new mode of tumor therapy using amanitin. Amanitin not only interrupts growth of cancer cells but also is capable of modifying antigen expression, and hence the immune system may be able to combat the cancer cells with augmented efficacy. The dosage of amanitin given is determined by monitoring the leukocyte cell count in blood. When the cell count rises, and the general condition of the patient is optimal, a higher dose is administered and vice versa.[12]

ANTIDOTES TO FUNGAL TOXINS

Mortality arising from poisoning by amanitin-producing mushroom species is escalating. The toxicity of alpha-AMA results from the arrest of protein synthesis in hepatocytes. Amanitin intoxication is as yet a problem that is difficult to tackle in the absence of a specific and effective antidote. A reduction of cell viability of human hepatocytes, an elevation of lactate dehydrogenase (LDH) activity, and decreased cell spreading and attachment were detected after exposure for 12, 24, and 36 h to 2 microM alpha-AMA. Benzylpenicillin (BPCN), acetylcysteine (ACC), and silibinin (SIL) added as antidotes to hepatocyte cultures promote cell proliferation and attachment. In hepatocyte cultures exposed simultaneously to alpha-AMA and the aforementioned antidotes, MTT and LDH (parameters indicating cytotoxicity) do not differ significantly from the control.[8]

Additionally, alpha-AMA exhibits prooxidant properties that may contribute to its severe hepatotoxicity. In human hepatocyte culture, alpha-AMA causes an elevation in superoxide dismutase activity and a rise in lipid peroxidation. The introduction of antidotes (ACC or SIL) to the culture medium more effectively protects against lipid peroxidation in human hepatocytes treated with alpha-AMA than BPCN, which has no antioxidant activity.[9]

REFERENCES

1. Coulet M, Guillot J. Poisoning by Gyromitra: a possible mechanism. *Med Hypotheses* 1982;**8**:325–34.
2. Du L, Lou L. PKS and NRPS release mechanisms. *Nat Prod Rep* 2010;**27**:255–78.
3. Hallen HE, Luo H, Scott-Craig JS, Walton JD. Gene family encoding the major toxins of lethal Amanita mushrooms. *Proc Natl Acad Sci USA* 2007;**104**:19097–101.
4. Holmdahl J, Ahlmen J, Bergek S, Lundberg S, Persson SA. Isolation and nephrotoxic studies of orellanine from the mushroom Cortinarius speciosissimus. *Toxicon* 1987;**25**:195–9.

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5. Holzapfel CW, Steyn PS, Purchase IF. Isolation and structure of aflatoxins M1 and M2. *Tetrahedron Lett* 1966;**25**:2799–803.
6. Kleinerova E, Kybal J. Ergot alkaloids. II. On the spectrum of ergot alkaloids contained in the sclerotia of *Claviceps purpurea* (Fr.) Tul. and *Claviceps paspali* Stev. et Hall. *Folia Microbiol (Praha)* 1969;**14**: 602–4.
7. Lindberg P, Bergman R, Wickberg B. Isolation and structure of coprine, the in vivo aldehyde dehydrogenase inhibitor in *Coprinus atramentarius*; syntheses of coprine and related cyclopropa4one derivatives. *J Chem Soc Perkin* 1977;**1**:684–91.
8. Magdalan J, Ostrowska A, Piotrowska A, Gomulkiewicz A, Podhorska-Okolow M, Patrzalek D, et al. Benzylpenicillin, acetylcysteine and silibinin as antidotes in human hepatocytes intoxicated with alpha-amanitin. *Exp Toxicol Pathol* 2010;**62**:367–73.
9. Magdalan J, Piotrowska A, Gomulkiewicz A, Sozanski T, Szelag A, Dziegiel P. Influence of commonly used clinical antidotes on antioxidant systems in human hepatocyte culture intoxicated with alpha-amanitin. *Hum Exp Toxicol* 2011;**30**:38–43.
10. May JP, Perrin DM. Tryptathionine bridges in peptide synthesis. *Biopolymers* 2007;**88**:714–24.
11. Meier JL, Burkart MD. The chemical biology of modular biosynthetic enzymes. *Chem Soc Rev* 2009;**38**:2012–45.
12. Riede I. Tumor therapy with Amanita phalloides (death cap): stabilization of B-cell chronic lymphatic leukemia. *J Altern Complement Med* 2010;**16**:1129–32.
13. Stoloff L, Nesheim S, Yin L, Rodricks JV, Stack M, Campbell AD. A multimycotoxin detection method for aflatoxins, ochratoxins, zearalenone, sterigmatocystin, and patulin. *J Assoc Off Anal Chem* 1971;**54**:91–7.
14. Sussmuth R, Muller J, von Dohren H, Molnar I. Fungal cyclooligomer depsipeptides: from classical biochemistry to combinatorial biosynthesis. *Nat Prod Rep* 2011;**28**:99–124.
15. Tsuji A, Fujisawa Y, Mino T, Yuasa K. Identification of a plant aminopeptidase with preference for aromatic amino acid residues as a novel member of the prolyl oligopeptidase family of serine proteases. *J Biochem* 2011;**150**:525–34.
16. Ueno Y, Ishii K, Sakai K, Kanaeda S, Tsunoda H. Toxicological approaches to the metabolites of Fusaria. IV. Microbial survey on "bean-hulls poisoning of horses" with the isolation of toxic trichothecenes, neosolaniol and T-2 toxin of *Fusarium solani* M-1-1. *Jpn J Exp Med* 1972;**42**:187–203.
17. Walton JD, Hallen-Adams HE, Luo H. Ribosomal biosynthesis of the cyclic peptide toxins of Amanita mushrooms. *Biopolymers* 2010;**94**: 659–64.

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Chapter 26

Plant Antifungal Peptides

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ABSTRACT

A large number of peptides isolated from different plant species seem to play an important role in plant defense against fungi. The antifungal activities of these compounds have been associated with interaction with components from walls, membranes, or nuclei of fungal cells. According to this view, antifungal peptides may cause physiological, morphological, and molecular disorders and in some cases kill the fungal cell, being dependent on several factors, including chemical and structural properties from peptides and genetic aspects of fungal host species, which consequently may determine susceptibility or resistance to peptides. In this chapter, the structural and functional properties of multiple plant antifungal peptide families, including defensins, cyclotides, and several others, will be analyzed, relating structural parameters with fungicidal activities. Moreover, the potential biotechnological use of these molecules for the treatment of fungal infections in humans and mainly in the protection of economically important crops will also be examined.

DISCOVERY

In 1942, the first antimicrobial peptide (AMP) was isolated by Ball and Hale in the wheat plant and named α-purothionin. Decades later, a large number of antifungal peptides have already been explored and described in many plant species in diverse parts of the world. They have been grouped into several families, and many of them display similar features according to their chemical parameters and mode of action. In short, plant peptides can target bacteria, virus, and fungi, although antifungal activity has been much more commonly reported.[11] The diversity of peptides with antifungal activity is enormous, where different classes have been identified and divided into distinct classes: cyclotides, defensins (previously named γ-thionins), lysine-rich, hevein-like, impatiens, knottins, lipid transfer proteins (LTPs), snakins, and thionins, among others (Table 1). Peptides can be constitutively produced or induced by the host plant in response to fungal infections. They have been reported in different plant species and different plant tissues (Figure 1), having been isolated from roots, corms, stems, bulbs, leaves, fruits, seeds, and flowers (Table 1). Because these peptides form the first barriers against a microorganism's infections, a really wide variability of structures and functional properties has been observed between these compounds.[5,23]

THREE-DIMENSIONAL STRUCTURES OF PLANT ANTIFUNGAL PEPTIDES

Over the years, many three-dimensional structures of antifungal peptides have been solved through X-ray crystallography or NMR.[23] Structural elucidation is extremely important for providing a better definition of protein families and also for understanding the mechanism of action of each class of biologically active peptides. It is difficult to establish a clear relationship between structure and activity in peptide classes with only a few members due to the absence of homologous structures or mutants with different activities. In these cases, the main explanation for activity has been related to the amphipathic surface of the peptide. Two examples are MiAMP1[17] and PAFP-S.[10] MiAMP1 was isolated from the nut kernels of the native Australian plant *Macadamia integrifolia* and is the only antifungal peptide that has been classified in the β-barrelins family. Its structure is unique among antifungal peptides and is comprised of a Greek key β-barrel with eight β-strands arranged into two Greek key motifs, stabilized by three disulfide bonds (Figure 2),[17] being its activity is restricted to plant pathogens. Structural analysis suggests that its activity may be related to its amphipathic and cationic surface, through electrostatic interactions with the membrane's anionic portion, followed by hydrophobic interactions. This is partially confirmed by bioassays in the presence of different concentrations of potassium and calcium salts. High salt concentrations deplete the inhibitory effects in plant pathogens. However, yeasts remain sensitive to MiAMP1, independently of salt concentrations, suggesting that this peptide may have a different target in yeasts, because the interaction between the cationic residues and the membrane is obstructed by high salt concentrations.[17]

Similar results were observed for PAFP-S, an antifungal peptide from *Phytolacca americana* seeds. PAFP-S is the only knottin antifungal peptide with a solved structure,[10]

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00026-9
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TABLE 1 Overview of the Wide Distribution, Localization, and Fungus Target of Antifungal Peptides

Class	Plants species	Tissue localization	Organism target
α-Helical hairpin	*Echinochloa crus-galli*	Seeds	*Alternaria alternata, Alternaria solani, Aspergillus niger, Bipolaris sorokiniana, Colletotrichum graminicola, Diplodia maydis, Fusarium graminearum, Fusarium oxysporum, Fusarium solani, Fusarium verticilloide, Phytophtora infestans, Phytophtora debaryanum, Polymyxa betae, Trichoderma album*
β-Barrelin	*Macadamia integrifolia*	Fruits	*Saccharomyces cerevisiae*
Cyclotides	*Chassalia parviflora, Oldenlandia affinis, Psychotria longipes, Viola hederacea*	Leaves, stems and lower in roots	*Candida albicans, Candida kefyr, Candida tropicalis*
Defensins	*Aesculus hippocastanum, Arabidopsis thaliana, Capsicum annuum, Nicotiana tabacum, Petunia hybrida, Pisum sativum, Raphanus sativus, Sinapis alba, Vigna radiata*	Epidermis and vascular bundles of pods, stems, roots, leaves, flowers and wet or dry seeds	*Alternaria brassicola, Aspergillus niger, Aspergillus versicolor, Botrytis cinerea, Cercospora beticola, Cladosporium sphaerospermum, Fusarium culmorum, Fusarium moniliforme, Fusarium oxysporum, Fusarium solani, Leptosphaeria maculans, Neurospora crassa, Penicillium digitatum, Pyricularia oryzae, Rhizoctonia solani, Saccharomyces cerevisae, Septoria tritiei, Trichophyton mentagrophytes,Trichoderma viride, Verticilium alboatrum*
Hevein-like	*Amaranthus caudatus, Amaranthus retroflexus, Beta vulgaris, Euonymus europaeus Eucommia ulmoides, Eutrema wasabi, Ipomoea nil, Fagopyrum esculentum*	Leaves, roots and seeds	*Alternaria brassicola, Alternaria consortiale, Alternaria nicotianae, Ascochyta lycopersici, Ascochyta pisi, Botrytis cinera, Cercospora beticola, Colletotrichum gossypii, Colletotrichum lindemuthianum, Fusarium culmorum, Fusarium moniliforme, Fusarium oxysporum, Fusarium solani, Geotrichum candidum, Gibberella zeae, Helminthosporium sativum, Magnaporte grisea, Mycosphaerella eumusae, Neurospora crassa, Phoma exigua, Phytophthora capsici, Phytophthora cryptogea, Phytophthora infestans, Phytophthora parasitica, Pythium ultimum, Rhizoctonia solani, Saccharomyces cerevisiae, Sclerotinia sclerotiorum, Trichoderma hamatum, Verticillium dahliae*
Impatiens	*Impatiens balsamina*	Seeds	*Alternaria longipes, Botrytis cinerea, Cladosporium sphaerospermum, Fusarium culmorum, Penicillium digitatum, Trichoderma viride, Verticillium alboatrum*
Knottins	*Mesembryanthemum crystallinum, Mirabilis jalapa, Phytolacca americana*	Leaves	*Alternaria brassicola, Alternaria panax, Ascochyta pisi, Botrytis cinerea, Cercospora beticola, Colletotrichum lindemuthianum, Fusarium culmorum, Fusarium oxysporum, Fusarium oxysporum, Nectria haematococca, Phoma betae, Pyrenophora tritici-repentis, Pyricularia oryzae, Rhizoctonia solani, Verticiliium dahliae, Venturia inaequalis*
Lipid transfer proteins	*Allium cepa, Leonurus Artemisia, Phaseolus aureus*	Seeds, cotyledons, shoot meristem, inter-cotyledon space, primary xylem and immature vascular elements	*Alternaria alternate, Alternaria brassicola, Ascockyta pisi, Aspergillus niger, Bipolaris maydis, Botrytis cinerea, Cerospora personata, Colletotrichum gloeosporiodes, Colletotrickum lindemutkianum, Fusarium culmorum, Fusarium graminearum, Fusarium oxysporum, Nectria haematococca, Penicillium digitatum, Pythium aphanidermatum, Phoma betae, Pyrenopkora tritici-repentis, Pyricularia grisea, Pyricularia oryzae, Rhizoctonia cerealis, Rhizoctonia solani, Saccharomyces cerevisiae, Sclerotinia sclerotiorum, Sclerotium rolfsii, Trichoderma harzianum, Verticillium dahliae*
Snakins	*Solanum tuberosum*	Tubers, stems, flowers, shoot apex and leaves	*Aspergillus flavus, Bipolaris maydis, Botrytis cinerea, Colletotrichum graminicola, Colletotrichum lagenarium, Fusarium solani, Fusarium culmorum, Fusarium oxysporum, Plectosphaerella cucumerina*
Thionins	*Aesculus hippocastanum, Arabidopsis thaliana, Beta vulgaris, Capsicum annuum, Hordeum vulgare, Medicago sativa, Nicotiana tabacum, Petunia hybrida, Phyllostachys pubescens, Pisum sativum, Raphanus sativus, Sinapis alba, Triticum aestivum, Triticum kiharae, Tulipa gesneriana, Vicia faba, Viscum album*	Leaves, flowers, roots and seeds	*Aspergillus niger, Aspergillus versicolor, Alternaria brassicola, Botrytis cinérea, Cercospora beticola, Cladosporium sphaerospermum, Fusarium moniliforme, Fusarium oxysporum, Fusarium solani, Geotrichum candidum, Leptosphaeria maculans, Neurospora crassa, Penicillium digitatum, Phytophtora infestans, Pyricularia oryzae, Saccharomyces cerevisiae, Sclerotinia sclerotiorum, Septoria tritiei, Trichoderma viride, Trichophyton mentagrophytes, Verticilium alboatrum, Verticillium dahliae*

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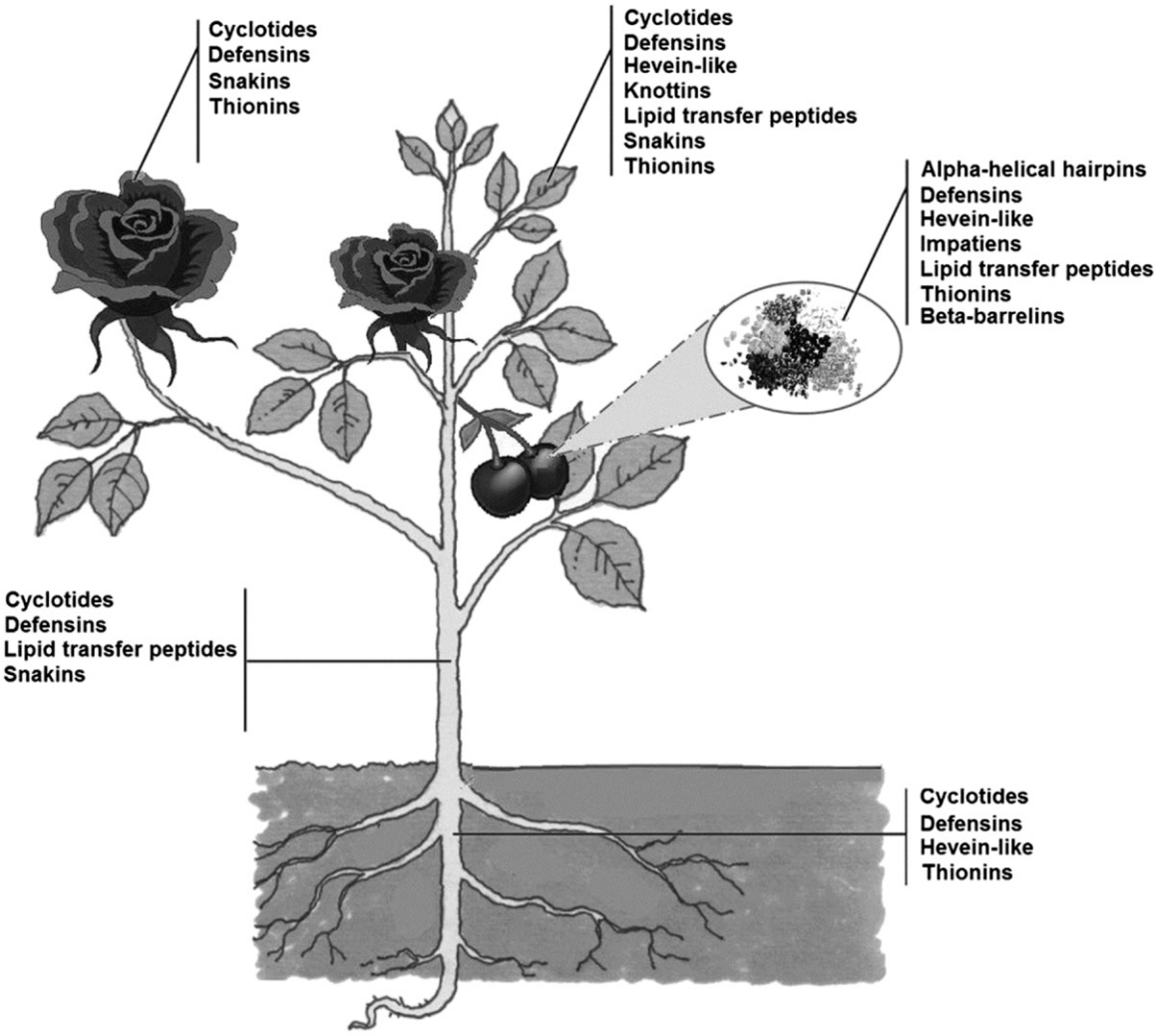

FIGURE 1 Illustrative figure showing the different plant tissues where antifungal action has been found.

comprising an antiparallel β-sheet with three β-strands, stabilized by three disulfide bonds (Figure 2). As well as MiAMP1, PAFP-S has an amphipathic surface, and its activity is also restricted to plant pathogens.

Even in cases where there are several three-dimensional structures available, the elucidation of regions or features responsible for antifungal activity is sometimes not completely possible, as observed for the cyclotides kalata B1, circulin A, and circulin B, extracted from plants of the Rubiaceae family[29] (Figure 2). The cyclotide family consists of multi-functional cyclic peptides with approximately 29 amino acid residues and a typical cysteine knot motif, showing an extremely conserved disulfide bonding pattern (Cys^{I}–Cys^{IV}, Cys^{II}–Cys^{V}, and Cys^{III}–Cys^{VI}). Cyclotides can be split into two subfamilies, bracelets and Möbius. The main difference between them is a cis-proline in the fifth loop, only observed in Möbius. Both subfamilies have members with elucidated structures and antifungal activity. Circulins A and B belong to the bracelet group, while B1 belongs to the Möbius one. Despite knowing these three structures, the mechanisms of antifungal activity have not been fully understood. Similar to that observed for MiAMP1 and PAFP-S, bioassays with high salt concentrations practically abolish the antifungal activity of cyclotides, suggesting once more the essentiality of ionic forces and also the amphipathic surface.

The α/β-thionins were the first class of antimicrobial peptides described in plants.[23] Like the classes reported so far in this section, α/β-thionins also show an amphipathic surface.[23] However, like cyclotides, α/β-thionins have a wide range of biological activities in addition to their antifungal property. These proteins also have bactericidal, insecticidal, and immunomodulatory functions and can inhibit protein synthesis in cell-free systems.[23] The overall shape of α/β-thionins resembles a capital letter L, where the α-helixes define the long arm and the short antiparallel β-sheet the short arm (Figure 2).[23] Besides having a probable electrostatic interaction with membranes, DNA binding has been

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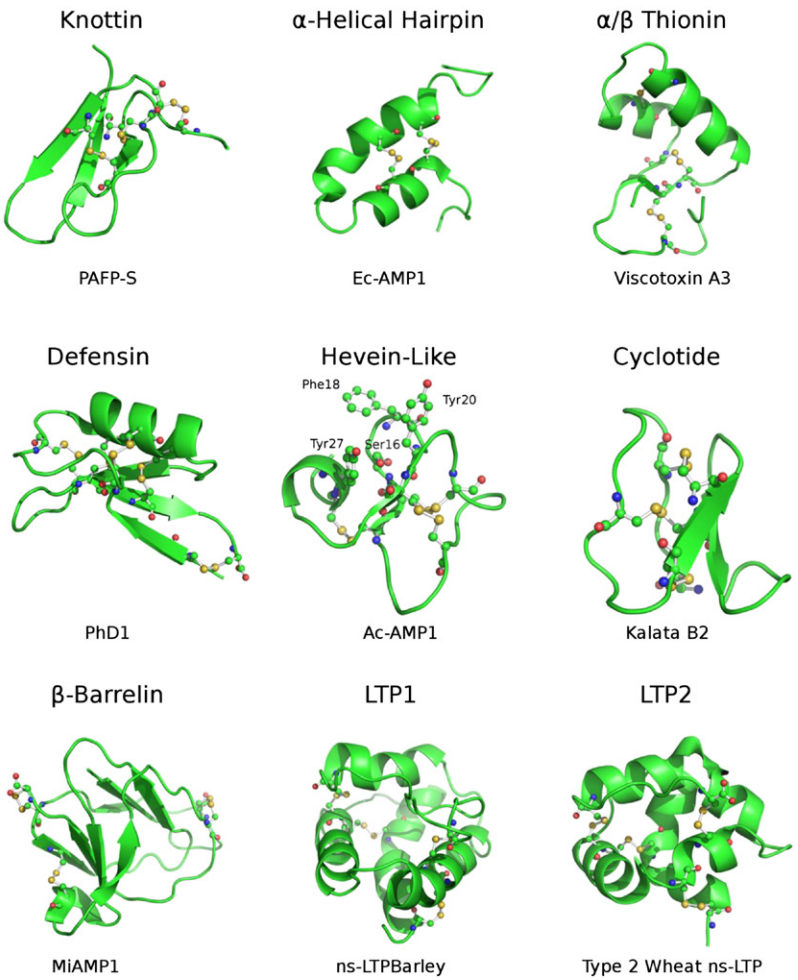

FIGURE 2 Three-dimensional structures of different antifungal plant peptide families. Disulfide bridges are shown as ball and stick. In hevein-like peptides, the active residues are also shown as ball and stick and labeled. See color plate 13.

also proposed. It has been suggested that DNA binding could occur through the structural helix-turn-helix motif, common in prokaryotic gene-regulatory proteins and homeodomain-containing proteins.[23]

Interestingly, the absence of structural information can lead to a wrong classification of peptide classes. Plant defensins are good examples of the first mistaken cataloging. In 1990, when the first plant defensin was described and no structures were available, they were classified as a subclass of thionins, named γ-thionins, because of their cationic and cysteine-rich composition similar to that of thionins. In subsequent years, with the discovery of related sequences in insects and the elucidation of first structures, it was observed that structures of α/β-thionins are different from those of defensins.[23] Although α/β-thionins have a fold that resembles the capital letter L, defensins have a typical cysteine stabilized αβ motif (CSαβ), consisting of an antiparallel β-sheet with three β-strands and one α-helix (Figure 2). This class is probably one of the most ancient antimicrobial peptides, present in the whole Eukaryote Domain and it is found in plants, animals, and fungi, which suggests that defensins have existed for one billion years.[23]

Overall, defensins can have three, four, or five disulfide bonds, but in the Plant Kingdom, they have been found only with four or five disulfide bonds and have several kinds of activities, such as α-amylase and protein synthesis inhibition as well as bactericidal and antifungal activities.[23] In contrast to other antifungal classes, the antifungal activity of plant defensins has not been related only to their amphipathic surface, although most, if not all, of them show this property. Actually, the antifungal activity is related to a structural motif that comprises the region of second and third β-strands. This region that comprises the second and third β-strands came to be called the γ-core by Yount and Yeaman.[35] The dextromeric isoform of the γ-core found in defensins is represented by the pattern [A–Z]{1,3}G[A–Z]C[A–Z]{3,9}C, where "[A–Z]" means any natural amino

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acid residue; and the numbers inside curly brackets represent the range of amino acid residues. Through this γ-core, the antimicrobial activity of brazzein,[35] a sweet-tasting protein from *Pentadiplandra brazzeana*, could be predicted and its deleterious activity confirmed against *Candida albicans*.

Another class with a well-defined antifungal active site is the hevein-like one. This class has a fold composed of a twisted antiparallel β-sheet with two β-strands, stabilized by three or five disulfide bonds,[8,16,34] and the γ-core also is observed in this class (Figure 2). The first solved structure from this class was Ac-AMP1 isolated from *Amaranthus caudatus*.[16] Ac-AMP1 shows a clear similarity to hevein domains. However, this peptide lacks a C-terminai portion of about 10 residues, including the fourth disulfide bond. The antifungal activity was related to a sugar binding domain composed of residues Ser^{16}, Phe^{18}, Tyr^{20}, and Tyr^{27}. Like Ac-AMP1, EAFP2 from *Eucommia ulmoides*[34] displays a similar sugar binding site of hevein, composed of residues Ser^{18}, Tyr^{20}, Tyr^{22}, and Tyr^{29}, and its structure is stabilized by five disulfide bonds. Nevertheless, EAFP2 also has inhibitory effects on cell wall chitin-free fungi, which may be related to the amphipathic surface of EAFP2. The third structure from this class is WAMP-1a,[8] which also possesses five disulfide bridges but with a different pattern when compared with that of EAFP2 (Figure 2).

Like hevein-like peptides, LTPs also have a well-defined structural scaffold. The overall tertiary structure is composed of a helix bundle with four α-helices, stabilized by four disulfide bonds.[23] There are two subfamilies of LTPs known as LTP1 and LTP2, which differ in mass but show similar disulfide connectivity. LTP1 displays 9 kDa and a disulfide pattern of Cys^{I}–Cys^{VI}, Cys^{II}–Cys^{III}, Cys^{IV}–Cys^{VII}, and Cys^{V}–Cys^{VIII}, LTP2 shows 7 kDa and the disulfide arrangement of Cys^{I}–Cys^{V}, Cys^{II}–Cys^{III}, Cys^{IV}–Cys^{VII}, and Cys^{VI}–Cys^{VIII} (Figure 2). Despite such differences, the two subfamilies exhibit a single hydrophobic cavity responsible for their activity. Molecular dynamics studies show that LTP penetration into the membrane leads to change in membrane conformation. However, the *in vivo* role of LTPs is still unknown.[23]

More recently, a novel structural class of antifungal peptides has been described and characterized as disulfide-stabilized α-helical hairpins[20] (Figure 2). EcAMP1 from *Echinochloa crusgalli* is the first prototype from this class with solved three-dimensional structure. As the name suggests, the fold comprises two antiparallel α-helixes connected by a short loop, resembling a hairpin. This fold is somewhat similar to the α/β-thionin fold, with different disulfide bond patterns (Figure 2). Interestingly, the mechanism of action of this peptide involves no membrane disturbance. It first acts by binding to the membrane and subsequently by internalizing cytoplasm.

Indeed, a better relation between structure and antifungal activity must be established when the majority of structure analogs for classes with few members become available. The amphipathic surface is the broadest explanation of peptide activity; however, it may have a secondary role. Actually, there must be a set of amino acid residues, such as that observed in hevein-like peptides, or a structural motif, such as the γ-core in defensins, which are responsible for the activity.

PROPOSED MECHANISMS OF ACTION OF PLANT ANTIFUNGAL PEPTIDES

The elucidation of mechanisms of action of multiple plant antifungal peptides has been sought in recent years. However, many of these mechanisms are still unknown or at best shadowy. Here, we discuss several possible situations that arise from interaction between plant antifungal peptides and fungal cells. To elucidate how the peptides can inhibit fungal growth, different strategies have been assessed.

Morphological effects of plant antifungal peptides on fungal cells have been commonly demonstrated through microscopic analyses. Investigations such as these have showed, for example, that beyond reducing growth, defensins such as RsAFP2 from *Raphanus sativus* and HsAFP1 from *Heuchera sanguine* may cause alterations in fungal hyphae, like reducing branching and elongation, whereas other defensins, such as $DmAMP_1$ and $DmAMP_2$ from *Dahlia merckii*, $AhAMP_1$ from *Aesculus hippocastanum*, and $CtAMP_1$ from *Clitoria ternatea*, reduce hyphal elongation without leading to drastic morphological distortions. Therefore, based on morphological effects, the first group was named morphogenic defensins, and the second group nonmorphogenic defensins. Interestingly, among many fungi evaluated for these effects, only *Neurospora crassa* was inhibited by two defensin groups, and no morphological alteration was observed, suggesting that peptides could have a different action mode according to fungal species.[5]

Studies have also shown that plant antifungal peptides may have inhibitory activity affected by the presence of monovalent and divalent cations in the culture medium. In summary, it has been suggested that cations could compete with peptides for fungal membrane receptors through electrostatic interactions. This could reduce the interaction between peptides and fungal cells, and consequently lead to a decline in antifungal activity.[4] Because plants and other organisms present intracellular and extracellular ion concentrations, the ability of antifungal peptides to resist ions under physiological levels is extremely important for their activity.

Besides morphological alterations, plant antifungal peptides can also induce several physiological fungal responses such as modification in Ca^{2+} uptake, K^{+} efflux, and pH of mycelial suspension. The defensins RsAFP2 and DmAMP1, for example, can induce a rapid Ca^{2+} uptake, K^{+} efflux and broth alkalinization in *N. crassa*. However, in

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the incubation medium supplemented with divalent cations, these effects were suppressed.[30]

Further efforts to understand the reason for the responses of fungi to plant antifungal peptides have demonstrated the relevance of interaction of antifungal peptides with receptors in fungal cells for antifungal activity. These targets have been found in the wall, membrane, and nuclei of fungal cells. The cell wall seems to be the goal for the peptide NaD1 (*Nicotiana alata* defensin), which is also able to permeate the plasma membrane and enter the fungal cytoplasm. Removal of components from the fungus cell wall, like β-glucan network or glycosylated proteins, prevents the permeation of NaD1 or hyphal death, thus indicating the importance of the cell wall for antifungal activity of NaD1.[32]

Moreover, sphingolipids present in fungal membranes are considered another target site for antifungal peptide interaction. These compounds act as binding-sites for antifungal peptides. Studies have shown which mutant fungi without genes responsible for biosynthesis of sphingolipids, like mannosyldiinositolphosphoryl-ceramide [M(IP)2C] and glucosylceramides (GlcCer), presented resistance to DmAMP1 and RsAFP2, respectively, when compared with that of wild-type fungi, demonstrating the importance of these sphingolipids to antifungal activity of DmAMP1 or RsAFP2. Interestingly, RsAFP2 can also interact with GlcCer isolated from *Pichia pastoris* but was unable to bind to soybean or human GlCer.[4] Probably, differences in ceramide structures among these three classes of organism (fungal, plant, and mammalian) may confer biding selectivity. It was later shown that RsAFP2 shows nontoxicity for mammalian cells.[31]

The interaction of antifungal peptides with fungal membrane also seems important for the inhibition of biofilm formation. Antifungal peptide TnAFP1 from *Trapa natans* can inhibit the biofilm formation of *Candida tropicalis*. This peptide may also affect the expression of the genes MDR1and ERG11, which are very important for ergosterol biosynthesis. The overexpression of these genes is commonly associated with biofim formation and consequently drug resistance.[14] Because most of the nosocomial infections caused by *Candida* species are associated with the formation of biofilms on medical devices, TnAFP1 is a promising potential candidate for biofilm control.

Proteins present in the nucleus can also interact with defensins and affect fungal development. Lobo et al.[13] reported which PsD1 (a defensin isolated from garden pea, *Pisum sativum*) can interact with several nuclear proteins, including a cyclin-like protein. This work was performed through a yeast two-hybrid screening system, using Psd1 as bait and proteins of *N. crassa* cDNA library as targets. Indeed, fluorescence microscopy analysis reveals colocalization of FITC-conjugated PsD1 and DAPI-stained fungal nuclei. This report also showed, through analysis of DNA content by flow cytometry, that PsD1 interferes in cell cycle progression.[13] In a study by Medeiros et al.,[18] the interaction of PsD1 with membrane was assessed by Nuclear Magnetic Resonance (NMR), showing which conformational selection is a binding mechanism and which regions around loop 1 of the peptide interact with carbohydrate and ceramide parts of GlcCer.[18,33]

Another investigation showed that some amino acid residues in specific positions are important for antifungal activity. Mutational analysis in RsAFP2, through several amino acid substitutions, resulted in a decrease or improvement of antifungal activity.[6] Parallel analysis of the three-dimensional structure of RsAFP2 allows prediction of the sites possibly relevant to antifungal activity. Similar studies were performed with MsDEF1, a morphogenic defensin from *Medicago sativa* and MtDef4, a nonmorphogenic defensin from *Medicago truncatula*.[28] In plant defensins, it has been observed that a γ-core motif is conserved, and as shown above, this site is associated with antifungal activity. Investigations using MsDef1 and MtDef4 have allowed the identification and functional characterization of the γ-core motifs for activity toward *Fusarium graminearum*. Interestingly, a γ-core substitution variant of MsDef1, containing the γ-core motif of MtDef4 (designated MsDef1- γ4) becomes a nonmorphogenic defensin. Moreover, this variant was more potent than MsDef1 and almost as potent as MtDef4. The variant MsDef1- γ4 presented a net charge that was more positive (+7) than MsDef1, net charge (+3). Interesting results were obtained about the activity of this variant toward *F. graminearum* lacking GlCer. First, it is important to know, as shown in a previous report, that *F. graminearum* lacking GlCer becomes resistant to MsDef1, but not to MtDef4.[26] As observed for MtDef4, *F. graminearum* lacking GlCer is highly sensitive to MsDef1- γ4. Based on this, the authors suggested that the γ-core motif of MsDef1 might be responsible for determining its morphogenicity and may possibly interact with GlCer in the fungal membrane.[28]

More recently, fungal growth inhibition by AFPs has been associated with fungal cell oxidative stress by reactive oxygen species (ROS) generation. This molecule can be produced by the activity of the mitochondria's electron transport chain. ROS can damage intracellular DNA, proteins, and lipids, consequently compromising cellular development. ROS have also frequently been associated with apoptosis induction. Defensins like RsAFP2 can induce ROS production in wild-type *C. albicans* and also lead to membrane permeation and fungus death.[1] However, in some *C. albicans* mutants, lacking GlcCe, this effect was not noticed, indicating that interaction between RsAFP2 and GlcCer is required for ROS generation. These studies also showed that ascorbic acid blocks RsAFP2-induced

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ROS production and also antifungal activity, indicating the existence of intracellular signaling induced by defensins.[1] Further studies showed that RsAFP2 can induce cell death by apoptosis.[3] These investigations involved yeast apoptosis assays, measuring apoptosis markers, including: ROS levels, phosphatidylserine externalization and DNA fragmentation of yeast cultures. Concomitant to apoptosis, RsAFP2 triggered the activation of caspases or caspase-like proteases, which are essential molecules in the apoptotic process.[3]

Although sphingolipids [M(IP)2C] and GlcCer seem to be indispensable for antifungal activity of the defensins RsAFP2 and DmAMP1, this is not a rule for all AFPs. HsAFP1, for example, does not require the presence of the above-mentioned sphingolipids.[2] To understand the action mode of HsAFP1, Aerts et al.[2] screened the *Saccharomyces cerevisiae* deletion mutant library for hypersensitivity and resistance toward HsAFP1. Several genes identified in this study were important for mitochondrial functionality. Based on genetic results, these authors demonstrated by *in vitro* experiment the involvement of mitochondrial function in the antifungal action of HsAFP1. This revealed that the respiration inhibitor sodium azide blocks mitochondrial function by HsFP1 antifungal action antagonization. Further, it was observed that HsAFP1 induces ROS accumulation in *C. albicans* cells. These investigations showed that adequate mitochondrial respiration is essential for antifungal activity of HsAFP1.[2]

Finally, all investigations to understand the antifungal action of peptides isolated from plants have shown the breadth of this process after initial interaction between plant peptides and receptors in the fungal cell[33] (Figure 3), making it impossible to detect a single mechanism of action. Many improvements have already been made to understand the mechanism of antifungal activity of plant peptides. But much remains to be done, because there are hundreds of antifungal peptides described in plants,[11] and few clarifications of the antifungal action mode of a considerable proportion of these plant peptides.

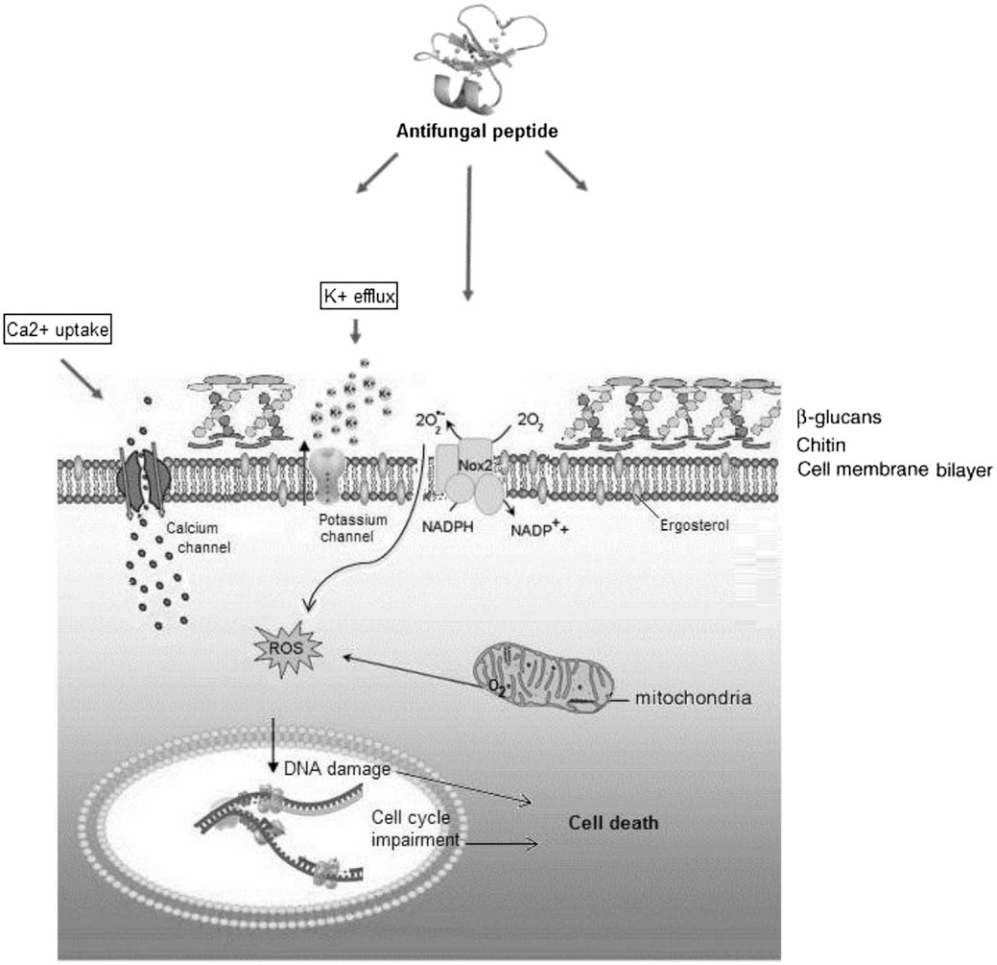

FIGURE 3 Schematic representing the possible action mechanisms of plant antifungal peptides and their biotechnological potential.

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TRANSGENIC PLANTS EXPRESSING ANTIFUNGAL PEPTIDES FROM PLANTS

Transgenic plants can be considered an attractive alternative for heterologous protein production due to lower production costs and less investment needed in appropriate infrastructure. The potential of plants to act as an expression vector or in bioindustry has not been much used commercially due to lower protein production levels and the difficulty in extracting and marketing any biopharmaceutical produced in transgenic plants. Nevertheless, tremendous progress has been made in the last decade in this transgenic plant field, reaching expression levels and offering a competitive alternative to the establishment of novel production technologies including bacteria, insects and mammalian cell cultures.[7] AMPs can also be produced in model and crop plants, establishing this as a valuable biotechnological approach. This tactic may offer a solution for yielding crops that are resistant to a wide range of bacterial and fungal pathogens.[22] Antimicrobial peptides from different origins have emerged as an option for improving crop disease resistance because proof of their activities against microbial phytopathogens has been widely provided.[19] Moreover, in the near future, the transgene probably could also be used as a systematic way of improving knowledge about AMPs, including mechanisms of action, cellular targets, and possible effects on the mammalian immune system, thus diversifying the arsenal against plant pathogens.

In plants, AMPs may be expressed in a stable manner and transformed via biolistics, agrobacterium-mediated, or transiently expressed via a plant virus carrying the transgene.[7] To date, several plants have been transformed with plant AMP genes to improve resistance toward various pathogenic fungi (Table 2). The first field trial using AMP genes produced complete protection against *Verticillium dahliae* in potato, with equivalent results to fumigant practices, successfully showing that the single-gene approach may be effective to control pathogen attack.[12] Transgenic model plants such as

TABLE 2 Fungus-Resistant Transgenic Plants Expressing Antifungal Peptides

Transgene	Source plant	Recipient plant	Promoter	Fungus tested
RsAFP2	*Raphanus sativus*	Tobacco	*Cauliflower mosaic virus (CaMV)*	*Alternaria longipes*
RsAFP2	*Raphanus sativus*	Tomato, oil rape	*CaMV 35S*	*Alternaria solani, Fusarium oxysporum, Phytophthora infestans, Rhizoctonia solani, Verticillium dahliae*
AlfAFP	*Medicago sativa*	Potato	*Figwort mosaic virus 35S*	*Verticillium dahliae*
Spi1	*Picea abies*	Tobacco, Norway Spruce embryonic cultures	*CaMV 35S*	*Heterobasidion annosum*
DRR230-a; DRR230-c	*Pisum sativum*	Tobacco	*Duplicated CaMV 35S*	*Fusarium oxysporum, Asochyta pinodes, Trichoderma reesei, Ascochyta lentis, Fusarium solani, Leptosphaeria maculans, Ascochyta pisi, Alternaria alternata*
BSD1	*Brassica rapa*	Tobacco	*CaMV 35S*	*Plasmodiophora parasitica*
WT1	*Wasabia japonica*	Rice	*Maize Ubiquitin*	*Magnaporthe grisea*
Snakin-1	*Solanum tuberosum*	Potato	*CAMV 35S*	*Rhizoctonia solani*
Thi2.1	Arabidopsis	*Arabidopsis thaliana*	*CAMV 35S*	*Fusarium oxysporum*
Vis-A3	*Viscum álbum*	*Arabidopsis thaliana*	*CAMV 35S*	*Plasmodiophora brassicae*
WjAMP-1	*Wasabia japônica*	Potato	*CAMV 35S*	*Botrytis cinerea*
		Melon	*CAMV 35S*	*Alternaria solani, Fusarium oxysporum*
RsAFP2	*Raphanus sativus*	Rice	*Maize Ubiquitin*	*Magnaporthe oryzae, Rhizoctonia solani*
DmAMP1	*Dahlia merckii*	Papaya	*CAMV 35S*	*Phytophthora palmivora*
pnAMP-h2	*Pharbitis nil*	Tobacco	*CAMV 35S*	*Phytophthora parasitica*

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TABLE 2 Fungus-resistant transgenic plants expressing antifungal peptides—*Cont'd*

Transgene	Source plant	Recipient plant	Promoter	Fungus tested
DRR206	*Pisum sativum*	Canola	*CAMV 35S*	*Leptosphaeria maculans*
*Bj*D	*Brassica juncea*	Tobacco	*Duplicated CaMV 35S*	*Fusarium moniliforme, Phytophthora parasitica pv. nicotianae*
		Peanut		*Pheaoisariopsis personata, Cercospora arachidicola*
SmAMP1.1a and SmAMP2	*Stellaria media*	Tobacco and arabidopsis	*CaMV 35S*	*Bipolaris sorokiniana and Thielaviopsis basicola*
CaAMP1	*Capsicum annuum*	Arabidopsis	*CaMV 35S*	*Hyaloperonospora parasitica*
DmAMP1	*Dahlia merckii*	Rice	*UB1*	*Magnaporthe oryzae, Rhizoctonia solani*
Mj-AMP1	*Mirabilis jalapa*	Tomato	*CaMV 35S*	*Alternaria solani*
PDF1.3	Arabidopsis	Arabidopsis	*MAR-CaMV 35S*	*Alternaria brassicicola, Botrytis cinerea, Cercospora beticola, Fusarium culmorum, Fusarium graminearum, Fusarium oxysporum, Plectosphaerella cucumerina, Trichoderma hamatum*
Puroindolines	*Triticum aestivum*	Rice	*UB1*	*Magnaporthe oryzae, Rhizoctonia solani*
LjAMP1	*Leonurus japonicas*	Tobacco	*CaMV 35S*	*Alternaria alternata, Alternaria brassicae, Aspergillus niger, Botrytis maydis, Botrytis cinerea, Cercospora personata, Colletotrichum gloeosporiodes, Fusarium oxysporum, Penicillium digitatum, Pyricularia grisea, Rhizoctonia solani, Rhizoctonia cerealis, Rhizopus nigrieans, Saccharomyces cerevisiae* *Sclerotinia sclerotiorum, Trichoderma harzianum, Verticillium dahliae*
AceAMP1	*Allium cepa*	Rose	*CaMV 35S*	*Sphaerotheca pannosa*
AceAMP1	*Allium cepa*	Geranium	*CaMV 35S*	*Botrytis cinerea*
RsAFP2	*Raphanus sativus*	Wheat	*Maize ubiquitin*	*Fusarium graminearum, Rhizoctonia cerealis*
RsAFP-1 & RsAFP-2	*Raphanus sativus*	Tobacco and Peanut		*Fusarium moniliforme* RsAFP-1 and RsAFP-2 of *Raphanus sativus Phytophthora parasitica*
Thionin Thi2.1	*Arabidopsis*	Tomato	*Thi2.1*	*Fusarium wilt (FW)*
DmAMP1 and RfAFP2	*Dahlia merckii* and *Raphanus sativus*	Arabidopsis	*CaMV 35S*	*Fusarium culmorum*

tobacco and *Arabidopsis*[19] have also been evaluated against fungi and bacteria due to transformation facilities. Moreover, several other transgenic crops expressing fungal resistance genes, such as rice and barley,[12,19] have been produced. An unusual study with the forestry tree *Picea abies* expressing *Pa18* encoding gene for a putative lipid transfer protein in somatic embryos showed inhibition of agrobacterium growth around the embryogenic callus.[27]

The *Wj-AMP*1 gene, isolated from wasabi leaves (*Wasabia japonica*), is the plant AMP most frequently found in transgenic plant studies so far. *Nicotiana benthamiana* transformed via viral vector expression and further expressing this gene was able to inhibit fungal and bacterial growth. This plant exhibited high resistance to *Magnaporthe grisea* and *Botrytis cinerea* but was weakly active against the phytopathogenic bacterium *Pseudomonas cichorii*. Further, transgenic potato expressing the same gene also showed antifungal activity against *B. cinerea*. Similar results have been obtained when the fungal defensin Wj-AMP1, expressed in the orchid *Phalaenopsis*, exhibited resistance against the phytopathogen *Erwinia carotovora*. Finally, resistant rice overexpressing wasabi defensin showed higher resistance toward the fungus *M. grisea*.[12,21]

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Nevertheless, it is important to remember that the mechanism of expression control by using different promoters seems to be essential for plant resistance improvement. Most transgenic plants carrying AMPs are driven by constitutive promoters such as cauliflower mosaic virus 35S (CaMV 35S) or ubiquitin (UBQ).[7] In both cases, transcripts are produced at high levels in a wide variety of plant tissues. Oard et al.[22] used a light-regulated leaf-specific promoter of chloroplast carbonic anhydrase (CA), which is considered a moderate promoter, to control the AMP β-purothionin fused to an extracellular rice endochitinase signal peptide (RSP). This unusual construction generates higher resistance against *Xanthomonas oryzae*, *Pseudomonas syringae*, and *Rhizoctonia solani*. These data suggest that the use of constitutive promoters is not essential to antimicrobial control. Patkar and Chattoo[24] used phenylalanine ammonia lyase (PAL), wound pathogen promoter and UBQ promoter to control Ace1AMP coding gene in transgenic rice, observing that resistance levels against the fungi *M. grisea* and *R. solani* and bacterium *X. oryzae* increased when a regulated promoter was used.

Moreover, the use of additional proteins or fused AMPs may involve an increase in antimicrobial activities. In Arabidopsis, it was found that the use of the specific CWP2 (chicken-derived specific antibody) for *Fusarium* cell wall protein fused to RsAFP from *R. sativus* enhanced resistance specifically against *Fusarium oxyosporum*.[25] However, constructions containing a polygene coding region for two AMPs, DmAMP1, and RsAFP2, produced a peptide that was cleaved after transduction. These plants exhibited greater resistance to *Fusarium culmorum* compared with that which was expressed with a single gene, demonstrating a synergistic effect.[9] These strategies are extremely valuable for the understanding of synergistic effects toward multiple pathogens.

CONCLUSIONS

In summary, the plant antifungal peptides described here and the novel peptides that certainly will be discovered in future could be used as important molecules in the development of biotechnological products. These peptides could be used as pharmaceuticals or in the production of resistant transgenic plants that could clearly contribute to agribusiness. Although some authors suppose that maintaining the stability of natural peptides outside the original organism may be a potential problem for transgenic plants, due to small size and susceptibility to protease degradation,[15] protein engineering applied to antifungal peptides could help with this issue. Moreover, these authors propose that rational design of peptides, including deletions or insertions into the original gene, could also improve peptide specificity, reducing toxicity to the host organism and finally modulating the spectrum of action. Prospects for using plant antifungal peptides to combat economic and social problems are excellent, despite the many intrinsic problems to be solved; indeed, this is an opportunity to bring together multiple techniques from biochemistry and molecular biology. Discovering novel antifungal peptides by genomic and peptidomic strategies, elucidating peptide structures unsolved until now, finding new promotors and expressing these molecules in different organisms will certainly boost the application of these remarkable compounds.

REFERENCES

1. Aerts AM, François IEJA, Meert EMK, Li Q-T, Cammue BPA, Thevissen K. The antifungal activity of RsAFP2, a plant defensin from *Raphanus sativus*, involves the induction of reactive oxygen species in *Candida albicans*. *J Mol Microbiol Biotechnol* 2007;**13**:243–7.
2. Aerts AM, Bammens L, Govaert G, Carmona-Gutierrez D, Madeo F, Cammue BPA, et al. The antifungal plant defensin HsAFP1 from *Heuchera sanguinea* induces apoptosis in *Candida albicans*. *Front Microbiol* 2011;**2**.
3. Aerts AM, Carmona-Gutierrez D, Lefevre S, Govaert G, Francois IE, Madeo F, et al. The antifungal plant defensin RsAFP2 from radish induces apoptosis in a metacaspase independent way in *Candida albicans*. *FEBS Lett* 2009;**583**:2513–6.
4. Aerts AM, Francois IE, Cammue BP, Thevissen K. The mode of antifungal action of plant, insect and human defensins. *Cell Mol Life Sci* 2008;**65**:2069–79.
5. Carvalho AO, Gomes VM. Plant defensins—prospects for the biological functions and biotechnological properties. *Peptides* 2009;**30**:1007–20.
6. De Samblanx GW, Goderis IJ, Thevissen K, Raemaekers R, Fant F, Borremans F, et al. Mutational analysis of a plant defensin from radish (*Raphanus sativus* L.) reveals two adjacent sites important for antifungal activity. *J Biol Chem* 1997;**272**:1171–9.
7. Desai PN, Shrivastava N, Padh H. Production of heterologous proteins in plants: strategies for optimal expression. *Biotechnol Adv* 2010;**28**:427–35.
8. Dubovskii PV, Vassilevski AA, Slavokhotova AA, Odintsova TI, Grishin EV, Egorov TA, et al. Solution structure of a defense peptide from wheat with a 10-cysteine motif. *Biochem Biophys Res Commun* 2011;**411**:14–8.
9. Francois IE, De Bolle MF, Dwyer G, Goderis IJ, Woutors PF, Verhaert PD, et al. Transgenic expression in *Arabidopsis* of a polyprotein construct leading to production of two different antimicrobial proteins. *Plant Physiol* 2002;**128**:1346–58.
10. Gao GH, Liu W, Dai JX, Wang JF, Hu Z, Zhang Y, et al. Solution structure of PAFP-S: a new knottin-type antifungal peptide from the seeds of *Phytolacca americana*. *Biochemistry* 2001;**40**:10973–8.
11. Hammami R, Ben Hamida J, Vergoten G, Fliss I. PhytAMP: a database dedicated to antimicrobial plant peptides. *Nucleic Acids Res* 2009;**37**:D963–8.
12. Jha S, Chattoo BB. Expression of a plant defensin in rice confers resistance to fungal phytopathogens. *Transgenic Res* 2010;**19**:373–84.
13. Lobo DS, Pereira IB, Fragel-Madeira L, Medeiros LN, Cabral LM, Faria J, et al. Antifungal *Pisum sativum* defensin 1 interacts with *Neurospora crassa* cyclin F related to the cell cycle. *Biochemistry* 2007;**46**:987–96.

This book belongs to Central Library BADJI Mokhtar-ANNABA UNIVERSITY Copyright Elsevier 2024

14. Mandal SM, Migliolo L, Franco OL, Ghosh AK. Identification of an antifungal peptide from *Trapa natans* fruits with inhibitory effects on *Candida tropicalis* biofilm formation. *Peptides* 2011;**32**:1741–7.
15. Marcos JF, Munoz A, Perez-Paya E, Misra S, Lopez-Garcia B. Identification and rational design of novel antimicrobial peptides for plant protection. *Ann Rev Phytopathol* 2008;**46**:273–301.
16. Martins JC, Maes D, Loris R, Pepermans HA, Wyns L, Willem R, et al. H NMR study of the solution structure of Ac-AMP2, a sugar binding antimicrobial protein isolated from *Amaranthus caudatus*. *J Mol Biol* 1996;**258**:322–33.
17. McManus AM, Nielsen KJ, Marcus JP, Harrison SJ, Green JL, Manners JM, et al. MiAMP1, a novel protein from *Macadamia integrifolia* adopts a Greek key β-barrel fold unique amongst plant antimicrobial proteins. *J Mol Biol* 1999;**293**:629–38.
18. Medeiros LN, Angeli R, Sarzedas CG, Barreto-Bergter E, Valente AP, Kurtenbach E, et al. Backbone dynamics of the antifungal Psd1 pea defensin and its correlation with membrane interaction by NMR spectroscopy. *Biochim Biophys Acta* 2010;**1798**:105–13.
19. Montesinos E. Antimicrobial peptides and plant disease control. *FEMS Microbiol Lett* 2007;**270**:1–1.
20. Nolde SB, Vassilevski AA, Rogozhin EA, Barinov NA, Balashova TA, Samsonova OV, et al. Disulfide-stabilized helical hairpin structure and activity of a novel antifungal peptide EcAMP1 from seeds of barnyard grass (*Echinochloa crusgalli*). *J Biol Chem* 2011;**286**:25145–53.
21. Ntui VO, Thirukkumaran G, Azadi P, Khan RS, Nakamura I, Mii M. Stable integration and expression of wasabi defensin gene in "Egusi" melon (*Colocynthis citrullus* L.) confers resistance to Fusarium wilt and Alternaria leaf spot. *Plant Cell Rep* 2010;**29**:943–54.
22. Oard SV, Enright FM. Expression of the antimicrobial peptides in plants to control phytopathogenic bacteria and fungi. *Plant Cell Rep* 2006;**25**:561–72.
23. Padovan L, Scocchi M, Tossi A. Structural aspects of plant antimicrobial peptides. *Curr Protein Pept Sci* 2010;**11**:210–9.
24. Patkar R, Chattoo B. Transgenic indica rice expressing ns-LTP-like protein shows enhanced resistance to both fungal and bacterial pathogens. *Mol Breed* 2006;**17**:159–71.
25. Peschen D, Li HP, Fischer R, Kreuzaler F, Liao YC. Fusion proteins comprising a *Fusarium*-specific antibody linked to antifungal peptides protect plants against a fungal pathogen. *Nat Biotechnol* 2004;**22**:732–8.
26. Ramamoorthy V, Cahoon EB, Li J, Thokala M, Minto RE, Shah DM. Glucosylceramide synthase is essential for alfalfa defensin-mediated growth inhibition but not for pathogenicity of *Fusarium graminearum*. *Mol Microbiol* 2007;**66**:771–86.
27. Sabala I, Elfstrand M, Farbos I, Clapham D, von Arnold S. Tissue-specific expression of Pa18, a putative lipid transfer protein gene, during embryo development in Norway spruce (*Picea abies*). *Plant Mol Biol* 2000;**42**:461–78.
28. Sagaram US, Pandurangi R, Kaur J, Smith TJ, Shah DM. Structure–activity determinants in antifungal plant defensins MsDef1 and MtDef4 with different modes of action against *Fusarium graminearum*. *PLoS ONE* 2011;**6**:e18550.
29. Tam JP, Lu YA, Yang JL, Chiu KW. An unusual structural motif of antimicrobial peptides containing end-to-end macrocycle and cystine-knot disulfides. *Proc Natl Acad Sci USA* 1999;**96**:8913–8.
30. Thevissen K, Ghazi A, De Samblanx GW, Brownlee C, Osborn RW, Broekaert WF. Fungal membrane responses induced by plant defensins and thionins. *J Biol Chem* 1996;**271**:15,018–25.
31. Thevissen K, Kristensen HH, Thomma BP, Cammue BP, Francois IE. Therapeutic potential of antifungal plant and insect defensins. *Drug Discov Today* 2007;**12**:966–71.
32. Van der Weerden NL, Hancock REW, Anderson MA. Permeabilization of fungal hyphae by the plant defensin NaD1 occurs through a cell wall-dependent process. *J Biol Chem* 2010;**285**:37,513–20.
33. Wilmes M, Cammue BPA, Sahl H-G, Thevissen K. Antibiotic activities of host defense peptides: more to it than lipid bilayer perturbation. *Nat Prod Rep* 2011;**28**:1350–8.
34. Xiang Y, Huang RH, Liu XZ, Zhang Y, Wang DC. Crystal structure of a novel antifungal protein distinct with five disulfide bridges from *Eucommia ulmoides* Oliver at an atomic resolution. *J Struct Biol* 2004;**148**:86–97.
35. Yount NY, Yeaman MR. Multidimensional signatures in antimicrobial peptides. *Proc Natl Acad Sci USA* 2004;**101**:7363–8.

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Chapter 27

Polysaccaride Krestin (PSK) and Polysaccharopeptide PSP

Jennifer Man-Fan Wan

ABSTRACT

Higher basidiomycetes fungi commonly known as mushrooms have been applied as folk medicine for thousands of years particularly in Asia. Today, fungi are becoming one of the important natural resources for the development of pharmaceutical agents, particularly, against cancer and autoimmune diseases. They are known to produce a variety of proteins with interesting biological activities. These include lectins, antifungal proteins, ribonucleases, ubiquitin-like proteins, hemolysis, and peptides. The 1-3-glycopeptides (polysaccharide–peptide complexes) isolated from mushrooms are well known for their immunomodulating and antitumor activities. Polysaccharopeptide (PSP) and Polysaccharide Kurcha (PSK) (commercial name Krestin) derived from *Coriolus vesicular* mycelia are probably the two most notable ones. Both have been discovered with very strong immunological values and are commercially available as biologic response modifiers. Other pharmacological activities of PSP and PSK include antitumor, antiviral, antimicrobial, and anti-inflammatory. This chapter discusses their discovery, structure, and chemical composition as well as their biological activities.

DISCOVERY

Polysaccharide Krestin (PSK) and polysccharopeptide (PSP) are two of the best-known commercial fungal polysaccharide–protein complexes derived from *Coriolus versicolor* mycelia with very important therapeutic values. These mushroom peptides have been used to treat infectious diseases and cancer.[2–6,15,16,23,25] PSK and PSP are derived from the CM-101 and COV-1 strains of the fungus *Coriolus versicolor* by Japanese[7] and Chinese[33] scientists, respectively. According to the *Shennong Emperor's Classic of Materia Medica*, a book published over 3000 years ago, *C. versicolor* (*L. Ex Fr.*) *Quel,* belonging to the higher basidiomycete fungi, was applied as folk medicine for thousands of years particularly in Asia. *C. versicolor* is a wood-decaying mushroom occurring in tropical, subtropical, and temperate zones. Because of its unique appearance, it is called "云芝" (Yun-Zhi) in Chinese, which means "cloud-like mushroom." It has also been named *Polyporous versicolor* (L.) Fr. or *Polysticutus versicolor*, and some taxonomists considered that this fungus belongs to the *Trametes* family.[4,23]

PSK was first isolated in Japan in the late 1960s, and PSP was isolated around 1983 in China.[2,4,23] Each compound was discovered with strong immunological values and has been used as an adjuvant for cancer treatment with no known side effects.[2,6,12,19,21,25] The fermentation for PSK lasts up to 10 days, whereas PSP production involves a 36- to 72-h culture. PSK is recovered from water extracts of the biomass by salting out with ammonium sulfate, whereas PSP is recovered from ethanol precipitation.[2,7,33] Both are dark brown powders, with a light odor and are soluble in hot water with a pH ranging from neutral to slightly acidic. The PSP/PSK polymers are only soluble in hot water and insoluble in methanol, pyridine, chloroform, benzene, and hexane.

STRUCUTRE AND CHEMICAL COMPOSITIONS

The composition and structure of PSK and PSP have been reviewed elsewhere.[2,4,15,23,25] PSK and PSP share very close chemical structures and biological functions. The presence of fucose in PSK and rhamnose and arabinose in PSP distinguishes the two protein-bound polysaccharides, which are otherwise chemically similar. PSK is composed of 34–35% soluble carbohydrate, 28–35% protein, 7% moisture, 6–7% ash, and the remainder is composed of free sugars and amino acids. PSP is made up of 46% soluble carbohydrate, 31% protein, and 15% of incombustible substances. Both have a molar mass of approximately 100 kDa. PSP and PSK contain β-1→3, β -1→6, α-1→4, and α-1→2 glucosidic linkages in their polysaccharide moieties with D-glucose being the major monosaccharide and are covalently linked to several proteins. The amino acid composition is 0.58% glutamine, 0.4% aspartic acid, 0.32% serine, 0.26% alanine, 0.26% glycine, 0.24% leucine, 0.23% lysine, 0.23% threonine,

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00027-0
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0.22% Isoleucine, 0.18% arginine, 0.18% valine, 0.17% trpyptophan, 0.15% phenylalanine, 0.15% tyrosine, 0.1% proline, 0.09% cystine, 0.07% histidine, and 0.04% methinione. The polysaccharide component is composed of 74% glucose, 4% xylose, 2.7% glalactose, 2.4% fructose, and 1.5% mannose.

BIOLOGICAL ACITVITIES

Biological Response Modifiers (BRMs) Activity

Both PSK and PSP have been classified as biological response modifiers (BRMs) because they can interact with the immune system and able to upregulate or downregulate specific aspects of the host response. The biological response activities of PSK and PSP include immunopotentiation by inducing production of interleukins (ILs) such as IL-8, IL-6, tumor necrosis factor-alpha (TNF-α), interferons,[13,18,25] macrophages,[11,14,18,20] T-lymphocytes,[12,14,17,18,21] counterimmunosuppressive effects of chemotherapy,[24] radiotherapy,[6] antagonism of immunosuppression induced by tumors, antiviral,[3,26] anti-inflammatory,[1,2] analgesic,[22] antihyperlipemic,[35] antioxidative, production of superoxide dismutase, and glutathione peroxidase.[10] The enhanced anticancer properties of PSK and PSP in combination with radiation, chemotherapy, or immunotherapy are associated with their ability to activate both cellular and humoral components of the host immune system.[2,4,5,12,14,15,24,27,29]

Modulate Both Innate (NonSpecific) and Adaptive (Specific) Immunity

Macrophages are involved in all stages of the immune response. They function in phagocytosis, antigen processing and presentation, secretion of cytokines, and antibody-dependent cell-mediated cytotoxcity. Both PSP and PSK can restore and increase phagocytic function of macrophages.[11,14,18,20] PSK can stimulate production of differentiation-inducing factor from macrophages, and activate the function of polymorphonuclear leukocytes (PMN), promote the phenotype and functional maturation of dendrite cells derived from human $CD4^+$ mononuclear cells, and resolve the immunosuppressive state of a cancer-bearing host.[11] PSK inhibits Epstein–Barr virus induced B-cell proliferation through T cells activated by the monocyte products IL-15 and IL-12.[18]

Natural killer cells (NKs) are a subset of bone marrow-derived lymphocytes that are capable of lysing or killing infected or tumor cells. Cytotoxicity and production of cytokines such as interferon-gamma (IFN-γ), TNF-α, and granulocyte-macrophage-colony stimulating factor (GM-CSF) that can modulate natural and specific immune responses are two relevant functions of NK cells. The ability of PSK to increase the production of human NK cells[21] makes it a unique biological response modifier for the treatment of cancer and viral infection. PSK inhibits human immunodeficiency virus infection *in vitro*.[26] PSP can activate macrophages to release mediators such as nitric oxide (NO) and TNF-α; prostaglandin E_2, IL-1, and IL-6[1,13] in response to bacterial infection.

PSK and PSP act as mitogens, and they play a role in the first step of activation signaling pathways in antigen-presenting cells (APCs). Subsequent to this activation and signaling is humoral- and cell-mediate-immunity induction. The biological abilities of PSP and PSK include stimulation on Th1- and Th2-dependent antibody production, promotion of Th1-dominant state and killer activities, and enhancement of both Th1- and Th-2-dependent cytokines such as IL-10, IL-4, and IL-2.[2,12,17] Both PSP and PSK are able to stimulate or suppress the production of cytokines that mediate innate immunity. These include proinflammatory cytokines (e.g., IL-1, IL-6, TNF-α, and regulatory cytokines such as IL-2 and IL-10).[13,17] In lipopolysaccharide (LPS)-treated mouse peritoneal macrophages, PSP potentiates the release of inflammatory mediator prostaglandin E2 and the proinflammatory cytokines TNF-α, IL-1β, and histamine.[1] Increased production of IL-1 may cause the production of various other cytokines such as TNF-α, which elicits the lysis of malignant cells and bacterial cells.

Maintaining the Correct Balance Between Th1- and Th2-Mediated Immunity

Maintaining the correct balance between cellular (mediated by T-helper 1 cells, Th1) and humoral (mediated by T-helper 2 cells,Th2) immunity, is critical to ensure an effective immune response to infection and human diseases based on the types of cytokines secreted. Uncontrolled Th1- and Th2-mediated responses can lead to autoimmune and inflammatory diseases, respectively. For instance, in cancer, the balance between Th1 and Th2 immune responses is shifted toward Th2 dominance resulting in a poor prognosis. Therefore, identifying agents that modulate the Th1/Th2 cytokines are therefore essential for controlling these diseases. PSK therapy shifts the Th1/Th2 balance toward Th1 dominance in patients with gastric or colorectal cancer.[12]

Our *ex vivo* study of human T lymphocyte proliferation showed that PSP exhibits similar characteristics as cyclosporine A (CsA) does in controlling aberrant T cell proliferation.[17] Differing from that of CsA, PSP reduces Th1 cytokines and CD3+/CD25+ cell expression but not Th2 cytokine expression, which could help the cytokine balance shift toward Th2 dominance. Molecular analysis

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indicates that the suppression of IL-2 expression by PSP, like that of CsA, was via Janus-kinase-signal transducers and activator of transcription pathways, and subsequently reduces the production of the IL-2 receptor, cytotoxic T cells, natural killer cells, and gamma interferon. The effect of PSP on Th1 cytokine suppression involves the activation of mitogen-activated protein kinase (MAPK) p38 and signal transducers and activators of transcription 5 (STAT5) on T cells. Unlike CsA, PSP does not induce an apoptotic influence on the proliferating T cells.[17]

CsA is also a microbial product, derived from the fungus *Tolypocladium inflatum* and is used as an immunosuppressive drug for the treatment of autoimmune diseases. It is capable of altering the Th1/Th2 cytokine production profiles in the control of autoimmune and inflammatory diseases through the inhibition of IL-2. However, CsA is not specific, and its long-term use is associated with significant side effects and toxicities. Whether PSP can be a more suitable candidate for maintaining the correct balance between Th1- and Th2-mediated immunity than CsA in treatment of autoimmune diseases.

Antitumor Activity

The antitumor activity of PSK and PSP has been extensively described.[2,5,6,15,16,22,23,25,27,29,31,32] Evidence relating to the therapeutic efficacy of PSK and PSP comes from numerous clinical trials conducted on hundreds of cancer patients. Three reviews of human studies with PSK have positive conclusions: The 1998 review by T.B. Ng concluded that PSK helps alleviate symptoms and prevents the decline in the immune-status of patients with esophageal, gastric, and lung cancers treated with radiotherapy and chemotherapy.[23] A review by Parris Kidd summarized the benefits of PSK on the quality-of-life aspects of the patients who underwent chemotherapy and radiation therapy.[15] Fischer and Yang summarized that the greatest amount of clinical evidence for the use of PSK was in the treatment of gastric cancer after curative resection.[5] The immune-protection of PSK has been greatly notified in cancer patients during chemotherapy and radiotherapy.[12–14] Cotreatment of PSK helped to restore the suppressed immune system of the patients caused by anticancer drugs during chemotherapy and radiotherapy. The immune restoration abilities of PSK/PSP include the increase of antibody and cytokine production and improvement of impaired antitumor activity of natural killer cells, T cells, macrophages, and peripheral blood lymphocytes.[2,6,12–14,19–21,24,29] Other antitumor activities include detoxification of carcinogens via increased expression and activity of phase I and phase II enzymatic–oxidative–radical–scavenging effect, induction of differentiation, direct cytotoxicity, angiogenesis, and antiproliferation.[5,9,10,25,32,34] PSP/PSK work in multiple steps of the malignant process by inhibiting adhesion, invasion, motility, and metastatic growth of tumor cells.[2,6]

Mechanistic studies at the cellular level with cancer cell line reveal that PSP and PSK act via the host to boost cellular immunity indirectly on tumor cells and directly on cancer cells. These fungal peptides can activate the cell death program (apoptosis), inhibit DNA synthesis, arrest cell cycle, and inhibit cell division,[5,9,27,28,34] which are all strategies for treatment of human cancer. The induction of apoptosis by PSP involves mitochondrial pathways and an increase of the Bax/BCl_2 ratio.[35] Induction of S-phase cell cycle arrest by PSP is positively associated with the accumulation of the cell cycle phase proteins, cyclin E, and B1.[9,28,35] PSP can synergistically enhance the apoptotic effect of the S-phase targeted drugs doxorubicin (Doxo) and etoposide (VP-16) via enhancing the sensitivity of cancer cells to PSP by creating an S-phase trap.[9,28] Bivariate bromodeoxyuridine (BrdURd)/DNA flow cytometry analysis estimated a longer DNA synthesis time (Ts) for the HL-60 cells cotreated with PSP, confirming that PSP can create an S-phase trap. A negative correlation between the increase in apoptotic cell population (pre-G1 peak) with their S-phase cell population expression ($R2=0.998$) and the expression of cyclin E expression ($R2=0.872$) and caspase 3 activity ($R=0.997$) suggest that PSP enhances the apoptotic machinery of Doxo and VP-16 in a cell cycle-dependent manner and is mediated, at least, in part, by the PSP-mediated modulation of the regulatory checkpoint cyclin E.[9]

OTHER BIOLOGICAL ACTIVITIES

In addition to the antitumor activity, PSP and PSK exhibit antiviral activity[336] and analgesic activity.[1,22] PSK can inhibit HIV infection by modifying the viral receptor or by stopping HIV from binding with lymphocytes.[3] Alpha-glucosidase is a Golgi complex enzyme involved in viral protein processing and HIV-1 gp 120 processing. PSP inhibits the interaction between HIV-1 reverse transcriptase, and inhibits a glycohydrolase enzyme associated with viral glycosylation.[26] PSP shows analgesic action in mice subjected to hot-plate-induced pain stress.[1] Other biological activities of PSK/PSP include strong scavenging effects on superoxide and hydroxyl radicals,[26] expressing the mimetic activity of superoxide dismutase (SOD), promoting oxidative stress relief for cancer-bearing hosts,[10,26] and protecting macrophages from lipoperoxide accumulation and foam cell formation caused by oxidative modified low density lipoprotein.[35] The antimicrobial activity of PSK has been shown in *Escherichia coli*, *Listeria monocytogenes* and *Candida*.[26] Phagocyte activation and reactive nitric oxide production of macrophages are suggested mechanisms contributing to the antimicrobial activity of PSK.[26]

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CONCLUSIONS

Polysaccharides-Krestin and polysaccharide peptide are two effective protein complexes derived from *C. versicolor* with very important therapeutic significance. They are considered as biological response modifiers because they can modulate both innate and specific immunity, induce multicytokine production and regulate their function, as well as to correct the balance between Th1 and Th2 immunity. Both PSP and PSK have the ability to modulate inflammation, immunity, and hematopoiesis thereby eliciting various therapeutic effects. Other pharmacological activities of PSP/PSK include analgesic, antimicrobial, antiviral, and antioxidant. They are sold in the form of capsules, ground biomass tablets, syrups, food additives, and teas around the world and are used as adjuvant for cancer therapy and neutraceuticals with no reported side effects.

REFERENCES

1. Chan SL, Yeung JHK. Polysaccharide peptides from COV-1 strain of *Coriolous versicolor* induce hyperalgesia via inflammatory mediator release in the mouse. *Life Sci* 2006;**78**(21):2463–70.
2. Cheng KF, Leung PC. General review of polysaccharopeptides (PSP) from *C. versicolor*: pharmacological and clinical studies. *Cancer Ther* 2008;**6**:117–30.
3. Collins RA, Ng TB. Polysaccharopeptide from *Coriolus versicolor* has potential for use against human immunodeficiency virus type I infection. *Life Sci* 1997;**60**(25):PL383–7.
4. Cui J, Chisti Y. Polysaccharopeptides of *Coriolus versicolor*: physiological activity, uses, and production. *Biotechnol Adv* 2003;**21**(2):109–22.
5. Fischer M, Yang LX. Anticancer effects and mechanisms of polysaccharide-K (PSK): implications of cancer immunotherapy. *Anticancer Res* 2002;**22**(3):1737–54.
6. Hayakawa K, Mitsuhashi N, Saito Y, Takahashi M, Katano S, Shiojima K, et al. Effect of krestin (PSK) as adjuvant treatment on the prognosis after radical radiotherapy in patients with non-small cell lung cancer. *Anticancer Res* 1993;**13**(5C):1815–20.
7. Hotta T, Enomoto A, Yoshikumi C, Ohara M, Ueno S. Protein-bound polysaccharides. US Patent 1981; 4,271,151.
8. Hu TX, Chen JW, Xu JY, Lu JY, Yang QY. Effects of polysaccharide-peptide of *Coriolus* and polysaccharide of *Ganoderma* on scavenging active oxygen species. *Acta Biochim Biophys Sinica* 1992;**24**:465–70.
9. Hui KP, Sit WH, Wan JM. Induction of S phase cell arrest and caspase activation by polysaccharide peptide isolated from *Coriolus versicolor* enhanced the cell cycle dependent activity and apoptotic cell death of doxorubicin and etoposide, but not cytarabine in HL-60 cells. *Oncol Rep* 2005;**14**(1):145–55.
10. Kamei H, Hashimoto Y, Koide T, Kojima T, Hasegawa M, Umeda T. Direct tumor growth suppressive effect of melanoidin extracted from immunomodulator-PSK. *Cancer Biother Radiopharm* 1997; **12**(5):341–4.
11. Kanazawa M, Mori Y, Yoshihara K, Iwadate M, Suzuki S, Endoh Y, et al. Effect of PSK on the maturation of dendritic cells derived from human peripheral blood monocytes. *Immunol Lett* 2004;**91**(2, 3): 229–38.
12. Kanazawa M, Yoshihara K, Abe H, Iwadate M, Watanabe K, Suzuki S, et al. Effects of PSK on T and dendritic cells differentiation in gastric or colorectal cancer patients. *Anticancer Res* 2005; **25**(1B):443–9.
13. Kariya Y, Inoue N, Kihara T, Okamoto N, Sugie K, Mori T, et al. Activation of human killer cells by the protein-bound PSK independently of interferon and interleukin 2. *Immunol Lett* 1992;**31**(3):241–5.
14. Kato M, Hirose K, Hakozaki M, Ohno M, Saito Y, Izutani R, et al. Induction of gene expression for immunomodulating cytokines in peripheral blood mononuclear cells in response to orally administered PSK, an immunomodulating protein-bound polysaccharide. *Cancer Immunol Immunother* 1995;**40**(3):152–6.
15. Kidd PM. The use of mushroom glucans and proteoglycans in cancer treatment. *Altern Med Rev* 2000;**5**(1):4–27.
16. Kobayashi H, Matsunaga K, Oguchi Y. Antimetastatic effects of PSK (Krestin), a protein-bound polysaccharide obtained from basidiomycetes: an overview. *Cancer Epidemiol Biomarkers Prev* 1995;**4**(3):275–81.
17. Lee CL, Sit WH, Jiang PP, So IW, Wan JM. Polysaccharopeptide mimics ciclosporin-mediated Th1/Th2 cytokine balance for suppression of activated human T cell proliferation by MAPKp38 and STAT5 pathways. *J Pharm Pharmacol* 2008;**60**(11):1491–9.
18. Liu A, Arbiser JL, Holmgren A, Klein G, Klein E. PSK and Trx80 inhibit B-cell growth in EBV-infected cord blood mononuclear cells through T cells activated by the monocyte products IL-15 and IL-12. *Blood* 2005;**105**(4):1606–13.
19. Liu JX, Zhou JY, Liu TF. Phase 3 clinical trails for Yun Zhi polysaccharide (PSP) capsules. In: Yang QY, editor. *Advanced research in PSP*. Hong Kong: The Hong Kong Association for Health Care Limited; 1999. p. 295–303.
20. Liu WK, Ng TB, Sze SF, Tsui KW. Activation of peritoneal macrophages by polysaccharopeptide from the mushroom, *Coriolus versicolor*. *Immunopharmacology* 1993;**26**(2):139–46.
21. Mizutani Y, Yoshid O. Activation by the protein-bound polysaccharide PSK (krestin) of cytotoxic lymphocytes that act on fresh autologous tumor cells and T24 human urinary bladder transitional carcinoma cell line in patients with urinary bladder cancer. *J Urol* 1991;**145**(5):1082–7.
22. Ng TB, Chan WY. Polysaccharopeptide from the mushroom *Coriolus versicolor* possesses analgesic activity but does not produce adverse effects on female reproductive or embryonic development in mice. *Gen Pharmacol* 1997;**29**(2):269–73.
23. Ng TB. A review of research on protein-bound polysaccharide (polysaccharopeptide, PSP) from the mushroom *Coriolus versicolor* (Basidiomycetes: Polyporacea). *Gen Pharmacol* 1998;**30**(1):1–4.
24. Ohwada S, Ikeya T, Yokomori T, Kusaba T, Roppongi T, Takahashi T, et al. Adjuvant immunochemotherapy with oral Tegafur/Uracil plus PSK in patients with stage II or III colorectal cancer: a randomized controlled study. *Br J Cancer* 2004;**90**(5):1003–10.
25. Ooi VE, Liu F. Immunomodulation and anti-cancer activity of polysaccharide–protein complexes. *Curr Med Chem* 2000;**7**(7):715–29.
26. Tochikura TS, Nakashima H, Hirose K, Yamamoto N. A biological response modifier, PSK, inhibits human immunodeficiency virus infection *in vitro*. *Biochem Biophys Res Commun* 1987;**148**(2):726–33.
27. Tsukagoshi S, Hashimoto Y, Fujii G, Kobayashi H, Nomoto K, Orita K. Krestin (PSK). *Cancer Treat Rev* 1984;**11**(2):131–55.
28. Wan JM, Sit WH, Louie JC. Polysaccharopeptide enhances the anticancer activity of doxorubicin and etoposide on human breast cancer cells ZR-75-30. *Int J Oncol* 2008;**32**(3):689–99.

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29. Wang HX, Ng TB, Ooi VE, Liu WK, Chang ST. A polysaccharide-peptide complex from cultured mycelia of the mushroom *Tricholoma mongolicum* with immunoenhancing and antitumor activities. *Biochem Cell Biol* 1996;**74**(1):95–100.
30. Yang QY, Jong SC, Li XY, Zgou JX, Chen RT, Xu LZ. Antitumor and immunomodulating activities of the polysaccharide-peptide (PSP) of *Coriolus versicolor*. *Eos-Riv Immunol* 1992;**12**:29–34.
31. Yang QY, Hu YJ, Li XY, Yang JC, et al. A new biological response modifier substance-PSP. In: Yang QY, Kwok CY, editors. *PSP international symposium 1993*. Shanghai: Fudan University Press; 1993. p. 56–72.
32. Yang QY. A brief introduction to the research of PSP. In: Yang QY, Kwok CY, editors. *PSP international symposium 1993*. Shanghai: Fudan University Press; 1993. p. 14–7.
33. Yuan C, Mei Z, Liu S, Yi L. PSK protects macrophages from lipoperoxide accumulation and foam cell formation caused by oxidatively modified low-density lipopotein. *Atherosclerosis* 1996;**124**(2):171–81.
34. Yang QY. A method for producing protein-bound polysaccharide of *Corilous versicolor* (PSP). Chinese patent 1989;CN 1034957A.
35. Yang X, Sit WH, Chan DK, Wan JM. The cell death process of the anticancer agent polysaccharide-peptide (PSP) in human promyelocytic leukemic HL-60 cells. *Oncol Rep* 2005;**13**(6):1201–10.

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Chapter 28

AKH/RPCH Peptides

Gerd Gäde and Heather G. Marco

ABSTRACT

Color change in the integument is the obvious action of the red pigment-concentrating hormone (RPCH), which is produced in the neurosecretory cells of the X-organ of crustaceans. Mobilization of various fuels used during energy-demanding processes is the classical regulatory role of the adipokinetic/hypertrehalosemic/hyperprolinemic hormones (AKHs), which are produced in neurosecretory cells of the insect corpus cardiacum. These neuropeptide hormones have common structural features: a chain length of 8–10 amino acids, blocked N- and C-termini, and aromatic amino acids at positions 4 and 8. The precursor is organized as signal peptide, AKH/RPCH peptide, amidation and processing sites, followed by another peptide of unknown function. Only one AKH receptor type is known yet, although certain insect species have up to three mature AKHs. Ligand–receptor interaction, receptor conformational changes necessary for receptor activation, and intracellular signaling pathways have been partly unraveled.

DISCOVERY

Already in the 1920s–1940s it was shown that extracts from certain glands of crustaceans and insects caused a change of integumental color from dark to light when injected into shrimps.[9,23] Complete purification of the active material from the eyestalks of the prawn *Pandalus borealis* (Panbo) was achieved mainly by gel chromatography on Sephadex LH-20 in water/butanol mixtures, and the structure of the active principle was elucidated as a blocked octapeptide by the use of quantitative amino acid analysis, proteolytic cleavage, Edman-dansyl sequencing, and high-resolution mass spectrometry. This resulted, in 1972, in the first complete primary structure of an invertebrate neuropeptide,[3] which was termed red pigment-concentrating hormone (RPCH; today denoted as Panbo-RPCH) (Table 1). In insects, extracts of the corpus cardiacum (CC) from either the American cockroach or the migratory (*Locusta migratoria*; Locmi) and desert locusts were shown, during the 1960s, to have metabolic effects: elevation of the hemolymph sugar trehalose (hypertrehalosemic effect) or of the hemolymph lipids (adipokinetic or hyperlipemic effect). In 1976, the complete primary structure of one of the locust's adipokinetic neuropeptides from the CC, today denoted as Locmi-AKH-I (Table 1), was obtained by enzymatic cleavage and mass spectrometry after purification by size-exclusion gel chromatography on controlled-pore glass and thin-layer chromatography on silica gel.[25] The sequence of the decapeptide Locmi-AKH-I showed close structural similarities with Panbo-RPCH; both peptides are crossreactive in the reciprocal biological system. Today, >50 members of this AKH/RPCH family of peptides are known by primary structure from the two arthropod sister groups, Crustacea and Insecta. Interestingly, individual crustaceans have only one family member, and only two AKH members have been identified to date in Crustacea: the highly conserved Panbo-RPCH in decapod crustaceans and Dappu-RPCH (Table 1) in branchiopod (cladoceran) crustaceans of the genus *Daphnia*.[1] Insecta, on the other hand, contain up to three AKHs in an individual and show a high degree of variability in the sequence. There is, however, group specificity in the insect AKHs and, moreover, insects of some orders synthesize the crustacean form Panbo-RPCH[5,7] (Table 1).

AKH STRUCTURE

Primary Structure

Commonly, AKHs are characterized by a chain length of 8–10 amino acids, a blocked N-terminus (pGlu), a blocked C-terminus (carboxyamide), and aromatic amino acids at position 4 (mostly Phe but sometimes also Tyr) and position 8 (Trp). A few AKHs even have a third aromatic amino acid (at position 2: see Scade-CC-I and -II in Table 1), and some have a negatively charged amino acid (Asp, as in Scade-CC-I and -II, and in Phote-HrTH, Table 1). In addition to the modified termini, other posttranslational modifications encompass an AKH with a phosphorylated Thr residue at

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00028-2
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TABLE 1 Primary Structures of Selected Peptides of the AKH/RPCH Family

Peptide code name	Primary structure*	Comments
Panbo-RPCH	pGlu-Leu-Asn-Phe-Ser-Pro-Gly-TrpNH_2	Color change hormone of *Pandalus borealis* and adipokinetic hormone of stink bug *Nezara viridula*
Dappu-RPCH	pGlu-Val-Asn-Phe-Ser-Thr-Ser-TrpNH_2	Peptide found in *Daphnia pulex* that has no color change effect in shrimps but adipokinetic effect in stink bug
Locmi-AKH-I Locmi-AKH-II Locmi-AKH-III	pGlu-Leu-Asn-Phe-Thr-Pro-Asn-Trp-Gly-ThrNH_2 pGlu-Leu-Asn-Phe-Ser-Ala-Gly-TrpNH_2 pGlu-Leu-Asn-Phe-Thr-Pro-Trp-TrpNH_2	Adipokinetic hormones of *Locusta migratoria*, three isoforms known
Emppe-AKH	pGlu-Val-Asn-Phe-Thr-Pro-Asn-TrpNH_2	Adipokinetic peptide of the mantid *Empusa pennata*
Tenmo-HrTH	pGlu-Leu-Asn-Phe-Ser-Pro-Asn-TrpNH_2	Hypertrehalosemic peptide of the beetle *Tenebrio molitor*
Declu-CC	pGlu-Leu-Asn-Phe-Ser-Pro-Asn-Trp-Gly-AsnNH_2	Peptide found in the meloid beetle *Decapotoma lunata*
Scade-CC-I Scade-CC-II	pGlu-Phe-Asn-Tyr-Ser-Pro-Asp-TrpNH_2 pGlu-Phe-Asn-Tyr-Ser-Pro-Val-TrpNH_2	Peptides of the scarabaeid beetle *Scarabaeus deludens*, very likely hyperprolinemic
Phote-HrTH	pGlu-Leu-Thr-Phe-Ser-Pro-Asp-TrpNH_2	Hypertrehalosemic peptide of *Phormia terraenovae* and also *Drosophila melanogaster*

**For sequence references, see Ref. 5.*

position 6 (synthesized in the CC of a cetoniid beetle),[10] an AKH with a hydroxylated Pro residue at position 6 found in the CC of a stink bug[11] and an as yet unknown modification in AKHs of various cicadas.[9] In two stick insect species, the Trp[8] residue is either modified to kynurenine or displays an unusual C-glycosylation.[9,19]

Three-Dimensional Structure

As is obvious from Table 1, the termini and the aromatic amino acids at positions 4 and 8 are conserved, suggesting an important feature for receptor binding. This was confirmed by structure activity studies using analogs (see, e.g., Ref. 8). Studies on the solution conformation of a few selected AKH peptides (mostly Locmi-AKHs, Emppe-AKH, Tenmo-HrTH, and Declu-CC) have been performed using either circular dichroism or nuclear magnetic resonance techniques and applying constrained molecular dynamics. All studies indicate a β-turn between residues 4–8, but they are at variance with each other about the N-terminus where β-sheet, "extended structure" and "extended PII conformation" have been proposed.[21]

AKH/RPCH Distribution

The results from several immunocytochemical and in situ hybridization studies have shown that AKHs are localized and synthesized mainly in the glandular lobe of the insect CC.[9] Interestingly, in the case of the migratory locust, mRNAs encoding the three Locmi-AKHs were shown to colocalize in neurosecretory cells of the locust CC,[9] suggesting that the three AKH peptides are synthesized and released from the same cells. Although some have also reported immunoreactivity in neurons of the brain and other parts of the central nervous system of insects, this nonCC immunoreactivity is often a nonspecific crossreaction to other unidentified peptides.[2] Such crossreactions are clearly abolished by the use of antisera preabsorbed with RPCH, whereas the specific immunoreactions in neurons of the CC remain unaffected by preabsorption.[2] Neuronal projections from AKH-producing cells in the CC onto the crop have been observed in dipterans, and it is postulated that AKH may play a role in such animals by stimulating crop emptying.[15,17]

Immunocytochemistry in crustaceans has identified RPCH in neurons of the X-organ and, to a lesser extent, in the medulla terminalis (in the eyestalks of decapods); RPCH immunoreactivity is also distributed in neurons of abdominal ganglia in a crayfish and in neurons that project to the neuropil of the stomatogastric ganglion of a crab and a spiny lobster.[9] This distribution pattern ties in well with the functional activity of RPCH in crustaceans. In situ hybridization studies have confirmed that RPCH is synthesized in neurons of the eyestalks of crustaceans.[18]

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AKH/RPCH Precursor Structure and Processing

Direct protein chemical methods and molecular cloning techniques have been used to show in detail the biosynthesis of up to three AKHs in locusts, including the characterization of the precursor.[9] The AKH gene/precursor is also known from all fully sequenced insect genomes, a number of other insects where genomic information is still lacking, such as the tobacco hornworm moth (*Manduca sexta*), and two blaberid cockroaches (*Blaberus discoidalis* and *Blattella germanica*).[14,15] The Panbo-RPCH precursor is known from a number of crab species, and the Dappu-RPCH gene is known from the *Daphnia pulex* genome data. Apparently, a distinct mRNA encodes each AKH or RPCH precursor, which is translated into a preprohormone with the following general organization: signal peptide, followed by the mature AKH or RPCH peptide, a Gly residue for amidation, a dibasic processing site, and C-terminally a so-called "tail" or "precursor-related peptide" of variable length (shorter in insects than in crustaceans).[23] Specific functions have not yet been annotated for these precursor-related peptides.

For the migratory locust, two independently translated monomers of the proLocmi-AKH-I are oxidized to a unique precursor dimer by the formation of a disulfide bond at the "tail peptide." Processing, therefore, results in two monomeric Gly-Lys-Arg-extended Locmi-AKH-I decapeptides in addition to the dimeric molecule of the "tail peptide." First, the two basic amino acids are enzymatically removed by carboxypeptidase, and the resulting Gly-extended Locmi-AKH-I is modified to the amidated form by a peptidylglycine-α-amidating monooxygenase (see Ref. 9 and the Peptide Biosynthesis/Processing section of this book). This processing scheme has not been verified for any RPCH, and it is not even known whether dimers of other AKHs are formed.

AKH/RPCH RECEPTORS AND THEIR SIGNALING

Although the mature peptides (Panbo-RPCH and Locmi-AKH-I) have been known since the 1970s, receptor studies for AKH/RPCH were scarce. The first report on an AKH receptor protein in insects mentioned specific binding studies with tritiated Manse-AKH on membrane fractions purified from the fat body of adult *M. sexta*, and later, the putative AKH receptor was cloned and sequenced.[27] In crustaceans, it has only been shown that unidentified membrane proteins from various neuronal tissues are able to bind Panbo-RPCH.[9] Less than 10 years ago, with the help of molecular biological methods, receptors for AKH peptides of the fruit fly *Drosophila melanogaster* and the silkworm *Bombyx mori* were cloned and characterized.[22,24] Interestingly, the G protein-coupled receptors (GPCRs) with 7 membrane-spanning domains are structurally related to receptors of the vertebrate gonadotropin-releasing hormone. The identification of the *D. melanogaster* AKH receptor and the subsequent sequencing data from the entire genome of several other insects/organisms led to the identification of similar receptor sequences from those genomic databases. Thus, to date, AKH receptors are known from all those insects that have had the entire genome sequenced. However, researchers have constrained themselves to searching only for homologous sequences (using BLAST programs and consensus cloning), and curiously, only one such AKH receptor per species has been found even in those species where >1 AKH ligand is present (e.g. *B. mori* with two AKHs).[24] It is conceivable that where the ligand (AKH gene) has been duplicated, there may also be a duplication (and modification) of the receptor and that this undiscovered second receptor is very different from the known AKH receptors and therefore not amenable to the current search strategies. The AKH receptor is expressed in insect fat body.[12,15,26,27] Receptor specificity and characterization of cloned receptors have all taken place via *in vitro* assays in which the AKH receptor was expressed in a heterologous system, namely, in vertebrate cell lines (e.g. Chinese hamster ovary cells, CHO; human embryonic kidney cells, HEK) or in frog oocytes.

For a number of insect species and AKH peptides, we have a good knowledge of the events after receptor binding, that is, the mode of action pathways.[6] In brief, for the activation of glycogen phosphorylase, which ultimately results in the increase of trehalose in the hemolymph (see below: biological function), a G_q protein is activated, phospholipase is stimulated, the second messenger inositol trisphosphate is produced, Ca^{2+} from internal stores are released but, apparently, entry of extracellular calcium into the cell is also promoted. For the activation of triacylglycerol lipase, which leads to the increase of diacylglycerol in hemolymph, a G_s protein is activated, adenylyle cyclase is stimulated, the second messenger cyclic AMP is increased, and intracellular Ca^{2+} also plays a role.

Later studies on the *B. mori* AKH receptor (Bommo-AKHR) used molecular and cell biology methods to characterize its signaling after stably expressing the receptor in HEK293 cells.[13,26] These experiments confirm that the ligand Manse-AKH binds to a G_s-coupled Bommo-AKHR and dose dependently increases cAMP and intracellular Ca^{2+}, which stimulates phosphorylation of extracellular signal-related kinase 1/2 (ERK 1/2); activation of the ERK 1/2 pathway is mediated via protein kinases A and C. After ligand-binding and activation of Bommo-AKHR, the receptor is rapidly internalized from the cell membrane in clathrin-coated pits and slowly recycled in endosomes after the removal of the ligand; internalization is mediated by GPCR kinase and β-arrestin-2, desensitizing the receptor after ligand-binding; three amino acids (Thr^{356}, Ser^{359}, and Thr^{362}) at the C-terminus of Bommo-AKHR are important for this internalization. In contrast to biochemical studies

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using fat body tissues of insects to study the signal transduction pathways of AKH/AKHR (see above), the heterologously expressed Bommo-AKHR does not involve a phosholipase. This may be a limitation of the HEK cell machinery versus that of the insect fat body and should serve as a reminder that the Bommo-AKHR studies should be repeated in *B. mori* fat body tissue to confirm that the homologous system does, indeed, operate via the same transduction pathways as seen with the HEK cell line.

Much less is known of the mode of action of Panbo-RPCH in crustaceans, but it seems to be clear that for both the aggregation of the integumentary pigment and the retraction of distal retinal pigment in the compound eye, internal and external Ca^{2+} are necessary and that phospholipase and inositol trisphosphate may be involved in the signaling processes.[9]

G protein–coupled receptors are classified into three classes (A–C) based on their cell signaling and trafficking pathways.[20] According to the current model, the insect AKH receptor belongs to Class A GPCRs along with μ-opioid receptor, endothelin type A and β-2-adrenergic receptor, among others.[13] The crystal structures of the two main subgroups of class A GPCRs are known and by homology modeling, two models were constructed for the three-dimensional structure of the *Anopheles gambiae* AKH receptor (Anoga-AKHR).[20] The β-2-adrenergic–based structure of Anoga-AKHR represents an "open" conformation, that is, the ligand Anoga-AKH has easy access to the hormone binding pocket (typifying the inactive, ligand-free AKHR state), whereas the rhodopsin-based structure is "closed" (inaccessible to the free ligand, equating the active receptor to which the ligand is bound). Ligand-docking calculations show a 30 times higher affinity for the endogenous ligand than for another AKH used in the study.

BIOLOGICAL ACTIONS

In crustaceans, the major biological function of Panbo-RPCH is surely the aggregation of pigment granules in the epithelial chromatophores. Thereby, one or more different types of chromatophore (and, thus, pigment colors) can be affected. Next, the peptide is also responsible for the retraction (aggregation) of the distal retinal pigment in the ommatidia. Extrapigmentary effects of Panbo-RPCH are known as well. For example, it is implicated in the stimulation of the release of methyl farnesoate from mandibular organs; other effects are neuromodulatory, which affect the rhythm of certain parts of the stomatogastric and swimmeret system; moreover, modulation of photoreceptor cells has been reported.[9] There is a claim that Panbo-RPCH increases the glucose level in the isopod crustacean *Porcellio scaber*; however, the article is not clear on the methods and concentrations used, and a number of necessary controls were omitted.[28]

In insects, the basic physiological action of AKH peptides is the increase of those metabolites (regardless of the nature of the metabolites, see below) that are used by the muscles (and other tissues) as a source of energy especially during exercise, such as flight and running. The AKH peptides target the fat body tissue to convert its stored triacylglycerols or glycogen to diacylglycerol or trehalose, respectively, or to synthesize the amino acid proline from alanine and fatty acids. Diacylglycerols, trehalose, or proline are released from the fat body, and their concentrations are consequently elevated in the hemolymph. There is no specific AKH peptide that is responsible for eliciting the increase of diacylglycerols in the hemolymph of locusts, for example, and another one that elevates the concentration of hemolymph trehalose in cockroaches or the level of proline in the tsetse fly. The activation of the respective pathways for lipid-, carbohydrate-, or proline mobilization is inherent in the enzymatic machinery of the fat body of the respective species and correlates with its main fuel for heavy locomotory activities, for example, flight activity.[4] Other actions of AKH peptides are on the hemolymph level to increase the concentration of lipophorins, that is, proteins that elevate the capacity of shuttling more lipids from fat body to muscles, or, on the flight muscle level where AKH peptides, are proposed to stimulate the oxidation of lipids. Other stimulatory actions are the acceleration of the rate of the heart beat, which is interpreted as a mechanism to facilitate faster distribution of metabolites, and the increase in the oxidative capacity of mitochondria. Inhibitory actions of AKH peptides are also known on the biosynthesis of protein, RNA and fatty acids.[4] The effect on fatty acids is logical, because the main effect of AKH is to stimulate lipid degradation, while the former effect is mainly discussed under the physiological aspect of reproduction where AKH peptides and the repression of the synthesis of vitellogenin (the egg yolk precursor protein) have been analyzed. The functional importance of AKHs has been investigated by molecular manipulations *in vivo* in *D. melanogaster* where mutant flies were generated by targeted genetic constructs and specific crossbreeding,[12,17] and in the cockroach *B. germanica* by the use of RNA interference (RNAi) methods.[14] The fruit fly studies confirm that AKH and its receptor play an important role in metabolism, specifically in mobilizing stored metabolites from the fat body. However, the absence of AKH and/or its receptor does not seem to have adverse conditions during larval development, and does not affect the normal reproductive ability of adults, despite the lowered levels of trehalose and lipids in the hemolymph. A possible explanation for these results may be that there is a compensatory mechanism or alternative pathway by which lipids are mobilized in insects. Evidence for such a dual, independent adipokinetic system is seen in *D. melanogaster*, where an adipose triglyceride lipase called Brummer lipase is as important as the AKH/AKHR in fat mobilization: dual loss of Brummer lipase and AKHR results in extreme obesity in flies, the stored lipid could not be mobilized by AKH (although the stored carbohydrates are

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accessed and depleted), and double mutant flies die quickly after food deprivation – all in contrast to single mutants (i.e. where either Brummer lipase or AKHR was lacking).[12] Inactivation of the AKH/AKHR system results in upregulation (hyperstimulation) of the Brummer lipase gene expression in starving flies. Conversely, overexpression of AKH in fat body suppresses Brummer lipase transcription, thus showing clear compensatory regulation between the two systems. Another triglyceride lipase has been characterized from moth fat body (a homolog is present in *D. melanogaster* too) as an active phospholipase AI.[12]

It seems that AKH peptides are somehow intricately linked with certain actions of Juvenile hormone in its inhibitory effect on vitellogenin production.[4] Blage-HTH is a member of the AKH family that causes hypertrehalosemia in *B. germanica*; adult female cockroaches display a higher concentration of trehalose in the hemolymph than the males, and this fluctuates in a cyclic pattern that correlates with the maturation of oocytes in the reproductive cycle. When the hypertrehalosemic hormone (HrTH) is silenced in female cockroaches via double-stranded RNA, the peak in circulating trehalose, as well as oviposition is significantly delayed in virgin females and in some instances, deformed ootheca were produced.[14]

AKH peptides also have functions that are not directly related to energy metabolism, such as an implied role in the immune response of locusts and activation of antioxidant mechanisms. These studies have been reviewed.[16] In certain insects (firebug, cricket), AKH peptides have an effect on the locomotory activity. Interestingly, AKH-deficient *D. melanogaster* shows normal circadian locomotory activity (peaks at dawn and dusk) but do not display starvation-induced hyperactivity that is observed in normal (wild-type) flies; this implies that AKH regulates "foraging" locomotion.[17]

In summary, although the mobilization of substrates for high-energetic phases is the major and most well-known function of AKH peptides in insects, these peptides are truly multifunctional and exert pleiotropic action as is also known from the majority of other hormones in invertebrates and vertebrates.

ACKNOWLEDGMENTS

The authors research was financially supported in part by the National Research Foundation (Pretoria, South Africa; latest grant numbers: FA 2007021300002 and IFR 2008071500048 to G.G. and GUN No. 61107 to H.G. Marco) and by the University of Cape Town in the form of block grants to the authors.

REFERENCES

1. Christie AE, Cashman CR, Brennan HR, Ma M, Sousa GL, Li L, et al. Identification of putative crustacean neuropeptides using in silico analyses of publicly accessible expressed sequence tags. *Gen Comp Endocrinol* 2008;**156**:246–64.
2. Eckert M, Gabriel J, Birkenbeil H, Greiner G, Rapus J, Gäde G. A comparative immunocytochemical study using an antiserum against a synthetic analogue of the corpora cardiaca peptide Pea-CAH-I (MI, neurohormone D) of *Periplaneta americana*. *Cell Tissue Res* 1996;**284**:401–13.
3. Fernlund P, Josefsson L. Crustacean color change hormone: amino acid sequence and chemical synthesis. *Science* 1972;**177**:173–5.
4. Gäde G. Regulation of intermediary metabolism and water balance of insects by neuropeptides. *Ann Rev Entomol* 2004;**49**:93–113.
5. Gäde G. Peptides of the adipokinetic hormone/red pigment-concentrating hormone family. A new take on biodiversity. *Ann NY Acad Sci* 2009;**1163**:125–36.
6. Gäde G, Auerswald L. Mode of action of neuropeptides from the adipokinetic hormone family. *Gen Comp Endocrinol* 2003;**132**: 10–20.
7. Gäde G, Auerswald L, Simek P, Marco HG, Kodrik D. Red pigment-concentrating hormone is not limited to crustaceans. *Biochem Biophys Res Commun* 2003;**309**:967–73.
8. Gäde G, Hayes TK. Structure–activity relationships for *Periplaneta americana* hypertrehalosemic hormone I: the importance of side chains and termini. *Peptides* 1995;**16**:1173–80.
9. Gäde G, Marco HG. Structure, function and mode of action of select arthropod neuropeptides. In: Atta-Ur-Rahmann, editor. *Studies in natural product chemistry. Bioactive natural products (part M)*, vol. 33. Amsterdam: Elsevier; 2006. p. 69–139.
10. Gäde G, Simek P, Clark KD, Auerswald L. Unique translational modification of an invertebrate neuropeptide: a phosphorylated member of the adipokinetic hormone peptide family. *Biochem J* 2006;**393**:705–13.
11. Gäde G, Simek P, Marco HG. An invertebrate [hydroxyproline]-modified neuropeptide: further evidence for a close evolutionary relationship between insect adipokinetic hormone and mammalian gonadotropin hormone family. *Biochem Biophys Res Commun* 2011; **414**:592–7.
12. Grönke S, Müller G, Hirsch J, Fellert S, Andreou A, Haase T, et al. Dual lipolytic control of body fat storage and mobilization in *Drosophila*. *PLOS Biol* 2007;**5**:e137.
13. Huang H, Deng X, He X, Yang W, Li G, Shi Y, et al. Identification of distinct c-terminal domains of the *Bombyx* adipokinetic hormone receptor that are essential for receptor export, phosphorylation and internalization. *Cell Signal* 2011;**23**:1455–65.
14. Huang J-H, Lee H-J. RNA interference unveils functions of the hypertrehalosemic hormone on cyclic fluctuation of hemolymph trehalose and oviposition in the virgin female *Blattella germanica*. *J Insect Physiol* 2011;**57**:858–64.
15. Kaufmann C, Merzendorfer H, Gäde G. The adipokinetic hormone system in Culicinae (Diptera: Culicidae): molecular identification and characterization of two adipokinetic hormone (AKH) precursors from *Aedes aegypti* and *Culex pipiens* and two AKH receptor variants from *A. aegypti*. *Insect Biochem Mol Biol* 2009;**39**:770–81.
16. Kodrik D. Adipokinetic hormone functions that are not associated with insect flight. *Physiol Entomol* 2008;**33**:171–80.
17. Lee G, Park JH. Hemolymph sugar homeostasis and starvation-induced hyperactivity affected by genetic manipulations of the adipokinetic hormone-encoding gene in *Drosophila melanogaster*. *Genetics* 2004;**167**:311–23.
18. Linck B, Klein JM, Mangerich S, Keller R, Weidemann WM. Molecular cloning of crustacean red pigment-concentrating hormone precursor. *Biochem Biophys Res Commun* 1993;**195**:807–13.

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19. Malik A, Gäde G, Lange AB. Sequencing and biological effects of an adipokinetic/hypertrehalosemic peptide in the stick insect, *Baculum extradentatum*. *Peptides* 2012;**34**:51–6.
20. Mugumbate G, Jackson GE, van der Spoel D. Open conformation of adipokinetic hormone receptor from the malaria mosquito facilitates hormone binding. *Peptides* 2011;**32**:553–9.
21. Nair MM, Jackson GE, Gäde G. Conformational study of insect adipokinetic hormones using NMR constrained molecular dynamics. *J Comput-Aided Mol Des* 2001;**15**:259–70.
22. Park Y, Kim Y-J, Adams ME. Identification of G protein-coupled receptors for *Drososphila* PRXamide peptides, CCAP, corazonin, and AKH supports a theory of ligand-receptor coevolution. *Proc Natl Acad Sci USA* 2002;**99**:11423–8.
23. Rao KR. Crustacean pigmentary-effector hormones: chemistry and functions of RPCH, PDH, and related peptides. *Amer Zool* 2001;**41**:364–79.
24. Staubli F, Jorgensen TJD, Cazzamali G, Williamson M, Lenz C, Sondergaard L, et al. Molecular identification of the insect adipokinetic hormone receptors. *Proc Natl Acad Sci USA* 2002;**99**:3446–51.
25. Stone JV, Mordue W, Batley KE, Morris HR. Structure of locust adipokinetic hormone, a neurohormone that regulates lipid utilisation during flight. *Nature* 1976;**263**:207–11.
26. Zhu C, Huang H, Hua R, Li G, Yang D, Luo J, et al. Molecular and functional characterization of adipokinetic hormone receptor and its peptide ligands in *Bombyx mori*. *FEBS Lett* 2009;**583**:1463–8.
27. Ziegler R, Isoe J, Moore W, Riehle MA, Wells MA. The putative AKH receptor of the tobacco hornworm, *Manduca sexta*, and its expression. *J Insect Sci* 2011;**11**:40.
28. Zrala J, Kodrik D, Zahradnickova H, Zemek R, Socha R. A novel function or red pigment-concentrating hormone in crustaceans: *Porcellio scaber* (Isopoda) as a model species. *Gen Comp Endocrinol* 2010;**166**:330–6.

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Chapter 29

Allatostatins

Stephen S. Tobe and William G. Bendena

ABSTRACT

There are three families of allatostatins (ASTs), peptides discovered on the basis of their ability to inhibit juvenile hormone (JH) production in insects: the cockroach type representing the FGLa family, the cricket type representing the $W(X)_6Wa$ family, and the *Manduca* type representing the PISCF family. Each of these families performs multiple functions, including the modulation of myotropic activity and neurotransmission. These effects are species and order specific, and each family inhibits JH production in only a limited number of related orders, suggesting that their original functions were probably not the inhibition of JH production. This chapter summarizes the expression and distribution of these peptides, their interaction with receptors, and their biological activities.

DISCOVERY

JH, produced by the insect corpora allata (CA), is the regulator of metamorphic and reproductive functions in most insects. The JHs are a family of sesquiterpenoid compounds, the most ubiquitous of which is JH III. The production of JHs and related compounds[1] are regulated by other compounds originating in the central nervous system, principally neuropeptides. Measurement of the production of JH has revealed large changes in the rates of biosynthesis, which are also believed to be reflected in changes in the titers in the hemolymph.[28] ASTs, inhibitors of production of JH, were hypothesized to exist in 1980,[27] after demonstrations by Scharrer and colleagues[24] that presumed that peptidergic factors in the brains of cockroaches modulate the volume of the CA, which in turn was believed to translate into changes in hormone production.

The first ASTs were discovered in the cockroach, *Diploptera punctata*, and are members of the 'FGLa' family (the common C-terminal sequence for all members of the family) (FGLa-related allatostatins—FGLa/ASTs).[5,34] Two years later, the first member of the 'PISCF' family was identified in the moth *Manduca sexta* (PISCF-related allatostatins—PISCF/AST).[5,16] In both instances, the ability of the peptides to inhibit the production of JH was the principal assay used for identification, after HPLC separation of brain extracts. Because the first function ascribed to the families was inhibition of JH production, the name 'allatostatin' was applied to the families. The FGLa/ASTs have numerous other biological actions, including modulation of myotropic activity and gut enzyme activity, and they also can act as neurotransmitters. The FGLa/AST family is now known to consist of >400 different peptides,[11] and members are found in many invertebrate phyla, including most arthropods examined to date, as well as in arachnids and nematodes (Table 1). However, only in cockroaches, crickets, and termites do they function as inhibitors of JH production.[25] DNA sequencing projects have indicated that the PISCF/AST family is also widely distributed, occurring in annelids, arachnids, molluscs, crustaceans, and insects. Evidence that PISCF/ASTs inhibit JH biosynthesis is limited to the dipteran *Aedes aegypti* and Lepidoptera.

A third family of peptides was subsequently discovered in crickets and is characterized by the consensus sequence $W(X)_6Wa$,[20] which is characteristic of the Myoinhibitory peptide (MIP) family of peptides. This family of peptides is widely distributed throughout the protostomes. The ability to inhibit JH production has been shown in *Gryllus* and *Tenebrio molitor*. Although this family does have allatostatic activity in a limited number of species, it is not classified as an allatostatin because of its prior discovery as a member of the MIP family. The peptides of this family are now known as the MIPs.

STRUCTURE OF ALLATOSTATIN PRECURSORS

The first preproallatostatin protein was characterized from a gene sequence obtained from the cockroach, *D. punctata*.[2,6] The *Diploptera* AST precursor has 370 amino acid residues and contains 13 unique ASTs that range from 6 to 18 amino acids and, when processed, share a COOH-terminal core sequence Y/FXFGL/I-amide. Each AST in the precursor is followed by glycine and a dibasic KR or RK endoproteolytic cleavage site. After cleavage, the terminal glycine functions as the substrate for peptidyl-glycine α-amidating monooxygenase. Purified FGLa/ASTs are C-terminally amidated and require amidation for biological activity, confirming

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00029-4
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TABLE 1 Sequences of Representative Members of the Three Allatostatin Families

Species	Designation	Sequence
FGLa/AST family		
Diploptera punctata	Dippu-AST 1	LYDFGLa
	Dippu-AST 2	AYSYVSEY**KR**LPVYNFGLa
	Dippu-AST 5	DRLYSFGLa
Drosophila melanogaster	Drome-AST 3	SRPYSFGLa
Bombyx mori	Bommo-AST 5	ARMYSFGLa
C. elegans	*nlp-5**	ALSTFDSLGGMGLa
	*nlp-6**	MAAPKQMVFGFa
		YKPRSFAMGFa
		AAMRSFNMGFa
		LIMGLa
W(X)$_6$Wa/MIP family		
Gryllus bimaculatus	Grybi-AST 1	AWRDLSGGWa
	Grybi-AST 2	AWNNLGSAWa
	Grybi-AST3	GWRDLNGGWa
	Gybi-AST4–6	GWQDLNGGWa
PISCF/AST family		
Manduca sexta	Manse-PISCF AST	pQVRFRQCYFNPISCF-OH
Drosophila melanogaster	Drome-PISCF AST	pQVRYRQCYFNPISCF-OH
Anopheles gambiae	Anoga-PISCF AST	pQIRYRQCYFNPISCF-OH

One dibasic cleavage site in Dippu-AST 2 is shown in bold. Substitutions in the PISCF family are underlined.
*C. elegans *AST-like peptides have no designation but are derived from the* nlp-5 *and* nlp-6 *genes.*

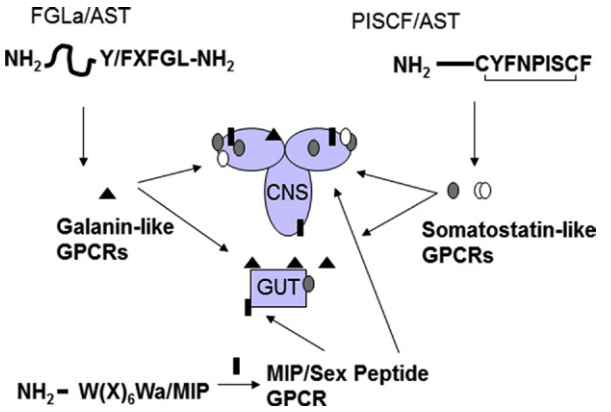

FIGURE 1 The FGLa/ASTs interact with two galanin-like G protein-coupled receptors (GPCRs). One type of receptor may be more prevalent in the CNS and the other more abundant in the gut tissue. PISCF/ASTs with oxidized cysteines C7–C14 binds with two somatostatin-like receptors. In *Drosophila*, these receptors seem to be localized to the pars intercerebralis of the brain and the optic lobe. Somatostatin-like receptors have not been found to be expressed in the gut although PISCF/AST immunoreactivity is present in gut tissues. WX_{6W} ASTs/MIP interact with MIP/sex peptide GPCRs although expression data are not yet available. The MIP/ASTs share sequence similarity with myoinhibitory peptides (MIPs) and galanin. As the N-terminus of galanin is required for receptor binding, MIP/ASTs and myoinhibitory peptides could hypothetically interact with the galanin-like GPCRs.

that these signals are utilized *in vivo*. The FGLa/ASTs are clustered in groups within the precursor, with each group separated by spacer regions containing acidic amino acids which effectively neutralize the basic charge contribution of the FGLa/ASTs and their processing sites (Fig. 1).

The structure and organization of the *Diploptera* precursor serve as a prototype for all FGLa/AST precursors identified to date. There is variation between different orders in the number of FGLa/ASTs present in their respective precursors. For example, Lepidoptera FGLa/AST precursors contain 8–10 peptides, Dipterans, 4–5, and crustacean AST precursors contain up to 50 peptides (see genome databases).

In crickets, the W(X)$_6$Wa/MIP precursor expresses multiple peptides structurally similar to the Dippu FGLa/AST precursor. The preproAST polypeptide sequence for the W(X)$_6$Wa cricket family of ASTs was first identified from cloned DNA in *Drosophila*[32] and the cricket, *Gryllus bimaculatus*.[31] The *Drosophila* W(X)$_6$Wa precursor contains sequences for five unique W(X)$_6$Wamide peptides, which are followed by GKR, indicating COOH-terminal amidation and cleavage. The *Gryllus* precursor contains sequences for six cricket ASTs, four of which are unique in sequence. The spacing between W residues is generally 6 amino acids but has been found to be 7 or 8 in select organisms.[12,29]

The sequence of the preproAST polypeptide of *Manduca* PISCF was deduced from the DNA sequence in the moth, *Pseudaletia unipuncta*[13] and in *Drosophila*.[23,33] A single 15 amino acid AST peptide flanked by dibasic endoproteolytic cleavage sites is released as a nonamidated peptide from the carboxyl terminus of the 125 amino acid precursor. The PISCF/AST differs between *Manduca* and *Drosophila* by one conservative amino acid substitution (Y^4 for F^4) and an additional substitution in *Anopheles*/*Aedes* (I^2 for V^2).[19] A more dramatic alteration occurs in *Acrythosiphon pisum* that differs from *Drosophila* through the loss of pQ^1 and C-terminal amidation.[12] In all PISCF/ASTs, the C residues at –2 and –9 from the C-terminus are maintained (Table 1).

DISTRIBUTION OF mRNA AND PEPTIDES

The distribution and expression of ASTs have been examined with antibodies specific to AST peptides, through tissue in situ hybridization whereby specific nucleic acid probes

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detect cells expressing AST mRNA and by quantitative PCR of individual tissues.[7,8] The following describes the extensive expression of FGLa/ASTs noted in *D. punctata*. Four medial cells of the pars intercerebralis express FGLa/ASTs and extend axons that arborize and terminate adjacent to 25–30 lateral neurosecretory cell bodies of the protocerebrum. These lateral cells project FGLa/AST-immunoreactive nerve fibers in the nervi corporis cardiaci (NCC) II and arborize within the corpus cardiacum and CA. Extensively arborizing AST-immunoreactive neurons are also detected in the optic, central and antennal brain lobes as well as in the pulsatile organ muscle.[26] Clearly, the brain and associated structures contain large amounts of FGLa/ASTs, but we do not know the relative percentage of this material that projects into the CC and CA, for inhibition of JH biosynthesis. However, these observations do suggest that the FGLa/AST peptides play other major roles in the brain.

FGLa/AST-immunoreactive neurons innervate the rectal dilator muscles, muscles of the rectum, anterior hindgut, midgut,[26] and the lateral and common oviducts. FGLa/AST-immunoreactive cells are also detected in open-type midgut endocrine cells. These cells extend from the basal lamina to the lumen of the midgut and are sites of AST mRNA synthesis. AST expression and production are also found in a population of circulating hemocytes that comprise approximately 5% of the total hemocytes[25] In other insects, such as flies, moths, and earwigs, FGLa/ASTs are detected in the cells of the brain and optic lobe but generally are not detected in nerve fibers extending within the CA. Immunoreactive nerve fibers have been detected in the foregut, midgut, and hindgut of *Locusta* but such localization is rare in other insects. However, expression in endocrine cells of the midgut is common.[2] Immunoreactive midgut cells for each family of AST have been mapped in *Drosophila*.[30]

Little data are available on the expression of the two FGLa/AST receptors in *Drosophila*. However, microarray suggests that one receptor subtype may predominate in brain and the other in the gut.[4]

PEPTIDE PROCESSING

The occurrence of multiple FGLa/ASTs in the preproallatostatins, with associated cleavage and amidation signals, suggests that all of the peptides are probably cleaved and present at the same time in any given tissue. Prohormone convertases are probably responsible for the liberation of the various AST peptides. There is no evidence for selective processing of the precursor or of tissue-specific processing.

Degradation of the FGLa/ASTs to terminate their biological actions occurs in several different ways and is dependent on the precise sequence of the peptides and the tissues in which degradation is studied. For example, endopeptidases as well as amino- and carboxypeptidases are the principal proteolytic enzymes for degradation of the ASTs.[9] In tissues, the FGLa/AST peptides are cleaved by carboxypeptidases to yield the corresponding nonamidated FG-OH peptides (removal of L-amide), resulting in inactive peptides. Hemolymph of *D. punctata* does not contain carboxypeptidases, but rather aminopeptidases, which produce peptides with an intact core sequence, and hence continued but reduced biological activity. Interestingly, changes in the sequence can also alter the half-life of the AST peptides. For example, Dippu-AST 5 has a much longer half-life than other ASTs in hemolymph. To date, the peptidases responsible for the cleavage of the ASTs have not been identified or cloned.

ALLATOSTATIN RECEPTORS

The sequencing of the *Drosophila* genome has led to the identification of at least 32 neuropeptide-encoding genes. These neuropeptides interact with G protein-coupled receptors (GPCRs) which are characterized as seven-transmembrane domain receptors (see Figure 1). *Drosophila* has approximately 160 GPCRs of which 44 have characteristics of receptors for peptide ligands.[10]

The presence of multiple FGLa/AST receptors was first suggested by ligand–membrane binding studies in the cockroach, *D. punctata*.[26] Two *Drosophila* genes encode GPCRs functionally specific for FGLa/AST peptides. Both GPCRs belong to the rhodopsin-like GPCR family and are most closely related to the vertebrate galanin receptors. The first, gene, CG2872, is located at chromosomal locus 3D6-E1 and encodes a GPCR (AlstR, Dar-1) of 394 amino acids that responds to FGLa/ASTs.[3] AlstR/Dar-1 mRNA is not expressed in embryos but appears in larvae, pupae, and adults. Expression is found in both heads and bodies of adults. The second *Drosophila* gene, CG10001 (Dar-2), is located at chromosomal locus 98DE and encodes a GPCR of 357 amino acids.[18] Dar-2 is probably a paralog of AlstR/Dar-1 as Dar-2 shares 47% overall sequence identity and 60% identity in the transmembrane regions. Dar-2 mRNA expression begins in 8- to 16-hour embryos and is found in all later stages of development. In third instar larvae and adults, mRNA expression in the gut/abdomen is greater than that in isolated brain/head. RNAi knockdown of either Dar-1 or Dar-2 does not alter juvenile hormone biosynthesis or release and therefore, they do not seem to be involved in the regulation of JH production in *Drosophila* (Bendena and Tobe, unpublished). AST receptor (Dar-1) orthologs have been sequenced in *D. punctata*, *Periplaneta americana*, and *Bombyx mori*. *Bombyx* fifth instar larvae express GPCR mRNA in the foregut, midgut, hindgut, and brain. Additional AST receptor orthologs have been identified through sequencing projects to include invertebrates such as *Apis mellifera*, *Anopheles gambiae*, and *Caenorhabditis elegans*.

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The *Drosophila* $W(X)_6W$/AST or MIP receptor gene has been identified as the sex peptide receptor (CG16752),[15,22] which corrects the original assignment (CG14484 = CG30106), and which is now known as a CCHamide receptor.

Two *Drosophila* PISCF/AST receptor genes (CG7285 and CG13702) have been identified in two separate functional assays.[17] CG7285 and CG13702 encode GPCRs of 483 amino acids (Drostar1) and 549 amino acids (Drostar2), respectively (Fig. 1). Both receptors share 60% overall identity and 76% identity in the transmembrane regions. These genes are paralogs located at cytological locus 75D1 and 75D2 and are separated by 80 kb. Drostar 1 and 2 are orthologs of the vertebrate somatostatin receptors sharing 42% amino acid identity in the transmembrane region. This identity is concentrated in those parts of the transmembrane regions facing the cytosol where the receptors couple with their heterotrimeric G-protein. In situ hybridization has localized the Drostar genes to cells in the lamina region of the optic lobes and to cells of the pars intercerebralis of the brain of adult flies.[17]

STRUCTURE ACTIVITY AND ACTIVE CONFORMATIONS

Structure–activity studies have shown that the core sequence for biological activity in the FGLa family is the C-terminal pentapeptide Y/FXFGL/I-a although some analogs of FGLa/AST (Y/FX region substituted with an aromatic group) do show activity.[14,35] Amidation is also essential for biological activity. The consensus sequence for the cricket family seems to be $W(X)_6Wa$ and amidation is similarly necessary. The minimal biologically active region for the PISCF family is unknown although the oxidation of the two cysteine residues is critical for receptor binding. Receptor binding activity was not dependent on the presence of a blocked N-terminus.[16]

The core region of the FGLa/AST family seems to possess secondary structural elements, which are probably essential for interaction with the receptors. In particular, this region probably contains a type- II β turn, with the most critical residues being F and L, at least in selected ASTs. Incorporation of moieties promoting a β turn in the core region permitted the synthesis of analogs of these peptides that exhibit high biological activity.[21]

BIOLOGICAL ACTIONS

Based on immunocytochemical distribution, isolation, and sequencing, or mining of genomic databases, it has become clear that the AST families are ubiquitous throughout the Class Insecta and many other invertebrate phyla, and regulate many processes in addition to JH production.[25]

Other actions of ASTs have been largely defined in individual species, and therefore, it is unknown at present if such actions can be inferred across the insects. In the case of the FGLa/AST family, such actions include 1. the inhibition of vitellogenin production by fat body in cockroaches, 2. the inhibition of myotropic activity, particularly in gut tissue of Dictyoptera and Orthoptera, 3. regulation of release of digestive enzymes in the midgut of cockroaches, 4. neurotransmission in crab stomatogastric ganglion, and possibly cockroach CNS. Treatment of cultured mammalian neurons expressing Dar-1 receptor with the FGLa/AST ligand is being used to selectively silence these neurons.[25]

Biological actions of the $W(X)_6Wa$/MIP family include 1. inhibition of ecdysteroid biosynthesis (prothoracicostatic activity) by prothoracic glands in silk moth and ovaries of crickets, 2. inhibition of locust oviduct muscle, and 3. inhibition of spontaneous muscle contraction in foregut of the cockroach.

The PISCF/AST family displays biological effects similar to the other two AST families, dramatically inhibiting contraction in larval *Drosophila* heart muscle for example, (hence, its name *flatline*) and its localization in gut suggests similar functions in this tissue. This peptide family may also play a role in the regulation of migratory flight in Lepidoptera. Although the PISCF/AST family seemed to inhibit JH production only in the members of the Lepidoptera, the discovery of a conservatively modified PISCF peptide in the *A. gambiae* genome database and its apparent ability to inhibit JH production in adults of another mosquito, *A. aegypti*[19] suggests that this peptide family may be important as an allatoregulator in at least some Diptera as well.

The modes of action of these three peptide families remain largely unexplored, although studies with the FGLa/AST family indicate that the second messengers cAMP, cGMP, and calcium are all effectors. With our present knowledge of second messenger cascades and signaling pathways, the time is appropriate for a reexploration of this area.

CONCLUDING REMARKS

As is the case with many invertebrate peptide families, the allatostatin families are multifunctional peptides. The very large number of peptides in the FGLa/AST family, with significant variation in length, but conservation of the core C-terminal pentapeptide, indicates that this family serves important functions across the invertebrates. However, the relatively limited number of orders in which they act as inhibitors of JH biosynthesis[25] also suggests that this function appeared secondarily; thus, the FGLa/AST family was coopted as inhibitors by cockroaches, crickets, and termites early in evolution. In these instances, specificity of function resides with the tissues and the cognate allatostatin receptors. Because the CA of these orders are heavily invested by FGLa-containing nerves, with multiple release sites, the local concentration of the peptides is probably very high.

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It is therefore conceivable that differing concentrations of the peptides or differing receptor affinity could confer multiple functionality. Receptor affinity in both the CA and other target tissues such as gut and oviduct muscle no doubt dictate responsiveness. The occurrence of at least two receptor types in insects and their similarity to the galanin GPCRs suggests an ancient origin. The same ideas can be applied to the evolutionary necessity of varying insects to adopt alternative peptides as ASTs. A clue may come from the cricket MIP/ASTs whereby the most potent molecule resembles the mammalian galanins and hence may interact with the galanin-like receptor as does the FGLa/AST. Subtle changes in the receptor "binding pocket" may have provided the "evolutionary pressure" to solicit other peptide molecules to perform the "allatostatic" function. The PISCF/AST is further removed in sequence yet has the same functional quality as the FGLa/ASTs in that a major role is inhibition of muscle contraction. As muscle contraction is the probable ancestral function, a reasonable hypothesis would be that all ASTs were coopted for allatostatic regulation somewhat later in evolutionary time.

REFERENCES

1. Belles X, Martin D, Piulachs MD. The mevalonate pathway and the synthesis of juvenile hormone in insects. *Annu Rev Entomol* 2005;**50**:181–99.
2. Bendena WG, Donly BC, Tobe SS. Allatostatins: a growing family of neuropeptides with structural and functional diversity. *Ann N Y Acad Sci* 1999;**897**:311–29.
3. Birgul N, Weise C, Kreienkamp HJ, Richter D. Reverse physiology in drosophila: identification of a novel allatostatin-like neuropeptide and its cognate receptor structurally related to the mammalian somatostatin/galanin/opioid receptor family. *EMBO J* 1999;**18**:5892–900.
4. Chintapalli VR, Wang J, Dow JAT. Using FlyAtlas to identify better *Drosophila melanogaster* models of human disease. *Nat Genet* 2007;**39**:715–20.
5. Coast GM, Schooley DA. Toward a consensus nomenclature for insect neuropeptides and peptide hormones. *Peptides* 2011;**32**:620–31.
6. Donly BC, Ding Q, Tobe SS, Bendena WG. Molecular cloning of the gene for the allatostatin family of neuropeptides from the cockroach *Diploptera punctata*. *Proc Natl Acad Sci U S A* 1993;**90**:8807–11.
7. Garside CS, Bendena WG, Tobe SS. Quantification and visualization of Dippu-AST mRNA in the brain of adult *Diploptera punctata*: mated females vs. virgin females vs. males. *J Insect Physiol* 2003;**49**:285–91.
8. Garside CS, Koladich PM, Bendena WG, Tobe SS. Expression of allatostatin in the oviducts of the cockroach *Diploptera punctata*. *Insect Biochem Mol Biol* 2002;**32**:1089–99.
9. Garside CS, Hayes TK, Tobe SS. Inactivation of Dip-allatostatin 5 by membrane preparations from the cockroach *Diploptera punctata*. *Gen Comp Endocrinol* 1997;**108**:258–70.
10. Hewes RS, Taghert PH. Neuropeptides and neuropeptide receptors in the *Drosophila melanogaster* genome. *Genome Res* 2001;**11**:1126–42.
11. Hult EF, Weadick CJ, Chang BSW, Tobe SS. Reconstruction of ancestral FGLamide-type insect allatostatins: a novel approach to the study of allatostatin function and evolution. *J Insect Physiol* 2008;**54**:959–68.
12. Huybrechts J, Bonhomme J, Minoli S, Prunier-Leterme N, Dombrovsky A, Abdel-Latief M, et al. Neuropeptide and neurohormone precursors in the pea aphid, *Acyrthosiphon pisum*. *Insect Mol Biol* 2010;**19**: 87–95.
13. Jansons IS, Cusson M, McNeil JN, Tobe SS, Bendena WG. Molecular characterization of a cDNA from *Pseudaletia unipuncta* encoding the *Manduca sexta* allatostatin peptide (Mas-AST). *Insect Biochem Mol Biol* 1996;**26**:767–73.
14. Kai Z, Huang J, Xie Y, Tobe SS, Ling Y, Zhang L, et al. Synthesis, biological activity, and hologram quantitative structure–activity relationships of novel allatostatin analogues. *J Agric Food Chem* 2010;**58**:2652–8.
15. Kim Y, Bartalska K, Audsley N, Yamanaka N, Yapici N, Lee J, et al. MIPs are ancestral ligands for the sex peptide receptor. *Proc Natl Acad Sci U S A* 2010;**107**:6520–5.
16. Kramer SJ, Toschi A, Miller CA, Kataoka H, Quistad GB, Li JP, et al. Identification of an allatostatin from the tobacco hornworm *Manduca sexta*. *Proc Natl Acad Sci U S A* 1991;**88**:9458–62.
17. Kreienkamp HJ, Larusson HJ, Witte I, Roeder T, Birgul N, Honck HH, et al. Functional annotation of two orphan G protein-coupled receptors, Drostar 1 and -2, from *Drosophila melanogaster* and their ligands by reverse pharmacology. *J Biol Chem* 2002;**277**:39937–43.
18. Lenz C, Williamson M, Grimmelikhuijzen CJ. Molecular cloning and genomic organization of a second probable allatostatin receptor from *Drosophila melanogaster*. *Biochem Biophys Res Commun* 2000;**273**:571–7.
19. Li Y, Hernandez-Martinez S, Fernandez F, Mayoral JG, Topalis P, Priestap H, et al. Biochemical, molecular and functional characterization of PISCF-allatostatin, a regulator of juvenile hormone biosynthesis in the mosquito *Aedes aegypti*. *J Biol Chem* 2006;**281**: 34048–55.
20. Lorenz MW, Kellner R, Hoffmann KH. A family of neuropeptides that inhibit juvenile hormone biosynthesis in the cricket, *Gryllus bimaculatus*. *J Biol Chem* 1995;**270**:21103–8.
21. Nachman RJ, Moyna G, Williams HJ, Tobe SS, Scott AI. Synthesis, biological activity, and conformational studies of insect allatostatin neuropeptide analogues incorporating turn-promoting moieties. *Bioorg Med Chem* 1998;**6**:1379–88.
22. Poels J, Van Loy T, Vandersmissen H, Van Hiel B, Van Soest S, Nachman R, et al. Myoinhibiting peptides are the ancestral ligands of the promiscuous Drosophila sex peptide receptor. *Cell Mol Life Sci* 2010;**67**:3511–22.
23. Price MD, Merte J, Nichols R, Koladich PM, Tobe SS, Bendena WG. *Drosophila melanogaster* flatline encodes a myotropin orthologue to *Manduca sexta* allatostatin. *Peptides* 2002;**23**:787–94.
24. Scharrer B. Neurosecretion 11. The effects of nerve section on the intercerebralis–cardiacum–allatum system of the insect *Leucophaea maderae*. *Biol Bull* 1952;**102**:261–72.
25. Stay B, Tobe SS. The role of allatostatins in juvenile hormone synthesis in insects and crustaceans. *Annu Rev Entomol* 2007;**52**:277–99.
26. Stay B, Bendena WG, Tobe SS. Allatostatins: identification, primary structures, functions and distribution. *Adv Insect Physiol* 1994;**25**: 267–338.
27. Tobe SS. Regulation of the corpora allata in adult female insects. In: Locke M, Smith DS, editors. *Insect biology in the future: VBW 80*. New York: Academic Press; 1980. p. 345–67.
28. Tobe SS, Ruegg RP, Stay BA, Baker FC, Miller CA, Schooley DA. Juvenile hormone titre and regulation in the cockroach *Diploptera punctata*. *Experientia* 1985;**41**:1028–34.

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29. Veenstra JA. Neuropeptide evolution: neurohormones and neuropeptides predicted from the genomes of *Capitella teleta* and *Helobdella robusta*. *Gen Comp Endocrinol* 2011;**171**:160–75.
30. Veenstra JA. Peptidergic paracrine and endocrine cells in the midgut of the fruit fly maggot. *Cell Tissue Res* 2009;**336**:309–23.
31. Wang J, Meyering-Vos M, Hoffmann KH. Cloning and tissue-specific localization of cricket-type allatostatins from *Gryllus bimaculatus*. *Mol Cell Endocrinol* 2004;**227**:41–51.
32. Williamson M, Lenz C, Winther AM, Nassel DR, Grimmelikhuijzen CJ. Molecular cloning, genomic organization, and expression of a B-type (cricket-type) allatostatin preprohormone from *Drosophila melanogaster*. *Biochem Biophys Res Commun* 2001;**281**:544–50.
33. Williamson M, Lenz C, Winther AME, Nassel DR, Grimmelikhuijzen CJP. Molecular cloning, genomic organization, and expression of a C-type (*Manduca sexta*-type) allatostatin preprohormone from *Drosophila melanogaster*. *Biochem Biophys Res Commun* 2001;**282**:124–30.
34. Woodhead AP, Stay B, Seidel SL, Khan MA, Tobe SS. Primary structure of four allatostatins: neuropeptide inhibitors of juvenile hormone synthesis. *Proc Natl Acad Sci U S A* 1989;**86**:5997–6001.
35. Xie Y, Kai ZP, Tobe SS, Deng XL, Ling Y, Wu XQ, et al. Design, synthesis and biological activity of peptidomimetic analogs of insect allatostatins. *Peptides* 2011;**32**:581–6.

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Chapter 30

Allatotropin

Frank M. Horodyski

ABSTRACT

Allatotropin (AT) was first characterized in the lepidopteran insect, *Manduca sexta*, based on its ability to stimulate juvenile hormone biosynthesis by adult female lepidopteran corpora allata *in vitro*. Since that initial discovery, a family of related peptides were isolated or predicted from genomic sequences from a large number of invertebrates. The AT gene is expressed in numerous defined neurons in the central and peripheral nervous systems, and in some insects is expressed in peripheral tissues, notably the gut and Malpighian tubules. Myotropic activity is stimulated in a wide range of invertebrates suggesting that this could be the ancestral role of this peptide. This widespread distribution of AT and its receptor is consistent with the multiple biological activities of AT which include myotropic and cardioacceleratory activities, regulation of fluid secretion, nutrient allocation, and entrainment of the circadian clock.

ISOLATION AND STRUCTURE OF ALLATOTROPIN

The precise regulation of juvenile hormone (JH) levels in the insect hemolymph is fundamental to the control of development, metamorphosis, and reproductive physiology. Among the mechanisms for controlling JH levels is the stimulation or inhibition of its biosynthesis. An *in vitro* radiochemical assay was developed to measure JH biosynthesis by the corpora allata (CA), the source of JH in insects.[25] In this assay, a radioactively labeled precursor to JH, ^{3}H- or ^{14}C-[methyl]-methionine, was incubated with the CA and the incorporation of label into the methyl ester of JH allowed its quantification after the separation of the precursor from the product. With this assay, the neuropeptide allatotropin (AT) was isolated based on its ability to stimulate JH biosynthesis by adult female CA.[13] This C-terminal amidated tridecapeptide is now referred to as Manse-AT (for *Manduca sexta* allatotropin).

Manse-AT is a member of a peptide family whose sequences have been determined by biochemical methods, deduced from gene sequences, or identified in silico from nucleotide sequence databases of insects whose genomes have been sequenced.[7,33] The rate of identification of new AT-related peptides has rapidly increased as new genomic and EST data are being acquired. Thus far, 34 unique sequences have been determined or predicted for this peptide family, and the number will undoubtedly increase as genome sequences from additional species are acquired (Table 1). The peptide sequences have rapidly evolved, but some defining features of the sequences are conserved. A glycine residue followed by phenylalanine and a basic amino acid is often present at or near the N-terminus. At the C-terminus, all peptides contain an aromatic residue followed by the C-terminal amidation, and this is often preceded by a basic amino acid (arginine or histidine). Truncated Manse-AT lacking the N-terminal 5 amino acids retains full biological activity on the CA.[13] However, the truncation of the N-terminal 6 amino acids results in a 10-fold decrease in biological activity. The C-terminal amide is essential for activity. Among the insects, it is noteworthy that a sequence encoding a peptide related to AT is not found in the genomes of the fly *Drosophila melanogaster*, the bee *Apis mellifera*, or the parasitic wasp *Nasonia vitripennis*; indicating that the AT gene was probably lost in these insects.[11] This suggests that either the regulatory functions of AT are performed by other peptides or that the regulatory functions of AT are missing in these insects.[19] Besides the insects, a related peptide was isolated and characterized or predicted in several species of mollusks, annelids, an arachnid, and in a crustacean.[5,7,12,31] Interestingly, several peptides have been isolated from mollusks and annelids based on their myotropic activity.[7,12] No evidence for a peptide structurally related to AT has been found invertebrates, so this peptide family probably exists only in invertebrates.

There are reports of other peptides that are structurally unrelated to Manse-AT, which have allatotropic activity as shown with the *in vitro* radiochemical assay. The 36 amino acid sex peptide from *D. melanogaster* stimulates JH biosynthesis in *D. melanogaster* and *Helicoverpa armigera*.[8] A predicted decapeptide, Spofr-AT 2, possesses allatotropic activity on *Spodoptera frugiperda* CA.[2] The C-terminal 6 amino acids of Spofr-AT 2 are identical to Manse-AS, an allatostatin peptide that inhibits JH biosynthesis. Thus,

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00030-0
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TABLE 1 Sequences of Allatotropins and Related Peptides Predicted from Insects and Other Invertebrates

Species	Acronym	Sequence	Phylum
Manduca sexta	Manse-AT	GFK-NVEMMTARGFa*	Arthropoda
Locusta migratoria	Locmi-myo-I	GFK-NVALSTARGFa*	Arthropoda
Rhodnius prolixus	Rhopr-AT	GFK-NVQLSTARGFa	Arthropoda
Homalodisca coagulata	Homca-AT	GFK-NHQLSTARGFa	Arthropoda
Acyrthosiphon pisum	Acypi-AT	GFK-NMDLSTARGFa	Arthropoda
Daphnia pulex	Dappu-ATR	GFK-TVGLATARGFa	Arthropoda
Ixodes scapularis	Ixosc-AT-I	GGFQ-KLRLSTARGFa	Arthropoda
Aedes aegypti	Aedae-ATL	APFR-NSEMMTARGFa*	Arthropoda
Phlebotomus papatasi	Phlpa-AT	APFR-NTEMMTARGFa	Arthropoda
Rhyncosciaria americana	Rhyam-AT	APFI-NTEMMTARGFa	Arthropoda
Samia cynthia ricini	Samcy-ATL-I	GTFKPNSNILIARGFa	Arthropoda
Manduca sexta	Manse-ATL-I	GTFKPNSNILIARGYa	Arthropoda
Helicoverpa armigera	Helar-ATL-I	GVFRPNSNVLIARGYa	Arthropoda
Tribolium castaneum	Trica-AT	GIEALKY-HNMDLGTARGYa*	Arthropoda
Ixodes scapularis	Ixosc-AT-II	GFR-KMKISTARGFa	Arthropoda
Fusinus ferrugineus	Fusfe-FEP4	GFRMNSSNRVAHGFa*	Mollusca
Manduca sexta	Manse-ATL-II	GTPTFK-SPTVGIARDFa	Arthropoda
Bombyx mori	Bommo-ATL-II	GTPTFK-SPSVGIARDFa	Arthropoda
Samia cynthia ricini	Samcy-ATL-II	GTPIFK-SPTVGIARDFa	Arthropoda
Eisenia foetida	Eisfo-ETP	GFKDGAADRISHGFa*	Annelida
Achatina fulica	Achfu-AEP3	GFRGDAASRVAHGFa*	Mollusca
Lymnaea stagnalis	Lymst-MATP	GFRANSASRVAHGYa*	Mollusca
Aplysia californica	Aplca-ATRP	GFRLNSASRVAHGYa*	Mollusca
Achatina fulica	Achfu-AEP2	GFRQDAASRVAHGYa*	Mollusca
Helobdella robusta	Helro-AT	SFDLYDNSRYAHGYa	Annelida
Pheretima vittata	Phevi-PTP	GFRDGSADRISHGFa*	Annelida
Capitella teleta	Capte-AT	GFRMGAADRFSHGFa	Annelida
Pomatoceros lamarckii	Pomla-AT	GFRMGAANRFSHGFa	Annelida
Alvinella pompejana	Alvpo-AT-I	GFRLGAADRFSHGFa	Annelida
Alvinella pompejana	Alvpo-AT-II	GFGLGAADRFSHGFa	Annelida
Spodoptera frugiperda	Spofr-ATL	FNPKSNLMVAYDFa	Arthropoda
Samia cynthia ricini	Samcy-ATL-III	FNPKNSLMVAYDFa	Arthropoda
Bombyx mori	Bommo-ATL-III	MKFTPKIGLMVAHDFa	Arthropoda
Manduca sexta	Manse-ATL-III	PWFNPKSKLLVSTRFa	Arthropoda

*Indicates that the peptide has been structurally characterized. Amino acids identical to those in Manse-AT are shaded.

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multiple unrelated peptides might function in an insect to stimulate JH biosynthesis.

THE ALLATOTROPIN GENE AND ITS EXPRESSION

The AT gene was first characterized in *M. sexta* and the peptide is processed as part of a larger propeptide.[7] In *M. sexta*, the AT gene is transcribed as three mRNAs that differ by alternative splicing. Each mRNA can encode Manse-AT, and the alternative exons contained in the larger mRNAs each encoding an allatotropin-like (ATL) peptide whose sequence is related to Manse-AT. A total of three ATL peptides are predicted from *M. sexta* cDNA sequences. Subsequently, gene sequences were determined or predicted from other insects, and alternative splicing of AT mRNAs with the predicted presence of ATL peptides was found in several lepidopteran insects. Outside the Lepidoptera, the predicted organization of the propeptide is conserved, but the only example of two potential AT-like peptides is in the tick, *Ixodes scapularis*, in which an EST sequence predicts two AT peptides (Accession: EEC06620).

Expression of the AT gene was first described in the lepidopteran insects *M. sexta* and *Pseudeletia unipuncta*, and later in multiple other lepidopterans.[7] In all cases, the gene is expressed in multiple cells throughout the central and enteric nervous systems. There are some differences in cell localization between insect species, but the most predominant expression in larvae is in the frontal ganglion and terminal abdominal ganglion followed by the brain and subesophageal ganglion. Additional cells contain AT mRNA in the adult including neurosecretory cells in each abdominal ganglia and brain, and a cluster of sex-specific cells in the posterior region of the terminal ganglion.[7,29] In *Bombyx mori*, AT mRNA is in the midgut,[23] and in *S. frugiperda*, AT mRNA is in the larval gut, in the adult testes and ovaries, and in the follicle cells between the oocytes.[1] Midgut and ovary expression is also present in *Samia cynthia ricini*.[28] The widespread expression of AT throughout the insect is consistent with multiple biological roles of the peptide throughout development.

The multiple Manse-AT mRNAs derived from alternative splicing are expressed in a stage- and tissue-specific manner.[15] Because each mRNA is predicted to encode AT and a unique set of ATL peptides, the diversity of peptides products from a single gene could vary in a dynamic manner between tissues and during development. Stage- and tissue-specific expression of alternatively spliced AT mRNAs is also documented in other lepidopteran insects.[7]

The localization of an AT-like peptide was characterized in a large number of insects by immunocytochemistry.[7] In general, there is a close correlation between AT-immunoreactive cells and those that contain AT mRNA. Additional information can be obtained with AT antiserum, such as the location of axons and the localization of release points around the adult CA in lepidopteran insects for the allatotropic action of the peptide.[6,29] The distribution of AT-immunoreactive cells can often provide clues for additional roles of the peptide, such as the presence in the *M. sexta* developing antennal lobe suggests a role in the formation of olfactory glomeruli.[30] The presence of AT-like immunoreactivity suggests the widespread presence of AT in insects, but the existence of crossreactive substances cannot always be ruled out.

BIOLOGICAL ACTIVITY OF ALLATOTROPIN

Activity in Lepidopteran Insects

This initial report on the identification of Manse-AT showed a specific stimulation of JH biosynthesis by CA from adult female lepidopteran insects, in which JH biosynthesis was stimulated by about 4-fold.[13] Subsequent studies showed the existence and activity of the peptide to be more widespread. In numerous lepidopteran insects, Manse-AT stimulates JH biosynthesis by the adult female CA. However, in some, but not all lepidopteran species, Manse-AT stimulates JH biosynthesis by larval CA.[3,7] The key to this finding is the observation that endogenous JH esterases in the CA can sometimes prevent the detection of biological activity by the use of ^{3}H-methylmethionine in the *in vitro* radiochemical assay, because the ^{3}H-methyl group is incorporated into the labile methyl ester of JH.[3] When ^{3}H-propionate is used as a precursor, the label is incorporated into the carbon skeleton of JH-I and JH-II, homologs that are synthesized by larval CA. However, the biological activity of Manse-AT is not detected on larval *M. sexta* CA using ^{3}H-propionate, so the activity of Manse-AT on larval CA is not universal in Lepidoptera. Together, these results show that an important function of AT in Lepidoptera is the stimulation of JH biosynthesis by the CA.

Because AT was first isolated based on bioactivity on the CA, there is an inherent bias in thinking of AT as solely an allatotropic peptide. We are limited to the use of established bioassays largely done *in vitro*, so important functions for peptides might be missed until additional assays are used to characterize the peptide. Even still, important roles for peptides might be unknown to this day because of the lack of suitable means to detect their function. Indeed, many neuropeptides possess multiple bioactivities that can differ during different life stages or target tissues. Manse-AT and its homologs stimulate muscle contractions and possess cardioacceleratory functions in multiple insect species.[7] Also, Manse-AT increases oscillations of the ventral diaphragm, which surrounds the nerve cord in the lepidopteran *P. unipuncta*.[14] This activity could enhance the circulation of the peptide in the hemolymph after its secretion.

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With midgut tissue mounted on a modified Ussing Chamber, Manse-AT inhibits active ion transport across the larval midgut epithelium.[17] This activity is only found in feeding *M. sexta*, and the peptide is inactive when tested on molting or wandering *M. sexta*, or on midguts of two other lepidopteran insects. This activity suggests a role for Manse-AT in regulating nutrient absorption from the diet. It is not known whether this bioactivity of AT is shared among other insect orders, because technical considerations prevent the use of the short circuit bioassay in most other insects, because a large tissue size is required to avoid experimental artifacts. AT also stimulate the release of digestive enzymes from the gut of *S. frugiperda*.[18]

Although the Malpighian tubules are not often thought of as a target of AT, Manse-AT inhibits fluid secretion by larval *M. sexta* Malpighian tubules (G.M. Coast, personal communication). The effect of Manse-AT on growth, development, and food consumption was investigated by injection into larval *Lacanobia oleracea*.[4] The lack of an effect in this assay was attributed to the short half-life of the peptide in the hemolymph, that is, <5 min. By contrast, injection of Manse-AT into the last instar *S. frugiperda* reduced weight gain, increased mortality, and reduced oviposition.[22]

The lack of AT in *D. melanogaster* precludes the investigation of AT function using genetic tools available in that insect. The technique of dsRNA-mediated RNA interference has the potential to knock down the expression of any gene in any insect provided the gene sequence is known. The challenges to be overcome include the delivery and uptake of dsRNA to the tissue of interest, the nervous system in the case of neuropeptides. The only study to test the effects of reducing AT expression thus far is the knockdown of Spofr-AT 2 mRNA in *S. frugiperda*.[9] This peptide shares identify with Manse-AS at the C-terminus and is structurally unrelated to Manse-AT. Feeding or injection of Spofr-AT 2 dsRNA to larvae leads to reduced mRNA levels in the brain and gut, and results in an increase in JH levels and a prolongation of the last larval stage, indicating that this peptide acts as an allatostatin in larvae. By contrast, injection of Spofr-AT 2 dsRNA to adults leads to reduced mRNA levels in the brain, and results in decreased JH levels in young adults and a decreased oviposition rate, supporting an allatotropic role in adults.

The Manse-ATL peptides, predicted from the cDNA sequences of alternatively spliced AT mRNAs, were tested for bioactivity using three different assays. Manse-ATL-I (the ATL peptide with the greatest structural identity to Manse-AT) stimulates JH biosynthesis by the adult female *M. sexta* CA at 20 nM.[15] All three Manse-ATL peptides inhibition transport across the midgut epithelium at 100 nM. Also, each Manse-ATL peptide stimulates foregut contractions in *L. oleracea*, with Manse-ATL-I being the most potent, and Manse-ATL-III the least potent (N. Audsley, personal communication). Thus, the expression of the longer alternatively spliced mRNAs would lead to the production of multiple peptides that might provide a more robust biological response.

Changes in Manse-AT mRNA levels can also provide clues to the functions of the peptide. In larval *M. sexta*, starvation, parasitism, or feeding of an ecdysteroid analog correlates with the upregulation of a specific alternatively spliced Manse-AT mRNA, implicating Manse-AT and/or Manse-ATL peptides in the physiological response to nutrient deprivation and increased JH levels that occur during these treatments.[16] In the monarch butterfly, *Danaus plexippus*, summer butterflies contain high JH levels compared with that of migrants which are JH deficient.[35] Summer butterflies also contain higher levels of AT mRNA, which invoke a role for AT as one of the factors that controls JH levels and prevents the triggering of migration.

Activity in Other Insect Orders

In *Aedes aegypti*, larval nutrition influences the activity of the CA at adult emergence, so that a mosquito reared under suboptimal conditions has low JH synthesis.[20] CA activity in the adult is correlated with AT levels in the head measured by ELISA, providing strong evidence for the role of AT in the control of JH levels in the mosquito.[10] Stimulation of JH biosynthetic activity by the CA *in vitro* has also been seen in dipteran, coleopteran, and dermapteran insects by Manse-AT or the endogenous peptide by the *in vitro* radiochemical assay, indicating that this peptide family can regulate JH levels in many insect orders.[7] A myotropic role for AT was found in many insects from the orders Orthoptera, Dictyoptera, Hemiptera, and Coleoptera, indicating that this is a conserved function for this peptide family.[7]

In the kissing bug, *Triatoma infestans*, an AT-like peptide is involved in the elimination of urine after ingestion of a blood meal.[27] The Malpighian tubules secrete an AT-like peptide that stimulates peristaltic contractions of the hindgut. This activity induces diuresis *in vitro* using a Malpighian tubule–hindgut preparation, because secretion can be blocked by an antiserum to *A. aegypti* AT. An effect was also observed *in vivo*, because injection of AT antiserum inhibits urine production in blood-fed insects.

A role for AT in the regulation of the circadian clock is seen in the cockroach, *Leucophaea maderae*.[24] AT-like immunoreactivity is present in the optic lobe, which sends processes into the accessory medulla. Injection of AT into the medulla results in phase shifts in circadian locomotor activity.

Activity in Other Invertebrates

As previously mentioned, peptides similar to Manse-AT have been identified and characterized in annelids and mollusks, phyla, which lack JH. In many cases, these peptides were

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isolated and characterized based on their myotropic activity. This finding led to the suggestion that the ancestral role of AT is myotropic, and its function to stimulate JH biosynthesis is secondary and was acquired in insects.[7] The function of an allototropin-related peptide (ATRP) is most extensively characterized in *Aplysia californica*.[12] The central actions of ATRP on a defined motoneuron that drive ingestive motor programs, and its peripheral actions on muscle contractions, implicate ATRP in the regulation of feeding.

THE ALLATOTROPIN RECEPTOR

The majority of neuropeptide receptors are members of a large family of structurally related proteins that contain seven hydrophobic membrane-spanning regions, the G protein-coupled receptors (GPCRs). Ligand binding to a GPCR leads to activation of a GTP-binding protein (G protein) that induces alterations in the level of second messengers which exert functional biochemical changes in the target cells and in the organism. The AT receptor (ATR) was first identified in the silkmoth, *B. mori* by a systematic screen to identify all GPCRs expressed in the corpora cardiaca-corpora allata complex, followed by functionally testing the response of GPCR-expressing *Xenopus laevis* oocytes elicited by known neuropeptides.[34] One expressed GPCR (BNGR-A16) specifically responds to AT in a dose-responsive manner. A highly related GPCR (BNGR-A5) shares a high degree of structural identity to BNGR-A16 but has not been tested for a functional response to AT. Thus, it is not yet known whether BNGR-A5 represents a second receptor for AT, or whether it responds to a different peptide. A similar approach was used to identify the ATR from *A. aegypti*, and its functional properties were confirmed in transfected mammalian cells tested with mosquito AT.[21]

PCR and genomics-based approaches were used to identify an ATR from *M. sexta*[11] and *Tribolium castaneum*.[32] In addition, similar sequences were identified in several additional insects by in silico screening of genomic or EST sequences. It is probable that these receptors recognize the endogenous AT, but their functional properties have not yet been tested. Consistent with the lack of AT in *D. melanogaster*, no sequence related to ATR was identified in the genome of that insect; however, a sequence related to ATR was identified in *A. mellifera* and *N. vitripennis*, suggesting that a hymenopteran peptide may have diverged from a Manse-AT-related sequence and might act through the identified GPCR.

The *M. sexta* ATR expressed in three separate transfected mammalian cell lines is specifically activated in a dose-responsive manner by low or sub-nanomolar concentrations of Manse-AT and each Manse-ATL peptide.[11] Interestingly, the Manse-ATL peptides are more potent than Manse-AT in each cell line. The expressed *T. castaneum* ATR is activated by low or sub-nanomolar concentrations of the endogenous Trica-ATL, and is also activated by the heterologous peptides Manse-AT and Locmi-AG-myotropin-I (Locmi-AT), but at a lower potency.[32] For both the Manse-ATR and Trica-ATR expressed in mammalian cells, the receptor is coupled through two second messenger pathways, resulting in increases in cAMP and intracellular calcium.[11,32] Early work showed that Manse-AT stimulates inositol phosphate production in adult female CC-CA, and the elevation of intracellular Ca^{2+} elicits an increase in JH biosynthesis.[26]

The biological actions of neuropeptides require the presence of a receptor in target cells and the availability of peptide to interact with the receptor. In larval *B. mori*, ATR mRNA is present at highest levels in the brain, CC-CA, epidermis, and testes, and the relative mRNA levels in tissues varies depending on the precise developmental stage.[34] In *M. sexta*, ATR mRNA is present at the highest level in larval Malpighian tubule, gut, testes, CA and CC; and is also present in the adult CC-CA complex.[11] In *T. castaneum*, ATR mRNA is present at highest levels in the head, fat body, and reproductive system, with a higher level in males compared with that in females.[32] In *A. aegypti*, ATR mRNA is present in the highest quantities in the CA, nervous system, and reproductive system.[21] The widespread distribution of ATR mRNA in these insects indicates multiple and diverse actions of AT that are elicited throughout the development of the insect. This is consistent with the results of biological assays, in which the target tissues of AT express the receptor. Additionally, novel functions for AT are suggested by the presence of ATR in tissues which are not known targets for AT, such as the reproductive system, and will drive further studies to more completely define the function of AT in insects and other invertebrates.

REFERENCES

1. Abdel-latief M. Expression and localization of the *Spodoptera frugiperda* allatotropin (Spofr-AT) and allatostatin (Spofr-AS) genes. *Arch Ins Biochem Physiol* 2004;**55**:188–99.
2. Abdel-latief M, Meyering-Vos M, Hoffmann KH. Characterization of a novel peptide with allatotropic activity in the fall armyworm *Spodoptera frugiperda*. *Reg Peptides* 2004;**122**:69–78.
3. Audsley N, Weaver RJ, Edwards JP. Juvenile hormone biosynthesis by corpora allata of larval tomato moth, *Lacanobia oleracea*, and regulation by *Manduca sexta* allatostatin and allatotropin. *Insect Biochem Molec Biol* 2000;**30**:681–9.
4. Audsley N, Weaver RJ, Edwards JP. *In vivo* effects of *Manduca sexta* allatostatin and allatotropin on larvae of the tomato moth, *Lacanobia oleracea*. *Physiol Ent* 2001;**26**:181–8.
5. Christie AE, McCoole MD, Harmon SM, Baer KN, Lenz PH. Genomic analysis of the *Daphnia pulex* peptidome. *Gen Comp Endocrinol* 2011;**171**:131–50.
6. Duve H, Audsley N, Weaver R, Thorpe A. Allatostatins and allatotropin in the corpus/cardiacum/corpus allatum complex of larval and adult lepidopterans studied by confocal laser scanning microscopy: correlation to juvenile hormone biosynthesis. *Cell Tiss Res* 2003;**314**: 281–95.

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7. Elekonich MM, Horodyski FM. Insect allatotropins belong to a family of structurally-related myoactive peptides present in several invertebrate phyla. *Peptides* 2003;**24**:1623–32.
8. Fan Y, Rafaeli A, Moshitzky P, Kubli E, Choffat Y, Applebaum SW. Common functional elements of *Drosophila melanogaster* seminal peptides involved in reproduction of *Drosophila melanogaster* and *Helicoverpa armigera* females. *Insect Biochem Molec Biol* 2000;**30**:805–12.
9. Griebler M, Westerlund SA, Hoffmann KH. Meyering-Vos M. RNA interference with the allatoregulating neuropeptide genes from the fall armyworm *Spodoptera frugiperda* and its effects on the JH titer in the hemolymph. *J Insect Physiol* 2008;**54**:997–1007.
10. Hernández-Martinez S, Mayoral JG, Li Y, Noriega FG. Role of juvenile hormone and allatotropin on nutrient allocation, ovarian development and survivorship in mosquitoes. *J Insect Physiol* 2007;**53**:230–4.
11. Horodyski FM, Verlinden H, Filkin N, Vandersmissen HP, Fleury C, Reynolds SE, et al. Isolation and functional characterization of an allatotropin receptor from *Manduca sexta*. *Insect Biochem Molec Biol* 2011;**41**:804–14.
12. Jing J, Sweedler JV, Cropper EC, Alexeeva V, Park J-H, Romanova EV, et al. Feedforward compensation mediated by the central and peripheral actions of a single neuropeptide discovered using representational difference analysis. *J Neurosci* 2010;**30**:16545–58.
13. Kataoka H, Toschi A, Li JP, Carney RL, Schooley DA, Kramer SJ. Identification of an allatotropin from adult *Manduca sexta*. *Science* 1989;**243**:1481–3.
14. Koladich PM, Tobe SS, McNeil JN. Enhanced haemolymph circulation by insect ventral nerve cord: hormonal control by *Pseudaletia unipuncta* allatotropin and serotonin. *J Exp Biol* 2002;**205**:3123–31.
15. Lee K-Y, Chamberlin ME, Horodyski FM. Biological activity of *Manduca sexta* allatotropin-like peptides, predicted products of tissue-specific and developmentally-regulated alternatively spliced mRNAs. *Peptides* 2002;**23**:1933–41.
16. Lee K-Y, Horodyski FM. Restriction of nutrient intake results in the increase of a specific *Manduca sexta* allatotropin (Manse-AT) mRNA in the larval nerve cord. *Peptides* 2002;**23**:653–61.
17. Lee K-Y, Horodyski FM, Chamberlin ME. Inhibition of midgut ion transport by allatotropin (Mas-AT) and *Manduca* FLRFamide peptides in the tobacco hornworm, *Manduca sexta*. *J Exp Biol* 1998;**201**:3067–74.
18. Lwalaba D, Hoffmann KH, Woodring J. Control of the release of digestive enzymes in the larvae of the fall armyworm, *Spodoptera frugiperda*. *Arch Ins Biochem Physiol* 2010;**73**:14–29.
19. Nässel DR, Winther ÅME. *Drosophila* neuropeptides in regulation of physiology and behavior. *Prog Neurobiol* 2011;**92**:42–104.
20. Noriega FG. Nutritional regulation of JH synthesis: a mechanism to control reproductive maturation in mosquitoes? *Insect Biochem Molec Biol* 2004;**34**:687–93.
21. Nouzova M, Mayoral JG, Brockhoff A, Goodwin M, Meyerhof W, Noriega FG. Functional characterization of an allatotropin receptor expressed in the copora allata of mosquitoes. *Peptides* 2012;**34**:201–8.
22. Oeh U, Dyker H, Lösel P, Hoffmann KH. *In vivo* effects of *Manduca sexta* allatotropin and allatostatin on development and reproduction in the fall armyworm, *Spodoptera frugiperda* (Lepidoptera, Noctuidae). *Invert Reprod Dev* 2001;**39**:239–47.
23. Park C, Hwang JS, Kang SW, Lee BH. Molecular characterization of a cDNA from the silk moth *Bombyx mori* encoding *Manduca sexta* allatotropin peptide. *Zool Sci* 2002;**19**:287–92.
24. Petri B, Homberg U, Loesel R, Stengl M. Evidence for a role of GABA and Mas-allatotropin in photic entrainment of the circadian clock of the cockroach *Leucophaea maderae*. *J Exp Biol* 2002;**205**:1459–69.
25. Pratt GE, Tobe SS. Juvenile hormones radiobiosynthesized by corpora allata of adult female locusts *in vitro*. *Life Sci* 1974;**14**:575–86.
26. Reagan JD, Miller WH, Kramer SJ. Allatotropin-induced formation of inositol phosphates in the corpora allata of the moth, *Manduca sexta*. *Arch Ins Biochem Physiol* 1992;**20**:145–55.
27. Santini MS, Ronderos JR. Allatotropin-like peptide released by Malpighian tubules induces hindgut activity associated with diuresis in the Chagas disease vector *Triatoma infestans* (Klug). *J Exp Biol* 2007;**210**:1986–91.
28. Sheng Z, Ma L, Cao M-X, Li S, Jiang R- J. Biochemical and molecular characterization of allatotropin and allatostatin from the Eri silkworm, *Samia cynthia ricini*. *Insect Biochem Molec Biol* 2007;**37**:90–6.
29. Truesdell PF, Koladich PM, Kataoka H, Kojima K, Suzuki A, McNeil JN, et al. Molecular characterization of a cDNA from the true armyworm *Pseudaletia unipuncta* encoding *Manduca sexta* allatotropin peptide. *Insect Biochem Molec Biol* 2000;**30**:691–702.
30. Utz S, Huetteroth W, Vömel M, Schachtner J. Mas-allatotropin in the developing antennal lobe of the sphinx moth *Manduca sexta*: distribution, time course, developmental regulation, and colocalization with other neuropeptides. *Dev Neurobiol* 2008;**68**:123–42.
31. Veenstra JA. Neuropeptide evolution: neurohormones and neuropeptides predicted from the genomes of *Capitella telata* and *Helobdella robusta*. *Gen Comp Endocrinol* 2011;**170**:582–8.
32. Vuerinckx K, Verlinden H, Lindemans M, Vanden Broeck J, Huybrechts R. Characterization of an allatotropin-like peptide receptor in the red flour beetle, *Tribolium castaneum*. *Insect Biochem Molec Biol* 2011;**41**:815–22.
33. Weaver RJ, Audsley N. Neuropeptide regulators of juvenile hormone synthesis. *Ann NY Acad Sci* 2009;**1163**:316–29.
34. Yamanaka N, Yamamoto S, Zitnan D, Watanabe K, Kawada T, Satake H, et al. Neuropeptide receptor transcriptome reveals unidentified neuroendocrine pathways. *PLoS ONE* 2008;**3**:e3048.
35. Zhu H, Casselman A, Reppert SM. Chasing migration genes: a brain expressed sequence tag resource for summer and migratory monarch butterflies (*Danaus plexippus*). *PLoS ONE* 2008;**3**:e1345.

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Chapter 31

B-Type Allatostatins and Sex Peptides

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ABSTRACT

In many species, mating induces many behavioral changes in the female. For *Drosophila melanogaster*, the sex peptide (SP) has been identified as the main molecular factor behind these responses. Recently, the sex peptide receptor (SPR), a GPCR activated by SP, has also been characterized as responsive to *Drosophila* myoinhibiting peptides, a conserved peptide family that is also known as B-type allatostatins. These peptides bear the C-terminal hallmark of –WX_6Wamide. Given this unexpected link between these peptides, they will be covered together in the following chapter.

DISCOVERY

Sex Peptides

Sex peptide (SP, also known as Accessory gland peptide 70A), was characterized in 1988 as a factor synthesized in the *Drosophila* male accessory glands and transferred to the female on copulation. The observed effects were an increase in egg laying and a loss of receptivity of the female toward new mating partners. The sequencing of the peptide and cDNA revealed a 36-aa long peptide, preceded in its precursor by a 19-aa hydrophobic signal sequence.[15]

The second of the sex peptides, ductus ejaculatorius peptide 99B (DUP99B), was not characterized until 2002. As its name implies, DUP99B is produced in the ejaculatory duct from a precursor that gives rise to a 31-aa mature peptide. The sequence of the peptide is almost identical to that of SP with regard to its C-terminal region. DUP99B was shown to elicit the same postmating responses as SP when injected into a female fly's hemolymph.[23]

Although both peptides have only been identified in *Drosophila* species, they also elicit physiological responses (see Biological Action) in the lepidopteran, *Helicoverpa armigera*. Therefore, it has been suggested that a similar sex peptide could be also be present in this species.[7,15] Because these sex peptides have only been characterized in various species of fruit flies, the following chapter on the sex peptides will be largely focused on these dipterans.

B-type Allatostatins

The B-type—or cricket type—allostatin family is characterized by a C-terminal WX_6Wamide sequence. The first identified B-type allatostatin was the myoinhibiting peptide from *Locusta migratoria.*[24] *Lom*-MIP was purified from an extract of brains, corpora cardiaca, corpora allata, and subesophageal ganglia. It was determined to repress spontaneous contractions of the hindgut and the oviduct in *L. migratoria*, as well as the hindgut of *Leucophaea maderae*. In the locust, however, the peptide does not display an inhibitory effect on juvenile hormone (JH) synthesis by the corpora allata. It was not until the discovery of the four peptide family members (*Grb*-AST1-4) from the cricket, *Gryllus bimaculatus*, that they were characterized as allatostatins.[16,17] The names "B-type allatostatin" and "myoinhibiting peptide" are synonymous to each other.

Although most results on this peptide family have been generated in insects, members of this peptide family have also been identified in a number of crustacean species. Most of these were discovered in decapod species, but a B-type allatostatin was also found in *Daphnia pulex.*[3,5]

STRUCTURE OF THE mRNA/GENE

Sex Peptides

Both genes encoding SP and DUP99B have been cloned and sequenced in *Drosophila melanogaster*, and both display an intron insertion at the same site. The highly similar C-terminal parts of the peptides are encoded by the second exon, whereas the first encodes the signal sequence and the N-terminal part of the peptide. It has been proposed that both genes have arisen by gene duplication. This is supported by the aa sequence similarities in not only the C-terminal regions of the peptides but also in the signal sequences.[15,23]

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00031-2
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B-type Allatostatins

Knowledge of the gene structure for the B-type allatostatins was not generated until the publication of the *Drosophila* genome. The fruit fly *myoinhibitory peptide* (*mip*) gene encodes a precursor in two exons, spaced by an intron. This preprohormone gives rise to five MIPs.[31] In *Rodnius prolixus*, the *Ast-B* gene shows more complexity, having four exons.[18] With five exons, the *AstB* gene features more complexity in *D. pulex*.[5]

PROCESSING OF THE PRECURSOR

Sex Peptides

SP synthesis occurs via a precursor, 55 aa in length, from which a 19-aa signal peptide is released. Amino acid sequence analysis of the SP from various *Drosophila* species has revealed two regions of high similarity. The five most N-terminal aa (WEWPW) are identical and the C-terminal region, encoded by the second exon, shows strong conservation.[15]

Like SP, DUP99B is synthesized from a precursor of 54 aa, from which 21 are cleaved off N-terminally as well as the two C-terminal aa (RK). The peptide displays a pyroglutamic acid modification in the N-terminus. Further, the N-terminal region was shown to undergo glycosylation, consisting of a difucosylated oligosaccharide structure. This latter modification decreases the amount of peptide required to induce ovulation, although this effect has not been observed in receptivity assays.[23]

The processed SP and DUP99B peptides are, respectively, 36 and 31 aa long and display a linear N-terminal region and a circular C-terminal part, resulting from a disulfide bridge formation. In between the two bridge-forming Cys residues, 9 out of the 11 aa are identical.

B-type Allatostatins

In *Drosophila*, the B-type allatostatin gene encodes a precursor of 211 aa that starts with a signal sequence and harbors five WX_6Wa peptides, all of which are flanked by dibasic or tribasic cleavage sites.[31] Results from *Rhodnius* indicate the presence of an *AST-B* gene encoding at least nine peptides.[18] For *Bombyx mori*, the precursor gives rise to eight peptides.[32] In the tick, *Ixodes scapularis*, the 132 aa MIP precursor likely gives rise to three peptides, of which two show the hallmarks for C-terminal amidation.[26]

DISTRIBUTION OF THE mRNAs AND PEPTIDES

Sex Peptides

The results generated in *D. melanogaster* reveal very similar temporal expression profiles for both the *sp* and the *dup99B* gene. Little to no transcript can be detected during development until the late pupal stages. Not surprisingly, expression levels are by far the highest in male adults. By contrast, female adults show no expression of *sp* or *dup99B*.[20]

The spatial expression patterns of *sp* and *dup99B* are equally constricted but notably different. Although *sp* transcripts are only found in the male accessory gland, the expression of *dup99B* is limited to the upper ductus ejaculatorius.[15,20,23] In addition, *dup99B* is also expressed in the cardia of both male and female flies.[15]

B-type Allatostatins

In contrast to the strictly limited expression profile of the sex peptides, the transcript for the B-type allatostatin is much more widely distributed. In *D. melanogaster*, the expression of the precursor has been found throughout all developmental stages.[20] The expression of *mip* is by far the greatest in the central nervous system (CNS). These results correspond with those obtained from immunohistochemical analyses, which point out the presence of MIP in the CNS of *Drosophila* and *Aedes aegypti*.[11]

Detailed immunohistological studies on *D. melanogaster* have revealed additional sites of strong MIP immunoreactivity in the larval midgut in the region before the copper cells and much weaker signals from between the copper cells. Also, a group of cells in the posterior part of the larval midgut is weakly MIP immunoreactive, and these decrease in density toward the middle of the posterior midgut. By contrast, MIP immunoreactivity is hard to pinpoint on the adult fly's midgut.[29] Immunohistochemistry in combination with Gal4 enhancers has allowed for the identification of MIP expression sites in the central complex of the *Drosophila* brain. Immunoreactivity appears in the ventral, central, and dorsal layers, with the latter showing strong labeling.[10]

Using in situ hybridization, the transcript for the *B. mori* prothoracicostatic peptides (PTSP) has been mapped to the CNS with the most intense signals in a pair of brain medial neurosecretory cells and also strong signals from the interneuron 704 and epiproctodeal glands. In the brain, the expression is detected during the feeding period but decreases before the time of each ecdysis. Transcript levels in the terminal ganglia, however, remained constant during the fourth and fifth instar stages. In the epiproctodeal glands, the expression is strong in pharate fifth instar larvae and just after ecdysis. It is at a low level during the first 5 days of the fifth instar stage, then rises. Immunohistological results are also available from *B. mori* and concur with the findings from the expression results.[32] These results generally correspond with what is known from *Manduca sexta*.[4]

Further, in *L. migratoria*, the presence of the peptide has been traced to approximately 12 neurons, including those innervating the glandular cells of the corpora cardiaca and the corpora allata.[24]

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Detection of the myoinhibiting peptide resulted in very similar patterns for the tick species *I. scapularis* and *Rhipicephalus appendiculatus*. In the synganglion, immunoreactivity could be detected in the neurons of the protocerebrum, palpal lobe, cheliceral lobe and the pedal lobes 2 and 3 (and 4 in the case of *I. scapularis*) and on the ventral side, the opistosomal lobe and a number of small neurons that were not detected in *R. appendiculatus*. Further, it was shown that the salivary gland in the tick is innervated by MIP-positive axonal projections.[25]

In decapods, the B-type allatostatins are widely distributed in the CNS as indicated by mass spectrometry analyses. In these animals, they are likely to serve as both autocrine or paracrine factors and as circulating hormones.[3] Using an antibody raised against *Cancer borealis* AST-B1, immunostaining reveals the distribution of this peptide throughout the stomatogastric nervous system of the Jonah crab. Immunoreactivity is detected in neurons and neuropil in the commissural ganglia, in somata in the esophageal ganglion, in fibers in the stomatogastric nerve and in neuropilar processes in the stomatogastric ganglion.[27]

RECEPTORS AND SIGNALING CASCADES

Studies in *Drosophila* show that SP activates the G-protein-coupled receptor encoded by CG16752, the sex peptide receptor, of which putative homologs have also been identified in various other insect species.[11,20,34] Transcript and protein presence of this receptor has been shown in the female reproductive organs, especially in the spermatheca, and in the CNS of both females and males in very similar patterns.[20,34] An in-depth study focusing on the neurons in which *spr* is expressed reveals that the presence of SPR in neurons expressing both *fruitless (fru)* and *pickpocket (ppk)* is necessary and sufficient for the mating-induced behavioral changes. This conclusion is warranted as *spr* knockdown in *ppk*$^+$ or *fru*$^+$ neurons is enough to eliminate postmating responses. In addition, specifically expressing *spr* in either of these neuron populations rescues the phenotype of *spr* null mutants in assays of egg laying, virgin receptivity, and remating frequency. Further, silencing the *ppk*$^+$ neuron signaling by means of specifically blocking the synaptic transmission of these neurons reveals a significant decrease in virgin receptivity and an increase in virgin egg laying. The sensory neurons innervating the uterus are thus far the only ones identified as expressing both *ppk* and *fru*.[8] Independently, other data support the importance of these neurons in SP signaling. Inhibition of PKA in these neurons raises the rate of egg laying by virgins and reduces the virgin's receptivity. Therefore, signaling through Gαi followed by PKA inhibition has been proposed as the *in vivo* path of action.[33]

Most interestingly, SPR from *Drosophila* has also been identified as the cognate receptor for MIPs, with MIP4 showing a lower EC50 value than SP (Fig. 1). As the C-terminal sequence similarity between MIPs and SP is limited to the $-WX_6W$ motif in MIPs compared with that of $-WX_8W$ in SP, it was hypothesized that the Trp residues present in these peptides might be essential for receptor activation. Indeed, truncated and Ala-substituted analogs of the peptides in which the Trp residues were eliminated show a strong loss of agonist activity.[11,20] Given the conserved nature of the receptor, in contrast to the limited number of species in which SP has been identified, it is sensible

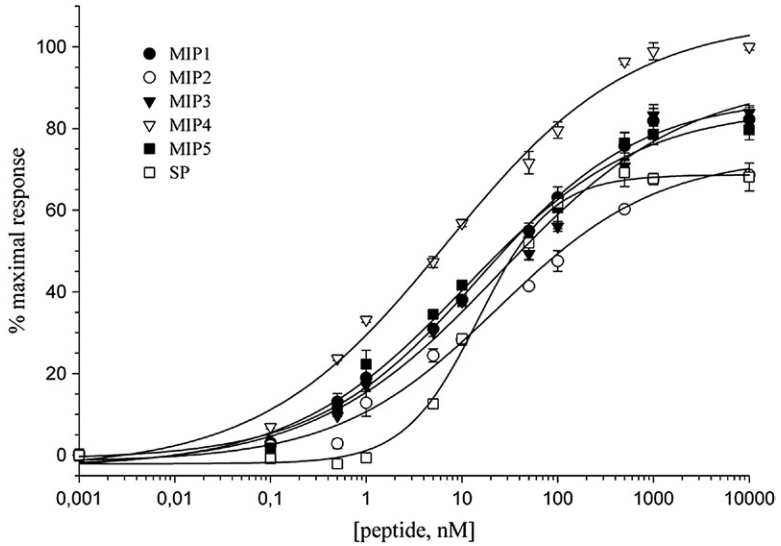

FIGURE 1 *Drosophila* SPR is a receptor for both SP and MIPs. CHO cells coexpressing aequorin, Gα16, and *Drosophila* SPR dose dependently increase intracellular Ca^{2+}-concentration when challenged with *Drosophila* SP and MIPs 1–5. Data points represent mean ± SD of three independent measurements performed in triplicate and are given as a percentage of the maximal response. The zero response level corresponds to the treatment with buffer only. (Figure reproduced from Ref. 20 with the publisher's permission.)

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to put forward the MIP family as the ancestral ligands for SPR. Further evidence from *B. mori* supports this theory. In this organism too, the SPR homolog has been identified as a cognate receptor for the *Bom*PTSPs (B-type allatostatins).[32]

BIOLOGICAL ACTIONS

Sex Peptides

The biological effects initially attributed to SP have been witnessed in both mated female flies and virgin females that were injected with SP. First, there is dramatic loss of receptivity of mated females compared with virgins. Once fruit fly females have copulated, they will actively reject new males that present themselves as additional mating partners. Second, after mating, the *Drosophila* female will display a strong increase in egg laying.

Further, it has been shown that SP induces the progression of oocytes to stage 10. This effect is likely mediated by juvenile hormone (JH) as SP is known to have allatotropic effects and because the oocyte progression can also be stimulated by methoprene, a JH mimic. Like SP, methoprene also induces the uptake of yolk protein (YP) into the ovary, in addition to strong increase of *yp* transcription in the ovary. However, the stimulation of egg laying and loss of receptivity cannot be mimicked by methoprene treatment.[15]

Further, female flies, having mated, significantly increase their food uptake. This effect has also been attributed to SP-transfer during copulation, without substantial involvement of sperm or the act of copulation.[2] A closer look at this aspect has made clear that this effect is probably an indirect consequence of the increased egg laying mentioned above. This is supported by the lack of feeding increase in sterile ovo^{D1} mutant females and the triggering of the effect in virgin females by silencing *fru* neurons rather than exposing them to SP.[1]

The observation that mated female's life spans are significantly shorter in comparison with their virgin counterparts raises the question as to whether this could result from adverse effects coupled with an increase in feeding. This has been refuted based on sterile ovo^{D1} mutant females displaying the same survival cost linked with mating, despite the observation that their food intake is not elevated.[1]

Results on feeding in *Drosophila* indicate that not only the quantity of the female's food intake will differ after copulation, but also its preference. In contrast to virgin females, mated females that have been deprived of a protein rich food source (i.e. yeast) for 3 days will show a strong preference for food containing yeast. SPR activity in the *ppk+* neurons is essential for this effect because expression of SPR in these neurons in an *SPR* mutant background largely restores the wildtype phenotype. Further, evidence suggests the involvement of TOR/S6K signaling and serotonin in the modulation of food preference.[22,28]

The presence of an intact C-terminal region of SP, including its disulfide bridge, is key to the biological activity of the peptide in receptivity and oviposition assays but it is not sufficient. In contrast, deletion of the 10 most N-terminal aa residues does not affect the peptide's activity in these assays.[15] However, the N-terminal part of the peptide has been shown to induce JH biosynthesis in the corpora allata of both *Drosophila melanogaster* and the moth, *H. armigera*. In concurrence with these results, DUP99B, which does not show strong similarity with SP in the N-terminal region, is not capable of eliciting allatal stimulation. For *Helicoverpa*, the C-terminal region of SP can reduce PBAN-induced pheromone production, which has been interpreted as a mechanism to lower the chance of a female participating in multiple copulations, thus increasing a male's chance for successful reproduction.[7,15]

Based on female postmating responses after mating with males that either produce no sperm-bound SP or SP that lacks the dibasic cleavage site at its N-terminal region, for the postmating responses to linger longer than 1 day (short-term postmating response), sperm-bound SP needs to be present and the long term postmating response requires the possibility of SP being cleaved from the sperm at the aforementioned dibasic cleavage site. The short-term postmating response, however, does not require this and is most likely primarily the result of unbound SP present in the seminal fluid.[19]

Although SP alone is capable of inducing a transient increase in egg production and laying, the presence of many conserved classes of seminal proteins is also required to reach the witnessed long term postmating effects.[15] A cascade unfolds in which the protease CG9997 is necessary for the transfer of lectins CG1652 and CG1656 to the female on mating. These lectins also require the presence of cysteine-rich secretory protein CG17575 to efficiently accumulate in the seminal receptacle. A lack of any of these factors will lead to a quick deficiency of SP in the female seminal receptacle.[21]

In addition to the postmating effects described above, copulation induces a number of other responses that have been attributed to the action of SP. Mating induces the expression of an array of antimicrobial peptide genes. Using SP^0 males and males lacking germline, it has been demonstrated that SP is the principal factor underlying this response. SP^0 males fail to induce transcription of the antimicrobial peptide genes *Metchnikowin*, *Drosomycin*, and *Diptericin.* However, the effect on the last two is weaker in comparison with the first. Further, continuous expression of SP in the fat body elevates the transcript levels of these genes above the level of mating-induced expression. SP is therefore believed to regulate the transcription of antimicrobial peptide genes in a dose-dependent manner.[19] Looking further into this aspect of SP functionality, it was discovered that the hydroxyproline-rich region in the middle of the peptide is the eliciting factor of the immune response. Given the particular pattern formed by the five hydroxyprolines,

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the hypothesis was raised that these motifs' actions result from its mimicking, on a molecular level, of sugar components within the microbial cell wall. In this case, pattern recognition receptors would be responsible for the elevated immune response on challenging by SP.[6]

A sexual dimorphism exists in *Drosophila* in relation to sleep–wake behavior. Male flies show an increased activity concentrated around the period of lights-on or lights-off, interspaced with periods of quiescence. A similar pattern is seen in virgin females. In contrast, females that have copulated only display a period of quiescence during the nighttime. These females are significantly more active during the daytime than males or virgin females. This effect is not sperm dependent, as mating of wildtype females with males lacking sperm does not abolish it. It is, however, also an effect of SP as this behavioral change is not found in females that are mated to SP^0 males. This loss of daytime rest has been proposed as one of the factors contributing to the decrease of lifespan observed in mated females.[9]

The link between *Drosophila* male reproductive success and SP has been studied in more detail, resulting in the notion that more SP is transferred to the female during mating in the presence of a competitor male. Additionally, males that have larger accessory glands, and therefore produce more SP, will have a greater reproductive success.[30]

B-type Allatostatins

The first characterized biological function of these peptides is the ability of *L. migratoria* MIP to inhibit spontaneous muscle contractions in the hindgut and ovaries of the locust.[24] Further, juvenile hormone biosynthesis in the cricket *G. bimaculatus* is inhibited by *Grb*-ASTs and, to a lesser extent, by *Lom*-MIP.[16]

In both *D. melanogaster* and *M. sexta*, the CCAP/MIP neurons have been determined as responsible for the initiation and/or execution of the ecdysis behavior. Specifically, MIP was proposed to silence neurons that are not required during the ecdysis program.[12,13]

In *Drosophila*, a novel type of MIP-producing neuron was identified, which has cell bodies anterior to the base of the optic lobe medulla. Because branches of these neurons are closely associated with branches of clock neurons a putative link between the circadian clock and MIP has been proposed.[14]

In vitro evidence from *B. mori* indicates that expression of the receptor is strongly upregulated after a sudden decline of the 20-hydroxyecdysone titer. Considering the observation of sudden PTSP release coinciding with a drop of the 20E titer, it has been proposed that PTSP/PTSPR signaling is involved in the fine-tuning of ecdysteroid titers by suppressing prothoracic gland activity.[32]

Little is known about the physiological effects of the B-type allatostatins in Crustacea. For *C. borealis*, three out of the ten *Cb*-AST-B peptides identified by mass spectrometric analysis affect the pyloric rhythm in a state-dependent manner.[27]

REFERENCES

1. Barnes AI, Wigby S, Boone JM, Partridge L, Chapman T. Feeding, fecundity and lifespan in female *Drosophila melanogaster*. *Proc Biol Sci* 2008;**275**:1675–83.
2. Carvalho GB, Kapahi P, Anderson DJ, Benzer S. Allocrine modulation of feeding behavior by the sex peptide of Drosophila. *Curr Biol* 2006;**16**:692–6.
3. Christie AE, Stemmler EA, Dickinson PS. Crustacean neuropeptides. *Cell Mol Life Sci* 2010;**67**:4135–69.
4. Davis NT, Blackburn MB, Golubeva EG, Hildebrand JG. Localization of myoinhibitory peptide immunoreactivity in *Manduca sexta* and *Bombyx mori*, with indications that the peptide has a role in molting and ecdysis. *J Exp Biol* 2003;**206**:1449–60.
5. Dircksen H, Neupert S, Predel R, Verleyen P, Huybrechts J, Strauss J, et al. Genomics, transcriptomics and peptidomics of *Daphnia pulex* neuropeptides and protein hormones. *J Proteome Res* 2011.
6. Domanitskaya EV, Liu H, Chen S, Kubli E. The hydroxyproline motif of male sex peptide elicits the innate immune response in Drosophila females. *FEBS J* 2007;**274**:5659–68.
7. Fan Y, Rafaeli A, Gileadi C, Kubli E, Applebaum SW. *Drosophila melanogaster* sex peptide stimulates juvenile hormone synthesis and depresses sex pheromone production in *Helicoverpa armigera*. *J Insect Physiol* 1999;**45**:127–33.
8. Hasemeyer M, Yapici N, Heberlein U, Dickson BJ. Sensory neurons in the Drosophila genital tract regulate female reproductive behavior. *Neuron* 2009;**61**:511–8.
9. Isaac RE, Li C, Leedale AE, Shirras AD. Drosophila male sex peptide inhibits siesta sleep and promotes locomotor activity in the post-mated female. *Proc Biol Sci* 2010;**277**:65–70.
10. Kahsai L, Winther AM. Chemical neuroanatomy of the Drosophila central complex: distribution of multiple neuropeptides in relation to neurotransmitters. *J Comp Neurol* 2011;**519**:290–315.
11. Kim YJ, Bartalska K, Audsley N, Yamanaka N, Yapici N, Lee JY, et al. MIPs are ancestral ligands for the sex peptide receptor. *Proc Natl Acad Sci U S A* 2010;**107**:6520–5.
12. Kim YJ, Zitnan D, Cho KH, Schooley DA, Mizoguchi A, Adams ME. Central peptidergic ensembles associated with organization of an innate behavior. *Proc Natl Acad Sci U S A* 2006;**103**:14211–6.
13. Kim YJ, Zitnan D, Galizia CG, Cho KH, Adams ME. A command chemical triggers an innate behavior by sequential activation of multiple peptidergic ensembles. *Curr Biol* 2006;**16**:1395–407.
14. Kolodziejczyk A, Nassel DR. A novel wide-field neuron with branches in the lamina of the Drosophila visual system expresses myoinhibitory peptide and may be associated with the clock. *Cell Tissue Res* 2011;**343**:357–69.
15. Kubli E. Sex-peptides: seminal peptides of the Drosophila male. *Cell Mol Life Sci* 2003;**60**:1689–704.
16. Lorenz MW, Kellner R, Hoffmann KH. A family of neuropeptides that inhibit juvenile hormone biosynthesis in the cricket, *Gryllus bimaculatus*. *J Biol Chem* 1995;**270**:21103–8.
17. Nichols R, Bendena WG, Tobe SS. Myotropic peptides in *Drosophila melanogaster* and the genes that encode them. *J Neurogenet* 2002;**16**:1–28.

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18. Ons S, Sterkel M, Diambra L, Urlaub H, Rivera-Pomar R. Neuropeptide precursor gene discovery in the Chagas disease vector Rhodnius prolixus. *Insect Mol Biol* 2011 Feb;**20**(1):29–44. doi: 10.1111/j.1365-2583.2010.01050.x.
19. Peng J, Zipperlen P, Kubli E. Drosophila sex-peptide stimulates female innate immune system after mating via the Toll and Imd pathways. *Curr Biol* 2005;**15**:1690–4.
20. Poels J, Van Loy T, Vandersmissen HP, Van Hiel B, Van Soest S, Nachman RJ, et al. Myoinhibiting peptides are the ancestral ligands of the promiscuous Drosophila sex peptide receptor. *Cell Mol Life Sci* 2010;**67**:3511–22.
21. Ram KR, Wolfner MF. A network of interactions among seminal proteins underlies the long-term postmating response in Drosophila. *Proc Natl Acad Sci U S A* 2009;**106**:15384–9.
22. Ribeiro C, Dickson BJ. Sex peptide receptor and neuronal TOR/S6K signaling modulate nutrient balancing in Drosophila. *Curr Biol* 2010;**20**:1000–5.
23. Saudan P, Hauck K, Soller M, Choffat Y, Ottiger M, Spörri M, et al. Ductus ejaculatorius peptide 99B (DUP99B), a novel *Drosophila melanogaster* sex-peptide pheromone. *Eur J Biochem* 2002;**269**: 989–97.
24. Schoofs L, Holman GM, Hayes TK, Nachman RJ, De LA. Isolation, identification and synthesis of locusta myoinhibiting peptide (LOM-MIP), a novel biologically active neuropeptide from *Locusta migratoria*. *Regul Pept* 1991;**36**:111–9.
25. Simo L, Slovak M, Park Y, Zitnan D. Identification of a complex peptidergic neuroendocrine network in the hard tick, *Rhipicephalus appendiculatus*. *Cell Tissue Res* 2009;**335**:639–55.
26. Simo L, Zitnan D, Park Y. Two novel neuropeptides in innervation of the salivary glands of the black-legged tick, *Ixodes scapularis*: myoinhibitory peptide and SIFamide. *J Comp Neurol* 2009;**517**:551–63.
27. Szabo TM, Chen R, Goeritz ML, Maloney RT, Tang LS, Li L, et al. Distribution and physiological effects of B-type allatostatins (myoinhibitory peptides, MIPs) in the stomatogastric nervous system of the crab Cancer borealis. *J Comp Neurol* 2011;**519**:2658–76.
28. Vargas MA, Luo N, Yamaguchi A, Kapahi P. A role for S6 kinase and serotonin in postmating dietary switch and balance of nutrients in *D. melanogaster*. *Curr Biol* 2010;**20**:1006–11.
29. Veenstra JA. Peptidergic paracrine and endocrine cells in the midgut of the fruit fly maggot. *Cell Tissue Res* 2009;**336**:309–23.
30. Wigby S, Sirot LK, Linklater JR, Buehner N, Calbodi FC, Bretman A, et al. Seminal fluid protein allocation and male reproductive success. *Curr Biol* 2009;**19**:751–7.
31. Williamson M, Lenz C, Winther AM, Nassel DR, Grimmelikhuijzen CJ. Molecular cloning, genomic organization, and expression of a B-type (cricket-type) allatostatin preprohormone from *Drosophila melanogaster*. *Biochem Biophys Res Commun* 2001;**281**:544–50.
32. Yamanaka N, Hua YJ, Roller L, Spalovská-Valachová I, Mizoguchi A, Kataoka H, et al. Bombyx prothoracicostatic peptides activate the sex peptide receptor to regulate ecdysteroid biosynthesis. *Proc Natl Acad Sci U S A* 2010;**107**:2060–5.
33. Yang CH, Rumpf S, Xiang Y, Gordon MD, Song W, Jan LY, et al. Control of the postmating behavioral switch in Drosophila females by internal sensory neurons. *Neuron* 2009;**61**:519–26.
34. Yapici N, Kim YJ, Ribeiro C, Dickson BJ. A receptor that mediates the post-mating switch in Drosophila reproductive behaviour. *Nature* 2008;**451**:33–7.

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Chapter 32

Crustacean Bioactive Peptides

H. Dircksen

ABSTRACT

Research on crustacean peptides has concentrated mainly on decapods and isopods, and a growing number of >200 peptides have been sequenced from these two groups, the majority from decapods, but recently, the annotation of the *Daphnia pulex* genome has contributed many more novel peptides many of which were also sequenced *de novo*. Identified and bioactive crustacean peptides — the only ones reported here — regulate a large range of physiological functions, including color change, activities of heart, exoskeletal and visceral muscles, metabolic function, development, metamorphosis, and reproduction.

INTRODUCTION

Crustaceans are classical model animals for endocrinological research from the beginning of the discovery of neurosecretion in vertebrates and invertebrates in the 1920s. Crustacean peptides particularly from commercially valuable species have gained interest because many of them are involved in the control of growth by molting, of many metabolic processes and reproduction. Genomic, transcriptomic (e.g. via expressed sequence tags, ESTs) and proteomic approaches especially with the aid of sensitive mass spectrometric techniques have led to a rapid increase in the number of precursors and derived identified peptides in crustacean species preferably from Decapoda, Isopoda, and more recently Branchiopoda, because *Daphnia pulex* became the first crustacean model organism with a fully annotated genome. This gave another boost of peptide discovery with especially notable similarity to insect peptides and protein hormones (see for further references (sffr)[9]). Although functional studies on identified peptides are mainly available from the first two groups, this may soon change rapidly. Unfortunately, physiological investigations of their bioactivity have not kept pace with the speed in the discovery of crustacean peptides, hence their names are often just inferred from homology to insect peptides, and, thus, only suggestive of similar function.

Chromatophorotropic hormones were the first sequenced invertebrate neuropeptides in the 1970s, the antagonistic red pigment concentrating hormone (RPCH) and pigment dispersing hormone (PDH), but about 30 years later, crustacean cardioactive peptide (CCAP) and corazonin had to be added to this functionality. The first two peptide families, along with the crustacean hyperglycemic hormone superfamily,[33] have been studied extensively in several crustacean model systems. Most of the peptides were discovered from the extracts of major crustacean neuroendocrine organs such as the X-organ-sinus gland system (XO-SG) in the decapod eyestalk or the isopod brain and the pericardial organs (POs), large neurohemal release sites of neurosecretory neurons in the ventral nerve cord (VNC).[33] Apart from suitable crustacean muscle systems such as the easily accessible heart, some exoskeletal muscles and the hindgut, well-established simple systems for electrophysiological studies of neuronal networks such as the stomatogastric and cardiac ganglia (STG, CG) controlling the foregut and the neurogenic heart served as bioassay systems.

This review considers in alphabetical order biochemically fully identified crustacean peptides for which bioactivity has been established even if only seen in heterologous bioassays. Where known, the few crustacean neuropeptide receptors are commented on. Further, with possible exceptions in cases of CHH-superfamily peptides, chromatophore activators, and some myotropic peptides (e.g. proctolin, FMRFamide-related peptides (FaRPs)), work has often just begun to unravel involvement of cyclic nucleotide, Ca^{2+}/calmodulin or Ca^{2+}/inositol-triphosphate (IP_3)-dependent mechanisms of intracellular signaling.

ANDROGENIC GLAND HORMONE (AGH)/ INSULIN-RELATED PEPTIDES

Discovery

The androgenic gland hormone (AGH) regulating male sexual differentiation in isopod and decapod crustaceans

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00032-4
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was originally identified from the pill bug *Armadillidium vulgare* as an insulin-like peptide typically composed of A and B chains. Its A-chain is glycosylated, contains an unusual intrachain cystine-bridge, but is connected to the B-chain via two cystine-bridges. Later on similar insulin-like AG (IAG) peptides have been identified from other isopods and more recently from crayfish, shrimps, and a crab.[32] In *D. pulex*, a single nonglycosylated brain insulin-related peptide (DIRP) has been identified, and three putative insulin-like growth factors (DIGFs) were predicted.[9]

Structure of the mRNA/Gene and Processing of the Precursor

All precursors of AGHs/IAGs and DIGFs contain the B-C-A chain configuration typical of insulins after the signal peptide (SP), whereas in the DIRP precursor, as derived from a complex 5-exon *irp*-gene, two additional identified peptides precede the B-C-A chains. From AGHs/IAGs and DIRP, B- and A-chains are cleaved from the C-peptides at dibasic and/or RxxR cleavage sites; however, there are no such sites in DIGFs (sffr and detailed comparisons[9]).

Distribution of the mRNA/Peptide

Although AGHs/IAGs are restricted to decapod AGs (sffr[32]), DIRP occurs in distinct brain neurons (Ref. 9; J. Strauß and H. Dircksen, unpublished).

Biological Actions

The AG regulates male sex differentiation, that is, development of testis and secondary external sex characters in males, and implantation of AGs into females can induce sex shifts and changes toward male-like behaviors (sffr[32]).

ALLATOSTATINS (SEE CHAPTER 29)

Allatostatin (AST) denominates a peptide with inhibitory activity on juvenile hormone production or release from insect corpora allata (CA). AST-like peptides are ubiquitous in arthropods and can be divided into three types A, B, C. Neither has any crustacean AST hitherto been tested in heterologous assays using CA, nor has any authentic AST an effect on the mandibular organs, the crustacean CA-homologs producing the juvenile hormone-precursor methyl farnesoate, and its precursor, farnesoic acid (sffr[15]). Nevertheless, these ASTs share the names because of sequence similarities. Of note, in many insects also, CA-stimulatory allatotropins exist; but a brain peptide slightly different from insect allatotropins has only recently been identified as GFKTVGLATARGFamide in *D. pulex* only,[9] while seemingly being absent from decapods.

Allatostatin-Like Peptides of Type A (ASTAs)

Discovery

A very large number of ASTAs sharing the Y/F-X-FGLamide motif have been identified in several crustacean species: first, 20 from the crab *Carcinus maenas*, 3 from the crayfish *Orconectes limosus*, 40 from the shrimp *Penaeus monodon* and a growing number from other crabs, lobsters, and even a copepod and *D. pulex*.[2,9]

Structure of the mRNA/Gene and Processing of the Precursor

A precursor mRNA from the crayfish *Procambarus clarkii* encodes, immediately after the SP, 27 ASTAs (thereof 22 different) all separated by dibasic cleavage sites and some intercalating precursor-related peptides (PRPs), two ASTAs among them occurring in multiple copies. In the branchiopod precursor as derived from a 3-exon gene, only single copies of six ASTAs occur, whereas the copepod precursor shows three (one in three copies) ASTA-like peptides with noncanonical Y/F-X-FGI/Vamide C-termini.

Distribution of the mRNA/Peptide

ASTA-immunoreactive peptides seem to occur throughout the entire CNS and peripheral nervous systems such as the STG and seem to be released from classical neurohemal organs such as SGs and POs (sffr[2,10]).

Biological Actions

Although most earlier studies only used heterologous cockroach ASTAs, biological activities of three authentic ASTAs have only been tested in the crayfish *O. limosus* in which they are the first identified inhibitory peptides of crustaceans, inhibiting frequency and amplitude of contractions of semiisolated hindguts (Carcinustatin-8, Orcostatins I, II) and STG neuronal activities (Carcinustatin-8).[10] Similar inhibitory effects on motor output of *Cancer borealis* STG and cardiac ganglion have been found but merely by testing insect ASTAs (sffr[2]).

Allatostatin-Like Peptides of Type B (ASTBs, = Myoinhibitory Peptides)

Discovery

Originally described in crickets, ASTBs share WX_6Wamide C-termini (X variable amino acids (aas)). After the first ASTB, VPNDWAHFRGSWamide (CbAST-B1), was

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identified from POs of two *Cancer* species, up to ten different ASTBs were identified in a single species of crabs, shrimp and lobsters (sffr[2]).

Structure of the mRNA/Gene and Processing of the Precursor

The structures of a complex 5-exon gene and the precursor containing 8 different ASTBs (5 of which fully identified) are only available from *D. pulex*. The peptides are flanked by double-basic cleavage sites interspersed between six varied PRPs.[9]

Biological Actions

In the STG of *C. borealis*, CbAST-B1 decreases the frequency of the pyloric motor output depending on the crab's physiological state (sffr[2]).

Allatostatin-Like Peptides of Type C (ASTCs)

Discovery

A 15-mer peptide containing the C-terminal nonamidated PISCF motif was first described in *Manduca sexta*. ASTCs are further characterized by a cystine bridge spanning 6 aas N-terminally from this motif. The pentadecapeptide pQIRYHQCYFNPISCF (CbAST-C1) apparently is the most common ASTC in 29 species of decapod crustaceans, but others have been found in *C. borealis* (e.g. CbAST-C2: SYWKQCAFNAVSCFamide; sffr[2]). In *D. pulex* three genes with distinctly different ASTC1-C3 were found,[9] only DappuASTC1 being identical to CbAST-C2.

Structure of the mRNA/Gene and Processing of the Precursor

EST-deduced precursors from shrimp, crab and lobster encode single copies of either CbAST-C1 or CbAST-C2 at the end between a dibasic (KR) and/or tribasic (RRK, RKR, KRK) cleavage sites.[2] Two of the three *astC1-C3*-genes in *D. pulex* occur clustered in head-to-tail tandem arrangement on the same scaffold, whereas the third one is separate. In all three precursors, single ASTCs occur close to or at the end and are flanked on one side of the peptides by a monobasic (R) and on the other side by a dibasic cleavage site.[9]

Distribution of the mRNA/Peptide

MALDI-based surveys of lobster tissues show that CbAST-C1 occurs in *C. borealis* POs. However, CbAST-C2 was found widely distributed in the central nervous system (eyestalk ganglia, brain, commissural ganglia) and occurs also in the posterior midgut caecum but has not been found in SGs and was hardly detectable in POs (sffr[2]).

Biological Actions

Both CbAST-C1 and CbAST-C2 inhibit dose dependently the cycle frequency of the pyloric motor output from the STG ($EC_{50} \sim 10^{-8}$ M) and seem to modulate heart rate in crabs and lobsters (sffr[2]).

CHH-SUPERFAMILY

Discovery

The crustacean hyperglycemic hormone (CHH) superfamily can be divided into type-I and type-II subgroups, that is, the CHH subfamily sensu stricto, which contains 72 aa with often N-terminal pyroglutamate and always C-terminal amidation, and the molt-inhibiting hormone (MIH)/gonad-inhibiting hormone (GIH)/vitellogenesis-inhibiting hormone (VIH)/mandibular organ-inhibiting hormone (MOIH) subfamily (MIH subfamily), nonprotected 77–83 aa (usually 78), respectively, based on their structures and bioactivities. The first CHH was identified from the SG of *C. maenas* and soon after an MIH of similar structure but no hyperglycemic activity from the same source. Further type-II GIHs, VIHs and MOIHs were later identified. Altogether, the CHH-superfamily now comprises >80 family members from about 40 crustacean species (sffr[33]).

Structure of the mRNA/Gene and Processing of the Precursor

Characteristically, several *chh*-genes often occur in multiple isoforms of 3-exon or 4-exon genes in decapods and *D. pulex* (except a 2-exon *chh*-gene for *P. monodon*), and *mih*- and *moih*-genes are only found as 3-exon genes in shrimps, giant tiger prawns and crabs. However, no specific *vih/gih*-genes have hitherto been described. Although multiple *chh*-genes occur in clusters of up to nine *chhA*- or *chhB*-genes in tandem in penaeid shrimps, in other species, several copies of slightly differing *chh*-genes have been found. The 4-exon *chh*-genes usually give rise to products of alternative splicing, that is, precursors encoding the long nonamidated or the short amidated isoforms, the latter being devoid of the message from the third exon. Two single *mih*-genes only have been found for several species, and slightly different *mih*- and *moih*-genes occur distinctly clustered (apparently only) in cancrid crabs. A clear-cut difference between of type-I and type-II subfamily precursors is the occurrence of PRPs restricted to CHH-subfamily precursors (sffr[33]).

Distribution of the mRNA/Peptide

CHH superfamily mRNAs and peptides are produced by neurosecretory cells in the classical XO-SG system and intrinsic cells of pericardial organs or astacuran thoracic

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roots, but they occur also as paraneurons in nonneural tissues, especially in the foregut and hindgut epithelium of crabs (sffr[33]).

Receptors and Signaling Cascades

Receptor-binding studies have clearly identified distinct receptors for CHHs and MIHs in crabs and lobster although their primary structures remain to be elucidated. Although both cAMP and cGMP have distinct but overlapping functions in the inhibition of Y-organ ecdysteroidogenesis by MIH and CHH, respectively, apparently cGMP acts as a major second messenger mediating diverse CHH actions.[33]

Biological Actions

Classically, the name-giving bioactivities characterized the diverse CHH-superfamily members, although type-II peptides have only restricted actions (hence their names) compared to those of the rather pleiotropic type-I peptides. CHHs exhibit at least eight different functions, that is, apart from upregulating hemolymph (1) glucose and (2) lipid levels, they stimulate (3) hepatopancreatic amylase secretion and (4) ion transport in gills and, transiently during premolt and ecdysis, (5) water uptake as necessary for successful molting, inhibit (6) ecdysteroidogenesis in Y-organs, (7) MF-synthesis in mandibular organs, and (8) AGH and protein synthesis in the androgenic glands (sffr[33]).

CORAZONIN

Discovery

The N- and C-terminally blocked undecapeptide Arg^7-corazonin (pQTFQYSRGWTNamide, CRZ) was first identified as a cardioactive factor in the American cockroach. In crustaceans, the highly conserved Arg^7-CRZ was much later identified in POs of the crab *C. borealis*, several other decapods (sffr[2]) and recently also in *D. pulex* brains.[9] It is to date the only known isoform in crustaceans.

Structure of the mRNA/Gene and Processing of the Precursor

Precursors have been predicted for the shrimp *Litopenaeus vannamei* and the water flea *Daphnia carinata* (sffr[2]). The only crustacean identified *crz*-gene (with three exons), its derived mRNA and precursor are presently known from *D. pulex*. Similar to RPCH/AKH precursors (see below), the peptide always follows immediately after the SPs and is separated by a double-basic cleavage site from a long PRP containing up to two cysteines. Apart from the CRZ region, however, insect, decapod and *D. pulex* precursors have only little in common.[9]

Distribution of the mRNA/Peptide

CRZ was sequenced from POs, brain and VNC tissues (sffr[2,9]), but there are currently no data about CRZ-expressing cells in crustaceans.

Biological Actions

CRZ leads to retraction of erythrophore pigment granules and dispersion of leucophore pigment granules in crayfish, and inhibits spermatogenesis in freshwater prawns, though at relatively high concentrations (sffr[23]).

CRUSTACEAN CARDIOACTIVE PEPTIDE (CCAP)

Discovery

Crustacean cardioactive peptide (CCAP; PFCNAFTG-Camide), a cyclic amidated nonapeptide hormone containing a cystine bridge between Cys^3 and Cys^9, was isolated via heart bioassays from *C. maenas*.[26] In crustaceans, this isoform only has now been identified in many decapods but an exceptional isoform (Ala^7-CCAP) has been predicted from *D. carinata* ESTs and the *D. pulex* genome (sffr[9]).

Structure of the mRNA/Gene and Processing of the Precursor

Precursor mRNAs have been cloned from several decapods[5] and recently been predicted from *D. carinata* ESTs and the *D. pulex* genome (sffr[9]). In the highly similar precursor, the peptide is flanked by two pairs of PRPs all separated by dibasic to tetrabasic cleavage sites.[5] The peptide message in the 3-exon *Ala^7-ccap*-gene of *D. pulex* is characteristically split by an intron as in insects.[9]

Distribution of the mRNA/Peptide

In crustaceans, CCAP-expressing serially homologous neurons usually occurring in almost every VNC segment as pairs of an interneuron and a neurosecretory neuron with peripheral release sites (e.g. crustacean POs). These cell types are highly conserved neurons in arthropods. In crabs, these cells start CCAP-expression already by 50% embryogenesis.[5] In crab and lobster, as in insects, the peptide occurs together with the dimeric bursicon homolog of the insect cuticle tanning and sclerotizing hormone (sffr[25]).

Biological Actions

Originally named for its excitatory activity on the shore crab heart, CCAP is excitatory on other invertebrate muscles and an established neuromodulator of STG and CG

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(sffr[2]). Chronotropic and inotropic effects on blue crab heart likely are brought about by four stimulatory actions on both the heart muscle and the CG, that is, contraction amplitude, motor neuron burst and contraction frequency, burst duration, and number of spikes per motor neuron burst (sffr[2]). Its dramatic release into the hemolymph during ecdysis indicates its involvement in coordinating physiological and behavioral processes, in particular increased hemolymph pressure and flow, and the potentiation of muscular force during this stage.[22] Although not clear at present, the role of bursicon in these crustacean CCAP neurons may be similar to that in insects (sffr[25]). In a freshwater shrimp, CCAP can even act as a chromatophorotropin inducing pigment dispersion and reducing RPCH-induced pigment aggregation albeit at relatively high effective concentrations.[19]

DIURETIC HORMONE 31

Discovery

A precursor of a typically 31aa calcitonin-like diuretic hormone (CLDH or DH31), well conserved in insects and identical in structure to CLDH of *Tribolium castaneum* (GLDLGLGRGFSGSQAAKHLMGLAAANFAGGPamide), has first been predicted and full-length cloned from an EST library of *Homarus americanus*, and the derived HomamDH31 has been tested for cardioactivity.[3] A very similar DappuDH31 (5 aa-exchanges only) was fully sequenced by mass spectrometry from *D. pulex* brains.[9]

Structure of the mRNA/Gene and Processing of the Precursor

The precursor structures of lobster and water flea precursors are very similar with regard to the DH31s even when compared with insect DH31 precursors typically encoding DH31 in the second last position flanked by dibasic (KR) and tetrabasic (RRRR, RKRR) cleavage sites, among 4 PRPs not related to any other arthropod peptide. The 3-exon *dh31*-gene in *D. pulex* contributes with all exons to the DappuDH31-precursor.[3,9]

Distribution of the mRNA/Peptide

RTPCR shows amplicons in the brain, eyestalk ganglia, commissural ganglia, and in motoneurons but not pacemaker neurons of the cardiac ganglion of the lobster,[3] and in the brain of *D. pulex*.[9]

Biological Actions

A synthetic lobster peptide shows profound stimulatory effects on the lobster heart at low threshold concentrations of 10^{-11}–10^{-10} M as probably brought about by HomamDH31-containing CG motoneurons and/or by direct peptide actions on the heart muscle. Perfusion of semiisolated hearts leads to increased frequency and amplitude but a decreased duration of contractions.[3]

ENKEPHALINS (SEE CHAPTER 219)

Discovery

Early evidence from immunocytochemistry and combined HPLC/radioimmunoassay studies strongly suggested Leu-enkephalin/Met-enkephalin (YGGFL/M) in eyestalk ganglia neurons of spiny lobster and shore crab. Both peptides were later Edman-sequenced from extracts of SGs and thoracic ganglia of *C. maenas* (sffr[16]).

Distribution of the mRNA/Peptide

Enkephalins occur throughout the medullae of the eyestalk ganglia and in the SG (in *C. maenas* even colocalized with MIH). Fibers in the medulla terminalis presumably interacting with dendrites of XO-SG neurons probably originate also in the brain and/or thoracic ganglia of *Panulirus interruptus* and *C. maenas* (sffr[16,33]).

Receptors

Three receptor types resembling the vertebrate δ- and κ-type opioid receptors have been characterized pharmacologically in nervous tissues (e.g. K_i = 18.9 nM for Leu-enkephalin) and localized by autoradiography at CHH terminals in the SG of *C. maenas*. Moreover, displacement studies using several opioids and antagonists have shown that these receptors competitively bind both Leu- and Met-enkephalins (sffr[2]).

Biological Actions

Enkephalins in crustaceans have been implicated in the regulation of stimulated release of chromatophorotropins (RPCH, PDH), inhibition of ovarian maturation, and in the control of carbohydrate metabolism and locomotory activity. The Effects of injected enkephalins, usually substantiated by the use of opioid receptor antagonists such as naloxone, on hemolymph glucose concentrations seem to be species specific, as either hypoglycemia (e.g. in *Uca pugilator*, *P. clarkii*, *O. limosus*) or hyperglycemia (e.g. in *Oziotelphusa senex senex*, *Penaeus indicus*, *Metapenaeus monocerus*) are reported. These effects are apparently caused by stimulation or inhibition of CHH release from XO-SG neurons via synaptic contacts on their dendrites in the medulla terminalis, explaining the often high threshold concentrations around 10^{-7} M used (sffr[2]).

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FMRFAMIDE-RELATED PEPTIDES (FARPS) (SEE CHAPTER 35)

Even though the family founder tetrapeptide, the molluscan cardioactive FMRFamide, does not occur in crustaceans, several distinct groups of peptides with members exhibiting RFamide or LRFamide C-termini and extended N-termini are known, but only a few are established bioactive. Five heart bioactive extended FaRPs from POs and STGs were the first to be identified in lobster, crayfish, and crab followed later by several other decapod isoforms from eyestalk shrimp ganglia and CNS of several crustaceans, which but await being tested for bioactivity (sffr[2,17]). In *D. pulex* only two variants extended FaRPs exceptionally exhibiting an antepenultimate Leu to Ile exchange, that is, an -FIRFamide motif, have been identified.[9] Other bioactive FaRPs such as sulfakinins and myosuppressins were identified much later as was the unique peptide GAHKNYLRFamide.[6] Although yet to be identified biochemically, synthetic lobster neuropeptide F-related peptides as predicted from cloned cDNAs are orexigenic (see below).

Extended FaRPs

Discovery

Heart bioactive extended FaRPs, that is, N-terminally extended FLRFamides such as TNRNFLRFamide (F1) and SDRNFLRFamide (F2) were first identified from lobster POs, followed by the closely related NRNFLRFamide (NF1) and DRNFLRFamide (DF2) from crayfish POs, and GYNRSFLRFamide from crab POs. Shortly after F1 and F2 were identified also neuromodulatory substances from crab STGs (sffr[2,17]).

Structure of the mRNA/Gene and Processing of the Precursor

The only known two extremely similar decapod precursor mRNAs from crayfish *P. clarkii* brain encode five single copies and one double copy of different decapeptide, nonapeptide, or heptapeptide RNFLRFamides, among which also the heptapeptides F1 and F2 occur and the first decapeptide is encoded as two variants AYSDRN(F/Y)LRFamide. The peptides are flanked by dibasic (KR) and monobasic (R) cleavage sites (except for the last one) and several spacer peptides of variable length with no similarity to any other known arthropod peptides.[35]

Distribution of the mRNA/Peptide

Although several immunocytochemical analyses usually detected crossreactive RFamides throughout the CNS of nearly all crustaceans, more specific ISH analyses of crayfish extended FaRPs mRNAs detected numerous distinct brain neurons (mainly cluster 9, a few in cluster 11) that were partially ISH reactive for mRNAs of ASTAs, orcokinins and tachykinin-related peptide precursors.[35] Similar extended FaRPs were identified, although with less spatial resolution, from crab STGs and brain sections by mass spectrometry. Several extended FaRPs have been identified from decapod POs suggesting hormonal release (sffr[2]).

Receptors and Signaling Cascades

Four enzymes such as calcium/calmodulin- dependent protein kinase, protein kinase C, cAMP- and cGMP-dependent protein kinases are involved in the complex differential effects of F1, F2, NF1, and DF2 on tonic and phasic lobster and/or crayfish muscles as measured *in vitro* via excitatory and inhibitory junction potentials (EJPs, IJPs; sffr[17]).

Biological Actions

Among the most extensively studied extended FaRPs F1, F2, NF1, DF2, and GYNRSFLRFamide, the first four affect various aspects of (1) circulation by altering the cardiac output and arterial valve performance thereby redirecting the organ distribution of hemolymph, stimulate (2) parts of the digestive system (gastric mill and pyloric activity via the STG motoneurons; circular and longitudinal hindgut muscles via terminal ganglion neurons), (3) temperature-compensated synaptic transmission, and (4) contraction amplitudes of exoskeletal muscles (dactyl opener, abdominal extensors, respectively) in lobster and crayfish. These effects usually occur at threshold concentrations of 10^{-10}–10^{-9} M that are in the range of hemolymph contents as measured in lobster and apparently require a C-terminal -RNFLRFamide motif. GYNRSFLRFamide is a powerful modulator of the blue crab heart at threshold concentrations of about 10^{-8} M, which required at least a heptapeptide sequence XXZFLRFamide for full potency (sffr[17]). Similar chronotropic and inotropic effects on crab heart by F1, F2, and GYNRSFLRFamide probably occur by dual actions on both the heart muscle and the CG. Further, SDRNFLRFamide increases intracellular Ca^{2+} via voltage-dependent Ca^{2+}-induced release from sarcoplasmic reticulum or through the sacrolemma (sffr[12]). In *C. borealis* STGs, stimulatory activities by TNRNFLRFamide elicited via distinct ion currents converge together with other peptides such as CCAP, proctolin, RPCH, Cab-TRP-Ia on specific neurons in the pyloric circuitry (sffr[30]).

GAHKNYLRFAMIDE

Discovery

Among the three identified decapod peptides sharing a -YLRFamide C-terminus in shrimps and several *Cancer* crabs, the last discovered but the only one extensively studied was the nonapeptide GAHKNYLRFamide (sffr[6]).

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Distribution of the mRNA/Peptide

GAHKNYLRFamide has been identified from brain, STGs, and POs of crabs and shrimps by mass spectrometry (sffr[2]), but no cell type-specific associations have hitherto been worked out.

Biological Actions

Similar to the identified crab FaRPs F1 and F2, GAHKNYLRFamide stimulates neurons in both *C. borealis* CGs and STGs inducing a concentration-dependent increase in burst frequency and duty cycle (burst duration/period) of large CG neurons at an EC_{50} around 10^{-8} M. Although GAHKNYLRFamide stimulates specific neurons in both pyloric and gastric mill circuits of the crab STG, responses of the gastric mill circuitry (alternating dorsal gastric, lateral gastric, and gastric mill neurons) are much more pronounced. However, in silent pyloric or isolated STGs, GAHKNYLRFamide can actually reinitiate a triphasic pyloric rhythm.[6]

KININS (SEE THE KININ CHAPTERS IN THIS AND OTHER SECTIONS OF THE BOOK)

Discovery

Kinin-family peptides in insects and crustaceans are characterized by an $FX_1X_2WGamide$ motif (X, variable aas). The first crustacean kinins were identified from the shrimp *Litopenaeus* (=*Penaeus*) *vannamei* that contains six Pev-kinins 1–6, among which three even have a C-terminal Ala-amide instead of a Gly-amide. Several kinins have later been found in the crab *C. borealis* (sffr[2]).

Distribution of the mRNA/Peptide

Kinin-immunoreactive fibers and terminals of unknown origin have been detected in *C. borealis* POs (sffr[2]).

Biological Actions

Apart from being myoactive in the heterologous cockroach hindgut bioassay, which led to their discovery, Pev-kinin 1, 3, and 4 have also stimulatory effect on the pyloric rhythm of the STG of C. borealis. Pev-kinins stimulate fluid secretion and muscle contraction in insect tissues, but have only an effect on crustacean hindgut muscle at micromolar concentration (sffr[2]).

MYOSUPPRESSIN

Discovery

A first incompletely sequenced crayfish decapeptide possessed the C-terminal consensus motif -HVFLRFa, a characteristic structure for all arthropod myosuppressins (MS) a subfamily of the FaRPs (sffr[17]). Only a single peptide with this C-terminus has thus far been fully identified as pQDLDHVFLRFamide in decapods, among which it seems to be widely conserved.[27] However, in *D. pulex*, an MS identical to cockroach leucomyosuppressin pQDVDHVFLRFamide has been identified.[9]

Structure of the mRNA/Gene and Processing of the Precursor

A first precursor was sequenced from *H. americanus* EST library clones, and later a similar RTPCR-amplified precursor derived from the 2-exon Dappu *ms*-gene in *D. pulex* (sffr[2,9]). In both precursors, the different decapod MS and DappuMS occur at the end of the ORF and were flanked by dibasic/monobasic (KR, R) or dibasic (KR, RR) cleavage sites, respectively.

Distribution of the mRNA/Peptide

Mass spectrometric analyses only suggest pQDLDHVFLRFamide to be widely distributed within decapod nervous systems and the midgut, where they probably serve both as a locally acting or circulating hormone (sffr[2]).

Biological Actions

The semiisolated lobster heart and its cardiac ganglion (CG) are strongly modulated by MS, that is, *in vitro* decreasing the frequency but increasing the amplitude of heart contractions likely brought about by a decrease in CG cycle frequency via hyperpolarization and direct actions on muscle tissue at threshold concentrations of 10^{-8}–10^{-7} M (sffr[2]).

NEUROPEPTIDE F-RELATED PEPTIDES

Discovery

Two precursors and likely splice variants encoding the so-called long neuropeptide F-related peptides KPDPSQLANMAEALKYLQELDKYYSQVSRPRFamide (LitvaNPF-I) and KPDPSQLANMAEALKYLQELDKYYSQVSRPSPRSAPGPASQIQALENTLKFLQLQELGKLYSLRARPRFamide (LitvaNPF-II) have been cloned for two penaeid species *L. vannamei* and *Melicertus marginatus* based on predicted sequences from an EST another penaeid *Marsupenaeus japonicus.*[1] The shorter isoform is identical in all three penaeids and the longer isoform is identical only in the former two penaeids. Two similar splice forms arise from alternative splicing of the Dappu *npf*-gene, as confirmed by RTPCR and mass spectrometric sequencing of the shorter isoform DappuNPF1.[9]

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Structure of the mRNA/Gene and Processing of the Precursor

As predicted for penaeid NPF precursors and identified for the DappuNPF1 precursor, the peptide sequences start directly after the signal peptide and are cleaved via a dibasic cleavage site KR from a single following carboxy-terminal PRP. Assuming that the penaeid NPF isoforms likely also arise from alternative splicing as in *D. pulex*, then the longer second NPF-II as DappuNPF2 represent isoform peptides containing the unspliced message "insert" (37 aas in penaeids and 27 aas in DappuNPF2) from a second exon.[1,9]

Distribution of the mRNA/Peptide

RTPCR-profiling has much stronger NPF-I- than NPF-II-specific signals in the penaeid eyestalk ganglia, brain, thoracic, and abdominal ganglia and even in the midgut, although the latter seemed to lack NPF-II amplicons.[1] DappuNPF1 was only sequenced from brain tissue.[9]

Biological Actions

Surprisingly, synthetic penaeid NPF-I can simply be fed to the animals as a diet supplement, which leads to a significant increase in food intake and faster growth relative to controls.[1]

ORCOKININ AND ORCOMYOTROPIN

Discovery

The orcokinin peptide family is established in crustaceans and insects, and predicted also for chelicerates and *Caenorhabditis elegans*. The first tridecapeptide orcokinin NFDEIDRSGFGFN (Asn^{13}-OK) was identified as a hindgut-myoactive peptide in 1992 from VNC of the crayfish, *O. limosus*, followed by homologs from *O. limosus* hindguts and from other decapods (*C. maenas*, *Cherax destructor*, *P. clarkii*, *C. borealis*, and *H. americanus*) (sffr[2,9]). Another even more potent hindgut-myoactive peptide orcomyotropin FDAFTTGFamide (OMT) was discovered in the hindgut extracts from *O. limosus*.[8] The first two precursors of the crayfish *P clarkii* contained different orcokinin isoforms, and one copy of an OMT-like undecapeptide FDAFTTGFGHS (OMTL). OMTs and OMTLs have been identified from several decapods and up to three OMTL/OK-precursors in *H. americanus* (sffr[2,27]), but a separate OMT-precursor is not known. In *D. pulex* only two different genes encode only two out of three identified OKs NLDEIDRSNFGTFA, NLDEIDRSDFGRFV, NLDEIDRSDFSRFV, and one out of two different OMTL-related peptides. Of note, DappuOKs are tetradecapeptides as are all insect OKs.[9]

Structure of the mRNA/Gene and Processing of the Precursor

The similar crayfish pre-pro-orcokinins A and B contain one copy of Ser^{11}-OMT followed by 10 and 11 copies of four distinct OKs (NFDEIDRSGFGFX with X = Asn, Val, or Ala; and $Thr^{8}His^{13}$-OK or Ser^{9}-OK). In the three closely related lobster precursors, one copy of Asn^{11}-OMTL followed similarly by the four OKs and further OK isoforms such as His^{13}-OK and SSEDMDRLGFGFN have been found (sffr[2]). Apart from some other unrelated PRPs, all OMTs and OKs are flanked by canonical dibasic KR cleavage sites (in *D. pulex* one additional KK site).[9]

Distribution of the mRNA/Peptide

In several crustaceans, OK immunoreactivity has been localized in the CNS, STNS, neurohemal structures, and in the hemolymph, suggesting both neural and hormonal roles.[2]

Biological Actions

Asn^{13}-OK, Val^{13}-OK, or Ala^{13}-OK exhibit similar myotropic effects most pronounced on crayfish hindguts (chronotropic and inotropic; sffr[8]), although Asn^{13}-OK was inactive on lobster hindgut. OMT is more potent than OKs in *O. limosus* hindgut assay, whereas OMTLs do not seem to be bioactive (e.g. on STG[7]). Exogenous application of OKs to the STG affects the pyloric rhythm in crabs and *H. americanus* (sffr[2]).

PIGMENT-DISPERSING HORMONE (PDH)

Discovery

Discovered as a light-adapting hormone in caridean shrimp (NSGMINSILGIPRVMTEA-amide; alpha-PDH) and later as a pigment-dispersing hormone in many other crustaceans (NSELINSILGLPKVMNDA-amide, beta-PDH), the 18mer peptides can occur in up to three different isoforms. A structurally quite deviating variant has been sequenced recently from *Daphnia magna* and *D. pulex* (sffr[29]).

Structure of the mRNA/Gene and Precursor Processing

Precursors derived from sequenced mRNAs are known from decapod and branchiopod crustaceans contain the PDH at the end separated by a dibasic cleavage site from a long PRP.

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In the 2- or 3-exon PDH genes, the PRP-encoding region is interrupted by the first and/or the only one intron, whereas the second intron splits the 3′ UTR-message (sffr[9,29]).

Distribution of the mRNA/Peptide

A first immunolocalization was described in crabs and crayfish, but it turned out recently that separate PDH-precursor mRNAs and derived slightly different PDHs are differentially distributed in eyestalks of *Cancer productus* (sffr[2,29]).

Receptors and Signaling Cascades

The first putative G protein-coupled receptors for PDH were cloned from the Kuruma prawn *Marsupenaeus japonicus* (MjPDHR, AB478163.1, 2009) and predicted for *D. pulex*.[31] Evidence from erythrophores of freshwater shrimp *Macrobrachium potiuna* suggests that alpha-PDH elicits pigment dispersion via G protein-coupled receptor-mediated increases in cAMP activating a cAMP-dependent protein kinase A. This phosphorylates a granule-bound 53- to 57-kDa protein, which then allows for kinesin-driven dispersion of granules (sffr[18]).

Biological Actions

PDH is a classical antagonist to RPCH in controlling chromatophore pigment dispersion in many crustaceans (sffr[28]) but seems to have no neuromodulatory roles in the STG.[2] As the insect homolog pigment-dispersing factor (PDF) is an established circadian clock output factor, evidence for similar roles of PDHs in controlling circadian rhythmicity in decapod, isopod, and branchiopod crustaceans is now emerging. Methyl farnesoate synthetic activity of crayfish mandibular organs is apparently inhibited by PDH *in vitro* (sffr[28,29,34]).

PROCTOLIN

Discovery

Identified as the first peptide in insects, but later also from lobster and crab pericardial organs (POs), proctolin (RYLPT), is one of the most conserved peptides in arthropods (sffr[2]). However, in *D. pulex*, authentic proctolin has recently been sequenced together with a novel isoform Met[4]-proctolin.[9]

Structure of the mRNA/Gene and Processing of the Precursor

In crustaceans, genes, mRNAs and derived precursors for the two identified proctolin isoforms have only recently been deduced from the *D. pulex* genome and are in both cases similar to the structures known from insect proctolin genes. The proctolins are immediately following the SPs but are separated from long PRPs only by a monobasic (R) cleavage site.[9]

Distribution of the mRNA/Peptide

In decapods, isopods, and branchiopods, proctolin-immunoreactive neurons occur almost throughout the entire CNS, among them also motoneurons coexpressing classical transmitters and in classical neurohemal organs such as the SG in the eyestalk ganglia and in projection neurons innervating the STG from commissural and the POs from thoracic ganglia (sffr[2]).

Receptors and Signaling Cascades

Proctolin enhances force of contractions as a cotransmitter via potentiation of Ca^{2+}-channel activity in postural motoneurons of decapods. However, in isopod skeletal muscles, proctolin apparently does not act via cAMP-dependent protein kinase A-mediated phosphorylation inhibiting non-voltage-dependent K^+-channels as initially shown, but via an activation of protein kinase C reducing cGMP contents by phosphorylating a cytoplasmic guanylyl cyclase (sffr and discussion[21]).

Biological Actions

Proctolin has myotropic effects not only on the hindgut (especially on ring muscles of the proctodeum, hence its name) but also on cardiac, skeletal, and visceral muscles also in isopods and barnacles. Cardioactivity in crustaceans is likely brought about by its neuromodulatory effects on CG neurons, but such effects are also well established for the STG, as brought about by at least three identified proctolinergic projection neurons from crab commissural ganglia (sffr[2,17]).

PYROKININ

Originally isolated as a N-terminally pyroglutamic acid (pQ)-bearing Leucopyrokinin pQTSFTPRLamide, many insect and fewer crustacean pyrokinins (PKs) without a pQ group became known as bearing the C-terminal motif FXPRLamide (with X being Gly, Ser, Thr or Val). In crustaceans, 5–10 aas long pyrokinins were first identified in as Pev-PK1 DFAFSPRLamide and Pev-PK2 ADFAFNPRLamide in shrimps *Litopenaeus (=Penaeus) vannamei*, later others also in crab and lobster (sffr[2]).

Distribution of the mRNA/peptide

Identified crab pyrokinins have hitherto only been localized to the commissural ganglia and STG of *C. borealis*,[24] but

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mass spectrometric mapping suggests a much wider distribution in the CNS of several crustaceans (sffr[2]).

Biological Actions

Both shrimp Pev-PKs stimulate hindgut contractions in cockroach and crayfish *in vitro*, and authentic crab PKs excite gastric mill rather than pyloric rhythms in *C. borealis* (sffr[24]).

RED PIGMENT CONCENTRATING HORMONE (RPCH)

Discovery

The first invertebrate peptide to be sequenced[11] and being extremely conserved in decapods and an isopod *Porcellio scaber* is the octapeptide pQLNFSPGWamide that shares considerable identities with many adipokinetic hormones (AKHs) from insects.[2,13] The recently discovered homolog pQVNFSTSWamide from *D. magna* and *D. pulex* is, however, much closer in structure to the insect AKHs.[9]

Structure of the Gene/mRNA and Processing of the Precursor

The decapod (crab *Callinectes sapidus*) and branchiopod (*D. pulex*) 2-exon RPCH/AKH genes contain the SP followed by the octapeptide separated by a dibasic (KR) processing site from a long PRP, which is interrupted by the intron (sffr[9]).

Distribution of the Peptide

In decapods and isopods, the RPCH-immunoreactive cells are mainly distributed in the eyestalk ganglia, especially prominent in the X-organ sinus gland (XOSG) system (sffr[2,20]). In crab embryos, another pathway leads from brain cells to possible postcomissural neurohemal organ structures of the VNC (sffr[4]).

Receptors and Signaling Cascades

To date, crustacean receptors for RPCH/AKH-like peptides are unknown, but the RPCH action on chromatophores is likely mediated by a complex interplay of decreased cAMP contents and increased Ca^{2+}-levels. In freshwater shrimp *M. potiuna*, a G-protein-coupled receptor is signaling through phospholipase C-driven inositol-triphosphate production, which elevates Ca^{2+} that in turn via a Ca^{2+}/Calmodulin-activated protein kinase C phosphorylates and activates a serine/threonine phosphatase. This then leads to dephosphorylation of granule-bound proteins followed by pigment aggregation (sffr[18]).

Biological Actions

Decapod RPCH is a chromatophorotropin leading to the concentration of pigment granules in red and other chromatophores of several shrimps and in crayfish distal retinal pigment cells.[13] The *Daphnia* homolog is apparently inactive in this regard but can mobilize lipids in an insect (sffr[9]). RPCH has profound effects in changing coupling of neuronal oscillators in crab STG and can stimulate release of methyl farnesoate from mandibular organs *in vitro*. Furthermore, it modulates the sensitivity of photoreceptor cells and retraction of distal retinal pigment (sffr[28]).

SIFAMIDE

Discovery

The first SIFamide in crustaceans was identified as FMRFamide-like peptide Pem-FLP7 (GYRKPPFNG-SIFamide) from *P. monodon*, a member of a peptide family whose first member was Neb-LFamide from the flesh fly *Neobellieria bullata*. Among crustaceans, Gly^1-SIFamide is the most common, whereas Val^1-SIFamide is found in two *Homarus* species, and both isoforms occur in *C. maenas* (sffr[2,27]). However, a very different and 1aa shorter TRKLPFNGSIFamide identified in *D. pulex* lacks the usual Tyr^2 and contains Leu^4-Pro^5 instead of the usual Pro-Pro motif in SIFamides of most insects and decapods.[9]

Structure of the mRNA/Gene and Processing of the Precursor

In all arthropod SIFamide mRNAs, the peptide is encoded directly after the SP and is followed by a conserved dibasic (KR) cleavage site separating SIFamide from a PRP containing two cysteines. The 2-exon *sifa*-gene in *D. pulex* encodes a 73 aas precursor (sffr[9]).

Distribution of the mRNA/Peptide

In crayfish *P. clarkii*, SIFamide mRNA or peptides are expressed in all nervous tissues, especially in brain projection neurons (cluster 10), but absent from the heart, hepatopancreas, and skeletal muscle but in *H. americanus* also in the midgut (sffr[2]).

Biological Actions

Val^1-SIFamide modulates the lobster pyloric neural network, and Gly^1-SIFamide controls male aggressive behavior in *Macrobrachium rosenbergii*.[2]

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SULFAKININS

Discovery

Sulfakinins (SKs), first identified in cockroaches, share a heptapeptide structure containing a sulfated tyrosine $XY_{(SO3H)}$GH**M/L**RFamide (X = D or E) in several arthropod groups. In decapod crustaceans, the first peptides pQFDEY$_{(SO3H)}$GHMRFamide (SKI) and AGGSGGVGGEY$_{(SO3H)}$DDY(SO3H)GHLRFamide (SKII) were identified from CNSs of shrimps *P. monodon* and *L. vannamei*, and later a shorter non-sulfated SKII isoform from lobster. Interestingly, in *D. pulex*, two unique SKI (containing a Tyr-amide C-terminus) and SKII were identified which, however, were not Tyr-sulfated (sffr[9]).

Structure of the mRNA/Gene and Processing of the Precursor

Lobster and *D. pulex* sulfakinin-mRNA precursors contain single copies of SKI and SKII in tandem close to or at the end of the ORFs, respectively. The *D. pulex sk*-gene is a 4-exon gene encoding a 207-bp long precursor carrying the SK1-SK2 tandem separated by dibasic (KR, RR) processing sites (sffr[9]).

Distribution of the mRNA/Peptide

Up to ten SK-specific neurons only in total are found in the brain and VNC of shrimp *P. monodon* (sffr[2]).

Biological Actions

Synthetically sulfated isoforms of SKs are cardioactive in the lobster *H. americanus*, increasing both frequency and amplitude of the heartbeat (sffr[2]).

TACHYKININ-RELATED PEPTIDES

Discovery

Tachykinin-related peptides (TRPs) form a large group of invertebrate peptides exhibiting a C-terminal -FX$_1$GX$_2$Ramide motif (X_1, X_2 variable) with slight resemblance to vertebrate tachykinins and neurokinins. Two TRPs have first been identified from *C. borealis*, the *nona*peptide CabTRP-Ia APSGFLGMRamide and a truncated isoform CabTRP-Ib SGFLGMRamide, the former being identified later in many other decapods. A third decapod *nona*peptide TRP, TPSGFLGMRamide, was later identified from *Cancer* crabs, followed by three further *nona*peptide TRPs identified from *L. vannamei* showing slightly different N-terminal sequences (APAGFLGMRamide, APSGFNGMRamide and APSFGLDMRamide; sffr[2]). Interestingly, merely three *deca*peptide TRPs have been identified from *D. pulex*.[9]

Structure of the mRNA/Gene and Processing of the Precursor

The first TRP precursors cloned from cDNA libraries of *P. clarkii* and *P. interruptus* are highly conserved, containing seven copies each of CabTRP-Ia. However, a very similar EST-deduced and full-length cloned precursor of *H. americanus* contained six copies of CabTRP-Ia and one copy of TPSGFLGMRamide at the end. The three quite unrelated decapeptide DappuTRPs (except perhaps for DappuTRP2 TP*N*S*RA*FLGMRa and TPS*G*FLGMRamide) as derived from exon 2 of the 3-exon Dappu *tkrp*-gene are somewhat closer related to insect TPRs than to those of decapods. Within the precursors, all TRPs are flanked by dibasic (KK, KR, RR) cleavage sites and spacer PRPs showing no sequence similarity among the species or resemblance to any other arthropod peptide (see Ref. 9 for more detailed comparisons).

Distribution of the mRNA/Peptide

TRPs are present within several neurons of the CNS partly entering the STG and in the midgut endocrine paraneurons of several crustaceans. In *P. clarkii*, eyestalk ganglia and CNS, especially brain neurons of clusters 9 and 11 and among posterior lateral cells are ISH reactive for TRP-mRNA, which was absent from midgut and hindgut tissue.[35] In the crayfish *Pacifastacus leniusculus*, TRPs occur in many neurons of the brain and the eyestalk ganglia, particularly associated with identified amacrine cells of the lamina, and in fibers terminating in the STG (sffr[14]). CabTRP-Ia is also found in likely open-type midgut endocrine cells of *Cancer* crabs (sffr[2]).

Signaling Cascades

Stimulatory activities of CabTRP-1a on pyloric circuitry in the STG of *C. borealis* are partly mediated via calmodulin-dependent enzyme cascades leading to alterations of voltage-dependent currents (sffr[30]).

Biological Actions

CabTRP-Ia and TPSGFLGMRamide activate pyloric (preferably) and gastric mill circuitries of decapod STGs together with other peptide modulators such as proctolin. When tested at 10^{-6} M, both the peptides equally lead to significant increases in frequency and amplitude of heart contractions (sffr[2,30]). Cockroach *Leucophaea maderae* TRP-4 (=Met^6-CabTRP-Ia) modulates photoreceptor sensitivity in combination with GABA by presynaptic inhibition from amacrine

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cells of the crayfish lamina.[14] CabTRP-Ia release from midgut endocrine cells seems to elevate circulating levels of the peptide in starved animals, and may lead to its myotropic action on the musculature of the foregut (sffr[2]).

CONCLUSIONS AND PERSPECTIVES

This brief survey of identified bioactive neuropeptides in crustaceans has omitted many novel crustacean neuropeptides for which hitherto no bioactivity profile has been available. In decapods, there are now altogether >200 different peptides including bioactive truncated versions known,[2] and the *D. pulex* genome contains at least 43 identified peptide and protein hormone genes encoding more than 70 different peptides, already half of which have now been sequenced.[9] Furthermore, exploration of other peptide sources such as the gut probably containing so-called "brain–gut peptides" (e.g. as CabTRP-Ia) has just begun. Thus, it is certain that other as yet unidentified neuropeptides, for example, also antimicrobial peptides, and neuropeptide families will soon be added to the list of bioactive crustacean neuropeptides.

REFERENCES

1. Christie AE, Chapline MC, Jackson JM, Dowda JK, Hartline N, Malecha SR, et al. Identification, tissue distribution and orexigenic activity of neuropeptide F (NPF) in penaeid shrimp. *J Exp Biol* 2011;**214**:1386–96.
2. Christie AE, Stemmler EA, Dickinson PS. Crustacean neuropeptides. *Cell Mol Life Sci* 2010;**67**:4135–69.
3. Christie AE, Stevens JS, Bowers MR, Chapline MC, Jensen DA, Schegg KM, et al. Identification of a calcitonin-like diuretic hormone that functions as an intrinsic modulator of the American lobster, *Homarus americanus*, cardiac neuromuscular system. *J Exp Biol* 2010;**213**:118–27.
4. Chung JS, Webster SG. Expression and release patterns of neuropeptides during embryonic development and hatching of the green shore crab, *Carcinus maenas*. *Development* 2004;**131**:4751–61.
5. Chung JS, Wilcockson DC, Zmora N, Zohar Y, Dircksen H, Webster SG. Identification and developmental expression of mRNAs encoding crustacean cardioactive peptide (CCAP) in decapod crustaceans. *J Exp Biol* 2006;**209**:3862–72.
6. Cruz-Bermudez ND, Fu Q, Kutz-Naber KK, Christie AE, Li L, Marder E. Mass spectrometric characterization and physiological actions of GAHKNYLRFamide, a novel FMRFamide-like peptide from crabs of the genus *Cancer*. *J Neurochem* 2006;**97**:784–99.
7. Cruz-Bermudez ND, Marder E. Multiple modulators act on the cardiac ganglion of the crab, *Cancer borealis*. *J Exp Biol* 2007;**210**:2873–84.
8. Dircksen H, Burdzik S, Sauter A, Keller R. Two orcokinins and the novel octapeptide orcomyotropin in the hindgut of the crayfish *Orconectes limosus*: identified myostimulatory neuropeptides originating together in neurones of the terminal abdominal ganglion. *J Exp Biol* 2000;**203**:2807–18.
9. Dircksen H, Neupert S, Predel R, Verleyen P, Huybrechts J, Strauss J, et al. Genomics, transcriptomics, and peptidomics of *Daphnia pulex* neuropeptides and protein hormones. *J Proteome Res* 2011;**10**:4478–504.
10. Dircksen H, Skiebe P, Abel B, Agricola H, Buchner K, Muren JE, et al. Structure, distribution, and biological activity of novel members of the allatostatin family in the crayfish *Orconectes limosus*. *Peptides* 1999;**20**:695–712.
11. Fernlund P. Synthesis of the red-pigment-concentrating hormone of the shrimp, *Pandalus borealis*. *Biochim Biophys Acta* 1974;**371**:312–22.
12. Fort TJ, Brezina V, Miller MW. Regulation of the crab heartbeat by FMRFamide-like peptides: multiple interacting effects on center and periphery. *J Neurophysiol* 2007;**98**:2887–902.
13. Gäde G. Peptides of the adipokinetic hormone/red pigment-concentrating hormone family: a new take on biodiversity. *Ann N Y Acad Sci* 2009;**1163**:125–36.
14. Glantz RM, Miller CS, Nässel DR. Tachykinin-related peptide and GABA-mediated presynaptic inhibition of crayfish photoreceptors. *J Neurosci* 2000;**20**:1780–90.
15. Kwok R, Zhang JR, Tobe SS. Regulation of methyl farnesoate production by mandibular organs in the crayfish, *Procambarus clarkii*: a possible role for allatostatins. *J Insect Physiol* 2005;**51**:367–78.
16. Lüschen W, Buck F, Willig A, Jaros PP. Isolation, sequence analysis, and physiological properties of enkephalins in the nervous tissue of the shore crab *Carcinus maenas* L. *Proc Natl Acad Sci U S A* 1991;**88**:8671–5.
17. Mercier AJ, Friedrich R, Boldt M. Physiological functions of FMRFamide-like peptides (FLPs) in crustaceans. *Microsc Res Tech* 2003;**60**:313–24.
18. Nery LEM, daSilva MA, Castrucci AMD. Role of cyclic nucleotides in pigment translocation within the freshwater shrimp, *Macrobrachium potiuna*, erythrophore. *J Comp Physiol B* 1998;**168**:624–30.
19. Nery LEM, DaSilva MA, Castrucci AMD. Possible role of non-classical chromatophorotropins on the regulation of the crustacean erythrophore. *J Exp Zool* 1999;**284**:711–6.
20. Nußbaum T, Dircksen H. Neuronal pathways of classical crustacean neurohormones in the central nervous system of the woodlouse, *Oniscus asellus* (L.). *Phil Trans R Soc B: Biol Sci* 1995;**347**:139–54.
21. Philipp B, Rogalla N, Kreissl S. The neuropeptide proctolin potentiates contractions and reduces cGMP concentration via a PKC-dependent pathway. *J Exp Biol* 2006;**209**:531–40.
22. Phlippen MK, Webster SG, Chung JS, Dircksen H. Ecdysis of decapod crustaceans is associated with a dramatic release of crustacean cardioactive peptide into the haemolymph. *J Exp Biol* 2000;**203**:521–36.
23. Poljaroen J, Tinikul Y, Phoungpetchara I, Kankoun W, Suwansa-ard S, Siangcham T, et al. The effects of biogenic amines, gonadotropin-releasing hormones and corazonin on spermatogenesis in sexually mature small giant freshwater prawns, *Macrobrachium rosenbergii* (De Man, 1879). *Aquaculture* 2011;**321**:121–9.
24. Saideman SR, Ma M, Kutz-Naber KK, Cook A, Torfs P, Schoofs L, et al. Modulation of rhythmic motor activity by pyrokinin peptides. *J Neurophysiol* 2007;**97**:579–95.
25. Sharp JH, Wilcockson DC, Webster SG. Identification and expression of mRNAs encoding bursicon in the plesiomorphic central nervous system of *Homarus gammarus*. *Gen Comp Endocrinol* 2010;**169**:65–74.

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26. Stangier J, Hilbich C, Beyreuther K, Keller R. Unusual cardioactive peptide (CCAP) from pericardial organs of the shore crab *Carcinus maenas*. *Proc Natl Acad Sci USA* 1987;**84**:575–9.
27. Stemmler EA, Cashman CR, Messinger DI, Gardner NP, Dickinson PS, Christie AE. High-mass-resolution direct-tissue MALDI-FTMS reveals broad conservation of three neuropeptides (APSGFLGM-Ramide, GYRKPPFNGSIFamide and pQDLDHVFLRFamide) across members of seven decapod crustaean infraorders. *Peptides* 2007;**28**:2104–15.
28. Strauss J, Dircksen H. Circadian clocks in crustaceans: identified neuronal and cellular systems. *Front Biosci Landmark* 2010;**15**:1040–74.
29. Strauss J, Zhang Q, Verleyen P, Huybrechts J, Neupert S, Predel R, et al. Pigment-dispersing hormone in *Daphnia* interneurons, one type homologous to insect clock neurons displaying circadian rhythmicity. *Cell Mol Life Sci* 2011;**68**:3403–23.
30. Swensen AM, Marder E. Modulators with convergent cellular actions elicit distinct circuit outputs. *J Neurosci* 2001;**21**:4050–8.
31. Tilden AR, McCoole MD, Harmon SM, Baer KN, Christie AE. Genomic identification of a putative circadian system in the cladoceran crustacean *Daphnia pulex*. *Comp Biochem Physiol Part D Genomics Proteomics* 2011;**6**:282–309.
32. Ventura T, Rosen O, Sagi A. From the discovery of the crustacean androgenic gland to the insulin-like hormone in six decades. *Gen Comp Endocrinol* 2011;**173**:381–8.
33. Webster SG, Keller R, Dircksen H. The CHH-superfamily of multifunctional peptide hormones controlling crustacean metabolism, osmoregulation, moulting, and reproduction. Gen Comp Endocrinol, in press [Epub Nov 2011].
34. Wilcockson DC, Zhang L, Hastings MH, Kyriacou CP, Webster SG. A novel form of pigment-dispersing hormone in the central nervous system of the intertidal marine isopod, *Eurydice pulchra* (Leach). *J Comp Neurol* 2011;**519**:562–75.
35. Yasuda-Kamatani Y, Yasuda A. Characteristic expression patterns of allatostatin-like peptide, FMRFamide-related peptide, orcokinin, tachykinin-related peptide, and SIFamide in the olfactory system of crayfish *Procambarus clarkii*. *J Comp Neurol* 2006;**496**:135–47.

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Chapter 33

Developmental Peptides: ETH, Corazonin, and PTTH

Michael E. Adams, Young-Joon Kim, Yoonseong Park and Dusan Zitnan

ABSTRACT

Ecdysis-triggering hormones (ETHs) are small, linear, C-terminally amidated peptides produced and released by endocrine Inka cells under the control of ecdysteroids and other peptides. Released into the blood at the end of each molt, they activate peptidergic central neuron ensembles to trigger a sequence of physiological and behavioral events culminating in ecdysis or shedding of the old cuticle. Corazonin is an N- and C-terminally blocked neuropeptide produced by neurosecretory cells in the brain and ventral nerve cord. It has many physiological actions that, depending on the insect, include cardioacceleration, melanization, and ecdysis initiation. PTTH is a large (22-kDa) homodimeric peptide hormone produced by two pairs of lateral neurosecretory cells in the brain. It acts on the prothoracic glands to promote synthesis and release of ecdysteroids. Precursors of all these peptide hormones contain associated peptides of unknown function.

ECDYSIS-TRIGGERING HORMONES

Discovery

Ecdysis-triggering hormones (ETHs) were first isolated and sequenced from epitracheal glands of the tobacco hawkmoth, *Manduca sexta*, and the silkmoth, *Bombyx mori*, and shortly thereafter were identified in *Drosophila*.[29,30] Epitracheal glands consist of a large, peptidergic Inka cell, a smaller putative endocrine cell, an exocrine cell, and a canal cell. Zitnan initially observed strong immunoreactivity with antiserum to the neuropeptide FMRFamide in Inka cells on the surface of tracheal tubes immediately adjacent to each spiracle in the waxmoth *Galleria mellonella*, and noticed that they lost staining after ecdysis. Epitracheal gland extracts trigger premature ecdysis when injected into pharate *M. sexta* larvae. The first isolation of an ETH was accomplished by reversed-phase HPLC using an extract of approximately 40 *M. sexta* epitracheal glands.[31] The peptide was pure after a 2-step purification guided by the ability of fractions to trigger premature ecdysis behavior in pharate *M. sexta* larvae on injection into the hemocoel. ETHs of *M. sexta* (MasETH) and *B. mori* are linear peptides with a conserved C-terminal sequence motif of –NIPRMamide (Fig. 1).[17] Preecdysis-triggering hormone (PETH), discovered through precursor analysis (see below), is identical in the two moth species.[32] The *Drosophila* peptides DrmETH1 and DrmETH2 were deduced using BLAST analysis of the fly genome database using the moth precursor sequences and confirmed by RT-PCR.[12] Subsequently, ETH-related peptides have been identified in a wide variety of insect orders through direct isolation of peptides, PCR, or *in silico* screening.[17] These include Orthoptera (cockroaches, crickets, grasshoppers), Hemiptera (aphids), Homoptera (planthoppers), Phthiraptera (lice), Coleoptera (beetles), Diptera (flies, mosquitoes), Lepidoptera (moths), and Hymenoptera (wasps, honeybee). In the honeybee *Apis*, only one ETH ligand is evident in the precursor. ETH-like peptides also occur in the related arthropod classes Crustacea (water flea, *Daphnia*) and Arachnida (tick, *Ixodes*).

Inka cells and associated ETH-like immunoreactivity occur in all insect Orders thus far examined, including Thysanura (silverfish), Orthoptera (crickets, grasshoppers, cockroaches, walking sticks), Plecoptera (stoneflies), Hemiptera and Homoptera (bugs), Megaloptera (alderflies), Neuroptera (antlions), Coleoptera (beetles), Lepidoptera (moths), Diptera (flies, mosquitos, crane flies), and Hymenoptera (bees, wasps, ants). Extracts of epitracheal glands or trachea containing Inka cells from many of these species induce ecdysis behavior in *B. mori*,[34] verifying that the ETH signaling system is widespread, functional, and conserved in insects.

Structure of the Precursor mRNA/gene and Peptide Processing

Manduca sexta *and* Bombyx mori

The MasETH and BomETH precursors contain a 23-amino-acid signal sequence and one copy each of PETH, ETH,

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00033-6
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and ETH-AP.[30] Dibasic proteolytic cleavage sites are depicted in Fig. 2. Direct isolation of peptides from *M. sexta* epitracheal glands yields, in addition to PETH, ETH, and ETH-AP, significant amounts of precursor peptides. These include the unprocessed precursor minus the signal sequence PETH-ETH-ETH-AP (PEA), PETH-ETH (PE), and ETH-ETH-AP (EA). Analysis of peptide content in Inka cells and circulating peptides in the hemolymph during ecdysis behavior reveal a 30% excess of PETH over ETH, despite the 1:1 stoichiometry of these peptides in the precursor. The relatively lower level of circulating ETH results from incomplete processing of the EA precursor, levels of which increase in the hemolymph during the ecdysis sequence.[32]

Ecdysteroid pulses that initiate the molt induce increased synthesis of ETH peptides in Inka cells. This is especially evident during the fifth (last) larval instar of *M. sexta*, where the pupal steroid commitment pulse is followed by a much larger second pulse that programs the pupal stage. Levels of ETHs and precursors increase in synchrony with the leading edges of each of these two steroid peaks,[35] indicating that expression of the ETH gene and precursor processing are steroid regulated.

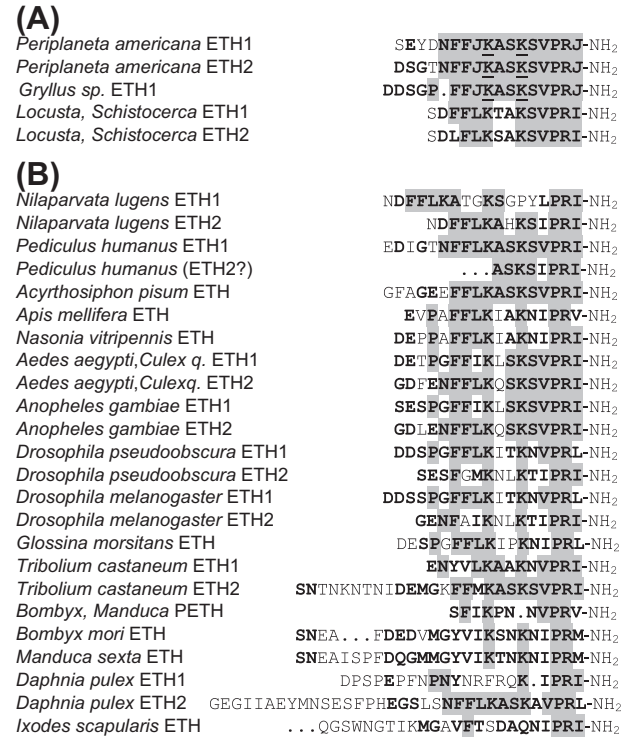

FIGURE 1 Amino acid sequence alignments of ecdysis-triggering hormones.

Drosophila melanogaster *and* Anopheles gambaie

The ETH precursor in *Drosophila* is encoded by the gene CG18105, which occurs on the right arm of the second chromosome at position 60E1–2. The preprohormone consists of a 21-amino-acid signal sequence and one copy each of DrmETH1 and DrmETH2, both of which have ecdysis-triggering biological activity.[12] Assignment of the ETH1 N-terminus is not obvious from inspection of the precursor sequence. However, synthesis and bioassay of the two most probable peptides (N-terminus beginning with either AIS– or DDS–) reveals that the DDS– sequence is significantly more bioactive in inducing adult ecdysis.[12] Three additional putative peptides occurring downstream of ETH2 have not been tested for biological activity. These include a 21-amino-acid peptide unblocked at the C-terminus (DrmETH-AP1), a 63-amino-acid peptide with a blocked C-terminus (DrmETH-AP2), and a 54-amino-acid peptide unblocked at the C-terminus.[12] Processing sites for the precursor are depicted in Figure 2.

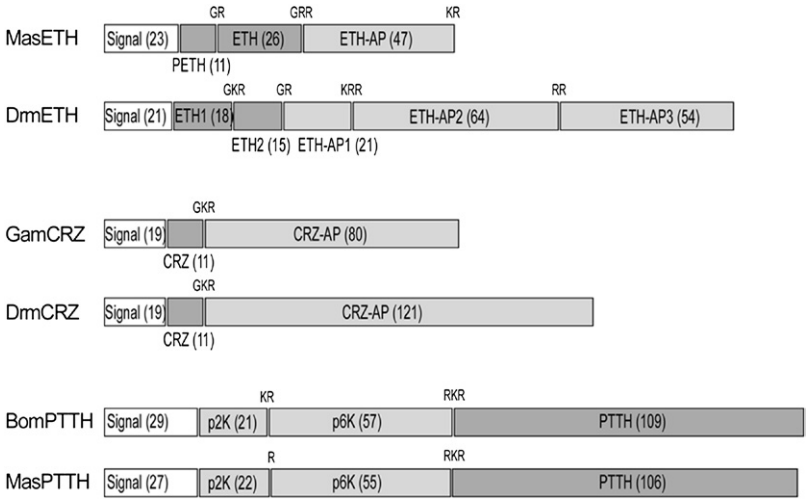

FIGURE 2 Schematic diagrams of precursors and processing sites for ETHs,[12,32] corazonins,[1] and PTTHs.[7]

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An ETH precursor has been deduced from the mosquito *A. gambiae* genome database.[34] Peptides closely related to DrmETH1 and DrmETH2 are evident in the mosquito precursor and associated peptides downstream of ETH2 (Fig. 2). Related peptides from *Aedes aegypti* (AeaETH1, AeaETH2) have been isolated and sequenced by mass spectrometry. These results verify the sequences inferred from genome analysis.[3]

Distribution of the mRNA and Peptides

The ETH gene and mRNA are detected only in Inka cells. Northern blots were used to detect ETH-encoding mRNA in *M. sexta* epitracheal glands; the signal is absent in the CNS, epidermis, muscle, and trachea.[8] Inka-cell-specific expression of ETH was shown using the ETH promoter to drive expression of EGFP in transgenic flies. A nucleotide sequence encoding the following fusion protein was constructed: the ETH gene upstream promoter region (~300 bp), the ETH gene, and a nucleotide sequence encoding EGFP. Transformed fly lines showed EGFP-associated fluorescence only in Inka cells.[10] The baculovirus expression system (Bac-to-Bac) was used to identify the ETH promoter in *B. mori*. The upstream promoter region (600 bp) with EGFP was subcloned into the bacmid DNA and the recombinant baculovirus was injected into larvae and pupae of *B. mori*. Strong and specific EGFP expression was detected only in Inka cells.[4]

Receptors

ETH receptors identified in *D. melanogaster* are G-protein-coupled receptors.[29,30] A single gene (CG5911) containing 5 exons encodes two ETH receptor subtypes through alternative splicing of two 3′ exons to generate CG5911a and CG5911b. Phylogenetic analysis shows that CG5911 is most closely related to vertebrate thyrotropin releasing hormone and neuromedin receptors, as well as to *Drosophila* receptors for PBAN-like pyrokinin receptors.

ETH receptors are coupled to Gαq, which mobilizes calcium from intracellular stores via the phosphoinositide turnover pathway. Pharmacological profiles of ETH receptors were determined by heterologous expression in mammalian CHO cells using aequorin as an intracellular calcium reporter. ETH receptors in *Drosophila* show high sensitivity and selectivity to fly and moth ETHs. Remarkably, CG5911b shows a considerably higher sensitivity to ETH1 and ETH2 as compared with CG5911a. Both DrmETH1 and DrmETH2 activate CG5911b with EC_{50} values of approximately 1 nM. By contrast, ETH1 and ETH2 activate CG5911a with EC_{50} values of approximately 400 nM and approximately 4 μM, respectively.[11] Thus, although CG5911b is more sensitive to the cognate ETH ligands than CG5911a, the latter is more discriminating between ETH1 and ETH2.

ETH receptors were subsequently sequenced and pharmacologically characterized in the moth, *M. sexta*.[9] As in *Drosophila*, two receptor subtypes (ETHR-A, ETHR-B) are produced through alternative splicing of 3′ exons. Both receptor subtypes are specific for ETH peptides; PETH and ETH show EC_{50} values for ETHR-A of 33 and 17 nM. For ETHR-B, PETH and ETH show EC_{50} values of 10 and 30 nM, respectively. Thus, unlike their *Drosophila* orthologs, *Manduca* ETH receptors have similar sensitivity to ETH ligands.

ETH receptors have been cloned and expressed in the yellow fever mosquito, *A. aegypti*.[3] As previously reported for *Drosophila* and *Manduca*, *Aedes* receptors occur as two subtypes (AeaETHR-A and AeaETHR-B) by alternative splicing of 3′ exons. Pharmacological profiling of the mosquito receptors revealed that, like the *Manduca* receptors, both receptor subtypes have comparable sensitivities to ETH ligands.

Expression of ETH receptors in *Manduca* and *Aedes* coincides with the surge of ecdysteroids that initiates molting. This association supports previous evidence for a causal relationship between ecdysteroid elevation and onset of sensitivity of the CNS to ETH in *Manduca*.[35]

ETHR-A receptors are expressed in discrete sets of central neurons in moths and flies. The majority of these are "peptidergic ensembles" that release peptides involved in scheduling of successive steps of the ecdysis behavioral sequence (see below). For example, subsets of ETH receptor neurons in *Drosophila* release FMRFamide, kinin, or eclosion hormone. Other ensembles co-release myoinhibitory peptides (MIPs), CCAP, and bursicon. In *Manduca*, one ensemble co-releases kinin and CRF-like diuretic hormone, whereas another releases CCAP, MIPs, sNPF and MIPs, or calcitonin-like DH31 and MIPs.

ETH receptors have been found widely in the Insecta using *in silico* screening[17] (see the chapter in the Peptide Biosynthesis/Processing section of the book). This approach also has led to their discovery in representative species from the Crustacea (Daphnia) and Arachnida (*Ixodes*). Interestingly, ETH receptor subtypes in *Ixodes* are predicted to arise through the expression of two distinct genes. This differs from all other species examined, where subtype diversity occurs through alternative splicing of pre-RNAs.

Biological Actions

ETHs released into the hemocoel by Inka cells act directly on the CNS to trigger a behavioral sequence culminating in ecdysis of the old cuticle. It is remarkable that ETH signaling mediates ecdysis in insects with widely divergent body plans. Even though the specific motor patterns may be different as

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a result, the signaling system and the behaviors seem to be conserved over millions of years.

Injection of MasETH into pharate *M. sexta* larvae initiates preecdysis contractions within minutes. The animal switches to ecdysis contractions after about one hour.[32] The peptide also elicits patterned motor output from the isolated CNS, the parameters of which (e.g., burst frequency, burst duration) correspond precisely to those governing contractions in the whole animal. This is known as "fictive" behavior, because although the pattern of motor output corresponds to the behavior, muscles have been removed and one can record only a "fictional" behavior. These observations indicate that the peptide acts directly on the CNS to elicit centrally patterned preecdysis and ecdysis behaviors. By contrast, injection of PETH into pharate *Manduca* larvae elicits preecdysis only. Indeed, the action of PETH helped to define two preecdysis behaviors: preecdysis I and preecdysis II.[32] Injection of PETH alone triggers preecdysis I only; thereafter, the animal ceases all contractions. Subsequent injection of ETH triggers preecdysis II followed by ecdysis behavior. Injection of a naive pharate larva with MasETH triggers simultaneously both preecdysis I and preecdysis II, followed by ecdysis behavior. Observations of natural behavior reveal that the animal begins the behavioral sequence with preecdysis, and 20 min later begins preecdysis II contractions. Both behaviors continue in parallel for the next 40 min, after which a transition to ecdysis behavior occurs. Therefore, it seems that the action of PETH dominates during the first 20 min of the ecdysis behavioral sequence. This may be explained by the observation that the concentration of PETH in the hemolymph is somewhat higher than that of ETH during early preecdysis.[32]

Injection of DrmETH1 into *Drosophila* larvae elicits the entire ecdysis behavioral sequence.[2,10] These steps include tracheal collapse and inflation, preecdysis behaviors consisting of anterior–posterior contractions and squeezing waves, and peristaltic ecdysis contractions. In contrast, ETH2 injection triggers tracheal collapse and inflation and ecdysis, but fails to elicit preecdysis behaviors. Deletion of the gene encoding *Drosophila* ETHs causes failure of successful ecdysis and lethality.[10] However, null mutants can be rescued by injection of either ETH1 or ETH2. In pharate adults, injection of ETH1 (>1 fmol) or ETH2 (>10 fmol) triggers the entire eclosion behavioral sequence, although ETH2 is less potent than ETH1. Steps in adult eclosion include head inflation (preeclosion), forward head thrusts (onset of ecdysis), bilateral, alternating contractions of the thorax, and strong peristaltic abdominal contractions pushing the body forward.

ETH controls similar behaviors associated with ecdysis or eclosion in *B. mori*, beetles, honeybee, and mosquito; this material is summarized in a recent review.[29]

CORAZONIN

Discovery

Corazonin, an undecapeptide blocked at both N- and C-termini, was first isolated from corpora cardiaca of the cockroach, *Periplaneta americana*.[24] The peptide is named after the Spanish word "corazon" or "heart," because of its signature cardioacceleratory activity in the cockroach. The identical peptide subsequently was identified in other cockroaches, crickets, and moths. A variant of corazonin, His(7)-corazonin, was isolated from the grasshopper *Schistocerca americana*, and subsequently from many other orthopterans, including *Schistocerca gregaria*, *Locusta migratoria*, and the stick insect, *Carousius morosus*. Additional isoforms have been identified in honeybees (Thr^4, His^7-corazonin), crane flies (Gln^{10}-corazonin), bumblebees (Tyr^3, Gln^7, Gln^{10}-corazonin), and in the new insect order Mantophasmatodea (His^4, Gln^7-corazonin).[13] The amino acid sequence of corazonin is somewhat related to adipokinetic hormone and red pigment concentrating hormone of crustaceans, suggesting a common ancestry.

Structure of the Precursor mRNA/Gene and Peptide Processing

The corazonin gene has been characterized in *D. melanogaster* (CG3302) and other *Drosophila* species, including *Drosophila virilis*, *Drosophila simulans*, *and Drosophila erecta*.[1] The cDNA encoding the precursor also has been characterized in the waxmoth, *G. mellonella* and in *B. mori*.[16] The precursor consists of a signal sequence, a single copy of the 11-mer corazonin peptide, and a corazonin-associated polypeptide (CRZ-AP or CAP) of approximately 120 amino acids in flies and a peptide of 80 amino acids in the waxmoth (Fig. 2). The genomic sequence indicates the presence of an intron in the region corresponding to the *D. melanogaster* CAP coding region. A second intron occurs in the 5′ UTR region of *D. virilis*. Although the CRZ sequence is highly conserved in all species examined, the CAP sequence is highly variable and hence seems to be rapidly evolving.

Distribution of the mRNA and Peptides

Corazonin mRNA and the peptide itself are confined to the central nervous system in all insects examined. The pattern of cellular expression depends on the particular insect and is stage dependent. In the cockroach *P. americana*, corazonin-like immunoreactivity (CRZ-IR) is found in 10 lateral neurosecretory cells of the brain, which project to release sites in the ipsilateral lobe of the corpus cardiacum. Bilaterally paired CRZ-IR neurons occur in all neuromeres of the ventral ganglia, in a DUM cell of the subesophageal ganglion,

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an interneuron in the optic lobes, and additional cells at the base of optic lobes, as well as in the tritocerebrum and deutocerebrum.[27] In the last larval instar of the waxmoth, *G. mellonella*, *in situ* hybridization detected mRNA in four pairs of brain lateral neurosecretory neurons. These same cells showed CRZ-IR, verifying the presence of both mRNA and peptide in the same cells. No other cells in the CNS were observed. Roller et al.[15] surveyed 6 orders of insects for CRZ-IR, and found central neurons, in both the brain and ventral nerve cord of most insects, except for Coleoptera and the albino strain of the grasshopper, *L. migratoria*. Extracts of ganglia displaying CRZ-IR induced melanization in the albino grasshopper, providing further evidence for the widespread presence of the peptide in the CNS.

In *Drosophila*, *in situ* hybridization showed a pattern of corazonin expression in central neurons that changes during the life history of the animal. During larval life, mRNA is present in 1 pair of brain dorsomedial neurons and 3 pairs of dorsolateral neurons. In addition, 8 bilateral pairs are observed in the ventral nerve cord.[1] In the adult, hybridization signals occurred in 6–8 pairs of brain dorsolateral neurons and widely on anterior surface in the optic lobe medulla, but none in the dorsomedial region or in the ventral nerve cord corresponding to cells previously observed in larvae. Despite the presence of mRNA in optic lobe neurons, CRZ-IR is absent. It has been suggested that the absence of immunoreactivity may result from either too rapid turnover of peptides or translational silencing.[1]

Receptors

The corazonin receptor (CRZR) in *D. melanogaster* is a GPCR encoded by the gene CG10698.[30] Phylogenetic analysis groups this receptor with adipokinetic hormone (AKH) and vasopressin receptors. Expressed either in *Xenopus* oocytes or mammalian CHO cells, CG10698 is highly selective for corazonin, whose EC_{50} is in the low nanomolar range. Other ligands tested at up to 10 μM concentration (including AKH) were inactive.

Corazonin receptors (CRZRs) in *M. sexta*[8] and *B. mori* are involved in initiation of ecdysis. Northern blots show CRZR expression in the CNS and epitracheal glands, but not in muscle, epidermis, or trachea. *In situ* hybridization revealed very strong and specific expression of CRZR that is restricted to Inka cells only 3–4 h before ecdysis in *B. mori*.[29] The *M. sexta* receptor is highly sensitive and selective for corazonin when expressed in *Xenopus* oocytes (EC_{50} = 200 pM) or CHO cells (EC_{50} = 75 pM). Inka cells respond to corazonin (≥10 pM) by releasing low levels of ETH. Interestingly, elevated concentrations of corazonin (>1 nM) suppress ETH release from Inka cells, indicating the likelihood that receptor desensitization plays a role in cellular responses. Such densensitization may place limits on the amount of ETH release, ensuring that only low levels are released in the initial stages of the ecdysis behavioral sequence.

Biological Actions

Corazonin is a potent cardioaccelerator in *P. americana* and in a small number of related cockroach species, but not in other insects. His^7-corazonin of locusts triggers melanization, and hence is also known as a "pigmentotropin."[23,25] The melanization activity of His^7-corazonin is confined to locusts. Corazonin signaling may be involved in responses to nutritional stress.[26]

Corazonin is involved in developmental events related to molting, ecdysis, and metamorphosis. For example, the peptide prolongs spinning behavior in fifth instar *B. mori* larvae, and delays adult development during the pupal stage in the same species.[22] As mentioned above, corazonin injection into moth larvae elicits ecdysis behaviors. Injection of corazonin (10 pmol) into pharate fifth instar larval *Manduca* induces preecdysis behavior within approximately 40 min through release of PETH and ETH. Subsequent release of eclosion hormone leads to depletion of PETH and ETH from Inka cells and ecdysis onset. Before natural ecdysis, the peptide was detected in the hemolymph of *Manduca* larvae just before onset of preecdysis behavior. Exposure of isolated Inka cells to corazonin leads to low level release of ETHs. It thus seems probable that, in *Manduca*, corazonin is involved in initiating the early stages of the ecdysis behavioral sequence by causing ETH release from Inka cells.[8]

Finally, corazonin is implicated in circadian rhythms and diapause. The peptide is colocalized with the PER gene product in lateral neurosecretory 1a cells of *Manduca*,[28] and these cells are implicated in regulation of circadian rhythms.[19] Ablation of these cells in *Manduca* suppresses diapause during short days, indicating their possible role in measuring day length.[20]

PROTHORACICOTROPIC HORMONE (PTTH)

Discovery

Stefan Kopec's demonstration of a brain hormone that initiates molting in insects was a propitious event that launched the field of insect endocrinology.[29] The brain hormone constituted the first definitive example of hormonal signaling in insects, yet it took >65 years for its molecular structure to be defined as PTTH. The hormone's molecular size was estimated initially to be approximately 25 kDa, and PTTH-like bioactivity was located in two pairs of lateral protocerebral neurosecretory cells projecting to release sites in the

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corpora allata.[21] Ultimate determination of molecular identity came through a combination of direct peptide sequencing and cDNA precursor analysis. Sequencing of the peptide was made possible by tissue preparation on a heroic scale. Initially, 500,000 silkmoth heads were processed to obtain the N-terminal 13 amino acids; subsequent processing of 1.8 million *Bombyx* heads yielded virtually the entire peptide sequence and the realization that the hormone is a dimer.[5,6] For elucidation of the precursor sequence, polyclonal antisera directed against the N-terminus were used to screen a cDNA expression library. The amino acid sequence of the predicted precursor was in complete agreement with that obtained by Kataoka et al.[7] (Genbank accession number D90082). *Bombyx* PTTH was thus defined as a homodimer consisting of identical 109 amino acid subunits. Nucleotide probes based on the *Bombyx* sequence were used to determine the primary structures of PTTH in many other lepidopteran species, including *Samia cynthia*, *Antheraea pernyi*, *Hyalophora cecropia*, and *Spodoptera exigua*. cDNAs encoding related peptide sequences have been determined in *Drosophila*, *Anopheles*, and *Tribolium*.[21] In *Drosophila*, PTTH and the protein encoded by *trunk* share structural similarities and a common receptor (see the section on Receptors below).

Structure of the Precursor mRNA/gene and Peptide Processing

The PTTH gene of *B. mori* consists of 5 exons that encode a 224-amino-acid precursor protein with three proteolytic cleavage sites (Fig. 2). Functions for the N-terminal peptides inferred from the precursor (p2K, p6K) have not been established. The C-terminal 109-amino-acid fragment of the precursor is processed to form the biologically relevant monomeric subunit, which contains seven cysteines (Fig. 2). Homodimerization of these subunits via a single disulfide bond formed between Cys15 of each monomer yields the active hormone. Disulfide bonds within each monomer are formed according to the following pattern: Cys17–Cys54, Cys40–Cys96, and Cys48–Cys98. Interestingly, the 109-amino-acid monomer is about 50% as active as the native dimeric hormone. Glycosylation accounts for the disparity between the predicted (13 kDa) and observed (17 kDa) molecular weights of the monomer, and also contributes to full activity of the native hormone, because the expression product from bacteria without glycosylation has lower biological activity than that of the native hormone. Two putative peptides of 2 and 6 kDa predicted from sequences upstream of the 109-mer fragment are flanked by canonical proteolytic cleavage sites. There is no evidence yet regarding possible functions for these peptides. PTTHs cloned and characterized from other moth species (*Samia*, *Hyalophora*, *Antheraea*, *Manduca*) and nonlepidopteran species (*Drosophila*, *Anopheles*) are summarized elsewhere.[18,21]

Distribution of the mRNA and Peptides

Expression and synthesis of PTTH are confined to two pairs of lateral neurosecretory cells in the brain. This was originally shown in *M. sexta* by single cell dissection and bioassay[21] and was substantiated in many species of moth (*B. mori*, *Antheraea polyphemus*, *M. sexta*) by *in situ* hybridization and immunohistochemical staining. Possibly homologous cells were shown in the *Drosophila* brain[33] and in the subesophageal and ventral ganglia of *Drosophila*, but these have not been confirmed by *in situ* hybridization or bioassay.

Receptors

The PTTH receptor of *Drosophila* is a tyrosine kinase encoded by the gene "torso."[14] This multifunctional receptor functions during early embryogenesis to specify correct patterning of anterior and posterior structures. During this period of development, the presumed ligand for *torso* is Trunk (trk), a dimeric protein that is structurally related to PTTH. After embryogenesis, trk disappears and PTTH functions as the ligand of *torso* to regulate ecdysone synthesis. RNA silencing of *torso* phenocopies ablation of PTTH neurons, that is, both manipulations lead to a prolonged third larval instar and increased pupal size.

Expression of *torso* is confined to the prothoracic gland during larval stages. Activation of *torso* leads to phosphorylation of extracellular signal-regulated kinase (ERK) via the MAPK pathway. ERK knockouts also phenocopy loss of PTTH.[14]

Biological Actions

The sole tissue target of PTTH is the prothoracic gland, which responds to the hormone at low to subnanomolar concentrations by elevating synthesis of ecdysteroid. Consistent with this function, hemolymph levels of PTTH increase immediately before ecdysteroid pulses, which initiate molting at each stage of development.[18]

REFERENCES

1. Choi YJ, Lee G, Hall JC, Park JH. Comparative analysis of Corazonin-encoding genes (Crzs) in Drosophila species and functional insights into Crz-expressing neurons. *J Comp Neurol* 2005;**482**:372–85.
2. Clark AC, del Campo ML, Ewer J. Neuroendocrine control of larval ecdysis behavior in Drosophila: complex regulation by partially redundant neuropeptides. *J Neurosci* 2004;**24**:4283–92.
3. Dai L, Adams ME. Ecdysis triggering hormone signaling in the yellow fever mosquito *Aedes aegypti*. *Gen Comp Endocrinol* 2009;**162**:43–51.
4. Daubnerova I, Roller L, Zitnan D. Transgenesis approaches for functional analysis of peptidergic cells in the silkworm *Bombyx mori*. *Gen Comp Endocrinol* 2009;**162**:36–42.

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5. Kataoka H, Nagasawa H, Isogai A, Ishizaki H, Suzuki A. Prothoracicotropic hormone of the silkworm, *Bombyx mori*: amino acid sequence and dimeric structure. *Agric Biol Chem* 1991;**55**:73–86.
6. Kataoka H, Troetschler RG, Kramer SJ, Cesarin BJ, Schooley DA. Isolation and primary structure of the eclosion hormone of the tobacco hornworm, *Manduca sexta*. *Biochem Biophys Res Commun* 1987;**146**:746–50.
7. Kawakami A, Kataoka H, Oka T, Mizoguchi A, Kimura-Kawakami M, Adachi T, et al. Molecular cloning of the *Bombyx mori* prothoracicotropic hormone. *Science* 1990;**247**:1333–5.
8. Kim YJ, Spalovska-Valachova I, Cho K-H, Zitnanova I, Park Y, Adams ME, et al. Corazonin receptor signaling in ecdysis initiation. *Proc Natl Acad Sci U S A* 2004;**101**:6704–9.
9. Kim YJ, Zitnan D, Cho KH, Schooley DA, Mizoguchi A, Adams ME. Central peptidergic ensembles associated with organization of an innate behavior. *Proc Natl Acad Sci USA* 2006;**103**:14,211–6.
10. Park Y, Filippov V, Gill SS, Adams ME. Deletion of the ecdysis-triggering hormone gene leads to lethal ecdysis deficiency. *Development* 2002;**129**:493–503.
11. Park Y, Kim YJ, Dupriez V, Adams ME. Two subtypes of ecdysis-triggering hormone receptor in *Drosophila melanogaster*. *J Biol Chem* 2003;**278**:17710–5.
12. Park Y, Zitnan D, Gill SS, Adams ME. Molecular cloning and biological activity of ecdysis-triggering hormones in *Drosophila melanogaster*. *FEBS Lett* 1999;**463**:133–8.
13. Predel R, Neupert S, Russell WK, Scheibner O, Nachman RJ. Corazonin in insects. *Peptides* 2007;**28**:3–10.
14. Rewitz KF, Yamanaka N, Gilbert LI, O'Connor MB. The insect neuropeptide PTTH activates receptor tyrosine kinase torso to initiate metamorphosis. *Science* 2009;**326**:1403–5.
15. Roller L, Tanaka Y, Tanaka S. Corazonin and corazonin-like substances in the central nervous system of the Pterygote and Apterygote insects. *Cell Tissue Res* 2003;**312**:393–406.
16. Roller L, Yamanaka N, Watanabe K, Daubnerova I, Zitnan D, Kataoka H, et al. The unique evolution of neuropeptide genes in the silkworm *Bombyx mori*. *Insect Biochem Mol Biol* 2008;**38**: 1147–57.
17. Roller L, Zitnanova I, Dai L, Simo L, Park Y, Satake H, et al. Ecdysis triggering hormone signaling in arthropods. *Peptides* 2010;**31**:429–41.
18. Rybczynski R. Prothoracicotropic hormone. In: Gilbert LI, Iatrou K, Gill SS, editors. *Comprehensive molecular insect science*. Oxford: Elsevier; 2005. p. 61–123.
19. Sauman I, Reppert SM. Circadian clock neurons in the silkmoth *Antheraea pernyi*: novel mechanisms of period protein regulation. *Neuron* 1996;**17**:889–900.
20. Shiga S, Davis NT, Hildebrand JG. Role of neurosecretory cells in the photoperiodic induction of pupal diapause of the tobacco hornworm *Manduca sexta*. *J Comp Neurol* 2003;**462**:275–85.
21. Smith W, Rybczynski R. Prothoracicotropic hormone. In: Gilbert LI, editor. *Insect endocrinology*. San Diego: Academic Press; 2012. p. 1–62.
22. Tanaka Y, Hua Y, Roller L, Tanaka S. Corazonin reduces the spinning rate in the silkworm, *Bombyx mori*. *J Insect Physiol* 2002;**48**:707–14.
23. Tawfik AI, Tanaka S, De Loof A, Schoofs L, Baggerman G, Waelkens E, et al. Identification of the gregarization-associated dark-pigmentotropin in locusts through an albino mutant. *Proc Natl Acad Sci USA* 1999;**96**:7083–7.
24. Veenstra JA. Isolation and structure of corazonin, a cardioactive peptide from the American cockroach. *FEBS Lett* 1989;**250**:231–4.
25. Veenstra JA. Presence of corazonin in three insect species, and isolation and identification of [His7]corazonin from *Schistocerca americana*. *Peptides* 1991;**12**:1285–9.
26. Veenstra JA. Does corazonin signal nutritional stress in insects? *Insect Biochem Mol Biol* 2009;**39**:755–62.
27. Veenstra JA, Davis NT. Localization of corazonin in the nervous system of the cockroach *Periplaneta americana*. *Cell Tissue Res* 1993;**274**:57–64.
28. Wise S, Davis NT, Tyndale E, Noveral J, Folwell MG, Bedian V, et al. Neuroanatomical studies of period gene expression in the hawkmoth, *Manduca sexta*. *J Comp Neurol* 2002;**447**:366–80.
29. Zitnan D, Adams ME. Neuroendocrine regulation of ecdysis. In: Gilbert LI, editor. *Insect Endocrinology*. San Diego: Academic Press; 2012. p. 253–309.
30. Zitnan D, Kim YJ, Zitnanova I, Roller L, Adams ME. Complex steroid–peptide–receptor cascade controls insect ecdysis. *Gen Comp Endocrinol* 2007;**153**:88–96.
31. Zitnan D, Kingan TG, Hermesman J, Adams ME. Identification of ecdysis-triggering hormone from an epitracheal endocrine system. *Science* 1996;**271**:88–91.
32. Zitnan D, Ross LS, Zitnanova I, Hermesman JL, Gill SS, Adams ME. Steroid induction of a peptide hormone gene leads to orchestration of a defined behavioral sequence. *Neuron* 1999;**23**:523–35.
33. Zitnan D, Sehnal F, Bryant PJ. Neurons producing specific neuropeptides in the central nervous system of normal and pupariation-delayed Drosophila. *Dev Biol* 1993;**156**:117–35.
34. Zitnan D, Zitnanova I, Spalovska I, Takac P, Park Y, Adams ME. Conservation of ecdysis-triggering hormone signalling in insects. *J Exp Biol* 2003;**206**:1275–89.
35. Zitnanova I, Adams ME, Zitnan D. Dual ecdysteroid action on the epitracheal glands and central nervous system preceding ecdysis of *Manduca sexta*. *J Exp Biol* 2001;**204**:3483–95.

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Chapter 34

Diuretic/Antidiuretic

Geoffrey M. Coast and David A. Schooley

ABSTRACT

A diverse group of peptides are implicated in the control of Malpighian tubule and hindgut function in insects. They are the products of neurosecretory cells in the brain and ventral nerve cord ganglia and are present in identified neurohemal structures from which they can be released into the circulation to function as diuretic and/or antidiuretic hormones. Receptors are known for most of them, and they couple to cAMP, cGMP, or Ca^{2+}-signaling pathways to regulate a variety of transport processes.

DISCOVERY

In the excretory process of insects, Malpighian (renal) tubules (MT) generate a flow of primary urine that is subsequently modified by reabsorptive and secretory processes in the hindgut (ileum and rectum). Diuretic hormones (DHs) accelerate primary urine production, whereas antidiuretic hormones either stimulate fluid reabsorption in the hindgut or reduce MT secretion; these are comprehensively reviewed elsewhere.[6,32]

Corticotropin-releasing Factor (CRF)-Like Peptides

To date, 34 CRF-related DHs are known from 26 species of insects representing 8 orders, plus two acarines and one crustacean. They comprise paralogous subfamilies of 'long' and 'short' peptides as exemplified by two of the CRF-related DHs from the tobacco hornworm *Manduca sexta*, Manse-DH_{41}[20] and Manse-DH_{30}[4] (Fig. 1), both of which act *via* cAMP to increase MT secretion. Manse-DH_{41} has 41% sequence identity with sauvagine, but somewhat less with CRF, and has only 9 residues identical to Manse-DH_{30}. *Bombyx mori* contains three paralogs, versus four paralogs in the vertebrate CRF superfamily.[32] "Short" paralogs are present only in lepidopteran, coleopteran, and dipteran (D.A. Schooley and D.A. Jensen, unpublished) insects. The CRF-related DH from beetles are mostly nonamidated (*Tenebrio molitor*-DH_{47} and Tenmo-DH_{37})[32] and Trica-DH_{37} (*Tribolium castaneum*), whereas Trica-DH_{47} is amidated.[32]

Calcitonin (CT)-Like Peptides

The first CT-like DH was identified in the Pacific beetle cockroach, *Diploptera punctata*.[13] Dippu-DH_{31} (Fig. 1) has low sequence identity to vertebrate CT, but it shares the unusual C-terminal Pro-Gly-NH_2. The 19 CT-like DHs that have been identified comprise a highly conserved family present in 15 species of insects, 3 crustaceans, and 2 acarines.[32]

Kinins

Fifty kinins are known from 18 species of insect and one acarine, and frequently (but not always) multiple isoforms are present. The first (Leuma-Ks; Fig. 1) were identified from the Madeira cockroach (*Leucophaea maderae*) by their myostimulatory action on the hindgut,[15] but were later shown to be potent diuretics. There are no kinins encoded by the genome of *T. castaneum*,[32] or hymenopteran insects (D.A. Schooley and Y. Park, unpublished).

CAP_{2b}

Manse-CAP_{2b} (Fig. 1) was first identified as a cardioacceleratory peptide (CAP)[16] but was later shown to stimulate secretion by MT from the fruit fly *Drosophila melanogaster*. Other CAP_{2b} peptides have been identified by mass spectral analysis of tissue extracts and by mining genomic databases. In the blood sucking bug *Rhodnius prolixus*, the yellow fever mosquito *Aedes aegypti*, and in the xeric insect *T. molitor*, CAP_{2b} has antidiuretic activity.[32]

Arginine Vasopressin (AVP)-like DH

An AVP-like peptide (Fig. 1) was first identified in the migratory locust (*Locusta migratoria*)[30] and the antiparallel homodimer (F2; Locmi-AVP-like DH; AVPL-DH) stimulates secretion by a semiisolated MT preparation that includes the ampullae attached to a short segment of

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00034-8
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CRF-related DH

Manse-DH        RMPSLSIDLPMSVLRQKLSLEKERKVHALRAAANRNFLNDI-NH2
Manse-DP2       ---SFSVNPAVDILQH-------RYMEKVAQ-NNRNNLNRV-NH2

CT-like DH

Dippu-DH31      GLDLGLSRGFSGSQAAKHLMGLAAANYAGGP-NH2

Kinin

Leuma-KI        DPAFNSWG-NH2

CAP2b

Manse-CAP2b     pQLYAFPRV-NH2

AVP-like peptide

Locmi-AVPL-DH   CLITNCPRG-NH2
                CLITNCPRG-NH2

ADFs

Tenmo-ADFa      VVNTPGHAVSYHVY-COOH
Tenmo-ADFb      YDDGSYKPHIYGF-COOH

ITP

Schgr-ITP       SFFDIQCKGVYDKSIFARLDRICEDCYNLFREPQLHSLC
                RSDCFKSPYFKGCLQALLLIDEEEKFNQMVEIL-NH2
Schgr-ITPL      SFFDIQCKGVYDKSIFARLDRICEDCYNLFREPQLHSLC
                RKDCFTSDYFKGCIDVLLLQDDMDKIQSWIKQIHGAEPGV-COOH
```

FIGURE 1 Sequences of representative diuretic and antidiuretic peptides. In each case, the sequence shown is that of the first member of the family to be described.

gut.[32] A peptide with a sequence identical to the Locmi-AVPL monomer (F1) is encoded in the genome of the red flour beetle (*Tribolium castaneum*), but homologous sequences are absent from the published genomes of dipteran and lepidopteran insects, and the honeybee (*Apis mellifera*), although an AVPL sequence is present in the genome of the jewel wasp (*Nasonia vitripennis*).[1]

Antidiuretic Factors (ADFs)

Tenmo-ADFa and Tenmo-ADFb (Fig. 1) were identified from head extracts of *T. molitor* pupae on the basis of their ability to increase cGMP production by MT from adult beetles.[11,12] Both are extremely potent in reducing secretion by the free portion of larval MT with an EC_{50} value of 10 fM for Tenmo-ADFa. To date, they have not been identified from any other insect, but Tenmo-ADFa is also a potent antidiuretic in *A. aegypti*.[23] Interestingly, Tenmo-ADFb has diuretic activity in the house cricket, *Acheta domesticus*.[32]

Ion Transport Peptide (ITP)

Schgr-ITP (Fig. 1) is the only identified ADH that stimulates fluid reabsorption from the insect hindgut and was isolated from the locust, *Schistocerca gregaria*, on the basis of its ability to increase electrogenic Cl^- transport across the ileum.[2] A cDNA encoding the prepropeptide was cloned and the C-terminal amidated peptide has 39–42% sequence similarity to crustacean hyperglycemic hormone (CHH) from the shore crab, *Carcinus maenas*.[24] A splice variant of Schgr-ITP encodes an Schgr-ITP-like peptide (ITP-L) with a nonamidated C-terminus (Fig. 1), which has no currently known biological function. cDNAs encoding ITP and ITP-L have been cloned from *M. sexta*, *B. mori*, and *A. aegypti*.[8] Similarly, in *Drosophila melanogaster* and *Tribolium castaneum*, ITP and two distinct ITP-L peptides have been identified.[32] Only the *T. castaneum* ITP is not amidated at the C-terminus.

STRUCTURE OF PRECURSOR mRNAs AND GENES

CRF-Related DH

The CRF-DH gene (*Dh*) of *D. melanogaster* (CG8348) is located in region 85E on chromosome 3R. It comprises four exons, with the prepropeptide (Fig. 2) encoded by exons 2, 3, and 4.[5] The sequence N– and C-terminal to the cleavage signals of Drome-DH_{44} resemble no known

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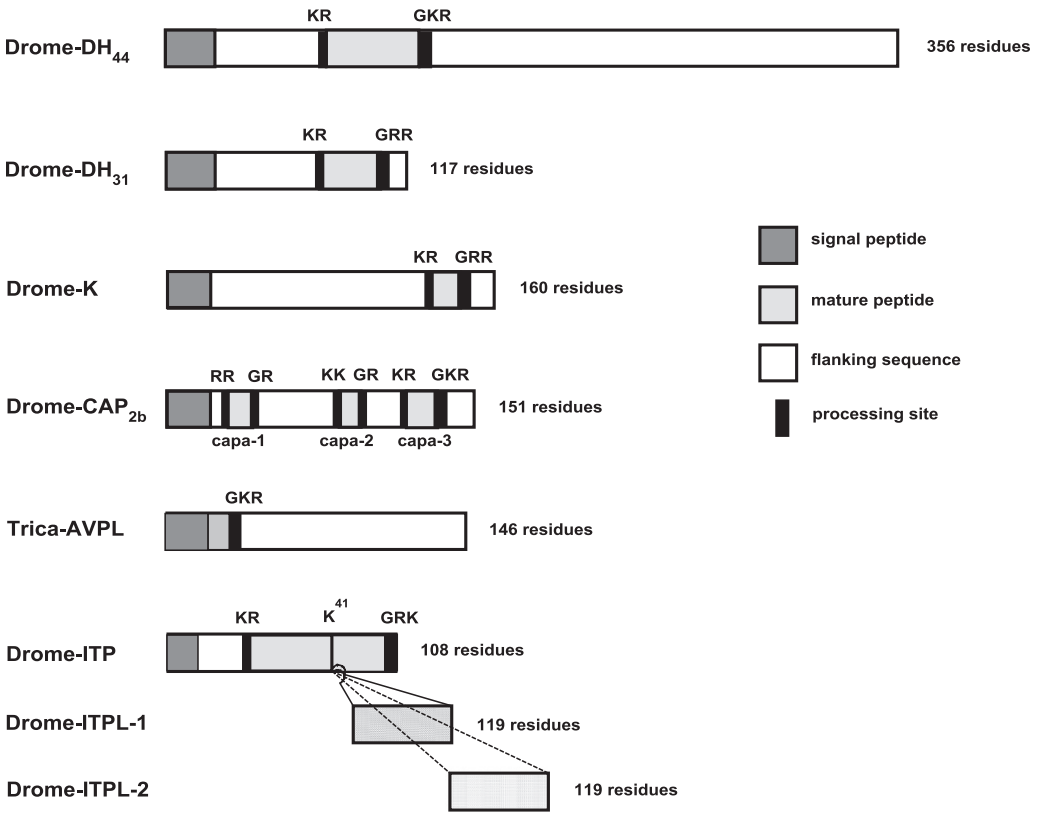

FIGURE 2 Schematic representations (approximately to scale) of precursor structures and processing sites for diuretic and antidiuretic peptides that are encoded by either the *D. melanogaster* genome or, for AVPL, the *T. castaneum* genome. The alternative C-termini of the precursors for Drome-ITP, ITPL1, and ITPL2 are shown as hatched and stippled boxes, respectively.

peptide. In *B. mori*, a single gene encodes three different peptides having 34, 41, and 45 residues; the latter is a paralog of Drome-DH_{44}, whereas the former are paralogs of Manse-DH_{30} and Manse-DH_{41}.[32] All three *B. mori* precursor peptides have 70 identical residues at the N-terminus.

CT-Like DH

The CT-like DH gene (*Dh31*) of *D. melanogaster* (CG13094) is located in region 29D2–3 on chromosome 2L and comprises five exons. The prepropeptide (Fig. 2) is encoded by exons 3, 4, and 5. The flanking sequences have no similarity to other known peptides.

Kinins

The kinin gene (*pp*; *leucokinin*) of *D. melanogaster* (CG13480) is located in region 70E2 of chromosome 3L. The prepropeptide (Fig. 2) is encoded by a single exon and contains one copy of Drome-K. By contrast, three kinins (Aedae-K-1, Aedae-K-2, and Aedae-K-3) are encoded on the corresponding gene of *A. aegypti*,[32] and six in *R. prolixus*.[33]

CAP_{2b}

The *capability* (*capa*) gene of *D. melanogaster* (CG15520) is located in region 99C6 of chromosome 3R. The prepropeptide (Fig. 2) is encoded by three exons, with exon 2 encoding two CAP_{2b} peptides (capa-1 and capa-2), and exon 3 encoding a peptide of the pyrokinin family.

AVP-Like DH

An arginine vasopressin-like (*avpl*) peptide gene is present in linkage group 8 of the *T. castaneum* genome. It comprises three exons and two small introns[1] and encodes a peptide that is identical to the Locmi-AVP-like peptide. Downstream of AVPL is a sequence that closely resembles mammalian neurophysin, which is required for the proper processing of AVP in mammals.[1] There is no gene homologous to *T. castaneum avpl* in the genome of *D. melanogaster*.

ADFs

The genome of *T. castaneum* contains five genes encoding Tenmo-ADFb and four closely related peptides, but none encoding Tenmo-ADFa.[32]

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ITP

The *ion transport peptide* (*itp*) gene of *D. melanogaster* (CG13586) encodes three different transcripts giving rise to Drome-ITP, Drome-ITPL1 and ITPL2 *via* alternative splicing of five exons.[9] Exons one and two are common to all three mRNAs and encode an unusually short (15 residues) signal peptide, a flanking sequence followed by a processing site (KR), and the first 41 residues of ITP, ITPL1, and ITPL2. The 46 residue nonamidated C-termini of ITPL1 and ITPL2 are encoded by exons 3 and 4, respectively, whereas the 32-residue C-terminus of ITP followed by an amidation processing site (GRK) is encoded by exon 5. The ITP genes in *M. sexta*, *B. mori*, and *A. aegypti* also involve three exons, but they differ in organization from that of *D. melanogaster*.[8]

DISTRIBUTION OF mRNA AND PEPTIDES

CRF-Related Peptides

In *D. melanogaster*, the *Dh* transcript is found exclusively in three bilateral pairs of cells in the *pars intercerebralis* (PI) of the larval and adult brain.[5] Drome-DH_{44} immunoreactivity (IR) is present in the cells and in their axons projecting to the retrocerebral complex. In other insects, CRF-DH-IR material is present in median neurosecretory cells (MNCs) of the brain, in posterior-lateral neurosecretory cells (NSCs) of the abdominal ganglia, and in associated neurohemal organs, corpora cardiaca (CC), perivisceral organs (PVO), and abdominal nerves.[6] CRF-DH-IR material is also present in the endocrine cells of the midgut and MT ampullae.

CT-Like Peptides

CT-like-IR material is present in lateral NSCs of the brain in *R. prolixus* and in axons projecting to the CC and dorsal vessel (heart).[34] In the mesothoracic ganglionic mass, CT-like-IR is present in bilaterally paired NSCs and in five dorsal unpaired medial (DUM) neurons, which also contain serotonin, an identified DH. Axons from CT-like-IR cells extend into abdominal and trunk nerves that form neurohemal release sites.

Kinins

Kinin-like-IR material is widely distributed in the CNS in both interneurons and NSCs. Kinin-IR is present in MNCs of the PI in *L. maderae*, and in axons to the CC,[27] but not in MNCs of locusts, houseflies, and honeybees. Kinin-IR is, however, consistently present in posterior-lateral NSCs of abdominal ganglia 1–7 and associated neurohemal areas.[6] These NSCs colocalize CRF-DH-IR.

CAP_{2b}

The *D. melanogaster* CAP_{2b} prepropeptide is present in three pairs of ventral NSCs in abdominal neuromeres, with neurohemal release sites on abdominal median nerves of larvae and on the dorsal surface of the thoracico-abdominal ganglion of adults.[21] It is also present in a pair of NSCs in the labial neuromere with axons projecting to the brain and retrocerebral complex.

AVP-Like DH

Locmi-AVPL-IR material is present in a pair of NSCs in the subesophageal ganglion (SEG) of *L. migratoria*.[6] Each NSC has a bifurcating axon with the anterior-directed branch extending into the brain and optic lobes, while the posterior-directed branch gives rise to arborizations in each VNC ganglion and to plexuses in peripheral nerves arising from the VNC. Blebbed processes in the proximal part of these nerves might form neurohemal release sites, although they are not closely associated with the nerve sheath in *L. migratoria*.[6] A similar pair of NSCs is present in the SEG of *T. castaneum* and the presence of AVPL has been confirmed by *in situ* hybridization.[1]

ADFs

Nothing is known of the distribution of Tenmo-ADFa, but Tenmo-ADFb-IR is present in two pairs of lateral NSCs in the protocerebrum and in another pair located anterior and lateral to these. Axons from these cells project to ADFb-ir varicosities that might form neurohemal release sites.[12]

ITP

Schgr-ITP mRNA is expressed in the brain and CC of adult locusts,[24] and ITP-like-IR material is present in the PI and CC of *S. gregaria*.[32] There are also ITP-IR NSCs in each thoracic and abdominal PVO. Many cells in the brain and ganglia of *M. sexta* and *B. mori* express both ITP and ITP-L in all life stages examined.[8]

PEPTIDE PROCESSING

The prepropeptides of identified DHs and of ITP contain an N-terminal signal sequence of about 20 amino acid residues (only 15 residues for ITP) followed by active and inactive peptide sequences separated by some combination of two basic amino acids (Fig. 2). However, to obtain Aedae-K-2 and Drome-CAP_{2b} peptides, the propeptide must be cleaved at single Arg residues by a mono-arginyl convertase. In vertebrates, PC1/PC3 convertase, which normally cleaves at Lys-Arg, can in an appropriate context cleave at single Arg residues (see the Peptide Biosynthesis/Processing section of

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this book). With the exception of three coleopteran CRF-like DH, coleopteran ITP, and ADFs, the mature peptides are amidated and the C-terminal of the propeptide ends with a cleavage and amidation signal –Gly–Xxx–Xxx (Xxx represents Arg or Lys) or –Gly–Arg for Drome-CAP_{2b} peptides (Fig. 2).

RECEPTORS

CRF-Related Peptides

Receptors for CRF-related DH belong to the secretin-like (Family B) family of G-protein-coupled receptors (GPCRs). Two orthologs are present in *D. melanogaster* (CG8422 and CG12370), and CG8422 encodes a functional receptor (Drome-DH_{44}-R1) for Drome-DH_{44}.[18] However, Drome-DH_{44}-R1-IR material is not present in MT principal cells, which suggests CG12370 encodes a receptor (Drome-DH_{44}-R2) that mediates the diuretic activity of Drome-DH_{44}.[19] Indeed, only the CG12370 transcript is enriched in MT compared with that of whole bodies.[35] The EC_{50} for Drome-DH_{44} is >100-fold higher than that for CG8422, consistent with a currently unknown CRF-like DH serving as the CG12370 ligand.[32] A separate receptor probably exists for the short paralogs in other species, but there are no data on this. Interestingly, a CRF-binding protein is also present in insects, providing further evidence for the long evolutionary history of the CRF system.[17]

CT-Like Peptides

The *D. melanogaster* genome encodes a Family B GPCR (CG17415) related to the calcitonin receptor-like receptor (CLR) family.[19] CG17415 signals in response to Drome-DH_{31}; in common with other CLR coexpression with accessory proteins, either mammalian or *D. melanogaster* receptor component protein or with mammalian receptor activity modifying proteins, enhances the potency of signaling. In MT, Drome-DH_{31}-R is present only in principal cells, and the gene transcript is enriched 17-fold compared with that of whole bodies.[35] Drome-DH_{31}-R is present also in corazonin-expressing neurons within the CNS, which also express Drome-DH_{44}-R1.[22] Drome-DH_{31} and Drome-DH_{44} may therefore control corazonin release, possibly at the time of ecdysis.

Kinins

The kinin receptors of *D. melanogaster*[14] and *A. aegypti*[32] belong to Group III-B of Family A GPCRs, and seem to be paralogs of the true neurokinin receptors. Drome-K–R is present only in stellate cells of MT, and the gene (*lkr*) transcript is enriched 3-fold compared with that of whole bodies.[35] The receptor is present throughout the larval and adult CNS, most notably on MNCs of the PI that express Drome-DH_{44} and on their neurohemal axons and terminals in the CC. Outside the CNS, the receptor is present in testes and ovary, and these tissues respond to Drome-K with an increase in intracellular Ca^{2+}.

CAP_{2b}

CAP_{2b} peptides have a C-terminal motif (PRX-NH_2) similar to that of neuromedin U (NMU) and arginine vasopressin (AVP), and receptors related to those for NMU and AVP are encoded in the *D. melanogaster* genome. One of these (CG14575; *capaR*) responds functionally to Drome-CAP_{2b} peptides ending with PRV-NH_2, but not to other PRX-NH_2 peptides.[28] This receptor probably mediates the diuretic activity of CAP_{2b}, and the gene transcript is enriched 11-fold in MT compared with that of whole bodies.[35]

AVP-Like DH

Analysis of the *T. castaneum* genome identified a gene (*avplr*) encoding putative AVP-like peptide receptor that is related to other members of the AVP receptor group of Family A GPCRs.[1] The expressed receptor is activated by low nanomolar concentrations of F1, the monomeric AVPL, and by the antiparallel (F2) and parallel (D2) dimers, although they are 7–12 and 60–80 times less potent than F1, respectively. The expression of *avplr* mRNA is mainly confined to the CNS and, significantly, is not detectable in the MT and hindgut.

Nothing is known of the receptors for ADFs or for ITP.

STRUCTURE–ACTIVITY AND ACTIVE CONFORMATION

CRF-Related Peptides

The N-terminus is required for receptor activation, while the C-terminal two-thirds of the peptide is needed for receptor binding.[32] The C-terminal amide is also important for high affinity binding, although the nonamidated Tenmo-DH_{37} is a potent stimulant of *T. molitor* MT.[12] Manse-DH_{41} and Manse-DH_{30} may adopt a folded helix-loop-helix conformation, bringing the C-terminal amide close to the N-terminus, and the constrained analog [$Cys^{3,40}$]Manse-DH has similar efficacy and potency to Manse-DH.[32]

Kinins

The C-terminal pentapeptide is highly conserved and is the minimum sequence required for activity, but residues outside of this 'active core' may be required for high affinity binding.[32] Within the active core, Phe^1 and Trp^4 are invariant and essential for activity. The active core adopts a *cis*

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Pro type VI β-turn when bound to receptors,[26] bringing the side chains of Phe[1] and Trp[4] together on one face of the molecule where they can interact with the receptor.

CAP_{2b}

The minimum sequence for full activity in *Musca domestica* MT is the C-terminal hexapeptide (YAFPRV-NH_2), within which the critical residues appear to be Arg and Val.[25]

AVP-Like DH

There are three isoforms of the AVP-like peptide; the monomeric F1 with an intramolecular disulfide bridge, and the parallel (D2) and antiparallel (F2) dimeric forms with intermolecular disulfide bridges. In the *L. migratoria* assay using semiisolated MT, only F2 was active and was therefore called the Locmi-AVP-like DH,[30] although it does not stimulate secretion by fully isolated MT.[6] All three isoforms activate the expressed AVP-like peptide receptor, with F1 being most potent, but F1 was the only isoform with diuretic activity in an *in vivo* bioassay using adult *T. castaneum*.[1]

ITP

At the N-terminus of Schgr-ITP, Ala substitutions at positions 1, 4, 5, and 6 have no effect on activity, but Phe[2] and Phe[3] are essential for receptor activation.[29] The C-terminal Leu-NH_2 is also essential as are the six Cys residues, and disulfide bridges probably fix the distance between essential sites at the N- and C- termini.[6]

There have been no structure/activity studies with ADFs.

BIOLOGICAL ACTIONS

MT fluid secretion is coupled to ion transport, and DHs stimulate KCl/NaCl secretion into the lumen. This is driven by active proton transport across the principal cell apical membrane by a V-type ATPase, with secreted protons returning to the cell *via* cation/H^+ antiporters. Drome-DH_{44} and Drome-DH_{31} act *via* cAMP to stimulate V-ATPase activity, possibly by increasing recruitment of the V-ATPase to the apical membrane or by promoting association of the catalytic (V_1) and proton-translocating (V_0) domains. The Na^+:K^+ concentration ratio of the secreted urine is probably determined by their uptake across the basolateral membrane, and the natriuretic response of mosquito MT to Anoga-DH_{31} (the Mosquito Natriuretic Peptide) seems to result from the opening of a cAMP-dependent Na^+ channel and stimulation of Na^+–K^+–$2Cl^-$ cotransport.[3,7]

Kinins open a Cl^--selective conductance pathway making more Cl^- available for KCl/NaCl secretion into the MT lumen.[3] The binding of Drome-K to Drome-K–R on stellate cells activates phospholipase Cβ, promoting the inositol trisphosphate (IP_3)-mediated release of Ca^{2+} from intracellular stores.[10] Secretion is stimulated by the opening of Ca^{2+}-activated 'maxi' Cl^- channels in the stellate cell apical membrane. By contrast, kinins seem to open a paracellular Cl^- conductance in *A. aegypti*.[3] A cocktail of *M. sexta* kinins and both CRF-like DH have been shown to activate pre-ecdysis behavior in this species; moreover, the ecdysis triggering hormone receptor A is expressed in neurons in abdominal ganglia 3–7 which coexpress kinins and CRF-like DH.[22]

Drome-CAP_{2b} also uses IP_3 as a second messenger but acts on principal cells and not on stellate cells.[10] The emptying of IP_3-sensitive Ca^{2+} stores opens store-operated channels and the resultant influx of extracellular Ca^{2+} activates a Ca^{2+}/calmodulin-sensitive nitric oxide (NO) synthase expressed only in principal cells.[10] The NO that is generated activates a soluble guanylate cyclase to elevate the levels of cGMP, which increase V-ATPase activity. At the same time, elevated levels of intracellular Ca^{2+} activate mitochondria in the apical region of the cell and hence increase the availability of ATP to the V-ATPase. However, CAP_{2b} activity in housefly MT is independent of NO and cGMP, and it leads to the opening of a Cl^- conductance pathway.[32]

CAP_{2b} acts *via* cGMP to reduce secretion by MT from *R. prolixus* and *T. molitor* larvae. It antagonizes the diuretic activity of serotonin (*R. prolixus*)[29] and Tenmo-DH_{37} (*T. molitor*),[34] which act *via* cAMP-dependent mechanisms. CAP_{2b} may therefore activate a cGMP-dependent cAMP phosphodiesterase, which will lower the levels of the diuretic cyclic nucleotide.[31] There is no evidence of NO involvement and CAP_{2b} may activate a membrane-bound guanylate cyclase.

Locmi-AVPL-DH (F2) stimulates cAMP production and fluid secretion by a semiisolated MT preparation that includes the ampullae and a short segment of the gut, but it has no effect on fully isolated MT, suggesting it may act as a releasing factor for Locmi-CRF-DH, which is present in ampullary endocrine cells.[6] Similarly, neither F1 nor F2 stimulates secretion by MT of *T. molitor* (used in place of *T. castaneum* because of its larger size) but, importantly, F1 (*not* F2) stimulates secretion when added to MT in the presence of intact CNS, although only when the CC and CA are attached.[1] This is consistent with the diuretic activity of F1 being indirect, acting as a releasing factor for a DH (possibly a CRF-related DH) in the CC/CA complex.

Tenmo-ADFa and Tenmo-ADFb act *via* cGMP to reduce secretion by *T. molitor* MT, and probably activate a cAMP-specific phosphodiesterase because ADFa antagonizes the actions of Tenmo-DH_{37}.[32] Tenmo-ADFa also reduces secretion by *A. aegypti* MT, but it has no effect on tubule electrophysiology or on the concentrations Na^+, K^+, and Cl^- in the secreted urine.[23] This is consistent with the reduced levels

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of cAMP and the down-regulation of a cAMP-dependent electroneutral transporter.

Different DHs can act synergistically (or cooperatively) with one another to control MT secretion. Thus the dose–response curve of Locmi-DH, a CRF-related peptide, becomes steeper and is displaced to the left when it is tested together with a low concentration (0.05 nM) of the native kinin (Locmi-K) in a diuretic assay using *L. migratoria* MT.[6] A major advantage of such synergism is that small changes in DH titer have a large impact on fluid secretion, thereby permitting more precise control of MT function. Synergism has also been reported between CRF-related (Dippu-DH_{46}) and CT-like (Dippu-DH_{31}) DH,[13] and between a CRF-related DH (Rhopr-DH_{49}) and serotonin.[32]

Fluid uptake from the locust ileum is coupled to active salt (NaCl/KCl) transport. Schgr-ITP increases transepithelial NaCl/KCl transport by stimulating an apical membrane electrogenic Cl^- pump.[29] Na^+ and K^+ cross the apical membrane through separate channels both of which are activated by cAMP. Although Schgr-ITP acts *via* cAMP, it inhibits active proton secretion into the lumen, whereas cAMP increases proton secretion into the medium.[29] The crustacean homologs of ITP, CHH, and MIH are known to act *via* cGMP.[32]

CONCLUDING REMARKS

Insect DH and ADH constitute a diverse group of peptides that have in common their role in controlling fluid and ion loss *via* the excretory system. Their effects on epithelial transport processes in MT and hindgut are reasonably well understood, but there is still much to be learned about the contribution these peptides make singly and collectively to the control of ion and fluid homeostasis *in vivo*. It is also clear that they may have additional roles within the CNS, as is evident from the presence of kinin receptors on NSCs expressing CRF-related DH, and of receptors for CRF-related and CT-like DH on corazonin-containing neurons.

REFERENCES

1. Aikins MJ, Schooley DA, Begum K, Detheux M, Beeman RW, Park Y. Vasopressin-like peptide and its receptor function in an indirect diuretic signaling pathway in the red flour beetle. *Insect Biochem Mol Biol* 2008;**38**:740–8.
2. Audsley N, McIntosh C, Phillips JE. Isolation of a neuropeptide from locust corpus cardiacum which influences ileal transport. *J Exp Biol* 1992;**173**:261–74.
3. Beyenbach KW. Transport mechanisms of diuresis in Malpighian tubules of insects. *J Exp Biol* 2003;**206**:3845–56.
4. Blackburn MB, Kingan TG, Bodnar WTK, Shabanowitz J, Hunt DF, Kempe T, et al. Isolation and identification of a new diuretic peptide from the hornworm, *Manduca sexta*. *Biochem Biophys Res Commun* 1991;**181**:927–32.
5. Cabrero P, Radford JC, Broderick KE, Costes L, Veenstra JA, Spana EP, et al. The Dh gene of *Drosophila melanogaster* encodes a diuretic peptide that acts through cyclic AMP. *J Exp Biol* 2002;**205**:3799–807.
6. Coast GM, Orchard I, Phillips JE, Schooley DA. Insect diuretic and antidiuretic hormones. In: Evans PD, editor. *Advanced Insect Physiology*. London: Academic Press; 2002. p. 279–409.
7. Coast GM, Garside CS, Webster SG, Schegg KM, Schooley DA. Mosquito natriuretic peptide identified as a calcitonin-like diuretic hormone in *Anopheles gambiae* (Giles). *J Exp Biol* 2005;**208**:3281–91.
8. Dai L, Žitňan D, Adams ME. Strategic expression of ion transport peptide gene products in central and peripheral neurons of insects. *J Comp Neurol* 2007;**500**:353–67.
9. Dircksen H, Kahsai L, Albus C, Mandali M, Nässel D. Identified *Drosophila* ion transport peptide splice forms are differentially expressed in central and peripheral neurons throughout post-embryogenesis. *Compar Biochem Physiol A Mol Integr Physiol* 2008;**150**:S134.
10. Dow JAT, Davies SA. Integrative physiology and functional genomics of epithelial function in a genetic model organism. *Physiol Rev* 2003;**83**:687–729.
11. Eigenheer RA, Nicolson SW, Schegg KM, Hull JJ, Schooley DA. Identification of a potent antidiuretic factor acting on beetle Malpighian tubules. *Proc Natl Acad Sci USA* 2002;**99**:84–9.
12. Eigenheer RA, Wiehart UM, Nicolson SW, Schoofs L, Schegg KM, Hull JJ, et al. Isolation, identification and localization of a second beetle antidiuretic peptide. *Peptides* 2003;**24**:27–34.
13. Furuya K, Milchak RJ, Schegg KM, Zhang J, Tobe SS, Coast GM, et al. Cockroach diuretic hormones: characterization of a calcitonin-like peptide in insects. *Proc Natl Acad Sci USA* 2000;**97**:6469–74.
14. Hewes RS, Taghert PH. Neuropeptides and neuropeptide receptors in the *Drosophila melanogaster* genome. *Genome Res* 2001;**11**:1126–42.
15. Holman GM, Cook BJ, Nachman RJ. Isolation, primary structure and synthesis of two neuropeptides from *Leucophaea maderae*: members of a new family of cephalomyotropins. *Comp Biochem Physiol C* 1986;**84**:205–11.
16. Huesmann GR, Cheung CC, Loi PK, Lee TD, Swiederek KM, Tublitz NJ. Amino acid sequence of CAP2b, an insect cardioacceleratory peptide from the tobacco hawkmoth, *Manduca sexta*. *FEBS Lett* 1995;**371**:311–4.
17. Huising MO, Flik G. The remarkable conservation of corticotropin-releasing hormone (CRH)-binding protein in the honeybee (*Apis mellifera*) dates the CRH system to a common ancestor of insects and vertebrates. *Endocrinology* 2005;**146**:2165–70.
18. Johnson EC, Bohn LM, Taghert PH. *Drosophila* CG8422 encodes a functional diuretic hormone receptor. *J Exp Biol* 2004;**207**:743–8.
19. Johnson EC, Shafer OT, Trigg JS, Park J, Schooley DA, Dow JA, et al. A novel diuretic hormone receptor in *Drosophila*: evidence for conservation of CGRP signaling. *J Exp Biol* 2005;**208**:1239–46.
20. Kataoka H, Troetschler RG, Li JP, Kramer SJ, Carney RL, Schooley DA. Isolation and identification of a diuretic hormone from the tobacco hornworm, *Manduca sexta*. *Proc Natl Acad Sci USA* 1989;**86**:2976–80.
21. Kean L, Cazenave W, Costes L, Broderick KE, Graham S, Pollock VP, et al. Two nitridergic peptides are encoded by the gene capability in *Drosophila melanogaster*. *Am J Physiol Regul Integr Comp Physiol* 2002;**282**:R1297–1307.

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22. Kim YJ, Žitňan D, Cho KH, Schooley DA, Mizoguchi A, Adams ME. Central peptidergic ensembles associated with organization of an innate behavior. *Proc Natl Acad Sci USA* 2006;**103**: 14,211–6.
23. Massaro RC, Lee LW, Patel AB, Wu DS, Yu M-J, Scott BN, et al. The mechanism of action of the antidiuretic peptide Tenmo ADFa in Malpighian tubules of *Aedes aegypti*. *J Exp Biol* 2004;**207**:2877–88.
24. Meredith J, Ring M, Macins A, Marschall J, Cheng NN, Theilmann D, et al. Locust ion transport peptide (ITP): primary structure, cDNA and expression in a baculovirus system. *J Exp Biol* 1996;**199**:1053–61.
25. Nachman RJ, Coast GM. Structure–activity relationships for *in vitro* diuretic activity of CAP_{2b} in the housefly. *Peptides* 2007;**28**:57–61.
26. Nachman RJ, Zabrocki J, Olczak J, Williams HJ, Moyna G, Scott AI, et al. *cis*-Peptide bond mimetic tetrazole analogs of the insect kinins identify the active conformation. *Peptides* 2002;**23**:709–16.
27. Nässel DR, Cantera R, Karlsson A. Neurons in the cockroach nervous system reacting with the antisera to the neuropeptide leucokinin I. *J Comp Neurol* 1992;**322**:45–67.
28. Park Y, Kim YJ, Adams ME. Identification of G protein-coupled receptors for *Drosophila* PRXamide peptides, CCAP, corazonin, and AKH supports a theory of ligand-receptor coevolution. *Proc Natl Acad Sci USA* 2002;**99**:11,423–8.
29. Phillips J, Meredith J, Wang Y, Zhao Y, Brock H. Ion Transport Peptide (ITP): structure, function, evolution. In: Goos HJ, Rastogi RK, Vaudry H, Pierantonio PV, editors, *Perspective in comparative endocrinology: unity and diversity*. Bologna: MEDIMOND Inc.; 2001. p. 745–52.
30. Proux JP, Miller CA, Li JP, Carney RL, Girardie A, Delaage M, et al. Identification of an arginine vasopressin-like diuretic hormone from *Locusta migratoria*. *Biochem Biophys Res Commun* 1987;**149**:180–6.
31. Quinlan MC, Tublitz NJ, O'Donnell MJ. Anti-diuresis in the blood-feeding insect *Rhodnius prolixus* Stål: the peptide CAP_{2b} and cyclic GMP inhibit Malpighian tubule fluid secretion. *J Exp Biol* 1997;**200**:2363–7.
32. Schooley DA, Horodyski FM, Coast GM. Hormones controlling homeostasis in insects. In: Gilbert LI, editor. *Insect endocrinology*. Oxford: Academic Press; 2012. p. 366–429.
33. Te Brugge V, Paluzzi J-P, Neupert S, Nachman RJ, Orchard I. Identification of kinin-related peptides in the disease vector, *Rhodnius prolixus*. *Peptides* 2011;**32**:469–74.
34. Te Brugge VA, Lombardi VC, Schooley DA, Orchard I. Presence and activity of a Dippu-DH_{31}-like peptide in the blood-feeding bug, *Rhodnius prolixus*. *Peptides* 2005;**26**:29–42.
35. Wang J, Kean L, Yang JL, Allan AK, Davies SA, Herzyk P, et al. Function-informed transcriptome analysis of *Drosophila* renal tubule. *Genome Biol* 2004;**5**:R69.

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Chapter 35

FMRFamide-Like Peptides (FLPs)

Ian Orchard and Angela B. Lange

ABSTRACT

Since the discovery of the molluscan cardioacceleratory peptide FMRFamide, a variety of neuropeptides that share the C-terminal RFamide have been characterized from cnidarians through to vertebrates. A particularly diverse array of families occurs in insects, including *N*-terminally extended FMRFamides and extended FL/IRFamides, myosuppressins, neuropeptide Fs, and short neuropeptide Fs. These peptide families are distinct and not related to one another and are now collectively referred to as FMRFamide-like peptides (FLPs). The FLPs are found throughout the central nervous system in a variety of neuronal types, in neurohemal organs, in the stomatogastric nervous system, and within endocrine cells of the midgut (at least in insects). The genes for the peptides and their receptors have been cloned and structure–activity relationships established in some cases. Many biological processes seem to be influenced by these peptides, but these peptides seem to be particularly involved in aspects of feeding, digestion, and food transport.

DISCOVERY

Since the discovery of the molluscan cardioacceleratory tetrapeptide FMRFamide,[24] vast arrays of neuropeptides that share the C-terminal RFamide have been characterized from cnidarians through to vertebrates. These FMRFamide-like peptides (FLPs) fall into several families of peptides (Tables 1 and 2) that are now considered unrelated and so the earlier collective term, FMRFamide-related peptides (FaRPs), is inappropriate. A particularly diverse array of FLPs occurs within insects, where there are *N*-terminally extended FMRFamides and extended FL/IRFamides, myosuppressins, neuropeptide Fs (NPFs), short neuropeptide Fs (sNPFs), and sulfakinins (discussed in the chapter on sulfakinins in this section of the book). Excellent reviews of FLPs have been published.[1,13,16,17,31]

Extended FMRFamides and Extended FL/IRFamides

The first FLP to be identified was the tetrapeptide FMRFamide, a cardioexcitatory peptide isolated from the nervous system of the clam, *Macrocallista nimbosa*.[24] Interestingly, authentic FMRFamide is only found in two phyla, the molluscs and annelids, and in these and other phyla, there are many more peptides that are *N*-terminally extended FMRFamides, FLRFamides, FIRFamides, or LRFamides.[31] Echinoderms do not have extended RFamide peptides but instead have peptides that terminate in Famide and Yamide. In insects, extended FMRFamides have only been identified in the dipterans and recently in the hemipteran *Rhodnius prolixus*,[20,21] by the sequencing or prediction from the gene. Other insects contain extended FLRFamides. In *Drosophila* species, single genes encoding 13 extended FMRFamides have been characterized and a number of the predicted peptides sequenced. Similarly, 13 extended FMRFamides have been sequenced from thoracic ganglia of the blowfly, *Calliphora vomitoria*, and are located on a single gene. The dipteran genes also code for some extended FIRFamides. *N*-terminally extended FM/L/IRFamides have been sequenced in many invertebrates (see Tables 1 and 2 for examples), and a comprehensive listing can be found in Walker et al.[31]

Myosuppressins

The first *N*-terminally extended RFamide to be sequenced in insects was leucomyosuppressin (LMS; pQDVDHVFLRFamide), which inhibits spontaneous contractions of *Leucophaea maderae* hindgut.[21] Other members of this so-called "myosuppressin" family have been sequenced,[21,22,31] some of which are shown in Tables 1 and 2. The myosuppressin family seems to be restricted to insects and crustaceans.[15,21,31] In insects, these peptides share the sequence $X^1DVX^4HX^6FLRFamide$ (where X^1 is pQ, Q, P, T, or A; X^4 is D, G, or V; and X^6 is V or S). In *R. prolixus*,[20] there is a variation wherein X^8 is M, resulting in the only FMRFamide to be found outside of the dipterans, and the only myosuppressin to not have the FLRFamide motif. In crustaceans,[31] the myosuppressins share the sequence $X^1X^2LDHVFLRFamide$ (where X^1 is pQ, Q, or P; and X^2 is D or E).

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00035-X
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TABLE 1 Sequences of Representative Members of FLPs in Molluscs

Peptide sequence	Species
FM/L/IRFamide	
FMRFamide	*Macrocallister nimbosa*
FLRFamide	*Lymnaea stagnalis*
FIRFamide	*Sepia officinalis*
AFLRFamide	*Octopus vulgaris*
NFLRFamide	*Mytilus edulis*
NDPFLRFamide	*Helix aspersa*
pQDPFLRFamide	*Helix aspersa*
SDPFLRFamide	*Lymnaea stagnalis*
ALSGDAFLRFamide	*Sepia officinalis*
Neuropeptide F	
DNSEMLAPPPRPEEFTSAQQLRQY	*Aplysia californica*
LAALNEYYSIMGRPRFamide	
TEAMLTPPERPEEFKNPNELRKYL	*Lymnaea stagnalis*
KALNEYYAIVGRPRFamide	

For details see Refs. 16, 31.

Neuropeptide Fs

The first member of the invertebrate neuropeptide F (NPF) family was identified in the tape worm *Monieza expansa* and shown to be related to the neuropeptide Y (NPY) family of vertebrate peptides (with a *C*-terminal F instead of a Y). Subsequently, NPFs were identified in molluscs.[13,31] These peptides have 32–36 amino acid residues and have a common *C*-terminal GRPRFamide. They are sometimes referred to as long NPFs. In insects,[3,31] many sequenced peptides have been referred to as NPF/NPY-like peptides. Some of these are now known to be members of a different family (short NPFs, see later) causing some confusion in the literature, which has recently been clarified.[16] "True" NPFs (Tables 1 and 2) are 28–45 amino acid residues with a consensus *C*-terminal RXRFamide (where X is commonly P or V). Shorter forms are known to exist but might represent degradation products. NPFs have been described in most invertebrate phyla, except for cnidarians and nematodes.[31] Examples of extended RYamides are known in a few species.

Short NPFs (sNPFs)

Short NPFs, also referred to as head peptides, were isolated (often from the head) by the use of an RIA with an antiserum

TABLE 2 Sequences of Representative Members of FLPs in Arthropods

Peptide sequence	Acronym	Species
Myosuppressin		
pQDVDHVFLRFamide	Leucomyosuppressin	*Leucophaea maderae*
PDVDHVFLRFamide	SchistoFLRFamide	*Schistocerca gregaria*
TDVDHVFLRFamide	Dromyosuppressin	*Drosophila melanogaster*
pQDVVHSFLRFamide	ManducaFLRFamide	*Manduca sexta*
pQDLDHVFLRFamide		*Carcinus maenas*
PELDHVFLRFamide		*Cancer borealis*
Extended FMRFamide		
SVKQDFMHFamide	*Drosophila* FMRFamide 1	*Drosophila melanogaster*
DPKQDFMRFamide	*Drosophila* FMRFamide 2	*Drosophila melanogaster*
TPAEDFMRFamide	*Drosophila* FMRFamide 7	*Drosophila melanogaster*
SDNFMRFamide	*Drosophila* FMRFamide 9	*Drosophila melanogaster*
SPKQDFMRFamide	*Drosophila* FMRFamide 10	*Drosophila melanogaster*
PDNFMRFamide	*Drosophila* FMRFamide 11	*Drosophila melanogaster*
SAPQDFVRSamide	*Drosophila* FMRFamide 12	*Drosophila melanogaster*
MDSNFIRFamide	*Drosophila* FMRFamide 13	*Drosophila melanogaster*

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TABLE 2 Sequences of Representative Members of FLPs in Arthropods—Cont'd

Peptide sequence	Acronym	Species
Extended FL/IRFamide		
GNSFLRFamide	MasFLRFamide II	*Manduca sexta*
DPSFLRFamide	MasFLRFamide III	*Manduca sexta*
GGRSNDNFIRFamide	Pea-FMRFa-4	*Periplaneta americana*
DRSDNFIRFamide	Pea-FMRFa-7	*Periplaneta americana*
ARPDNFIRFamide	Pea-FMRFa-11	*Periplaneta americana*
SDRNFLRFamide	FLI3	*Homarus americanus*
TNRNFLRFamide	FLI4	*Homarus americanus*
DRNFLRFamide	DF_2	*Procambarus clarkii*
NRNFLRFamide	NF_1	*Procambarus clarkii*
Neuropeptide F		
LVAARPQDSDAASVAAAI-		
RYLQELETKHAQHARPRFamide	Ang-NPF	*Anopheles gambiae*
SFTDARPQDDPTSVAEAI-		
RLLQELETKHAQHARPRFamide	Aea-NPF	*Aedes aegypti*
SNSRPPRKNDVNTMADAY-		
KFLQDLDTYYGDRARVRFamide	Drm-NPF	*Drosophila melanogaster*
KPDPSQLANMAEALKYLQ-		
ELDKYYSQVSRPRFamide		*Marsupenaeus japonicas*
DGDVMGGGEGGEMTAMADAIK-		
YLQGLDKVYGQAARPRFamide		*Daphnia magna*
Short Neuropeptide F		
AQRSPSLRLRFamide	*Drosophila* sNPF1	*Drosophila melanogaster*
SPSLRLRFamide	*Drosophila* sNPF2	*Drosophila melanogaster*
ARGPQLRLRFamide	Led-NPF1	*Leptinotarsa decemlineata*
APSLRLRFamide	Led-NPF2	*Leptinotarsa decemlineata*
ANRSPSLRLRFamide	*Periplaneta* head peptide	*Periplaneta americana*
PSLRLRFamide		*Carcinus maenas*
APALRLRFamide		*Macrobrachium rosenbergii*

For details see Refs. 16,21,31.

against the tape worm NPF or FMRFamide. Short NPFs are not related to NPFs and hence the confusion in the literature.[16] They have only been described in insects and crustaceans and no other invertebrates.[16,22,31] They typically consist of 8–10 amino acid residues with the consensus sequence XPXLRLRFamide.

STRUCTURE OF THE mRNA/GENE

Extended FMRFamides and Extended FL/IRFamides

Genes for this family were first identified in molluscs. In pulmonates, there is a single gene, whereas other molluscs have

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two genes. Typical of molluscs is the presence of multiple copies of peptides on the same gene. The FMRFamide precursor gene of *Aplysia californica* was the first sequence[31] and found to consist of repeated stretches of 15 or 16 amino acids, each segment containing one FMRFamide, its associated processing signals, and an acidic sequence, which may only be a spacer. All together, the gene encodes 28 copies of FMRFamide, one copy of FLRFamide, and one copy of an apparently related peptide ending in –YLRFamide. In *Lymnaea stagnalis*, the peptides are encoded by a multiexon gene that undergoes alternative mRNA splicing to generate two mRNAs. Exon I is shared by both mRNAs, along with exon II for type 1 mRNA, and exons III–V for type 2 mRNA. Type 1 mRNA codes for tetrapeptides, whereas type 2 mRNA codes for heptapeptides and hexapeptides. In *Helix aspersa*, there are also alternatively spliced mRNAs. The cDNA has also been isolated from the cuttlefish, *Sepia officinalis*, which has a precursor protein encoding 14 peptides, 11 of which are FMRFamide.

In insects, the *D. melanogaster* FMRFamide gene was identified through oligonucleotide isolated DNA clones, using the sequence DPKQDFMRFamide.[18] The gene contains 2 exons and 1 intron. The precursor is 347 amino acids in length and contains a predicted signal sequence of 20 residues. This is a single copy gene that encodes 5 copies of DPKQDFMRFamide, 2 of TPAEDFMRFamide, and 1 each of SDNFMRFamide, SPKQDFMRFamide, and PDNFMRFamide, all bounded by conventional proteolytic sites. Precursors containing *N*-terminally extended FMRFamides have been identified in the dipterans *D. melanogaster*, *Drosophila virilis*, *C. vomitoria*, and *Lucillia cuprina*.[21,31] Precursors in *C. vomitoria* and *L. cuprina* specify 16 potential extended FMRFamides and 2 potential extended FIRFamides. There is no evidence for alternative splicing of the mRNA transcript; however, expression data indicate that the intron of *D. melanogaster* may contain regulatory enhancer sequences. By tandem mass spectrometry, extended RFamides have been structurally elucidated in *Periplaneta americana* and sequence information used for subsequent cloning of the gene.[23] This precursor gene encodes for 24 putative peptides, 23 of which were detected by mass spectrometric methods. Many of these are *N*-terminally extended FIRFamides, although the gene is referred to as the cockroach FMRFamide gene. The cDNA encodes for a protein of 505 amino acids that has several features typical of the *D. melanogaster* FMRFamide cDNA, including a 22 amino acid signal peptide, N- and C-terminal sequences not coding for FMRFamides, and a central region with 24 putative peptide sequences. Although clearly similar in sequence to the extended dipteran FMRFamides, none of the encoded peptides contains the C-terminal FMRFamide. Instead, 10 FIRFamides, 3 FIRLamides, 2 FVRFamides, and peptides with an individual C-terminus of FVRLamide, LVRIamide, TLRVamide, SVRLamide, and LMRFamide are found. The remaining 4 putative peptides are nonamidated peptides. The genes have now also been identified in the genomes of other nondipteran insects such as *Tribolium castaneum*, *Acyrthosiphon. pisum*, *Apis mellifera*, and *R. prolixus*.[7,8,12,20]

cDNAs (AB158642, AB158643) have been cloned in the crayfish *Procambarus clarkii*.[34] The two prepropeptide sequences each consist of 323 residues, encoding 7 copies of extended LRFamide peptides, representing 6 different sequences.

Myosuppressins

The myosuppressin precursor has been determined through cloned DNA or predicted from the genomes in a wide range of insects, including *Diploptera punctata*, *Blattella germanica*, *Pseudaletia unipuncta*, and *Manduca sexta*, *D. melanogaster*, *T. castaneum Bombyx mori*, *R. prolixus*, and *Anopheles gambiae*.[3,12,20,21,30] In all species studied, only a single decapeptide myosuppressin is released from the C-terminus of the precursor upon cleavage at dibasic and tribasic endoproteolytic processing sites and amidation. In *D. punctata*, the myosuppessin gene has 3 exons. There is no evidence to suggest alternative splicing in the expression of the myosuppressin gene, although there may be unusual processing in the midgut of parasitized *M. sexta* larvae.[21] The organization of the *D. melanogaster* myosuppressin gene is similar to the myosuppressin genes in other insect species. Analysis of the nucleotide sequence shows that a 100 amino acid translation product contains a signal peptide of 24 amino acid residues. A phylogenetic analysis has been described.[30] An EST (FE188748) in the Pacific white shrimp *Litopenaeus vannamei* has been identified as encoding a putative precursor for a myosuppressin.[14]

Neuropeptide Fs

Typically, there are either one or two genes encoding NPFs in invertebrates, although there are examples of multiple genes.[3,16,19,28,31] These NPF genes contain only one copy of the mature NPF, although in species with two genes one of these NPFs ends with RGRYamide. The prepropeptide encoded by the *D. melanogaster* NPF gene has 96 amino acids of which the first 26 constitute a putative signal peptide.[3,16] Removal of the signal peptide, posttranslational processing and amidation produces the 36 amino acid *D. melanogaster* NPF. This peptide shares sequence similarity with mammalian NPY and related peptides, although it is more closely related to invertebrate NPFs. The *D. melanogaster* NPF gene is present as a single copy in the genome. Similarly, the genomes of a variety of species of annelids and insects contain sequences encoding an NPF each.[16,31] No NPF gene has been identified in the genome of *T. castaneum*.[1]

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Short Neuropeptide Fs

The gene for *Aedes aegypti* head peptides encodes a protein containing 3 copies of bioactive peptides, whereas the sNPF genes of *A. gambiae* and *D. melanogaster* encode 5 and 4 peptides, respectively, with differing but related sequences.[3,16] Multiple sNPF copies are a common, but not universal, feature. One sNPF is encoded on the prepropeptide in ticks, spiders, and crustaceans, two sNPFs are encoded in many lepidopterans, with other lepidopterans, dipterans, and coleopterans having multiple copies.[16,31] The sNPF gene (CG13968) in *D. melanogaster*[11,16] has 4 exons in the coding region of its transcript. The cDNA encodes a 281 amino acid protein that contains a 30 amino acid signal peptide and 2 putative extended RLRF peptides and two extended RLRWamides, flanked by pairs of basic residues. However, mass spectrometry has revealed that most of the peptides are not processed in the predicted way and that possibly only the first sNPF actually occurs as predicted.[16] The amino acid sequences of the sNPFs are quite different from vertebrate NPY, in contrast to NPF that has 31% homology with NPY. Thus, the NPFs (not the sNPFs) are considered as homologs of the vertebrate NPY.[16]

PROCESSING OF THE PRECURSOR

The genes for enzymes that process or degrade regulatory peptides have been identified in *D. melanogaster* and *A. gambiae*.[25,29] Peptides are synthesized as a larger precursor that is subsequently processed into the active substances. After cleavage of the signal peptide, further proteolytic processing occurs predominantly at basic amino acid residues. Some rules have been proposed that allow a prediction of which putative proteolytic sites are actually used in insects.[29] The great majority of known or predicted insect peptides, including the extended RFamides, are α-amidated, and so the significance of amidating mechanisms for peptide sequencing is great. Peptide signaling is terminated by a combination of mechanisms that include receptor desensitization or internalization, peptide diffusion, and enzymatic degradation.[29] A number of peptidases responsible for neuropeptide inactivation have been identified in insects,[25] but little work has examined FLPs.

Extended FMRFamides and Extended FL/IRFamides

In molluscs, the FMRFamide gene can undergo alternative mRNA splicing to generate two mRNAs, as mentioned earlier. Interestingly, in *Lymnaea stagnalis*, it has been shown that the gene can be differentially spliced in neurons, such that type 1 mRNA is found in neurons within all ganglia, whereas type 2 mRNA is only found in neurons in the visceral and right parietal ganglia. In *Helix aspersa*, there are also alternatively spliced mRNAs that can be expressed in different neurons. Thus, neurons can express different complements of FMRFamide peptides.[13,31]

There is considerable conservation of gene organization for the extended FMRFamides in dipterans. The single copy gene encodes a precursor containing multiple copies of *N*-terminally extended FMRFamides liberated by endoproteolytic and amidation enzymes. In *D. melanogaster*, there is 1 monofunctional peptidylglycine-α-hydroxylating monooxygenase gene and 2 monofunctional peptidyl-α-hydroxyglycine α-amidating lyase genes, which are required to produce α-amidated bioactive products.[5] The DNA regulatory sequences controlling *D. melanogaster* FMRFamide neuropeptide gene expression are a model for insect neuropeptide gene expression.[21] There is some debate over cell-specific processing of the *D. melanogaster* FMRFamide gene. Immunohistochemistry with antisera generated against 3 of the *D. melanogaster* FMRFamides shows a unique, nonoverlapping cellular expression pattern indicative of differential processing of the precursor. By contrast, others have concluded that there is a single predominant pattern of expression, and it is unlikely that there are widespread cell-specific differences in precursor processing.[18,21] The *P. americana* FMRFamide gene[23] liberates 24 putative peptides with appropriate endoproteolytic and amidation processing.

Myosuppressins

Endoproteolytic and amidation enzymes liberate a single myosuppressin neuropeptide from the precursor protein. Alternative processing might exist in parasitized *M. sexta* larvae because 2 extended myosuppressins have been identified in the midgut.[5]

Neuropeptide Fs

The long NPFs are encoded on single genes that contain one copy of the neuropeptide following endoproteolytic and amidation enzyme processing.[16]

Short Neuropeptide Fs

The sNPF genes contain multiple copies of sNPFs that are predicted to be liberated by endoproteolytic and amidation enzymes.[16]

DISTRIBUTION OF mRNA/PEPTIDE

Immunohistochemical studies have indicated the presence of FMRFamide-like immunoreactivity distributed throughout the central nervous system (CNS) and peripheral nervous system (PNS) of invertebrates, and within endocrine cells of

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the insect midgut. Most of these studies used antisera raised against the C-terminal RFamide and potentially indicate the distribution of multiple members of the extended RFamide families. The specific localization of mRNA by Northern blot and/or in situ hybridization is described here.

Extended FMRFamides and Extended FL/IRFamides

Identifiable neurons express the FMRFamide precursor in molluscs. For example, in *L. stagnalis*, these include heart excitatory motor neurons, visceral white interneurons, and a pleural interneuron. With alternative slicing, 280 neurons distributed among all the ganglia, express the tetrapeptides, while the heptapeptides are found in about 57 neurons in the visceral and parietal ganglia.[13,31]

In situ hybridization reveals that the *D. melanogaster* FMRFamide gene is expressed in approximately 44 neurons of the 10,000 in the larval CNS.[21] In *P. americana*, a nested reverse-transcription PCR analysis for the extended FIRFamides shows expression in the brain, and in thoracic and abdominal ganglia.[23] In the crayfish, *P .clarkii*, the gene is expressed in brain neurons associated with the olfactory system.[15,34]

Myosuppressins

In situ hybridization[21] shows that expression of the *D. punctata* myosuppressin gene is most abundant in the brain and optic lobes and in the frontal and subesophageal ganglia. In the brain, expression is found in cells of the pars intercerebralis, a region known to contain the medial neurosecretory cells. In *P. unipuncta*, 57% of the cells producing the myosuppressin gene transcript are found in the optic lobe. Within the digestive tract, *D. punctata* myosuppressin mRNA-positive endocrine cells are found in the anterior portion of the midgut, as they are in *P. unipunta*. In situ hybridization also reveals a variety of cell types within the CNS, a peripheral neurosecretory cell, and midgut endocrine cells in *M. sexta* that express the myosuppressin gene.

Neuropeptide Fs

In situ hybridization indicates that the NPF gene is widely expressed in the CNS of molluscs.[13,31] In *H. aspersa*, the NPF transcript is found in neurons in most ganglia of the circumesophageal ring, and in all CNS ganglia except the buccal ganglia in *L. stagnalis*. Most of these neurons are found in the cerebral and pedal ganglia, and their distribution suggests a role in reproduction and possibly growth and development. NPF is found in many neurons in the CNS of *A. californica* and also in the abdominal bag cells. Their location in the buccal and cerebral ganglia suggests a role in feeding, and their association with bag cells suggests a role in reproduction.

In situ hybridization for *D. melanogaster* NPFs reveals medial and lateral cell pairs in the larval and adult brains, although the lateral cells are only weakly stained in the adult.[3,16] All of these are interneurons. No NPF neurons were observed in the ventral nerve cord (VNC). There are about 15 endocrine cells in larval midgut and 75–150 endocrine cells in the adult midgut. The transcript expression of *A. gambiae* NPF shows that the PCR product is present in eggs and in head, thorax, and abdomen of 4th instar larvae, and in pupae. It is present in the head and abdomen of adult males and females. Northern blot analysis has also revealed the presence of *A. gambiae* NPF transcript in the brain and midgut of adult female mosquitoes. The *A. aegypti* cDNA is expressed in the brain and midgut,[3] and in the foregut, midgut, and all ganglia of the CNS in *Reticulitermes flavipes*.[19]

Short Neuropeptide Fs

Expression of sNPF has essentially only been reported in *D. melanogaster*. Here, sNPF mRNA has been studied by Northern blot analysis and in situ hybridization. Similar amounts of the transcript are expressed throughout all developmental stages of embryos and larvae. In situ hybridization reveals expression of the sNPF transcript in the nervous system from stage 14 embryos onward and its presence in neurons of the brain and ventral ganglia. No expression is apparent in the midgut. A large number of neurons express sNPF in the larval and adult CNS, in addition to over 1000 mushroom body Kenyan cells. Most of these are unidentified small interneurons, but others are identified, including olfactory sensory neurons, lateral clock neurons, and lateral neurosecretory cells. The *A. aegypti* head peptide gene is expressed predominantly in the brain, terminal ganglion, and midgut.[11,16]

RECEPTORS AND SIGNALING CASCADE

There is little molecular information on these receptors for invertebrates, other than insects. Studies in these other invertebrates have concentrated on the mode of action of the peptide and receptor cascade. Publication of the genomes for a variety of insect species has enabled analysis of putative G-protein-coupled receptors (GPCRs) with "reverse pharmacology" identifying the natural ligand of functionally expressed orphan receptors.

Extended FMRFamides and Extended FL/IRFamides

Molluscs seem to use both GPCRs and gated ion channel receptors to mediate the biological effects of FMRFamide

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and related peptides.[13,31] A radioligand-binding assay identified an FMRFamide receptor in the brain and heart of *H. aspersa* that was most sensitive to YGGFMRFamide. The cDNA of the FMRFamide-gated receptor/channel protein from *H. aspersa* has been cloned and sequenced and shares 93% sequence identity with the FMRFamide-gated sodium channel in *L. stagnalis*. Structure/activity properties have recently been reviewed.[31] This family of peptides has been shown to directly gate amiloride-sensitive sodium channels in C2 neurons in *H. aspersa*, resulting in excitation, while inhibiting others through increasing permeability to potassium. FMRFamides have also been implicated in transmitter mobilization, in presynaptic calcium entry, and in blocking L-type calcium currents and potentiating T-type calcium currents.[31]

A receptor for the extended FMRFamides (CG2114) has been cloned, sequenced, and characterized in *D. melanogaster*[7,26] and predicted in *A. gambiae*, *T. castaneum*, and *A. mellifera*. The cDNA sequence codes for a protein of 549 amino acid residues, which contains 7 transmembrane domains and potential N-glycosylation sites. Reverse pharmacology by the use of the receptor in Chinese hamster ovary (CHO) cells reveals that the receptor responds to the *D. melanogaster* extended FMRFamides, the most potent of which is PDNFMRFamide. Interestingly, other extended FMRFamides are much less effective. These results imply that there are other receptors for the remaining extended FMRFamides. This receptor can also be activated by some other RFamide peptides, including the *D. melanogaster* sNPF and the *D. melanogaster* myosuppressin, which leads to some debate on the true physiological relevance of this FMRFamide receptor. Northern blot analysis reveals that the *Drosophila* receptor is expressed in all stages but mostly in larvae and adults. Expression analysis by RT-PCR illustrates that the receptor is present in the trachea, which might therefore contaminate the positive results for CNS, fat body, intestine, and Malpighian tubules.

There have been a few studies examining the structure activity of the dipteran-extended FMRFamides. The question of functional redundancy has been addressed in *D. melanogaster* and *C. vomitoria* where multiple extended FMRFamides are encoded on the same gene.[18,21] By examination of twitch tension of body wall muscles, it was concluded that the *D. melanogaster* FMRFamides are functionally redundant. By contrast, the use of a heart rate or crop assay showed that some of the *D. melanogaster* FMRFamides are functionally distinct with some showing activity and others not. The *D. melanogaster* FMRFamide receptor expressed in CHO cells shows differential potency to the extended FMRFamides.[26] A similar functional distinctiveness has been observed in *C. vomitoria* where the semiisolated heart and salivary glands respond to some of the *C. vomitoria* FMRFamides but not to others.[21]

Myosuppressins

Two *D. melanogaster* GPCRs have been cloned and characterized (CG8985, CG13805), which are specific for the *D. melanogaster* myosuppressin and which do not react with other *D. melanogaster* neuropeptides.[26] The corrected genes from the database (coding for receptor 1 and 2) have multiple exons and introns. The cDNA codes for a protein of 478 amino acid residues (receptor1) and 488 amino acids (receptor 2), which each contains 7 transmembrane domains. The extracellular *N*-terminus contains potential N-glycosylation sites. Northern blot analysis illustrates that receptor 1 is strongly expressed in the adult head (essentially absent in the thorax and abdomen), with receptor 2 present in the adult head, thorax, and abdomen. Similarly, a GPCR closely related to the *D. melanogaster* myosuppressin receptor has also been identified in the *A. gambiae* Genome Project database and subsequently cloned and characterized.[26] *Anopheles gambiae* seems to have only one myosuppressin receptor, as does *T. castaneum* and *A. mellifera*,[6,7] in contrast to the two sequenced from *D. melanogaster.* An *in vitro* binding assay has been used to characterize putative receptors for *L. migratoria* myosuppressin associated with the oviduct and CNS of *L. migratoria*.[21] The specific binding for oviducts suggests the presence of 2 receptor types of high and low affinity.

The structure–activity relationships of the myosuppressins have been examined in *L. maderae* hindgut and *L. migratoria* oviduct.[21] In hindgut, VFLRFamide seems to be the active core for inhibitory biological activity. In oviduct, the amide is critical for both binding with the receptor and inhibitory biological activity. Interestingly, VFLRFamide is the minimum sequence required for receptor binding, whereas HVFLRFamide is the minimum sequence for comparable inhibitory biological activity. The nonpeptide benzethonium chloride (Bztc) shares several chemical features with the sequence VFLRFamide, and Bztc mimics the biological activity of myosuppressins in a variety of, but not all, target tissues.[21] A molecular dynamic/modeling study identified plausible structural explanations for this activity. Myosuppressin analogs containing restricted conformational components have also been developed and shown to antagonize the myosuppressin receptors.[21]

Neuropeptide Fs

The *Drosophila* genome has a genomic sequence with similarity to NPY receptors.[3,16] This gene [CG 1147] for *D. melanogaster* NPF receptor 1 has been cloned and sequenced. The deduced protein is 481 amino acids long and is predicted to have 7 transmembrane domains. This receptor resembles the vertebrate Y_2 receptors and is most closely related to the *Lymnaea* NPY receptor. CHO-K1

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cells expressing the RNA of the NPF receptor 1 have been used in radioreceptor binding assays. The NPF receptor 1 also inhibits forskolin-stimulated adenylyl cyclase activity in transfected CHO cells. In situ hybridization indicates that the transcripts for this receptor are found in many cells of the larval CNS and midgut. The *A. gambiae* genome contains sequences encoding for an NPF receptor, and the cDNA has been cloned and sequenced.[3] The deduced protein is 424 amino acids and is predicted to have seven transmembrane domains. This receptor has also been stably expressed for radioligand-binding analysis and exhibits high affinity for *A. gambiae* NPF. RT-PCR analysis reveals that the receptor transcript is present in all life stages. A phylogenetic analysis of NPY/NPF receptors has been performed.[3]

Structure–activity studies have not been reported in any detail for the NPFs. Functional expression of the receptors was used to identify the ligands for the receptor, so the NPF receptors are, by definition, more responsive to NPFs than to other extended RFamides. By functional expression and radioligand-binding analysis, the *A. gambiae* NPF receptor exhibits high-affinity binding for *A. gambiae* NPF but lower affinity for the NPFs from *A. aegypti* and *D. melanogaster*.[3]

Short NPF

A gene from the *Drosophila* Genome Project database has been cloned and represents a seven transmembrane GPCR that shows some structural similarity to vertebrate NPY_2 receptors and is activated by the sNPFs of *D. melanogaster*.[3] This sNPF receptor (CG7359), expressed in CHO-K1 cells, is not activated by other peptides. Functional expression of the sNPF receptor illustrates that the activated receptor produces inward rectifying potassium currents by a pertussis toxin-sensitive G-protein-mediated pathway. The *D. melanogaster* sNPFs are more potent than long NPFs.[3] The sNPF receptor is enriched in the brain, eye, VNC, and spermatheca of adults. The sNPF receptor has now been identified in the fire ant *Solenopsia invita*, and mosquito *A. gambiae*, and predicted *in silico* from a number of other insect species genomes.[16] In fire ant queens, expression is highest in the brain and is also found in the midgut, hindgut, Malpighian tubules, fat body, and ovaries. Similarly, in the mosquito, the receptor is expressed in the head, dorsal thorax, ventral thorax, abdominal wall, midgut, hindgut, and Malpighian tubule.[16]

BIOLOGICAL ACTIONS

A range of biological actions have been established for FLPs by the use of *in vitro* bioassays, injection, and molecular techniques. It is often difficult to correlate these actions with the particular families, because of the likelihood of the higher doses of peptides crossreacting with receptors for a different family. However, many target tissues for FLPs have been identified, and many biological processes seem to be influenced by these peptides, including reproduction, growth, feeding, circulation, ecdysis, and development.[3,10,13,15–17,21,31] The FLPs seem to act as neurotransmitters, neuromodulators, or neurohormones.

Extended FMRFamides and Extended FL/IRFamides

The extended FMRFamides influence heart rate, gut mobility, and skeletal muscle activity in a variety of invertebrates.[10,15,16,31] The first FLP, FMRFamide, was identified as a cardioexcitatory peptide in *M. nimbosa*.[24] Further work in molluscs, revealed that heart excitatory motoneurons contain FMRFamide and FLRFamide and that these regulate heart activity in *L. stagnalis*.[31] The visceral white interneurons contain extended FLRFamides (along with other extended RFamides), and these regulate respiration and heart rate.[31] Other suggested effects in molluscs include osmoregulation, reproduction, feeding, and chromatophore migration.[13]

In arthropods, DRNFLRFamide is released from the POs of the crayfish *P. clarkii* and is thought to act as a neurohormone.[15] This FLP increases the amplitude of EJPs in the deep extensor muscles (an action thought to be presynaptic) and also is mysostimulatory on superficial abdominal extensor muscles and hindgut.[15] In the lobster, *Homarus americanus*, the gastropyloric receptor neurons contain FLPs suggesting a sensory role in the stoatogastric system. FLPs are also associated with crustacean eyestalk and so might play a role in the visual system.[2] In insects, such as *D. melanogaster*, these various extended FMRFamides have differing effectiveness.[18,21] In *C. vomitoria*, a similar functional distinctiveness is suggested by the fact that only 2 of the 6 *C vomitoria* extended FMRFamides tested are active on the semiisolated heart, and only 3 of the 6 tested on salivary gland are active sialogogs.[21] The extended FL/IRFamides also influence oviduct contractions in *L. migratoria*, contractions of the antennal heart in *P. americana*, and reduce spike frequency of dorsal unpaired median neurons in *P. Americana*.[21,23]

Myosuppressins

Myosuppresins, as their name implies, reduce the frequency of spontaneous contractions of visceral and cardiac muscle.[18,21] In some insect species, they have also been shown to enhance the force of neurally evoked contractions of skeletal muscle. Myosuppressins also stimulate enzyme secretion and inhibit short-circuit current in midgut, and inhibit the release of adipokinetic hormones. Injection of the cockroach myosuppressin into *B. germanica* inhibits food intake, with food accumulating in the foregut.[30]

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Neuropeptide Fs

The NPY signaling pathway is implicated in the stimulation of food uptake in the vertebrates and in the regulation of food conditioned foraging behaviors in *Caenorhabditis elegans*.[16] The presence of NPF in buccal and cerebral ganglia of *A. californica* suggests a role in feeding circuitry and modulation of the feeding motor program, and there is now good evidence for this.[13,31] In addition, NPF is present in bag cells (together with egg-laying hormone) implying a role in reproduction and renal functions that are under the control of bag cells. Similarly, in *H. aspersa*, NPF is associated with the neurons that release ovulation and egg-laying hormone, again implying an action in reproductive behavior.[13,31]

In *D. melanogaster*, NPFs have been implicated in diverse functions, such as foraging, feeding and motivation, ethanol sensitivity, stress responses, reproduction, clock function, and learning.[16] For example, the NPF neuronal network in *D. melanogaster* has been described, and its food-dependent modifications have been examined. Gustatory stimulation by sugar is sufficient to trigger long-term changes in the NPF neuronal circuit.[27] Expression of *D. melanogaster* NPF is high in the brain of larvae attracted to food, and its downregulation coincides with food aversion, hypermobility, and cooperative burrowing in the older larvae. Young transgenic larvae that have lost their NPF signaling prematurely display the behavioral phenotype associated with the older larvae. Overexpression of NPF in older larvae prolongs feeding and suppresses hypermobility and cooperative burrowing behaviors.[32] NPF is also expressed in male clock neurons, and it has been suggested that NPF might be involved in clock-regulated sex dimorphic behavior and in male aggression.[16] The hemolymph titer of *A. aegypti* NPF changes in females shortly after a blood meal and during the course of the reproductive cycle.[3] Bioassays have shown that NPFs can inhibit muscle contraction of hindgut preparations in *R. prolixus* and *R. flavipes*.[4,19]

Short NPFs

The involvement of sNPFs in feeding has also drawn some attention, but sNPFs are also implicated in growth and development, osmotic and metabolic stress, locomotion, the circadian clock, olfaction, and hormone release.[16] For example, in *D. melanogaster*, sNPF mutants in which the pathway is overexpressed have elevated food intake, whereas mutants in which the pathway is suppressed have reduced food intake.[11] Injection of *A. aegypti* head peptide inhibits host-seeking by females and its hemolymph titer is low before a blood meal but peaks at 36–48h post blood meal.[28] Blood feeding also results in an increased titer of RFamide peptides in the hemolymph of *R. prolixus* that peaks at 3, 6, and 10h postfeeding.[21] A sNPF in *L. decemlineata* is a potent gonadostimulin in *L. migratoria*, stimulating oocyte length after injection.[9] Within, *L. decemlineata* itself, the 2 sNPFs are absent from the brains of insects in diapause.[9] In *B. mori*, sNPF is found in the intrinsic cells of the corpora cardiaca,[33] and the NPF receptor is located to the corpus allatum (CA), which is the site of synthesis and release of juvenile hormone (JH). sNPF is an allatostatin, inhibiting the production of JH. Receptors for allatotropin are found on the sNPF cells, and it has been suggested that allatotropin might inhibit the sNPF cells, thereby removing inhibition to the CA.[33]

Clearly, the extensive distribution of FLPs throughout the invertebrates (and indeed the vertebrates) illustrates the importance that this group of peptides has in integration and regulation of biological functions. Numerous biological processes are influenced by these peptides, including circulation, reproduction, ecdysis, and development; however, these peptides seem to be particularly involved in aspects of feeding, digestion, and/or food transport.

REFERENCES

1. Espinoza E, Carrigan M, Thomas SG, Shaw G, Edison AS. A statistical view of FMRFamide neuropeptide diversity. *Mol Neurobiol* 2000;**21**:35–56.
2. Fan Y, Sun P, Wang Y, He X, Deng X, Chen X, et al. The G protein-coupled receptors in the silkworm, *Bombyx mori*. *Insect Biochem Mol Biol* 2010;**40**:581–91.
3. Garczynski SF, Crim JW, Brown MR. Characterization of neuropeptide F and its receptor from the African malaria mosquito, *Anopheles gambiae*. *Peptides* 2005;**26**:99–107.
4. Gonzalez R, Orchard I. Physiological activity of neuropeptide F on the hindgut of the blood-feeding hemipteran, *Rhodnius prolixus*. *Peptides* 2009;**9**:57.
5. Han M, Park D, Vanderzalm PJ, Mains RE, Eipper BA, Taghert PH. *Drosophila* uses two distinct neuropeptide amidating enzymes, dPAL1 and dPAL2. *J Neurochem* 2004;**90**:129–41.
6. Hauser F, Cazzamali G, Williamson M, Park Y, Li B, Tanaka Y, et al. A genome-wide inventory of neurohormone GPCRs in the red flour beetle *Tribolium castaneum*. *Front Neuroendocrinol* 2008;**29**: 142–65.
7. Hauser F, Cazzamali G, Williamson M, Blenau W, Grimmelikhuijzen CJP. A review of neurohormone GPCRs present in the fruitfly *Drosophila melanogaster* and the honey bee *Apis mellifera*. *Prog Neurobiol* 2006;**80**:1–9.
8. Huybrechts J, Bonhomme J, Minoli S, Prunier-Leterme N, Dombrovsky A, Abdel-Latief M, et al. Neuropeptide and neurohormone precursors in the pea aphid, *Acyrthosiphon pisum*. *Insect Mol Biol* 2010;**19**:87–95.
9. Huybrechts J, De Loof A, Schoofs L. Diapausing Colorado potato beetles are devoid of short neuropeptides FI and FII. *Biochem Biophys Res Commun* 2004;**317**:909–16.
10. Keller R. Crustacea neuropeptides: structure, function and comparative aspects. *Experientia* 1992;**48**:442–8.
11. Lee KS, You KH, Choo JK, Han YM, Yu K. *Drosophila* short neuropeptide F regulates food intake and body size. *J Biol Chem* 2004;**279**:50781–9.

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12. Li, B, Predel R, Neupert S, Hauser F, Tanaka Y, Cazzamali G, et al. Genomics, transcriptomics, and peptidomics of neuropeptides and protein hormones in the red flour beetle *Tribolium casteneum*. *Genome Res* 2008;**18**:113–22.
13. López-Vera E, Aguilar MB, Heimer de la Cotera EP. FMRFamide and related peptides in the phylum mollusca. *Peptides* 2008;**29**:310–7.
14. Ma M, Gard AL, Xiang F, Wang J, Davoodian N, Lenz PH, et al. Combining *in silico* transcriptome mining and biological mass spectrometry for neuropeptide discovery in the Pacific white shrimp *Litopenaeus vannamei*. *Peptides* 2010;**31**:27–43.
15. Mercier J, Doucet D, Retnakaran A. Molecular physiology of crustacean and insect neuropeptides. *J Pestic Sci* 2007;**32**:345–59.
16. Nässel DR, Wegener C. A comparative review of short and long neuropeptide F signaling in invertebrates: any similarities to vertebrate neuropeptide Y signaling? *Peptides* 2011;**32**:1335–55
17. Nässel DR, Taghert PH. Invertebrate neuropeptides. *eLS* 2006;1–10
18. Nichols R. Signaling pathways and physiological functions of *Drosophila melanogaster* FMRFamide-related peptides. *Annu Rev Entomol* 2003;**48**:485–503.
19. Nuss AB, Forschler BT, Crim JW, TeBrugge V, Pohl J, Brown MR. Molecular characterization of neuropeptide F from the eastern subterranean termite *Reticulitermes flavipes* (Kollar) (Isoptera: Rhinotermitidae). *Peptides* 2010;**31**:419–28.
20. Ons S, Sterkel M, Diambra L, Urlaub H, Rivera-Pomar R. Neuropeptide precursor gene discovery in the Chagas disease vector *Rhodnius prolixus*. *Insect Mol Biol* 2011;**20**:29–44.
21. Orchard I, Lange AB, Bendena WG. FMRFamide-related peptides: a multifunctional family of structurally related neuropeptides in insects. *Adv Insect Physiol* 2001;**28**:267–329.
22. Predel R, Neupert S, Garczynski SF, Crim JW, Brown MR, Russell WK, et al. Neuropeptidomics of the mosquito *Aedes aegypti*. *J Proteome Res* 2010;**9**:2006–15.
23. Predel R, Neupert S, Wicher D, Gundel M, Roth S, Derst C. Unique accumulation of neuropeptides in an insect: FMRFamide-related peptides in the cockroach, *Periplaneta americana*. *Eur J Neurosci* 2004;**20**:1499–513.
24. Price DA, Greenberg EJ. Structure of a molluscan cardioexcitatory neuropeptide. *Science* 1977;**197**:670–1.
25. Riehle MA, Garczynski SF, Crim JW, Hill CA, Brown MR. Neuropeptides and peptide hormones in *Anopheles gambiae*. *Science* 2002;**298**:172–5.
26. Schöller S, Belmont M, Cazzamali G, Hauser F, Williamson M, Grimmelikhuijzen CJP. Molecular identification of a myosuppressin receptor from the malaria mosquito *Anopheles gambiae*. *Biochem Biophys Res Commun* 2005;**327**:29–34.
27. Shen P, Cai HN. *Drosophila* neuropeptide F mediates integration of chemosensory stimulation and conditioning of the nervous system by food. *J Neurobiol* 2001;**47**:16–25.
28. Stanek DM, Pohl J, Crim JW, Brown MR. Neuropeptide F and its expression in the yellow fever mosquito, *Aedes aegypti*. *Peptides* 2002;**23**:1367–78.
29. Taghert PH, Veenstra JA. *Drosophila* neuropeptide signaling. *Adv Genet* 2003;**49**:1–65.
30. Vilaplana L, Castresana J, Bellés X. The cDNA for leucomyosuppressin in *Blatella germanica* and molecular evolution of insect myosuppressins. *Peptides* 2004;**25**:1883–9.
31. Walker RJ, Papaioannou S, Holden-Dye L. A review of FMRFamide- and RFamide-like peptides in metazoa. *Invert Neurosci* 2009;**9**: 111–53.
32. Wu Q, Wen T, Lee G, Park JH, Cai HN, Shen P. Developmental control of foraging and social behavior by the *Drosophila* neuropeptide Y-like system. *Neuron* 2003;**39**:147–61.
33. Yamanaka N, Yamamoto S, Zitnan D, Watanabe K, Kawada T, Satake H, et al. Neuropeptide receptor transcriptome reveals unidentified neuroendocrine pathways. *PLoS ONE* 2008;**3**:e3048.
34. Yasuda-Kamatani Y, Yasuda A. Characteristic expression patterns of allatostatin-like peptide, FMRFamide-related peptide, orcokinin, tachykinin-related peptide, and SIFamide in the olfactory system of crayfish *Procambarus clarkii*. *J Comp Neurol* 2006;**496**:135–47.

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Chapter 36

Free-Living Nematodes

Edward.P. Masler

ABSTRACT

Nematodes use a wide array of peptide messengers to control nearly all life processes. Molecular similarities and differences among nematode species with differing life histories, such as free-living and parasitic types, facilitate the application of research results across the phylum and advance our understanding of the roles, complexity, and diversity of biologically active peptides in nematodes. Processes as diverse as hatching and locomotion, mating and feeding, defense, and metabolism are controlled and influenced by nematode peptides comprising exceedingly complex families. This complexity results from a large number of genes encoding related amino acid sequences, as in the case of the FMRFamide-like peptides, from intricate variations of molecular architectures, as seen among members of the insulin-like peptides family and from the broad variety of peptides comprising neuropeptide-like proteins, including those peptides that have antimicrobial functions. These peptide families are briefly described for free-living nematodes, along with their established and proposed functions, including receptor information.

INTRODUCTION

Nematodes are second only to insects in the number of species, breadth of distribution, and amount of biomass, occupying every ecological niche. They include parasites of animals, insects and plants, as well as nonparasitic, microbivorous free-living species. Most are microscopic, although some animal-parasitic species are quite large. Nematodes are unsegmented roundworms having a longitudinal nerve cord and nerve ring. The nervous system is accompanied by a simple anatomy comprising muscular mouthparts with a pharyngeal pump to facilitate feeding, an intestine that serves as a metabolic organ somewhat analogous to the mammalian liver, a muscular body wall, and cuticle, gonads, and sexually dimorphic features such as the uterus. All nematodes have an egg stage, three external larval stages, and one or more adult forms.[11a] As parasitic species are covered elsewhere in this volume, this chapter focuses on free-living species.

A discussion of peptides in free-living nematodes necessarily focuses on *C. elegans*, which contains the first completely sequenced animal genome and has become an important organism for molecular genetic and cellular biology research. Peptides in other free-living species have been studied, including the related *C. briggsae* and other *Caenorabditis* species, *Oscheius* spp., *Panagrellus redivivus*, and *Pristionchus pacificus*, but the bulk of peptide-related information from free-living nematodes has been generated from work with *C. elegans*. Studies on biologically active peptides in nematodes are extensive, but the number of different peptide families discovered thus far is small compared with higher invertebrates and vertebrates. Nevertheless, complexity and variety of peptide sequences within each family can be quite high, and genomic analysis suggests that the actual number of families is substantial. Here, we consider three large families of peptides in free-living nematodes, one of which has been the quite extensively studied biologically.

FMRFAMIDE-LIKE PEPTIDES (FLPS)

1. Discovery and structure. By far, the most extensively studied family of peptides in nematodes comprises the FMRFamide-like peptides (FLPs). They are hormones affecting neuromuscular, behavioral, reproductive and other activities and have been comprehensively reviewed and cataloged.[12–14,16,18] Free-living nematode FLPs are neuropeptides of 4 to approximately 15+ amino acids, characterized by an amidated C-terminus with an RF_{NH2} motif. This is a common posttranslational modification of biologically active peptides and requires a glycine residue at the C-terminus of the precursor. Thus, the "RFG" pattern has been used to scan precursor peptides for the presence of potential FLPs. Beyond this signature, there is sequence variation toward the N-terminus. Nematodes possess more than one *flp* gene,[13,18] with many genes expressing multiple FLP sequences, presenting a broad peptide diversity. This is best illustrated in *C. elegans*, where 31 *flp* genes have

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Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00036-1
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been characterized, encoding precursor peptides predicting over 80 unique FLP sequences. Most predicted FLPs have been confirmed, many of the predicted peptides have been isolated, functions assigned, and some receptors identified (Table 1).

Ten of the *C. elegans flp* genes each encode a single unique FLP (Category I, Table 1), and six genes (*flp-6, flp-8, flp-9, flp-14, flp-20, flp-22*) encode multiple copies of single amino acid sequence. The majority of *flp* genes encode multiple unique peptides (Categories III and IV), with two genes encoding seven peptides (*flps-1*, *-13*) and *flp-13* encoding nine unique sequences. In addition to encoding multiple unique amino acid sequences, some *flp* genes in Category IV also code multiple copies of some sequences as well. Most of the genes that encode multiple sequences produce peptides in which the last four residues are identical. Exceptions are *flp*-11, *flp*-13, *flp*-17, *flp*-19. All FLPs have neuromuscular effects, but not all the effects are the same, leading to the hypothesis that a variety of FLP receptors exists. This argument is supported by the large number of unique sequences identified.

Among the many FLPs identified, KHEYLRF (*flp-14*) is present in all nematode species examined and is the most abundant FLP in *C. elegans* and *P. redivivus*. In *C. elegans*, *flp-14* encodes four copies of the peptide. KSAYMRF (*flp-6*) is also present in all species examined, and six copies are encoded by *C. elegans flp-6*. Also, SADPNFLRF and SDPNFLRF (*flp-1*) are widely distributed. The abundance and/or distribution of these FLPs indicate a fundamental importance.

2. Expression. At least nineteen *flp*s are expressed throughout larval and adult stages in *C. elegans*, including both hermaphrodite and male adult stages.[8,14,28] Expression is distributed among various sets of neurons and is conservatively estimated to be present in approximately 160 of the 300 total neurons in the worm. A number of *flp*s are expressed in male-specific neurons associated with mating, as well as in other neurons. Although all detected *flp*s are expressed in neurons, some are also expressed in nonneuronal cells including head muscle (*flp-2*, *flp-11*), pharyngeal muscle (*flp-5*, *flp-15*), sheath cells (*flp-11*, *flp-15*), vulval cells (*flp-10*) and uterine cells (*flp-2*, *flp-11*). *flp-15* is also detected in late embryos. *flp-4* and *flp-8* expression increases in the developmentally quiescent dauer, or diapause, stage. When the worm exits dauer diapause and resumes development, expression of these two genes ceases in a specific set of neurons. The large number and variety of unique FLPs indicate redundancy to ensure that critical physiological and developmental events occur and may also enable flexible and specific regulation of such events. Receptors, for example, may use multiple ligands to modulate responses to various similar, but distinct, messages.[8]

3. Receptors and function. Over a dozen receptors have been characterized from *C. elegans*. All are G protein-coupled, 7-transmembrane members of the rhodopsin family.[17] NPR-1 (gene **C39E6.6**)[10,28] is a 457aa protein that specifically binds ligand products of *flp-18* (EMPGVLRF) and *flp-21* (GLGPRPLRF). Two naturally occurring versions of the receptor differ by a single residue at position 215 (NPR-1-215F, NPR-1-215V), with NPR-1-215F coded by the ancestral gene. *C. elegans* strains exhibit either social or solitary feeding behaviors, and NPR-1-215V suppresses social feeding and is functionally dominant over NPR-1-215F. Each variant, expressed in *Xenopus laevis* oocytes, binds FLP-21 equally, whereas only NPR-1-215V binds FLP-18 (EMPGVLRF). In contrast, both receptors expressed in *C. elegans* pharyngeal muscle bind each ligand equally. This suggests that the receptors may respond differently to the cellular environment. Overexpression of *flp-21* or injection of FLP-21 suppresses social feeding in NPR-1-215F animals, whereas *flp-21* deletion promotes social feeding in animals expressing either receptor variant. A homolog is predicted by *C. briggsae* gene CBG14540.

FLP-15 peptides GGPQGPLRF and RGPSGPLRF are ligands for receptor NPR-3, encoded by **C10C6.2**.[11] The 376 aa receptor protein was expressed in Chinese hamster ovary cells and exhibited low ligand binding at 37°C. Only after a shift of the culture to 28°C was robust binding obtained, as was also observed with NPR-1.[11] Gene knockout using RNAi produced phenotypes with greatly impaired locomotion and paralysis.[7] A homolog is predicted by *C. briggsae* gene CBG06153.

The gene **C26F1.6** coding for receptor VRFa-1 was cloned and expressed in a human kidney cell line.[22] The expressed protein (360aa) was selectively activated by *flp-7* product TPMQRSSMVRF and AMRNALVRF from *flp-11* at similar ligand doses (1–1.3μM). The receptor specificity was determined by the entire length of the ligand, indicating that variations toward the N-terminus are functionally important. RNAi gene knockout in *C. elegans* produced an increase in egg laying and progeny. A homolog is encoded by *C. briggsae* gene CBG20584.

FLPs SPREPERF and LRGEPIRF (*flp-2*) were confirmed as ligands for **T19F4.1.**[21] Two alternative splice variants of the receptor were isolated (**T19F4.1a**, **T19F4.1b**). The **T19F4.1b** protein (432aa) and **T19F4.1a** protein (402aa) differ primarily in the C-terminal region, suggesting different specificities in G protein coupling. SPREPER-F_{NH2} activated both receptor isoforms at approximately 50nM EC_{50}, whereas EC_{50} for $LRGEPIRF_{NH2}$ was >6μM for each variant. In contrast to other *C. elegans*, FLP receptors expressed in mammalian systems, **T19F4.1** is active at 37°C. A homolog is encoded by *C. briggsae* CBG19036. The orphan GPCR **Y59H11AL.1**, similar to insect tachykinin and neuropeptide-Y receptors, was expressed in human

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TABLE 1 Arbitrary *C. elegans* FLP Categories

Gene	WormBase ID	Peptide	No.	Receptors
Category I				
flp-10	T06C10.4	QPKARSGYIRFG		
flp-12	C05E11.8	RNKFEFIRFG[C]		
flp-21	C26F1.10	GLGPRPLRFG		C39E6.6, C25G6.5
flp-23	F22B7.2	TKFQDFLRFG		
flp-24	C24A1.1	VPSAGDMMVRFG		
flp-27	C25H3.5	GLGGRMRFG		
flp-28	W07E11.4	APNRVLMRFG		
flp-32	R03A10.2	AMRNSLVRFG		
flp-33	T07D10.6	APLEGFEDMSGFLRTI-DGIQKPRFG		
flp-34	R09A1.5	ALNRDSLVASLNNAE-RLRFG		
Category II				
flp-6	F07D3.2	KSAYMRFG[C, P]	6×	
flp-8	F31F6.4a	KNEFIRFG[C]	3×	
flp-9	C36H8.3	KPSFVRFG[C]	2×	Y59H11AL.1
flp-14	Y37D8A.15	KHEYLRFG[C, P]	4×	C25G6.5, C16D6.2
flp-20	E01H11.3	AMMRFG	2×	
flp-22	F39H2.1	SPSAKWMRFG	3×	Y59H11AL.1
Category III				
flp-1	F23B2.5a	SADPNFLRFG[C, P] SQPNFLRFG[C] ASGDPNFLRFG[C] SDPNFLRFG[C, P] AAADPNFLRFG[C] KPNFLRFG[C] AGSDPNFLRFG		C25G6.5, C16D6.2
flp-2	W07E11.3a	SPREPIRFG LRGEPIRFG		T19F4.1
flp-3	W07E11.2	SPLGTMRFG TPLGTMRFG EAEEPLGTMRFG ASEDALFGTMRFG NPLGTMRFG EDGNAPFGTMRFG SAEPFGTMRFG SADDSAPFGTMRFG NPENDTPFGTMRFG		C53C7.1a, C16D6.2
flp-4	C18D1.3	PTFIRFG ASPSFIRFG		C16D6.2
flp-5	C03G5.7	GAKFIRFG AGAKFIRFG APKPKFIRFG		C25G6.5

(Continued)

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TABLE 1 Arbitrary *C. elegans* FLP Categories—Cont'd

Gene	WormBase ID	Peptide	No.	Receptors
flp-11	K02G10.4a	AMRNALVRFG NGAPQPFVRFG ASGGMRNALVRFG		C16D6.2, C26F1.6, Y59H11AL.1
flp-15	ZK525.1	GGPQGPLRFG RGPSGPLRFG		C10C6.2, C16D6.2
flp-19	M79.4	WANQVRFG ASWASSVRFG		
flp-25	K04H4.7	DYDFVRFG ASYDYIRFG		
flp-26	R173.4	FNADDLTLRFG GGAGEPLAFSPDMLSLRFG		
Category IV				
flp-7	F49E10.3	SPMQRSSMVRFG TPMQRSSMVRFG SPMERSAMVRFG SPMDRSKMVRFG	 3× 2×	C26F1.6, Y59H11AL.1
flp-13	F33D4.3	SDRPTRAMDSPLIRFG AADGAPLIRFG[C] APEASPFIRFG[C] ASPSAPLIRFG SPSAVPLIRFG SAAAPLIRFG ASSAPLIRFG	 2× 2×	Y59H11AL.1
flp-16	F15D4.8	AQTFVRFG GQTFVRFG	2×	
flp-17	C52D10.11	KSAFVRFG KSQYIRFG	2×	
flp-18	Y48D7A.2	DFDGAMPGVLRFG EMPGVLRFG SVPGVLRFG[C] EIPGVLRFG SEVPGVLRFG DVPGVLRFG	 3×	C25G6.5, C39E6.6, Y58G8A.4

The number following a gene is the WormBase sequence identifier. The C-terminal glycine predicts amidation of the mature peptide. The number in brackets following a peptide sequence is the copy number per gene. The number in parentheses following peptide sequence is confirmed receptor. [C]amidated product isolated from *C. elegans*, [P]amidated product isolated from *P. redivivus*. Categories used: I-single sequence, single copy; II-single sequence, multiple copies; III-multiple sequences, single copies; IV-mixed multiple and single copies. Data from WormBase, GenBank and references cited in the text.

(HEK-293) kidney cells and activated by a number of nematode FLP ligands.[20] Products of *flps-1*, *-7*, *-9*, *-11*, *-13*, and *-22* bound to the receptor, with *flp-7* SPMERSAMVRF $_{NH2}$ the most potent ($EC_{50} < 1.1\mu M$) effector.

NEUROPEPTIDE-LIKE PEPTIDES (NLPS)

1. Discovery and structure. Not all nematode neuropeptides are FLPs. Immunochemical screens using antisera against vertebrate and invertebrate antigens provide circumstantial evidence for numerous other neuropeptides in nematodes. More directly, however, a rigorous examination of the *C. elegans* genome was made for neuropeptide-like protein encoding genes (*nlp* genes) using a combination of structural and alignment searches,[19,24] and evaluation of related ESTs in other species. The search actively eliminated any genes encoding peptides ending in RFG; that is, *flps*. Forty-seven *C. elegans nlp* genes encode over 130 unique putative neuropeptides.[13,18] The peptides sort into eleven families based upon conserved motifs and similar ESTs and neuropeptides in other species. The authors[24] list the families in decreasing order of

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confidence that neuropeptides are encoded: **(1)** homology to orcokinin myotropic neuropeptide: *nlp-14* and *nlp-15* contain a C-terminal GFxGF, *nlp-8* and *nlp-15* encode peptides structurally related to those of *nlp-14* and *-15*; **(2)** homology to myomodulin peptides: *nlp-2*, *nlp-22*, *nlp-23* contain FRPG, with the glycine predicting amidation; **(3)** homology to buccalin neuropeptide neurotransmitter: *nlp-1*, *nlp-7*, *nlp-13* contain C-terminal MSFG and related motifs; **(4)** homology to insect allatostatin, buccalin: *nlp*-5 and *nlp*-6 contain C-terminal MGLG and MGFG; **(5)** *nlp-24*, *nlp-25*, and *nlp-27* through *nlp-32* encode YGGWG and YGGYG C-terminal motifs: similar encoded peptides in other nematode and insect ESTs; **(6)** *nlp-9* and *nlp-21* encode GGARAF: similar motifs in other nematodes; **(7)** *nlp-18* and *nlp-20* code for FAFA and related motifs, similar to motifs predicted in other nematode ESTs; *nlp-20* (**F45E4.8**) may reside in an operon with an endopeptidase (**F45E4.7**); **(8–11)** *nlp-4*, *nlp-10* through *nlp-12*, *nlp-17*, and *nlp-19* encode peptides sorted into 4 groups based upon C-terminal motifs and have hallmarks of neuropeptides but are not clearly related to established neuropeptides.

2. Expression and function. Postembryonic expression in *C. elegans* was detected for a number of *nlp*s. Neurons in the ventral nerve cord express *nlp-7*, *-9*, *-14*, *-15*, and *-21*. Pharyngeal neurons express *nlp-3*, *-8*, *-13*, *-18*, *-19*, *-20*, *-21*, and *-24*. Two *nlp*s, *nlp-21* and *nlp-31*, are expressed in embryos. NLP-37 **(F48B9.4)** is expressed in head neurons and affects locomotion and acts through receptor **C13B9.4**. In addition, receptors for *nlp-12* (**T23B3.4**, **Y39A3B.5**) and *nlp-44* (**K10B4.4**) have been identified.[15] Beyond the potential functions inferred from homologous neuropeptides, at least two NLPs (NLP-29, **B0213.4** and NLP-31, **B0213.6**) are proposed to function in the immune response (section 5.2.).

INSULINS (INSS)

1. Discovery and structure. Thirty-nine insulin-like peptides (INSs) are predicted from the *C. elegans* genome. They range in length from 67 to 218 residues and exhibit remarkably little sequence homology. Multiple alignments of INS-1 through INS-38 (WormBase) show that only 2 sequences have as much as 60 percent homology (INS-7, INS-8), 2 are 47 percent homologous (INS-25, INS-29), and two pairs are 40 percent homologous (INS-22, INS-23; INS-25, INS-29). All other alignments reveal <39% homology, with the majority <30%. Clearly, this family of peptides is not characterized by sequence identity. Only through the combined use of an extensive array of multiple sequence search tools, expression searches and molecular modeling were the INS sequences identified.[26]

Caenorhabditis elegans insulin genes predict three basic peptide precursor structures.[25] All structures contain a signal sequence and the B-chain and A-chain peptides characteristic of vertebrate insulin. INS-1 and INS-18 precursors have an intervening C-peptide between the A- and B-chain peptides, similar to human insulin precursor. INSs 2–9 have an F-peptide between the signal sequence and B-chain peptide but no C-peptide. The A- and B-chain peptides are contiguous. Notable here is a dauer-associated (see 3. Receptors and function below) gene, *daf-28* (**Y116F11B.1**), encoding an insulin-like peptide with INSs 2–9 precursor structure.[15] All the remaining INSs have only the signal sequence and contiguous A- and B-peptides.

In addition to signal sequences, and the A-chain and B-chain peptides shared with vertebrate insulins, all *C. elegans* INSs have highly conserved cysteines essential to formation of interchain and intrachain disulfide bonds.[19,20] Based upon the architectures of disulfide bond formation, and the comparison of these architectures with that of human insulin, *C. elegans* INSs have been sorted into three structural groups-α, β, and γ.[20] These are illustrated in Fig. 1 using representative *C. elegans* INS sequences and human insulin as reference. Because sequence homologies are so low, only those portions of the molecules containing the important A-chain peptide and B-chain peptide insulin signatures are compared. α- and β-group members form 3 interchain bonds (B1–A2, B2–A4, B3–A5) and one intrachain bond (A1–A3). The disulfide intrachain bond (A1–A3) is absent from the α-group, but it is suggested that substitute residues (italics, Fig. 1) form hydrophobic interactions to stabilize the structure.[19] The γ-group has two interchain bonds and one intrachain bond and in this respect is most similar to vertebrate insulins. All *C. elegans* INS sequences have residues predicted to enable the formation of α-helices (Fig. 1, underlined). Despite little primary structural homology among their group, and to vertebrate insulin, all *C. elegans* INSs are capable of forming stable tertiary structures recognized by insulin receptors. In fact, INS-6 (**ZK84.6**) can bind to the human insulin receptor.[4]

2. Expression. Insulin genes are widely expressed throughout development and in a number of tissues.[26] Expression patterns for fifteen *ins* genes (*ins-1* through *ins-9*, *ins-11*, *ins-18*, *ins-21*, *ins-22*, *ins-23*) reveal that all except *ins-9* are expressed in the embryo, all are expressed in larval stages, and all but *ins-2* in the adult. All *ins* genes are expressed to varying degrees in the nerve ring and sensory neurons. All except *ins-9* are expressed in nonsensory neurons. Six genes (*ins-1*, *ins-2*, *ins-4*, *ins-5*, *ins-8*, *ins-18*) are also expressed in nonneuronal tissues including vulva, intestine, pharynx, and hypodermis.

3. Receptors and function. A single insulin receptor (*daf-2*; Y55D5A.5) has been described in *C. elegans*, although additional receptors are proposed.[2] DAF-2 is a transmembrane receptor tyrosine kinase homolog of the mammalian insulin/IGF receptor family.[26] It is a central

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                    *    30      *    40         *    50          *   90   *      100  *    110  *
Hu : --------------FVNQHLC---GSHLVEALYLVC------GERGFF-----  -------------KRGIVEQCCTS--ICSLYQLENY-CN-----------

α-type
24 : -------MGLIRANQGPQKAC---GRSMMMKVQKLCA-----GGCTIQNDD--  ----------------LTIKSCST--GYTDAGFISA-CCPSGFVF-----
26 : -------IGNHHHGTKAGLTC---GMNIIERVDKLCN-----GQCTRNYDA--  ----------------LVIKSCHR--GVSDMEFMVA-CCPTMKLFIH---
30 : -------REPVVAAQGAKKTC---GRSLLIKIQQLCH-----GICTVHADD--  ----------------LHETACMK--GLTDSQLINS-CCPPIPQTPFVF-
36 : ------IRKRHPEGKLVIRDC---KRYLIMYSRTICK-----EKCEKFD----  -----------FIFTDLLVEGCHSNQTLSNERTREL-CCPNAGSN-----

β-type
 1 : ----------------SIRLC---GSRLTTTLLAVCRN----QLCTGLTAFKR  ------------KRGGIATECCEK--RCSFAYLKTF-CCNQDDN------
 2 : --------------VQKRLC---GRRLILFMLATC------GECDTD-----  ------------SSEDLSHICCIK--QCDVQDIIRV-CCPNSFRK-----
 3 : --------------GDKVKIC---GTKVLKMVMVMCG-----GECSS------  ------------TNENIATECCEK--MCTMEDITTK-CCPSR--------
 4 : ------------VPAGEVRAC---GRRLLLFVWSTC-----GEPCTPQ-----  ------------EDMDIATVCCTT--QCTPSYIKQA-CCPEK--------
 6 : -----------VPAPGETRAC---GRKLISLVMAVC-----GDLCNPQ-----  ------------EGKDIATECCGN--QCSDDYIRSA-CCP----------
 7 : -----------VPDEKKIYRC---GRRIHSYVFAVCG-----KACESN-----  ------------TEVNIASKCCRE--ECTDDFIRKQ-CCP----------
 8 : -----------VP-EQKNKLC---GKQVLSYVMALCE-----KACDSN-----  ------------TKVDIATKCCRD--ACSDEFIRHQ-CCP----------
 9 : -----------TLETEKIYRC---GRKLYTDVLSACN-----GPCEPG-----  ------------TEQDLSKLCCGN--QCTFVEIRKA-CCADKL-------

γ-type
17 : ---------------GSLKLCPPGGASFLDAFNLICP-----MRRRRR-----  SVSENYNDGGGSLLGRTMNMCCET--GCEFTDIFAI-CNPFG--------
19 : YIIDSSESYEVLMLFGYKRTC---GRRLMNRINRVCVK-------DID-----  -------PADIDPKIKLSEHCCIK--GCTDGWIKKHICSEEVLNFGFFEN
                          1             2        3                          12     3         45
```

B-peptide **A-peptide**

FIGURE 1 Multiple alignment (ClustalW) amino acid sequences encompassing the B-peptide and A-peptide portions of human insulin precursor (Hu; SwissProt P01308) and selected insulin-like peptides from *Caenorhabditis elegans*; INS-1 (**F13B12.5**), INS-2 (**ZK75.2**), INS-3 (**ZK75.3**), INS-4 (**ZK75.1**), INS-6 (**ZK84.6**), INS-7 (**ZK1251.2**), INS-8 (**ZK1251.11**), INS-9 (**C06E2.8**), INS-17 (**F56F3.6**), INS-18 (**T28B8.2**), INS-24 (**ZC334.3**), INS-26 (**ZC334.1**), INS-30 (**ZC334.2**), INS-36 (**Y53H1A.4**); gene identifiers from WormBase. Numbers at the top are position numbers for human insulin. Numbers at the bottom indicate cysteines (shaded) participating in disulfide bonds (see text in Insulins (INSS)/Discovery and structure). Dark shading indicates additional cysteines not found in human or γ-group sequences. Underline indicates residues predicted to enable formation of α-helices. Residues that substitute for cysteines in intrachain bonding of type α-group insulins are italicized.

member of a complex pathway regulating a number of basic events including nematode development, longevity, stress response, and dauer formation. *Caenorhabditis elegans* larvae respond to environmental stress, such as crowding or lack of food, by entering the arrested dauer stage. Upon return of favorable conditions, larvae resume development. Stimulation of DAF-2 promotes increased metabolism, growth, and aging. Downregulation of the receptor can induce larval dauer formation or extended adult lifespan.[9,15,19,26,29] Insulins are proposed to regulate metabolism as ligands of DAF-2. INS-1 (**F13B12.5**) is an antagonist of DAF-2, enhancing dauer arrest,[26] whereas INS-18 (**T28B8.2**) activates DAF-2 and regulates lifespan.[15] Another insulin-like peptide (DAF-28; **Y116F11B.1**) also may be a DAF-2 agonist and prevent dauer arrest. Expression of *ins* genes in sensory neurons and their interaction with DAF-2 support their roles as regulators of response to external and internal cues. Including insulins, nearly 120 genes encoding over 250 neuropeptides have so far been reported in free-living nematodes, but these numbers are most likely conservative.[13]

ANTIMICROBIAL PEPTIDES

1. Discovery and structure. Animals respond to pathogen challenge through the mobilization of complex molecular defenses in the forms of adaptive and/or innate mechanisms. Adaptive systems are characterized by the ability to remember specific antigens and render long-lasting immunity, as in the higher vertebrates. Innate immunity is a more basic, rapid response to infection characterized by phagocytosis and production of antimicrobial peptides. Both types of immunity are present in higher vertebrates, while lower animals, including nematodes, depend solely on innate immunity as defense against pathogens. In nematodes, the production of antibacterial peptide factors (ABFs) in response to bacterial challenge was demonstrated in the animal parasite *Ascaris suum*,[27] in which a family of antibacterial factor like peptides (ASABFs) has been reported.[5,27] The screening of sequence databases with the ASABF amino acid sequence revealed homologies to two peptides both in the *C. elegans* database.[6] Genes for the *C. elegans* homologs (*abf-1* and *abf-2*) were isolated and characterized.[6] They are organized sequentially, produce a polycistronic mRNA, and are thought to form an operon. The product peptides ABF-1 and ABF-2 possess structural properties generally found in antimicrobial peptides, including conserved cysteine residues necessary to stabilize the molecule and preserve activity.[5] Antimicrobial peptides are a rapidly evolving group and sequence comparisons usually reveal limited homologies with no significant sequence homology outside of the nematodes.[30] A BLAST search done for this report using *C. elegans* ABF-1 (BAA89489) and ABF-2 (BAA89490) detected significant homologs outside of *C. elegans*. Two examples of high scoring

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```
AsABF   MKTAIIVVLLVIFASTNAAVDFSSCARMDVEGLSKVAQGLCISSCKFQNCGTCHCEKRGGRPTCVCDRCGRGGGEWPSVPMPKGRSSRGRRHS
ABF-1   --MLYFCLLLVLLLPNNGVSSEASCARMDVEVMQRIAQGLCTSSCTAQKCMTCICKKVDSHPTCFCGGCSN-ANDVSLDTLIS----QLPHN-
ABF-2   MFVRSLFLALLLATIVAADIDFSTCARMDVEILKKAAQGLCITSCSMQNCGTCSCKKRSGRPTCVCYRCANGGGDIPLGALIK----RG----
CbABF   MLFRFLLFTFLCVSHISAGIDFSTCARMDVEVLDKAARGLCITSCSMQNCGTCYCEKRRGRPTCVCSRCANGGGNIPLDSLIK----RGKH--
```

FIGURE 2 Antibacterial peptides. Multiple alignment (ClustalW) of full length amino acid sequences of antibacterial peptides from *Ascaris suum* (AsABF, BAA11943), *Caenorhabditis elegans* (ABF-1, BAA89489; ABF-2, BAA89490), and *C. briggsae* (CbABF, **CAP31030**). Identical positions are shaded and include 8 conserved cysteines; signal peptides are underlined and italicized. Predicted signal sequence is underlined only. Accession numbers are from GenBank.

sequences from *A. suum* ASABF (BAA11943) and *C. briggsae* **CAP31030** (CBR-ABF2), are compared with the *C. elegans* sequences in Fig. 2. Sequence homology of ABF-1 is low (35–36 percent to the other 3 peptides), while homology of ASABF is 52 and 54 percent, respectively, to ABF-2 and CAP31030. ABF-2 has antibacterial activity,[6] and is 72 percent homologous to CAP31030. Signal peptides are present in ASABF and both *C. elegans* ABFs (Fig. 2, underlined, italicized), and a signal sequence is suggested for the *C. briggsae* peptide (underlined only). All 4 sequences share a number of identical residues in 13 locations (Fig. 2, shaded boxes), including the conserved cysteines (8 total).

2. Expression and function. ABF-2 was detected immunologically only in the pharyngeal region of larval and adult hermaphrodite stages of *C. elegans*.[6] *abf-1* and *abf-2* transcripts were detected in adult hermaphrodites and eggs. Protein expression was detected in larval and adult hermaphrodite stages but not in pre-hatch embryos. ABF-2 was observed in the pharynx, pharyngeal neurons, and excretory cells, whereas ABF-1 was detected in the pharynx and body wall muscle.[6] Only in *A. suum* has *in vivo* bacterial challenge been shown to induce production of nematode antibacterial peptides,[27] but *in vitro* experiments and structural analyses of candidate peptides argue strongly that *C. elegans* ABFs are true antibacterial peptides.[6,30] It is interesting that expression of two neuropeptide-like proteins (NLP-29 and NLP-31) was induced in *C. elegans* exposed to fungal or bacterial pathogens,[1] and that synthetic NLP-31 has both potent antifungal and moderate antibacterial activities. NLP-29 and NLP-31 each have <22 percent homology to any of the peptides in Fig. 2 and lack the conserved cysteines. Also, the DAF-2 signaling pathway seems to function in pathogen resistance.[3,23]

TABLE 2 Overview of 3 Peptide Gene Families in *C. elegans*

Measure	FLP	NLP	INS
Number of genes	31	48	42
Total peptides	101	153**	84
Percent unique peptides	71.3	89.5	100
Percent genes encoding multiple peptides*	77.4	81.3	100
Known receptors	16	5	1
Number of genes encoding known ligands†	14	4	8

Data used for summaries obtained from WormBase and references cited in the text.
*Includes unique sequences and multiple copy sequences.
†9 flp genes encode some sequences associated with more than one receptor, and some receptors are associated with more than one gene.
**Includes antimicrobial sequences.

SUMMARY

Free-living nematode peptides occupy 3 large families containing 31–42 genes that encode 84–153 peptides. Most of the genes encode multiple peptides (Table 2). One family (NLPs) contains a subset of antimicrobial peptides that might be considered as a fourth, smaller family. Information on gene expression, receptor recognition and identification, and biological activity continues to grow. With this information, analyses of the complex and varied environment of nematode peptides will continue to add to our understanding of the development, evolution, and mechanism of signaling systems.

REFERENCES

1. Couillault C, Pujol N, Reboul J, Sabatier L, Guichou J-F, Kohara Y, et al. TLR-independent control of innate immunity in *Caenorhabditis elegans* by the TIR domain adaptor protein TIR-1, an ortholog of human SARM. *Nat Immunol* 2004;**5**:488–94.
2. Dlakic M. A new family of putative insulin receptor-like proteins in *C. elegans*. *Curr Biol* 2002;**12**:155–7.
3. Garsin DA, Villanueva JM, Begun J, Kim DH, Sifri CD, Calderwood SB, et al. Long-lived *C. elegans daf-2* mutants are resistant to bacterial pathogens. *Science* 1921;**2003**:300.
4. Hua Q-x, Nakagawa SH, Wilken J, Ramos RR, Jia W, Bass J, et al. A divergent INS protein in *Caenorhabditis elegans* structurally resembles human insulin and activates the human insulin receptor. *Genes Dev* 2003;**17**:826–31.
5. Kato Y, Komatsu S. ASABF, a novel cysteine-rich antibacterial peptide isolated from the nematode *Ascaris suum*. *J Biol Chem* 1996;**271**:30493–8.

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6. Kato Y, Aizawa T, Hoshino H, Kawano K, Nitta K, Zhang H. *abf-1* and *abf-2*, ASABF-type antimicrobial peptide genes in *Caenorhabditis elegans*. *Biochem J* 2002;**361**:221–30.
7. Keating CD, Kriek N, Daniels M, Ashcroft NR, Hopper NA, Siney EJ, et al. Whole-genome analysis of 60 G protein-coupled receptors in *Caenorhabditis elegans* by gene knockout with RNAi. *Curr Biol* 2003;**13**:1715–20.
8. Kim K, Li C. Expression and regulation of an FMRFamide-related neuropeptide gene family in *Caenorhabditis elegans*. *J Comp Neurol* 2004;**475**:540–50.
9. Kimura KD, Tissenbaum HA, Liu Y, Ruvkun G. *daf-2* an insulin receptor-like gene that regulates longevity and diapause in *Caenorhabditis elegans*. *Science* 1997;**277**:942–6.
10. Kubiak TM, Larsen MJ, Nulf SC, Zantello MR, Burton KJ, Bowman JW, et al. Differential activation of "social" and "solitary" variants of the *Caenorhabditis elegans* G-protein-coupled receptor NPR-1 by its cognate ligand AF9. *J Biol Chem* 2003;**278**: 33724–9.
11. Kubiak TM, Larsen MJ, Zantello MR, Bowman JW, Nulf SC, Lowery DE. Functional annotation of the putative orphan *Caenorhabditis elegans* G-protein-coupled receptor C10C6.2 as a FLP-15 peptide receptor. *J Biol Chem* 2003;**278**:42115–20.

11a. Lee DL, editor. *The biology of nematodes*. London: Taylor & Francis; 2002. p. 648.

12. Li C. The ever expanding neuropeptide gene families in the nematode *Caenorhabditis elegans*. *Parasitology* 2005;**131**:S109–27.
13. Li C, Kim K. Neuropeptide gene families in *Caenorhabditis elegans*. In: Geary TG, Maule AG, editors. *Neuropeptide systems as targets for parasite and pest control*. New York: Springer Science+Business Media; 2010. p. 98–137.
14. Li C, Kim K, Nelson L. FMRFamide-related neuropeptide gene family in *Caenorhabditis elegans*. *Brain Res* 1999;**848**:26–34.
15. Li W, Kennedy SG, Ruvkun G. *daf-28* encodes a *C. elegans* insulin superfamily member that is regulated by environmental cues and acts in the DAF-2 pathway. *Genes Dev* 2003;**17**:844–58.
16. Maule AG, Mousley A, Marks NJ, Day TA, Thompson DP, Geary TG, et al. Neuropeptide signaling systems-potential drug targets for parasite and pest control. *Curr Topics Med Chem* 2002;**2**: 733–58.
17. McVeigh P, Geary TG, Marks NJ, Maule AG. The FLP-side of nematodes. *Trends Parasitol* 2006;**22**:385–96.
18. McVeigh P, Leech S, Mair GR, Marks NJ, Geary TG, Maule AG. Analysis of FMRFamide-like peptide (FLP) diversity in phylum Nematoda. *Int J Parisitol* 2005;**35**:1043–60.
19. McVeigh P, Alexander-Bowman S, Veal E, Mousley A, Marks NJ, Maule AG. Neuropeptide-like protein diversity in phylum Nematoda. *Int J Parisitol* 2008;**38**:1493–503.
20. Mertens I, Clinckspoor I, Janssen T, Nachman R, Schoofs L. FMRFamide related peptide ligands activate the *Caenorhabditis elegans* orphan GPCR Y59H11AL.1. *Peptides* 2006;**27**:1291–6.
21. Mertens I, Meeusen T, Janssen T, Nachman R, Schoofs L. Molecular characterization of two G protein-coupled receptor splice variants as FLP2 receptors in *Caenorhabditis elegans*. *Biochem Biophys Res Commun* 2005;**330**:967–74.
22. Mertens I, Vandingenen A, Meeusen T, Janssen T, Luyten W, Nachman RJ, et al. Functional characterization of the putative orphan neuropeptide G-protein coupled receptor C26F1.6 in *Caenorhabditis elegans*. *FEBS Lett* 2004;**573**:55–60.
23. Millet ACM, Ewbank J. Immunity in *Caenorhabditis elegans*. *Curr Opin Immunol* 2004;**16**:4–9.
24. Nathoo AN, Moeller RA, Westlund BA, Hart AC. Identification of neuropeptide-like protein gene families in *Caenorhabditis elegans* and other species. *PNAS* 2001;**98**:14000–5.
25. Nelson DW, Padgett RW. Insulin worms its way into the spotlight. *Genes Dev* 2003;**17**:813–8.
26. Pierce SB, Costa M, Wisotzkey R, Devadhar S, Homburger SA, Buchman AR, et al. Regulation of DAF-2 receptor signaling by human insulin and *ins-1*, a member of the unusually large and diverse *C. elegans* insulin gene family. *Genes Dev* 2001;**15**:672–86.
27. Pillai A, Ueno S, Zhang H, Kato Y. Induction of ASABF (*Ascaris suum* antibacterial factor)-type antimicrobial peptides by bacterial injection: novel members of ASABF in the nematode *Ascaris suum*. *Biochem J* 2003;**371**:663–8.
28. Rogers C, Reale V, Kim K, Chatwin H, Li C, Evans P, et al. Inhibition of *Caenorhabditis elegans* social feeding by FMRFamide-related peptide activation of NPR-1. *Nat Neurosci* 2003;**6**:1178–85.
29. Tatar M, Bartke A, Antebi A. The endocrine regulation of aging by insulin-like signals. *Science* 2003;**299**:1346–51.
30. Zhang H, Kato Y. Common properties specifically found in the CSαβ-type antimicrobial peptides in nematodes and mollusks: evidence for the same evolutionary origin? *Dev Comp Immunol* 2003;**27**:499–503.

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Chapter 37

FXPRLamide (Pyrokinin/PBAN) Family

Miriam Altstein, Aliza Hariton and Ronald J. Nachman

ABSTRACT

The pyrokinin/pheromone biosynthesis-activating neuropeptide (PK/PBAN) family is one of the most studied neuropeptide (Np) families in insects and is one of the most important in insect physiology regulation. Physiological functions regulated by the PK/PBAN peptides include development, mating, muscle contraction, and tanning. Members of the family are found in many Lepidopteran and other insect species. In this chapter, we briefly present a historical perspective of the discovery of the PK/PBAN peptides, provide details on the structure of the PK/PBAN genes and the processing of their prohormone to bioactive peptides, describe the distribution of the mRNA and peptides in the insect nervous system, and summarize the current knowledge on the PK/PBAN receptors, their signaling mechanisms, and their biological activity. Employment of the PK/PBAN family of peptides as a basis for designing a novel generation of insect-control agents based on Np antagonists is also briefly discussed.

INTRODUCTION

The pyrokinin/pheromone biosynthesis-activating neuropeptide (PK/PBAN) family of peptides is one of the most studied neuropeptide (Np) families in insects and is one of the most important in regulating their physiology. Physiological functions regulated by the PK/PBAN peptides include development and mating, as well as muscle contraction activities and induction of cuticular melanization. The family is present in a wide variety of insects and comprises the following C-terminally amidated peptides: PKs; myotropins (MTs); PBANs; diapause hormone (DH); and β- and γ-subesophageal Nps (SGNPs), all of which share a common sequence (FXPRLa). PK/PBAN peptides have been studied for nearly 30 years, mainly in moths, during which many new members have been identified, the genes of the peptides and their receptors have been cloned and characterized, and their distribution in the nervous system studied in many moth species; their physiological roles have been determined through physiological and behavioral analyses at the whole-organism level, complemented by molecular and cellular studies. The PK/PBAN peptides also form a basis for the development of new-generation insect-control agents based on their antagonists.

In this chapter, we review the current knowledge of the PK/PBAN family of peptides, most of which is based on studies with PBAN and DH in Lepidopterans and describe the application of this knowledge to the generation of highly potent, selective, and readily bioavailable potential insect-control agents. The amount of information accumulated on this family is too large to be covered within the framework of this chapter, and the reader is referred to the reviews cited in the text. For the convenience of the reader, and to minimize confusion that might arise from the complexity of the peptide terminology, each of the main sections below is divided into subsections based on *pban* or *capa* gene-encoded peptides and their characteristics in Lepidopteran or non-Lepidopteran species.

DISCOVERY OF THE PK/PBAN FAMILY OF PEPTIDES: HISTORICAL PERSPECTIVE

PBAN

The first member of the family to be discovered was leucopyrokinin (LPK), identified in the cockroach *Leucophaea maderae* in 1986.[14] It was followed in 1989 by the pheromone biosynthesis activation neuropeptide (PBAN), first reported by Raina and Klun[29] as a Np that regulates sex pheromone production in the female moth *Helicoverpa zea*; Raina et al.[28] isolated and characterized it as a 33 amino acid, C-terminally amidated Np, which was designated *Hez*-PBAN. Since 1989, the primary sequences of 24 PBAN molecules from many other moth species have been determined[3] (Table 1). PBAN molecules were also found in insects belonging to orders other than Lepidoptera, for example, *Drosophila melanogaster*, *Anopheles*

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00037-3
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TABLE 1 Amino Acid Sequence of PBAN and Other Peptides of the PK/PBAN Family

Code name	Insect species	Amino acid sequence
Hez-PBAN	*Helicoverpa zea*	LSDDMPATPADQEMYRQDPEQIDSRTKY**FS** **PRLa**
Bom-PBAN-I	*Bombyx mori*	LSEDMPATPADQEMYQPDPEEMESRTRY**FS** **PRLa**
Bom-PBAN-II	*Bombyx mori*	RLSEDMPATPADQEMYQPDPEEMESRTRY**FS** **PRLa**
Bma-PBAN	*Bombyx mandarina*	LSEDMPATPADQEIYQPDPEVMESRTRY**FS** **PRLa**
Lyd-PBAN	*Lymantria dispar*	LADDMPATMADQEVYRPEPEQIDSRNKY**FS** **PRLa**
Has-PBAN	*Helicoverpa assulta*	LSDDMPATPADQEMYRQDPEQIDSRTKY**FS** **PRLa**
Agi-PBAN	*Agrotis ipsilon*	LADDTPATPADQEMYRPDPEQIDSRTKY**FS** **PRLa**
Mab-PBAN	*Mamestra brassicae*	LADDMPATPADQEMYRPDPEQIDSRTKY**FS** **PRLa**
Spl-PBAN	*Spodoptera littoralis*	LADDMPATPADQELYRPDPDQIDSRTKY**FS** **PRLa**
Plx-PBAN	*Plutella xylostella*	RLKDSGLAPPDEYRTPELLDARAQY**FS** **PRLa**
Har-PBAN	*Helicoverpa armigera*	LSDDMPATPADQEMYRQDPEQIDSRTKY**FS** **PRLa**
Hev-PBAN	*Heliothis virescens*	ADDMPATPADQEMYRQDPEQIDSRRTKY**FS** **PRLa**
Spe-PBAN	*Spodoptera exigua*	LSDDMPATPADQELYRPDPDQIDSRTKY**FS** **PRLa**
Anp-PBAN	*Antheraea pernyi*	LSDDMPATPKDQEMYHQDPEQVDTRTRY**FS** **PRLa**
Scr-PBAN	*Samia cynthia ricini*	LTEDMPATPTDQEMFDQDPEQIDTRTRY**FS** **PRLa**
Ado-PBAN	*Adoxophyes sp*	QSEAVTSSDEQVYRQDMSPVDGRLKY**FS** **PRLa**
Mas-PBAN	*Manduca sexta*	ISEDMPATPSDQEYPMYHPDPEQIDTRTRY**FS** **PRLa**
Assc-PBAN	*Ascotis selenaria cretacea*	QLVDDVPQRQQIEEDRLGSRTRF**FS** **PRLa**
Cla-PBAN	*Clostera anastomosis*	LADDMPATPSDQEYYRQDPEQIDSRSNY**FS** **PRLa**
Ort-PBAN	*Orgyia thyellina*	LSDDMPATPPDQEYYRPDPEQIDSRTKY**FS** **PRLa**
Sol-PBAN	*Solenopsis invicta*	SYGDAYEVDEDDHPL**F**V**PRL**a
	Solenopsis richetri	SYGDAYEVDEDDHPL**F**V**PRL**a
	Solenopsis geminate	SYGDAYEVDEDDHPL**F**V**PRL**a
	Solenopsis pergandii	SYGDAYEVDEDDHPL**F**V**PRL**a
	Solenopsis carolinesis	SFGDAYEVDEDDHPL**F**V**PRL**a
Bom-DH	*Bombyx mori*	TDMKDESDRGAHSERGALW**FG** **PRLa**
Hez-DH	*Helicoverpa zea*	NDVKDGAASGAHSDRLGLW**FG** **PRLa**
Has-DH	*Helicoverpa assulta*	NDVKDGAASGAHSDRLGLW**FG** **PRLa**
Har-DH	*Helicoverpa armigera*	NDVKDGAASGAHSDRLGLW**FG** **PRLa**
Agi-DH	*Agrotis ipsilon*	NDVKDGGADRAHSDRGGMW**FG** **PRLa**
Spl-DH	*Spodoptera littoralis*	NEIKDGGSDRGAHSDRAGLW**FG** **PRLa**
Lom-PK-I	*Locusta migratoria*	pEDSGDGWPQQP**F**V**PRLa**
Lom-PK-II	*Locusta migratoria*	pESVPT**FT** **PRLa**
Lem-PK	*Leucophaea maderae*	pETS**FT** **PRLa**

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TABLE 1 Amino Acid Sequence of PBAN and Other Peptides of the PK/PBAN Family—Cont'd

Pea-PK-5	*Periplaneta americana*	GGGGSGETSGMW**FG**PRLa
Pea-PK-6	*Periplaneta americana*	SESEVPGMW**FG**PRLa
Lom-MT-I	*Locusta migratoria*	GAVPAAQ**FS**PRLa
Lom-MT-II	*Locusta migratoria*	EGD**FT**PRLa
Lom-MT-III	*Locusta migratoria*	RQQP**FV**PRLa
Lom-MT-VI	*Locusta migratoria*	RLHQNGMP**FS**PRLa
Scg-MT-1	*Schistocerca gregaria*	GAAPAAQ**FS**PRLa
Pss-β-SGNP (PT)	*Pseudaletia separata*	KLSYDDKVFENVE**FT**PRLa
Hez-β-SGNP	*Helicoverpa zea*	SLAYDDKSFENVE**FT**PRLa
Has-β-SGNP	*Helicoverpa assulta*	SLAYDDKSFENVE**FT**PRLa
Har-β-SGNP	*Helicoverpa armigera*	SLAYDDKSFENVE**FT**PRLa
Agi-β-SGNP	*Agrotis ipsilon*	SLSYEDKMFDNVE**FT**PRLa
Mab-β-SGNP	*Mamestra brassicae*	SLAYDDKVFENVE**FT**PRLa
Spl-β-SGNP	*Spodoptera littoralis*	SLAYDDKVFENVE**FT**PRLa
Bom-β-SGNP*	*Bombyx mori*	SVAKPQTHESLE**FI**PRLa
Drm-β-SGNP (PK-2)*	*Drosophila melanogaster*	SVP**FK**PRLa
Hez-γ-SGNP	*Helicoverpa zea*	TMN**FS**PRLa
Has-γ-SGNP	*Helicoverpa assulta*	TMN**FS**PRLa
Har-γ-SGNP	*Helicoverpa armigera*	TMN**FS**PRLa
Agi-γ-SGNP	*Agrotis ipsilon*	TMN**FS**PRLa
Mab-γ-SGNP	*Mamestra brassicae*	TMN**FS**PRLa
Spl-γ-SGNP	*Spodoptera littoralis*	TMN**FS**PRLa
Bom-γ-SGNP	*Bombyx mori*	TMS**FS**PRLa

Bold letters indicate conserved amino acid sequences. DH – diapause hormone; PBAN – pheromone biosynthesis-activating neuropeptide; PK – pyrokinin; MT – myotropin; PT – pheromonotropin; SGNP – subesophageal ganglion neuropeptide.
**A member of the family but with the difference that X = Lys or Ilu. The sequence of α-SGNP is the same in all Lepidopterans (VIFTPKLa). References for the listed peptides can be found in Ref. 3 Ort-PBAN and Sol-PBAN were published in Refs. 8, 34, respectively. Additional sequences of PBAN, DH, α-, β-, and γ-SGNP can be found in Ref. 16.*

gambiae, *Aedes aegypti*, *Tribolium castaneum*, and aphids. These peptides were not cloned but were predicted from the genome sequences of the insects.[16] Comparisons among the primary sequences of the PBAN molecules revealed that the Lepidopteran peptides share a high degree of homology (Table 1). Regardless of their length and degree of homology, all PBANs contain the C-terminal pentapeptide signature sequence FSPRLa.

Other Peptides of the Family

Further studies of insect Nps revealed that other peptides also have a very similar C-terminal sequence: FXPRLa (X = S, T, G or V). To mention just a few, PKs (e.g. LPK,[14] Lom-PK-I and Lom-PK-II) and the myotropins (e.g., Lom-MT-I to IV), which are myotropic peptides isolated from the migratory locust, *Locusta migratoria*; PK

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peptides of *Periplaneta americana* —Pea-PK-5 and Pea-PK-6—and the myotropic peptide of *Schistocerca gregaria* (Scg-MT-1). Additional peptides that were found to share the same consensus sequence are β- and γ-SGNPs; pheromonotropin (PT)—an 18 amino acid peptide isolated from *Pseudaletia (Mythimna) separate*—which is now included in the β-SGNP group; and DH, which induces embryonic diapause in *Bombyx mori* and terminates pupal diapause in *Helicoverpa/Heliothis* moths. For review, see Ref. 3 and Table 1. As the number of available sequences has increased, the *X* in the consensus sequence has been extended to include I (as in *Bom*-β-SGNP) and K (as in *Drm*-β-SGNP, see Table 1). All peptides sharing the above sequences were grouped into one family, which was designated the FXPRLa family or the PK/PBAN family, named after PBAN and Lem-PK, which was the first member of this family to be identified[14] and had myostimulatory ("kinin"-like) action and a pGlu residue at its N-terminus, (and thus termed pyrokinin).

More recently, in light of genomic database records, sequence similarities to previously annotated genes and actual gene sequencing, it became apparent that PK/PBAN peptides are encoded by two genes, which are found in many Lepidopteran and non-Lepidopteran insect species.[16,26] These findings help to categorize the FXPRLa peptides into two main groups that are processed from either the *pban* gene or the *capa* gene. To avoid terminological confusion, in this chapter, these peptides will be designated as *pban* gene-encoded peptides (PBAN, DH, β-, and γ-SGNPs) or *capa* gene-encoded peptides (CAPA-PKs), respectively. Exploration of the *capa* gene also reveals two additional periviscerikinin peptides sharing just the PRLa C-terminal sequence; they are termed CAPA-1 and 2 or PVK-1 and 2, and are not addressed in this chapter. For further information on the *pban* and *capa* gene peptides, the reader is referred to Refs. 16, 24, 26 and references therein.

STRUCTURE OF THE mRNA/GENE

PK/PBAN peptides are encoded by two genes: the *pban* gene (also termed *dh-pban* gene)[3,16] and the *capa* gene, which are present in almost all insects.[16,26]

pban Gene

The *pban* gene was first isolated from *H. zea* in 1989 by Raina[28] and encodes the PBAN preprohormone and three additional peptides—DH, β-SGNP and γ-SGNP—all of which contain the family's consensus sequence and one FXPKLa peptide (α-SGNP)[16] (Table 1). To date, the *pban* gene has been cloned from 19 species of Lepidopteran moths, in all of which it encodes all five PK/PBAN peptides. PBAN, DH, and β-SGNP are also produced in many other—non-Lepidopteran insects (e.g., the fire ant, the mosquito and the red flour beetle *Tribolium castaneum*), whereas α- and γ-SGNP are not always encoded.[16] In *Drosophila*, only two peptides are encoded by the *pban* gene (termed *hugin* gene).[16]

capa Gene

The *capa* gene was first identified in *D. melanogaster*. It encodes a CAPA-PK and two periviscerokinins—PVK-1 and PVK-2—which contain a PRLa sequence at their carboxy terminus.[16,26] CAPA-PK is also termed DH-like PK (because of the C-terminal-consensus WFGPRLa sequence typical of all DH Nps), CAPA-3 or myotropin-1 in *Drosophil*a, PK-1 in *Manduca sexta*, and PK-5 and PK-6 in *P. americana* (Table 1). The *capa* gene is present in many insect species, including Lepidopterans, and it encodes CAPA-PK in all tested insects other than *Apis mellifera.*[16] To the best of our knowledge, the *pban* or *capa* genes have not yet been cloned from *L. migratoria*, *S. gregaria* or *P. americana* although CAPA-PKs have been isolated, or predicted in light of sequence homologies, and localized in various parts of the nervous system.

PROCESSING OF THE PRECURSOR

pban Gene and *capa* Gene

The *pban* gene and the *capa* gene are translated into typical Np preprohormones, which contain, at their N-terminus, a signal sequence that is removed before their transfer to the Golgi apparatus. In both genes, all bioactive peptides in the prohormone are separated by single or pairs of basic amino acids at their N-terminus, and by a Gly followed by a single or pair of basic amino acids at the C-terminus, which serve as post-translational sites for processing and C-terminal amidation enzyme, respectively (e.g., see a *pban* gene structure and processing sites of *Orgyia thyellina*[34] and a *capa* gene of *M. sexta*).[19]

Peptides derived from the *capa* gene undergo cell-type-specific sorting and packaging and were the first insect Nps shown to be differentially processed. Differential processing has been shown in *D. melanogaster* and in *B. mori* and in *P. americana* in which it exhibits an interesting and uncommon phenomenon, that different peptides originating from the *capa* gene could be individually packaged and fuse with each other and with other peptide-containing vesicles in the cytoplasm, after leaving the trans-Golgi network. A detailed description of the *capa* gene prohormone processing, alternative-splicing mechanism, sorting, and packaging is presented in Refs. 26, 30.

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DISTRIBUTION OF THE PEPTIDE/mRNA

Peptides

pban Gene-Derived Peptides

PBAN in Lepidoptera

PBAN distribution was studied by immunochemical methods (radioimmunoassay, enzyme-linked immunosorbent assays (ELISA) and immunocytochemical (ICC) methods) in adult females, males and larvae of various Lepidopteran species (e.g., *H. zea*, *M. sexta*, *B. mori*). These studies found PBAN-immunoreactive (IR) neurons in the brain and subesophageal ganglion (SOG) that projects to the corpora cardiaca (CC) and in ganglia of the ventral nerve cord (VNC). In the SOG, PBAN is found in seven pairs of neurosecretory cells. PBAN-IR is also found in the CC and in a pair of cells in the thoracic and abdominal ganglia. The ICC studies also reveal two pairs of axons originating from cell bodies in the SOG, which extend the entire length of the VNC and terminate in the terminal abdominal ganglia. ELISA studies indicate that the brain–SOG complex contains the highest level of PBAN-like IR, followed by the CC and the thoracic ganglia, and that abdominal ganglia contains low levels of the peptide. A more detailed analysis of PBAN-IR-containing cells, by means of matrix-associated laser desorption/ionization combined with mass spectrometry (MALDI/MS), reveal a basically similar distribution. For review, see Refs. 11, 27.

DH in Lepidoptera

DH distribution was studied by ICC analyses in *H. armigera* and *B. mori*. In *H. armigera*, DH is localized in the mandibular, maxillary, and labial cell clusters of the SOG, in a pair of ventral midline lateral neurons, and in each thoracic ganglion at all the developmental stages (embryo, larva, pupa, and adults).[33] In *B. mori*, immunoreactive neural processes with varicosities are in the CC and the corpus allatum (CA); as in *H. armigera*, IR is detected at all developmental stages.[21]

PBAN in Non-Lepidopteran Species

ICC testing of the distribution of PBAN in the fire ant *Solenopsis invicta* found that the peptide-producing neurons are located in the SOG and CC in all adult sexual forms. PBAN-IR is also detected in two segmented thoracic and four segmented abdominal ganglia, which project to the neurohemal perisympathetic organs (PSOs), indicating release of PBAN to the hemolymph.[9]

Other FXPRLa-Containing Peptides in Non-Lepidopteran Species

More recently, detailed bioinformatic studies were combined with MALDI-TOF and electrospray ionization quadrupole (ESI-Q)-TOF MS to make an inventory of Nps containing the PK/PBAN consensus sequence in different parts (ganglia and nerves) of the central nervous systems (CNSs) of *L. migratoria* and *S. gregaria*. In both species, the peptides are detected in the SOG, in the retrocerebral complex, which comprises the CC and the CA, and in the hypocerebral ganglion, which is part of the stomatogastric nervous system. In *S. gregaria* FXPRLa-containing peptides are also detected in the recurrent and esophageal nerves (which are also part of the stomatogastric nervous system).[10] Because the *pban* gene and *capa* gene have not yet been cloned from locusts, it is difficult to relate these peptides (termed PKs in the above study) to any of the PK/PBAN family gene products.

capa Gene-Derived Peptides

CAPA-PKs in Lepidopteran and Non-Lepidopteran Species

CAPA-PK peptides are the characteristic and most abundant Nps in the abdominal neurohemal system. The location and projection of CAPA neurons was studied in D. melanogaster, P. americana and M. sexta. So far, CAPA-PKs are expressed only in the CNS and not in any other tissue. The peptides are synthesized in median neurosecretory neurons of the abdominal ganglia and are released from the PSOs into the hemolymph. In Drosophila, a truncated form of CAPA-PK was detected in the neurosecretory cells of the SOG, in the retrocerebral complex, and in the larval ring gland, it was also found in the SOG of M. sexta. In P. americana, CAPA-PKs also occur in interneurons, from which they may be released into the CNS. A detailed review on the distribution of CAPA peptides by MS is presented in Ref. 26.

Gene/mRNA

pban Gene Expression in Lepidopteran and Non-Lepidopteran Species

pban gene distribution and expression were determined by Northern blotting, real-time polymerase chain reaction (RT-PCR), and in situ hybridization, and the results are similar to those obtained by immunochemistry. PBAN mRNA is expressed in both male and female moths and is detected in the SOG as well as in the brain, thoracic ganglia, and abdominal ganglia of larvae, pupae, and adults of several moth species (*H. zea*, *H. armigera*, *M. sexta*, *and B. mori*). Expression is predominantly in the SOG, where gene transcripts are located in the same DH-PBAN-producing neurosecretory cells as detailed above (Ref. 27 and references therein).

pban gene transcripts are also found in the head, thorax, and abdominal ganglia of the female fire ant, *S. invicta*. The strongest transcriptional signal and highest number of *pban* gene copies are detected in the head, and much weaker and

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fewer ones in the thoracic and abdominal ganglia, where the numbers of copies were almost negligible.[9]

capa Gene Transcripts in Lepidopteran and Non-Lepidopteran Species

In situ hybridization of CAPA mRNA was performed in Lepidopteran and non-Lepidopteran species. In *M. sexta*, about 15 pairs of CAPA interneurons were detected in the brain of first-instar larvae. The number of neurons decreased during postembryonic development and only one pair expressed CAPA mRNA in the adult brain.[19]

RECEPTORS AND SIGNALING CASCADE

PK/PBAN Receptors

A variety of techniques—bioinformatic, biochemical, molecular, physiological, pharmacological, and histochemical—were used to predict, clone, isolate, locate, and characterize the PK/PBAN receptors in various insects. The great majority of the studies focused on the receptors that mediate the pheromonotropic activity in moths. Early studies had suggested that PBAN might act on a target other than the pheromone gland (see Ref. 3 and references therein), but since the late 1990s, it became clear, beyond any doubt, that in most moth species, PBAN acts directly on the pheromone gland via the PK/PBAN receptors. A histochemical study of the pheromone gland cells of *H. peltigera* yielded the first direct indication of the presence of the PK/PBAN receptors in the pheromone gland.[1]

Similarly to Nps of other vertebrates and invertebrates, the PK/PBAN Nps activate cellular processes via G-protein-coupled receptors (GPCRs). At the turn of the century, many studies on cloning of insect Np GPCRs, including those of the PK/PBAN family, were stimulated by the *Drosophila* Genome Project,[13] and by completion of genome sequences of other insects. In light of these data, it is now apparent that all the PK/PBAN receptors fall into two groups: receptors for *capa* gene peptides—herein termed PK-1/DH-receptors (PK-1/DH-R); and receptors for the *pban* gene peptides—herein termed PK-2/PBAN-R. Both receptor types are found in a wide variety of insects and are activated by PK/PBAN/DH peptides, although at differing affinities. In general, PK-1/DH-R exhibits high affinity (at low nM doses) toward WFPRLa-containing peptides (DH signature sequence), whereas other PK/PBAN peptides activate the receptors at much higher concentrations; the PK-2/PBAN-R exhibit high (nanomolar) affinity to PBAN and lower affinity toward other FXPRLa peptides.

So far, 13 PK-2/PBAN-Rs from moths, *Drosophila* and mosquitoes and 2 PK-1/DH-Rs (from *B. mori* and from *Drosophila*) were cloned and expressed in cell lines. Another 12 were annotated according to sequence homologies with already-cloned PK-2/PBAN-R or PK-1/DH-R genes (Table 2). The first receptors were cloned in light of the assumption of Hewes and Taghert[13] that PK/PBAN-R might be homologous to the mammalian neuromedin U receptor (NmU-R) because their ligands—NmU and the PK/PBAN Nps—contain similar motifs at their C-termini, that is, FRPRNa and FSPRLa, respectively, and PBAN was found to activate an NmU-R in *Drosophila*. Primers based on the consensus sequences of NmU and on sequences of genes CG8784 and CG8795, CG9918 and CG14575—which, in the *Drosophila* Genome Project were predicted as coding for GPCRs were designed and were successfully used to clone two *D. melanogaster* receptors (PK-2-1/PBAN and PK-2-2/PBAN, originally termed CG8784 and CG8795, respectively), two *Anopheles* receptors (PK-1/DH and PK-2/PBAN), the *D. melanogaster* PK-1 receptor (PK-1/DH-R also termed, CG9918), and the *B. mori* and *H. zea* PBAN-Rs (Table 2). A few additional PBAN receptors from pheromone glands of adult female moths were cloned later, based on the already published PK/PBAN-R sequences (for example, *H. virescens*, *H. peltigera*, *H. armigera*, *Plutella xylostella*, and *Spodoptera exigua*), and from several different larval instars (for example, *S. littoralis*, *H. virescens*). A DH-R was cloned from pupal ovaries of *B. mori* (Table 2). Interestingly, multiple isoforms were identified in *H. virescens* and *M. sexta* and, most recently, an isoform of the *H. zea* PBAN-R, which contains a long C-terminal tail similar to that of *B. mori* was annotated (*Hez*-PBAN-R-iso; Table 2). In all of the studies, other than that on the *B. mori* pupal ovary, expression was monitored by observing Ca^{2+} influx; in the case of the *B. mori* pupal ovary receptor, it was monitored by voltage clamping. The occurrence of PBAN-R in adult male moths has also been reported.[4] Receptor characterization is detailed in Refs. 3, 16–18, 20 and references therein.

A detailed evolutionary trace analysis, based on sequence alignments of the actually cloned and the predicted PK-2/PBAN-Rs and PK-1/DH-Rs, aimed to discover highly conserved and group-specific amino acids that might predict global and group-specific putative ligand recognition and docking sites was conducted. In both groups, the study reveals several highly conserved trans-membrane (TM) amino acid residues that form, most probably, the binding sites that activate the receptors and some group-specific sequences in the extracellular domains, which could indicate specificity toward different *pban* gene and *capa* gene peptide ligands.[16] A preliminary verification of this hypothesis already was obtained from computer-modeled structure analysis of mutated and/or modified chimeric receptors. All of the studies add important information on the receptor's structure/activity relationship (SAR) and reveal the extracellular and intracellular domains responsible for receptor stability, ligand recognition, and binding.[6,7,17]

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TABLE 2 Summary of PK-1/DH and PK-2/PBAN Cloned Receptors

Insect	Code	Tissue	GenBank Acc. #	Expression sys.
Actually Cloned and Expressed				
Pheromone gland				
Helicoverpa zea	*Hez*-PBAN-R	Pheromone gland	AY319852	Sf-9
Bombyx mori	*Bom*-PBAN-R	Pheromone gland	AB181298	Sf-9
Heliothis virescens	*Hev*-PBAN-R (C)	Pheromone gland	EU000527	CHO
Heliothis peltigera	*Hep*-PBAN-R	Pheromone gland	JN648826	Sf-9
Plutella xylostella	*Plx*-PBAN-R	Pheromone gland	AY974334	HeLa
Spodoptera exigua	*Spe*-PBAN-R	Pheromone gland	ABY62317	–
Helicoverpa armigera	*Har*-PBAN-R	Pheromone gland	AY792036	–
Larvae				
Drosophila melanogaster	*Drm*-PK-1/DH-R (CG9918)	Whole larvae (3rd instar)	AF368273	CHO
Spodoptera littoralis	*Spl*-PBAN-R	Whole larvae (5th instar)	DQ407742	NIH3T3, HEK293 Sf-9
Heliothis virescens	*Hev*-PBAN-R (A)	Larvae CNS (5th instar)	EU000525	CHO
Heliothis virescens	*Hev*-PBAN-R (B)	Larvae CNS (5th instar)	EU000526	CHO
Pupae				
Bombyx mori	*Bom*-DH-R	Developing ovary (pupa)	AB164386	Xenopus oocytes
Whole insect				
Drosophila melanogaster	*Drm*-PK-2-1/PBAN-R (CG8784)	Whole insect	AY277898	CHO
Drosophila melanogaster	*Drm*-PK-2-2/PBAN-R (CG8795)	Whole insect	AY277899	CHO
Anopheles gambiae	*Ang*-PK-1/DH-R	Adult whole insect	AY900218	CHO
Anopheles gambiae	*Ang*-PK-2/PBAN-R	Adult whole insect	AY900219	CHO
Annotated Based on Predicted Sequence Homology				
Aedes aegypti	*Aea* PK-2/PBAN-R	–	XP_001657210	–
Aedes aegypti	*Aea* PK-1/DH-R		XP_001662936	–
Culex quinquefasciatus	*Cug* PK-2/PBAN-R	–	XP_001861460	–
Culex quinquefasciatus	*Cug* PK-1/DH-R	–	XP_001864163	–

(Continued)

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TABLE 2 Summary of PK-1/DH and PK-2/PBAN Cloned Receptors—Cont'd

Insect	Code	Tissue	GenBank Acc. #	Expression sys.
Apis mellifera	*Apm* PK-2/PBAN-R	–	NP_001091688 XP_001122475	–
Apis mellifera	*Apm* PK-1/DH-Ra	–	NP_001157480 XP_623966	–
Tribolium castaneum	*Trb* PK-2/PBAN-R	–	XP_968501	–
Tribolium castaneum	*Trb* PK-1/DH-R	–	XP_968729 XP_968803	–
Acyrthosiphon pisum	*Acp* PK-2/PBAN-R	–	XP_001950091	–
Orgyia thyellina	*Ort*-DH-R	–	AB283041	–
Manduca sexta	*Mas*-PBAN-R-(A)	–	ACQ90219	–
Manduca sexta	*Mas*-PBAN-R-(B)	–	ACQ90220	–
Manduca sexta	*Mas*-PBAN-R-(C)	–	ACQ90221	–
Manduca sexta	*Mas*-PBAN-R-(D)	–	ACQ90222	–
Helicoverpa zea (isoform)	*Hez*-PBAN-R-iso	–	JN206677	–

CHO – Chinese hamster ovary cells; HEK293 – Human embryonic kidney cells.

SIGNALING CASCADE

PK-2/PBAN-R in Lepidopterans

Response to PBAN in Sex Pheromone Biosynthesis

In all moth species tested so far, Ca^{2+} is necessary for the biological response to PBAN and activation of the PBAN-R by the ligand results in an influx of extracellular calcium and a subsequent increase in cytosolic Ca^{2+}. Depletion of Ca^{2+} causes loss of pheromonotropic activity (for review, see Ref. 20).

Although extracellular Ca^{2+} has proved to be absolutely necessary for the bioactivity of PBAN in all moth species tested so far, the intracellular signal transduction cascade stimulated by the Np differs among moth species. In some moths, for example, *Heliothinae* species and *Argyrotaenia velutinana*, the cyclic nucleotide second messenger, cAMP, is a critical component in PBAN signaling, whereas in others, (e.g., *B. mori* and *S. litura*), cAMP do not seem to be involved, hinting at a possible correlation between the steps in the sex pheromone biosynthetic pathway affected by PBAN (early or late) and its ability to activate cAMP. The signaling pathways affected by PBAN in the pheromone gland were extensively studied in *B. mori* and several *Heliothinae* species; detailed reviews on PBAN signal transduction and its downstream cascade of events in biosynthesis of bombykol (the *B. mori* sex pheromone) and other sex pheromones of other moth species can be found in Refs. 17, 20.

Response to PBAN in Cuticular Melanization

PBAN signaling was also examined in a larval receptor of *S. littoralis*, where it induces activation of a matrix-associated protein (MAP) kinase via a signaling mechanism that was protein kinase C (PKC)-dependent but Gαi-independent. As with the pheromone gland receptors, the larval receptor is dependent on Ca^{2+} and responds to PBAN analogs and PT (β-SGNP) by MAP kinase activation.[35]

PK-2/PBAN-R in Non-Lepidopterans

Response to pban Gene–Derived Peptides

Ca^{2+} is also involved in the activation of the PK-2/PBAN-R in *D. melanogaster* and *A. gambiae*, as indicated by Ca^{2+} elevation in CHO cells that express the cloned *Drm*- or *Ang*-PK-2/PBAN-R in response to nanomolar concentrations of *Drm*- or *Ang-pban gene*-derived peptides.[25,31]

PK-1/DH-R in Lepidopterans

Response to DH in Embryonic Diapause

PK-1/DH-R signaling was tested with a *B. mori* pupal ovary receptor, which regulates embryonic diapause. The receptor is also dependent on Ca^{2+} and is coupled to a Gq protein with downstream signaling action involving protein kinase C.[15]

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PK-1/DH-R in Non-Lepidopterans

Response to CAPA-PK

Although the biological function of the CAPA-PK peptides is still unknown, similar results were obtained regarding involvement of Ca^{2+} in activation of the PK-1/DH-R by nanomolar concentrations of *Drm*- or *Ang*-CAPA-PK.[5,25]

BIOLOGICAL ACTIONS

The PK/PBAN family of peptides is a ubiquitous, multifunctional family that plays a major physiological role in regulating a wide range of developmental processes in insects: pupariation, diapause, cuticular melanization, feeding, that is, gut muscle contraction and mating behavior, that is, sex pheromone production. For a review, see Ref. 3. The wide distribution of these peptides and their receptors in all insects hints at the possibility that additional functions of the *pban* gene and *capa* gene peptides may be found in the future.

pban Gene-Derived Peptides

PBAN in Sex Pheromone Biosynthesis in Females

The first function that was discovered to be mediated by a member of the PK/PBAN family was stimulation of sex pheromone biosynthesis in female moths.[29] Now, after nearly 30 years of research, it is well established that PBAN regulates sex pheromone production in a circadian manner in many, but not all, moth species. PBAN is released to the hemolymph via the CC and reaches its target organ, the pheromone gland, where it exerts its activity. PBAN regulates either the initial or the terminal steps in the pheromone biosynthetic pathway, as indicated in the signaling cascade section. The cellular mechanisms by which PBAN regulates sex pheromone production were studied in detail and are reviewed in Refs. 17, 20.

PBAN in Sex Pheromone Biosynthesis in Males

A putative role of PBAN in the regulation of free fatty acids in male moths is hypothesized to be involved in chemical communication between the two sexes.[4] The mechanism by which PBAN affects male attractants is still unclear.

PBAN in Melanization

PBAN peptides are involved in cuticular melanization—the last step in the molting processes—in many noctuid moths. The possible involvement of this family of Nps in the control of larval cuticular melanization was first raised by Ogura and coworkers in studies of the common army worm, *Leucania separata*. The hormone—termed melanization and reddish coloration hormone (MRCH)—was later found to be identical with *Bom*-PBAN, which initiates melanization of the integument of many moth larvae. For a detailed review, see Ref. 3 and references therein.

DH in Embryonic and Pupal Diapause

As studies on the bioactivity of the *pban* gene peptides progressed, it became apparent that a *pban gene*-encoded peptide, DH, plays a major role in development processes. Studies since the late 1990s have found that DH induces embryonic diapause in *B. mori* and terminates pupal diapause in *Heliothinae* species. The effect of DH on embryonic and pupal diapause is reviewed in Ref. 32. DH is an important inducer of seasonal reproductive polyphenism in *O. thyellina*, inducing diapause that might be orchestrated via seasonal signaling pathways to accomplish seasonal adaptation.[34] Other functions of *pban* gene-encoded peptides are summarized in Ref. 3.

Bioactivity of Other FXPRLa-Containing Peptides in Lepidopteran and Non-Lepidopteran Insects

PK/PBAN Nps also accelerate pupariation in wandering larvae of the fleshfly (*Sarcophaga bullata*). Lem-PK (LPK) accelerate the switch from wandering behavior to immobilization/retraction behavior, and subsequently affect puparial tanning. By accelerating both aspects of puparium formation, Lem-PK mimick the effects of the pupariation factors by affecting the central motor neurons.[22] Studies by Altstien et al.[2] reveal that LPK and Lom-MT-II can also stimulate sex pheromone biosynthesis in *H. peltigera* adult females and cuticular melanization in *S. littoralis* larvae.

capa Gene-Derived Peptides

In contrast to the *pban gene*-encoded peptides, the physiological effects of the CAPA-PKs have not been investigated in detail and their role(s) in insect physiology is/are currently unknown.

The involvement of *pban*-gene-encoded Nps in the above functions, with the exception of myotropic activity, was determined by means of a variety of *in vivo* bioassays, for example, pheromonotropic, melanotropic, diapause, and pupariation assays. The myotropic activity was performed using an isolated cockroach hindgut. These studies revealed that most of the above functions could be stimulated by more than one member of this peptide family, sometimes at differing doses, and that the peptides are not species specific; this subject is reviewed in Ref. 3. Gene silencing was used to study the role of PBAN in sex pheromone biosynthesis in *P. xylostella*,[18]

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PBAN signaling pathways regulating sex pheromone synthesis in *B. mori*,[20] and the role of PBAN in male moths.[4]

THE PK/PBAN PEPTIDES FAMILY AS POTENTIAL TARGETS FOR PEST MANAGEMENT

As is evident from the studies described above, PK/PBAN Nps regulate many physiological and behavioral processes during development and mating and maintain visceral activities. Peptides involved in the regulation of vital functions are prime targets for advancement of the understanding of insect physiology and, therefore, are also targets for the development of novel insect-control strategies based on interference with their activity by means of receptor-selective antagonists.

Despite the vast scientific and insecticidal/insect-control potential of antagonists and, to some extent, of agonists, their application in insects has not been widely implemented so far. This is mainly because of lack of defined methods for obtaining antagonists based on known Np agonists, and because of the inability to predict which conformation will lead to a highly potent inhibitory or stimulatory receptor-selective activity. In addition, peptides are highly susceptible to proteolytic degradation and have poor bioavailability. Therefore, their conversion into an insecticide prototype necessitates rendering them resistant to peptidase degradation, and designing them with high bioavailability. For many years, the approach most commonly used by the agrochemical industry for insecticide discovery was based on random screening of large chemical or natural libraries of nonpeptide compounds and then optimizing a lead molecule with respect to the desired properties. In the past decade, a parallel approach, based on rational drug design—or structure-based design—has evolved which integrates and implements the vast amount of information on the genes encoding the peptides and their receptors, as well as their SARs.

In the past years, we have applied an integrated strategy, termed **I**nsect **N**p **A**ntagonist **I**nsecticide (INAI), based on a rational design approach of small-molecule antagonists based on a known Np agonist for the design of antagonists and, especially, to determine the properties that give them high stability and bioavailability. We have used the PK/PBAN Np family as a model system. A large variety of cyclic and linear peptides have been designed, synthesized, and tested for antagonistic activity on PK/PBAN-mediated functions, and their metabolic stability and bioavailability, that is, ability to penetrate the insect cuticle and to reach and activate the target organ, have been extensively examined.

Highly potent and highly bioavailable antagonists that can penetrate the insects cuticle and effectively inhibit several key functions in moths were discovered; also—most important—a large amount of information has been gained on the agonistic and antagonistic structural requirements of the PK/PBAN peptides. For instance, experiments with restricted-conformation analogs provide compelling evidence that PK/PBAN peptides adopt a *trans*Pro, type I β-turn within the shared C-terminal pentapeptide region to achieve an agonist receptor response.[23] Information has also been gleaned on the requirements needed to obtain high metabolic stability and cuticular and digestive tract penetrability. For example, PK/PBAN analogs featuring enhanced biostability and bioavailability efficiently penetrate the insect cuticle[3,12,23] and gut and have demonstrated pheromonotropic activity when fed to *H. virescens*.[23] In other studies, potent biostable PK/PBAN agonists[23] have been shown to prevent pupal diapause in treated *H. zea* larvae that had been programmed for diapause, and a novel antagonist with a dihydroimidazole *trans*Pro motif[23] inhibited the termination of *H. zea* pupal diapause initiated by the DH. The results suggest potential for the development of novel control strategies that derail the success of overwintering in pest insects.[23] In addition, two PK/PBAN-Rs—one from *S. littoralis* larvae and one from the pheromone gland of *H. peltigera* adult females—have been cloned and stably expressed in a cell line. These receptors are currently being characterized and used in the development of microplate high-throughput binding assays for screening libraries obtained from various sources for occurrence of potential insect-control agents capable of binding to these receptors.

Knowledge on the conformational requirements needed to acquire highly potent, metabolically stable, and bioavailable antagonists, and the availability of stably expressed PK/PBAN cloned receptors, together with the many *in vivo* and *in vitro* assays that we have developed to monitor bioactivity and bioavailability, enable to proceed toward the design and synthesis of insecticide prototypes based on already available antagonists. In this context, the overall know-how, together with the generic nature of the strategy, will facilitate application to other insect Np families, in the development of Np-based insect-control agents. For detailed reviews on these topics, see Refs. 3, 12, 23 and references therein.

SUMMARY AND FUTURE PROSPECTS

Intensive studies of the PK/PBAN peptides have yielded a vast amount of important information on the chemical and molecular nature of the peptides in Lepidopteran and non-Lepidopteran insects: their genes, post-translational processing, distribution, receptors, signaling mechanisms, and bioactivity. Nevertheless, many questions about these peptides are still unresolved, especially regarding their mode(s) of action, and much remains to be learned about the structural, chemical, and cellular basis of their activity and also about downstream cellular events, species specificity, receptor heterogeneity, and the mechanisms that underlie their functional diversity.

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The investigation of the transcriptional factors that regulate the *pban* gene and *capa* gene expression, which might be influenced by a circadian clock, is still in its infancy, as is that of the mechanisms of Np biosynthesis. The finding of the differential processing of the CAPA prohormone has provided a good opportunity to study the still poorly understood mechanism of packaging and sorting of Nps in the insect, and of the stimuli that regulate their release. The large number of cloned receptors provides a good basis for further studies of their SARs, and their stable expression in different cell lines invites further studies on the signaling pathways of the *pban*- and *capa* gene-encoded peptides—studies that might provide insights into the full range of cellular mechanisms that underlie the bioactivity of this multifunctional family in Lepidopteran and non-Lepidopteran insects. The vast number of insects—from diverse species and taxa—that all contain FXPRLa peptides and exhibit differential expression of the gene products calls for further studies on the bioactivity of the peptides, especially in non-Lepidopteran species, where knowledge is currently very scarce. Such studies could improve our understanding of their physiological role and shed light on their phylogenetic relationships during the course of evolution. The information that has already been accumulated, together with the currently available and developing bioinformatic, peptidomic, molecular, biological, and chemical analytical tools offer great potential for further exploration of the above issues. Apart from—and beyond—the high scientific value of the above findings, the strategies and approaches that were developed in the course of PK/PBAN research also offer great potential for practical application, by providing a basis for generation of insect Np antagonist-based insect-control agents, as described in this chapter.

REFERENCES

1. Altstein M, Ben-Aziz O, Bhargava K, Li QJ, Martins-Green M. Histochemical localization of the PBAN receptor in the pheromone gland of *Heliothis peltigera*. *Peptides* 2003;**24**:1335–47.
2. Altstein M, Ben-Aziz O, Zeltser I, Bhargava K, Davidovitch M, Strey A, et al. Inhibition of PK/PBAN-mediated functions in insects: discovery of selective and non-selective inhibitors. *Peptides* 2007;**28**:574–84.
3. Altstein M, Hariton A. Rational design of insect control agents: the PK/PBAN family as a study case. In: Ishaaya I, Horowitz R, editors. *Biorational control of arthropod pests: application and resistance management*. New York: Springer; 2009. p. 49–81.
4. Bober R, Rafaeli A. Gene-silencing reveals the functional significance of pheromone biosynthesis activating neuropeptide receptor (PBAN-R) in a male moth. *Proc Natl Acad Sci USA* 2010;**107**(39):16858–62.
5. Cazzamali G, Torp M, Hauser F, Williamson M, Grimmelikhuijzen CJP. The *Drosophila* gene CG9918 codes for a pyrokinin-1 receptor. *Biochem Biophysical Res Commun* 2005;**335**:14–19.
6. Choi MY, Fuerst EJ, Rafaeli A, Jurenka R. Role of extracellular domains in PBAN/pyrokinin GPCRs from insects using chimera receptors. *Insect Biochem Mol Biol* 2007;**37**:296–306.
7. Choi MY, Jurenka RA. Site-directed mutagenesis and PBAN activation of the *Helicoverpa zea* PBAN-receptor. *FEBS Lett* 2010;**584**:1212–16.
8. Choi MY, Meer RKV, Valles SM. Molecular diversity of PBAN family peptides from fire ants. *Arch Insect Biochem Physiol* 2010;**74**(2):67–80.
9. Choi MY, Vander Meer RK, Shoemaker D, Valles SM. PBAN gene architecture and expression in the fire ant, *Solenopsis invicta*. *J Insect Physiol* 2011;**57**(1):161–5.
10. Clynen E, Schoofs L. Peptidomic survey of the locust neuroendocrine system. *Insect Biochem Mol Biol* 2009;**39**(8):491–507.
11. Davis NT, Homberg U, Teal PEA, Altstein M, Agricola HJ, Hildebrand JG. Neuroanatomy and immunocytochemistry of the median neuroendocrine cells of the subesophageal ganglion of the tobacco hawkmoth, *Manduca sexta*: immunoreactivities to PBAN and other neuropeptides. *Microsc Res Tech* 1996;**35**:201–29.
12. Hariton A, Ben-Aziz O, Davidovitch M, Altstein M. Bioavailability of backbone cyclic PK/PBAN antagonists: inhibition of sex pheromone biosynthesis elicited by the natural mechanism in *Heliothis peltigera* females. *FEBS Lett* 2009;**277**:1035–44.
13. Hewes RS, Taghert PH. Neuropeptides and neuropeptide receptors in the *Drosophila melanogaster* genome. *Genome Res* 2001;**11**:1126–42.
14. Holman GM, Cook BJ, Nachman RJ. Isolation, primary structure and synthesis of A blocked myotropic neuropeptide isolated from the cockroach, *Leucophaea-Maderae*. *Comp Biochem Physiol C Pharmacol Toxicol Endocrinol* 1986;**85**:219–24.
15. Homma T, Watanabe K, Tsurumaru S, Kataoka H, Imai K, Kamba M, et al. G protein-coupled receptor for diapause hormone, an inducer of *Bombyx* embryonic diapause. *Biochem Biophysical Res Commun* 2006;**344**:386–93.
16. Jurenka R, Nusawardani T. The pyrokinin/pheromone biosynthesis-activating neuropeptide (PBAN) family of peptides and their receptors in Insecta: evolutionary trace indicates potential receptor ligand-binding domains. *Insect Mol Biol* 2010 Dec.
17. Jurenka R, Rafaeli A. Regulatory role of PBAN in sex pheromone biosynthesis of heliothine moths. *Front Endocrinol* 2011;**2**: 1–8.
18. Lee DW, Shrestha S, Kim AY, Park SJ, Yang CY, Kim Y, et al. RNA interference of pheromone biosynthesis-activating neuropeptide receptor suppresses mating behavior by inhibiting sex pheromone production in *Plutella xylostella* (L.). *Insect Biochem Mol Biol* 2011;**41**(4):236–43.
19. Loi PK, Tublitz NJ. Sequence and expression of the CAPA/CAP2b gene in the tobacco hawkmoth, *Manduca sexta*. *J Exp Biol* 2004;**207**(21):3681–91.
20. Matsumoto S, Ohnishi A, Lee JM, Hull JJ. Unraveling the pheromone biosynthesis activating neuropeptide (PBAN) signal transduction cascade that regulates sex pheromone production in moths. *Vitam Horm Pherom* 2010;**83**:425–45.
21. Morita A, Niimi T, Yamashita O. Physiological differentiation of DH-PBAN-producing neurosecretory cells in the silkworm embryo. *J Insect Physiol* 2003;**49**:1093–102.
22. Nachman RJ, Strey A, Zubrzak P, Zdarek J. A comparison of the pupariation acceleration activity of pyrokinin-like peptides native to the flesh fly: which peptide represents the primary pupariation factor? *Peptides* 2006;**27**:527–33.

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23. Nachman RJ. Agonists/antagonists of the insect kinin and pyrokinin/PBAN neuropeptide classes as tools for rational pest control biorational control of arthropod pests. In: Ishaaya I, Horowitz AR, editors. Netherlands: Springer; 2009. p. 21–48.
24. Nassel DR, Winther AME. *Drosophila* neuropeptides in regulation of physiology and behavior. *Prog Neurobiol* 2010;**92**(1):42–104.
25. Olsen SS, Cazzamali G, Williamson M, Grimmelikhuijzen CJP, Hauser F. Identification of one capa and two pyrokinin receptors from the malaria mosquito *Anopheles gambiae*. *Biochem Biophysical Res Commun* 2007;**362**:245–51.
26. Predel R, Wegener C. Biology of the CAPA peptides in insects. *Cell Mol Life Sci* 2006;**63**(21):2477–90.
27. Rafaeli A. Pheromone biosynthesis activating neuropeptide (PBAN): regulatory role and mode of action. *Gen Comp Endocrinol* 2009;**162**:69–78.
28. Raina AK, Jaffe H, Kempe TG, Keim P, Blacher RW, Fales HM, et al. Identification of a neuropeptide hormone that regulates sex-pheromone production in female moths. *Science* 1989;**244**:796–8.
29. Raina AK, Klun JA. Brain factor control of sex-pheromone production in the female corn-earworm moth. *Science* 1984;**225**:531–3.
30. Roller L, Yamanaka N, Watanabe K, Daubnerova I, Zitnan D, Kataoka H, et al. The unique evolution of neuropeptide genes in the silkworm *Bombyx mori*. *Insect Biochem Mol Biol* 2008;**38**(12):1147–57.
31. Rosenkilde C, Cazzamali G, Williamson M, Hauser F, Sondergaard L, DeLotto R, et al. Molecular cloning, functional expression, and gene silencing of two *Drosophila* receptors for the *Drosophila* neuropeptide pyrokinin-2. *Biochem Biophys Res Commun* 2003;**309**:485–94.
32. Schiesari L, Kyriacou CP, Costa R. The hormonal and circadian basis for insect photoperiodic timing. *FEBS Lett* 2011;**585**(10):1450–60.
33. Sun JS, Zhang QR, Zhang TY, Zhu ZL, Zhang HM, Teng MK, et al. Developmental expression of FXPRLamide neuropeptides in peptidergic neurosecretory cells of diapause- and nondiapause-destined individuals of the cotton bollworm, *Helicoverpa armigera*. *Gen Comp Endocrinol* 2005;**141**:48–57.
34. Uehara H, Senoh Y, Yoneda K, Kato Y, Shiomi K. An FXPRLamide neuropeptide induces seasonal reproductive polyphenism underlying a life-history tradeoff in the Tussock moth. *Plos One* 2011;**6**(8).
35. Zheng L, Lytle C, Njauw C-N, Altstein M, Martins-Green M. Cloning and characterization of the pheromone biosynthesis activating neuropeptide receptor gene in *Spodoptera littoralis* larvae. *Gene* 2007;**393**:20–30.

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Chapter 38

Insulin-Like Peptides

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ABSTRACT

Insulin-like peptides (ILPs) are encoded by multiple, distinct genes in insects as in other metazoans. This chapter summarizes our understanding of the diversity, secretion, signaling, and function of ILPs in insects. Studies of the fruit fly, *Drosophila melanogaster*, and other insects showed that ILPs activate insulin receptor orthologs and a specific cascade of intracellular proteins, known as the insulin signaling pathway. This pathway is responsive to nutritional status as modulated through the "target of rapamycin" pathway. Together, these pathways help regulate the growth, development, aging, and reproduction of insects. Little is known about how the diverse ILPs act as hormones, neurotransmitters, or growth factors to regulate these processes and others necessary for survival, such as nutrient apportionment, diapause, and innate immunity. Nevertheless, the diversity of ILP structure and function known for insects is remarkably similar to that characterized for mammals, suggesting a strong evolutionary conservation across the metazoan phyla.

INTRODUCTION

Insulin-like peptides (ILPs) epitomize the conservation of hormone structure and function across higher invertebrate and vertebrate animals. They are encoded by multiple, distinct genes within each species and, on secretion, serve as hormones, neurotransmitters, and growth factors. The unique structure of the ILP propeptide with continguous B, C, and A chains defines this superfamily. Bioactivity depends on the formation of two disulfide links between the B and A chains, a third intra-A chain disulfide bond, and cleavage of the C chain, with the exception of the vertebrate insulin-like growth factors (IGFs). Cysteine positions in the B and A chains are remarkably conserved across the superfamily, as are proteolytic cleavage sites for the C chain. The sequence divergent ILPs are transduced in target cells by either insulin receptor tyrosine kinases or G protein-coupled receptors to networks of signaling proteins that directly affect biochemical pathways and gene expression. Of the higher invertebrate groups, our accumulated knowledge of ILP endocrinology is the deepest for insects.

Studies of the model insect, *Drosophila melanogaster* (referred to as *Drosophila*) and other insects continue to elucidate the remarkable complexities of the insulin-signaling pathway (ISP) through its key role in metabolism, growth, reproduction, and aging. Still, little is known about the secretion of ILPs, receptor interactions, and their direct effects on specific processes in the target tissues of insects. Previous reviews of insect ILP endocrinology provide an extensive collation of earlier studies,[3,16,23,29,30,34] and herein, we offer of a summary of these reviews and more recent studies. Companion reviews are available in this handbook for the endocrinology of insulin and related peptides in vertebrates.

CHARACTERIZATION OF INSECT AND INVERTEBRATE ILPS

The first invertebrate ILP was discovered in the silkworm *Bombyx mori*,[20,22] and now >38 ILPs are known for this species, far more than in other lepidopteran species.[3] Only single ILPs were isolated and characterized to date for locust species.[5] Genomic and EST sequencing combined with bioinformatics has identified multiple ILP genes in other insect species.[3] This strategy first revealed seven ILP genes in *Drosophila* (DILP1-7, and recently DILP-8,[8,12] that presumably are ligands for the single insulin receptor (IR) homolog.[3,16,17,23,29,30,35] Their homologs are now known for another 11 *Drosophila* species.[3] In *Anopheles, Aedes* and *Culex* mosquitoes, five to eight ILPs were characterized in each of the species examined.[3,18,21,33] Strong structural conservation of ILPs across the Phylum Arthropoda is supported by the recent identification of ILP encoding genes in the water flea *Daphnia pulex*,[11] American dog tick *Dermacentor variabilis*, deer tick *Ixodes scapularis*, pea aphid *Acyrthosiphon pisum*, kissing bug *Rhodnius prolixus*, human body louse *Pediculus humanus humanus*, flour beetle *Tribolium castaneum*, honeybee *Apis melifera*, parasitic wasps *Nasonia longicornis* and *Nasonia giraulti*, and leaf cutter ant, *Atta cephalotes*[28] (see Ref. 3 for all others).

Multiple ILPs have been identified in species of another major invertebrate phylum, Mollusca, and in species

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00038-5
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representing early groups of the Deuterostomia.[3] More than 200 ILPs are identified for these groups and arthropods; well-supported clades arose from closely related species, and there is evidence for ILPs in the common protostomian ancestor of Arthropoda and Mollusca.[3] Structural comparisons also indicate that at least two vertebrate ILP subfamilies, insulin, and insulin-like growth factors (IGFs), are conserved among insects, but there is no clear evidence for relaxin/ILP homologs.

EXPRESSION, PROCESSING AND SECRETION OF ILPS

Approximately 40 genes encoding a variety of peptide hormones, including ILPs, are expressed in specific cells in both the nervous system and midgut of insects.[32] The ILPs are the only peptide hormones encoded as multiple genes, whereas others are proteolytically cleaved and processed from a single propeptide into smaller, mature forms (e.g., FMRFamides). One or more of the ILP genes in an insect are expressed in specific bilateral cell clusters, called medial neurosecretory cells (MNCs), in the *pars intercerebralis* region of the brain.[3,23] Axons from the cells in each cluster crossover and extend as separate bundles out of the brain to neurohemal release sites associated with the aorta, corpora cardiaca (CC), and along the midgut.[3,23,34] Another putative ILP source is the gut, which contains neuroendocrine cells and hundreds to thousands of isolated endocrine cells. However, few studies have detected ILP expression in this tissue.[3,23,34] As peptide hormones, ILPs are stored or secreted from their cellular sources in a bioactive form, thus transcript levels do not necessarily correlate with release or storage. Release of ILPs into the hemolymph for transport to target tissues likely rises and falls in response to internal and external cues mediated in the brain, and ILPs probably are degraded by proteases in the hemolymph or after binding to receptors and being internalized.

Studies of *Drosophila* provide a comprehensive survey of the expression of *DILP* and other peptide hormone genes, as well as their known receptor genes, in the adult nervous system and all regions of both the larval and adult gut in *Drosophila*.[13,17,23,24,32,35] The *DILP1-5* genes are expressed in the MNCs, probably reflecting their clustering within a 26 kb section of chromosome III. Only *DILP3* expression is detected in the larval and adult midgut, and it is localized to muscle bands in the anterior region.[32] As well, *DILP2*, *DILP4*, *DILP5*, and *DILP6* are expressed in the embryonic or larval gut, and *DILP5* transcripts are observed in the follicle cells of ovaries and Malpighian tubules.[3,16,23,32] Expression of the individual *DILP* genes varies in the MNCs during development, in response to different cues and *DILP* gene deletions, suggesting different promoter/enhancer elements. DILP2 and DILP5 accumulate in the MNCs of nutrient-deprived larvae, and their secretion is enhanced by feeding, targeted depolarization, and a fat-body-derived specific factor.[13] DILP secretion from MNCs is mostly complete after 30 min, as quantified by immunofluorescence microscopy, and there is no increase in *DILP* expression. Other studies have implicated serotonin and short neuropeptide F[23] in the regulation of DILP secretion based on the proximity of stained axons/neurons and genetic manipulations.

The MNCs originate from a single pair of neuroblasts that divide and differentiate into *DILP*-expressing cells at the end of embryogenesis.[7,23] The site of MNC origin is adjacent to other neuroblasts that separate and differentiate into CC cells that are part of the ring gland and produce adipokinetic hormone (AKH). Both sets of neuroblasts originate in a domain of the neuroectoderm of *Drosophila* embryos that shares homology with the hypothalamic–pituitary axis in the vertebrate brain. Vertebrate IGFs and relaxins are expressed and functional in the brain, whereas insulin production is shifted to gut endocrine cells in the mammalian pancreas. Organization and function of insulin-secreting cells in the pancreas are regulated by the transcription factor PAX6, a homolog of the *Drosophila* gene *eyeless* required for DILP MNC differentiation.[7] This finding is a striking example of functional conservation across animals and tissues.

DILP6 and DILP7 differ from the other DILPs in their structure and tissue expression pattern. *DILP6* expression surges and falls in the fat body during the pupal stage, and its deletion blocks development.[24] Based on similar structure, source, and function, DILP6 is an apparent IGF homolog. DILP7, a putative relaxin-like peptide, is localized in eight pairs of cells in the thoracic/abdominal ganglia.[3,16,17,23,35] Axons from these cells project to the *DILP* MNCs and out of the ganglia to other tissues. Based on its neuronal distribution, DILP7 may function more as a neurotransmitter than a peptide hormone.

Similar expression patterns are known for ILPs in other insects.[3,34] In mosquitoes, most *ILP* genes are expressed in the brain (head) across all life stages.[3,18,21,33] Specific ILPs were localized by immunocytochemistry in either the MNCs or more lateral brain neurosecretory cells and in other cells along the ventral ganglia of female mosquitoes.[3,21] Transcripts for mosquito *ILP2*, *ILP5* (DILP7 ortholog), and *ILP6* (IGF homolog) are expressed in other female tissues and body regions. Midgut endocrine cells in female *Aedes aegypti* are immunostained with an ILP antiserum but not in *Anopheles gambiae* or *Anopheles stephensi*.[3,21] In all three species, ILP immunostained axons extend from the brain MNCs along the anterior midgut in adults. This tissue is the site of sugar digestion and absorption, which may be regulated by ILPs released from the axons. Brain MNCs are the probable sources of two ILPs structurally characterized for the cotton leafworm, *Spodoptera littoralis*,[20] and an

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ILP isolated and characterized for the desert locust, *Schistocerca gregaria*.[5] Transcript expression of the locust *ILP* is highest in the brain and fat body of both sexes. The two ILP genes in the honeybee, *Apis mellifera*, are transcribed in the brains of different castes,[2,9] but expression in other tissues was not examined. An IGF-like peptide was isolated from the hemolymph of *B. mori* pupae as a single unprocessed 8 kDa peptide with B, C, and A domains.[24] It is similar in structure to the dipteran ILP6 and vertebrate IGFs and secreted by the fat body.

The dynamics of ILP processing, storage, and release are not fully characterized for insects. One possibility is that unprocessed ILPs may be secreted by undifferentiated cells or tissues during embryogenesis and metamorphosis, as reported for proinsulin during vertebrate development.[3] Dose-responsive bioactivity or receptor binding exists for one *B. mori* ILP (bombyxin-II), two *Samia cynthia* ILPs (ILP-A1, ILP-B1), and two *Ae. aegypti* ILPs (ILP3 and ILP4).[3,33] A recombinant form of DILP5 competitively displaces radioactively labeled insulin bound to human and *Drosophila* cells.[25]

Before secretion, ILPs are packed in secretory granules presumably with their processing enzymes. The C peptide of vertebrate proinsulin is cleaved by two prohormone convertases and a carboxypeptidase. These same enzymes process proforms of numerous neuropeptides in vertebrates, and their homologs likely have similar functions in insects. In *Drosophila*, the gene encoding a prohormone convertase 2 (PC2) homolog (*amontillado*) is expressed in neurosecretory cells through the nervous system and gut, and coexpression of a helper protein, 7B2, is probably required for activity.[3] Although *PC2* expression is required for *Drosophila* development and in particular for AKH function and processing, its role in ILP processing is yet to be determined.

After secretion into the hemolymph, ILPs may be transported to target cells by binding proteins (BPs) that facilitate ILP action. In vertebrates, specific BPs protect and store circulating IGFs (IGFBPs) and also activate IGF receptor signaling and other intracellular pathways. The first putative IGFBP characterized for insects is a 27 kDa protein secreted by *Sp. fugiperda* cells that binds human insulin and IGFs in culture.[3] Its native function has not been resolved. Studies of the *Drosophila* homolog, imaginal morphogenesis protein-late 2 (Imp-L2), suggest a role in ILP signaling. Imp-L2 is expressed in brain DILP MNCs and other tissues, and binds unprocessed, tagged DILPs in solution.[3,4] *Imp-L2* expression is not required for normal development, but it is essential for larval survival during starvation. Imp-L2 protein also binds to a homolog of the vertebrate acid labile subunit (ALS), which in vertebrates stabilizes the IGFBP/IGF complex in circulation. *Drosophila* ALS is expressed in the brain MNCs and in the fat body. Overexpression of tagged ALS and DILP2 in larval fat body yielded a putative complex in hemolymph, whereas reduced *ALS* expression limits carbohydrate and lipid mobilization in fed larvae.[4] In the tick, *Amblyomma americanum*, expression of *IGFBP* genes in different tissues was responsive to blood feeding.[3] Silencing the genes by RNAi resulted in the ticks taking smaller blood meals and increased mortality. A locust neuropeptide, neuroparsin, has limited sequence similarity to IGFBPs and binds a locust ILP in solution.[5] Homologs are known for all insect and arthropod groups examined to date, except for the *melanogaster* subgroup of *Drosophila*.[5] Neuroparsin and ILPs are secreted by different, but neighboring, brain MNCs in locusts, other *Drosophila spp.*, and other insects. Alternatively spliced transcripts of the neuroparsin gene are expressed in the nervous system and other tissues, thus complicating the assessment of its many functional roles.[5] Other putative IGFBP genes were identified in the genome of a mosquito baculovirus or in transcripts from the venom gland of the wasp *Eumenes pomiformis*, suggesting other levels of ILP interaction.[3]

Circulating ILPs probably are degraded by proteases, but once ILPs are bound by receptors, the complex may be internalized, where the ILP is degraded and the receptor recycled to the surface. Another possibility is that internalized ILPs may interact with cytoplasmic and nuclear proteins and activate signaling pathways in insects, as shown for insulin, IGFs, and IGFBPs in mammalian cells. An insulin-degrading enzyme (IDE, a zinc metalloendopeptidase) is highly conserved from yeast to mammals, and a *Drosophila* homolog is expressed in all life stages and cell lines.[31] *Drosophila* and human IDE bind and cleave mammalian insulin under the same conditions, but DILPs have not been tested. Homozygous *IDE* knockout renders viable and fertile flies with increased trehalose levels,[31] suggesting a specific effect on DILP regulation of metabolism.

INSULIN SIGNALING

Although insect genomes contain multiple ILPs, typically they encode only one IR, which is an approximately 500 kDa homodimer membrane protein consisting of two extracellular α subunits and two intracellular β tyrosine kinase subunits linked by disulfide bonds. ILP binding to the IR activates either the canonical ISP or the MAPK pathway in insect and mammalian cells. However, it is unknown for most insects whether some or all ILPs bind to the native IR, alternatively spliced/hybrid IR forms, or other receptor types. To date, specific binding of insect ILPs to native receptors has been shown only for two mosquito ILPs. *Ae. aegypti* ILP3 binds to the native IR (~500 kDa), and ILP4 to a yet unknown 55 kDa membrane protein.[33] Putative relaxin GPCR homologs are known for this species and *Drosophila*, but they remain orphan receptors.[3]

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Mining the genomes of insects has revealed all components of the mammalian ISP, even though insect ISPs are less complex due to fewer protein isoforms.[3] Insulin signaling unfolds when binding of an ILP to the IR initiates a sequence of phosphorylation events resulting in activation of either the ISP (Fig. 1) or MAPK branches. Additionally, components of the ISP branch activate the target of rapamycin (TOR) signaling pathway (Fig. 1), which is also activated through amino acid sensing.[3,18,29] The convergence of these pathways regulates metabolism, cell survival and differentiation, longevity, and fertility. Signaling through the MAPK branch is activated primarily by IGF binding to an IR ortholog in mammalian cells and results in activation of ERK-1/2, JNK, and p38 proteins kinases regulating cell proliferation, differentiation, and cell survival.[3,29] Crosstalk between MAPK and PI3K has been demonstrated in *Drosophila*, where a component of the MAPK pathway, GTPase Ras, is required for maximal PI3K signaling.[29]

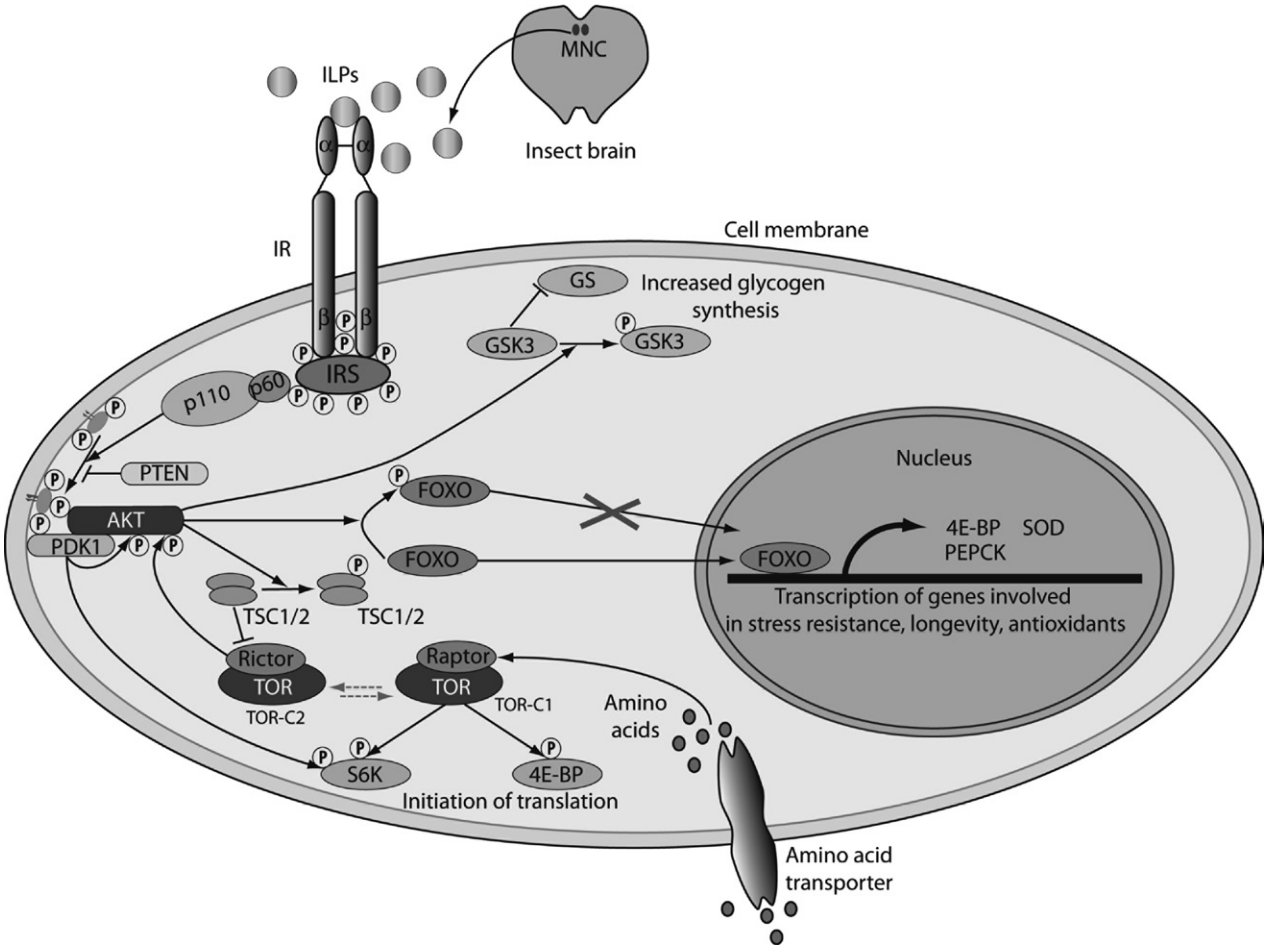

FIGURE 1 Activation of insulin and TOR signaling by insulin-like peptides and amino acids in insects. Insulin-like peptides (ILPs) are secreted by medial neurosecretory cells (MNC) in the brain into the hemolymph. Insulin signaling is activated when an ILP binds to the α-subunits of the membrane-associated insulin receptor (IR). This binding causes a conformational change and autophosphorylation of tyrosine residues on the β-subunits of the IR. In turn, the β-subunits phosphorylate an adaptor protein, insulin receptor substrate (IRS), providing docking sites for the regulatory subunit of phosphatidylinositol 3-kinase (PI3K), p60. Binding of p60 to IRS activates the catalytic subunit of PI3K, p110, which phosphorylates membrane-anchored phosphatidylinositol bisphosphate (PIP_2) and converts it into phosphatidylinositol trisphosphate (PIP_3). Both, 3-phosphoinositide-dependent protein kinase 1 (PDK1) and Akt kinase, bind to PIP_3 at the membrane, where PDK1 phosphorylates and activates both Akt and p70 S6 ribosomal kinase (S6K). For full activation, Akt also requires phosphorylation by rapamycin-insensitive companion of "target of rapamycin" (TOR) kinase (Rictor) in the TOR-complex 2 (TOR-C2). Activated Akt is a nexus for many downstream molecules including glycogen synthase kinase-3 (GSK-3) that increases glycogen synthesis, tuberous sclerosis complex (TSC1/2)—a negative regulator of the TOR kinase pathway, and FOXO transcriptional factors. When phosphorylated by Akt, FOXO cannot enter the cell nucleus and initiate transcription of a variety of genes, such as eukaryotic translation initiation factor 4E-binding protein (4E-BP), phosphoenolpyruvate carboxykinase (PEPCK), and supper-oxide dismutase (SOD).The ISP inhibitor phosphatase and tensin homolog (PTEN) dephosphorylates PIP_3 to PIP_2, thereby resetting the ISP to a preactivated state. The TOR pathway is activated by both the uptake of amino acids and by Akt (see above). TOR kinase participates in two molecular complexes, TOR-C1 and TOR-C2. Rapamycin-sensitive companion of TOR (Raptor) is associated with TOR-C1, whereas Rictor is associated with TOR-C2. TOR-C1 phosphorylates S6K and an inhibitor of translation, 4E-BP, leading to initiation of translation. TOR-C1 is activated autonomously by an influx of free amino acids and changes in the ATP: ADP ratio and oxygen levels.

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REGULATORY ROLES OF INSULIN-LIKE PEPTIDES

Through genetic manipulation, a great range of physiological processes in *Drosophila* were found to be regulated by ILPs through the ISP and TOR pathway.[3,16,17,23,29,30,35] However, evidence that endogenous DILPs directly affect any physiological process in *Drosophila* is still lacking. Mammalian insulin is used as a substitute for native ILPs in this and most other insects, but it is bioactive at relatively high concentrations. A few recent advances establish functional roles for ILPs or show direct bioactivity of native ILPs in insects.

GROWTH AND DEVELOPMENT

Growth and postembryonic development of insects are regulated through diverse signaling pathways activated by juvenile hormone (JH), ecdysteroid hormones (ECDs), and neuropeptides, including ILPs. Over the past decade, a general consensus has emerged that growth and development are regulated jointly by the ISP and TOR pathway.

The functional redundancy of DILPs is seen by knockout of the expression of individual and combinations of *DILPs* to determine the effects on growth, metabolism, reproduction, and lifespan.[17,35] Single *DILP* mutants are viable and generally exhibit normal growth/adult body weight, with *DILP6* mutants being the smallest. Combined *DILP* knockouts reveal that one lacking all but *DILP6* develop into adults, whereas combinations with the *DILP6* knockout are lethal, suggesting that fat body expressed DILP6 and DILPs expressed in the MNCs act redundantly. Mutation combinations of *DILP1-5*, all expressed in the MNCs, render viable flies with reduced growth/body weight, again supporting the redundancy of developmental and growth-controlling DILP action.

ILP treatment directly stimulates the growth or proliferation of cells in different organs and imaginal discs.[3,27,34] In Lepidoptera, hemocyte discharge by hematopoietic organs *in vitro* is stimulated in a dose-responsive manner by synthetic ILP (bombyxin-II) and bovine insulin, and addition of 20-hydroxyecdysone (20-ECD) enhances the effects. Wing discs from last instar larvae of the lepidopterans, *Precis coenia* or *Manduca sexta*, are synergistically stimulated to grow *in vitro* by 20-ECD and an ILP (bombyxin-II or partially purified brain extract) treatment. Commitment to pupal development in wing discs *in vivo* is stimulated by the injection of mammalian insulin or purified *M. sexta* ILP into newly molted, starved last instar *M. sexta* larvae, and further indicates that these ILP treatments suppress JH action later in the instar. The native *B. mori* IGF-like peptide, isolated from the larval hemolymph, stimulates the growth of genital imaginal discs *in vitro*.[24] Secretion of this peptide by the fat body occurs only in early pupae and is regulated by a surge of ECD. The structural homolog in *Drosophila*, DILP6, also promotes growth based on the negative effects of *DILP6* mutations, and its expression in pupae is regulated by ECDs.[24] The receptor and signaling pathways activated by these putative IGFs are yet to be determined.

In the *Drosophila* adult midgut, replacement of damaged/dead cells by stem cell division is reduced in IR, IRS, and MNC-ablated mutants, but is restored in mutants overexpressing IR or *DILP2*.[1] Midgut stem cells of lepidopteran larvae also proliferate after treatment with synthetic ILP (bombyxin-II) and insulin, and this effect is enhanced by the presence of fat body extract, suggesting the presence of an IGF.[15] As shown by these studies, insect tissues and cells are amendable to culture and experiments to investigate the direct effects of ILPs on the ISP and their specific functions.

ECDYSTEROID HORMONE PRODUCTION

Ecdysteroid hormones are the primary regulators of insect development, molting, and metamorphosis. In the larval stages, ECDs are synthesized from dietary cholesterol in the prothoracic glands (PG) and released into the hemolymph for circulation to target tissues. Several neuropeptides stimulate or inhibit ECD production.[20] Prothoracicotropic hormone (PTTH) was the first one structurally and functionally characterized, and its receptor tyrosine kinase, Torso, more recently identified. The first invertebrate ILP, known as "small PTTH" and later bombyxin, was isolated from *B. mori* based on its stimulation of pupal molting and ECD production by the PG of another silkworm, *Sa. cynthia*. Bovine insulin also stimulates ECD production by the PG of last instar *B. mori*, and activates the ISP but not MAPK signaling. Two other native ILPs stimulated the pupal–adult molt of a related silkmoth, *Sa. cynthia*. Cross species activity was shown for a synthetic *B. mori* ILP that stimulates ECD production by the PG of the hemipteran *Rhodnius prolixus.* These results suggest ILPs may regulate ECD production by the PG in the larval/nymphal instars of insects.

METABOLISM

The small size and high-energy demands of insects require that the regulation of metabolism be highly sensitive, so that nutrients are allocated between immediate energy needs and storage to facilitate growth, development and reproduction. Two neuropeptides and their signaling pathways balance the energetic needs of insects between storage and mobilization of nutrients. Of the multiple ILPs encoded in an insect, one or more are probably functional homologs of vertebrate insulin, as first shown for mosquitoes.[3,33] Insulin induces cells to take up glucose and convert it to glycogen, and to shift from catabolic to anabolic lipid and protein metabolism. AKH is the structural and functional

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homolog of mammalian glucagon, an insulin antagonist, in many different insects.[26] Acting through a G protein-coupled receptor and cAMP/inositol triphosphate signaling, AKH activates the mobilization of carbohydrate, proline, and lipid stores.

The fat body is the primary site of intermediary metabolism and nutrient storage, and thus responsive to ILP/TOR and AKH signaling. These processes also occur in the midgut and in hemocytes distributed throughout the body and govern the fat body through unknown mechanisms. The particulars of ILP and AKH signaling in the fat body during larval and adult stages are best understood for *Drosophila*.[3,29] Studies suggest that DILP1-5 either alone or in combination regulate carbohydrate and lipid metabolism in an insulin-like fashion, based on single and combinatorial *DILP* deletions.[17,35]

Synthetic ILPs directly affects metabolic processes in insects. A silkmoth ILP (bombyxin-II), when injected into neck-ligated larvae, lowered trehalose and glycogen levels, apparently promoting consumption of carbohydrate reserves and not the accumulation of reserves, as for insulin in mammals.[3,34] Synthetic *Ae. aegypti* ILP3 injected into decapitated, sugar fed female *Ae. aegypti* stimulates trehalose depletion and glycogen and lipid storage in a dose-responsive manner.[33]

Quantitative analyses of ILP and ISP gene expression have been used to determine the effect of feeding or starvation in *Drosophila* and to identify other genes affected by nutrient intake.[3,29] Notably, none of the *DILPs* show any significant change in expression for up to 13 h after starved females are fed, nor do *Akt*, *FOXO*, or *TOR* (see Fig. 1 for acronyms). However, the expression of *IR*, *IRS*, and *PDK1* is greatly decreased within 2 h after feeding and for the whole period. These trends suggest that the regulation of *DILP* and ISP gene expression is not uniformly responsive to nutrient intake.

REPRODUCTION

As with growth and development, JH, ECDs, and neuropeptides, including the ILPs, are implicated in the regulation of insect reproduction. For most insect groups, JH is considered the primary gonadotropic hormone in females, and for dipteran species that require protein food to produce eggs, ECDs assume this function. Ovaries are the primary source of circulating ECDs in females, because the PG degenerate in transition to the adult stage, and follicle cells surrounding the oocytes are the site of ECD biosynthesis.[3,18,33,34] These steroids are either stored in the oocytes for use during embryonic development or released into the hemolymph. The gonadotropic action of JH or ECDs is to activate the fat body and follicle cells to secrete vitellogenin and other yolk proteins (YP) for uptake by the maturing oocytes.

The interaction between nutrient, JH, ECD, and ILP signaling is best characterized for *Drosophila* and mosquito females,[3,18,30] and for both, JH is required for the development of the fat body and ovaries. A study comparing the effects of knocking out single and multiple *DILPs* found that flies lacking only *DILP6*, the IGF homolog, or the combination of *DILP2-3-5* have the greatest reduction in lifetime fecundity compared with wild-type flies, and other single or combined *DILP* mutations or had less of an effect.[17] The mechanisms by which DILPs regulate processes associated with egg maturation in *Drosophila* are not well defined, but both JH and the ISP are implicated in the activation of ovarian ECD production. Females with a mutation in the *IRS* gene are small and sterile but maintain a normal range of JH and ECD production given their size, thus suggesting the ISP plays a primary role in egg maturation. Oogenesis is dependent on DILP activation of the ISP in the stem cells and their progeny to form egg chambers.[3] The TOR pathway affects this process via ISP dependent and independent mechanisms. Synthesis of YPs by the fat body and follicle cells is dependent on ISP and ECD signaling.

The role of ILPs in the endocrine cascade that regulates egg maturation is well defined for female *Ae. aegypti*.[3,18,33] The mosquito IR homolog is expressed in ovaries, and mammalian insulins stimulate ovary ECD production *in vitro* through the ISP. Insulin similarly stimulates this process in the ovaries of the blowfly *Phormia regina.*[3,34] Of the eight ILPs encoded in *Ae. aegypti,* ILP3 is most similar in structure to mammalian insulin and produced in the brain MNCs.[33] In response to the ingestion of blood, ILP3, and possibly other ILPs, are released and stimulate three key processes required for egg maturation: ovary ECD production, serine protease digestion of the blood meal, and expression of vitellogenin for uptake by oocytes.[18] Full activation of these processes *in vivo* and *in vitro* by ILP3 is dependent on IR expression and the presence of amino acids, affirming the interaction of insulin and TOR pathways. Both synthetic ILP3 and ILP4 stimulate ECD production by ovaries in a dose-responsive manner, at concentrations 100-fold less than required for mammalian insulin, but ILP4 interacts with a different receptor.[33]

DIAPAUSE

Diapause is an endocrine-mediated metabolic and developmental arrest induced by changes in abiotic cues that indicate the onset of adverse environmental conditions.[10] This arrest occurs in a species-specific life stage, and in adult diapause, reproduction halts. Injection of mammalian insulin terminates diapause and promotes development in the pupae of two lepidopterans, *Sa. cynthia* and *Pieris brassicae* pupae, as well as stimulates ECD production.[10,34] Likewise, alterations in the ISP affect adult diapause in dipterans. Female *Culex pipiens* mosquitoes enter diapause in the autumn with large fat reserves and arrested ovarian

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development. Knockdown of the IR by RNAi in nondiapausing females arrests primary oocytes development, as in the diapause state.[10] By contrast, knockdown of *FOXO* expression in females preprogrammed for diapause greatly decreases lipid storage and lifespan, and RNAi knockdown of *ILP1* halts ovarian development in nondiapausing females. The above studies suggest that inactivation of the ISP facilitates diapause induction and its activation terminates or prevents diapause. This concept seems to contradict the anabolic role played by the ISP in nondiapausing insects, but it is in accord with its role in the nematode, *Caenorhabditis elegans*.

BEHAVIOR

Different ILPs are expressed in the central nervous system of insects and probably activate the ISP in specific neuronal pathways that govern behaviors associated with nutrient acquisition.[3,16,23,29] Downregulation of *DILP* expression and the ISP in fed *Drosophila* larvae increases food ingestion and acceptance of food containing noxious chemicals, whereas overexpression reduces these behaviors in starved larvae. This pathway also negatively interacts with neuropeptide F signaling that promotes foraging over a range of aversive conditions.

Caste determination and behavioral differentiation in the honeybee *Apis mellifera* are in part regulated through ILP expression and the ISP.[2,9] Expression levels of *ILP-1* and the *IR* are higher in queen larvae than in specific worker larval instars. By contrast, *ILP-2* is expressed at greater levels in workers than in queens. Worker duties shift from young bees nursing larvae to older bees foraging for nectar and pollen. Foragers have significantly higher transcript levels of *ILP-1* in heads and *InR1* and *InR2* in abdomens than nurse bees.

IMMUNITY

The innate immune system in insects coordinates pathogen recognition with induction of defenses to block infection. The ISP plays a role in balancing the cost of an immune response with survival and reproduction.[3,6,16,19,21] In *Drosophila, Mycobacterium marinum* infection reduces Akt and glycogen synthase kinase-3 phosphorylation, and infected flies die sooner than *FOXO* mutants. *IRS* mutants also display increased resistance to bacteria and increased lifespan. Starvation attenuates the ISP in *Drosophila* and activates the immune response without pathogen challenge, as shown by increased antimicrobial peptide (AMP) expression. Mutations disrupting ISP, feeding wild-type adult flies with an inhibitor of PI3K signaling, or starvation of larvae all increase AMP expression in noninfected animals. By contrast, AMP expression is reduced in *FOXO* mutant flies, starved flies, and flies treated with PI3K inhibitor, suggesting a direct link between FOXO signaling and activation of AMP expression.

The link between ISP and immunity has also been shown in the midgut of mosquitoes consuming *Plasmodium falciparum* infected blood. Feeding high concentrations of human insulin to *An. stephensi* and *An. gambiae* results in a twofold increase in the number of *Plasmodium* oocysts on their midguts.[21] *Plasmodium*-derived glycosylphosphatidyl inositol is an insulin mimetic in mammals, and when fed to Anopheline females, stimulates Akt phosphorylation and nitrogen oxide (NO) synthase expression in the midgut epithelium.[19] This enzyme produces toxic levels of NO, a potent anti-*Plasmodium* molecule. Human insulin induces NO and hydrogen peroxide production in cultured *An. stephensi* cells and in the midgut, resulting in increased oxidative stress that is harmful for the malaria parasite. Moreover, *An. stephensi* fed *P. falciparum* infected blood has increased expression of the genes encoding *AnsILP2* and *AnsILP3* in heads and midguts and *AnsILP5* in the head.[21] *Ae. aegypti* ILP3 stimulates proliferation of hemocytes, major mosquito immune cells.[6] Male dragonflies, *Libellula pulchella*, infected with gut parasites (Apicomplexa, *Hoplorhynchus*) exhibit poor mating success and increased trehalose levels in hemolymph and thoracic lipid stores.[3] Injection of human insulin reduced trehalose levels in healthy males and increased levels in infected ones. Together, these studies suggest that insects chronically infected with pathogens also display the "metabolic syndrome" associated with chronic infection, obesity, and insulin resistance of type-2 diabetes in humans.

AGING

Dietary, genetic, and molecular interventions that reduce insulin signaling increase lifespan in many organisms including *Drosophila* and mosquitoes.[3,16,17,30] Altering the ISP in the nervous system, fat body, and midgut directly affects the dynamics of aging in *Drosophila*. Reduced expression of *DILP2* in the nervous system increases lifespan, as do mutations in genes for ISP proteins in other tissues, e.g. *IR*. Similarly, overexpression of the ISP antagonists *FOXO* and *PTEN* in the nervous system and fat body also extends lifespan. Studies in the mosquito *An. stephensi* show that ILP expression may be age dependent.[21] Also the midgut acts as a "signaling center" for the regulation of aging in mosquitoes, and overexpression of an active Akt specifically in the midgut of transgenic mosquitoes significantly reduced adult lifespan.[21]

CONCLUSIONS

The great diversity and success of insects results in part from the central regulation exerted by multiple ILPs through the ISP and associated pathways that coordinate growth, development, and reproduction, in response to external and internal cues regarding food ingestion, nutritional state, and

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pathogen infection. Studies of *Drosophila*, mosquitoes, and other insects reveal the structural conservation of ILPs and signaling proteins and substantiated their regulatory properties, largely through changes in gene expression in response to nutritional or physiological manipulations. Still, few studies have confirmed direct and dose-responsive effects of ILPs and their binding to the IR or other receptors in insects or other invertebrates. Little is known for insects about the dynamics of ILP secretion and titer, particularly the internal and external cues that govern ILP release and binding to the IR or other proteins. An overarching question is how do multiple ILPs interact with a single IR to regulate and coordinate diverse physiological and behavioral processes in different tissues, so that growth, development, and reproduction can be successfully completed?

Our understanding of the intricacies of insulin signaling in diverse insects has also revealed its interactions with other signaling pathways, including activin/transforming growth factor beta signaling.[14] Substantive evidence indicates that ILPs acting through the ISP may regulate ECD and JH secretion, and thus direct or coordinate development and reproduction. Sensing of nutrients acquired through ingestion or mobilized/stored internally through different mechanism affects both the ISP and TOR pathway in an apparent positive feedback loop, and modulates expression of many genes or any number of different biochemical processes in tissues. Another question is how does pathogen infection alter ISP regulation of metabolic homeostasis and modulate innate immune responses? With the genomes of insects and invertebrates being characterized at an unprecedented pace, the expansion of genetic techniques into nonmodel organisms, and improvements in ILP synthesis and biochemical approaches, our understanding of this fundamental signaling cascade should significantly advance in the next few years.

REFERENCES

1. Amcheslavsky A, Jiang J, Ip YT. Tissue damage-induced intestinal stem cell division in *Drosophila*. *Cell. Stem Cell* 2009;**4**:49–61.
2. Ament SA, Wang Y, Robinson GE. Nutritional regulation of division of labor in honey bees: toward a systems biology perspective. *Wiley Interdiscip Rev Syst Biol Med* 2010;**2**:566–76.
3. Antonova Y, Arik A, Moore W, Riehle M, Brown M. Insulin-like peptides: structure, signaling, and function. In: Gilbert L, editor. *Insect endocrinology*. UK, USA: Elsevier; 2012. p. 63–92.
4. Arquier N, Geminard C, Bourouis M, Jarretou G, Honegger B, Paix A, et al. *Drosophila* ALS regulates growth and metabolism through functional interaction with insulin-like peptides. *Cell Metab* 2008;**7**:333–8.
5. Badisco L, Claeys I, Van Hiel M, Clynen E, Huybrechts J, Vandersmissen T, et al. Purification and characterization of an insulin-related peptide in the desert locust, *Schistocerca gregaria*: immunolocalization, cDNA cloning, transcript profiling and interaction with neuroparsin. *J Mol Endocrinol* 2008;**40**:137–50.
6. Castillo J, Brown MR, Strand MR. Blood feeding and insulin-like peptide 3 stimulate proliferation of hemocytes in the mosquito *Aedes aegypti*. *PLoS Pathog* 2011;**7**:e1002274.
7. Clements J, Hens K, Francis C, Schellens A, Callaerts P. Conserved role for the *Drosophila* Pax6 homolog Eyeless in differentiation and function of insulin-producing neurons. *Proc Natl Acad Sci U S A* 2008;**105**:16183–8.
8. Columbani J, Andersen DS, Leopold P. Secreted peptide DILP8 coordinates *Drosophila* tissue growth with developmental timing. *Science* 2012;**336**:582–5.
9. de Azevedo SV, Hartfelder K. The insulin signaling pathway in honey bee (*Apis mellifera*) caste development—differential expression of insulin-like peptides and insulin receptors in queen and worker larvae. *J Insect Physiol* 2008;**54**:1064–71.
10. Denlinger D, Yocum G, Rinehart J. Hormonal control of diapause. In: Gilbert L, editor. *Insect endocrinology*. UK, USA: Elsevier; 2012. p. 430–63.
11. Dircksen H, Neupert S, Predel R, Verleyen P, Huybrechts J, Strauss J, et al. Genomics, transcriptomics, and peptidomics of *Daphnia pulex* neuropeptides and protein hormones. *J Proteome Res* 2011;**10**: 4478–504.
12. Garelli A, Gontijo AM, Miguela V, Caparros E, Dominguez M. Imaginal discs secrete insulin-like peptide 8 to mediate plasticity of growth and maturation. *Science* 2012;**336**:579–82.
13. Geminard C, Rulifson EJ, Léopold P. Remote control of insulin secretion by fat cells in *Drosophila*. *Cell Metab* 2009;**10**:199–207.
14. Gibbens YY, Warren JT, Gilbert LI, O'Connor MB. Neuroendocrine regulation of *Drosophila* metamorphosis requires TGFβ/activin signaling. *Development* 2011;**138**:2693–703.
15. Goto S, Loeb MJ, Takeda M. Bombyxin stimulates proliferation of cultured stem cells derived from *Heliothis virescens* and *Mamestra brassicae* larvae. *In Vitro Cell Dev Biol Anim* 2005;**41**:38–42.
16. Grönke S, Partridge L. The functions of insulin-like peptides in insects. In: Clemmons D, Robinson I, Christen Y, editors. *IGFs: local repair and survival factors throughout life span*. Berlin, Heidelberg: Springer; 2010. p. 105–24.
17. Grönke S, Clarke DF, Broughton S, Andrews TD, Partridge L. Molecular evolution and functional characterization of *Drosophila* insulin-like peptides. *PLoS Genet* 2010;**6**:e1000857.
18. Gulia-Nuss M, Robertson AE, Brown MR, Strand MR. Insulin-like peptides and the target of rapamycin pathway coordinately regulate blood digestion and egg maturation in the mosquito *Aedes aegypti*. *PLoS One* 2011;**6**:e20401.
19. Luckhart S, Riehle MA. The insulin signaling cascade from nematodes to mammals: insights into innate immunity of Anopheles mosquitoes to malaria parasite infection. *Dev Comp Immunol* 2007;**31**:647–56.
20. Marchal E, Vandersmissen HP, Badisco L, Van de Velde S, Verlinden H, Iga M, et al. Control of ecdysteroidogenesis in prothoracic glands of insects: a review. *Peptides* 2010;**31**:506–19.
21. Marquez AG, Pietri JE, Smithers HM, Nuss A, Antonova Y, Drexler AL, et al. Insulin-like peptides in the mosquito *Anopheles stephensi*: identification and expression in response to diet and infection with plasmodium falciparum. *Gen Comp Endocrinol* 2011;**173**:303–12.
22. Nagata K. Studies of the structure-activity relationships of peptides and proteins involved in growth and development based on their three-dimensional structures. *Biosci Biotechnol Biochem* 2010;**74**:462–70.

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23. Nässel D. Insulin signaling and its regulation in physiology and behavior of *Drosophila*. *Can J Zool* 2012;**90**:476–88.
24. Okamoto N, Yamanaka N, Yagi Y, Nishida Y, Kataoka H, O'Connor MB, et al. A fat body-derived IGF-like peptide regulates postfeeding growth in *Drosophila*. *Dev Cell* 2009;**17**:885–91.
25. Sajid W, Kulahin N, Schluckebier G, Ribel U, Henderson HR, Tatar M, et al. Structural and biological properties of the *Drosophila* insulin-like peptide 5 show evolutionary conservation. *J Biol Chem* 2011;**286**:661–73.
26. Schooley D, Horodyski F, Coast G. Hormones controlling homeostasis in insects. In: Gilbert L, editor. *Insect endocrinology*. UK, USA: Elsevier; 2012. p. 366–429.
27. Shingleton AW. The regulation of organ size in *Drosophila*: physiology, plasticity, patterning and physical force. *Organogenesis* 2010;**6**:76–87.
28. Suen G, Teiling C, Li L, Holt C, Abouheif E, Bornberg-Bauer E, et al. The genome sequence of the leaf-cutter ant *Atta cephalotes* reveals insights into its obligate symbiotic lifestyle. *PLoS Genet* 2011;**7**:e1002007.
29. Teleman AA. Molecular mechanisms of metabolic regulation by insulin in *Drosophila*. *Biochem J* 2009;**425**:13–26.
30. Toivonen JM, Partridge L. Endocrine regulation of aging and reproduction in *Drosophila*. *Mol Cell Endocrinol* 2009;**299**:39–50.
31. Tsuda M, Kobayashi T, Matsuo T, Aigaki T. Insulin-degrading enzyme antagonizes insulin-dependent tissue growth and abeta-induced neurotoxicity in *Drosophila*. *FEBS Lett* 2010;**584**:2916–20.
32. Veenstra JA. Peptidergic paracrine and endocrine cells in the midgut of the fruit fly maggot. *Cell Tissue Res* 2009;**336**:309–23.
33. Wen Z, Gulia M, Clark KD, Dhara A, Crim JW, Strand MR, et al. Two insulin-like peptide family members from the mosquito *Aedes aegypti* exhibit differential biological and receptor binding activities. *Mol Cell Endocrinol* 2010;**328**:47–55.
34. Wu Q, Brown MR. Signaling and function of insulin-like peptides in insects. *Annu Rev Entomol* 2006;**51**:1–24.
35. Zhang H, Liu J, Li CR, Momen B, Kohanski RA, Pick L. Deletion of *Drosophila* insulin-like peptides causes growth defects and metabolic abnormalities. *Proc Natl Acad Sci U S A* 2009;**106**:19617–22.

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Chapter 39

Molluscan Bioactive Peptides

Anna Di Cosmo and Gianluca Polese

ABSTRACT

In this chapter, we try to provide a detailed summary of the studies reporting: discovery, gene organization, distribution, receptors, and biological action of peptides in the phylum Mollusca. Peptides have been collected in families or groups according to molecular structure. In separate subsections, we group structurally different peptides sharing a common biological action. According to these criteria, the RFamide peptides, and tachykinins, have been discussed as members of one peptide family. Other peptides are presented according to their involvement in energy flow, growth, feeding behavior, osmoregulation, and reproduction. Finally, a detailed analysis of peptides involved in egg laying and sexual behavioral repertoires is also reported.

INTRODUCTION

During the past six years, the discovery of novel peptides and hormones from Mollusc phylum, enabled by the availability of whole genomes, transcriptomes, and peptidomes, now provides ample tools for studying the structural evolution of peptides and hormones and redefining the phylogenetic relationships of key families of peptide and hormones across ecdysozoans, lophotrochozoans, and chordates. In this framework, the article of Veenstra J.A. (2010) that reports the genome sequence of limpet *Lottia gigantea* and the initiated large-scale molluscan transcriptome projects (i.e. Di Cosmo A. and Moroz L., "Euroceph Meeting", 2011), offer an excellent opportunity to look at the neuropetides and neurohormone genes. These are the fastest evolving intercellular signals; in particular, cephalopods developed a number of specific innovations in their repertoire of secretory molecules that might support their unique features. Most of the older references are present in our chapters in the first edition of this book.[11,12]

FMRFamide, FMRFamide-RELATED PEPTIDES (FaRPs)

Discovery

The tetrapeptide Phe-Met-Phe-Arg-NH_2 (FMRFamide) was discovered in the ganglia of the clam *Macrocallista nimbosa* (Price and Greenberg, 1977), and it is the first member of the extended family of FaRPs (also named RFamide peptides). They are known as molluscan cardioexcitatory peptides, but their roles as neurotrasmitters and neuromodulators in the central nervous system have been done. Sequences variations of RFamide family members occur mainly in the N-terminal region. Lately, novel RFamide neruopeptides have been characterized in *Haliotis asinina*[8] and in *Sepia officinalis*,[35] so that peptides of the RFamide family have been described through all the molluscan classes.[19] See related RF chapters in this and the Brain Peptides section of the book.

Structure of the Precursor mRNA/Gene

Characteristics common to all the RFamide peptides is the shared sequence Arg-Phe-NH_2 (exactly RFamide) at the C-terminus. These peptides are translated from specific genes as propeptides. These contain multiple copies of either the same or different peptides, the latter sharing in almost all cases only the C-terminal RFamide. Each peptide contained in the propeptide is proteolytically cleaved and amidated during posttranslational processing. The gene coding for FMRFamide has been identified in the gastropods *Lymnaea stagnalis*, *Aplysia californica*, *Helix aspersa*, and *Haliotis asinine*,[8] in the bivalve *Mytilus edulis*, and in the cephalopods *Loligo pelaeii*, *Loligo opalescens*, *Sepia officinalis*, *Octopus vulgaris*, and *Idiosepius notoides*.[11,34,35] The FMRFamide locus of *Lymnaea* is one of the best characterized (see Santama and Benjamin, 2000). This gene consists of five exons (I–V) punctuated by four introns. Two differentially spliced transcripts have been identified: one that consists of exons I and II (precursor 1) and another that comprises exons I, III, IV, and V (precursor 2). Exons II encodes a battery of tetra- and pentapeptides species. The sequence of exon III contains the heptapeptides and pentapeptides. Exon V encodes two hexapeptides, whereas the sequence of the heptapeptide SKPYMRFamide is formed at the splicing junction between exons II and IV. Posttranslational processing of the two *Lymnaea* FaRP precursor allow the formation of two

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00039-7
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long non-FaRP peptides: the 22aa peptide, called SEEPLY, encoded in precursor 1 and the 35aa peptide, named acidic peptide, present in precursor 2.

Distribution of the mRNA/Peptide

In *Lymnaea*, the alternate mRNA expression of FMRFamide gene is specific to single identified neurons. The two different transcripts are expressed in a mutually exclusive manner in different neurons of adult CNS (Santama and Benjamin, 2000). By *in situ* hybridization and immunocytochemistry with peptide-specific antibodies, it was revealed that all neurons that express precursor 1 mRNA contain SEEPLY immunoreactivity and, similarly, all precursor 2 mRNA-expressing neurons contain the acidic peptide. In the developing stages of *Sepia officinalis* FaRP immunoreactivity is detected in fibers located in the neuropile and in cell bodies located in the posterior chromatophore lobe and in the liovisceral lobe. Furthermore, *in situ* hybridization with high density of strongly stained cells in the optic tract region suggests the involvement of FaRPs in all parts of the chromatophore control pathway.[1] Similar results were also obtained in *Idiosepius notoides*.[34] In *Limax valentianus*, immunoreactivity against FMRFamide is found in the procerebrum cell bodies, within the cell mass, and fibers in the terminal mass and internal mass layers.[18]

Receptors and Signaling Cascades

A G protein-coupled receptor (GPRC) was cloned from the mollusc *Lymnaea stagnalis*. The ligand for this receptor was purified and identified as an RFamide peptide, the *Lymnaea* cardioexcitatory peptide (LyCEP), which displays significant similarity to the *Achatina* cardioexcitatory peptide (ACEP-1). In the *Lymnaea* brain, the cells that produce the egg-laying hormone represent the predominant site of RFamide receptor gene expression.

Biological Actions

The cardioacceleratory action of RFamide peptides has been documented in numerous gastropods. The well-characterized central network controlling cardiorespiration in *Lymnaea* represents a good system to study the physiological consequences of alternative splicing of this neuropeptide gene. Most of the effects of the peptides from both precursors are inhibitory, and it has been suggested that the global inhibitory action of a released mixture of peptides may be more effective than the specific action of each peptide. FMRFamide also affects feeding in *Lymnaea*, *Aplysia*, *Limax*, and *Helisoma*. Moreover, FaRPs seem to be involved in osmoregulation in *Helisoma trivolvis*, *Helisoma duryi*, and *Melampus bidentatus*. In *Aplysia*, FMRFamide inhibits the 30-min period of firing of the bag cell neurons of *Aplysia californica* (after discharge), which causes the animal to undergo a long-lasting sequence of stereotypical reproductive behaviors (Fisher et al., 1993) and presynaptically inhibits the sensory neurons of the gill- and siphon-withdrawal reflex of *Aplysia* (Mackey et al., 1987). In the slug, *Limax valentianus*, FMRFamide regulates oscillatory activity of the olfactory center.[18] In cephalopods, FMRFamide exerts a positive contractile action on muscles of both male and female reproductive system.[24] In the CNS, this tetrapeptide seems to be involved in the inhibition of both secretory activity of the endocrine optic gland[10] and the screening pigment migration in combination with dopamine.[15] Also in cephalopods, involvement of FaRPs may occur in all parts of the chromatophore control pathway. Several FaRPs are potent stimulants of the chromatophore muscles, regulating their contraction.[29]

INSULIN-RELATED PEPTIDES AND NPY

Discovery

The neuroendocrine light green cells (LGCs) in the cerebral ganglia of *Lymnaea stagnalis* have been suggested to be involved in the control of growth. In these neuroendocrine cells, molluscan insulin-related peptides (MIPs) have been found. To date, many members of MIP family in the *Lymanea*, from pedal ganglions of *Anodonta cygnea*, and lately in *Lottia gigantea* have been identified and characterized (reviewed in Ref. 33).

Differential screening of cDNA libraries of the CNS of parasitized and nonparasitized snails also reveal specific alteration in neuropeptide encoding gene expression. Among these, one of the significantly upregulated genes encodes for *Lymanea* neuropeptide Y homolog (LyNPY). Moreover, peptides with striking similarity to NPY/peptideYY (PYY)/polypeptideY (PY)/pancreatic polypeptide (PP) have also been isolated in other molluscs.[33]

Structure of the Precursor mRNA/Gene

Seven MIP have been characterized from the pond snail *L. stagnalis*, one in *Aplysia californica*, whereas in *Lottia gigantea* genome, two genes encode for the homolog of *Lymnaea* MIP, insulin 1 and insulin 2, and other two insulin genes: one encodes a peptide quite similar to vertebrate insulin, and the fourth encodes a homolog of *Drosophila* insulin-like peptide.[33] In *L. stagnalis*, the MIPs are all produced by a single cell type in the cerebral ganglia, which has been extensively characterized. Also see the chapter on insulin-like peptides in this section of the book.

The invertebrate counterpart of NPY peptides resembles more neuropeptide F (NPF), which shares structural similarity to members of the vertebrate NPY/PYY/PY/PP

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superfamily. NPY genes have been characterized in *L. stagnalis*, *Aplysia*, and *Lottia gigantea* (reviewed in Ref. 33).

Distribution of mRNA/Peptide

In *Lymnaea*, LGCs express genes encoding the molluscan MIPs: type-A cells express all MIP genes, whereas type-B cells do not express the MIP-I gene. Gene III is relatively strongly expressed in type-B cells. Genes II and V are moderately expressed in both cell types. Uniquely, the molluscan MIP VII gene is also expressed in neurons that may form part of the feeding circuitry in *Lymnaea*. *In situ* hybridization and specific immunoreactivity for NPY have been reported in *Lymnaea* and *Aplysia*. In *Lymnaea*, LyNPY-positive cells contact in a nonsynaptic way the neuroendocrine centers involves in the regulation of reproduction, ovulation, egg laying, and growth. In *Aplysia*, bag cell neurons are positive for NPY, and Northern blot analysis indicates that the peptide is abundantly expressed in the CNS.[21]

Receptors

Receptors for both MIP and NPY have been found in the nervous system of *Lymnaea*. Typical insulin receptor features, including a cysteine-rich domain, a single transmembrane domain, and a tyrosine-kinase domain, are conserved in the cloned putative MIP receptor. The *Lymnaea* cloned NPY receptor is a typical GPCR.

Biological Actions

In contrast to vertebrate insulins, the MIPs are produced by neuroendocrine cells of the CNS. The MIPs of LGCs are involved in the regulation of growth of both the soft body parts and the shell, as well as for protein and carbohydrate metabolism. MIP VII may also be involved as a neuromodulator in the feeding circuitry. In *Lymnaea*, female reproductive effort is decreased early by NPY action on caudodorsal cells. Growth is also suppressed almost immediately by the action of NPY on the MIPs-producing LGCs. By contrast, after treatment with NPY, food consumption is unchanged, suggesting a different role for this peptide in invertebrates.[11,21]

PEPTIDES AND FEEDING BEHAVIOR

Discovery

In gastropods, three families of peptides are directly involved in the control of the accessory radula closer (ARC) muscle in *Aplysia*: myomodulins, buccalins, and small cardioactive peptides (SCPs). All these peptides are localized in the motoneurons B15 and B16 of the buccal ganglia of *Aplysia* (reviewed in Elliott and Vehovszky, 2000). Myomodulin is also present in several other molluscs, and three myomodulin-related peptides has been purified and sequenced from extracts of snails *Helix aspersa*. Small cardioactive peptides (SCP_A, SCP_B) have been isolated from a wide range of molluscan groups. In *Aplysia*, other peptide families have been added to the list of feeding behavior controlling peptides: the enterins, the feeding circuit-activating peptides (FCAPs), cerebrin, and the pentapeptide PRQFVamide. There is also a novel peptide "allatotropin-related peptide" (ATRP) in *Aplysia* feeding motor system that contributes to a form of feedforward control.[16]

Structure of the Precursor mRNA/Gene

Myomodulin (A-I) has been characterized from the motor neuron B16 and ARC muscle in *Aplysia californica*. The complete sequence of this cDNA encodes a 42-kD prepropeptide. The myomodulin-related peptides have been also characterized in *Aplysia*. In the snail *Lymnaea*, a single gene encodes five structurally similar forms of myomodulin. Buccalin cDNA from *Aplysia* contains 19 distinct buccalin-related peptides, several of which are present in multiple copies. The small cardioactive peptide (SCP) gene of *Aplysia* encodes a precursor protein that contains two peptides related in structure and other unrelated peptides. In the snail *Lymnaea*, the SCP gene encodes a prepropeptide containing two structurally related SCPs. Enterin cDNA codes for a total of 35 copies of 20 distinct but related amidated peptides named enterin A (ENa) through enterin T (ENt). Analysis of the precursor structure encoded in the FCAPs cDNA predicts eight different structurally related peptides, named FCAPs through FCAPh. Cerebrin cDNA codes for a precursor containing a single copy of the peptide and one additional peptide. The C-terminal glycine indicates that the peptide is amidated at the C-terminus. PRQFVamide cDNA codes for a total of 33 copies of PRQFVamide and four related pentapeptides.

Distribution of mRNA/peptides

The myomodulin and buccalin mRNA are localized in specific neurons in each ganglia of the *Aplysia* CNS, mainly in the motoneurons B16. Buccalin transcript is also localized in the motor neurons B15. *In situ* hybridization analysis indicates that in *Lymnaea* the myomodulin gene is widely and abundantly expressed in all ganglia of the brain. In *Aplysia* and *Lymnaea*, SCPs are present mainly in the buccal ganglion. In *Lymnaea*, SCP and myomodulin genes are expressed within some large motoneurons (B1–B4). Both the enterin and FCAP mRNA are unevenly distributed throughout the central ganglia of

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Aplysia. Cerebrin RNA distribution studies performed on *Aplysia* showed detectable levels only in the cerebral ganglia, whereas the PRQFVamide precursor is abundant in the abdominal ganglion.

Another peptide seems to be involved in a feedforward compensatory mechanism mediated by its central and peripheral actions: the ATRP. *In situ* hybridization and immunostaining detection show that ATRP is mainly present in the buccal and cerebral ganglia and it is also present in the abdominal, pleural, and pedal ganglia.[16]

Biological Actions

In *Aplysia*, several of these peptides act in concert to enhance acetylcholine-induced contractions in a specific muscle of the buccal mass. Myomodulins induce potentiation, depression of the amplitude of the contractions, and acceleration of the relaxation rate of ARC muscle, whereas buccalins decrease the size of contractions of the ARC muscle. SCPa and SCPb are capable of acting postsynaptically on the muscle to potentiate the action on the primary neurotransmitter, acetylcholine. The concerted action of SCPs and myomodulin on the feeding behavior and the innervation of the foregut have also been demonstrated in *Lymnaea*.

Enterins are active in both the gut and the CNS. They play an inhibitory role on the contractions of the gut, inducing the switch from egestive to ingestive behavior. Conversely, FCAPs are capable of initiating rhythmic feeding motor programs mainly ingestive. Also, cerebrin has a profound effect on the feeding motor pattern, dramatically shortening the duration of the radula protraction in a concentration-dependent manner. PRQFVamide decreases the excitability of some but not all neurons of the buccal feeding circuit. A novel peptide that regulates feeding behavior is ATRP. ATRP produces a dose-dependent increase in the tonic contraction of the I2 muscle, promotes short protraction and long retraction, and has higher activity of protraction motoneurons.[16]

PEPTIDES AND RENAL FUNCTION

Discovery

Apart from the evidence that FaRP are involved in osmoregulations, other peptides are involved in maintaining the water balance in molluscs. A bradykinin peptide is present in the left upper quadrant cells in *Aplysia californica*, which extensively innervate the kidney. Moreover, peptides contained in the neuron R15 present in the abdominal ganglion of *Aplysia* increase the water content in this gastropod. In *Lymnaea*, the peptide lymnokinin PSFHSWSamide, which belongs to a leukinin family of peptides and the sodium-influx-stimulating peptide, has been characterized.

Structure of the Precursor mRNA/Gene

A bradykinin clone encodes a putative 16.3-kD precursor peptide, which contains potential proteolytic cleavage sites that could generate smaller mature peptides. A cDNA cloned from R15 neurons encoded for a propeptide contains three major peptides that are named R15α, R15β, and R15γ peptides. R15 mRNA undergoes alternative RNA splicing to yield two different mature mRNA species, R15-1 and R15-2. Lymnokinin was only purified with classical HPLC/MALDI approaches, and a cDNA clone, encoding to prohormone of the sodium-influx-stimulating peptide of the freshwater snail *Lymnaea stagnalis* has been isolated and characterized.

Distribution of mRNA/Peptides

In *Aplysia*, the expression of bradykinin RNA is seen only in neuron L5 among the LUQ cells, whereas R15 peptide is mainly localized in that neuron. *In situ* hybridization showed that the *Lymnaea* sodium-influx-stimulating peptide gene is expressed by the neuroendocrine Yellow Cells of the central nervous system.

Receptors

The cloned GPCR receptor GRL104 from *Lymnaea* represents the leucokinin-like peptide receptors, and only lymnokinin is able to activate GRL104 at the nanomolar level (Cox et al., 1997).

Biological Actions

In *Aplysia*, bradykinin is supposed to regulate some renal functions. On the contrary, substantial evidence indicates that neuron R15 is involved in the regulation of extracellular water volume or ionic composition. Unfortunately, there is a paucity of data concerning the role of leucokinin-like peptides (lymnokinin) in molluscs. Indeed, the *Lymnaea* sodium-influx-stimulating peptide controls the activity of sodium pumps in the integument, pericardium, ureter, and nephridial gland.

TACHYKININ

Discovery

Tachykinins (TKs) constitute the largest vertebrate neuropeptide family with multifunctions in central and peripheral tissues. See the chapter on tachykinin in this section of the book. In several invertebrate species, two types of structurally related peptides, tachykinin-related peptides (TKRPs) and invertebrate tachykinins (inv-TKs), have been identified.[32] The first tachykinin to be sequenced was isolated from the posterior salivary glands of cephalopods *Eledone moschata*

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and *Eledone aldrovandi*. Two other TKs were sequenced from the *Octopus* salivary gland and named OctTK-I and OctTK-II. Seven tachykinin-related peptides (from OctTKRP-I to OctTKRP-VII) were isolated from the *Octopus vulgaris*.[17] Finally, a type of tachykinin-related peptide receptor (TKRPR) was isolated from the bivalve *Anodonta cygnea*, and more recently, a novel TKRPR from the cephalopods *Octopus vulgaris* has been described.[17]

Structure of the Precursor mRNA/Gene

The cDNA of preproOctTK-I and preproOctTK-II are very similar.[17,32] OctTKs were included in the sequences flanked by a typical dibasic signal at the N-terminal and an amidation signal and a single basic residue at the C-terminal. Most inv-TKs peptides possess a characteristic C-terminal-FX1GX2R-NH2 consensus sequence that has never been found to exist in vertebrates.[32]

Distribution of mRNA

The mRNA of preproOctTK-I is expressed in some mucous-containing cells of (type A) epithelium of the salivary glands of *Octopus vulgaris*. Oct-TKRP receptor is abundantly expressed in the brain, buccal ganglion, gastric ganglia, olfactory and peduncle lobes, and optic lobe.[17]

Biological Actions

Functioning as paracrine or autocrine factors or as endocrine messengers, tachykinins are also involved in the regulation of various other physiological processes such as intestinal motility, smooth muscle contraction, and cardiovascular function.[32] Inv-TKs are present exclusively in the salivary glands of several species. Furthermore, inv-TKs do not exhibit any action on invertebrate tissues at physiological concentrations. Therefore, despite high sequence similarity to vertebrate TKs, inv-TKs are not anticipated to be the functional counterparts of vertebrate TKs.[32] The undecapeptide eledoisin that shows vasodilatory and smooth muscle stimulatory actions in mammals has an obscure presence in the *Octopus* salivary glands; it may have no function in the nervous system of cephalopods: OctTK I and - II show immediate contractile activities when applied on the longitudinal muscle of the guinea ileum. The OctTKs, in particular OctTK-I, are secreted in mucous saliva as a venomous substance acting on vertebrates such as fishes that are prey and/or natural enemies of the *Octopus*.

PEPTIDES AND REPRODUCTION

Egg-laying Hormones (ELHs)

Discovery

The caudodorsal cell (CDC) system of *Lymnaea* and the bag cells of *Aplysia* function as a peptidergic command center that initiates and coordinates ovulation and egg mass production with a fixed sequence of overt behaviors: cessation of locomotion, turning, modulation of feeding movements, oviposition, and inspection. In *Lymnaea*, egg laying and correlated processes are initiated and coordinated by CDC, neuroendocrine cellS located in the cerebral ganglia near the intercerebral commissure (COM), the neurohemal area of the CDC. Around the time of ovulation, all CDC display a synchronous spiking activity (discharge), which allows peptide release. In a similar way, the bag cells of *Aplysia*, two clusters of neuroendocrine cells located in the abdominal ganglion, show a prolonged and synchronous burst of electrical activity (after discharge) that precedes egg-laying behavior. At least nine peptides are released during this activity, including the egg-laying hormone (ELH), αBCP, βBCP, δBCP, and γBCP. Two additional peptides, the acidic peptide (AP) and εBCP, have been chemically characterized. Peptides A and B, as well as three peptide complexes, have been isolated from the atrial gland in the reproductive tract of *Aplysia*. The two peptides, and the complexes as well, are able to induce egg-laying behavior. Three other biologically active peptides, califin A, B, and C, were isolated and characterized from the atrial gland. Immunolocalization of ELH-like peptide in the neuroendocrine bag cells and their processes in *Aplysia dactylomela* confirms the key role of this peptide in *Aplysia* reproduction.[7]

Structure of the Precursor mRNA/Gene

In *Lymnaea*, the CDCs express three different caudodorsal cell hormone (CDCH) genes: CDCH-1, -2, and -3. The CDCH-1 gene leads to nine different peptides, including the caudodorsal cell hormone-l, the ε-peptide, the calfluxin, the α-caudodorsal cell peptide, the δ-peptide, β1- and β2-peptides, and the carboxyl terminally located peptide, from the cerebral commissure. The CDCH-2 gene encodes the same peptides, except for caudodorsal cell hormone-2, which is different from the ovulation hormone. At least five distinct genes for ELH exist in *Aplysia*. Transcription of this small multigene family results in the expression of at least five distinct RNA transcripts encoding ELH. The pattern of transcripts differs strikingly in different tissues: bag cells express three distinct mRNA species, whereas the atrial gland expresses two distinct mRNAs. AlI cDNAs contain an exon that spans the coding region (exon III) and one or two additional exons. Genomic clones encoding peptides A and B have been isolated and subjected to nucleotide sequence analysis.

Two genes coding homologous hormones are located close to each other in the genome of *Lottia gigantea*.[33] A single EST was found for ELH 2, none for ELH 1, and an EST encoding a homologous *Crassostrea* hormone was also found. The *Lottia* ELH genes seem to be quite different from those in *A. californica*. The transcript isolated from *A. dactylomela*[7] encoded a partial ELH precursor protein

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containing an *A. dactylomela* ε-BCP (δ-BCP), acidic peptide (dAP), and a 36 amino acid ELH (dELH) that are each flanked by paired basic cleavage sites. An alignment of the dELH with other Aplysiidae ELHs and *Lymnaea* ELH shows high conservation. The predicted amino acid sequence of *A. dactylomela* as shown by phylogenetic analysis is 91.7% identical to *A. californica* and *A. brasiliana* ELH. Comparing the primary structure of *Aplysia* ELH-related peptides reveals a pattern of highly conserved regions near both the N- and C-termini. This is critical for receptor recognition/binding site; the removal of the N-terminal amino acid (ELH2–36) or extension of the C-terminus by one residue (ELH-Gly37) results in a loss of egg-laying activity. Based on MALDI-TOF MS, performed on bag cell segments of the abdominal ganglion, a peptide of 3193 Da represents dELH cleavage at Leu26. Finally, a recombinant egg-laying hormone was generated.

Distribution of mRNA

In *Lymnaea*, *in situ* hybridization experiments show CDCH genes expressed in the CDC, while a group of small neurons in the cerebral ganglia express only the CDCH-I gene. There is widespread expression of the CDCH genes in peripheral tissues. Sensory neurons in the head skin and mantle edge express the CDCH-I gene. In *Aplysia*, >90% of the ELH genes family expression occurs in the bag ceIls, but these genes are expressed also in the atrial gland and in an extensive system of neurons distributed in four of the five ganglia of the central nervous system.

Biological Actions

In *Lymnaea stagnalis*, during discharges, CDCH-I is released into the blood, where it acts as a hormone that stimulates ovulation and egg mass formation and synthesis of secretory products in the female accessory sex glands and affects neurons in the neuronal circuits controlling locomotion and feeding. During the discharge, calfluxin stimulates the release and/or synthesis of perivitelline fluid and, in addition, affects feeding movements, and α-CDCP elicits electrical discharge activity in all CDCs, causing the maximal release of hormone and induces the transition from cessation of locomotion to the turning phase of egg-laying behavior. Egg laying in *Aplysia californica* is controlled by the bag cells, which have similar morphological and electrophysiological characteristics to the CDCs. They release a battery of peptides, including ELH and bag cell peptides αBCP, βBCP, and γBCP. ELH induces ovulation and alters the electrical activities of several neurons in the abdominal, buccal, and pedal ganglia. αBCP mimics bag cell-induced inhibition of several identified and unidentified neurons. Two additional peptides released by bag cells the acidic peptide and εBCP have no known biological or pharmacological activities. Egg-laying procedures can induce *A. dactylomela* to lay eggs by injection of *A. dactylomela* recombinant ELH.[7] Similar to all the mature ELH peptides identified, dELH functions by inducing egg laying when injected into animals.

APGWamide

Discovery

APGWamide is a tetrapeptide with the sequence Ala-Pro-Gly-Trp-NH_2 and is cleaved from a longer precursor peptide. It was isolated from ganglia of prosobranch mollusc *Fusinus ferrugineus*. In *Lymnaea stagnalis* and in *Helix aspersa*, APGWamide seems to be localized mainly in the neurons of asymmetrical right lobe of the cerebral ganglion. APGWamide-related peptides are also found in bivalves. In the cephalopod *Sepia officinalis*, the dipeptide GWamide has been purified from the optic lobe. Immunoreactivity occurs in the nervous system and in the oviducal gland of *Octopus vulgaris* APGWamide and in neurons and fibers of *Idiosepius pygmaeus* with a greater number of immunoreactive cells in the male.[28] It is a common neurotransmitter/neuromodulator peptide found in many species of molluscs and is often related to sex organ growth or reproductive behavior.

Structure of the Precursor mRNA/Gene

The complementary DNA encoding this tetrapeptide has been cloned from *Lymnaea stagnalis*, *Mytilus edulis*, and *A. californica*, whereas an APGW-related peptide-containing cDNA has been found in *Mytilus edulis*. In *Lymnaea stagnalis*, the primary structure of the APGW preprohormone deduced from the cDNA sequence predicts that the prohormone can be endoproteolytically cleaved to give rise to 10 copies of the sequence APGWG and a C-terminal peptide of 40 residues, called CALP (C-terminal anterior lobe peptide). Both the APGWG and a CALP are posttranslationally amidated.

In *Aplysia*, sequence analysis of cDNA clone show that it contains a 209 amino-acid APGWamide precursor. In *Mytilus*, the nucleotide sequence of the APGWamide related peptide cDNA clone encodes a 196 amino acid preprotein (reviewed in Ref. 21). The full processing of the *Mytilus* precursor has the potential to generate five RPGWamide peptides, one KPGWamide, one TPGWamide, and a C-terminal peptide, but no copies of APGWamide.

Distribution of mRNA/Peptide

The gene encoding the precursor of APGWamide is mainly expressed in the neurons of the right anterior lobe of the cerebral ganglia of *Lymnaea stagnalis* (Smit et al., 1992). In addition, gene expression was detected in the left cerebral ganglion and in several neurons in other parts of the CNS.

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In *Aplysia*, Northern blot analysis of total RNA indicates that a major transcript was expressed predominantly in the right cerebral and right pedal ganglia (Fan et al., 1997). In *Idiosepius pygmaeus*, APGWamide immunoreactivity is found in both neurons and fibers in the palliovisceral lobe of the posterior subesophageal mass, in the optic tract region, in the olfactory lobe, the vertical lobe and the dorsal basal lobe while in the supraesophageal mass, APGWamide immunoreactivity is detected only in neurons. Most immunoreactive neurons are found in the palliovisceral and olfactory lobes of both sexes.[28]

Biological Action

In *Lymnaea*, APGWamide inhibits contraction of the penis retractor muscle and functions as a neurotransmitter within the CNS, inhibiting the activity of certain neuroendocrine cells and coordinating the activity of different populations of penial motoneurons. APGWamide and related peptides have also been suggested to play a key role in the control of male copulation behavior in other gastropods such as *Helix aspersa* and to function as an inhibitory neurotransmitter in the CNS of both *Helix aspersa* and *Achatina fulica*. APGWamide in *Haliotis asinina*, besides its function inducing spermiation,[6] may regulate somatic growth: exogenous administration of APGW amide significantly stimulates body growth in juveniles.[5] APGWamide-related peptide of *Mytilus* shows similar potency on contraction of both the pedal retractor and anterior bissus retractor bivalve muscles.[21] The cephalopod *Sepia officinalis*, the peptide GWamide purified from the optic lobe shows a strong inhibiting action on the motility of the mature oviduct. APGWamide in *Octopus vulgaris* is strictly linked and associated to those systems and lobes that directly process close and distant chemical signals (reviewed in Ref. 21). In *Idiosepius pygmaeus*, based on the significantly higher number of APGWamide-immunoreactive neurons in male brain versus female brain, it has been supposed its a major role in the control of male reproduction.[28]

GnRH AND RELATED PEPTIDES

Discovery

Gonadotropin-releasing hormone (GnRH) is a decapeptide neurohormone crucial for the regulation of reproductive and neural functions in vertebrates[25] (also see chapter in the Brain Peptides section of the book). Multiple reports indicate the presence of bioactive molecules that immunologically and biochemically resemble vertebrate GnRH in gastropods, bivalves, and cephalopods.[30] Many groups have immunolocalized GnRH-like peptides in diverse species of molluscs. Using antimammalian (m)GnRH antibody it has been detected neurons in the pedal ganglion and cerebral ganglion of both sexes of *Patinopecten yessoensiis*.[22] Using an antiserum against oct-GnRH, other authors have localized the peptide in the dorso-lateral basal, olfactory lobes, the two neuropeptidergic centers controlling the activity of the gonadotropic optic gland, and ovary of *Sepia officinalis*.[14] Minakata et al. (2009), according to our previous findings,[10] first identified GnRH in the central and peripheral system of *Octopus vulgaris*, they found Oct-GnRH immunoreactive signals in the glandular cells of the mature optic gland. Using the same antiserum, immunoreactive neurons in all CNS ganglia of *Aplysia californica* were found. Two putative isoforms of GnRH-like peptides have been localized in neural ganglia and ovary of female abalone, *Haliotis asinina*, using antilamprey (l)GnRH-III and antitunicate (t) antibodies.[23] Amano et al. (2008, 2010) found in some lobes of the nervous system of *Loligo bleekeri*, in some ganglia of *Haliotis discus hannai* and in the cerebrobuccal ring of *Acanthopleura japonica* cell bodies and fibers crossreacting with a mouse monoclonal LRH13 and cGnRH-II antibodies suggesting the existence of two putative isoforms of GnRH in these species of molluscs. In *Aplysia californica*, Tsai et al.[31] showed by immunohistochemistry and radioimmunoassay analyses that pedal ganglia, followed by cerebral ganglia, abdominal ganglia, and then buccal, contain the highest level of ap-GnRH peptide. Treen et al.,[30] using antioctopus GnRH-like peptide antibody, showed the presence of GnRH-like peptide in neural tissue of *Crassostrea gigas*. Bigot et al.[4] characterized two GnRH-related peptides (Cg-GnRH-a and Cg-GnRH-G) by mass spectrometry from extracts of the visceral ganglia of the Pacific oyster *Crassostrea gigas*. These peptides show a high degree of sequence identity with GnRHs of other molluscs and annelids and to a lesser extent with those of vertebrates or with AKH and corazonins of insects. Significant differences in transcriptional activity of Cg-GnRH encoding gene are recorded in the ganglia along the reproductive cycle and according to trophic conditions suggesting the involvement of Cg-GnRHs as synchronizers of nutritional status (reviewed by Minakata, 2012).

Structure of the Precursor mRNA/Gene

A GnRH precursor cDNA has been cloned from the cephalopod *Octopus vulgaris*.[20] The precursor peptide contains 87 amino acids. The *Octopus* GnRH-like peptide contains two additional amino acids at positions 2 and 3, but the general motif of a pyroglutamyl residue at the N-terminus and amidated glycine residues at the C-terminus is conserved. A peptide similar to the chicken I form of GnRH (cGnRH-I) has been partially purified in the same species of *Octopus*. It is probable that *Octopus* GnRH-like peptide is different from the peptide described by Di Cristo et al.,[13] since it is unlikely that these two peptides share identical immunological and biochemical properties given the marked structural differences. cDNAs encoding GnRH-like precursors have been described

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from *A. californica* differing from oct-GnRH by only two amino acids (Zhang et al., 2008) representing one of the only two GnRH-like molecules found outside phylum *Chordata*. These results provide valuable support for an ancient origin of GnRH during metazoan evolution. A GnRH sequence cloned from the brain, ovary, and eggs of *Sepia officinalis* of 269-bp PCR product[14] shares 100% sequence identity with the open reading frame of GnRH isoform isolated from *Octopus vulgaris*. This sequence encodes a peptide that is organized as a preprohormone from which, after enzymatic cleavage, a dodecapeptide is released. Onitsuka et al. (2009) isolated the full-length cDNA of a GnRH-like molecule from the central nervous system of the squid *Loligo edulis*. This GnRH is identical to *Octopus* GnRH in the deduced amino acid sequence of the peptide and 80.5% similar in base sequence. In *Lottia* genome, a gene codes for a similar peptide, and its expression is shown by two ESTs.[33] Treen et al. (2012) cloned the cDNA transcripts of prepro GnRH-like peptides from two species of bivalve molluscs, the Yesso scallop *Patinopecten yessoensis*, and the Pacific oyster *Crassostrea gigas*.

Distribution of mRNA

In situ hybridization analysis in *Octopus vulgaris* shows Oct-GnRH mRNA-expressing cell bodies in the supraesophageal and subesophageal parts of the CNS. GnRH neurons are mainly present in the posterior olfactory lobule. These neurons send axons to the secretory cells of the optic gland. GnRH is also present in the subpedunculate area that controls the same gland. GnRH neurons have also been localized in both the subesophageal and supraesophageal masses, as well as in the plexiform layer of the deep retina in the optic lobe. Recently, Zhang et al. (2008) found the transcript for *Aplysia* GnRH widely expressed in the CNS, the ovotestis, and the atrial gland. In *Loligo edulis*, the prepro-GnRH mRNA was expressed mainly in the CNS (Onitsuka et al., 2009). Using a set of experiments aimed at manipulating a putative glutamate/NMDA/NO signal transduction pathway, Di Cristo et al.[14] showed by quantitative real-time PCR that NMDA enhances the expression of GnRH mRNA in a dose–response manner. The reverting effect of a selective antagonist of NMDA receptors (NMDARs), 2-amino-5-phosphopentanoic acid (D-APV), confirms that such an enhancing action is an NMDA receptor-mediated response. The results suggest that in *Octopus* l-glutamate could be a key molecule in the nervous control of sexual maturation.

Receptor

A GPCR expressed in the gonad of the pacific oyster *Crassostrea gigas* named Cg-GnRH-related receptor (Cg GnRHR) exhibits a high degree of amino acid sequence identity with both vertebrate GnRH receptors and insect AKH receptors. Quantitative RT-PCR shows a specific expression of Cg-GnRH-R in both male and female gonads during the reproductive cycle. This finding gives an insight into the involvement of a GnRH receptor ortholog in the control of reproduction in a protostomian invertebrate.[26] However, the characterization of multiple transcripts encoding GnRH-R orthologs (Cg-GnRH-RII-L/Cg-GnRH-RII-S) includes a truncated receptor (Cg-GnRH-R-TF) generated by the alternative splicing of a single mRNA precursor. The differential structure of these receptors suggests the existence of two receptor subtypes with regard to ligand specificity. Pattern of expression of these transcripts suggests that Cg-GnRH-R cognate ligand is specifically involved in the control of gametogenesis while Cg-GnRH-RII-L and Cg-GnRH-RII-S ones likely do not control reproductive functions.[26,27] A GnRH receptor (oct-GnRHR) specific for oct-GnRH from *Octopus* brain that includes domains and motifs typical of vertebrate GnRH receptors has been identified by Kanda et al. (2006) and Minakata et al., (2009). The intron-inserted positions are conserved between oct-GnRHR and the chordate GnRHR genes. The oct-GnRHR was responsive to oct-GnRH but not to any other HPLC fractions of the *Octopus* brain extract. These results show that oct-GnRHR is an authentic receptor for oct-GnRH. Southern blotting of reverse-transcription PCR products revealed that the oct-GnRHR mRNA was widely distributed in the central and peripheral nervous systems and in several peripheral tissues. *In situ* hybridization showed that oct-GnRHR mRNA was expressed in lobes deputed to the control of feeding, memory, and motor coordination. In a recent review, Tsai et al.[31] presented preliminary proof for a cognate *Aplysia californica* GnRH receptor (ap-GnRHR) detecting an abundant putative ap-GnRHR transcript in the central nervous system and the foot.

Biological Action

In *Helisoma trivolvis*, CNS extract was capable of stimulating gonadotrophin release from dispersed goldfish pituitary cells. In addition, the application of mammalian GnRH to *Helisoma* CNS elicited diverse electrophysiological and morphological effects on individual neurons. Application of vertebrate GnRH to bag cells of *Aplysia californica* inhibited the electrical response, a finding supported by a previous study on *Lymnaea* stagnalis. The reproductive and neural involvement of the molluscan GnRH was further corroborated by studies on the bivalves and cephalopods. These studies demonstrated a strong mitogenic action of GnRH on bivalve gonadal cells and a spatial distribution of GnRH-IR in the CNS that is highly suggestive of reproductive regulation. In *Octopus*, GnRH was proposed to act on the secretory cells of the gonadotropic optic gland. Interestingly, GnRH neurons in *Octopus* are located in the olfactory lobe consistently with the known relationship between GnRH and chemosensory system observed in vertebrates. These results

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are reinforced by the presence of GnRH in the osphradium of *Aplysia californica*. It has also been proposed that GnRH in *Octopus* is involved in the control of reproductive ducts and the contractions of the heart.[12]

Oct-GnRH was shown by Kanda et al. (2006) to induce steroidogenesis of testosterone, progesterone, and 17β-estradiol in *Octopus* ovary and testis, where oct-GnRHR was abundantly expressed. These results suggest that functions of the GnRH family are evolutionarily conserved between octopuses and chordates according to the recent findings of De Lisa et al.[9] Nakamura et al. (2007) demonstrated through *in vitro* experiments the increase of mitotic activity of spermatogonia at phase II induced by mGnRH in the scallop *Patinopecten yessoensiss*. The same mitotic activity was observed in the hemocyte lysate. Tsai et al.[31] in an elegant review discussed the deviation of *Aplysia californica* GnRH from a traditionally assumed role in reproductive stimulation, calling for a reassessment of the phylogenetic reconstruction of the physiological role played by ap-GnRH. They proposed that "protostomian GnRHs, assuming they are orthologous to chordate GnRH, have taken different functional trajectories during 600–800 million years of evolution and are not universally important to the reproductive activation in extant protostomes." However, they compared the results to the available functional data from *Octopus vulgaris* and concluded that protostomian GnRH-like molecules are likely to assume a wide range of physiological roles, and reproductive activation is not an evolutionarily conserved role of these molecules. In this context, we would like to highlight that in a recent article,[9] it has been demonstrated that the stimulation of the olfactory lobes of the *Octopus*, neuropeptidergic centers controlling the activity of the gonadotropic optic gland, with 17β-estradiol increases the oct-GnRH mRNA, confirming the existence of a strong coupling between 17β-estradiol and the transcriptional activity of oct-GnRH neurons. This finding is in agreement with the existence of an axis "area subpedunculate-olfactory lobe-optic gland" responsible for the reproductive behavior of *O. vulgaris*, in which the activity of oct-GnRH neurons is regulated by 17β-estradiol. The *Octopus* may therefore be an ideal model to further investigate the functional role of oct-GnRH revealing conserved roles of the protostomian GnRH-like molecules. Finally, Treen et al.[30] demonstrated that putative GnRH-like peptide stimulated spermatogonial cell division in cultured Yesso scallop *Patinopecten yessoensis* testis.

VP/OP

Discovery

The members of the vasopressin (VP) and oxytocin (OT) superfamily are widely distributed throughout the animal kingdom. In the snail *Lymnaea stagnalis*, Lys-conopressin is the only member present, whereas cephalotocin (CT) and octopressin (OP) were found in *Octopus vulgaris*.[12] The structure of the precursor mRNA/Gene in *Lymnaea stagnalis*, conopressin cDNA, encodes far a precursor that is organized very similarly to the prohormones of the vasopressin/oxytocin superfamily with a hydrophobic signal peptide, a nominal peptide, a neurophysin domain, and a C-terminal copeptin homologous extension of the neurophysin domain. In *Octopus vulgaris*, the OP and CT precursor cDNAs predict a polypeptide of 145 and 152 amino acids, respectively. The two genes of the oxytocin/vasopressin superfamily in *Octopus vulgaris* lack introns and consist of a single exon in their protein-coding regions, unlike most vertebrates and Lys-conopressin precursors.[12] The N-terminal sequences of the OP precursor and the CT precursor are different from each other. Both the remaining parts are identical except for two nucleotide bases. Bardou et al.,[3] by using antibodies raised against mammalian OT and VP, immunolocalized in the nervous system of *Sepia officinalis* several populations of OT-like and VP-like immunoreactive cell bodies and fibers widely distributed in lobes involved in learning, behavioral communication, feeding, reproduction, and motor coordination. No double-immunolabeled cell bodies or fibers were found in any lobes of the CNS, indicating, the existence of distinct oxytocinergic-like and vasopressinergic-like systems.[20]

Distribution of mRNA

In situ hybridization on *Lymnaea* CNS showed that the conopressin gene is expressed in a few neurons in both pedal ganglia. In *Octopus*, *in situ* hybridization using CT- and OP-specific cRNA antisense probes on adjacent sections was performed. The expression of CT mRNA was primarily limited within the ventral median vasomotor lobe of the subesophageal brain. OP mRNA was expressed in the superior buccal, anterior basal, median basal, dorsal basal, vertical, subvertical, posterior brachial, and palliovisceral lobes.[12]

Receptor

A receptor for vasopressin-related Lys-conopressin has been cloned in *Lymnaea stagnalis* (LSCPRl). Expression of this receptor mRNA was detected in central neurons and peripheral muscles associated with reproduction. In addition, a novel Lys-conopressin receptor subtype, named LSCPR2, was found. In *Octopus* brain, a GPCR specific to CT (CTRl) was cloned. This cDNA coded for an orphan receptor that was activated by CT. An additional CT receptor, CTR2, and a novel OP receptor, OPR, were later found in the CNS of *Octopus vulgaris*. CTR2 mRNA is present in

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various peripheral tissues, and OPR mRNA is detected in both the nervous system and peripheral tissues.

Biological Actions

Very little is known about the functions of these peptides in molluscs. In *Lymnaea*, Lys-conopressin excites the vas deferens *in vitro* in a dose-dependent manner. Upon application, Lys-conopressin also excites pacemaker neurons in the anterior lobe of the right cerebral ganglion, acting on the inward current. Bardou et al.[2] showed the role of two VP/OT superfamily peptides, octopressin (OP) and cephalotocin (CT), on long-term memory (LTM) formation of a passive avoidance task in a cephalopod mollusc, the cuttlefish, *Sepia officinalis*.

REFERENCES

1. Aroua S, Andouche A, Martin M, Baratte S, Bonnaud L. FaRP cell distribution in the developing CNS suggests the involvement of FaRPs in all parts of the chromatophore control pathway in *Sepia officinalis* (Cephalopoda). *Zoology* 2011;**114**:113–22.
2. Bardou I, Leprince J, Chichery R, Vaudry H, Agin V. Vasopressin/oxytocin-related peptides influence long-term memory of a passive avoidance task in the cuttlefish, *Sepia officinalis*. *Neurobiol Learn Mem* 2010;**93**:240–7.
3. Bardou I, Maubert E, Leprince J, Chichery R, Cocquerelle C, Launay S, et al. Distribution of oxytocin-like and vasopressin-like immunoreactivities within the central nervous system of the cuttlefish, *Sepia officinalis*. *Cell Res* 2009;**336**:249–66.
4. Bigot L, Zatylny-Gaudin CL, Rodet F, Bernay B, Boudry P, Favrel P. Characterization of GnRH-related peptides from the Pacific oyster *Crassostrea gigas*. *Peptides* 2012;**34**:303–10.
5. Chansela P, Saitongdee P, Stewart P, Soonklang N, Hanna PJ, Nuurai P, et al. The tetrapeptide APGW-amide induces somatic growth in *Haliotis asinina* Linnaeus. *J Shellfish Res* 2010;**29**:753–6.
6. Chansela P, Saitongdee P, Stewart P, Soonklang N, Stewart M, Suphamungmee W, et al. Existence of APGWamide in the testis and its induction of spermiation in *Haliotis asinina* Linnaeus. *Aquaculture* 2008;**279**:142–9.
7. Cummins SF, Nuurai P, Nagle GT, Degnan BM. Conservation of the egg-laying hormone neuropeptide and attractin pheromone in the spotted sea hare, *Aplysia dactylomela*. *Peptides* 2010;**31**:394–401.
8. Cummins SF, Tollenaere A, Degnan BM, Croll RP. Molecular analysis of two FMRFamide-Encoding transcripts expressed during the development of the tropical abalone *Haliotis asinina*. *J Comp Neurol* 2011;**519**:2043–59.
9. De Lisa E, Paolucci M, Di Cosmo A. Conservative nature of oestradiol signalling pathways in the brain lobes of *Octopus vulgaris* involved in reproduction, learning and motor coordination. *J Neuroendocrinol* 2012;**24**:275–84.
10. Di Cosmo A, Di Cristo C. Neuropeptidergic control of the optic gland of *Octopus vulgaris*: FMRF-amide and GnRH immunoreactivity. *J Comp Neurol* 1998;**398**:1–2.
11. Di Cosmo A, Di Cristo C. Chapter 36 – Molluscan bioactive peptides. In: Abba JK, editor. *Handbook of biologically active peptides*. Burlington: Academic Press; 2006. p. 235–40.
12. Di Cosmo A, Di Cristo C. Chapter 37 – Molluscan peptides and reproduction. In: Abba JK, editor. *Handbook of biologically active peptides*. Burlington: Academic Press; 2006. p. 241–6.
13. Di Cristo C, De Lisa E, Di Cosmo A. Control of GnRH expression in the olfactory lobe of *Octopus vulgaris*. *Peptides* 2009;**30**:538–44.
14. Di Cristo C, De Lisa E, Di Cosmo A. GnRH in the brain and ovary of *Sepia officinalis*. *Peptides* 2009;**30**:531–7.
15. Di Cristo C, Delli Bovi P, Di Cosmo A. Role of FMRFamide in the reproduction of *Octopus vulgaris*: molecular analysis and effect on visual input. *Peptides* 2003;**24**:1525–32.
16. Jing JA, Sweedler JV, Cropper EC, Alexeeva V, Park JH, Romanova EV, et al. Feedforward compensation mediated by the central and peripheral actions of a single neuropeptide discovered using representational difference analysis. *J Neurosci* 2010;**30**:16545–58.
17. Kanda A, Takuwa-Kuroda K, Aoyama M, Satake H. A novel tachykinin-related peptide receptor of *Octopus vulgaris* – evolutionary aspects of invertebrate tachykinin and tachykinin-related peptide. *FEBS J* 2007;**274**:2229–39.
18. Kobayashi S, Hattori M, Karoly E, Ito E, Matsuo R. FMRFamide regulates oscillatory activity of the olfactory center in the slug. *Eur J Neurosci* 2010;**32**:1180–92.
19. Lopez-Vera E, Aguilar MB, de la Cotera EPH. FMRFamide and related peptides in the phylum mollusca. *Peptides* 2008;**29**:310–7.
20. Minakata H. Oxytocin/vasopressin and gonadotropin-releasing hormone from cephalopods to vertebrates. In: Vaudry H, Shioda S, editors. *Phylogenetic aspects of neuropeptides: from invertebrates to humans*; 2010. p. 33–42.
21. Morishita F, Furukawa Y, Matsushima O, Minakata H. Regulatory actions of neuropeptides and peptide hormones on the reproduction of molluscs. *Can J Zool-Rev Can Zool* 2010;**88**:825–45.
22. Nakamura S, Osada M, Kijima A. Involvement of GnRH neuron in the spermatogonial proliferation of the scallop, *Patinopectenyessoensiss*. *Mol Reprod Dev* 2007;**74**:108–15.
23. Nuurai P, Poljaroen J, Tinikul Y, Cummins S, Sretarugsa P, Hanna P, et al. The existence of gonadotropin-releasing hormone-like peptides in the neural ganglia and ovary of the abalone, *Haliotis asinina* L. *Acta Histochem* 2010;**112**:557–66.
24. Paolucci M, Di Cristo C. Progesterone receptor in the liver and oviduct of the lizard Podarcis sicula. *Life Sciences* 2002;**71**:1417–27.
25. Roch GJ, Busby ER, Sherwood NM. Evolution of GnRH: Diving deeper. *General and Comparative Endocrinology* 2011;**171**:1–6.
26. Rodet F, Lelong C, Dubos MP, Costil K, Favrel P. Molecular cloning of a molluscan gonadotropin-releasing hormone receptor orthologue specifically expressed in the gonad. *Biochimica et Biophysica Acta-Gene Structure and Expression* 2005;**1730**:187–95.
27. Rodet F, Lelong C, Dubos MP, Favrel P. Alternative splicing of a single precursor mRNA generates two subtypes of Gonadotropin-Releasing Hormone receptor orthologues and their variants in the bivalve mollusc *Crassostrea gigas*. *Gene* 2008;**414**:1–9.
28. Sirinupong P, Suwanjarat J, van Minnen J. Distribution of APGWamide-immunoreactivity in the brain and reproductive organs of adult Pygmy squid, *Idiosepius pygmaeus*. *Invertebrate Neuroscience* 2011;**11**:97–102.
29. Suzuki M, Kimura T, Ogawa H, Hotta K, Oka K. FMRFamide elicits chromatophore expansion and retraction depending on its type and development in the squid, *Sepioteuthis lessoniana*. *Invertebrate Neuroscience* 2009;**9**:185–93.

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30. Treen N, Itoh N, Miura H, Kikuchi I, Ueda T, Takahashi KG, et al. Mollusc gonadotropin-releasing hormone directly regulates gonadal functions: A primitive endocrine system controlling reproduction. *General and Comparative Endocrinology*.
31. Tsai PS, Sun B, Rochester JR, Wayne NL. Gonadotropin-releasing hormone-like molecule is not an acute reproductive activator in the gastropod, *Aplysia californica*. *General and Comparative Endocrinology* 2010;**166**:280–8.
32. Van Loy T, Vandersmissen HP, Poels J, Van Hiel MB, Verlinden H, Vanden Broeck J. Tachykinin-related peptides and their receptors in invertebrates: A current view. *Peptides* 2010;**31**:520–4.
33. Veenstra JA. Neurohormones and neuropeptides encoded by the genome of *Lottia gigantea*, with reference to other mollusks and insects. *General and Comparative Endocrinology* 2010;**167**: 86–103.
34. Wollesen T, Cummins SF, Degnan BM, Wanninger A. FMRFamide gene and peptide expression during central nervous system development of the cephalopod mollusk, *Idiosepius notoides*. *Evolution & Development* 2010;**12**:113–30.
35. Zatylny-Gaudin C, Bernay B, Zanuttini B, Leprince J, Vaudry H, Henry J. Characterization of a novel LFRFamide neuropeptide in the cephalopod *Sepia officinalis*. *Peptides* 2010;**31**:207–14.

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Chapter 40

Parasitic Nematode Peptides

Nikki J. Marks and Aaron G. Maule

ABSTRACT

Nematode parasite peptides and/or their encoding gene transcripts have been characterized from a small number of species including the animal parasites *Ascaris suum*, *Haemonchus contortus*, and *Trichostrongylus colubriformis* and from the plant parasites *Globodera pallida*, *Meloidogyne incognita*, and *Bursaphelenchus xylophilus*. While peptide purification and sequencing efforts seeded the early peptide discovery phase database mining and mass spectrometric approaches in *A. suum* have provided significant impetus to the discovery of FMRFamide-like peptides (FLPs) and neuropeptide-like proteins (NLPs). Recent genome annotations have provided information on the complement of FLPs and NLPs in *M. incognita* and *B. xylophilus*. Insulin-like peptides (INSs) have only been reported from the genome annotation efforts in *B. xylophilus*. Functional data are centered on FLPs, and the vast majority derive from physiology studies in *A. suum* and some reverse genetic studies in plant parasitic nematodes. Neuropeptides play important roles in many aspects of parasitic nematode biology.

INTRODUCTION

In spite of its structural simplicity (e.g., comprising only 298 neurons in the female *Ascaris suum*), the nematode nervous system exhibits remarkable biochemical diversity, encompassing a diverse array of neuropeptides. At present, three peptide groupings have been identified within the parasitic members of the phylum Nematoda, namely, FMRFamide-like peptides (FLPs), neuropeptide-like proteins (NLPs), and insulin-like peptides (INSs). Although FLPs and INSs encompass recognized peptide families, the NLPs are an amalgamation of non-FLP or INS neuropeptides from numerous distinct families that have been identified in nematodes. In addition to peptides belonging to these large groupings, a putative peptide fragment of chromogranin has been reported. Antibacterial peptides, some of which in *Caenorhabditis elegans* have been designated NLPs, are considered elsewhere.

Parasitic nematode peptides have been discovered largely through peptide sequencing approaches in the large gastrointestinal parasite of pigs, *Ascaris suum* with complementary small-scale studies in the sheep gastrointestinal parasite, *Haemonchus contortus*.[18] These efforts have been expanded markedly through the combined exploitation of nucleic acid sequence resources and mass spectrometric methods.[9,10,34] Large-scale expressed sequence tag (EST) analyses across phylum Nematoda have revealed the occurrence of putative orthologs of numerous known FLPs[21] and NLPs,[23] providing evidence for the conservation of peptide sequences between species. Targeted mass spectrometric methods applied to *A. suum* have generated data on both peptide structure and localization. The genomes of two plant parasitic nematodes, the sedentary endoparasitic cyst nematode *Meloidogyne incognita*[1] and the migratory endoparasitic pine wood nematode *Bursaphelenchus xylophilus*,[12] have had their *flp* and *nlp* complements annotated with *ins* genes also being recorded in the latter species.

Functional data center on the FLPs, with most arising from whole animal, tissue, or cell level physiology studies in adult *A. suum*.[4,20,27,28,31,35] Two small-scale studies report the impact of *flp* gene RNA interference (RNAi) in *M. incognita* and the potato cyst nematode *Globodera pallida*.[5,16] In all the functional studies undertaken, FLPs have dramatic effects on nematode parasite physiology/behavior.

FMRFamide-LIKE PEPTIDES (FLPs)

FLP Discovery – Structure of the Precursor mRNA/Gene

The majority of peptides known from parasitic nematodes are FLPs, neuropeptides that encompass a C-terminal signature sequence comprising Xi-Xii-RFamide, where Xi is any amino acid except cysteine and Xii is any hydrophobic amino acid except cysteine. Currently, FLPs comprise the largest known peptide family in parasitic nematodes.

Some 40 distinct FLPs have been structurally characterized from *A. suum* and designated AF1–AF11 and AF13–AF41 (only a partial sequence is available for AF12; note that AF17 comprises a FMHFamide C-terminus, the only member which deviates from the FLP signature and is only tentatively assigned) (Table 1).[2–4,6,7,9,29,34,35] Two of these peptides (KHEYLRFamide, AF2 and KSAYMRFamide,

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00040-3
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TABLE 1 *Ascaris suum* FMRFamide-Like Peptides (FLPs) and Their Encoding Transcript Designators Derived from Peptide Structure Determinations, Gene Cloning, and or Putative Peptide Predictions from Genbank Resources

Neuropeptide name	Transcript designation *A. suum*/ *C. elegans*	Amino acid sequence
AF1	*afp-7/As-flp-8*	KNEFIRF.NH_2
AF2	*afp-4/As-flp-14*	KHEYLRF.NH_2
AF3	*afp-1/As-flp-18*	AVPGVLRF.NH_2
AF4	*afp-1/As-flp-18*	GDVPGVLRF.NH_2
AF5	*afp-2/As-flp-4*	SGKPTFIRF.NH_2
AF6		FIRF.NH_2
AF7		AGPRFIRF.NH_2
AF8	*afp-3/As-flp-6*	KSAYMRF.NH_2
AF9	*afp-14/As-flp-21*	GLGPRPLRF.NH_2
AF10	*afp-1/As-flp-18*	GFGDEMSMPGVLRF.NH_2
AF11	*afp-11/As-flp-1*	SDIGISEPNFLRF.NH_2
AF12	*afp-1/As-flp-18*	FGDEMSMPGVLRF.NH_2
AF13	*afp-1/As-flp-18*	SDMPGVLRF.NH_2
AF14	*afp-1/As-flp-18*	SMPGVLRF.NH_2
AF15	*afp-9/As-flp-16*	AQTFVRF.NH_2
AF16	*As-flp-29*	ILMRF.NH_2
AF18		*NKFFLRKPXXX
AF19	*afp-13/As-flp-13*	AEGLSSPLIRF.NH_2
AF20	*afp-1/As-flp-18*	GMPGVLRF.NH_2
AF21	*afp-6/As-flp-11*	AMRNALVRF.NH_2
AF22	*afp-6/As-flp-11*	NGAPQPFVRF.NH_2
AF23	*afp-6/As-flp-11*	SGMRNALVRF.NH_2
AF24	*As-flp-12*	RNKFEFIRF.NH_2
AF25	*afp-11/As-flp-1*	NNFLRF.NH_2
AF26	*afp-11/As-flp-1*	KPNFLRF.NH_2
AF27	*afp-11/As-flp-1*	PADPNFLRF.NH_2
AF28	*afp-11/As-flp-1*	SAEPNFLRF.NH_2
AF29	*afp-11/As-flp-1*	NAEPNFLRF.NH_2
AF30	*afp-10/As-flp-28*	APNKILMRF.NH_2
AF31	*afp-11/As-flp-1*	TPSNNFLRF.NH_2
AF32	*afp-11/As-flp-1*	GSDPNFLRF.NH_2

TABLE 1 *Ascaris suum* FMRFamide-Like Peptides (FLPs) and Their Encoding Transcript Designators Derived from Peptide Structure Determinations, Gene Cloning, and or Putative Peptide Predictions from Genbank Resources—Cont'd

Neuropeptide name	Transcript designation *A. suum*/ *C. elegans*	Amino acid sequence
AF33	*afp-11/As-flp-1*	SNQAQNFLRF.NH_2
AF34	*afp-13/As-flp-13*	DSKLMDPLIRF.NH_2
AF35	*afp-13*	DPQQRIVTDETVLRF.NH_2
AF36	*afp-12/As-flp-24*	VPSAADMMIRF.NH_2
AF37	*As-flp-2*	FRGEPIRF.NH_2
AF38	*As-flp-2*	AQREPIRF.NH_2
AF39	*As-flp-5*	SPKQKFIRF.NH_2
AF41	*afp-11/As-flp-1*	KPNFIRF.NH_2
	As-flp-3	GGPLGTMRF.NH_2
	As-flp-4	ASFIRF.NH_2
	As-flp-4	*KASFIRL.NH_2
	As-flp-10a	LPHMEKISGFIRF.NH_2
	As-flp-10b	SCYMIRF.NH_2
	As-flp-10c	TVYAVIRF.NH_2
	As-flp-17	KSAFVRF.NH_2
	As-flp-17	KSSYIRF.NH_2
	As-flp-19	*WASQLRL.NH_2
	As-flp-19	ASWASKVRF.NH_2
	As-flp-25	TSYQYFRF.NH_2
	As-flp-31	GRPRGPLRF.NH_2

*Peptides not conforming to recognized FLP signature. Note that those peptides not assigned names are putative peptides predicted from Genbank database searches.[10,26,28,34,35]

AF8) and one novel peptide (LQPNFLRFamide) have also been structurally characterized from the third larval stage of *H. contortus* a common parasite of the abomasum of sheep.[8,13,18] In both parasites, AF2 is the most abundant peptide detected.

FLPs are encoded on *flp* genes and a genome wide BLAST survey identified ESTs encoding known *C. elegans* FLPs in numerous parasitic nematode species.[21] More than 1000 putative FLP-encoding ESTs have been identified across 32 parasite species with the identification of homologs of 29 *C*

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elegans flp genes and two additional *flp* genes (*flp-30* and *flp-31*) that appear to be unique to some sedentary endoparasites of plants; note that a *flp-31* homolog (*As-flp-31*) has recently been reported in *A. suum*.[10] Cloning and sequencing-based confirmation of *flp* gene transcripts have only been performed for selected genes in adult *A. suum*[17,35] and second stage larval *G. pallida*.[14] In *A. suum*, full length *flp* gene sequences have been designated *afp-X* (*Ascaris* FMRFamide-like Precursor proteins; X represents the gene number assigned chronologically upon discovery) genes; here, each will be co-designated by its *C. elegans* homolog, that is, *As-flp-X*. FLP-encoding genes characterized from *A. suum* include *afp-1* (*As-flp-18*), *afp-3* (*As-flp-6*), *afp-4* (*As-flp-14*), *afp-6* (*As-flp-11*), *afp-12* (*As-flp-24*), and *afp-13* (*As-flp-13*); these encode 15 FLPs and, in addition to FLPs, *afp-13* encodes three non-FLP neuropeptides (TPPEEDLLGRFT, TNIMGENRLNRNL, GNSYRSFFNPNEYTVVE).[9,26,35] Other *afp* genes have been predicted from sequence resources available for *A. suum*.[10] Five *flp*-encoding genes have been characterized from *G. pallida*, namely, *Gp-flp-1*, *Gp-flp-6*, *Gp-flp-12*, *Gp-flp-14*, *and Gp-flp-18*[14]; these encode 13 distinct FLPs (Table 2). Another four have been characterized from *M. incognita* (*Mi-flp-1*, *Mi-flp-7*, *Mi-flp-12*, *Mi-flp-14*) and one from *Meloidogyne minor* (*Mm-flp-12*) (Table 2).[11]

Published genome sequences for two plant parasitic nematodes have provided preliminary data on the neuropeptide complements for *M. incognita*[1] and *B. xylophilus*.[12] Both have reduced complements of FLP-encoding genes compared with *C. elegans* with *M. incognita* having 19 *flp* genes and *B. xylophilus* having 21 *flp* genes compared to *C. elegans* 29 *flp* genes (Table 3). There seems to be general consistency in the complement of *flps* occurring across nematode parasites.

FLP Distribution/Expression

Carboxy-terminal FLP antisera (mostly with undetermined specificity) promiscuous for a diverse array of FLP ligands have provided an insight into the widespread distribution of FLPs in nematode parasite nervous systems. Up to 75% of the *A. suum* nervous system expresses FLPs with staining occurring in all neuronal subtypes including interneurons, motorneurons and sensory neurons.[17] A number of studies report the localization/expression of individual FLPs or *flp* genes using a variety of methods including immunocytochemistry (ICC) with specific antisera, *in situ* hybridization (ISH) of the gene transcript and cell/ganglion specific mass spectrometry.[17] Although these studies are relatively few in number, they provide significant added value to peptide functional data based on physiology or phenotype.

The first study to use an antiserum specific for an individual FLP localized AF2 to a subset of neurons in the brain and pharynx as well as single neurons in the dorsal and ventral nerve cord that synapse with muscle and inhibitory motorneurons.[6] A mass spectrometric map of the major neuronal elements in adult *A. suum* reveals the complement of FLPs in each of the major neuronal elements (circumpharyngeal nerve ring, dorsal ganglion, lateral line ganglia, retrovesicular ganglion, ventro-dorsal commissures, ventral ganglion, segments of the dorsal and ventral nerve cords) and confirms that individual FLPs have distinct and variably overlapping expression patterns that provide for unique FLP fingerprints in each neuron/neuronal subset.[34]

The most compelling results on FLP localization have been provided for three distinct FLPs encoded on the *afp-6* (*As-flp-11*) gene, which were identified in the nerve ring and ventral ganglion of *A. suum* using mass spectrometry and by ICC and ISH in a single ventral ganglion neuron designated as an RIS-like neuron based on the *C. elegans* neuronal map.[35] Transcripts for *afp-1* (*As-flp-18*), *afp-3* (*As-flp-6*), and *afp-4* (*As-flp-14*) were each localized to a distinct subset of neurons using ISH.[26] A combination of mass spectrometry methods and ICC using a specific AF1 (KNEFIRFamide) antiserum localized this peptide to the paired URX and RIP head neurons, two pairs of lateral ganglion neurons, unpaired PQR, PDA, and PDB tail neurons that extend along the ventral nerve cord and three pairs of pharyngeal neurons.[29] A series of *afp* genes have been localized to individual neurons from the dorsal ganglion of *A. suum* using mass spectrometry.[10]

ISH was used to examine the expression pattern of five *flp* genes from the plant parasitic nematode, *G. pallida*.[15] Four *flp* genes (*Gp-flp-1*, *Gp-flp-6*, *Gp-flp-12*, and *Gp-flp-14*) are expressed in neurons of the infective J2 stage of *G. pallida*. Expression of *Gp-flp-1*, which encodes peptides terminating in the C-terminal motif FLRFamide, is localized to VA/VD and DA/DB-like motorneurons and RIF-, RIG-, AVG-, and SAB-like interneurons. *Gp-flp-6*, which encodes KSAYMRFamide, is expressed in neurons associated with the anterior nerve ring, PHA- and PHB-like sensory neurons and lumbar ganglion interneurons. In comparison, the expression of *Gp-flp-12* encoding KNKFEFIRFamide is localized to the retrovesicular ganglion D/V-like motorneurons, preanal ganglion motorneurons, lumbar ganglia interneurons associated with body wall, and vulval motorneurons, the BDU-like interneurons that are associated with the HSN vulval motorneuron, and an ADE-like sensory neuron. *Gp-flp-14* which encodes AF2 (KHEYLRFamide) is expressed in the anterior ganglion and several paired cells, which are posterior to the circumpharyngeal nerve ring; specifically the RMED-, RME-, and RMG-like cells associated with the head motorneurons and ADA-like interneurons. The study however did not report the expression of *Gp-flp-18*. Surprisingly, ISH of *Mi-flp-12* and *Mi-flp-14* indicated expression patterns that differed from those reported for the same transcripts in *G. pallida*.[11]

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TABLE 2 Parasitic Nematode FMRFamide-Like Peptides (FLPs) Characterized or Predicted from Confirmed Gene Sequences in *Haemonchus contortus*, *Globodera pallida*, *Meloidogyne incognita*, and *Meloidogyne minor*

Parasitic nematode	Encoding gene	Amino acid sequence
Haemonchus contortus		KHEYLRF.NH_2
		KSAYMRF.NH_2
		LQPNFLRF.NH_2
Globodera pallida	*Gp-flp-1*	TSSNFLRF.NH_2
	Gp-flp-1	SSASMSTSSEPNFLRF.NH_2
	Gp-flp-1	GGVDPTFLRF.NH_2
	Gp-flp-1	ANNNFLRF.NH_2
	Gp-flp-1	QANPNFLRF.NH_2
	Gp-flp-6	4x KSAYMRF.NH_2
	Gp-flp-12	KNKFEFIRF.NH_2
	Gp-flp-14	2x KHEYLRF.NH_2
	Gp-flp-18	DEFVAPGVLRF.NH_2
	Gp-flp-18	MPGVLRF.NH_2
	Gp-flp-18	AVPGVLRF.NH_2
	Gp-flp-18	AEVPGVLRF.NH_2
	Gp-flp-18	MPQVLRF.NH_2
Meloidogyne incognita	*Mi-flp-1*	NSLLMSGPWANLSWSDADPNFLRF.NH_2
	Mi-flp-1	AAGQSANFLRF.NH_2
	Mi-flp-1	SAPYDPNFLRF.NH_2
	Mi-flp-1	GLVGQSYLRF.NH_2
	Mi-flp-1	GNNFLRF.NH_2
	Mi-flp-7	APLDRSALVRF.NH_2
	Mi-flp-7	APLDRSALVRF.NH_2
	Mi-flp-7	2x APLDRAAMVRF.NH_2
	Mi-flp-7	APFDRSSMVRF.NH_2
	Mi-flp-12	KNNKFEFIRF.NH_2
	Mi-flp-14	2x KHEYLRF.NH_2
Meloidogyne minor	*Mm-flp-12*	KNNKFEFIRF.NH_2

The three *H. contortus* peptides listed have been structurally characterized, and all others are putative peptides predicted from characterized gene transcripts.[8,11,13,14,18]

Although only a small number of species have been studied, one remarkable feature of the FLP localization data in parasitic nematodes is that identical or near-identical peptides display unique distributions in individual nematode species. This variation in expression may contribute to the neuronal plasticity seen across nematodes in spite of a structurally very simple nervous system.

FLP Receptors

Although the peptide PF4 (KPNFIRFamide) seems to activate a ligand-gated chloride channel,[20] most other FLPs appear to associate with G protein-coupled receptors (GPCRs). Although currently no FLP receptors have been fully characterized from a parasitic nematode, a number of

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TABLE 3 Neuropeptide-Encoding *nlp* (Neuropeptide-Like Protein), *ins* (Insulin-Like Peptide), and *flp* (FMRFamide-Like Peptide) Genes Identified within the *Meloidogyne incognita* and *Bursaphelenchus xylophilus* Genome Sequences

Gene number	*Meloidogyne incognita*		*Bursaphelenchus xylophilus*		
	flp	*nlp*	*Flp*	*nlp*	*ins*
1	X	X	X		X a,b
2		X	X	X	
3	X	X	X	X	
4			X		
5	X		X		
6	X	X	X		X
7	X		X	X	
8		X	X	X	
9		X		X	
10		X		X	
11			X		
12	X	X	X	X	
13	X	X	X	X	
14	X	X	X	X	
15		X		X	
16	X		X		
17		X	X		X?
18	X	X	X		X
19	X		X		
20	X		X	X	
21	X	X	X		
22	X	X	X		
23					
24		NA		NA	
25	X	NA		NA	
26		NA		NA	
27	X	NA		NA	
28		NA		NA	
29		NA		NA	
30	X	NA		NA	
31	X	NA		NA	X
32	X	NA	X	NA	X?
33		NA	X	NA	
34	NA	NA	NA	NA	
35	NA		NA		
36	NA	X	NA		
37	NA	X	NA	X	
38	NA	X	NA	X	
39	NA		NA		
40	NA	X	NA	X	
41	NA		NA		NA
42	NA	X	NA	X	NA
43	NA		NA		NA
44	NA	X	NA		NA
45	NA		NA		NA
46	NA		NA	X	NA
47	NA		NA	X	NA

X indicates the presence of the ortholog of *Caenorhabditis elegans* gene; NA indicates genes not annotated in *C. elegans* and/or genes not relevant here; a,b denotes the existence of two *ins-1* subtypes; ? denotes variant *ins*-like sequences. Data derived from Refs. 1, 12.

Ascaris FLPs have however been identified as ligands to a G protein-coupled FLP receptor from *C. elegans*, namely, the NPR-1 receptor. Both AF9 (GLGPRPLRFamide) and PGV-LRFamides, which are encoded on *afp-1* (*As-flp-18*), were identified as ligands for this receptor.[24] A few FLPs have been identified as ligands (although potency was low) for the latrophilin-like receptor in the sheep parasite *H. contortus*.[25]

BIOLOGICAL ACTIONS OF FLPs

Ascaris suum Body Wall Muscle

Much data pertaining to the bioactivity of FLPs have been amassed using either innervated or denervated *A. suum* somatic body wall muscle strips. The bioactivity recorded for at least 25 distinct FLPs has been assigned to four response types, designated as body wall response types

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(bwRT) 1– 4. Bioactivity comprises an array of diverse activities ranging from bwRT-1, which consists of both presynaptic and postsynaptic inhibition causing a slow prolonged response. By contrast, bwRT2 comprises a fast and transient response, whereas bwRT3 is excitatory inducing increased contractility. Finally, bwRT4 produced a biphasic response comprising an initial transient relaxation followed by a period of increased contractility.[24]

AF1 (KNEFIRFamide) and AF2 (KHEYLRFamide) induce qualitatively similar biphasic activities on somatic muscle that comprise a transient relaxation followed by a sustained period of contractility (sequential, rapid contractions and relaxations) consistent with bwRT4,[2,4,20,30] although AF2 is up to 1000-fold more potent than AF1. Denervation of the muscle strip abolishes the excitatory phase of the biphasic response suggesting the involvement of distinct receptors;[20] AF2 also enhances membrane potential responses to the excitatory neurotransmitter acetylcholine believed to be through its interaction with muscarinic-type acetylcholine receptors.[31] Further studies reveal that AF2 increases transient inward calcium currents in *A. suum* muscle.[33]

AF3 (AVPGVLRFamide) and AF4 (GDVPGVLRFamide) cause bwRT3-like contractions of *A. suum* dorsal body wall muscle strips.[3] Other FLPs including AF5 (SGKPTFIRFamide), AF9 (GLGPRPLRFamide), AF10 (GFGDEMSMPGVLRFamide), AF13 (SDMPGVLRFamide), AF14 (SMPGVLRFamide), AF17 (FDRDFMHFamide), and AF20 (GMPGVLRFamide) also induce bwRT3 responses.[3,6] AF11 (SDIGISEPNFLRFamide), AF6 (FIRFamide), and AF19 (AEGLSSPLIRFamide) cause slow and persistent inhibition of body wall muscle typical of bwRT1, whereas bwRT4 effects have been reported for AF8 (KSAYMRFamide).[6,20] AF7 (AGPRFIRFamide) and AF16 (ILMRFamide) have little or no effects on *A. suum* somatic body wall preparations.[6]

More recently, AF21 (AMRNALVRFamide), AF22 (NGAPQPFVRFamide), and AF23 (SGMRNALVRFamide) have all been shown to induce transient dorsal body wall muscle relaxations in *A. suum.*[35] The largest relaxations are induced by AF22 as are the largest muscle cell hyperpolarizations.

Ascaridia galli and *Haemonchus contortus* Body Wall Muscle

A small number of peptides are active on the body wall muscle of the sheep parasite *H. contortus* and the chicken parasite *Ascaridia galli*.[18,32] In *A. galli*, AF3 and AF4 elicit an increase in the propagation of phasic spontaneous activity and cause contractions of body wall muscle, actions reported as being independent of the cholinergic system. In *H. contortus*, the injection of AF2 inhibits acetylcholine-induced contractions, whereas AF8 enhances both spontaneous and acetylcholine-induced contractions.[18] Selective application of AF8 to *H. contortus* isolates sensitive or susceptible to the cholinergic agonist levamisole revealed differential susceptibilities to AF8 consistent with the hypothesis that it modulates cholinergic signaling.[18]

Ascaris suum Ovijector

A wide variety of FLPs modulate the activity of the *A. suum* ovijector, which regulates egg output. As with body wall muscle effects, multiple ovijector response types have been recorded (ovRT1, inhibitory; ovRT2, excitatory; ovRT3, transient contraction; ovRT4, transient contraction/paralysis; ovRT5, contraction frequency increase). AF2 and AF8 induce ovRT1 effects, AF3, AF4, and AF9 induce ovRT2 effects, whereas AF1 induces ovRT4 effects. The other ovRT categories were recorded after the addition of FLPs identified from the free-living nematode *Caenorhabditis elegans* FLPs.[24]

Ascaris suum Pharynx

Two studies report the effects of six FLPs on serotonin-induced pharyngeal pumping activity in adult *A. suum*. Although AF1 and AF8 inhibit the serotonin-induced excitation, AF2, AF3, AF4, AF6, and AF16 have no significant effects on this activity.[24] More recently, the impact of AF21, AF22, and AF23 on both spontaneous small amplitude spikes that occur in the absence of pumping and on larger, serotonin-induced, larger amplitude spikes was reported.[35] All three FLPs reversibly inhibit both spontaneous small and induced large amplitude spiking. The AF22 effects are the most persistent, lasting for 8–9min compared to the 1–3min effects of AF21 and AF23.

Parasite Behavior, Body Length, Phenotype

The influence of peptide injection into the body cavity (pseudocoel) of adult female *A. suum* on body length and behavior/posture has been examined for a variety of FLPs. Most commonly, test peptides are administered via injection into the pseudocoelomic cavity of female *A. suum* that have been ligatured by the head to initiate an undulating waveform. The injected peptides disrupt locomotory behavior in one of two ways. First, by either completely abolishing all rhythmical movements and producing a straight worm or secondly, reducing the number and propagation of locomotory waveforms. [28]

The injection of AF1 or AF2 inhibits locomotory wave propogation, reduces waveform formation, and diminishes body length;[4,27] AF2 induces occasional abrupt, jerky movements.[7] Further, injections of AF1, AF2, AF5, or AF7 reduce locomotory activity with AF1 and AF5 also causing a linearization of body posture.[7] AF8 injections abolish locomotory activity and induce ventral coiling and head tremors.[7,27] AF9

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reduces worm motility and head searching activity with postural changes comprising anterior linearization and highly variable posterior coiling phenotypes; AF17 also reduces worm movement but increased body length. By contrast, the peptide products of *afp-1* (*As-flp-18*), including AF3, AF4, AF10, AF13, AF14, and AF20, induced exaggerated postures while increasing waveform frequency, head-thrashing and coiling; head searching activity is blunted and AF10 also decreases body length.[7,27]

Head searching and locomotory activities are abolished by AF15, AF17, AF19, AF21, AF22, and AF23;[7,27,35] with the injection of AF15, AF17, or AF19 the resultant inactive worms display a curved body posture. Additional observations on the effects of AF21, AF22, and AF23 center on persistence with AF21 effects diminishing within 2–3h, whereas those for AF22 and AF23 persisted for >11h and for 6–9h, respectively.[35] AF6 and AF16 injections have little obvious impact on worm posture/behavior.

One study showed that FLRFamide enhances body movement frequency in the plant parasite *M. incognita* although there is no effect on stylet activity.[19]

The development of RNAi-based reverse genetics approaches in some parasitic nematodes has facilitated the selective knockdown of *flp*-encoding genes and subsequent assessment of the phenotypic consequences using behavioral assays. At this time, two studies report that *flp* gene knockdown alters normal behavior in the infective J2 stage of the plant parasitic nematodes *G. pallida* and *M. incognita*.[5,16] The silencing of any one of five *flp* genes in *G. pallida* J2 worms (*Gp-flp-1*, *Gp-flp-6*, *Gp-flp-12*, *Gp-flp-14*, *Gp-flp-18*) or of *Mi-flp-18* in *M. incognita* J2 worms trigger profound motor dysfunction with a subsequent reduction in the ability of worms to migrate through a sand column. After 24h of continuous exposure to the specific RNAi trigger (double-stranded RNA or short interfering RNA complementary to the target gene transcript), migration is dramatically reduced by between 71% and 100% for all genes examined (*Gp-flp-1*, 98%; *Gp-flp-6*, 94%; *Gp-flp-12*, 100%; *Gp-flp-14*, 71%; *Gp-flp-18*, 100%; *Mi-flp-18*, 98%). The influence of silencing genes that encode a single FLP (*Gp-flp-6*, *Gp-flp-12*, *Gp-flp-14*) is as dramatic as RNAi of *flp* genes encoding multiple structurally related FLPs (*Gp-flp-1*, *Gp-flp-18*, *Mi-flp-18*). Observations of the *G. pallida* J2 worms post-RNAi reveal distinctive phenotypes associated with the knockdown of each gene transcript, with worms appearing as slow, stationary, twitching, coiled, or straight. Taken together, these RNAi data support critical roles for each of these *flp* genes in worm migratory behavior.

Ascaris suum Motorneuron Activity

Since demonstration of the inhibitory effects of AF1 on *A. suum* dorsal (DI) and ventral inhibitory (VI) motorneurons,[4] the effects of AF5, AF7, and AF11 were tested on multiple motorneuron subtypes,[6] a further 14 *Ascaris* FLPs (AF2, AF3, AF4, AF6, AF8, AF9, AF10, AF13, AF14, AF15, AF16, AF17, AF19, AF20) were tested on *A. suum* DI and dorsal excitatory 2 (DE2) nerves[7] and a further three FLPs (AF21, AF22, AF23) were tested on DI, dorsal excitatory 1 (DE1), and DE2 motorneurons (35). A wide variety of FLP effects on *A. suum* motorneurons have been shown and, before the work on AF21, AF22, and AF23, these were subdivided into five motorneuron response types (designated here as mnRT1 to mnRT5).

The first response type (mnRT1) occurs upon the addition of the FIRFamides (AF1, AF5, AF6, and AF7), which induces depolarizing effects on DE2 and hyperpolarizing effects on DI motorneurons. Although AF1 causes the most dramatic reductions in DE2 input resistance, the effects of AF6 and AF7 on both DE2 and DI are weak. The mnRT2 is observed upon superfusion with AF2, which induces a unique biphasic response in the input resistance of DE2 motorneurons; this response comprises an early transient increase followed by a sustained increase in input resistance. The AF2-induced DE2 depolarization is the largest recorded for any FLP. AF2 has no significant effects on DI motorneurons. A weak, transient DE2 hyperpolarization followed by a prolonged depolarization, sustained in the absence of input resistance, changes typifies the mnRT3 effects of AF9. AF9 effects on DI comprise a weak, transient hyperpolarization. AF8 induces mnRT4, which encompasses a profound depolarization of DI and a small hyperpolarization of DE2 motorneurons. AF17 and AF19 both induce mnRT5 dominated by strong hyperpolarizations and input resistance decreases in both DE2 and DI. Indeed, the strongest DE2 hyperpolarizations are seen with AF19.

Delineation of the effects of the *afp-6* (*As-flp-11*) encoded peptides AF21, AF22, and AF23 on *A. suum* motorneurons does not include mnRT assignments.[35] Although AF21 and AF23 cause marked depolarizations of DE1, DE2, and DI motorneurons concomitant with strong input resistance increases, AF22 causes hyperpolarization of the same three motorneuron subtypes with associated, small input resistance increases. Notably, AF23 induces hyperpolarizations of DE2 and DI motorneurons completely reverse AF21 and AF23 induced depolarizations of these neurons and seem to involve a calcium-dependent mechanism. It has been hypothesized that the apparently antagonistic actions of the hyperpolarizing AF22 versus the depolarizing AF21/AF23 could be allayed by the differential distribution of their cognate receptors/sites of action.

Second Messenger Signaling Components

The influence of numerous parasitic nematode FLPs on levels of the second messengers cyclic adenosine monophosphate

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(cAMP) and cyclic guanosine monophosphatre (cGMP) has been examined in *A. suum*; additionally, the influence of AF3 on cAMP levels in *A. galli* have been reported.[27,28,30,32] Levels of cAMP in *A. suum* muscle strips or whole worms increase significantly after treatment with the FLPs AF1 or AF2; although the effects of AF2 on cAMP are the most dramatic, it has no effects on cGMP whereas AF1 significantly increases the levels of both cAMP and cGMP in whole worms.[28] AF1 increases cAMP levels in the head region of *A. suum*. By contrast, AF3, AF5, and AF17 induce decreases in cAMP levels, although the effects of AF5 are small; AF3 also decreases cAMP levels in *A. galli*.[27,28,32] Surprisingly, *afp-11* (*As-flp-1*) encoded peptides have a variety of effects on cAMP levels in *A. suum*, including no significant effect (AF11, AF27 {PADPNFLRFamide}, AF28 {SAEPNFLRFamide}, AF32 {GSDPNFLRFamide}, AF33 {SNQAQNFLRFamide}), reduced cAMP levels (AF25 {NNFLRFamide}, AF26 {KPNFLRFamide}, AF29 {NAEPNFLRFamide}, AF41 {KPNFIRFamide}) or increased cAMP levels (AF31); AF26, AF28, AF33, and AF41 all reduce cGMP levels in whole worms.[28] Of the other FLPs tested on *A. suum* whole worms, AF9, AF19, AF30, and AF34 (DSKLNDPLIRFamide) significantly diminish cAMP levels; AF19 also reduces cGMP levels.[28] AF21, AF22, and AF23 all caused a significant reduction in whole *Ascaris* cAMP levels, but only AF23 has an effect on cGMP levels, also causing a significant reduction. AF3, AF4, AF8, AF10, AF11, AF13, AF15, AF27, AF32, AF35 (DPQQRIVTVETVLRFamide), and AF36 (VPSAADMMIRFamide) have no significant effects on the levels of *A. suum* cAMP or cGMP in whole-worm preparations.[27,28,30]

NEUROPEPTIDE-LIKE PEPTIDES (NLPs)

Discovery—Structure of the Precursor mRNA/Gene

Originally described in *C. elegans*, NLPs comprise a disparate group of neuropeptides that encompasses peptides from a diverse array of conserved peptide families and other peptides that appear to be unique to nematodes.[17] Currently, the total number of annotated *C. elegans nlp* genes is 47. A targeted BLAST survey of nematode parasite genomes and transcriptomes for a repertoire of 45 *C. elegans nlp* genes identified 26 of these as occurring in multiple nematode species[23]; the discovery of *nlp-46* in *C. elegans* and multiple nematode parasites was also reported (Table 4). Note that *nlp-24* to *nlp-33*, inclusive, are proposed to be antibacterial peptides and will not be discussed further here. Of the remaining 36 putative NLP-encoding genes in *C. elegans*, genes/gene transcripts encoding structurally similar NLPs from 26 of these genes have been identified in parasitic nematodes, including *nlp-1*, *nlp-2, nlp-3*, *nlp-5*, *nlp-6*, *nlp-7*, *nlp-8*, *nlp-9*, *nlp-10*,

TABLE 4 Putative *Ascaris suum* Neuropeptide-Like Proteins (NLPs) Identified by Mass Spectrometry or Predicted from Nucleotide Sequence Resources Including Expressed Sequence Tags and Genome Survey Sequences

Encoding gene	Predicted amino acid sequences*
As-nlp-1	MDASRFYLGFG
As-nlp-2	SLASGRWGLRPG
As-nlp-3	**SNFDGREASLPRF**
	AINPFTDSI.NH$_2$
	NTGIAAERDLPKRYFDALAGQSL.NH$_2$
	YFDALAGQSLA.NH$_2$
As-nlp-5	**TSISRSKDAKKPSLDILEGAGFSPL**
	PSLDILEGAGFSPL
	ALDTLEGSGFGFS
	ALDDLEGVGFGGML
	ALDSLEGTDFGL
	ALDYLEGSGFGLM
	AFDAIEGADIGFH
	ALDMLEGSGFGL
	ALDSLEGTGFGL
As-nlp-6	YASRGGFGQ
	ASMRFSDRVAAQ
As-nlp-7	FADEWDDPRFLSSAF.NH$_2$
	SGMMEFDDPRFLSSAF.NH$_2$
	NHLIGFDDPRFLSSAF.NH$_2$
	YDAAFDDPRFLSLAF.NH$_2$
As-nlp-8	**SFDRIDGSAFGPHRH**
	AFDRIEGSGFGLD
	AFDRIEGAGFGLS
	AFDRIEGAGFG
As-nlp-9	AGARFFGPRIYDVESYLYPTN
	FGSFSPYYFYQQPK
	GGGRTFASYWIPPSGNG
As-nlp-10	SSIPFHGGIYG
	TAALPFSGGLYG
	AGWIPFSGGLYG
As-nlp-12	DYRPLQF.NH$_2$
	DGYRPLQF.NH$_2$
	SYRPLQF.NH$_2$

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TABLE 4 Putative *Ascaris suum* Neuropeptide-Like Proteins (NLPs) Identified by Mass Spectrometry or Predicted from Nucleotide Sequence Resources Including Expressed Sequence Tags and Genome Survey Sequences—Cont'd

Encoding gene	Predicted amino acid sequences*
As-nlp-13/afp-5	EFDRDFMHF.NH_2
	SNAFDRNFMNF.NH_2
	ESQFSRDFLNF.NH_2
	(AF17) DFDRDFMHF.NH_2
	SDAFSRNFMNF.NH_2
	DDAFSRDFLSF.NH_2
As-nlp-14	ALDGLEGDGFGFT
	ALDSLEGTDFDFG
As-nlp-15a	AFDSLTGAGFTGFD
	AFDSLTGSGFTGFD
	SLDSPANRGFTHFD
As-nlp-15b	AFDSLTGAGFTGFD
	SFDSLNSRGFTG
	AFDSLVGHGFTG
As-nlp-16	NNDISSSGEHEHQ
As-nlp-17	NILSNMMRIG
As-nlp-18a	FAYSFAFA
As-nlp-18b	ASGQQNELSFAFA
	APFA
As-nlp-20a	NKDANSVYAFSFA
As-nlp-20b	AFANFAFA
As-nlp-21	KHYLQTDE
	GGGRSFRLSSLGE
	AGARSFPGAGGLT
As-nlp-22	SIALGRFSLRPG
As-nlp-23a	SMALGRLAFRPG
	SLALGRVDFRPG
	NVDIADKDIEYLMEDSMG
	SAAFGRFHFRPG
	SLALGRSGFRPG
	SLALGRVGFRPG
As-nlp-23b	GIAPYAGMRPG
As-nlp-35	DSKRAFLQQLMQRLKPRF

TABLE 4 Putative *Ascaris suum* Neuropeptide-Like Proteins (NLPs) Identified by Mass Spectrometry or Predicted from Nucleotide Sequence Resources Including Expressed Sequence Tags and Genome Survey Sequences—Cont'd

Encoding gene	Predicted amino acid sequences*
As-nlp-37	NNAEVVNHILKNFGALDRLGDVG
As-nlp-38	GHSWQTANGLWG
	SSWETANGLWG
As-nlp-43	KFYAWAG
	KFYQWAG
As-nlp-44	NDRPQSSAAVLVPYPRVG
As-nlp-46	NIAIGRGDGFRPG

**Peptides in bold typeface have been confirmed by mass spectrometry. a,b, indicate more than one genome survey sequence predicted to encode some common and some different NLPs. For NLP sequences in bold typeface, C-terminal amides or Gly residues are shown where these have been confirmed by mass spectrometry; C-terminal amides have been inserted on those predicted peptide sequences, which are coencoded with known amidated peptides, that is, C-terminal Gly residues in sequences not given in bold could indicate amidation. Data derived from Refs. 10, 22, 23, 34.*

nlp-11, *nlp-12*, *nlp-13*, *nlp-14*, *nlp-15*, *nlp-17*, *nlp-18*, *nlp-19*, *nlp-20*, *nlp-21*, *nlp-22*, *nlp-37*, *nlp-38*, *nlp-42*, *nlp-43*, *nlp-44*, and *nlp-46*. The vast majority of these await confirmation of peptide sequence and expression.

Mass spectrometric–based peptidomic studies on neuronal ganglia of *A. suum* were the first to report identification of some non-RFamide peptides including peptides that were NLP-12-like (DYRPLQFamide, DGYRPLQFamide, KSYRPLQFamide, SYRPLQFamide, qDRDYRPLQFamide) and two WNamides (NRRRNAAARWNamide and NRRRNATNRWNamide) that were also identified on an *A. suum* EST.[34] The *nlp-12* transcripts from *A. suum* (*As-nlp-12*) and the sheep parasite *Trichostrongylus colubriformis* (*Tc-nlp-12*) were cloned and sequence confirmed.[22] More recently, a combination of database mining and tandem mass spectrometry identified multiple NLPs in adult *A. suum*.[10] This study identified a variety of putative *A. suum nlp* genes within the genome survey sequences using BLAST and peptides encoded on nine of these *As-nlp* genes were verified by mass spectrometric methods (Table 4).

As with *flp* genes, analysis of two published plant parasitic nematode genome sequences[1,12] for *nlp* genes reveal a reduced NLP complement compared to *C. elegans* with *M. incognita* having 21 and *B. xylophilus* having 17 *nlp* genes compared with *C. elegans* 47 *nlp* genes (Table 3).

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NLP Distribution/Expression

The polymerase chain reaction (PCR) was used to detect *Tc-nlp-12* transcript in both the adult and infective third larval stage (L3) of *T. colubriformis*; ISH detected transcript in the tail, matching the expression pattern known for *C. elegans.*[22] Peptide masses matching those predicted on *As-nlp-3*, *As-nlp-5*, *As-nlp-7*, *As-nlp-8*, *As-nlp-9*, *As-nlp-15*, *As-nlp-21*, and *As-nlp-23* were identified in *A. suum* head extracts.[10] Mass spectrometry detected NLP-12 peptides in the *A. suum* nerve ring, retrovesicular ganglion, and ventral and dorsal nerve cords; the same approach detected RWNamides in the nerve ring, ventral ganglion, and retrovesicular ganglion.[34]

Biological Actions of NLPs

To date, a small number of *A. suum* NLPs have been tested for biological activity.[22,23] The NLP DYRPLQFamide (encoded on *As-nlp-12*) causes contraction of both dorsal and ventral *A. suum* body wall muscle strips, effects, which are not nerve cord dependent.[22] This peptide is inactive on the *A. suum* ovijector. Selected *C. elegans* NLPs are also active on *A. suum* body wall muscle including AVNPFLD-SIamide (*nlp-3*) and AFAAGWNRamide (*nlp-23*), which are both inhibitory, and AIPFNGGMYamide (*nlp-10*), which is excitatory.

INSULIN-LIKE PEPTIDES (INSs)

Discovery—Structure of the Precursor mRNA/Gene

INS peptides are typically characterized by A and B chain polypeptides, and in *C. elegans*, they modulate development, growth, and reproduction and are intrinsically linked with the development of the dauer or resting stage of this nematode. Currently, data on INSs in parasitic nematodes are restricted to the identification of putative *ins* genes in the genome of the plant parasitic nematode *B. xylophilus;*[12] these authors reported only three *ins* genes in *M. incognita* (unpublished). The *B. xylophilus* genome encodes seven *ins* gene orthologs, significantly less than *C. elegans*, which has 40 (including 39 *ins* genes and *daf-28*) (Table 3). Although this is only a small snapshot of INS diversity in parasitic nematodes, it could suggest that compared with *C. elegans* the INS peptides are even more dramatically constricted within the parasitic nematodes than the FLPs or NLPs.

REFERENCES

1. Abad P, Gouzy J, Aury J-M, Castagnone-Sereno P, Danchin EGJ, Deleury E, et al. Genome sequence of the metazoan plant–parasitic nematode *Meloidogyne incognita*. *Nat Biotechnol* 2008;**26**(8):909–15.
2. Cowden C, Stretton AOW. AF2, an *Ascaris* neuropeptide: isolation, sequence, and bioactivity. *Peptides* 1993;**14**:423–30.
3. Cowden C, Stretton AOW. Eight novel FMRFamide-like neuropeptides isolated from the nematode *Ascaris suum*. *Peptides* 1995;**16**: 491–500.
4. Cowden C, Stretton AOW, Davis RE. AF1, a sequenced bioactive neuropeptide isolated from the nematode *Ascaris suum*. *Neuron* 1989;**2**:1465–73.
5. Dalzell JJ, McMaster S, Fleming CC, Maule AG. Short interfering RNA-mediated gene silencing in *Globodera pallida* and *Meloidogyne incognita* infective stage juvenilles. *Int J Parasitol* 2010;**40**:91–100.
6. Davis RE, Stretton AOW. The motornervous system of *Ascaris*: electrophysiology and anatomy of the neurons and their control by neuromodulators. *Parasitology* 1996;**114**:S97–117.
7. Davis RE, Stretton AOW. Structure–activity relationships of 18 endogenous neuropeptides on the motornervous system of the nematode *Ascaris suum*. *Peptides* 2001;**22**:7–23.
8. Geary TG, Marks NJ, Maule AG, Bowman JW, Alexander-Bowman SJ, Day TA, et al. Pharmacology of FMRFamide-related peptides in helminths. *Ann NY Acad Sci* 1999;**897**:212–27.
9. Jarecki JL, Andersen K, Konop CJ, Knickelbine JJ, Vestling MM, Stretton AOW. Mapping neuropeptide expression by spectrometry in single dissected identified neurons from the dorsal ganglion of the nematode *Ascaris suum*. *ACS Chem Neurosci* 2010;**1**:505–19.
10. Jarecki JL, Frey BL, Smith LM, Stretton AOW. Discovery of neuropeptides in the nematode *Ascaris suum* by database mining and tandem mass spectrometry. *J Proteome Res* 2011;**10**:3098–106.
11. Johnston MJ, McVeigh P, McMaster S, Fleming CC, Maule AG. FMRFamide-like peptides in root knot nematodes and their potential role in nematode physiology. *J Helminthol* 2010;**84**(3):253–65.
12. Kikuchi T, Cotton JA, Dalzell JJ, Hasegawa K, Kanzaki N, McVeigh P, et al. Genomic insights into the origin of parasitism in the emerging plant pathogen *Bursaphelenchus xylophilus*. *PLoS Pathog* 2011;**7**:e1002219.
13. Keating C, Thorndyke MC, Holden-Dye L, Williams RG, Walker RJ. The isolation of a FMRFamide-like peptide from the nematode *Haemonchus contortus*. *Parasitology* 1995;**111**:515–21.
14. Kimber MJ, Fleming CC, Bjourson A, Halton DW, Maule AG. FMRFamide-related peptides in potato cyst nematodes. *Mol Biochem Parasitol* 2001;**116**:199–208.
15. Kimber MJ, Fleming CC, Prior A, Jones JT, Halton DW, Maule AG. Localisation of *Globodera pallida* FMRFamide-related peptide encoding genes using *in situ* hybridization. *Int J Parasitol* 2002;**32**: 1095–105.
16. Kimber MJ, McKinney S, McMaster SM, Day TA, Fleming CC, Maule AG. *flp* Gene disruption in a parasitic nematode reveals motor dysfunction and neuronal sensitivity to RNA interference. *FASEB J* 2007;**21**(4):1233–43.
17. Marks NJ, Maule AG. Neuropeptides in helminths: occurrence and distribution. *Adv Exp Med Biol* 2010;**692**:49–77.
18. Marks NJ, Sangster NC, Maule AG, Halton DW, Thompson DP, Geary TG, et al. Structural characterisation and pharmacology of KHEYLRFamide (AF2) and KSAYMRFamide (PF3/AF8) from *Haemonchus contortus*. *Mol Biochem Parasitol* 1999;**100**:85–194.
19. Masler EP. Responses of *Heterodera glycines* and *Meloidogyne incognita* to exogenously applied neuromodulators. *J Helminthol* 2007;**81**:421–7.
20. Maule AG, Geary TG, Bowman JW, Marks NJ, Blair KL, Halton DW, et al. Inhibitory effects of nematode FMRFamide-related peptides (FaRPs) on muscle strips from *Ascaris suum*. *Invertebr Neurosci* 1995;**1**:255–65.

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21. McVeigh P, Leech S, Mair G, Marks NJ, Geary TG, Maule AG. Analysis of FMRFamide-like peptide (FLP) diversity in phylum *Nematoda*. *Int J Parasitol* 2005;**35**:1043–60.
22. McVeigh P, Leech S, Marks NJ, Geary TG, Maule AG. Gene expression and pharmacology of nematode NLP-12 neuropeptides. *Int J Parasitol* 2006;**36**:633–40.
23. McVeigh P, Alexander-Bowman S, Veal E, Mousley A, Marks NJ, Maule AG. Neuropeptide-like protein diversity in phylum Nematoda. *Int J Parasitol* 2008;**38**:1493–503.
24. Mousley A, Novozhilova E, Kimber MJ, Day TA. Neuropeptide physiology in helminths. *Adv Exp Med Biol* 2010;**692**:78–97.
25. Mühlfeld S, Schmitt-Wrede HP, Harder A, Wunderlich F. FMRFamide-like neuropeptides as putative ligands of the latrophilin-like HC110-R from *Haemonchus contortus*. *Mol Biochem Parasitol* 2009;**164**(2):162–4.
26. Nanda J, Stretton AOW. *In situ* hybridization of neuropeptide-encoding transcripts *afp-1, afp-3*, and *afp-4* in neurons of the nematode *Ascaris suum*. *J Comp Neurol* 2010;**518**:896–910.
27. Reinitz CA, Herfel HG, Messinger LA, Stretton AOW. Changes in locomotory behaviour and cAMP produced in *Ascaris suum* by neuropeptides from *Ascaris suum* or *Caenorhabditis elegans*. *Mol Biochem Parasitol* 2000;**111**:185–97.
28. Reinitz CA, Pleva AE, Stretton AOW. Changes in cyclic nucleotides, locomotory behaviour, and body length produced by novel endogenous neuropeptides in the parasitic nematode *Ascaris suum*. *Mol Biochem Parasitol* 2011;**180**:27–34.
29. Sithigorngul P, Jarecki JL, Stretton AOW. A specific antibody to neuropeptide AF1 (KNEFIRFamide) recognises a small subset of neurons in *Ascaris suum*: differences from *Caenorhabditis elegans*. *J Comp Neurol* 2011;**519**:1546–61.
30. Thompson DP, Davis JP, Larsen MJ, Coscarelli EM, Zinser EW, Bowman JW, et al. Effects of KHEYLRFamide and KNEFIRFamide on cyclic adenosine monophosphate levels in *Ascaris suum* somatic muscle. *Int J Parasitol* 2003;**33**:199–208.
31. Trailovic SM, Clark CL, Robertson AP, Martin RJ. Brief application of AF2 produces long lasting potentiation of nAChR responses in *Ascaris suum*. *Mol Biochem Parasitol* 2005;**139**:51–64.
32. Trim N, Boorman JE, Holden-Dye L, Walker RJ. The role of cAMP in the actions of the peptide AF3 in the parasitic nematodes *Ascaris suum* and *Ascaridia galli*. *Mol Biochem Parasitol* 1998;**93**:263–71.
33. Verma S, Robertson AP, Martin RJ. The nematode neuropeptide, AF2 (KHEYLRF-NH_2), increases voltage-activated calcium currents in *Ascaris suum* muscle. *Br J Pharmacol* 2007;**151**(6):888–99.
34. Yew JY, Kutz KK, Dikler S, Messinger L, Li L, Stretton AO. Mass spectrometric map of neuropeptide expression in *Ascaris suum*. *J Comp Neurol* 2005;**8**:396–413.
35. Yew JY, Davis R, Dikler S, Nanda J, Reinders B, Stretton AO. Peptide products of the *afp-6* gene of the nematode *Ascaris suum* have different biological actions. *J Comp Neurol* 2007;**502**:872–82.

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Chapter 41

Pigment Dispersing Factor

Liesbet Temmerman, Ellen Meelkop and Liliane Schoofs

ABSTRACT

Pigment-dispersing hormones (PDHs) and pigment-dispersing factors (PDFs) make up a conserved family of signaling molecules thus far only identified from invertebrate sources. They all act on their respective G-protein-coupled receptors and can act both as neurotransmitters and neurohormones. Although PDF receptors are homologs of the vertebrate VPAC2 receptor, PDF sequences have never been found in vertebrates and only a functional homology to VIP has been described. In all invertebrate PDF systems studied, a clear role in the modulation of and response to environmental signals has been described. In crustaceans, PDH is an activator of rapid pigment dispersion in response to bright light. Insect PDFs, and possibly crustacean PDH, are of crucial importance to the endogenous biological clock that keeps track of the day/night rhythm. In nematodes, PDFs exert an overt role in the regulation of locomotion. This chapter summarizes the different invertebrate PDH-like signaling systems.

DISCOVERY

The first evidence of the existence of pigment dispersion controlling hormones dates from the beginning of the twentieth century. Injection of hemolymph from a dark colored shrimp (*Crangon crangon*) into a pale one caused the latter to darken by pigment dispersion as well. The eyestalks turned out to be the source of a peptide now known as pigment-dispersing hormone (PDH).[17] The first PDH peptide to be fully characterized at the protein level was isolated from *Pandalus borealis*.[4,10] This octadecapeptide belongs to the family of α-PDH peptides, which are distinguished from β-PDH peptides by a Gly and Met at positions three and four respectively. Although the occurrence of α-PDH seems to be restricted to *Pandalus* and *Macrobrachium* species, β-PDH peptides could be identified in a wide variety of (mostly decapod) crustaceans.[20]

The first PDH-like peptide in insects was identified in the grasshopper *Romalea microptera* by proving its pigment-dispersing activity in the crab *Uca pugilator*.[19] It is more closely related to β-PDH than to α-PDH and was named pigment-dispersing factor (PDF), as indeed are all other identified PDH-like peptides in insects and nematodes.

Although the first PDH-like peptides in crustaceans and insects were identified by their pigment-dispersing abilities, more advanced techniques such as mass spectrometry and the molecular cloning of *pdh* and *pdf* genes in EST databases led to the vast expansion of known PDH-like peptides among arthropods. The elucidation of the complete genome of the nematode *Caenorhabditis elegans* allowed for the identification of these peptides via reverse pharmacology. The predicted PDF receptor was cloned and subsequently used to search for the nematode's PDF ligands.[8] Although some crustaceans express both α- and β-PDH peptides—or several β-PDHs—by alternative splicing of their mRNA precursor, most insect species seem to contain only a single *pdf* gene. Nematodes, in contrast, express two different *pdf* genes: *pdf-1* and *pdf-2*, encoding for three PDF peptides: PDF-1a, PDF-1b, and PDF-2.[9]

STRUCTURE OF THE mRNA/GENE

Although cDNA sequences are available for several decapod crustaceans, the only crustacean with a completely sequenced genome is the cladoceran *Daphnia pulex*. The *pdh* gene of *D. pulex* contains two exons which give rise to a 169 amino acid (aa) long precursor protein (Fig. 1). This precursor contains a 28 aa long signal peptide, a 120 aa long PDH precursor related peptide (PPRP), and an 18 aa long PDH peptide.[22]

The *Drosophila melanogaster* PDF-encoding gene is intronless and encodes the 102 aa long preproPDF. This precursor contains a 16 aa long signal peptide, followed by a sequence of 63 aa termed PDF-associated peptide (PAP) (Fig. 1). The PAP region contains another cleavage site that could result in peptides of 18 and 43 amino acids.[16] The PAP region is highly diverged from those of the crustacean precursors, although the primary structure of the mature PDF is highly conserved among all members of the pigment-dispersing hormone family.

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00041-5
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Dp-pdh        1 ---------MHQLSAKLSHLSIALFVLLVSFAT---DAESAPPSISSNNRPEAQMSIQEM
Dm-pdf        1 -------------MARFTYL-VALVLI----------------AICCQWGYCGAMAMPDE
Ce-pdf-1a     1 MIQTFFFLPNSNFSISFSLYFSQISVIFSPRVPGRFKSGRVAPSWCAGREAESKTREIC
Ce-pdf-1b     1 MIQTFFFLPNSNFSISFSLYFSQISVIFSPRVPGRFKSGRVAPSWCAGREAESKTREIC
Ce-pdf-2      1 --------MSSRISVSLLLLAVVATMFFTANVV-----------------DATPRSQGNM

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Ce-pdf-1b    61 LSLGCAHSFYSTCA--------------SQPSEQAFPALPLPSSLNVNMNRFIISMIALL
Ce-pdf-2     36 MRYGNSLPAY--------------------------------------------------

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Dm-pdf       63 --------CRYPLI------LENSLGPSV--------------------PIRKR-NSELIN
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Ce-pdf-1b   107 AVFCAVSTASPLLYRAPQ--MYDDVQFVKRSNAELINGLIGMDLGKLSAVGKRSNAELIN
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Ce-pdf-1b   165 GLL---SMNLNKLSGAGFR
Ce-pdf-2     73 HILKNFGA-LDRLGDVGK-
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FIGURE 1 Alignment of PDH and PDF precursor proteins. *D. pulex* (*Dp*), *D. melanogaster* (*Dm*), and *C. elegans* (*Ce*) precursor proteins are similarly structured, starting with a signal peptide (italic), followed by a PDH precursor related peptide (PPRP), PDF-associated peptide (PAP), or linking peptide (bold) and the actual PDH/PDF peptides at the C-terminal end. Identical amino acids are highlighted in black, conserved amino acids in dark gray. Proteolytic cleavage and amidation sites are in lower case, and PDH/PDF peptides are underlined (aligment made with MUSCLE[2] and BoxShade v3.21).

The *C. elegans pdf-1* gene is a three exon containing gene generating two different transcripts through alternative splicing: *pdf-1a* and *pdf-1b*. Both precursors contain both PDF-1a and PDF-1b peptides (Fig. 1).[9] In addition, *pdf-1a* and *pdf-1b* precursors encompass a 29 aa signal sequence and a linking peptide, which were found in the HPLC fractions of *C. elegans* peptide extracts (SPLLYRAPQMYDDVQFV and SPLLYRAPQYQMYDDVQFV). The two different precursors differ from each other in two amino acids in this linking peptide of unknown function. The *pdf-2* gene also includes three exons but generates only one transcript and one pre-pro-protein containing one PDF-2 peptide. No linking peptide was found in the PDF-2 precursor.[9]

PROCESSING OF THE PRECURSOR

All PDH and PDF precursor proteins are similarly structured, containing a signal peptide on the N-terminal side followed by a PPRP (crustaceans), PAP (insects), or linking peptide (nematodes) of unknown function, a cleavage site, the mature peptide and a processing site. Note that the PPRP of *D. pulex* and the PAP of *D. melanogaster* include potential cleavage sites. The processing sites of the fly's preproPDF seem to be different from those of crustacean PDH and nematode PDF precursors. The proteolytic cleavage site preceding the mature PDF consists of triple basic amino acids (RKR), in contrast to the dibasic crustacean and nematode cleavage site (KR). Also, the α-amidation signal for decapod crustacean PDHs and *C. elegans pdf-1a* and *b* (GRR) is different from that of the fly's PDF and *C. elegans pdf-2* (GK). *D. pulex* forms an exception by containing a single Gly.

DISTRIBUTION OF THE mRNA/PEPTIDE

The PDF system probably represents the best studied invertebrate neuropeptide network. For several decades now, numerous studies of detailed *pdf* mRNA and/or PDF peptide localization have been conducted. Even though substantial interspecies differences exist, there is always a tight association with the visual system for crustaceans and insects. Initial studies of PDH localization have logically focused on the eyestalk expression patterns, but later studies also included the entire brain and the abdomen. Comparison of

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these localization studies reveals that the majority of PDH-immunoreactive cells and projections are indeed in the eyestalks and the median protocerebrum.[1,5] The identity and number of abdominal PDF-positive neurons tend to vary most between species. It has been suggested that in the abdomen, PDF could be released into the hemolymph, subsequently acting as a hormone.[18] Both PDH and PDF are observed throughout all developmental stages into adulthood.[5,6]

Nearly all hemimetabolan insects studied so far are hallmarked by three distinct PDF-positive neuron clusters per brain hemisphere: one at the anterior edge of the medulla (PDFMe) and two at either the posterior dorsal or the posterior ventral edge of the lamina (PDFLa) (Fig. 2). Nevertheless, the number of neurons in a cluster and the individual neuron size may differ between species. The contralateral projections of the PDFMe neurons are believed to be involved in the coupling of the two brain hemispheres, which is essential for the proper functioning of the biological clock (see below).[15] This is also true for holometabolan organisms, which however do not seem to express PDF in the PDFLa clusters. The PDFMe clusters comprise the only PDF-positive neurons in the brain of adult *D. melanogaster*, but in other holometabolans, additional clusters can be present. The fruitfly differs slightly from the general holometabolan PDF neuron architecture in that the PDFMe neurons do not project to the lamina. Therefore, although *D. melanogaster* is by far the best-studied insect considering the PDF system, here the more general holometabolan network organization is exemplified by the blowfly PDF system (Fig. 2).[11,15]

As a result of the close association with the visual system and the regulation of biological rhythms (see below), researchers have put effort into adding information on temporal changes of *pdf* mRNA/PDF peptide to the spatial distribution patterns. However, no general conclusions can be made. For some species, daily changes in mRNA and/or peptide levels have been reported, but for several others, no such changes could be detected.[12]

Because of the different body architecture, a comparison of crustacean PDH/insect PDF patterns with *C. elegans* localization studies is not directly possible. Via use of endogenous transcription of fluorescent reporter proteins under control of the *pdf-1/2* and *pdfr-1* promoters, several cells belonging to the nematode PDF system could be identified. PDF peptides are mainly detected in (chemo)sensory neurons, interneurons, and motorneurons, as well as in a few nonneuronal cells. *pdf-1* and

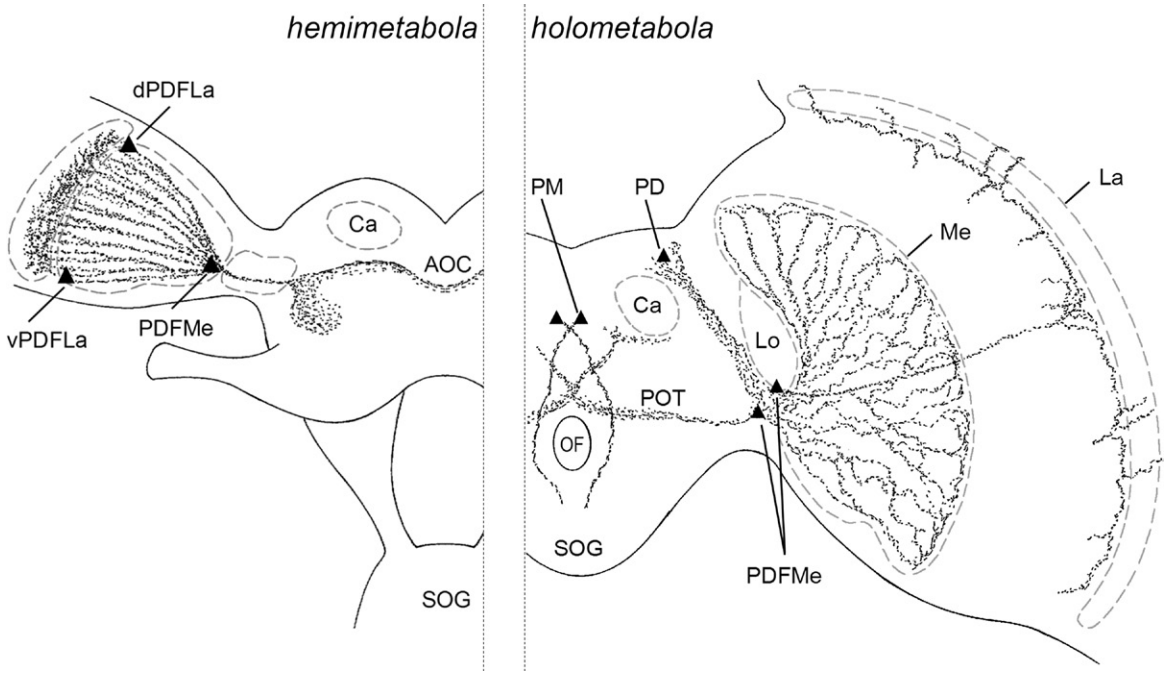

FIGURE 2 General PDF architecture of the hemi- and holometabolan brain. Three clusters of hemimetabolan PDF-producing cells (dPDFLa, dorsal PDFLamina cells; vPDFLa, ventral PDFLamina cells; PDFMe, PDFMedulla cells) provide fanlike ramifications in lamina (La), medulla (Me), and lobula (Lo) of the optic lobe and the lateral regions of the protocerebrum, and connect both hemispheres (AOC, anterior optic commissure). No PDFLa clusters are present in holometabolan representatives, PDFMe project in the lamina and the lateral region and connect both hemispheres (POT, posterior optic tract). Blowflies have two additional PDF-producing clusters per hemisphere in the posterior dorsal (PD) and medial (PM) regions of the brain. Ca, calyx of the mushroom body; OF, esophageal foramen; SOG, subesophageal ganglion; triangles represent cell clusters and not individual neurons (image adapted from Ref. 12 with permission from Elsevier).

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pdf-2 patterns only partially overlap, suggesting at least in part specific functions. Expression of *pdfr-1* was also observed in several sensory and motorneurons, some of which coexpress the peptides. Prominent receptor expression is observed in all muscles of the body wall. *C. elegans pdf* mRNA levels do not vary over the course of the day.[8,9]

RECEPTORS AND SIGNALING CASCADES

The discovery of the *D. melanogaster* PDF receptor, several years after the peptides had been identified, represented an immense breakthrough to the field.[14] So far, of all invertebrates studied, only the fruitfly and worm PDF receptors have been found. They are all class B G-protein-coupled receptors that influence adenylate cyclase via Gαs (*D. melanogaster* PDFR and *C. elegans* PDFR-1a/b) or via Gαi (*C. elegans* PDFR-1c).[8]

D. melanogaster pdfr mutants phenocopy *pdf* mutant defects (see below), thus confirming the role of the PDF signaling system as a whole. In addition, several of the PDF-producing cells also present PDFR at their surfaces, supporting the hypothesis that the system feeds back on itself. Similar to the earlier mentioned *C. elegans* receptor, fruitfly PDFRs have been observed in several non–neuronal cells.[7]

Detailed studies in the fruitfly have added more insight into the "upstream" regulation of the PDF signal itself, thus contributing to the further elucidation of the signaling pathways. Molecular changes of several components of the endogenous biological clock (Cryptochrome, Clock, Cycle. PAR-domain protein-1) control the PDF signal. In addition other genes (Neurofibromin-1, Disconnected, Lark) play a role in this signaling system.[12] As PDF is contained within dense core vesicles, it is not surprising that membrane excitability, which regulates vesicle release, is an important level of control. As such, the ion channels Shaw, Narrow abdomen and Slowpoke, and the Slowpoke-binding protein, which interacts with the latter channel, are important in PDF signal regulation.[12]

After the peptide has been released, it may act directly as a neuromodulator or diffuse to more distant sites, functioning in a nonsynaptic, paracrine way.[13] Such a neurohormonal action is supported by the finding of PDFR at relatively remote sites.[7]

PDF signaling not only triggers further molecular changes, in flies the peptide also influences daily changes in size and shape of the L1 and L2 interneurons, which receive direct input from the photoreceptor cells of the retina.[13] Regulating neuron plasticity could also participate in the control of downstream (visual) information transmission.[3]

One of the major insights that the identification of the PDFR brought into the field, concerns the level to which this signaling system is conserved in vertebrates. Although the invertebrate peptides do not have sequence homologs in vertebrates, the PDFR sequence finds well-conserved vertebrate counterparts in the VPAC2 and calcitonin receptors. VPAC2 is a receptor for both vasoactive intestinal peptide (VIP) and pituitary adenylate-cyclase-activating peptide (PACAP). VIP is considered the functional homolog of PDF, and the VIP-VPAC2 system is of crucial importance to the vertebrate circadian clock.[12]

BIOLOGICAL ACTIONS

Logically inferred from its name, the peptide must have some connection with invertebrate pigment dispersion. It is indeed this exact function that caused the naming of crustacean PDH, which was found to regulate both integumental and eye pigment migrations. Integumental pigment dispersion caused by PDH is involved in rapid reversible color changes in crustaceans in response to several changes such as light condition, background color, temperature, stress, molting, or even mating. A circadian rhythm in these pigment migrations has been described, which is in part regulated by PDH.[12]

In addition, crustaceans have pigments in the eyes that regulate light sensitivity, acting as a natural pair of sunglasses. Daily migrations of two subsets of the eye pigments, named the distal and the reflecting pigments, are under the control of PDH.[12] The actions of PDH are nonetheless not limited to the control of pigment dispersion. PDH influences the sensitivity of the retinal response to light over the day–night cycle by interfering directly with photoreceptor depolarization.[25]

The identification of PDH-like peptides in insects soon made it clear that although these molecules could elicit pigment-dispersing responses when applied to crustaceans, they do not serve a similar purpose in insects. Because at that time, there was doubt about the endogenous mode of action in insects, the peptides were designated pigment-dispersing factors (PDFs), rather than hormones. Even though there is increasing evidence for a hormonal function in insects as well,[18] the terminology remained.

PDF has been studied as the major output factor of the insect circadian (24 h) clock, where it confers time information to the periphery. The circadian clock can most easily be seen as an endogenous rhythm generator consisting of several so-called clock neurons, which communicate with each other and with all other tissues in an animal to adapt an organism's physiology to the time of the day. PDF's output function is exemplified by the observation that both *pdf* and *pdfr* mutants gradually lose their daily activity rhythms when kept under constant darkness. Wild-type flies under these conditions have no trouble in communicating with their endogenous clocks, which keeps

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them rhythmic in the absence of the external light–dark cycle.[21] PDF serves not only as an output factor but it also functions as a crucial network coordinator, enhancing the communication between different individual clock cells. When acting as a network regulator, PDF signaling adjusts circadian periods of target clock neurons, with a shortening or lengthening effect depending on the specific target cell.[27] The clock's systemwide modulating actions are taken to the next level when looking at social insects, where there is plasticity of circadian rhythms among colony members, depending on their specific tasks in the hierarchy. For example, small bumblebees stay in the nest to tend brood arrhythmically around the clock, whereas large bees display strong circadian rhythmicity and work outside the colony during daytime. It has been suggested that PDF could be responsible for regulating the system plasticity in such societies.[26] A function for PDH in the crustacean circadian clock has been proposed as well, but it needs further investigations.

In insects, PDF is widely studied, and apart from its actions in the central brain clock, additional functions in activity, reproduction, arousal, and geotaxis have been proposed. Geotaxis refers to the intriguing phenotype that induces a preference of movement away from or towards the source of gravity. Both *pdf* and *pdfr* mutant *D. melanogaster* display a higher tendency toward negative geotaxis (flying upwards) than wild-type flies do.[14,24] So far, no functional consequences of such preference could be assigned, and the phenotype, observed for a few other and apparently non-related gene mutations as well, remains a curiosity.

Deciphering the nematode PDF system is a slightly more challenging task, because the worms not only contain three distinguishable peptides but also produce three different receptor isoforms, which can all be activated by all peptides. *C. elegans pdf* genes are mainly expressed in neurons that fulfill a task in sensing and integration of environmental stimuli. Although these worms can detect light, they do not have a clear visual system such as that of insects or crustaceans. Nevertheless, based on its localization in neurons active in mechanosensation, oxygen sensing, chemosensation, or the control of locomotion, one can expect similar modulating actions of PDF on environmental perception and associated behavior. Although *pdf-1* mutants show an obvious locomotion phenotype, it has not yet been shown that the PDF peptides are also involved in the regulation of circadian activity, and the exact function of the PDF signaling system in this phylum remains elusive.[8,9,23]

ACKNOWLEDGMENTS

L. Temmerman benefits from a grant from FWO-Flanders and E. Meelkop is supported by IWT Flanders. For a comprehensive review on PDF, readers are referred to Ref. 12 of this work.

REFERENCES

1. Dircksen H, Zahnow CA, Gaus G, Keller R, Rao KR, Riehm JP. The ultrastructure of nerve-endings containing pigment-dispersing hormone (PDH) in crustacean sinus glands—identification by an antiserum against a synthetic PDH. *Cell Tissue Res* 1987;**250**(2):377–87.
2. Edgar RC. MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res* 2004;**32**:1792–7.
3. Fernandez MP, Berni J, Ceriani MF. Circadian remodeling of neuronal circuits involved in rhythmic behavior. *PLoS Biol* 2008;**6**:e69.
4. Fernlund P. Structure of a light-adapting hormone from the shrimp, *Pandalus borealis*. *Biochim Biophys Acta* 1976;**439**:17–25.
5. Harzsch S, Dircksen H, Beltz BS. Development of pigment-dispersing hormone-immunoreactive neurons in the American lobster: homology to the insect circadian pacemaker system?. *Cell Tissue Res* 2009;**335**:417–29.
6. Helfrich-Forster C, Tauber M, Park JH, Muhlig-Versen M, Schneuwly S, Hofbauer A. Ectopic expression of the neuropeptide pigment-dispersing factor alters behavioral rhythms in *Drosophila melanogaster*. *J Neurosci* 2000;**20**:3339–53.
7. Im SH, Taghert PH. PDF receptor expression reveals direct interactions between circadian oscillators in *Drosophila*. *J Comp Neurol* 2010;**518**:1925–45.
8. Janssen T, Husson SJ, Lindemans M, Mertens I, Rademakers S, Ver Donck K, et al. Functional characterization of three G protein-coupled receptors for pigment dispersing factors in *Caenorhabditis elegans*. *J Biol Chem* 2008;**283**:15,241–9.
9. Janssen T, Husson SJ, Meelkop E, Temmerman L, Lindemans M, Verstraelen K, et al. Discovery and characterization of a conserved pigment dispersing factor-like neuropeptide pathway in *Caenorhabditis elegans*. *J Neurochem* 2009;**111**:228–41.
10. Kleinholz LH. Purified hormones from the crustacean eyestalk and their physiological specificity. *Nature* 1975;**258**:256–7.
11. Kula-Eversole E, Nagoshi E, Shang Y, Rodriguez J, Allada R, Rosbash M. Surprising gene expression patterns within and between PDF-containing circadian neurons in *Drosophila*. *Proc Natl Acad Sci USA* 2010;**107**:13497–502.
12. Meelkop E, Temmerman L, Schoofs L, Janssen T. Signalling through pigment dispersing hormone-like peptides in invertebrates. *Prog Neurobiol* 2011;**93**:125–47.
13. Meinertzhagen IA, Pyza E. Neurotransmitter regulation of circadian structural changes in the fly's visual system. *Microsc Res Technol* 1999;**45**:96–105.
14. Mertens I, Vandingenen A, Johnson EC, Shafer OT, Li W, Trigg JS, et al. PDF receptor signaling in *Drosophila* contributes to both circadian and geotactic behaviors. *Neuron* 2005;**48**:213–9.
15. Nässel DR, Shiga S, Wikstrand EM, Rao KR. Pigment-dispersing hormone-immunoreactive neurons and their relation to serotonergic neurons in the blowfly and cockroach visual system. *Cell Tissue Res* 1991;**266**:511–23.
16. Park JH, Hall JC. Isolation and chronobiological analysis of a neuropeptide pigment-dispersing factor gene in *Drosophila melanogaster*. *J Biol Rhythms* 1998;**13**:219–28.
17. Perkins EB. Color changes in crustaceans, especially in Palaemonetes. *J Exp Zool* 1928;**50**:71–105.
18. Persson MG, Eklund MB, Dircksen H, Muren JE, Nassel DR. Pigment-dispersing factor in the locust abdominal ganglia may have roles as circulating neurohormone and central neuromodulator. *J Neurobiol* 2001;**48**:19–41.

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19. Rao KR, Mohrherr CJ, Riehm JP, Zahnow CA, Norton S, Johnson L, et al. Primary structure of an analog of crustacean pigment-dispersing hormone from the lubber grasshopper *Romalea microptera*. *J Biol Chem* 1987;**262**:2672–5.
20. Rao KR, Riehm JP. Pigment-dispersing hormones. *Ann N Y Acad Sci* 1993;**680**:78–88.
21. Renn SC, Park JH, Rosbash M, Hall JC, Taghert PH. A pdf neuropeptide gene mutation and ablation of PDF neurons each cause severe abnormalities of behavioral circadian rhythms in Drosophila. *Cell* 1999;**99**:791–802.
22. Strauss J, Zhang Q, Verleyen P, Huybrechts J, Neupert S, Predel R, et al. Pigment-dispersing hormone in *Daphnia* interneurons, one type homologous to insect clock neurons displaying circadian rhythmicity. *Cell Mol Life Sci* 2011;**68**:3403–23.
23. Temmerman L, Meelkop E, Janssen T, Bogaerts A, Lindemans M, Husson SJ, et al. *C. elegans* homologs of insect clock proteins: a tale of many stories. *Ann N Y Acad Sci* 2011;**1220**:137–48.
24. Toma DP, White KP, Hirsch J, Greenspan RJ. Identification of genes involved in *Drosophila melanogaster* geotaxis, a complex behavioral trait. *Nat Genet* 2002;**31**:349–53.
25. Verde MA, Barriga-Montoya C, Fuentes-Pardo B. Pigment dispersing hormone generates a circadian response to light in the crayfish, *Procambarus clarkii*. *Comp Biochem Physiol A Mol Integr Physiol* 2007;**147**:983–92.
26. Weiss R, Dov A, Fahrbach SE, Bloch G. Body size-related variation in Pigment Dispersing Factor-immunoreactivity in the brain of the bumblebee *Bombus terrestris* (Hymenoptera, Apidae). *J Insect Physiol* 2009;**55**:479–87.
27. Yoshii T, Wulbeck C, Sehadova H, Veleri S, Bichler D, Stanewsky R, et al. The neuropeptide pigment-dispersing factor adjusts period and phase of *Drosophila*'s clock. *J Neurosci* 2009;**29**:2597–610.

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Chapter 42

Proctolin

Angela B. Lange and Ian Orchard

ABSTRACT

The neuropeptide proctolin (RYLPT) was discovered in the cockroach, *Periplaneta americana*, where it was considered a neurotransmitter associated with hindgut visceral muscle. Proctolin was the first insect neuropeptide to be sequenced and is now known to be widely distributed in the nervous systems of arthropods and to be extremely active on a variety of arthropod muscle preparations. The genes for proctolin and its receptor have been cloned in a variety of insects but seem to be absent from the genomes of some other insects. Structure–activity studies have identified the critical positions in the proctolin structure for binding and biological activity, and agonists and antagonists have been designed. Many biological processes are influenced by proctolin, and proctolin is involved in regulating central neurons, and muscles associated with posture, and the digestive, reproductive, and circulatory systems. Proctolin may also be a neurohormone and may also act as a hormone-releasing factor.

DISCOVERY

The pentapeptide proctolin (RYLPT) was the first insect neuropeptide to be sequenced and synthesized.[26] The name is derived from its ability to stimulate hindgut (proctodeum) contractions in *Periplaneta americana* and by its presence within axons of the proctodeal nerve. Proctolin is present in a number of arthropods (insecta, crustacea, and chelicerata), identified more recently by mass spectrometry, where it is extensively distributed throughout the nervous system. Proctolin seems to be unique to arthropods, and there is no convincing evidence for its presence in other invertebrates or vertebrates. Unlike many biologically active peptides, proctolin is nonamidated and is not considered to be a member of an extended family of peptides; it has an identical amino acid sequence in all arthropods studied. There is one report, however, of the presence of proctolin along with an alternative sequence (AYLPT) in *Leptinotarsa decemlineata.*[25]

STRUCTURE OF THE mRNA/GENE

Proctolin is a small peptide, and early searches of the *Drosophila* genome databases failed to identify the proctolin gene.[28] However, several expressed sequenced tags (ESTs) were recognized in the *Drosophila melanogaster* genome database that encodes predicted genes containing the sequence RYLPT. One of these (CG7105) probably encodes the *Drosophila* proctolin precursor.[28] The predicted protein sequence for this gene has potential peptide cleavage sites flanking the RYLPT sequence (Fig. 1). The gene has 3 exons, and the preproprotein is 140 amino acids in length. This conceptual preproproctolin contains a secretion signal peptide (amino acids 1–38) followed by a single copy of the proctolin sequence, which is *C*-terminally flanked by a 94-amino acid peptide. A similar protein structure is found in *D. pseudoobscura.*[7] Interestingly, proctolin is yet to be chemically identified in *Drosophila*. The proctolin gene has now been predicted or cloned from a small number of insect species, including the red flour beetle *Tribolium castaneum* and the pea aphid *Acyrthosiphon pisum*.[5,6,15,29] No proctolin gene has been identified in searches of the genomes of honey bee *Apis mellifera*, mosquito *Aedes aegypti*, or silkmoth *Bombyx mori*, in which proctolin (and its receptor) are now considered absent.[24] Similarly, the gene for proctolin was suggested to be absent in the kissing bug *Rhodnius prolixus*,[19] but a reexamination has recently identified the gene (Fig.1) and authentic proctolin.[21] Recently, the first noninsect arthropod proctolin gene was reported, by way of an EST (FE183480), in the Pacific white shrimp, *Litopenaneus vannamei.*[16]

PROCESSING OF THE PRECURSOR

The biochemistry of proctolin biosynthesis and processing from its precursor in *Drosophila* has been suggested.[7,28] A signal peptidase probably cleaves Gly38-Arg39 and Gly31-Arg32 peptide bonds of the preproproctolin in *D. melanogaster* and *D. pseudoobscura*, respectively. This exposes the *N*-terminal Arg of proctolin without the need for a proprotein convertase (PC)-like endoprotease. It has been proposed that a second cleavage occurs at Arg44-Ser45 by a PC, since 2 of the 3 *D. melanogaster* PCs (FUR1 and FUR2) recognize and cleave between Arg–Ser of prodynorphin. This

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00042-7
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R.prolixus      ------MATTTQSKVMSREVIVVAVLMMVLLSSSMVQSRYLPTRGADDRILRLRQLLKDL MENDLDPIME 64
T.castaneum     ---------------MFDRK-LVFALVFVVFATLAVEGRYLPTRSNGDRIEKLRELLKDL FENEVE-KEE 53
A.pisum         -----------MAGKFSALFLVGFVAAVVVAPYMMAEARYLPTRGNDDRITRLKELLTDL LESGAQPNLE 59
D.melanogaster  MGVPRSHGTGIGCGSGHRWLLVWMTVLLLVVPPHLVDGRYLPTRSHGDDIDKLREFLMLQI LELSNEDPQQ 70

R.prolixus      HPA---APNG---QYDPR-------LYKRAAPPVQWDAVGAQFAGN------------------------  97
T.castaneum     YQA---DAPP---RWHPESK----LFYKREAP--------AH----------------------------  77
A.pisum         MERPYVDVNGDFSRLRPREY----NIPEKSIMELFNPTVPHHQRPRS------------------------ 102
D.melanogaster  QQQQQQQQQHPQLRLHNEATGGSSSSSNINNPRVSNGNSNAAWLQKLSAMGALDELGGDGARFGPNYGRY 140
```

FIGURE 1 Alignment of predicted proctolin-encoding prepropeptides in insects. Sequences were aligned with Clustal X for *Rhodnius prolixus*, *Tribolium castaneum*, *Acyrthosiphon pisum*, and *Drosophila melanogaster*. Amino acids highlighted in black are identical; those in gray are similar. Black bar indicates a possible second peptide. Note the conserved RYLPT for proctolin.

cleavage generates an Arg-extended proctolin intermediate that would need to be converted to proctolin in secretory granules by the action of a carboxypeptidase D. In all of the preproproctolin sequences, there is a suggestion of a second peptide immediately following the proctolin sequence, as shown in Fig. 1. It is unknown if this proctolin precursor associated peptide is actually processed or if it has any biological relevance.

DISTRIBUTION OF mRNA/PEPTIDE

In arthropods, immunohistochemical studies have indicated the presence of proctolin-like immunoreactive neurons distributed throughout the nervous system, but in insects, unlike many neuropeptides, no proctolin-like immunoreactive endocrine cells are associated with the midgut. Proctolin-like immunoreactivity is found in a variety of neuronal cell types, including interneurons, identified motoneurons projecting to visceral and skeletal muscles, and neurosecretory cells projecting to neurohemal areas.[2,14,20]

With the identification of the putative *Drosophila* proctolin gene, the power of in situ hybridization has now been combined with immunohistochemistry to provide a complete map of proctolinergic neurons in *D. melanogaster*. In situ hybridization with a probe against the *Drosophila* CG7105 transcript results in staining of cell bodies in the brain and ventral nerve cords of the third instar larvae of *D. melanogaster*.[28] The pattern of labeled cell bodies is very similar to that reported for proctolin-like immunoreactivity in *D. melanogaster*.[18,28] Neither immunohistochemistry nor in situ hybridization reveals any endocrine-like cells in the midgut. With regard to the ventral nerve cord, in situ hybridization reveals both lateral and medial cell body clusters, with both clusters showing stronger signals in the abdominal neuromeres than in the thoracic neuromeres. In the brain, there are hybridization signals in 2–3 lateral cell bodies in the protocerebrum and a set of 3 cell bodies lying ventrally within the tritocerebrum near the esophageal foramen. Interestingly, proctolin-like immunoreactive staining reveals that the 2–3 lateral cells of the protocerebrum are neurosecretory cells with axons projecting to the corpora cardiaca (CC) portion of the ring gland. In the CC, there are numerous varicose terminals displaying proctolin-like immunoreactivity.[28] Similarly, in crustaceans, proctolin-like immunoreactive neurosecretory cells project to the neurohemal pericardial organs (POs).[10]

RECEPTORS AND SIGNALING CASCADE

Early attempts at isolating proctolin receptors[7,12,20] made use of tritiated proctolin to bind to crude membrane preparations from locust hindgut and oviduct (two tissues highly responsive to proctolin). Proctolin binding was found to be time dependent, proportional to the concentration of membrane protein, saturable, specific, and reversible. Further work isolated proctolin-binding fractions from hindgut and from oviduct tissues by isoelectric focusing, anion exchange, HPLC, and size exclusion HPLC. The specific binding was detected in fractions with an elution volume equivalent to a molecular weight of 50 kDa. The G protein-coupled receptor (GPCR) CG6986 from the *Drosophila* genome database has been identified as a putative proctolin receptor[3,9] and verified by use of G protein-coupled receptors-β-arrestin2 interactions.[8] This *Drosophila* proctolin receptor is a typical GPCR with seven transmembrane regions. The unmodified protein has a mass of 62 kDa and shows only weak identity with other known GPCRs. The *D. pseudoobscura* genome possesses a highly homologous gene. The cloned DNA of the *D. melanogaster* proctolin receptor gene has been expressed in mammalian cells, and screening with neuropeptides reveals that the expressed receptor is specific for proctolin with an EC_{50} of 0.3 or 0.6nM.[3,9] Membrane preparations of mammalian cells transfected with the proctolin receptor have been used in competition studies. The putative proctolin receptor binds proctolin with high affinity (IC_{50}, 4nM).[9] Microarray analyses reveal the receptor transcript in head mRNA of different genotypes and under different environmental conditions. Northern blot analyses indicate that the proctolin receptor is only weakly expressed in embryos, larvae, pupae, and in the thorax and abdomen

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of adult flies but is strongly expressed in the heads of adult flies.[3]

Immunohistochemistry with antibodies against the receptor reveals immunoreactive signals in the hindgut, heart, and in distinct neuronal populations within the CNS, and Western blot analyses reveal a band in tissue homogenates similar to the predicted size of the protein. Immunohistochemistry reveals the receptor is present in several neuropil regions and is associated with neurosecretory cell bodies in the adult and larval brain, but there is little evidence of proctolin receptor expression in thoracic and abdominal ganglia. Strong immunostaining is obtained in muscle layers of the *Drosophila* adult and larval hindgut, the aorta, and on neuronal endings in adult heart.[7]

Screening of the *A. gambiae*, *A. mellifera*, and *B. mori* genome databases has failed to reveal any potential proctolin receptor,[3,7] matching the absence of a proctolin gene in these species.

A few studies have examined the signaling cascade leading to target cell responses, and the majority of these have focused on the effects of proctolin upon muscle contraction. Proctolin uses different second messengers depending upon the target tissue. Thus, cAMP seems to mediate proctolin-induced increases in Ca^{2+} channel activity in crayfish muscle, proctolin-induced changes in voltage-dependent Ca^{2+} activity and nonvoltage sensitive K^+ activity in isopod muscle, and proctolin-induced increases in myogenic rhythm in some specialized locust muscle.[23] In several other arthropod muscles, proctolin increases inositol 1,4,5-trisphopsphate (IP_3) content and activates protein kinase C (PKC). Proctolin requires extracellular Ca^{2+} to elicit contractions in oviduct of *L. migratoria*. A model for proctolin's action on this preparation proposes that proctolin acts on a receptor to mediate an activation of receptor and voltage-operated Ca^{2+} channels, thereby resulting in an influx of extracellular Ca^{2+}.[30] Proctolin also stimulates the production of diacylglycerol and IP_3 suggesting the involvement of intracellular Ca^{2+} stores. The increased levels of intracellular Ca^{2+} act via calmodulin to stimulate muscle contraction. The increasing levels of Ca^{2+} are then buffered by an increase in efflux via a Na^+/Ca^{2+} exchanger and by sequestration into intracellular stores. In isopod muscle, proctolin induces a decrease in cGMP content via a PKC-dependent inhibition of guanylate cyclase, resulting in an increase in contraction.[23]

Numerous analogs have been synthesized in an attempt to gain an insight into the structure–activity requirements of proctolin and its receptor. These experiments have focused on the contractions of foregut, hindgut, oviduct, and cardiac muscle in a variety of insect species. Radioactively labeled proctolin has also been used to study the binding characteristics of receptors in membranes prepared from target tissues. These results have been well reviewed by a variety of authorse[12,22] In early studies,[27] it was found that removal of the *C*-terminal Thr, or its replacement with Ser, resulted in a loss of 99% of the peptide's activity. Replacement of any of the amino acids with its D isomer or with Ala illustrated that each of them are necessary for full activity with activities for the analogs ranging from 5 to 16% of the activity of equimolar proctolin. A number of di-, tri-, and tetra-peptide fragments are also inactive. One analog with significant activity, three times more potent as proctolin, was [Phe(*p*-OMe)^2]-proctolin. Other super analogs have also been made by modifying the second residue, and indeed, some antagonists have also been generated.[11,12] These latter observations, combined with the observation that Phe^2-proctolin has only 15% of proctolin's potency, leads to the conclusion that the aromatic ring in the second position is important in receptor binding. Other studies have also concluded the importance of the aromatic ring in the second position and further evidence for this has been derived from a theoretical analysis.[1] Thus, the total energy for the 5 most probable conformations of proctolin indicates the preferred conformation is a quasi-cyclic structure with the tyrosyl side chain pointing outwards. This would place it in an ideal position to interact with active sites on the receptor.

The majority of the published results concern the exchange of one or more of the 5 amino acids of proctolin with other natural or non-natural α-amino acids. In addition, some analogs have been generated, which modify the peptide backbone. As summarized,[12] of 100 synthetic proctolin analogs produced, 45 have myotropic activity on various insect visceral muscles. Of these, 40 are agonists, and 5 are antagonists, with [α-MeTyr^2]-proctolin being the most potent antagonist. The agonistic property is dependent on the *para* substitution at position 2, and on the presence of the peptide bond hydrogen atom between Arg^1 and Tyr^2 and between Tyr^2 and Leu^3. Additional substitution at the *meta* position of the Tyr phenyl ring facilitates interaction with the receptor. The Arg^1, Leu^3, and Pro^4 residues are essential for myotropic activity.

Classical structure–activity studies have certainly provided an insight into the possible interaction between the proctolin molecule and its receptor, and the importance of the individual amino acid residues in these interactions. They have also been important for identifying super analogs and antagonists of the proctolin receptor for applied purposes. Unfortunately, no studies have examined the effects of these analogs on functionally expressed receptors.

Once proctolin has activated its receptor, there must be some means of inactivating, removing, or otherwise terminating its action. Proctolin, injected into the hemolymph of *P. americana*, has a half-life of about 4min.[20] By following the fate of radioactively labeled proctolin, it was suggested that the initial inactivation step is cleavage of the Arg–Tyr bond followed by the cleavage of the

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Tyr–Leu bond, indicating an aminopeptidase as the primary inactivation mechanism. A number of *in vitro* studies have further indicated peptidases as responsible for the metabolic inactivation of proctolin.[7,20] By the use of a variety of tissue homogenates from *P. americana* (hindgut, leg muscle, brain, and terminal abdominal ganglion), a soluble aminopeptidase was identified as a key proctolin inactivating enzyme. These tissues also contained another peptidase that removed the *N*-terminal dipeptide of proctolin by the cleavage of the Tyr–Leu peptide bond. The Tyr–Leu cleavage site seems to be more important in tissues that are richer in proctolin, leading to the suggestion of the existence of a proctolinase. Hemolymph also contains the soluble aminopeptidase activity, but the second peptidase seems mainly responsible for proctolin degradation in cockroach hemolymph *in vitro* at neutral pH. Similar peptidases are found in homogenates of brain and thoracic ganglia of *Schistocerca gregaria*.[7] The enzyme responsible for cleaving the *N*-terminal dipeptide is mainly cytosolic, whereas the aminopeptidase is enriched in locust synaptic membranes and might therefore be considered a neuronal proctolinase. This proctolinase has a K_m of 23μM and is inhibited by amastatin. A similar amastatin-sensitive aminopeptidase is also considered to be the major proctolin degrading enzyme associated with locust oviduct and hindgut membranes. The enzyme responsible for removing the *N*-terminal dipeptide of proctolin has activity resembling that of mammalian dipeptidyl-aminopeptidase III (EC 3.4.14.4, DPP-III), which is an enzyme associated with the metabolism of enkephalin and angiotensin. Two proteins with relative molecular masses of 80 and 76K have been isolated from foregut membranes of the cockroach, *Blaberus craniifer*, and identified as insect orthologs of mammalian DPP-III.[17] The purified cockroach enzyme hydrolyzes proctolin with a K_m of about 4μM. The cockroach DPP-III is a metalloenzyme, inhibited by EDTA and by tynorphin, a peptide inhibitor of mammalian DPP-III. Subsequent analysis of the *D. melanogaster* DPP-III gene (CG7415) and cDNA sequences predicts the expression of two insect variants (89,194 and 81,937Da) resulting from different translational start sites.[17] When the smaller cDNA is expressed in S2 cells, there is the generation of the 82kDa DPP-III and an 86kDa protein, suggesting that some DPP-III undergoes posttranslational modification that influences protein mobility in SDS-PAGE. The majority of the recombinant DPP-III is located in the soluble fraction of transfected S2 cells, but a significant amount is also detected in washed membranes. A search of the genome databases of *D. pseudoobscura* and *A. gambiae* reveals DPP-III proteins that, like *D. melanogaster*, have amino acid sequences similar to the mammalian DPP-III with complete conservation of key catalytic and zinc-coordinating residues.[7]

BIOLOGICAL ACTIONS

Proctolin was originally isolated from *P. americana* based on its myostimulatory action on the hindgut and subsequently shown to be a potent stimulator of contraction of a variety of arthropod visceral and skeletal muscle.[20] Much of the research on proctolin has focused on its effects on muscle contraction, prompted in part because of the presence of proctolin within identified motoneurons projecting to various muscles. However, proctolin's relevance extends to other physiological and behavioral processes.

In addition to the cockroach hindgut, proctolin stimulates contractions of the foregut in *S. gregaria* and the midgut of *Diploptera punctata* and *L. migratoria* and therefore might be regarded as a major neuromuscular modulator in the gut of insects and crustacea.[10,12,20,22] Proctolin is also a potent modulator of reproductive tissue, stimulating contractions of the oviducts in *P. americana*, *Leucophaea maderae*, *L. migratoria*, and spermathecae in *L. migratoria* and *R. prolixus*. In addition, proctolin influences egg-laying activity of *A. mellifora* queens and promotes the onset of vitellogenesis in *B. craniifer*.[7,12] Proctolin also seems to be a cardioacceleratory peptide, increasing the rate and amplitude of contractions of hearts in several insects and crustacea, although interestingly the hearts of *Manduca sexta* and *Stomoxys calcitrans* are not responsive to proctolin. In addition, proctolin enhances contractions of skeletal muscle, such as the extensor tibiae in *L. migratoria*, the coxal depressor in *P. americana*, the ventral protractor muscle and the ovipositor opener muscle of *L. migratoria*. *In vivo* application of antiproctolin antiserum in *Gryllus bimaculatus* indicates that proctolin, which is present in antennal motoneurons, stabilizes the prolonged antennal forward position during flight.[4] Proctolin seems to be necessary to induce a large enough muscle tension to hold the antenna in a forward position during flight. This matches the observation that proctolin is found in slow motoneurons that control skeletal muscle involved in maintaining posture.[18] Within the skeletal and visceral muscle preparations described above, it is accepted that proctolin is a cotransmitter that acts alongside a more classical neurotransmitter, such as glutamate.

Immunohistochemistry and in situ hybridization indicate that proctolin is present within interneurons within the CNS. Dorsal unpaired median neurons can be activated by proctolin, and proctolin influences ovipositional digging behaviors when applied to abdominal ganglion VIII and alters the rhythmic bursting of centrally generated action potentials recorded from the oviducal nerve in *L. migratoria*.[13,14,18] However, the functional roles of proctolin centrally are not well understood in insects,[18] although more is known about proctolin's central actions in crustaceans. For example, proctolin activates the rhythm generating networks in the crayfish swimmeret system and the crab stomatogastric and cardiac ganglion. Proctolin is often colocalized along with other neuroactive chemicals that act in different

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combinations as neuromodulators in these crustacean preparations.[10,18]

As mentioned earlier, immunoreactivity and *in situ* hybridization for proctolin reveal its presence in insect and crustacean neurosecretory cells. This, coupled with the detection of proctolin in insect hemolymph, is consistent with proctolin acting as a neurohormone.[2,7,14] A number of tissues that are not innervated (e.g., Malpighian tubules and upper lateral oviducts of *L. migratoria* and the heart of *P. americana* and *Tenebrio molitor*) are sensitive to proctolin, and so proctolin may be acting upon these tissues in its capacity as a neurohormone. Similarly, proctolin is found in the POs of crustaceans suggesting a neurohormonal role, but proctolin is yet to be demonstrated in the hemolymph. Criticism of this neurohormonal concept has been made because of the short half-life of proctolin when injected into the hemolymph. However, it has been pointed out that the concentration of any active peptide in the vicinity of a receptor is determined by a number of factors, including the rate of release, hemolymph flow, and distance from the release site to the receptor and therefore is not only a function of the rate of metabolism.[7] These points, along with the fact that responsive tissues are activated very quickly by proctolin at low concentrations suggest that a half-life of 4min does not argue against proctolin as a neurohormone. The role of proctolin in lateral neurosecretory cells projecting to the CC/CA in *L. migratoria* and to the CC in *D. melanogaster* has been investigated. In *L. migratoria*, proctolin may well be a neurohormone[2,14] but may also be a releasing factor for other hormones associated with the CC/CA. Thus, proctolin stimulates the release of adipokinetic hormones from the glandular lobe of the CC and also stimulates the production and release of juvenile hormone from the CA in *L. migratoria*.[2] In *D. melanogaster*, proctolin is colocalized with corazonin in the lateral neurosecretory cells in the brain. Corazonin has different actions in different insect species and seem to be involved in the control of heart, morph-specific color changes, silk gland activity, larval ecdysis behavior, and the circadian clock system. Ectopic expression of the proctolin gene in the CNS and midgut of *D. melanogaster* has been used to overexpress proctolin in pupae. Within these pupae, there is a 14% increase in the heart rate suggesting a cardioacceleratory endocrine function for proctolin.[28] Thus, there can be no doubt as to the importance of proctolin as a neuroactive chemical in arthropods, where it functions in quite diverse roles, roles that far exceed the original discovery of it being a cockroach hindgut neurotransmitter.

REFERENCES

1. Betins JR, Nikiforovich GV. Theoretical conformational analysis of proctolin molecule. *Bioorg Khim* 1979;**5**:1581–3.
2. Clark L, Zhang JR, Tobe S, Lange AB. Proctolin: a possible releasing factor in the corpus cardiacum/corpus allatum of the locust. *Peptides* 2006;**27**:559–66.
3. Egerod K, Reynisson E, Hauser F, Williamson M, Cazzamali G, Grimmelikhuijzen CJP. Molecular identification of the first insect proctolin receptor. *Biochem Biophys Res Comm* 2003;**306**:437–42.
4. Gebhardt M. In vivo application of anti-proctolin-antiserum affects antennal flight posture in crickets. *J Comp Physiol A* 2004;**190**:359–64.
5. Hauser F, Cazzamali G, Williamson M, Park Y, Li B, Tanaka Y, et al. A genome-wide inventory of neurohormone GPCRs in the red flour beetle *Tribolium castaneum*. *Front Neuroendocrinol* 2008;**29**:142–65.
6. Huybrechts J, Bonhomme J, Minoli S, Prunier-Leterme N, Dombrovsky A, Abdel-Latief M, et al. Neuropeptide and neurohormone precursors in the pea aphid, *Acyrthosiphon pisum*. *Insect Molec Biol* 2010;**19**:87–95.
7. Isaac RE, Taylor CA, Hamasaka Y, Nässel DR, Shirras AD. Proctolin in the post-genomic era: new insights and challenges. *Invert Neurosci* 2004;**5**:51–64.
8. Johnson EC, Bohn LM, Barak LS, Birse RT, Nässel DR, Caron MG, et al. Identification of *Drosophila* neuropeptide receptors by G protein-coupled receptors-β-Arrestin2 interactions. *J Biol Chem* 2003;**278**:52172–8.
9. Johnson EC, Garczynski SF, Park D, Crim JW, Nässel DR, Taghert PH. Identification and characterization of a G protein-coupled receptor for the neuropeptide proctolin in *Drosophila melanogaster*. *PNAS* 2003;**100**:6198–203.
10. Keller R. Crustacea neuropeptides: structure, function and comparative aspects. *Experientia* 1992;**48**:442–8.
11. Konopińska D. Insect neuropeptide proctolin and its analogues: an overview of the present literature. *J Pept Res* 1997;**49**:457–66.
12. Konopińska D, Rosiński G. Proctolin, an insect neuropeptide. *J Pept Sci* 1999;**5**:533–46.
13. Kwok R, Orchard I. Central effects of the peptides, SchistoFLRFamide and proctolin, on locust oviduct contraction. *Peptides* 2002;**23**:1925–32.
14. Lange AB. A review of the involvement of proctolin as a cotransmitter and local neurohormone in the oviduct of the locust, *Locusta migratoria*. *Peptides* 2002;**23**:2063–70.
15. Li B, Predel R, Neupert S, Hauser F, Tanaka Y, Cazzamali G, et al. Genomics, transcriptomics, and peptidomics of neuropeptides and protein hormones in the red flour beetle *Tribolium casteneum*. *Genome Res* 2008;**18**:113–22.
16. Ma M, Gard AL, Xiang F, Wang J, Davoodian N, Lenz PH, et al. Combining *in silico* transcriptome mining and biological mass spectrometry for neuropeptide discovery in the Pacific white shrimp *Litopenaeus vannamei*. *Peptides* 2010;**31**:27–43.
17. Mazzocco C, Fukasawa KM, Auguste P, Puiroux J. Characterization of a functionally expressed dipeptidyl aminopeptidase III from *Drosophila melanogaster*. *Eur J Biochem* 2003;**270**:3074–82.
18. Nässel DR. Neuropeptides in the nervous system of *Drosophila* and other insects: multiple roles as neuromodulators and neurohormones. *Prog Neurobiol* 2002;**68**:1–84.
19. Ons S, Sterkel M, Diambra L, Urlaub H, Rivera-Pomar R. Neuropeptide precursor gene discovery in the Chagas disease vector *Rhodnius prolixus*. *Insect Molec Biol* 2011;**20**:29–44.
20. Orchard I, Belanger JH, Lange AB. Proctolin: a review with emphasis on insects. *J Neurobiol* 1989;**20**:470–96.
21. Orchard I, Lee DH, da Silva R, Lange AB. The proctolin gene and biological effects of proctolin in the blood-feeding bug, *Rhodnius prolixus*. *Front Endocrin* 2011;**2**:59.

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22. Osborne RH. Insect neurotransmission: neurotransmitters and their receptors. *Pharmacol Ther* 1996;**69**:117–42.
23. Philipp B, Rogalla N, Kreissl S. The neuropeptide proctolin potentiates contraction and reduces cGMP concentration via a PKC-dependent pathway. *J Exp Biol* 2006;**209**:531–40.
24. Predel R, Neupert S, Garczynski SF, Crim JW, Brown MR, Russell WK, et al. Neuropeptidomics of the mosquito *Aedes aegypti*. *J Proteome Res* 2010;**9**:2006–15.
25. Spittaeles K, Vankeerberghen A, Torrekens S, Devreese B, Grauwels L, Van Leuven F, et al. Isolation of Ala[1]-proctolin, the first natural analogue of proctolin, from the brain of the Colorado potato beetle. *Mol Cell Endocrinol* 1995;**110**:119–24.
26. Starratt AN, Brown BE. Structure of pentapeptide proctolin, a proposed neurotransmitter in insects. *Life Sci* 1975;**17**:1253–6.
27. Starratt AN, Brown BE. Analogs of the insect myotropic peptide proctolin: synthesis and structure activity studies. *Biochem Biophys Res Comm* 1979;**90**:1125–30.
28. Taylor CAM, Winther AME, Siviter RJ, Shirras AD, Isaac RE, Nässel DR. Identification of a proctolin preprohormone gene (*Proct*) of *Drosophila melanogaster*: expression and predicted prohormone processing. *J Neurobiol* 2004;**58**:379–91.
29. Weaver RJ, Audsley N. Neuropeptides of the beetle, *Tenebrio molitor* identified using MALDI-TOF mass spectrometry and deduced sequences from the *Tribolium castaneum* genome. *Peptides* 2008;**29**:168–78.
30. Wilcox CL, Lange AB. Role of extracellular and intracellular calcium on proctolin-induced contractions in an insect visceral muscle. *Reg Peptides* 1995;**56**:49–59.

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Chapter 43

Sulfakinins

Liliane Schoofs, Tom Janssen and Ronald J. Nachman

ABSTRACT

Sulfakinins constitute a family of arthropod neuropeptides that typically contain the C-terminal heptapeptide Y(SO_3H)GHMRFamide. They display structural and functional similarities to the vertebrate gastrin-cholecystokinin family of peptides. Sulfakinins are synthesized by a limited number of neurosecretory cells and exhibit several effects, including myotropic activities, inhibition of food intake, and stimulation of digestive enzyme release. In many cases, the sulfate group is a requirement for biological activity. The gastrin/CCK and sulfakinin signaling system is a good example of the coevolution of neuropeptides and their receptors.

DISCOVERY

Sulfakinins are arthropod neuropeptides that typically contain a sulfated tyrosine residue—hence their name. They are characterized by the C-terminal heptapeptide XY(SO_3H) GHMRFamide (X = D or E) and display structural and physiological similarities to gastrin and cholecystokinin (CCK), their sulfated neuropeptide counterparts in vertebrates.[27] The initial evidence indicating that gastrin/CCK-like peptides are present in invertebrates came from immunocytochemical studies using gastrin and CCK antibodies on the insect neuroendocrine system and on various tissue extracts of 26 other species of invertebrates, including the phyla of Mollusca, Annelida, Arthropoda, and Ectoprocta. The first sulfakinins were isolated by conventional bioassay-monitored chromatographic purification of thousand(s) of nervous tissue extracts.[6,27] The release of insect genomes (from *Drosophila melanogaster*, *Anopheles gambiae*, *Aedes aegypti*, *Bombyx mori*, *Apis mellifera*, *Triboleum castaneum*) combined with very sensitive mass spectrometry has provided a powerful tool for peptidomic experiments. These reduce not only the starting material needed but also the time required to isolate and sequence the peptides from years to days and allowed the identification of sulfakinins in numerous arthropods, except for the parasitic wasp, *Nasonia vitripennis* and the pea aphid.[1,3,12–14,31,32,34] Sulfakinins have also been identified in chelicerate[2,7] and crustaceans.[4,5,16,20,33] Interestingly, one of the isolated forms in *Litopenaus vannamei* is disulfated,[33] a feature also found in cionin, the CCK in the protochordate *Ciona intestinalis*. At present, around 40 sulfakinin sequences are known (Fig. 1).

STRUCTURE OF THE mRNA/GENE

To date, genes encoding sulfakinins have been identified in *D. melanogaster*, *Calliphora vomitoria*, *Anopheles maculates*, *A. gambiae*, *A. mellifera*, and *Gryllus bimaculatus*.[6,13,22,28,31] Northern blot analysis in *Gryllus* showed that the sulfakinin gene is only expressed in the brain, as a single transcript.[22] The translation product generally contains an N-terminal signal peptide, followed by a long interspace sequence, ending with the highly conserved sulfakinins (two) encoding region, directly followed by a stop codon. The sulfakinin-encoding sequences are separated by conventional dibasic cleavage sites. Recognition of other dibasic endoproteolytic cleavage sites in nonconserved regions would result in the processing of additional peptides that are not similar to each other, nor to the sulfakinin peptides. Such an additional neuropeptide (DSK 0) in *Drosophila* shares the SFamide C-terminus with myotropins found in nematodes and sea urchins.[29]

PROCESSING

After cleavage from their inactive propeptide sulfakinin precursor (most likely by subtilisin-like proprotein convertases), sulfakinins undergo further posttranslational modification.[30] The tyrosine residue can be sulfated, most likely by a tyrosyl protein sulfotransferase. Both nonsulfated and sulfated forms have been biochemically isolated, although it is not clear whether this is due to the loss of the unstable sulfate group during isolation procedures. The N-terminal glutamate can be modified into a pyroglutamate residue. Further, the presence of a naturally occurring perisulfkinin with O-methylated glutamic acid has also been shown. Finally, all sulfakinins are amidated at their C-terminus (most likely by a peptidyl-amidating monooxygenase).

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00043-9
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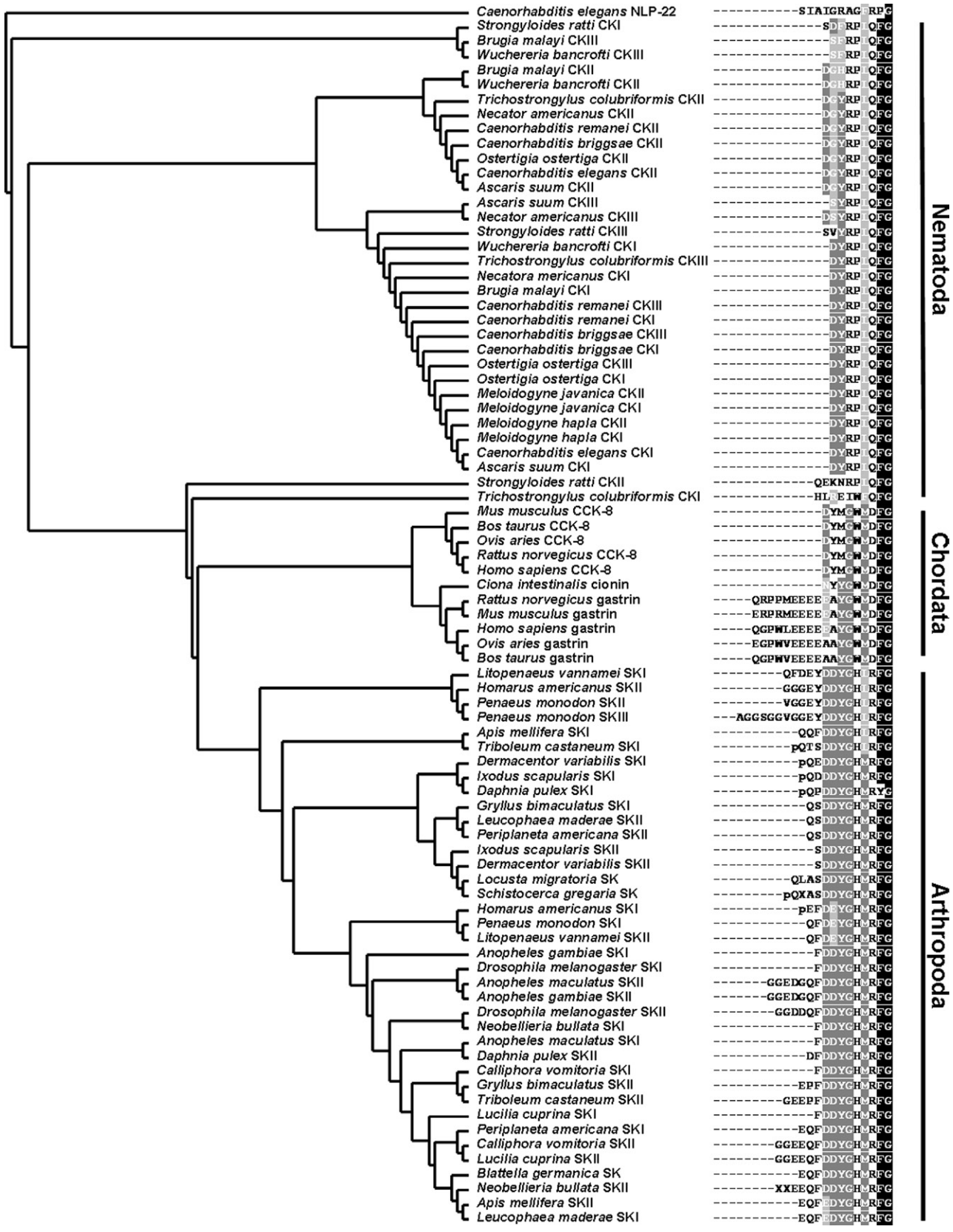

FIGURE 1 Alignment and phylogenetic analysis of the sulfakinins and gastrin/CCKs of arthropods, nematodes, and chordates. Identical amino acids are shown in black, amino acids that are conserved or similar are shown in gray. pQ indicates a pyroglutamic acid residue. X indicates an undetermined amino acid. The C-terminal glycine is amidated *in vivo*.

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TISSUE DISTRIBUTION

Sulfakinins are expressed in the CNS of all developmental stages in dipterans and decrease with age in female crickets.[22,28] In Diptera, they occur in a limited set of neurons in the brain (four pairs in *C. vomitoria*) and in extensive arborizations of dendrites in the CNS neuropile (Fig. 2), suggesting a neurotransmitter or neuromodulator function. Such a neurotransmitter function for sulfakinins seems to be a general characteristic feature across insect orders. In contrast to flies and crickets, the expression of sulfakinins does not seem to be restricted to neurons and axons in the CNS and are also localized in the sympathetic nervous system innervating the crop in the cockroach *Periplaneta americana*.[10,30] In the latter and in the mosquito, *A. aegypti*, endocrine cells of the midgut were immunostained as well. Mass spectrometric profiling unambiguously identified sulfakinins in the brain and in the stomatrogastric nervous system of adult locusts, including esophageal nerves innervating the foregut, but neither in the corpora cardiaca nor in the thoracic or abdominal ganglia.[3] The hormonal role of sulfakinins is questioned because no evidence for a release site has been found.[3,6]

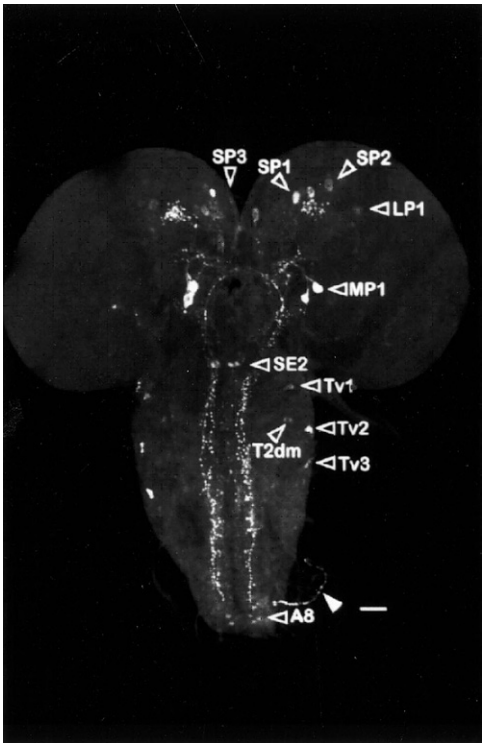

FIGURE 2 DSKI/II immunoreactivity in larval neural tissue at 96 h. Immunoreactive material is expressed in particular neurons, that is, SP1, SP2, SP3, MP1, SE2, Tv1–3, T2dm, and A8 cells. An immunoreactive fiber projects away from the central nervous system in an abdominal ganglion (filled arrow). Bar: 50 μm. From R. Nichols and I.A. Lim, Spatial and temporal immunocytochemical analysis of drosulfakinin (Dsk) gene products in the *Drosophila melanogaster* central nervous system. *Reproduced from Ref. 28, with the kind permission of Springer Science and Business Media.*

RECEPTORS

CCK and sulfakinin G-protein-coupled receptors share a very high degree of sequence similarity (>60%) and are considered to originate from a common ancestor. *Drosophila* has two sulfakinin receptors (DSKR), encoded by two loosely linked genes (~30 kb apart).[17] Interestingly, the two orthologous *Caenorhabditis elegans* receptors represent splice isoforms encoded by the same gene.[15] So far, sulfakinin receptors have also been identified in the insects *A. aegypti*, *A. gambiae*, *Culex quinquefasciatus, T. castaneum* and are predicted in the crustacean *Daphnia pulex* and the chelicerates *Ixodes scapularis* and *Dermacentor variabilis* as well as several nematodes, including *Brugia malayi* and in the flatworm, *Clonorchis sinensis*.[7,15]

In a cellular system, expressing the *Drosophila* sulfakinin receptor, unsulfated sulfakinin is 3000 times less potent than the sulfated form, indicative of the importance of the sulfate moiety for receptor activation. However, for the nematode receptors, no difference in response to sulfated and nonsulfated peptides was found.[15] The observed calcium signaling in cells expressing the DSKR was PTX insensitive, indicating that the receptor couples exclusively to the Gq type of Gα subunit. This is in agreement with mammalian CCK receptors where CCK-8 activates phospholipase C, triggering an increase in IP_3 production. The minimum sequence (active core) capable of eliciting a response is the C-terminal hexapeptide, Tyr(SO_3H)–Gly–His Met–Arg–Phe–NH_2, the His (or alternate aromatic residue), Arg (or alternate basic residue), and Phe residues being critical for activity. Human CCK-8 and gastrin II were unable to activate the drosulfakinin receptor.[17] However, replacement of the Asp residue with Arg in both gastrin and CCK can confer activity in the insect bioassay.[24] For myotropic activity, the sulfated tyrosine is a strict requirement for activity, although its position within the sequence[26] is not critical. This indicates that the sulfakinin receptor site demonstrates a degree of flexibility with respect to the interaction with the sulfate group and suggests that the movement of this group could occur during the molecular evolutionary process. The sulfate group is also a requirement for food intake inhibition activity in locusts and cockroaches.[18,26,35] Replacement of the acidic, unstable Tyr(SO_3H) group in sulfakinins by stable, acidic α-aminosuberic acid, which lacks a phenyl ring, is accompanied by the retention of cockroach myotropic and locust food intake–inhibition activity.[26] Replacement of the basic Arg in the sulfakinins and acidic Asp in the analogous position of CCK with neutral Pro is accompanied by a significant retention of bioactivity,[25] leading to an argument that these residues do not directly interact with the receptor but may stabilize the active conformation. This is consistent with an aqueous solution conformation study of a sulfakinin, which revealed a

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relatively rigid a-helical loop structure stabilized by alternating salt bridges between Arg and two acidic residues in the N-terminal region and between His and Tyr(SO_3H).

BIOLOGICAL ACTIONS

Sulfakinins affect several biological processes. They increase the frequency and amplitude of foregut,[18] hindgut,[27,30] and heart[4,19,29] contractions in arthropods. Comparable with the satiety effect of CCK observed in mammals, the injection of sulfakinins inhibits food intake in locusts[26,35] and cockroaches[18] and inhibits carbohydrate (but not protein) feeding in the fly, *Phormia regina*[8] and blood feeding in the horse fly, *Tabanus nigrovittatus*.[9] Silencing sulfakinin gene expression by RNAinterference results in an increase of food intake in *G. bimaculatus*.[21] In addition, sulfakinins stimulate the release of the digestive enzyme amylase from the insect digestive tract in the beetle *Rhynchophorus ferrugineus* and in the moth *Opisina arenosella*.[11,23] Both sulfated and nonsulfated sulfakinins stimulate the heart contractions but differ in their activities depending on the developmental stage.[29] The pleiotropic effects of sulfakinins are illustrated in a study showing that in the beetle *Zophobas atratus*, sulfakinin decreases the contractions of the heart, ejaculatory duct, and oviduct and also displays hyperglycemic activity.[19] The absence of sulfakinin and its receptor in the pea aphid[14] and in the parasitic wasp, *N. vitripennis*[12] indicates that these species may have lost the sulfakinin signaling system during evolution, causing or being the result of specific adaptations in behavior, feeding or habitats, compared with other insects.

Two CCK/sulfakinin receptors in the nematode, *C. elegans* and their activating ligands, two unsulfated sulfakinin-related peptides encoded by the NLP-12 prescursor protein, have been identified and characterized.[15] Interestingly, the amylase secretion is significantly lower in the receptor mutants. In addition, the lack of either receptor or peptide results in a highly increased fat content in the worm, which is in agreement with a similar phenotype in mice lacking the CCK-2 receptor.

In sum, the gastrin/CCK/sulfakinin signaling system represents a good example of the coevolution of neuropeptides and their receptors. The sulfakinins do not only seem to display structural but also physiological similarities to the vertebrate peptides gastrin and CCK. So far, sulfakinin peptides have only been isolated from arthropods and nematodes, but related peptides have been detected by immunological methods in annelids and snails. The elucidation of additional sulfakinins in species belonging to nonecdysozoan phyla of the protostomian lineage will shed light on the (still debated) common evolutionary origin of CCK/gastrin and sulfakinins.

REFERENCES

1. Baggerman G, Cerstiaens A, De Loof A, Schoofs L. Peptidomics of the larval *Drosophila melanogaster* central nervous system. *J Biol Chem* 2002;**277**:40,368–74.
2. Christie AE. Neuropeptide discovery in Ixodoidea: an in silico investigation using publicly accessible expressed sequence tags. *Gen Comp Endocrinol* 2008;**157**:174–85.
3. Clynen E, Schoofs L. Peptidomic survey of the locust neuroendocrine system. *Insect Biochem Mol Biol* 2009 Aug;**39**(8):491–507.
4. Dickinson PS, Stevens JS, Rus S, Brennan HR, Goiney CC, Smith CM, et al. Identification and cardiotropic actions of sulfakinin peptides in the American lobster *Homarus americanus*. *J Exp Biol* 2007;**210**:2278–89.
5. Dircksen H, Neupert S, Predel R, Verleyen P, Huybrechts J, Strauss J, et al. Genomics, transcriptomics, and peptidomics of *Daphnia pulex* neuropeptides and protein hormones. *J Proteome Res* 2011;**10**: 4478–504.
6. Duve H, Thorpe A, Scott AG, Johnsen AH, Rehfeld JF, Hines E, et al. The sulfakinins of the blowfly *Calliphora vomitoria*—peptide isolation, gene cloning and expression studies. *Euro J Biochem* 1995;**232**:633–40.
7. Donohue KV, Khalil SM, Ross E, Grozinger CM, Sonenshine DE, Michael Roe R. Neuropeptide signaling sequences identified by pyrosequencing of the American dog tick synganglion transcriptome during blood feeding and reproduction. *Insect Biochem Mol Biol* 2010;**40**:79–90.
8. Downer KE, Haselton AT, Nachman RJ, Stoffolano Jr JG. Insect satiety: sulfakinin localization and the effect of drosulfakinin on protein and carbohydrate ingestion in the blow fly, *Phormia regina* (Diptera: Calliphoridae). *J Insect Physiol* 2007;**53**:106–12.
9. Downer KE, Nachman RJ, Stoffolano JG. Effect of seasonality and perisulfakinin on engorgement by *Tabanus nigrovittatus* (Diptera: Tabanidae) in the laboratory. *Ann Entomol Soc America* 2006;**100**:251–6.
10. East PD, Hales DF, Cooper PD. Distribution of sulfakinin-like peptides in the central and sympathetic nervous system of the American cockroach, *Periplaneta americana* (L) and the field cricket, *Teleogryllus commodus* (Walker). *Tissue Cell* 1997;**29**:347–54.
11. Harshini S, Nachman RJ, Sreekumar S. In vitro release of digestive enzymes by FMRF amide related neuropeptides and analogues in the lepidopteran insect *Opisina arenosella* (Walk.). *Peptides* 2002;**23**:1759–63.
12. Hauser F, Neupert S, Williamson M, Predel R, Tanaka Y, Grimmelikhuijzen CJ. Genomics and peptidomics of neuropeptides and protein hormones present in the parasitic wasp *Nasonia vitripennis*. *J Proteome Res* 2010;**9**:5296–310.
13. Hummon AB, Richmond TA, Verleyen P, Baggerman G, Huybrechts J, Ewing MA, et al. From the genome to the proteome: uncovering peptides in the *Apis* brain. *Science* 2006;**314**:647–9.
14. Huybrechts J, Bonhomme J, Minoli S, Prunier-Leterme N, Dombrovsky A, Abdel-Latief M, et al. Neuropeptide and neurohormone precursors in the pea aphid, *Acyrthosiphon pisum*. *Insect Mol Biol* 2010;**19**:87–95.
15. Janssen T, Meelkop E, Lindemans M, Verstraelen K, Husson SJ, Temmerman L, et al. Discovery of a cholecystokinin-gastrin-like signaling system in nematodes. *Endocrinology* 2008;**149**: 2826–39.

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16. Johnsen AH, Duve H, Davey M, Hall M, Thorpe A. Sulfakinin neuropeptides in a crustacean—isolation, identification and tissue localization in the tiger prawn *Penaeus monodon*. *Euro J Biochem* 2000;**267**:1153–60.
17. Kubiak TM, Larsen MJ, Burton KJ, Bannow CA, Maritn RA, Zantello MR, et al. Cloning and functional expression of the first *Drosophila melanogaster* sulfakinin receptor DSK-R1. *Biochem Biophys Res Commun* 2002;**291**:313–20.
18. Maestro JL, Aguilar R, Pascual N, Valero ML, Piulachs MD, Andreu D, et al. Screening of antifeedant activity in brain extracts led to the identification of sulfakinin as a satiety promoter in the German cockroach—are arthropod sulfakinins homologous to vertebrate gastrins-cholecystokinins? *Eur J Biochem* 2001;**268**:5824–30.
19. Marciniak P, Kuczer M, Rosinski G. New physiological activities of myosuppressin, sulfakinin and NVP-like peptide in *Zophobas atratus* beetle. *J Comp Physiol B* 2011;**181**:721–30.
20. Ma M, Chen R, Sousa GL, Bors EK, Kwiatkowski M, Goiney CC, et al. Mass spectral characterization of peptide transmitters/hormones in the nervous system and neuroendocrine organs of the American lobster *Homarus americanus*. *Gen Comp Endocrinol* 2008;**156**:395–409.
21. Meyering-Vos M, Müller A. RNA interference suggests sulfakinins as satiety effectors in the cricket *Gryllus bimaculatus*. *J Insect Physiol* 2007;**53**:840–8.
22. Meyering-Vos M, Müller A. Structure of the sulfakinin cDNA and gene expression from the Mediterranean field cricket *Gryllus bimaculatus*. *Insect Mol Biol* 2007;**16**:445–54.
23. Nachman RJ, Giard W, Favrel P, Suresh T, Sreekumar S, Holman GM. Insect myosuppressins and sulfakinins stimulate release of the digestive enzyme a-amylase in two invertebrates: the scallop *Pecten maximus* and insect *Rhynchophorus ferrugineus*. *Ann NY Acad Sci* 1997;**814**:335–8.
24. Nachman RJ, Holman GM, Haddon WF, Vensel WH. Effect of sulfate position on myotropic activity of the gastrin/CCKlike insect leucosulfakinins. *Int J Pept Prot Res* 1989;**33**:223–9.
25. Nachman RJ, Holman GM, Haddon WF. Structural aspects of the gastrin-cholecystokinin-like insect sulfakinins. *Pept Res* 1989;**2**:171–7.
26. Nachman RJ, Vercammen T, Williams H, Kaczmarek K, Zabrocki J, Schoofs L. Aliphatic amino diacid Asu functions as an effective mimic of Tyr(SO3H) in sulfakinins for myotropic and food intake-inhibition activity in insects. *Peptides* 2005;**26**:115–20.
27. Nachman RJ, Holman GM, Haddon WF, Ling N. Leucosulfakinin, a sulfated insect neuropeptide with homology to gastrin and cholecystokinin. *Science* 1986;**234**:71–3.
28. Nichols R, Lim IA. Spatial and temporal immunocytochemical analysis of drosulfakinin (DSK) gene products in the *Drosophila melanogaster* central nervous system. *Cell Tissue Res* 1996;**283**:107–16.
29. Nichols R, Manoogian B, Walling E, Mispelon M. Plasticity in the effects of sulfated and nonsulfated sulfakinin on heart contractions. *Front Biosci* 2009;**14**:4035–43.
30. Predel R, Brandt W, Kellner R, Rapus J, Nachman RJ, Gade G. Post-translational modifications of the insect sulfakinins—sulfation, pyroglutamate-formation and O-methylation of glutamic acid. *Eur J Biochem* 1999;**263**:552–60.
31. Riehle MA, Garczynski SF, Crim JW, Hill CA, Brown MR. Neuropeptides and peptide hormones in *Anopheles gambiae*. *Science* 2002;**298**:172–5.
32. Roller L, Yamanaka N, Watanabe K, DaubnerovÃ¡ I, Zitnan D, Kataoka H, et al. The unique evolution of neuropeptide genes in the silkworm *Bombyx mori*. *Insect Biochem Mol Biol* 2008 Dec;**38**(12):1147–57.
33. Torfs P, Baggerman G, Meeusen Nieto TJ, Nachman RJ, Calderon J, De Loof A, et al. Isolation, identification, and synthesis of a disulfated sulfakinin from the central nervous system of an arthropod, the white shrimp *Litopenaeus vannamei*. *Biochem Biophys Res Commun* 2002;**299**:312–20.
34. Weaver RJ, Audsley N. Neuropeptides of the beetle, *Tenebrio molitor* identified using MALDI-TOF mass spectrometry and deduced sequences from the *Tribolium castaneum* genome. *Peptides* 2008;**29**(2):168–78.
35. Wei Z, Baggerman G, Nachman RJ, Goldsworthy G, Verhaert P, De Loof A, et al. Sulfakinins reduce food intake in the desert locust, *Schistocerca gregaria*. *J Insect Physiol* 2000;**46**:1259–65.

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Chapter 44

Tachykinin Peptides

Dick R. Nässel

ABSTRACT

Peptides related to vertebrate tachykinins have been identified in many invertebrates, including annelid worms, mollusks, crustaceans, and insects. Two major types can be distinguished, tachykinin-related peptides (TKRPs) with an FXGXRamide C-terminus and invertebrate tachykinins (invTKs) with FXGLMamide. The TKRPs are present in the nervous system and intestine, whereas the invTKs have been found only in the salivary glands. Receptors for TKRPs, ancestrally related to vertebrate neurokinin receptors, have been identified in many invertebrates. In some species, two different receptors are known. The TKRPs have a wide neuronal distribution and a broad spectrum of actions including myostimulatory, diuretic, and neuromodulatory actions. In *Drosophila*, genetic interference with TKRP signaling reveals roles in locomotor control, olfactory sensory processing, stress responses, and regulation of insulin signaling.

DISCOVERY

Tachykinins constitute a large and structurally varied family of multifunctional peptides present in the nervous system, gastrointestinal tract, and many other tissues. Members of this family have been identified in annelids, mollusks, arthropods, as well as in vertebrates (reviewed in Refs. 3, 5, 21, 22, 27, 33). Already in 1931 the first tachykinin, substance P, was isolated from horse brain. Substance P was followed by a large number of related peptides isolated from various vertebrates, all characterized by a common C-terminal sequence FXG$\underline{L}$Mamide.[22,23] The first tachykinin to be identified from an invertebrate was eledoisin, isolated from the salivary glands of the cephalopod *Eledone moschata* in 1962.[6] In 1990, the first insect peptides with similarities to tachykinins were identified from the locust *Locusta migratoria*.[28] The amino acid sequences of these two locust nonapeptides, designated locustatachykinin-I and -II, differ in a major way from vertebrate tachykinins and eledoisin by having a C-terminal pentapeptide FXG$\underline{V}$Ramide. Subsequently, it became apparent that tachykinins derived from the nervous system and intestine of insects and several other invertebrates share this structural feature (Table 1).[19,20,27] Thus, these invertebrate peptides are not referred to as tachykinins but as tachykinin-related peptides (TKRPs). Interestingly, vertebrate type of FXGLMamide-containing tachykinins are produced in the salivary glands of mosquitoes and two cephalopod species but not in the CNS of any invertebrate.[14,27] The salivary gland-derived tachykinins are henceforth referred to as invertebrate tachykinins (invTKs), as suggested by Satake et al.[27]

The first insect TKRPs were identified by their myostimulatory actions in the cockroach hindgut muscle contraction bioassay, an assay used for the isolation of a large number of novel peptides from HPLC-purified tissue extracts.[9,28] Most invertebrate TKRPs have been identified either by this hindgut bioassay, a combination of this and an immunoassay with antiserum to locust TKRP, or by searching genome or EST data bases, followed by mass-spectrometric identification in tissue. Thus, sequence data are now available for TKRPs from mollusks, annelids, the annelid-like worm *Urechis unicinctus*, a tardigrade, many insects, a few arachnids, some decapod crustaceans, and the cladoceran crustacean *Daphnia* (Table 1).[4,5,7,21,27,32,33] Many of the predicted peptide sequences in these organisms, but not all, have been confirmed by mass-spectrometric analysis of tissue.[5,7]

The invTKs have been identified in the salivary glands of two cephalopods and a mosquito, *Aedes aegypti*. In one of the cephalopods, *Octopus vulgaris*, typical TKRPs have in addition been identified in the CNS,[14,27] suggesting that TKRPs are neuronal (and intestinal) peptides, whereas invTKs are nonneuronal and can exist in the same organism.

STRUCTURE OF PRECURSOR mRNAs AND GENES

The three principal mammalian tachykinins, substance P, neurokinin A, and neurokinin B, are processed from two precursors, preprotachykinin A and B.[23] More recently additional tachykinins, the hemokinins, were identified on a third precursor gene, preprotachykinin C, expressed in hematopoietic cells.[23]

The first invertebrate cDNA to be identified for a TKRP precursor was isolated from the worm *U. unicinctus*.[27] This precursor consists of 243 amino acid residues, from which 7 different TKRPs can be liberated. Next, a gene

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00044-0
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TABLE 1 Sequences of Representative Members of the Extended Tachykinin Family

Species	Designation	Sequence
TKRPs (FXGXRamide)		
Octopus vulgaris[*]	Oct-TKRP-III	TVSANAFLGSRa
Hypsibius dujardini[#]	TKRP	AKPSGFWGARa
Decapod crustaceans	CabTRP-I	APSGFLGMRa
Daphnia pulex[$]	DappuTKRP-1	TPNSRAFLGMRa
	DappuTKRP-2	KMHGEKFLGMRa
	DappuTKRP-3	APSSNSFMGMRa
L. maderae[**]	LemTRP-1	APSGFLGVRa
	LemTRP-2	APEESP**KR**APSGFLGVRa
	LemTRP-3	NGERAPGS**KK**APSGFLGTRa
	LemTRP-3[11–19]	APSGFLGTRa
Drosophila[***]	DTK-1	APTSSFIGMRa
	DTK-2	APLAFVGLRa
	DTK-3	APTGFTGMRa
	DTK-4	APVNSFVGMRa
	DTK-5	APNGFLGMRa
	DTK-6	AALSDSYDLRGK**Q**QRFADFNSKFVAVRa
Stomoxys calcitrans[†]	StcTK-I	APTGFFAVRa
Anodonta cygnea[*]	AncTK	PQYGFHAVRa
InvTKs and substance P (FXGLMamide)		
Eledone moschata[*]	Eledoisin	PEPSKDAFIGLMa
Octopus vulgaris[*]	Oct-TK-II	KPPSSSEFVGLMa
Aedes aegypti[†]	Sialokinin I	SGNTGDKFYGLMa
C. elegans	nlp-8[‡]	SFDRMGGTEFGLMa
Mammals	Substance P	RPKPQQFFGLMa

The dibasic cleavage sites in LemTRPs and putative cleavage site in DTK-6 are indicated in bold. The G to A substitution in some TKRPs is indicated by underlining. Note that in ***Octopus***, both TKRPs and invTKs have been identified.
[*]*Mollusca.*
[#]*Tardigrade.*
[$]*Phyllopod crustacean.*
[**]Leucophaea maderae *(cockroach).*
[***]D. melanogaster.
[†]*Mosquito.*
[‡]*The* C. elegans *putative invTK has no designation but is derived from the* nlp-8 *gene.*

(*Dtk*; CG14734) coding for multiple TKRPs was identified in *Drosophila*.[29] The *Drosophila* precursor consists of 289 amino acids derived from an 870-bp open reading frame. There are 6 predicted TKRPs (DTK-1 to DTK-6) in the *Drosophila* precursor, five of which could be confirmed in the brain by mass spectrometry[35] the bona fide DTK-6 has not yet been identified (see however Ref. 24).

Through data mining and classical cloning techniques, we now know the sequences of many additional TKRP precursors from annelids, insects, crustaceans, arachnids, and mollusks.[3,5,26,27,32,34] Thus, there are, for example, 7 TKRPs on the precursor from the Honeybee *Apis mellifera*, 3 in the mosquito *Anopheles gambiae*, 13 in each of the cockroaches *Periplaneta americana* and *L. maderae*, 7 in *Octopus vulgaris*,

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and 7 each in the crayfish *Procambarus clarkii* and spiny lobster *Panulirus interruptus*, whereas in *Daphnia* 3 different types were found.[5,20,25,27]

The crayfish and lobster precursors, which are very similar to each other, each contain 7 identical TKRPs (CabTRP-I) and no further peptides. This is unique, so far, among the TRP precursors. In all other organisms (including the phyllopod crustacean *Daphnia*) multiple, closely related TKRP isoforms were found on the precursors. The decapod crustacean precursors may provide insights into the evolution of tachykinin isoforms. Possibly precursors, such as the decapod ones (but not *Daphnia*), with several identical copies of a peptide are more ancestral, and such a precursor could evolve by diversification into one with multiple closely related peptides.

An interesting feature of the cockroach TKRPs is that two N-terminally extended TKRPs exist that have internal dibasic cleavage sites (Table 1). These sites seem more likely to be used by processing enzymes in the brain than in the intestine. As a consequence, the nonapeptides LemTKRP-1 and LemTKRP-3[11–19] are much more abundant in the brain than LemTKRP-2 (17mer) and LemTKRP-3 (19mer), whereas the extended forms are prominent in the intestine.[25] Thus, there might be a differential posttranslational processing of these peptides in the brain and the gut. In total, the *L. maderae* precursor can yield up to 15 TKRP isoforms, as confirmed by MALDI-TOF mass spectrometry. Two of these are produced posttranslationally.

Judging from cDNAs, the sequences of the TKRP precursors of different invertebrates are conserved mainly in the peptide progenitor regions. The sequences providing spacing between peptide progenitors vary in length between species: from being just the dibasic cleavage sites and amidation signals (*Urechis*), to substantial spacer sequences (several of the insects). In cockroaches, there is a mix between the two spacer lengths. The genomic organization of the TKRP precursor gene, *Dtk*, in *Drosophila* reveals three exons separated by introns.[29] The DTK-1 to DTK-4 peptides are located on exon 2 and DTK-5 and 6 on exon 3. No evidence for alternative splicing of the precursor mRNA is available.

The precursor of the invTK of *Aedes* is small (96 amino acids, 406-bp cDNA) and codes for a single peptide, Sialokinin. The Sialokinin gene consists of three exons. Also the *Octopus* invTK precursor, OctTK-I is small (87 residues, 489 bp cDNA) and predicts one peptide.[14,27] The *Caenorhabditis elegans* gene *nlp-8* predicts three peptides, one of which has the C-terminal tetrapeptide identical to that of substance P (Table 1).[17] Because the other two peptides of the precursor are clearly unrelated, it is not probable that *nlp-8* is ancestrally related to genes coding for tachykinins and TKRPs.

DISTRIBUTION OF mRNA AND PEPTIDES

The localization of TKRP precursor transcript and/or peptides is known for several insects and crustaceans. In *Drosophila*, in situ hybridization has revealed a distribution of *Dtk* transcript in interneurons of the CNS and endocrine cells of the intestine. Immunocytochemistry confirms the presence of TKRPs (DTKs) in the same set of neurons. The number of neurons is relatively small in late third instar larvae (~40–50 neurons in the total CNS, of these 30–36 are in the brain) and somewhat higher (~200 neurons in the total CNS, ~150 in brain) in adult *Drosophila*.[35] Immunocytochemistry reveals neuronal arborizations in specific brain neuropils, such as optic lobes, antennal lobes, central body, and pars intercerebralis (area of branches of protocerebral neurosecretory cells). Additionally, a set of 10 large neurosecretory cells are in the brain; their axon terminations are found in neurohemal release sites on the aorta and in the corpora cardiaca.[12]

The principal distribution of TKRP containing neuron processes follows a basic pattern in the insects investigated.[19,20] In all insects studied, the same neuropils as listed for *Drosophila* are supplied by TKRP immunoreactive branches, but there is some variation between species in some other parts of the brain. In honeybees, there is additional expression of the TKRP gene transcript in intrinsic neurons (Kenyon cells) of the mushroom bodies.[31] The mushroom bodies are higher olfactory centers and also important for olfactory learning and memory. Thus, TKRPs may have a role in higher olfactory processing or even in olfactory learning in honeybees.

In the brain of decapod crustaceans, the distribution of TKRP immunoreactive neurons is similar to that of insects: in olfactory interneurons, optic lobe interneurons, and in the central body complex.[19] The neuronal distribution of TKRPs has also been studied in the snails *Helix pomatia* and *Lymnaea stagnalis*.[19] Numerous TKRP containing neurons were found especially in cerebral ganglia, and neuronal processes arborize in every ganglion. There is no report on the distribution of TKRPs in *U. unicinctus*, but mass spectrometry analysis has indicated that the TKRPs are expressed in the CNS and not in the intestine.

The invTK precursor distribution has been mapped by in situ hybridization in *Aedes* and *Octopus*. In the mosquito, the transcript is present only in adult female salivary glands, and in the cephalopod, it is found in a portion of the salivary glands only. The distribution of the *C. elegans* "invTK" encoded by *nlp-8* is seen in sets of identified neurons throughout the animal and processes on the gut.[15]

PEPTIDE PROCESSING

The TKRPs are probably liberated from their precursor proteins by common endoproteolytic enzymes such as prohormone convertases.[11] Enzymatic inactivation is an important

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means of terminating neuropeptide action. Several enzyme activities are known to cleave insect TKRPs and thus render them inactive: dipeptidyl peptidase IV (DPP IV), angiotensin converting enzyme (ACE), and nephrilysin (NEP)[11] (see the Peptide Biosynthesis/Processing section of the book).

RECEPTORS OF TKRPS

In mammals, three types of G protein–coupled receptors (GPCRs) for tachykinins have been identified,[22,23] as has been discussed in the tachykinin chapter of the Brain Peptides section of this book. These are designated NK1-3 and display preferential affinities for substance P, neurokinin A, and neurokinin B, respectively. The hemokinins seem to associate with the NK1 receptor. In invertebrates GPCRs for the TKRPs have been identified in several species of insect, and in the worm *U. unicinctus*.[8,20,27] Two main types of GPCRs with TKRPs as ligands have been distinguished in *Drosophila* among a set of six GPCRs that fall in the class of neurokinin-like receptors.[1,8,24] The first to be identified, designated DTKR, has been fully characterized with endogenous *Drosophila* TKRPs (DTK-1 to 6). GPCRs closely related to DTKR have been identified in most insects. The second type of TKRP receptor, NKD, can be activated by DTK-6 only in *Drosophila*[24] and has an ortholog in *Anopheles* and a few other insects.

The DTKR type of tachykinin receptors display sequence similarities to mammalian tachykinin receptors and the gene organization is similar in terms of location of intron-exon boarders.[27] Thus, the invertebrate and mammalian receptors are probably ancestrally related. The *Stomoxys* receptor STKR has been extensively investigated in different cellular expression systems.[32] It responds to its native ligand, StcTK, of *Stomoxys*, and also to other insect TKRPs. In heterologous expression systems, the STKR can couple both to calcium and cyclic AMP signaling systems. The *Drosophila* receptor DTKR when expressed in HEK-293 cells could be activated equally well with the six native DTKs (DTK-1 to DTK-6), and it coupled both to calcium and cyclic AMP signaling. On prolonged exposure of DTKR to DTKs β-arrestin, translocation is initiated and the receptor internalizes.

Antisera to a portion of DTKR were used for immunocytochemical mapping of the receptor protein in the CNS and intestine of *Drosophila*.[1] The distribution of DTKR immunoreactivity in the CNS matches that of the DTK peptide distribution. High density of DTKR is seen in the antennal lobes, central body, and pars intercerebralis. Peripheral sites of DTKR expression seem to be the intestine and Malpighian tubules. Both the intestine and the tubules are known to respond to TKRPs in several insect species. The protein of the other TKRP receptor, NKD, is distributed in *Drosophila* in a set of neurons distinct from those expressing DTKR and in intestinal endocrine cells.[24]

STRUCTURE ACTIVITY AND ACTIVE CONFORMATION

The insect and *U. unicinctus* TKRPs have been investigated with respect to structure activity relations. At least, the C-terminal hexapeptide is required for full biological activity of insect TKRPs in a few bioassays,[19,27] whereas in cells transfected with the *Stomoxys* STKR a pentapeptide seems sufficient for activation. Several amino acid substitutions are possible in this C-terminus, but in principle, FX_1GX_2Ramide is required. However, a substitution of the G for an A in this sequence changes the bioactivity only slightly. Natural isoforms with an $FX_1\underline{A}X_2$Ramide do exist in *Stomoxys* (Stc-TK) and *Drosophila* (DTK-6) as well as in mollusks (Table 1).[27] The Ramide is very critical for biological activity: substitution of Ramide to Mamide renders the invertebrate TKRPs inactive on invertebrate receptors such as STKR and UTKR (*U. unicinctus*).[27] Conversely, if the Mamide of a mammalian tachykinin is changed to an Ramide, the peptide can activate the invertebrate receptors.

A comparison was made of the aqueous solution conformations of a conformationally restricted bioactive analog of an insect TKRP and an analogous substance P conformer.[16] NMR spectra and molecular dynamics calculations show that the insect and mammalian peptides adopt similar active conformations.

BIOLOGICAL ACTIONS

A range of biological actions of TKRPs has been established in insects, using *in vitro* bioassays: modulation of different types of muscle, weak diuretic activity, neuromodulatory activity, and induction of adipokinetic hormone release.[19,20] TKRPs increase spontaneous muscle contractions in cockroach foregut, *Drosophila* midgut, cockroach hindgut, locust and cockroach oviduct, and the heart (dorsal vessel) of beetles and to modulate contractile properties of hindleg muscles in a locust. A neuromodulatory action occurs on locust dorsal unpaired median neurons. These neurons respond to TKRPs by depolarization, possibly mediated by activation of adenylate cyclase.

In arthropods, TKRPs seem to be generally involved in olfactory processing because insects and crayfishes express this type of peptide in olfactory interneurons. The TKRPs are colocalized with the inhibitory neurotransmitter γ-aminobutyric acid (GABA) in olfactory interneurons (at least in cockroaches and *Drosophila*). Thus, the peptide may act as cotransmitter and/or neuromodulator in GABA signaling in antennal lobes. In *Drosophila*, the receptor DTKR is expressed in the olfactory sensory neurons (OSNs) of the antennae and the DTK-expressing antennal lobe interneurons presynaptically modulate the sensitivity of OSNs to certain odors.[10] The DTKs also act as neuromodulators in the central complex (CX) of the *Drosophila*

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brain. When DTK levels are genetically knocked down in specific neurons innervating the CX locomotor activity patterns are specifically altered.[13]

In crustaceans, the neuromodulatory action of TKRPs has also been established. In the lamina of the crayfish visual system amacrine neurons contain colocalized TKRP and GABA and application of these two substances to retinal photoreceptors produces a dose-dependent hyperpolarization.[20] This action follows two time courses, a short latency hyperpolarization in the order of seconds and another evolving over minutes. The TKRP action involves a reduction of the photoreceptor potential and the light-elicited current and a potentiation of the presynaptic inhibitory action of GABA. The GABA and TRP actions may subserve lateral inhibition or feedback inhibition of the primary visual synapse (over seconds) and possibly light adaptation of photoreceptors (over minutes).

In another crustacean, the crab *Cancer borealis*, a TKRP has been identified in the neurons of the stomatogastric ganglion, STG.[18] Different overlapping subsets of STG neurons form networks that generate rhythms controlling chewing and filtering behaviors of the foregut. An endogenous TKRP localized in one of the neurons innervating the STG triggers a specific pyloric motor pattern. In this neuron, the TRP is colocalized with proctolin and GABA and the three substances act together to elicit the appropriate response.[18]

A set of 10 large neurosecretory cells in the *Drosophila* brain expresses DTK together with short neuropeptide F (sNPF) and ion transport peptide.[12] Targeted knockdown of DTK (or sNPF) in these cells renders the flies more sensitive to desiccation as determined by survival and water retention capacity, suggesting a role of these peptides in water and ion balance.[12] In *Drosophila*, DTKs also regulate the release of insulin-like peptides (ILPs) from insulin producing cells in the brain and Malpighian tubules.[2,30] In both cases, the peptidergic regulation of ILP signaling via the DTKR receptor affects organismal stress responses, and in the brain, ILP transcript levels were affected by DTKR knockdown.[2]

CONCLUDING REMARKS

Tachykinins, invTKs, and TKRPs constitute a large family of multifunctional neuropeptides whose signaling mechanisms seem to be partially conserved through evolution.[21,23,27] Although the vertebrate tachykinins and invertebrate TKRPs and their precursors display only limited sequence identities, their GPCRs display more striking similarities, suggesting ancestral relationships.[27] The invTKs, which display strong similarities to vertebrate tachykinins, seem to have evolved separately in the salivary glands of some invertebrates that use saliva in their interactions with vertebrate prey organisms. Thus, these invTKs may have evolved by mimicry to cause vasodilation or other actions in the prey vertebrates.

In invertebrates, the complete spectrum of functions of TKRPs remains to be determined. With the development of powerful genetic tools, *Drosophila* has emerged as a good model for analysis of peptide function. Findings so far suggest that the TKRPs have multiple functions distributed in different parts of the CNS, intestine, renal tubules, and maybe in other tissues.

REFERENCES

1. Birse RT, Johnson EC, Taghert PH, Nässel DR. Widely distributed *Drosophila* G protein-coupled receptor (CG7887) is activated by endogenous tachykinin-related peptides. *J Neurobiol* 2006;**66**:33–46.
2. Birse RT, Söderberg JA, Luo J, Winther ÅM, Nässel DR. Regulation of insulin producing cells in the adult *Drosophila* brain via the tachykinin peptide receptor DTKR. *J Exp Biol* 2011;**214**:4201–8.
3. Christie AE. Neuropeptide discovery in Ixodoidea: an in silico investigation using publicly accessible expressed sequence tags. *Gen Comp Endocrinol* 2008;**157**:174–85.
4 Christie AE, Nolan DH, Garcia ZA, McCoole MD, Harmon SM, Congdon-Jones B, et al. Bioinformatic prediction of arthropod/nematode-like peptides in non-arthropod, non-nematode members of the Ecdysozoa. *Gen Comp Endocrinol* 2011;**170**:480–6.
5 Dircksen H, Neupert S, Predel R, Verleyen P, Huybrechts J, Strauss J, et al. Genomics, transcriptomics, and peptidomics of *Daphnia pulex* neuropeptides and protein hormones. *J Proteome Res* 2011;**10**: 4478–504.
6. Erspamer V, Anastasi A. Structure and pharmacological actions of eledoisin, the active endecapeptide of the posterior salivary glands of *Eledone*. *Experientia* 1962;**18**:58–9.
7 Hauser F, Neupert S, Williamson M, Predel R, Tanaka Y, Grimmelikhuijzen CJ. Genomics and peptidomics of neuropeptides and protein hormones present in the parasitic wasp *Nasonia vitripennis*. *J Proteome Res* 2010;**9**:5296–310.
8 Hewes RS, Taghert PH. Neuropeptides and neuropeptide receptors in the *Drosophila* melanogaster genome. *Genome Res* 2001;**11**:1126–42.
9 Holman GM, Nachman RJ, Wright MS. Comparative aspects of insect myotropic peptides. *Prog Clin Biol Res* 1990;**342**:35–9.
10. Ignell R, Root CM, Birse RT, Wang JW, Nässel DR, Winther ÅM. Presynaptic peptidergic modulation of olfactory receptor neurons in *Drosophila*. *Proc Natl Acad Sci U S A* 2009;**106**:13070–5.
11 Isaac RE, Bland ND, Shirras AD. Neuropeptidases and the metabolic inactivation of insect neuropeptides. *Gen Comp Endocrinol* 2009;**162**: 8–17.
12. Kahsai L, Kapan N, Dircksen H, Winther AM, Nässel DR. Metabolic stress responses in *Drosophila* are modulated by brain neurosecretory cells that produce multiple neuropeptides. *PLoS ONE* 2010;**5**:e11480.
13. Kahsai L, Martin JR, Winther AM. Neuropeptides in the *Drosophila* central complex in modulation of locomotor behavior. *J Exp Biol* 2010;**213**:2256–65.
14. Kanda A, Iwakoshi-Ukena E, Takuwa-Kuroda K, Minakata H. Isolation and characterization of novel tachykinins from the posterior salivary gland of the common octopus *Octopus vulgaris*. *Peptides* 2003;**24**:35–43.
15. Keating CD, Kriek N, Daniels M, Ashcroft NR, Hopper NA, Siney EJ, et al. Whole-genome analysis of 60 G protein-coupled receptors in *Caenorhabditis elegans* by gene knockout with RNAi. *Curr Biol* 2003;**13**:1715–20.

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16. Nachman RJ, Moyna G, Williams HJ, Zabrocki J, Zadina JE, Coast GM, et al. Comparison of active conformations of the insectatachykinin/tachykinin and insect kinin/Tyr-W-MIF-1 neuropeptide family pairs. *Ann N Y Acad Sci* 1999;**897**:388–400.
17 Nathoo AN, Moeller RA, Westlund BA, Hart AC. Identification of neuropeptide-like protein gene families in *Caenorhabditis elegans* and other species. *Proc Natl Acad Sci U S A* 2001;**98**:14000–5.
18. Nusbaum MP, Blitz DM, Swensen AM, Wood D, Marder E. The roles of co-transmission in neural network modulation. *Trends Neurosci* 2001;**24**:146–54.
19. Nässel DR. Tachykinin-related peptides in invertebrates: a review. *Peptides* 1999;**20**:141–58.
20 Nässel DR. Neuropeptides in the nervous system of *Drosophila* and other insects: multiple roles as neuromodulators and neurohormones. *Prog Neurobiol* 2002;**68**:1–84.
21. Nässel DR, Winther AM. *Drosophila* neuropeptides in regulation of physiology and behavior. *Prog Neurobiol* 2010;**92**:42–104.
22. Otsuka M, Yoshioka K. Neurotransmitter functions of mammalian tachykinins. *Physiol Rev* 1993;**73**:229–308.
23. Pennefather JN, Lecci A, Candenas ML, Patak E, Pinto FM, Maggi CA. Tachykinins and tachykinin receptors: a growing family. *Life Sci* 2004;**74**:1445–63.
24. Poels J, Birse RT, Nachman RJ, Fichna J, Janecka A, Vanden Broeck J, et al. Characterization and distribution of NKD, a receptor for *Drosophila* tachykinin-related peptide 6. *Peptides* 2009;**30**:545–56.
25. Predel R, Neupert S, Roth S, Derst C, Nässel DR. Tachykinin-related peptide precursors in two cockroach species. *FEBS J* 2005;**272**:-3365–75.
26. Roller L, Yamanaka N, Watanabe K, Daubnerova I, Zitnan D, Kataoka H, et al. The unique evolution of neuropeptide genes in the silkworm *Bombyx mori*. *Insect Biochem Mol Biol* 2008;**38**:1147–57.
27. Satake H, Kawada T, Nomoto K, Minakata H. Insight into tachykinin-related peptides, their receptors, and invertebrate tachykinins: a review. *Zool Sci* 2003;**20**:533–49.
28. Schoofs L, Holman GM, Hayes TK, Nachman RJ, De Loof A. Locustatachykinin I and II, two novel insect neuropeptides with homology to peptides of the vertebrate tachykinin family. *FEBS Lett* 1990;**261**:397–401.
29. Siviter RJ, Coast GM, Winther ÅM, Nachman RJ, Taylor CA, Shirras AD, et al. Expression and functional characterization of a *Drosophila* neuropeptide precursor with homology to mammalian preprotachykinin A. *J Biol Chem* 2000;**275**:23273–80.
30. Söderberg JA, Birse RT, Nässel DR. Insulin production and signaling in renal tubules of *Drosophila* is under control of tachykinin-related peptide and regulates stress resistance. *PLoS ONE* 2011;**6**:e19866.
31. Takeuchi H, Yasuda A, Yasuda-Kamatani Y, Sawata M, Matsuo Y, Kato A, et al. Prepro-tachykinin gene expression in the brain of the honeybee *Apis mellifera*. *Cell Tissue Res* 2004;**316**:281–93.
32. Van Loy T, Vandersmissen HP, Poels J, Van Hiel MB, Verlinden H, Vanden Broeck J. Tachykinin-related peptides and their receptors in invertebrates: a current view. *Peptides* 2010;**31**:520–4.
33. Veenstra JA. Neurohormones and neuropeptides encoded by the genome of *Lottia gigantea*, with reference to other mollusks and insects. *Gen Comp Endocrinol* 2010;**167**:86–103.
34. Veenstra JA. Neuropeptide evolution: neurohormones and neuropeptides predicted from the genomes of *Capitella teleta* and *Helobdella robusta*. *Gen Comp Endocrinol* 2011;**171**:160–75.
35. Winther ÅM, Siviter RJ, Isaac RE, Predel R, Nässel DR. Neuronal expression of tachykinin-related peptides and gene transcript during postembryonic development of *Drosophila*. *J Comp Neurol* 2003;**464**:180–96.

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Chapter 45

Bradykinin Peptides

J. Michael Conlon

ABSTRACT

Bradykinin (BK)-related peptides are synthesized sporadically in the skin of certain frog species belonging to the families Bombinatoridae, Heleophryninae, Hylidae, Leiopelmatidae, and Ranidae. BK, identical in structure to the mammalian peptide, larger molecular forms of BK extended from either the N- or C-terminus by additional amino acids, and molecular variants of BK containing amino acid substitutions have been identified. The frog skin BK peptides are not generated by the activation of the kallikrein–kinin system, as in mammals, but by the action of processing enzymes cleaving at the site of single arginyl residues in precursors that generally contain multiple tandem copies of a BK containing domain. The frog skin BK-related peptides show a wide range of potencies in eliciting contractile or relaxant responses in mammalian smooth muscle preparations and peptides derived from probradykinins with weak antagonistic and BK-potentiating activities have also been identified.

DISCOVERY AND DISTRIBUTION

The skin of amphibians has proved to be a remarkably rich storehouse of myotropic peptides, including kinins. A BK-like ability to elicit a hypotensive response and to contract rat uterine and gastrointestinal smooth muscle has been found in the skin extracts of a wide range of frogs from Africa,[25] North and South America,[13] Australia, Papua New Guinea,[14] and Europe[26] by Erspamer and coworkers. In particular, the skin of certain frogs from the genus *Rana* are associated with very high concentrations of such BK-like bioactivity, and the skin of the European common frog *Rana temporaria* contains a peptide whose amino acid sequence is identical to that of mammalian BK.[2] The occurrence of BK and related peptides among the different families of frogs, and even among species belonging to the same genus, is sporadic. For example, the skins of the European frog *R. temporaria* and the African frog *R. galamensis* contain BK equivalent to >150 μg/g tissue, but BK was undetectable in the skins of *R. dalmatina, R. graeca and R. latastei*.[25,26] Similarly, among the North American ranids, the skin secretions *R. muscosa* contain high concentrations of BK although the peptide is undetectable in the secretions from those of its sister taxa *R. aurora* and *R. draytonii*.[24] The observation that high concentrations of BK are present in the skin of the Japanese brown frog *R. tagoi okiensis* but not *Rana tagoi tagoi* provides evidence to support the proposal that these frogs should be regarded as separate species rather than conspecific subspecies.[11,29]

As well as from frogs belonging to the family Ranidae, BK and/or BK-related peptides have been isolated from the species belonging to the families, Bombinatatoridae,[6–8,15–17] Heleophryninae,[23] Hylidae,[1,30] and Leiopelmatidae.[12] The tailed frog *Ascaphus truei* (Leiopelmatidae) occupies a unique position in phylogeny as the most primitive extant anuran and is regarded as the sister taxon to the clade of all other living frogs. Consequently, the identification of BK in skin secretions from this species demonstrates that dermal BK production arose early in anuran evolution.

BIOSYNTHESIS OF BRADYKININ AND RELATED PEPTIDES

The pathways of biosynthesis of BK-related peptides in frog skin are quite different from those in the blood and tissues of mammals. The kallikrein–kinin system in mammals involves the sequential action of several well-characterized proteolytic enzymes and is illustrated schematically in Fig. 1 (see also bradykinin chapters in the Cardiovascular Peptides section of this volume). The activation of Factor XII (Hageman factor) in blood results in the activation of plasma prekallikrein and subsequent generation of BK by the cleavage of high molecular mass kininogen. This protein contains only a single copy of the BK sequence. Alternative splicing of the primary transcript of the kininogen gene gives rise to a second mRNA that directs the synthesis of low molecular mass kininogen. This protein is a substrate for glandular or tissue kallikrein, a serine protease that is localized predominantly in kidney, pancreas, and pituitary, generating either [Lys^0]-BK in the human or [Arg^0]-BK in the rat. BK-related peptides have been generated in the

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00045-2
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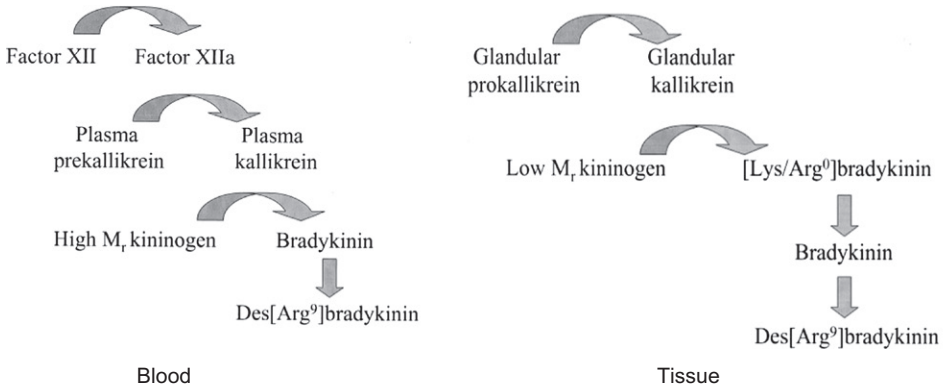

FIGURE 1 Generation of BK in the plasma and tissues of mammals by the activation of the kallikrein–kinin system.

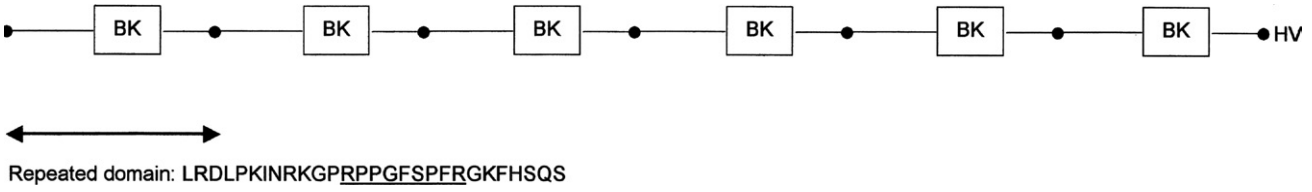

FIGURE 2 A schematic representation of the biosynthetic precursor of BK deduced from the nucleotide sequence of a cloned cDNA from the skin of *Bombina maxima.* The probradykinin contains six identical repeated copies of the domain shown. The BK sequence is underlined.

plasma of several species of reptile and fish by incubation with porcine pancreatic kallikrein and/or trypsin but similar attempts to generate BK in the plasma of a range of frog species have been unsuccessful.[9]

The primary structure of the biosynthetic precursor of a frog skin BK was first determined for the Chinese red-bellied toad *Bombina maxima*.[15] As shown schematically in Fig. 2, the probradykinin comprises six copies of the 28 amino acid residue repeated domain LRDLPKINRKGPRPPGFSPFRGKFHSQS. Thus, the generation of BK (sequence underlined) involves cleavage at the site of single arginyl residues by an as yet uncharacterized processing enzyme(s) rather than by the action of the kallikrein–kinin system as in mammals. As well as BK, the posttranslational processing of *B. maxima* probradykinin generates the N-terminally extended peptide, bombinakinin M (DLPKINRKGPRPPGFSPFR). It has been further shown that several preprobradykinin genes are expressed in the skin of *B. maxima*[6,16,17] and a cDNA encoding a bombinakinin M variant (DLSKMSFLHGIPPGFSPFR) that does not contain the full sequence of BK has been identified. It has been suggested that environmental factors may influence which particular bombinakinin M gene is expressed.[17] Subsequent work has shown that structural organization of probradykinin in the skins of the piebald odorous frog *Odorrana schmakeri*[18] the Yunnanfu frog *Odorrana grahami*,[5] the pickerel frog *Lithobates palustris*,[21] and the fire-bellied toad *Bombina orientalis*[8] is similar to that of the *B. maxima* precursor, comprising multiple BK-containing tandem repeats.

Amplification of a preprobradykinin cDNA encoding two copies of the analog [Arg^0,Leu^1,Thr^6,Trp^8] BK from a total RNA preparation of the skin of *Rana sakuraii* was accomplished by a 3′-RACE procedure with a primer that was designed from conserved regions of the 5′-UTR of *R. temporaria* preprotemporins.[28] The temporins are antimicrobial peptides widely distributed in the skins of ranid frogs (see the chapter on this peptide in this section of the book). The signal peptide region of the preprobradykinin is identical to that of preprotemporin-1SKa from *R. sakuraii* except for the substitution Ser^{18} → Asn (Fig. 3). At this time, there is no evidence to suggest that the genes encoding the BK-related and temporin-related peptides are derived from a common ancestral gene but the nucleotide sequence similarity in the 5′-UTR and the signal peptide regions suggests the possibility of a linked evolutionary history.

MOLECULAR VARIANTS OF BK

The different pathways of posttranslational processing of probradykinin in frog skin give rise to peptides in which the BK sequence is extended from either the N- or the C-terminus by additional amino acid residues. As shown in Fig. 4, a peptide comprising BK extended from its N-terminus by -Gly-Val-Ile-Pro-Leu-Leu and three peptides comprising BK extended from its C-terminus by -Ile-Ala, -Ile-Ala-Pro-Ala-Ser-Thr-Leu, and -Ile-Ala-Pro-Ala-Ser-Ile-Leu were purified from *R. temporaria*.[10] Related studies in other species have led to the isolation of BK-related peptides

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Preprotemporin-1SKa   MFTLKKSLLLLFFLGTINLSLCEEERNA*EEERRDDPEERDVEVEKR

PreproBK              MFTLKKSLLLLFFLGTISLSLCEQERDADEDEYAGEAKAEDVKRAGY

                      FLPVILPVIGKLLNGILGK

                      SRMIRLPPGFTPWRIAPASTLKRDEDEYTGEAKAEDVKRAGYSRMIRLPPGFTPWRIAPAIV
```

FIGURE 3 A comparison of the deduced amino acid sequences of the biosynthetic precursors of [Leu[1],Thr[6],Trp[8]] BK and temporin-1SKa from *R. sakuraii*. Conserved residues are shaded and the mature sequences are underlined.

```
Ascaphus truei                  RPPGFSPFRVD

Rana temporaria           LLPIVGRPPGFSPFR
                                RPPGFSPFRIA
                                RPPGFSPFRIAPASTL
                                RPPGFSPFRIAPASIL

Pelophylax nigromaculatus       RPPGFSPFRVAPAS

Phyllomedusa rohdei             RPPGFSPFRIY(SO3H)

Bombina orientalis              RPPGFSPFRGKFH

Bombina maxima         DLPKINRKGPRPPGFSPFR
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FIGURE 4 The primary structures of frog skin BK-related peptides that contain the full sequence of BK (residues underlined) extended from either the N- or the C-terminus by additional amino acid residues.

extended from the C-terminus by -Ile-Tyr(SO_3) (phyllokinin from *Phyllomedusa rohdei*),[1] by -Gly-Lys-Phe-His (bombinakinin O from *B. orientalis*),[32] by -Val-Ala-Pro-Ala-Ser (ranakinin N from *Pelophylax nigromaculatus*),[31] and by Val-Asp (peptide RD-11 from *A. truei*).[12] Metabolites of BK, identified as the (1–7) and the (1–8) fragments, have been isolated in high yield from the skins of *R. temporaria* and *R. esculenta* and [hydroxyprolyl[3]]-BK has been purified from *R. temporaria* and from the South African frog *Heleophryne purcelli* (Heleophryninae).[9]

In general, the primary structure of BK has been poorly conserved in vertebrates with only two amino acid residues (Gly[4] and Arg[9]) remaining invariant during the evolution of the peptide.[9] As shown in Fig. 5, BK-related peptides (and their N- and C-terminally extended forms) whose amino acid sequences are not the same as mammalian BK include [Thr[6]]-BK from *Glandirana rugosa*,[33] *R. tagoi okiensis*,[29] and *B. orientalis*;[32] [Val[1],Thr[6]]-BK from *P. nigromaculatus*,[22] *Hylarana nigrovittata*,[20] *Amolops loloensis*,[19] and *Phyllomedusa hypochondrialis*;[30] [Thr[6],Leu[8]]BK from *L. palustris*;[3] [Leu[5],Thr[6]]BK from *O. grahami*;[5] [Ala[3],Thr[6]]-BK and [Val[1],Thr[3],Thr[6]]-BK from *B. variegata*;[7] [Leu[1],Thr[6],Trp[8]] BK from *R. sakuraii*;[28] and [Pro[1],Val[2],Leu[5]]-BK from *A. truei*[11] (Fig. 5). [Arg[0],Trp[5],Leu[8]]BK, a peptide that is generated in the plasma of several teleost fish by activation of the kallikrein–kinin system,[9] together with [Thr[6],Leu[8]]BK was identified in the skin of *P. lessonae*.[27] Among the frog skin BK-related peptides only Gly[4], Pro[7], and Arg[9] are invariant.

BIOLOGICAL ACTIVITIES

The myotropic activities of several of the naturally occurring larger molecular forms of BK have been investigated. Phyllokinin [BK-Ile-Tyr(SO_3H)] was two to three times more potent and longer acting than BK in producing a hypotensive response in the anesthetized dog, but the peptide was less potent than BK in contracting a range of extravascular smooth muscle preparations. Removal of the sulfate group from phyllokinin resulted in an appreciable loss in potency.[1] By contrast, removal of the C-terminal extension to the BK sequence in ranakinin N with trypsin results in a 20-fold increase in potency in smooth muscle bioassays.[31] *Rana sakuraii* BK ([Arg[0],Leu[1],Thr[6],Trp[8]] BK) is 28-fold less potent than mammalian BK in effecting B2 receptor-mediated relaxation of mouse trachea.[28] BK-Val-Asp from *A. truei* is a full agonist at the B2 receptor in mouse trachea but the BK-related peptide AR-10 (APVPGLSPFR) and its C-terminally extended form AV-12 (APVPGLSPFRVV) function as low potency, partial agonists.[12]

Bombinakinin M from *B. maxima* is about fourfold less potent than BK in eliciting contraction of the guinea pig ileum. By contrast, the bombinakinin M variant containing the substitution Arg[1] → Ile in the BK domain is itself inactive in this preparation but potentiates the action of BK up to two-fold.[17] The predicted amino acid sequence of one of the multiple biosynthetic precursors of bombinakinin M contains at its extreme C-terminus the domain Arg-Gln-Ile-Pro-Gly-Leu-Gly-Pro-Arg-Gly. A peptide derived from this domain (pGlu-Ile-Pro-Gly-Leu-Gly-Pro-Arg.NH_2) has been termed kinestatin and acts as a weak antagonist at the B2 receptor in the rat tail artery.[6] Similarly, the peptide Asp-Tyr-Thr-Ile-Arg-Thr-Arg-Leu-His.NH_2 encoded by a different *B. maxima* probradykinin gene also weakly inhibits the contractile activity of bombinakinin M at the guinea pig ileum.[16] As well as the bombinakinin M precursor that contains six tandem repeats,[15] cDNA cloning has identified a larger precursor that contains eight identical copies of the bombinakinin M sequence plus one copy of a 28 amino acid residue peptide, termed bombinakinin M gene associated peptide.[16]

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Glandirana rugosa                RPPGFTPFR
                                 RPPGFTPFRIAPEIV

Bombina orientalis               RPPGFTPFR
Pelophylax nigromaculatus        VPPGFTPFR
Hylarana nigrovittata         RAEAVPPGFTPFR
Amolops loloensis              RAPVPPGFTPFR
Phyllomedusa hypocondrialis      VPPGFTPFRLT
                                 VPPGFTPFRQS
Bombina variegata                VPTGFTPFR
                                 RPAGFTPFR

Odorrana grahami                 RPPGLTPFR

Pelophylax lessonae              RPPGFTPLR
                               RRPPGWSPLR

Lithobates palustris             IRRPPGFTPLR
                            AGIRRPPGFTPLRIA

Rana sakuraii                    RLPPGFTPWR

Ascaphus truei                   APVPGLSPFR
                                 APVPGLSPFRVV
```

FIGURE 5 Molecular variants of BK isolated from skin extracts or skin secretions of frogs. Those amino acid residues in the BK domain that differ from mammalian BK are shaded.

This component shows limited structural similarity to mammalian cocaine- and amphetamine-regulated transcript (CART) and intracerebroventricular administration of the peptide induces a significant decrease in food intake in rats.

The biological function of myotropic peptides, such as BK, in the skin of amphibia is unclear, but it is reasonable to speculate that they may represent an important component of the organism's survival strategy, protecting the animal against ingestion by predators. Release of large amounts of BK, or a related peptide, into the lumen of the stomach of a predator will stimulate gastric and esophageal motility, which may lead to activation of the vomiting reflex and ejection of the prey unharmed.

REFERENCES

1. Anastasi A, Bertaccini G, Erspamer V. Pharmacological data on phyllokinin (bradykinyl-isoleucyl-tyrosine O-sulphate) and bradykinyl-isoleucyl-tyrosine. *Br J Pharmacol* 1966;**27**:479–85.
2. Anastasi A, Erspamer V, Bertaccini G. Occurrence of bradykinin in the skin of *Rana temporaria*. *Comp Biochem Physiol* 1965;**14**:43–52.
3. Basir YJ, Knoop FC, Dulka J, Conlon JM. Multiple antimicrobial peptides and peptides related to bradykinin and neuromedin N isolated from the skin secretions of the North American pickerel frog, *Rana palustris*. Biochim Biophys Acta 2000;**1543**:95–105
5. Chen M, Che Q, Wang X, Li J, Yang H, Li D, et al. Cloning and characterization of the first amphibian bradykinin gene. *Biochimie* 2010;**92**:226–31.
6. Chen T, O'Rourke M, Orr DF, Coulter DJ, Hirst DG, Rao P, et al. Kinestatin: a novel bradykinin B2 receptor antagonist peptide from the skin secretion of the Chinese toad, *Bombina maxima*. *Regul Pept* 2003;**116**:147–54.
7. Chen T, Orr DF, Bjourson AJ, McClean S, O'Rourke M, Hirst DG, et al. Novel bradykinins and their precursor cDNAs from European yellow-bellied toad (*Bombina variegata*) skin. *Eur J Biochem* 2002;**269**:4693–700.
8. Chen T, Orr DF, Bjourson AJ, McClean S, O'Rourke M, Hirst DG, et al. Bradykinins and their precursor cDNAs from the skin of the fire-bellied toad (*Bombina orientalis*). *Peptides* 2002;**23**:1547–55.
9. Conlon JM. Bradykinin and its receptors in non-mammalian vertebrates. *Regul Pept* 1999;**79**:71–81.
10. Conlon JM, Aronsson U. Multiple bradykinin-related peptides in the skin of the frog, *Rana temporaria*. *Peptides* 1997;**18**:361–5.
11. Conlon JM, Coquet L, Jouenne T, Leprince J, Vaudry H, Iwamuro S. Evidence from the primary structures of dermal antimicrobial peptides that *Rana tagoi okiensis* and *Rana tagoi tagoi* (Ranidae) are not conspecific subspecies. *Toxicon* 2010;**55**:430–5.
12. Conlon JM, Jouenne T, Cosette P, Cosquer D, Vaudry H, Taylor CK, et al. Bradykinin-related peptides and tryptophyllins in the skin secretions of the most primitive extant frog, *Ascaphus truei*. *Gen Comp Endocrinol* 2005;**143**:193–9.
13. Erspamer V, Falconieri Erspamer G, Cei JM. Active peptides in the skins of two hundred and thirty American amphibian species. *Comp Biochem Physiol* 1986;**85C**:125–37.
14. Erspamer V, Falconieri Erspamer G, Mazzanti G, Endean R. Active peptides in the skins of one hundred amphibian species from Australia and Papua New Guinea. *Comp Biochem Physiol* 1984;**77C**:99–108.

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15. Lai R, Liu H, Hui Lee W, Zhang Y. A novel bradykinin-related peptide from skin secretions of toad *Bombina maxima* and its precursor containing six identical copies of the final product. *Biochem Biophys Res Commun* 2001;**286**:259–63.
16. Lai R, Liu H, Lee WH, Zhang Y. Bombinakinin M gene associated peptide, a novel bioactive peptide from skin secretions of the toad *Bombina maxima*. *Peptides* 2003;**24**:199–204.
17. Lee WH, Liu SB, Shen JH, Jin Y, Zhang Y. Cloning of bradykinin precursor cDNAs from skin of *Bombina maxima* reveals novel bombinakinin M antagonists and a bradykinin potential peptide. *Regul Pept* 2005;**127**:207–15.
18. Li L, Bjourson AJ, He J, Cai G, Rao P, Shaw C. Bradykinins and their cDNA from piebald odorous frog, *Odorrana schmackeri*, skin. *Peptides* 2003;**24**:863–72.
19. Liang J, Han Y, Li J, Xu X, Rees HH, Lai R. A novel bradykinin-like peptide from skin secretions of rufous-spotted torrent frog, *Amolops loloensis*. *Peptides* 2006;**27**:2683–7.
20. Liu X, You D, Chen L, Wang X, Zhang K, Lai R. A novel bradykinin-like peptide from skin secretions of the frog, *Rana nigrovittata*. *J Pept Sci* 2008;**14**:626–30.
21. McCrudden CM, Zhou M, Chen T, O'Rourke M, Walker B, Hirst D, et al. The complex array of bradykinin-related peptides (BRPs) in the peptidome of pickerel frog (*Rana palustris*) skin secretion is the product of transcriptional economy. *Peptides* 2007;**28**:1275–81.
22. Nakajima T. New vasoactive peptides of nonmammalian origin. In: Pisano JJ, Austen KF, editors. *The chemistry and biology of the kallikrein–kinin system in health and disease*. Bethesda: Fogarty International Center Proceedings; 1974. p. 165–8.
23. Nakajima T, Yasuhara T, Falconieri Erspamer G, Visser J. Occurrence of Hyp^{3}-bradykinin in methanol extracts of the skin of the South African leptodactylid frog *Heleophryne purcelli*. *Experientia* 1979;**35**:1133–4.
24. Rollins-Smith LA, Woodhams DC, Reinert LK, Vredenburg VT, Briggs CJ, Nielsen PF, et al. Antimicrobial peptide defenses of the mountain yellow-legged frog (*Rana muscosa*). *Dev Comp Immunol* 2006;**30**:831–42.
25. Roseghini R, Falconieri Erspamer G, Severini C. Biogenic amines and active peptides in the skin of fifty-two African amphibian species other than Bufonids. *Comp Biochem Physiol* 1988;**91C**:281–6.
26. Roseghini R, Falconieri Erspamer G, Severini C, Simmaco M. Biogenic amines and active peptides in extracts of the skin of thirty-two European amphibian species. *Comp Biochem Physiol* 1989;**94C**: 455–60.
27. Samgina TY, Gorshkov VA, Vorontsov YA, Artemenko KA, Zubarev RA, Lebedev AT. Mass spectrometric study of bradykinin-related peptides (BRPs) from the skin secretion of Russian ranid frogs. *Rapid Commun Mass Spectrom* 2011;**15**(25):933–40.
28. Suzuki H, Iwamuro S, Ohnuma A, Coquet L, Leprince J, Jouenne T, et al. Expression of genes encoding antimicrobial and bradykinin-related peptides in skin of the stream brown frog *Rana sakuraii*. *Peptides* 2007;**28**:505–14.
29. Tazato S, Conlon JM, Iwamuro S. Cloning and expression of genes enocoding antimicrobial peptides and bradykinin from the skin and brain of Oki Tago's brown frog, *Rana tagoi okiensis*. *Peptides* 2010;**31**:1480–7.
30. Thompson AH, Bjourson AJ, Shaw C, McClean S. Bradykinin-related peptides from *Phyllomedusa hypochondrialis azurea*: mass spectrometric structural characterisation and cloning of precursor cDNAs. *Rapid Commun Mass Spectrom* 2006;**20**:3780–8.
31. Yanaihara N, Yanaihara C, Sakagami M, Nakajima T, Nakayama T. Synthesis of bradykininyl-Val-Ala-Pro-Ala-Ser-OH and its biological properties. *Chem Pharm Bull (Tokyo)* 1973;**21**:616–21.
32. Yashuhara T, Hira M, Nakajima T, Yanaihara N, Yanaihara C. Active peptides on smooth muscle in the skin of *Bombina orientalis* Boulenger and characterization of a new bradykinin analog. *Chem Pharm Bull (Tokyo)* 1973;**21**:1388–91.
33. Yashuhara T, Ishikawa O, Nakajima T, Araki K, Tachibana S. The studies on the active peptide on smooth muscle in the skin of *Rana rugosa*, Thr^{6}-bradykinin and its analogous peptide, ranakinin R. *Chem Pharm Bull (Tokyo)* 1979;**27**:486–91.

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Chapter 46

Bombesin Peptides

Eliot R. Spindel

ABSTRACT

The bombesin-like peptides were originally isolated from frog skin, but the discovery of extensive effects in mammals led to the search for and discovery of mammalian homologs. In frogs, four groupings of bombesin-like peptides have been described, the bombesins, the ranatensins, and the phyllolitorins, which are present in skin and gastrin-releasing peptide (GRP) which is not present in skin. In mammals, two bombesin-like peptides have been described, GRP and neuromedin B (NMB). Whether mammals have additional bombesin-like peptides related to the frog skin bombesin-like peptides remains to be determined. The receptors for amphibian bombesin-like peptides are 7-transmembrane G protein-coupled receptors linked to the generation of IP3. The bombesin-like peptides in frog skin are located at very high concentrations in myoepithelial glands, and secretion can be triggered by norepinephrine. Despite investigation, the exact role of bombesin-like peptides in frog skin remains unknown.

INTRODUCTION

The bombesin-like peptides are one of the classic examples of peptides first discovered in frog skin and then later found to be widely distributed in mammals. The prototypical bombesin-like peptide, bombesin, was isolated from the skin of the frog *Bombina bombina* by Anastasi et al.[2] in 1971. The bombesin-related peptides ranatensin and Leu-8 phyllolitorin were subsequently isolated from the skin of *Rana* and *Phyllomedusa* species by Nakajima et al.[22] and by Yasuhara et al.[29] As discussed below, different genera of frogs contain different, characteristic, bombesin-like peptides. In frog skin the bombesin-like peptides are located in cutaneous granular glands (Fig. 1). These are myoepithelial glands under adrenergic regulation such that subcutaneous injection of norepinephrine causes massive secretion of the contents of the glands.[1,3,5] Typically, the levels of peptides found in frog skin are 100- to 1000-fold higher than the levels in mammalian tissues. Peptides are probably secreted from frog skin as a defensive function or potentially as a means of communication. The exact function of bombesin-like peptides in frog skin remains unknown.

The mammalian bombesin-like peptides are gastrin-releasing peptide (GRP) and neuromedin B (NMB). Phylogenetic analysis of the prohormones encoding the bombesin-like peptides suggests that there are three distinct branches of bombesin-like peptides, the GRP branch, the NMB branch, and a branch that contains the multiple bombesin-like peptides found in amphibian skin. This raises two interesting questions: "Do frogs have bombesin-like peptides that fall in the NMB phylogenetic branch?" and "Do mammals have bombesin-like peptides that fall in the skin peptide branch?" To some extent, the grouping of the skin-derived amphibian bombesin-like peptides into different families is primarily historic because it is becoming increasingly clear that many amphibians have multiple bombesin-like peptides of differing structures in their skin[9,19] and that all the skin bombesin prohormones show close evolutionary relationships as discussed further below.

With the advent of more sophisticated mass spectrometry techniques and transcriptome sequencing by "Next Gen" sequencing techniques, the numbers of known bombesin-like peptides from amphibian species will probably increase dramatically in the coming years.

AMPHIBIAN BOMBESINS

Bombesin was originally isolated from the skin of the fire-bellied toad, *B. bombina* by Anastasi, Erspamer, and coworkers.[2] Bombesin is a tetradecapeptide with the structure shown in Fig. 2. Bombesin is also found in the skin of other *Bombina* species including *Bombina variegata* and *Bombina orientalis.* The original form of bombesin as isolated by Anastasi et al.[2] is characterized by a Leu as its penultimate residue. This penultimate residue was initially believed to differentiate the bombesin-like peptides isolated from *Bombina* species from the bombesin-like peptides isolated from *Rana* species that contain a Phe residue as the penultimate residue. This is not the case, however, as many frog species contain multiple forms of bombesin-like peptides in their skin with both Leu and Phe as penultimate residues. This is illustrated by the presence

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00046-4
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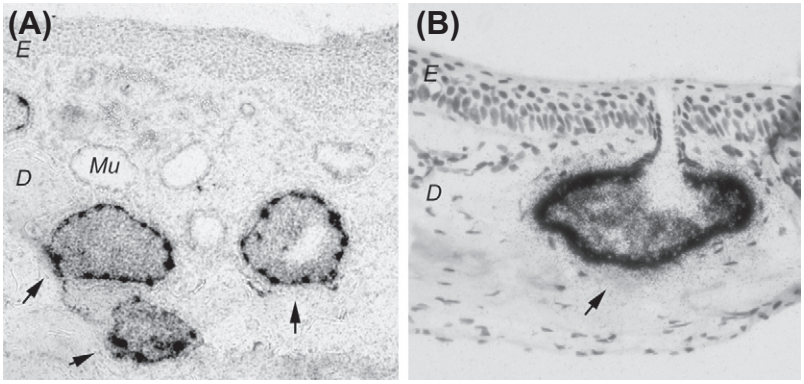

FIGURE 1 Bombesin-like peptide mRNA expression in frog skin. A. Low power view showing ranatensin mRNA expression in cutaneous granular glands in the skin of *Rana pipiens* (100×). B. Higher power view showing bombesin expression in the skin of *Bombina orientalis* (200×). E = epidermis, D = Dermis, Mu = mucus gland, arrows point to peptide-containing glands.

(A) Bombesins, alytesin and gastrin-releasing peptide (GRP)

Leu-13 bombesin (*B. orientalis*, 1)	pGlu-Gln-Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met.NH2
Phe-13 bombesin (*B. orientalis*)	pGlu-Gln-Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Phe-Met.NH2
Phe bombesin (*R. ridibunda*, 2)	Asn-Leu-Gly-Lys-Gln-Trp-Ala-Val-Gly-His-Phe-Met.NH2
SAP bombesin (*B. orientalis*)	pGlu-Gln-Ser-Leu-Gly-Asn-Gln-Trp-Ala-Arg-Gly-His-Phe-Met.NH2
Bombesin B1 (*O. grahami*, 3)	pGlu-Arg-Glu-Tyr-Arg-Ala-Pro-His-Trp-Ala-Ile-Gly-His-Phe-Met.NH2
Bombesin B4 (*O. grahami*, 3)	pGlu-Asn-Thr-Tyr-Arg-Ala-Pro-Gln-Trp-Ala-Val-Gly-His-Leu-Met.NH2
Bombesin-S (*S. varians*, 4)	pGlu-Met-Ile-Phe-Gly-Ala-Pro-Met-Trp-Ala-Leu-Gly-His-Leu-Met.NH2
Bombesin-R (*R. shuchinae*, 5)	pGlu-Thr-Ser-Phe-Met-Ala-Pro-Ser-Trp-Ala-Leu-Gly-His-Leu-Met.NH2
Proline-rich bombesin (*B. maxima*, 6)	Pro-Pro-Arg-Pro-Pro-Gln-Trp-Ala-Arg-Gly-His-Phe-Met.NH2
Alytesin (*Alytes obstetricans*)	pGlu-Gly-Arg-Leu-Gly-Thr-Gln-Trp-Ala-Val-Gly-His-Leu-Met.NH2
Frog GRP-10 (*B. orientalis*)	Gly-Ser-His-Trp-Ala-Val-Gly-His-Leu-Met.NH2
Human GRP-10	Gly-Asn-His-Trp-Ala-Val-Gly-His-Leu-Met.NH2

(B) Phyllolitorins

Leu-8 phyllolitorin (*Phyllomedusa sauvagei*)	pGlu-Leu-Trp-Ala-Val-Gly-Ser-Leu-Met.NH2
Phe-8 phyllolitorin (*P. sauvagei*)	pGlu-Leu-Trp-Ala-Val-Gly-Ser-Phe-Met.NH2

(C) Ranatensins, litorins and neuromedin B (NMB)

Ranatensin (*R. pipiens*)	pGlu-Val-Pro-Gln-Trp-Ala-Val-Gly-His-Phe-Met.NH2
Rohdei-litorin (*P. rhodei*)	pGlu-Leu-Trp-Ala-Thr-Gly-His-Phe-Met.NH2
Litorin (*L.aurea*)	pGlu-Gln-Trp-Ala-Val-Gly-His-Phe-Met.NH2
Ranatensin-R (*R. rugosa*)	Ser-Asn-Thr-Ala-Leu-Arg-Arg-Tyr-Asn-Gln-Trp-Ala-Thr-Gly-His-Phe-Met.NH2
Human Neuromedin B	Gly-Asn-Leu-Trp-Ala-Thr-Gly-His-Phe-Met.NH2

FIGURE 2 Structures of amphibian bombesin-like peptides. Amino acids conserved across all members of the family are shown in gray. A. Peptides classically designated as bombesin-like. B. Peptides classically designated as phyllolitorin-like. C. Peptides classically designated as ranatensin like. Notes: 1, also the same structure in *Bombina bombina* and *Bombina variegata*; 2, listed here as bombesin-like as per Samgina et al.;[24] 3, examples of 15 amino acid bombesins from *Odorrana grahami*;[9] 4, from skin of *Sanguirana varians* from Miao et al.;[16] 5, from Wang et al.;[27] 6, from Shen et al.[25] though the exact N-terminal cleavage site is not established. As more bombesin-like peptides are isolated from more diverse amphibian species, it is not clear how useful this historic classification will remain. More useful classification will however require more receptor binding and biological function information than is presently available.

of three forms of bombesin in the skin of *B. orientalis*, Leu-13 bombesin, Phe-13 bombesin, and Ser-3, Arg-10, Phe-13 bombesin (SAP bombesin) (Fig. 2) as reported by Nagalla et al.[19] The Analysis of genomic DNA shows that these three forms of bombesin are derived from separate genes. As well as being expressed in the skin at high levels, lower levels of these peptides are also expressed in the brain, lung, and gut.[19]

The presence of multiple form of bombesin in a single frog has similarly been reported by Li et al.[9] who found nine different bombesin-like peptides in the skin of *Odorrana grahami* including both Leu- and Phe-forms. Notably some

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of the peptides (bombesin B4, Fig. 2) seem to have different effects on smooth muscle contraction than expected.[25] The bombesins from *O. grahami* are 15 amino acids as opposed to the canonical 14, but similar 15 amino acid forms of bombesin have also recently been isolated from *Sanguirana varians*[16] and from *Rana suchinae*[27] (Fig. 2). Samgina et al.[24] have also described a Phe-form of bombesin from *Rana ridibunda.* Shen et al.[25] described a novel proline-rich variant of bombesin from *Bombina maxima* (Fig. 2) though how its biological functions may differ from typical bombesins is not yet known.

In the bombesin prohormones, the sequence of bombesin follows a small N-terminal extension peptide that follows the signal peptide. A Gln residue provides the signal for the C-terminal pyroglutamate, a Gly residue provides the nitrogen for the N-terminal amide followed by two basic amino acids to signal the C-terminal cleavage. After the two basic amino acids, is a C-terminal extension peptide of varying length.[19] Frog skin is rich in enzymes similar to the mammalian prohormone convertases, so processing of frog skin bombesin is likely to be highly homologous to processing of neuropeptides in mammals.

The receptor for Phe-13 bombesin was cloned by Spindel and coworkers.[18] It has 377 amino acids and is a 7-transmembrane, G protein-coupled receptor linked to increased IP3 formation. The amphibian Phe-13 bombesin receptor is clearly homologous to the mammalian receptors for bombesin-like peptides. In *B. orientalis*, the Phe-13 bombesin receptor is expressed primarily in brain. The affinity of the Phe-13 bombesin receptor is 0.2 nM for Phe-13 bombesin, 0.9 nM for Leu-13 bombesin, and 100 nM for SAP bombesin.[18] These differing affinities suggest that distinct receptors for the other forms of amphibian bombesin will also be present in the frog. Consistent with this, partial cDNA sequences of receptors homologous to the Phe-13 bombesin receptor have been identified in frog brain by PCR.[18] In terms of the standardized nomenclature for bombesin receptors, the receptor for Phe-13 bombesin has been designated as the BB4 receptor. Interestingly, the Phe-13 bombesin receptor is most homologous to the BRS-3 receptor raising the possibility that the BRS-3 ligand in mammals when characterized may fall in the same phylogenetic branch as the frog skin bombesin-like peptides. This is discussed more in Section 6.

Although the function of bombesin in frogs is unknown, bombesin produces diverse physiologic effects in mammals in the brain, GI tract, and lung. Within the GI tract, bombesin stimulates the secretion of many GI hormones including gastrin, somatostatin, VIP, GIP, and glucagon as well as the release of pancreatic exocrine enzymes.[14] Within the CNS, bombesin is a potent regulator of central homeostatic mechanisms including regulation of appetite, blood sugar, cardiac function, and gastric acidity[7,13,26] and in lung, bombesin stimulates lung cell growth.[10] These effects of bombesin in mammals are mediated by the interaction of bombesin with the mammalian GRP and NMB receptors as discussed in other chapters of this book. Because the multiple forms of bombesin occur in frog brain together with their receptors, it is safe to conclude that bombesin-like peptides also function as neurotransmitters in frog brain.

AMPHIBIAN PHYLLOLITORINS

The phyllolitorins were initially characterized by Yasuhara et al.[29] from the skin of *Phyllomedusa sauvagei*. They form a distinct class of bombesin-like peptides characterized by the Ser residue at the third position from the C-terminus as opposed to the His residue which characterizes all other bombesin-like peptides (Fig. 2). Just like bombesin, the phyllolitorins exist in multiple forms distinguished by their penultimate amino acid, a Phe-8 form resembling ranatensin and a Leu-8 form resembling bombesin. Generally, the Leu-8 form shows more bioactivity.[12] The phyllolitorins have distinct pharmacologic profiles that differentiate them from the other bombesin-like peptides and, even though their binding to the known bombesin receptors is lower than for other related bombesin-like peptides, they produce similar effects as bombesin on the induction of scratching and grooming behavior and effects on hypothermia.[12] Peripherally, phyllolitorins lower systemic blood pressure[23] in dogs and monkeys in contrast to the hypertensive effects of bombesin and ranatensin.

In an unusual mechanism, the two forms of phyllolitorin are produced by RNA editing of a single gene that encodes Phe-8 phyllolitorin.[20] The single primary transcript of the Phe-8 phyllolitorin gene is either processed unedited to the Phe-8 phyllolitorin mRNA or the uracil (U) residue in the codon encoding the Phe-8 is edited in the RNA to a cytosine (C) residue to create the codon encoding the Leu residue in Leu-8 phyllolitorin. Such U to C RNA editing while less common than C to U RNA editing has been reported in other systems including insect sodium channels.[11]

The phyllolitorins like bombesin are found in the highest levels in skin in the cutaneous granular glands. Lower levels of phyllolitorin mRNA are also present in brain and GI tract.[20] The receptors for phyllolitorins have not been characterized, but almost certainly are 7-transmembrane receptors that will be highly homologous to the receptor for Phe-13 bombesin. As for bombesin, the function of phyllolitorin in frog skin remains unknown.

RANATENSIN-LIKE PEPTIDES

At about the same time that Anastasi et al.[2] described the isolation of bombesin from the skin of *B. bombina*, Nakajima

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et al.[22] independently described the isolation of the related peptide ranatensin from the skin of *Rana pipiens*. As shown in Fig. 2, bombesin and ranatensin are highly homologous, the most critical difference being the penultimate residue which is Leu in bombesin and Phe in ranatensin. Different *Rana* and *Litora* species have slightly different but highly related ranatensin-like sequences, though peptides more closely related to bombesin have also been isolated from *Rana* species.[24] In mammals, ranatensin has a variety of effects. Ranatensin has strong effects on smooth muscle and blood pressure[4] and also replicates many of bombesin's effects on GI and CNS function, but with less potency.[17]

The ranatensin cDNA is similar in structure to the cDNAs for the other bombesin-like peptides. As in the bombesin prohormone, the sequence of ranatensin follows a short N-terminal extension peptide that immediately follows the signal peptide. If ranatensin is not cleaved from its N-terminal extension it gives a peptide highly similar to ranatensin-R (Fig. 2B) and to NMB-32, the larger form of mammalian NMB. In *R. pipiens*, the highest levels of ranatensin mRNA are found in the skin, but the ranatensin mRNA is also clearly detectable in brain. Highly homologous RNAs are also found in other *Rana* species including *Rana catesbeiana* and *Rana esculenta*. A full length cDNA for the ranatensin receptor has not been characterized, but sequence analysis of cDNA fragments amplified from RNA prepared from *R. pipiens* brain suggests that the ranatensin receptor will be highly homologous to the Phe-13 bombesin receptor.

GASTRIN-RELEASING PEPTIDE

Shortly after amphibian bombesin was isolated, its ability to stimulate the release of gastrin was used by McDonald and coworkers[15] to isolate a homologous peptide from porcine stomach that was named GRP. For many years GRP was considered the mammalian homolog of amphibian bombesin until Kim et al. showed that *Xenopus laevis* expressed a 29 amino acid peptide in stomach highly homologous to mammalian GRP[5] and Nagalla et al.[21] cloned cDNAs encoding GRP from *B. orientalis* to demonstrate that the same frog expressed different mRNAs to encode both bombesin and GRP. Phylogenetic analysis shows that frog GRP is far more closely related to mammalian GRP than to frog bombesin (Fig. 3). The prohormone structure of amphibian GRP is also highly similar to the prohormone structure of mammalian GRP, with the sequence for GRP immediately after the signal peptide. As for mammalian GRP, there is an Arg residue in frog GRP that allows processing of the 29 amino acid large form of GRP to the C-terminal decapeptide of GRP (GRP-10). The distribution of frog GRP is also quite distinct from that of bombesin in that frog GRP is not present in skin but rather is present in the highest quantities in gut and brain. In situ hybridization analysis of GRP expression shows expression of the frog GRP mRNA in cells in the basal layers of the gastric pits.[21] Kim et al.[26] have also shown that in *X. laevis* GRP effects contractility of stomach muscle. The localization and function of GRP in stomach suggest that amphibian GRP likely has similar functions in the GI tract of frogs as mammalian GRP does in mammals. Sequence analysis of partial cDNAs isolated from *B. orientalis* suggests that the amphibian GRP receptor is highly homologous to the mammalian GRP receptor.

PHYLOGENETIC CONSIDERATIONS AND IMPLICATIONS FOR ADDITIONAL MAMMALIAN BOMBESIN-LIKE PEPTIDES

Despite the 40 years of study of bombesin-like peptides in both frogs and mammals, there are several fundamental unanswered questions about the peptide family. In mammals, there are three receptors, the GRP receptor, the NMB receptor, and the BRS-3 receptor. The ligand for the BRS-3 receptor is unknown, and phylogenetic analysis shows that the Phe-13 bombesin receptor is closest to the BRS-3 receptor. Thus, it is tempting to speculate that the ligand for the BRS-3 receptor will be the true mammalian bombesin and phylogenetically more closely related to amphibian bombesin than to GRP or NMB. If this is the case, this suggests that one or more mammalian bombesin-like peptides closely related to the frog skin bombesins remain to be characterized. This would be similar to the characterization of the urocortins,[8] the mammalian homologs of amphibian sauvagine (see the CRH/Urocortin chapter

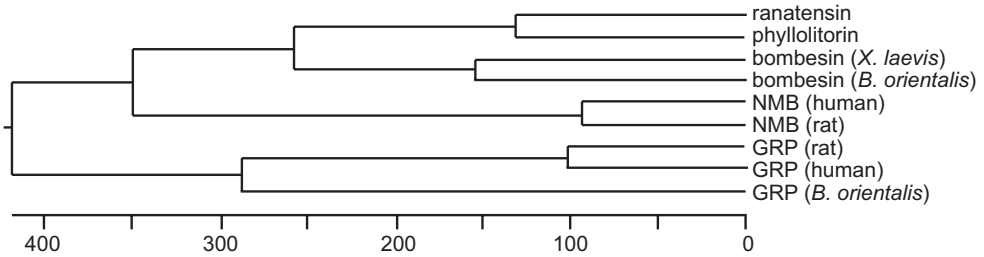

FIGURE 3 Phylogenetic tree derived from multiple comparisons of the known bombesin-like prohormones using MegAlign by DNAstar. As can be seen, the skin peptides are all closely related and distinct from GRP and neuromedin B.

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in the Brain Peptides section of the book). Given that frog species tend to have several forms of bombesin, this would suggest that the true mammalian bombesin, like the urocortins, will exist in multiple forms. Another possibility is that frogs also have a BRS-3 receptor in which case frogs would also have a BRS-3 ligand that is distinct from the skin bombesin-like peptides. Another question is if frogs have a NMB like peptide distinct from the skin bombesin-like peptides in that an amphibian homolog of NMB has yet to be definitively characterized, though *Xenopus* have a peptide in brain that resembles NMB but is phylogenetically more closely related to *B. orientalis* bombesin.[28] However, because frogs have Phe-form bombesin-like peptides present in brain (Phe-13 bombesin, Phe-8 phyllolitorin), it is possible that these peptides take the place of NMB in amphibians.

REFERENCES

1. Alvarez BB, Delfino G, Nosi D, Terreni A. Ultrastructure of poison glands of South American frogs: a comparison between *Physalaemus albonotatus* and *Leptodactylus chaquensis* (Anura: Leptodactylidae). *J Morphol* 2005;**263**:247–58.
2. Anastasi A, Erspamer V, Bucci M. Isolation and structure of bombesin and alytesin, two analogous active peptides from the skin of the European amphibians *Bombina* and Alytes. *Experientia* 1971;**27**:166–7.
3. Chen T, Farragher S, Bjourson AJ, Orr DF, Rao P, Shaw C. Granular gland transcriptomes in stimulated amphibian skin secretions. *Biochem J* 2003;**371**:125–30.
4. Clineschmidt BV, Geller RG, Govier WC, Pisano JJ, Tanimura T. Effects of ranatensin, a polypeptide from frog skin on isolated smooth muscle. *Br J Pharmacol* 1971;**41**:622–8.
5. Dockray GJ, Hopkins CR. Caerulein secretion by dermal glands. *J Cell Biol* 1975;**64**:724–33.
6. Kim JB, Johansson A, Holmgren S, Conlon JM. Gastrin-releasing peptides from *Xenopus laevis*: purification, characterization, and myotropic activity. *Am J Physiol Regul Integr Comp Physiol* 2001;**281**:R902–8.
7. Ladenheim EE, Hamilton NL, Behles RR, Bi S, Hampton LL, Battey JF, et al. Factors contributing to obesity in bombesin receptor subtype-3-deficient mice. *Endocrinology* 2008;**149**:971–8.
8. Lewis K, Li C, Perrin MH, Blount A, Kunitake K, Donaldson C, et al. Identification of urocortin III, an additional member of the corticotropin-releasing factor (CRF) family with high affinity for the CRF2 receptor. *Proc Natl Acad Sci USA* 2001;**98**:7570–5.
9. Li J, Yu H, Xu X, Wang X, Liu D, Lai R. Multiple bombesin-like peptides with opposite functions from skin of *Odorrana grahami*. *Genomics* 2007;**89**:413–8.
10. Li K, Nagalla SR, Spindel ER. A rhesus monkey model to characterize the role of gastrin-releasing peptide (GRP) in lung development. Evidence for stimulation of airway growth. *J Clin Invest* 1994;**94**:1605–15.
11. Liu Z, Song W, Dong K. Persistent tetrodotoxin-sensitive sodium current resulting from U-to-C RNA editing of an insect sodium channel. *Proc Natl Acad Sci USA* 2004;**101**:11862–7.
12. Masui A, Kato N, Itoshima T, Tsunashima K, Nakajima T, Yanaihara N. Scratching behavior induced by bombesin-related peptides. Comparison of bombesin, gastrin-releasing peptide and phyllolitorins. *Eur J Pharmacol* 1993;**238**:297–301.
13. McArthur AJ, Coogan AN, Ajpru S, Sugden D, Biello SM, Piggins HD. Gastrin-releasing peptide phase-shifts suprachiasmatic nuclei neuronal rhythms in vitro. *J Neurosci* 2000;**20**:5496–502.
14. McDonald TJ, Ghatei MA, Bloom SR, Adrian TE, Mochizuki T, Yanaihara C, et al. Dose–response comparisons of canine plasma gastroenteropancreatic hormone responses to bombesin and the porcine gastrin-releasing peptide. *Regul Pept* 1983;**5**:125–37.
15. McDonald TJ, Jornvall H, Nilsson G, Vagne M, Ghatei M, Bloom SR, et al. Characterization of a gastrin releasing peptide from porcine non-antral gastric tissue. *Biochem Biophys Res Commun* 1979;**90**:227–33.
16. Miao Y, Li W, Duan L, Xiao Y. A bombesin-like peptide from skin of *Sanguirana varians*. *Comp Biochem Physiol B Biochem Mol Biol* 2010;**155**:106–9.
17. Mukai H, Kawai K, Suzuki Y, Yamashita K, Munekata E. Stimulation of dog gastropancreatic hormone release by neuromedin B and its analogues. *Am J Physiol* 1987;**252**:E765–71.
18. Nagalla SR, Barry BJ, Creswick KC, Eden P, Taylor JT, Spindel ER. Cloning of a receptor for amphibian [Phe^{13}]bombesin distinct from the receptor for gastrin-releasing peptide: identification of a fourth bombesin receptor subtype (BB4). *Proc Natl Acad Sci USA* 1995;**92**:6205–9.
19. Nagalla SR, Barry BJ, Falick AM, Gibson BW, Taylor JE, Dong JZ, et al. There are three distinct forms of bombesin: identification of [Leu^{13}] bombesin, [Phe^{13}] bombesin and [Ser^{3}, Arg^{10}, Phe^{13}] bombesin in the frog *Bombina orientalis*. *J Biol Chem* 1996;**271**:7731–7.
20. Nagalla SR, Barry BJ, Spindel ER. Cloning of cDNAs encoding the amphibian bombesin-like peptides Phe^{8} and Leu^{8} phyllolitorin from *Phyllomedusa sauvagei*: potential role of U to C RNA editing in generating neuropeptide diversity. *Mol Endocrinol* 1994;**8**:943–51.
21. Nagalla SR, Gibson BW, Tang D, Reeve Jr JR, Spindel ER. Gastrin-releasing peptide (GRP) is not mammalian bombesin: identification and molecular cloning of a true amphibian GRP distinct from amphibian bombesin in *Bombina orientalis*. *J Biol Chem* 1992;**267**:6916–22.
22. Nakajima T, Tanimura T, Pisano JJ. Isolation and structure of a new vasoactive polypeptide. *Fed Proced* 1970;**29**:282.
23. Negri L, Improta G, Broccardo M, Melchiorri P. Phyllolitorins: a new family of bombesin-like peptides. *Ann NY Acad Sci* 1988;**547**:415–28.
24. Samgina TY, Artemenko KA, Gorshkov VA, Ogourtsov SV, Zubarev RA, Lebedev AT. De novo sequencing of peptides secreted by the skin glands of the Caucasian Green Frog *Rana ridibunda*. *Rapid Commun Mass Spectrom* 2008;**22**:3517–25.
25. Shen JH, Liu SB, Zhang YX, Jin Y, Lee WH, Zhang Y. Cloning of novel bombesin precursor cDNAs from skin of *Bombina maxima*. *Regul Pept* 2005.
26. Tache Y, Brown M. On the role of bombesin in homeostasis. *Trends Neurosci* 1982;**5**:431–3.
27. Wang H, Bian J, Chen Z, Miao Y, Li W. A novel bombesin-like peptide from skin of *Rana shuchinae*. *Mol Biol Rep* 2011;**38**:3599–603.
28. Wechselberger C, Kreil G, Richter K. Isolation and sequence of a cDNA encoding the precursor of a bombesin-like peptide from brain and early embryos of *Xenopus laevis*. *Proc Natl Acad Sci USA* 1992;**89**:9819–22.
29. Yasuhara T, Nakajima T, Nokihara K, Yanaihara C, Yanaihara N, Erspamer V, et al. Two new frog skin peptides, phyllolitorins, of the bombesin-ranatensin family from *Phyllomedusa sauvagei*. *Biomed Res* 1983;**4**:407–12.

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Chapter 47

Bombinins

Vincenzo Luca, Donatella Barra and Maria Luisa Mangoni

ABSTRACT

The skin secretion of *Bombina* species contains antimicrobial peptides with interesting biological properties. These peptides can be grouped into two distinct subfamilies, the bombinin and the bombinin H. The former comprises 27 amino acid residue peptides with a large spectrum of antimicrobial activity but virtually devoid of lytic effect on human erythrocytes. The latter includes 20 or 17 residue hemolytic peptides, some of which contain a D-amino acid in the second position of their sequence, as a result of a posttranslational modification. A brief description of the genes coding for these two peptide families, their biological activities along with the underlying molecular mechanism, are reported. Importantly, the presence of a single D-amino acid substitution represents a new approach naturally developed not only to modulate peptide stability *in vivo*, but also to confer the all-L peptide and its diastereomer distinctive biological features.

BOMBININS: GENERAL FEATURES

From the skin secretion of amphibian Anura, a multiplicity of antimicrobial peptides (AMPs) have been isolated and characterized.[1,25,31] Here we report a brief overview on the structure and function of AMPs from *Bombina* species. These are members of an ancient group of frogs which, according to some herpetologists, belongs to a separate family, the Bombinatoridae of the suborder Archaeobatrachia.[4] Besides the three main species, *Bombina bombina*, *B. variegata* and *B. orientalis*, a few others, most notably the Chinese red belly toad *B. maxima*, have been identified. Studies on the skin secretions of the two European species *B. bombina* and *B. variegata* started in the early sixties, when the pentapeptide Glu-His-Phe-Ala-Asn-amide was purified from this source.[24] About ten years later, it was shown that it corresponds to the carboxyl-end of a larger peptide (24 residues long) extracted from *B. variegata* and named bombinin.[5] Subsequently, by means of peptide sequencing and screening of a cDNA library prepared from the skin of *B. variegata*[23] and *B. orientalis*,[6] a family of AMPs related to bombinin was discovered. Members of this family (bombinin-like peptides, BLPs) contain 27 amino acids. They are amidated at the C-terminus and differ, at some positions in their N-terminal half, while the rest are identical.[23] It is possible that the original sequence of bombinin, derived from a mixture of similar peptides, contained some sequencing errors. The primary structure of BLPs is reported in Table 1. Interestingly, from the analysis of the BLP precursors by cDNA cloning, the existence of several variants of another peptide prototype was predicted. These peptides were then isolated by reversed-phase HPLC from the skin secretions of these frog species and named bombinins H,[20,23] with reference to their high hydrophobic character with respect to that of BLPs (Table 1) and because of their hemolytic activity (Table 2). Differences among the various bombinins H occurs at 3 positions, but the most surprising outcome in some of them is the presence of a D-alloisoleucine at the second N-terminal residue (Table 1), as a result of a posttranslational modification of the respective gene-encoded L-isoleucine (there is no codon for a D-amino acid).[12,22] Although peptides endowed with a D-amino acid have been found in the skin of other frogs[13] and in the venom of the male platypus,[28] and in a variety of invertebrate species,[7,10,11,27] bombinins H represent the first case of AMPs containing such a modification. An enzyme responsible for the change of chirality of an amino acid in peptide linkage was first described by Heck et al.[7] from the venom of a funnel web spider. This enzyme catalyzes the partial isomerization from the L- to the D-isomer of a serine close to the carboxyl-end of ω-agatoxin IV.[13] A peptidyl-aminoacyl-L/D-isomerase converting L-isoleucine to D-alloisoleucine at position 2 of mature peptides has been characterized from *Bombina* skin secretions.[9] The inversion of chirality occurs via de-protonation/protonation at the alpha carbon, and the reaction apparently proceeds without the addition of any cofactor.[9] Surprisingly, this enzyme has a rather low selectivity for residues at position 2, but the presence of a hydrophobic amino acid at position 1 and a small residue at position 3 of the substrate are essential.[8] From the skin of *B. orientalis*, two shorter (17 amino acids), more hydrophobic isoforms of the previously identified bombinins H, were also isolated.[6,21] They have an L or D-leucine

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00047-6
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TABLE 1 Sequences of Some BLPs and Bombinin H-Related Peptides

Bombinin-like peptides (BLP)	
Bombinin	GIG-A-LSA-KGALKGLAKGLAEHFAN-NH_2.
Clone 7*	GIGGALLSAGKSALKGLAKGLAEHFAN-NH_2.
9*	GIGGALLSAAKVGLKGLAKGLAEHFAN-NH_2.
13*	GIGGALISAGKSALKGLAKGLAEHFAN-NH_2.
14*	GIGGALLSDAKVGLKGLAKGLAEHFAN-NH_2.
33*	GIGASILSAGKSALKGFAKGLAEHFAN-NH_2.
42*	GIGGALLSAAKVGLKGLAKGLAEHFAN-NH_2.
BLP-1	GIGASILSAGKSALKGLAKGLAEHFAN-NH_2.
BLP-2	GIGSAILSAGKSALKGLAKGLAEHFAN-NH_2.
BLP-3§	GIGAAILSAGKSALKGLAKGLAEHF---NH_2.
BLP-5	GIGATILSVGKSALKGLAK----HF---NH_2.
BLP-6	GIGAALLSAGKSALKGLAKGLAEHFAN-NH_2.
BLP-7§	GIGGALLSAGKSALKGLAKGLAEHFAN-NH_2.
Maximins	
Maximin 1	GIGTKILGGVKTALKGALKELASTYAN-NH_2.
Maximin 2	GIGTKILGGVKTALKGALKELASTYVN-NH_2.
Maximin 3	GIGGKILSGLKTALKGAAKELASTYLH.
Maximin 4	GIGGVLLSAGKAALKGLAKVLAEKYAN-NH_2.
Maximin 5	SIGAKILGGVKTFFKGALKELASTYLQ.
Bombinins H	
H1	IIGPVLGMVGSALGGLLKKI-NH_2.
H2	IIGPVLGLVGSALGGLLKKI-NH_2.
H3	I/GPVLGMVGSALGGLLKKI-NH_2.
H4#	I/GPVLGLVGSALGGLLKKI-NH_2.
H5#	L/GPVLGLVGSALGGLLKKI-NH_2.
H6	IIGPILGLVSNALGGLL----NH_2.
H7#	I*L*GPILGLVSNALGGLL----NH_2.
GH-1§	IIGPVLGLVGKPLESLLE.
GH-2§	ILGPVLDLVGRALRGLLKKI-NH_2.
Maximin H	
Maximin H1	ILGPVISTIGGVLGGLLKNL-NH_2.
Maximin H2	ILGPVLSMVGSALGGLIKKI-NH_2.
Maximin H3	ILGPVLGLVGNALGGLIKKI-NH_2.
Maximin H4	ILGPVISKIGGVLGGLLKNL-NH_2.
Maximin H5*	ILGPVLGLVSDTLDDVLGIL-NH_2.

TABLE 1 Sequences of Some BLPs and Bombinin H-Related Peptides—Cont'd

Bombinin-like peptides (BLP)	
Maximin S	
Maximin S1	GSNTGFNFKTLDKE-NH_2.
Maximin S2	GSNKGNFMVDMIQALSN-NH_2.
Maximin S3	GSNKGFNFMVDMINALSN-NH_2.

**Sequences deduced from cDNA.*
§Sequences deduced from BLP-3[19] and BLP-7[18] genes, respectively.
#D-amino acids in position 2 are italicized.

as the second residue and lack the lysine-lysine-isoleucine motif at the C-terminus (Table 1, H6 and H7). Moreover, from the sequences of two BLP genes,[18,19] two additional peptides related to bombinins H, were predicted (Table 1, GH-1 and GH-2), but they were never detected in skin secretions. In contrast to other amphibian peptides bearing a D-amino acid, that is, the opioid dermorphin and deltorphins (see the chapter on these peptides in this section of the book and the Opioid Peptides Section), where the all-L isoform is apparently not detectable as a mature peptide and is not active (the presence of D-amino acids is essential for the interaction with opioid receptors), the all L-bombinins H are considerably active, although with different potency and cell selectivity from the corresponding D-amino acid–containing isoforms.

In 2002, from the skin of *B. maxima*, two other families of AMPs equivalent to bombinins and bombinins H were characterized. They are designated as maximins and maximins H, respectively (Table 1).[14] Remarkably, they have been found in the brain of *B. maxima*, but their function in this anatomical area is not yet clear.[15] More recently, from the skin of this *Bombina* species, a new group of AMPs consisting of 14 (maximin S1) or 18 (maximin S2–S5) amino acids has been discovered 29 (Table 1). The presence of a D-amino acid in maximin peptides has not been investigated.[14]

SOLUTION STRUCTURE

Either bombinins (net charge +4/+5) or bombinins H (net charge ranging from +1 to +3) are able to adopt α-helical conformations in hydrophobic environments such as 70% trifluoroethanol. More specifically, the helical conformation of BLPs is mostly amphipathic, as they contain polar residues properly spaced in the sequence, while bombinins H have basic residues confined at the C-terminal region (H1-H5). Furthermore, bombinins H are particularly rich in glycines (25%), which should make the formation of an amphipathic α-helix rather unlikely. As found in NMR solution structure, both H2 and H4 display well-defined

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TABLE 2 Antimicrobial and Cytolytic Activity of Bombinin/Bombinin H-Related Peptides

Peptide	BLP-1	BLP-3	H2	H4	H6	H7	GH-1_D	GH-1_L
				Lethal concentration (μM)				
Gram-negative bacteria								
Escherichia coli D21	1.7	3.0	21.4	4.7	NA	NA	NA	NA
Escherichia coli D22	1.5	0.9	4.4	3.1	NA	NA	NA	NA
Yersinia pseudotuberculosis YPIII	0.8	0.5	7.3	2.0	NA	NA	NA	NA
Pseudomonas aeruginosa ATCC 15692	6.3	9.2	NA	NA	NA	NA	NA	NA
Areomonas hydrophila Bo-3N	ND	NA	NA	NA	NA	NA	NA	NA
Pseudomonas syringae pv tobaci	ND	ND	32	8.2	NA	NA	NA	NA
Gram-positive bacteria								
Bacillus megaterium Bm11	0.3	0.8	1.4	0.8	NA	25	NA	NA
Staphylococcus aureus Cowan I	3.4	1.7	4.7	3.0	NA	NA	NA	NA
Staphylococcus lentus	ND	ND	2.0	0.2	NA	NA	ND	ND
Yeasts								
Candida albicans ATCC 10231	0.4	0.4	3.1	1.6	NA	NA	NA	NA
Candida guiller mondii	ND	ND	1.3	0.7	NA	NA	ND	ND
% Hemolysis at 15μM	<10	<10	11.0	28.0	78.0	20.0	NA	NA

ND, not determined; NA, not active.

α-helices between residues 5 and 18, whereas a distorted conformation is present at both termini.[30] However, the presence of several glycine residues confer bombinins H a high structural flexibility. Indeed, β-sheet conformers have been detected by Fourier transformed infrared spectroscopy in oriented bilayers of different lipid composition, with an aggregation tendency for the all-L-bombinin H2.[17]

THE GENE STRUCTURE OF BLPS AND BOMBININS H

Genes encoding the precursors of BLPs were cloned from *B. orientalis*.[18,19] These contain two exons separated by large introns (1–2kb). The first exon codes for the signal peptide, whereas the second exon codes for the mature AMP and for acidic and spacer peptides. The genetic information differs between the two genes that have been characterized: The first one (*BLP-3*) contains the information for two copies of a bombinin-like peptide (BLP-3) and a single copy of a bombinin H-related peptide; the second gene (BLP-7) codes only for a single copy of each peptide. The same organization was found in the cDNA clones isolated from *B. variegata*. Canonical Lys–Arg sequences flank the N-terminal end of the mature products for their correct processing, while a Gly residue adjacent the carboxyl-terminus of the peptide is necessary for its amidation. In the promoter regions of these genes, binding sites for NF-κB and NF-IL6 are present. A common regulatory pathway for the expression of AMP genes occurs in amphibians involving cooperation of a large number of transcription factors, such as those acting in the activation of the mammalian acute-phase response. In contrast with bombinins and maximins, the precursors of the homolog maximins S show a completely new arrangement: not a simple assembly of one or two copies of a BLP (maximin-like) plus a bombinin H (maximin H-like) peptide but a complex number and position of copies of active and spacer peptides.[29]

BIOLOGICAL PROPERTIES (ANTIBACTERIAL, ANTIFUNGAL, ANTIPARASITIC, AND CYTOLYTIC ACTIVITIES)

The antimicrobial activity of BLPs and bombinins H was initially distinguished on the basis of their cytolytic properties. BLPs are specifically active against Gram-positive (*Bacillus megaterium*, *Staphylococcus aureus*) and Gram-negative (*Escherichia coli*, *Yersinia pseudotuberculosis*,

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Pseudomonas aeruginosa) bacteria, as well as against *Candida albicans*, with no appreciable hemolytic capacity (Table 2). Differently, bombinins H have a lower antibacterial potency but a higher hemolytic activity.[20,21,23] Afterward, the interest of researchers addressed the functional significance of the incorporation of a D-amino acid in the sequence of bombinins H (either the 20 or the 17 residue peptides). These studies, based on the comparison of the activity of pairs of isomers (e.g. the all-L peptide and the corresponding D-amino acid-containing diastereomer), led to a deeper knowledge of their biological features.[16] Interestingly, when tested in liquid medium assay to determine the MIC value, the D-isoform H4 had a higher activity on Gram-negative bacteria, while an opposite outcome was found against Gram-positive strains, displaying the all-L H2 a 2-4 lower MIC (Table 3). However, our previous results using the inhibition zone assay had shown a stronger antimicrobial activity of H4 with respect to H2, against the Gram-positive *S. aureus.*[16] This could result from a different solubility and diffusion of the peptides through a solid medium. Furthermore, when the two isomers were analyzed in liquid medium for their bactericidal activity on *S. aureus*, both of them were able to cause 99.9% killing of the microbial population at the same concentration.[3]

Regarding the hydrophobic H6 (all-L) and H7 (the D-isomer), they behave in a different way, because only H7 could kill some bacterial strains.[16] An increase in hydrophobicity does not enhance the efficacy of bombinins H and the single positive charge of H6/H7 is not an essential requirement for their antimicrobial activity.[16] In support, the anionic maximin H5 is endowed with antimicrobial properties.[14] Antifungal activity against *Phytophthora nicotianae* spores shows a minimal fungistatic and fungicidal concentration, corresponding to 18 or 10μM, for H2 and H4, respectively. In addition, H2 has a 50% inhibition of *P. nicotianae* hyphae growth, at 18μM.[26] The couple H2/H4 was then tested against the worldwide spread protozoa *Leishmania*: H4 is the most active peptide, being lethal to both the insect stage (promastigote) and the mammalian pathological form (amastigote) of this parasite, at concentrations significantly lower than H2.[17]

To evaluate the cytotoxic effect of the peptides on mammalian cells, the hemolytic activity on human erythrocytes was investigated in physiological solution at different peptide concentrations. Bombinin H6 has the greatest activity, causing approximately 78% hemolysis at 15μM, whereas a hemolytic capacity around 20% was detected for its diastereomer H7 (Table 2). Regarding the couple H2/H4, a higher level of hemolysis is reached by the D-amino acid–containing H4 (28% compared with 11% of the all-L H2). GH-1_L and GH-1_D peptides encoded by the BLP-3 gene, are both devoid of cytotoxic effect at all concentrations used. By contrast, significant lysis was manifested by the maximins H[14]

TABLE 3 Minimal Inhibitory Concentration (MIC) of Bombinin H on Different Bacterial Strains

Peptide	H2	H4
	MIC (μM)	
Gram-negatives		
Acinetobacter baumannii 1	50	12.5
Escherichia coli O111:B4	>50	50
Escherichia coli ATCC 25922	>50	50
Pseudomonas aeruginosa ATCC 27853	>50	>50
Yersinia pseudotuberculosis YPIII	12.5	12.5
Gram-positives		
Staphylococcus aureus ATCC 29213	12.5	25
Staphylococcus aureus Cowan I	12.5	50
Staphylococcus aureus 43300	12.5	50
Staphylococcus capitis n.1	12.5	25
Staphylococcus epidermidis n. 18	>50	>50

BOMBININS H: MECHANISM OF ACTION OF ANTIMICROBIAL ACTIVITY

Previous studies described the ability of H2 and H4 to permeate the membrane of Gram-negative bacteria causing the release of intracellular enzymes, such as β-galactosidase, with a stronger effect for the D-isoform H4, according to its higher growth inhibitory activity on Gram-negative strains.[16] Analogously, the membrane perturbation activity of these two diastereomers on Gram-positive bacteria (i.e. *S. aureus* ATCC 29213) determined by measurement of the intracellular influx of SYTOX Green, shows that H4 increases the membrane permeability more than H2, although H4 has a 2-fold higher MIC than H2 against this strain (Table 3). Presumably, at low concentrations, H2 is more efficient than H4 in inhibiting the growth of Gram-positive bacteria, without affecting the integrity of their plasma-membrane and, as a result, their viability. Differently, at higher concentrations, H4 would have an easier capability to produce membrane breakages causing cell death, compared with H2. This would explain the achievement of a bactericidal effect equal to that exhibited by H2 (99.9% killing of the microbial population at 50μM) despite the higher MIC of H4 with respect to H2. The ability of the two peptides to destabilize bacterial membranes by a nonstereospecific process was confirmed by their capacity to cause the release of calcein from calcein-loaded phosphatidylethanolamine/phosphatidylglycerol (PE/PG) and phosphatidylglycerol/cardiolipin

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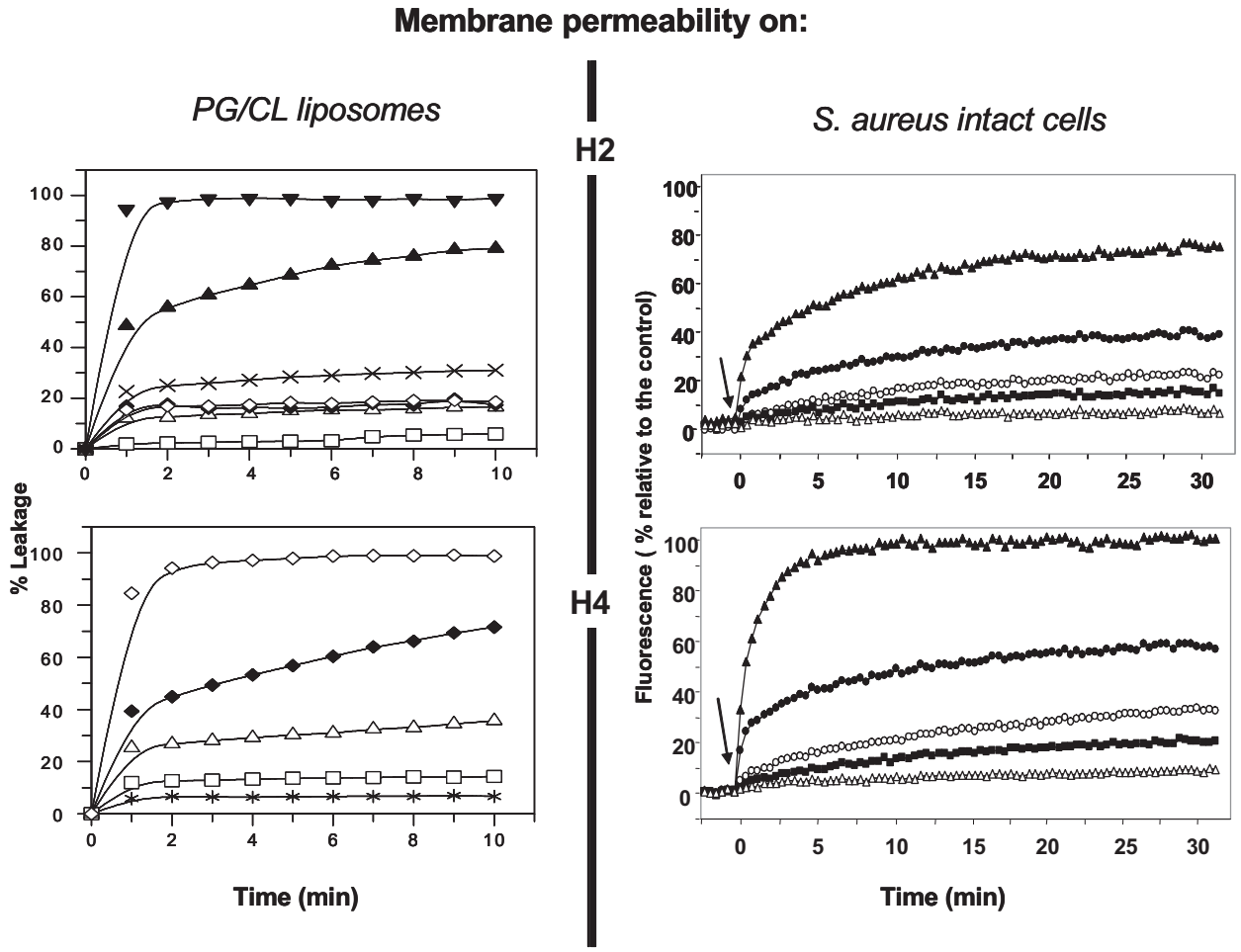

FIGURE 1 Effect of bombinins H2 and H4 on the membrane permeation of PG/CL liposomes (left panels) and of *S. aureus* ATCC 29213 (right panels). Left panels: Calcein leakage from PG/CL (6:4, w/w) vesicles, after peptide treatment. Calcein release from liposomes (50μM final concentration) was detected fluorimetrically ($\lambda_{exc}=490$nm, $\lambda_{ems}=517$nm). The percentage of leakage was calculated as described in Ref. 3. Peptide concentrations used were the following: 0.25μM (○); 0.5μM (*); 0.75μM (●); 1μM (□); 1.5μM (□); 2.5μM (Δ); 3μM (u); 3.5μM (◊); 7.5μM (×); 10μM (▲); 15μM (▼). Right panels: Intracellular influx of SYTOX Green. Cells were incubated with 1μM probe in PBS. Once basal fluorescence reached a constant value, the peptide was added (arrow, $t=0$) and changes in fluorescence were monitored ($\lambda_{exc}=485$nm, $\lambda_{ems}=535$nm) and plotted as the percentage of fluorescence, relative to that of bacteria fully permeated by the addition of Triton X-100 (1%, v/v, final concentration). All readings were normalized by subtracting fluorescence values of control (bacteria without peptide). Peptide concentrations used were the following: 3.125μM (Δ); 6.25μM (□); 12.5μM (○); 25μM (●) and 50μM (▲).

(PG/CL) liposomes.[3] These two lipid compositions were used to mimic the membrane of Gram-negative and Gram-positive bacteria, respectively. The higher affinity of H4 toward PG/CL phospholipid bilayers and its higher perturbing activity on this type of model membrane (Fig. 1), with respect to H2, supports the notion that the higher activity of the D-isoform is not mediated by recognition of a chiral target. Interestingly, working with PE/PG vesicles to mimic the lipid composition of *E. coli* inner membrane, both diastereomers similarly disturb the structure of these vesicles.[3] This suggests that the discrepancies found between the two isoforms in permeating the membrane of intact Gram-negative bacteria result from differences in their diffusion process through the microbial cell wall before reaching the target cytoplasmic membrane. A possible reason is that the D-amino acid–containing peptide is less aggregated in solution compared with the all L-bombinin H; therefore, it may be easier for it to penetrate through the peptidoglycan layer into the plasma-membrane. Indeed, the stereochemical isomerization of residue 2 to D-alloisoleucine in H4 peptide drastically decreases its tendency to form intrapeptide contacts,[2] causing conformational changes that may also alter the formation of interpeptide interactions. In the past years, lethal activity against promastigotes and amastigotes of Leishmania protozoa has been studied in depth for H2 and H4. The peptides cause extensive membrane blebbing with increased intracellular vacuolization, loss of electron dense intracellular space, while cytoplasmic organelles lose their regular shape (Fig. 2). Also against this pathogen, both diastereomers have a membrane-perturbing activity correlated to the degree of parasite's death.

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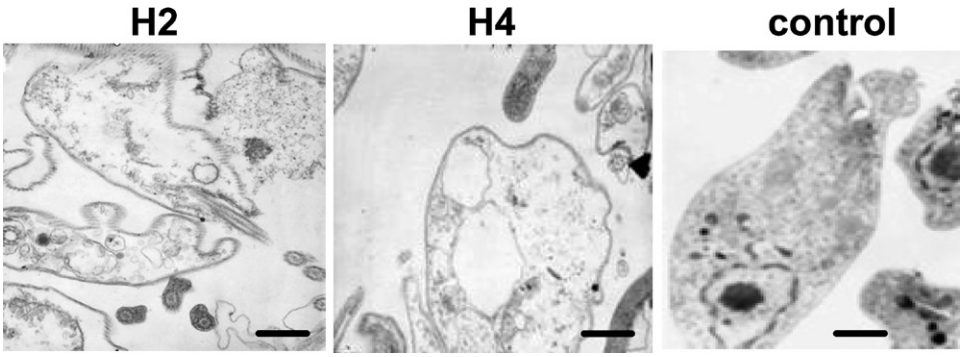

FIGURE 2 Electron microscopy of *Leishmania* promastigotes treated with bombinins H. Parasites were incubated for 1h with bombinins H2 and H4 at the concentration causing 80% killing (bar = 0.5μm).

Differently, for the other pair H6/H7, only the D-amino acid containing H7 permeates the membrane, but in a milder way than the more cationic H2/H4.[16] Indeed, it allows the intracellular influx of small molecules, for example, the chromogenic β-galactosidase substrate (*o*-nitrophenyl-β-galactoside, ONPG) but not the extracellular leakage of large compounds, such as the β-galactosidase.[17]

ACKNOWLEDGMENTS

This work was supported in part by grants from Università La Sapienza and the Italian Ministero dell'Università e della Ricerca Scientifica e Tecnologica.

REFERENCES

1. Bevins CL, Zasloff M. Peptides from frog skin. *Annu Rev Biochem* 1990;**59**:395–414.
2. Bozzi A, Mangoni ML, Rinaldi AC, Mignogna G, Aschi M. Folding propensity and biological activity of peptides: the effect of a single stereochemical isomerization on the conformational properties of bombinins in aqueous solution. *Biopolymers* 2008;**89**:769–78.
3. Coccia C, Rinaldi AC, Luca V, Barra D, Bozzi A, Di Giulio A, et al. Membrane interaction and antibacterial properties of two mildly cationic peptide diastereomers, bombinins H2 and H4, isolated from *Bombina* skin. *Eur Biophys J* 2011;**40**:577–88.
4. Conlon JM. Structural diversity and species distribution of host-defense peptides in frog skin secretions. *Cell Mol Life Sci* 2011;**68**:2303–15.
5. Csordàs A, Michl H. Isolation and structure of a haemolytic polypeptide from the defensive secretion of European *Bombina* species. *Monatsh Chem* 1970;**10**:182–9.
6. Gibson BW, Tang DZ, Mandrell R, Kelly M, Spindel ER. Bombinin-like peptides with antimicrobial activity from skin secretions of the Asian toad, *Bombina orientalis*. *J Biol Chem* 1991;**266**:23103–11.
7. Heck SD, Siok CJ, Krapcho KJ, Kelbaugh PR, Thadeio PF, Welch MJ, et al. Functional consequences of posttranslational isomerization of Ser46 in a calcium channel toxin. *Science* 1994;**266**:1065–8.
8. Jilek A, Mollay C, Lohner K, Kreil G, , et al. Substrate specificity of a peptidyl-aminoacyl-L:/D:-isomerase from frog skin. *Amino Acids* 2012;**42**:1757–64.
9. Jilek A, Mollay C, Tippelt C, Grassi J, Mignogna G, Mullegger J, et al. Biosynthesis of a D-amino acid in peptide linkage by an enzyme from frog skin secretions. *Proc Natl Acad Sci USA* 2005;**102**:4235–9.
10. Jimenez EC, Olivera BM, Gray WR, Cruz LJ. Contryphan is a D-tryptophan-containing Conus peptide. *J Biol Chem* 1996;**271**:28002–5.
11. Kamatani Y, Minakata H, Kenny PT, Iwashita T, Watanabe K, Funase K, et al. Achatin-I, an endogenous neuroexcitatory tetrapeptide from *Achatina fulica* Ferussac containing a D-amino acid residue. *Biochem Biophys Res Commun* 1989;**160**:1015–20.
12. Kreil G. Conversion of L- to D-amino acids: a posttranslational reaction. *Science* 1994;**266**:996–7.
13. Kreil G. D-amino acids in animal peptides. *Annu Rev Biochem* 1997;**66**:337–45.
14. Lai R, Zheng YT, Shen JH, Liu GJ, Liu H, Lee WH, et al. Antimicrobial peptides from skin secretions of Chinese red belly toad *Bombina maxima*. *Peptides* 2002;**23**:427–35.
15. Liu R, Liu H, Ma Y, Wu J, Yang H, Ye H, et al. There are abundant antimicrobial peptides in brains of two kinds of *Bombina* toads. *J Proteome Res* 2011;**10**:1806–15.
16. Mangoni ML, Grovale N, Giorgi A, Mignogna G, Simmaco M, Barra D. Structure-function relationships in bombinins H, antimicrobial peptides from *Bombina* skin secretions. *Peptides* 2000;**21**: 1673–9.
17. Mangoni ML, Papo N, Saugar JM, Barra D, Shai Y, Simmaco M, et al. Effect of natural L- to D-amino acid conversion on the organization, membrane binding, and biological function of the antimicrobial peptides bombinins H. *Biochemistry* 2006;**45**:4266–76.
18. Miele R, Borro M, Fiocco D, Barra D, Simmaco M. Sequence of a gene from *Bombina orientalis* coding for the antimicrobial peptide BLP-7. *Peptides* 2000;**21**:1681–6.
19. Miele R, Ponti D, Boman HG, Barra D, Simmaco M. Molecular cloning of a bombinin gene from *Bombina orientalis*: detection of NF-kappaB and NF-IL6 binding sites in its promoter. *FEBS Lett* 1998;**431**:23–8.
20. Mignogna G, Simmaco M, Kreil G, Barra D. Antibacterial and haemolytic peptides containing D-alloisoleucine from the skin of *Bombina variegata*. *Embo J* 1993;**12**:4829–32.
21. Mignogna GSM, Barra D. Defence peptides in the amphibian immune system. In: Jollès P, editor. *D-amino acids in sequences of secreted peptides of multicellular organisms*. Basel: Birkhauser Verlag; 1998. p. 29–36.

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22. Mor A, Amiche M, Nicolas P. Enter a new post-translational modification: D-amino acids in gene-encoded peptides. *Trends Biochem Sci* 1992;**17**:481–5.
23. Simmaco M, Barra D, Chiarini F, Noviello L, Melchiorri P, Kreil G, et al. A family of bombinin-related peptides from the skin of *Bombina variegata*. *Eur J Biochem* 1991;**199**:217–22.
24. Simmaco M, Kreil G, Barra D. Bombinins, antimicrobial peptides from *Bombina* species. *Biochim Biophys Acta* 2009;**1788**: 1551–5.
25. Simmaco M, Mignogna G, Barra D. Antimicrobial peptides from amphibian skin: what do they tell us? *Biopolymers* 1998;**47**: 435–50.
26. Simmaco MMM, Miele R, Borro M, Fiocco D, Barra D. Defence peptides in the amphibian immune system. In: Ascenzi P, Polticelli F, Visca P, editors. *Bacterial, plant and animal toxins*. Kerala, India: Research Signpost; 2003. p. 155–67.
27. Soyez D, Van Herp F, Rossier J, Le Caer JP, Tensen CP, Lafont R. Evidence for a conformational polymorphism of invertebrate neurohormones. D-amino acid residue in crustacean hyperglycemic peptides. *J Biol Chem* 1994;**269**:18295–8.
28. Torres AM, Menz I, Alewood PF, Bansal P, Lahnstein J, Gallagher CH, et al. D-Amino acid residue in the C-type natriuretic peptide from the venom of the mammal, *Ornithorhynchus anatinus*, the Australian platypus. *FEBS Lett* 2002;**524**:172–6.
29. Wang T, Zhang J, Shen JH, Jin Y, Lee WH, Zhang Y, et al. A novel group of antimicrobial peptides from toad *Bombina maxima*. *Biochem Biophys Res Commun* 2005;**327**:945–51.
30. Zangger K, Gossler R, Khatai L, Lohner K, Jilek A. Structures of the glycine-rich diastereomeric peptides bombinin H2 and H4. *Toxicon* 2008;**52**:246–54.
31. Zasloff M. Antimicrobial peptides of multicellular organisms. *Nature* 2002;**415**:389–95.

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Chapter 48

Caeruleins

John H. Bowie

ABSTRACT

Caerulein [pEQDY(SO_3H)TGWMDF-NH_2] is a common host-defense peptide of tree frogs of the genus *Litoria*, which has also been identified in several other frog genera. Caerulein is a hypertensive and smooth muscle active peptide that shows gastrin-like bioactivity similar to that of cholecystokinin 8 (CCCK-8), namely, smooth muscle activity, reduction of blood pressure, together with modification of satiety, sedation, and thermoregulation. Many modified caerulein-type peptides also occur in tree frogs, while some of these, *Litoria splendida*, *Litoria rothii*, *and Litoria peronii* change their caerulein content seasonally. The preprocaerulein sequence from *L. peronii* is different from those of other active skin peptides from tree frogs of the genus *Litoria*, suggesting that the ancestral gene producing caerulein is different from that producing other active peptides from *Litoria*.

INTRODUCTION

Amphibians have rich chemical arsenals that form an integral part of their defense systems and also assist with the regulation of dermal physiological action. In response to a variety of stimuli, host-defense compounds including peptides are secreted from specialized glands onto the dorsal surface and into the gut. The peptides exhibit a range of bioactivities, including antimicrobial, anticancer, antiviral, antifungal, and smooth muscle activity.[3,11,18]

Skin secretions may be obtained by electrical stimulation of the glands on the dorsal skin.[23] This benign process may be repeated at monthly intervals and does not harm the frogs. The active form of the peptide is stored in inactive form [as the pronative peptide (derived from the original prepropeptide or signal-spacer-peptide)] in the skin gland and is released in active form onto the skin with the spacer section removed. Often, some peptides, particularly antimicrobial peptides, are cytotoxic to the frog and after a suitable period on the skin (usually about 5–10 min) a protease degrades the active peptide to deactivate it.[18,19] In the particular case of the tree frog *Litoria splendida*, the two major peptides formed in the breeding season are caerulein and the antimicrobial caerin 1.1 hinged peptide [GLLSVLGSVAKHVLPHVVPVIAEHL-NH_2], and the frog can deactivate these peptides by removing the sulfate from caerulein and clipping off the first two amino acid residues (GL) from caerin 1.1.[18] Normally, the bioactive peptides may be identified using HPLC separation of the components of one secretion from one animal. The sequence of each peptide is usually identified by a combination of Edman sequencing and electrospray mass spectrometry. Caerulein-type molecules have a posttranslationally modified N-terminal group, which means that Edman sequencing is not appropriate. Caerulein also has a Tyr (SO_3H) group; the sulfate group is labile using positive ion mass spectrometry, and negative ion mass spectrometry should be used to identify such peptides.[6]

THE CAERULEIN PEPTIDES

Caerulein (Table 1) was first isolated from dried skins of the common Australian Green Tree Frog (*Litoria caerulea*) by Erspamer and colleagues in 1968[2] and subsequently from the African Clawed Frog (*Xenopus laevis*) and *Leptodactylus labyrinthicus*.[1] Erspamer's early survey of bioactive peptides from anurans leads to the isolation of phyllocaerulein from the South American hylid frog *Phyllomedusa sauvagei*, Asn^2Leu^3 caerulein from the South African hyperoliid frog *Hylambates maculata*, [$Glu(OMe)^2$ caerulein from *Nyctimystes disrupta* and Leu^3 caerulein 3–10 from the Philippine ranid frog *Rana erythraea* (for sequences, see Table 1).[11] Many caerulein precursor fragments have recently been identified from several other *Xenopus* species.[8] Caerulein has been identified, after electrical stimulation of the skin glands, in the following *Litoria* species: *L. aurea*, *L. dahlii*, *L. ewingi*, *L. genimaculata*, *L. gilleni*, *L. gracilenta*, *L. infrafrenata*, *L. lesueuri*, *L. raniformis*, *L. splendida*, *L. subglandulosa*, and *L. xanthomera*.[3] More recently, caerulein together with the modification caerulein 1.2 (see Table 1) have been identified from *L. splendida* in winter[27] and also *L. rothii*.[22] A variety of modified caerulein-type peptides (see Table 1) have been isolated from *L. citropa*[26] and *L. subglandulosa*.[7]

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00048-8
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TABLE 1 The Caerulein Family of Neuropeptides and some Mammalian Analogs*

Name	Sequence	Species
Caerulein	pEQDY(SO_3H)TGWMDF-NH_2	various *Litoria*
		Xenopus laevis
		Leptodactylus labyrinthicus
Caerulein 1.2	pEQDY(SO_3H)TGWFDF-NH_2	*L. splendida*
		L. rothii
		L.c., L. sub
Phyllocaerulein	pEEY(SO_3H)TGWMDF-NH_2	*Phyllomedusa sauvagei*
[Asn^2Leu^5]caerulein	pENDY(SO_3H)LGWMDF-NH_2	*Kassina maculata*
[$Glu(OMe)^2$]caerulein	pEE(OMe)DY(SO_3H)TGWMDF-NH_2	*Nyctimystes disrupta*
[Leu^3]caerulein (3-10)	DY(SO_3H)LGWMDF-NH_2	*Rana erythraea*
Caerulein 2.1	pEQDY(SO_3H)TGAHMDF-NH_2	*L.c., L. sub*
Caerulein 2.2	pEQDY(SO_3H)TGAHFDF-NH_2	"
Caerulein 3.1	pEQDY(SO_3H)GTGWMDF-NH_2	"
Caerulein 3.2	pEQDY(SO_3H)GTGWFDF-NH_2	"
Caerulein 4.1	pEQDY(SO_3H)TGSHMDF-NH_2	"
Caerulein 4.2	pEQDY(SO_3H)TGSHFDF-NH_2	"
Mammalian analogs of caerulein		
Cholecycstokinin 8	DY(SO_3H)MGWMDF-NH_2	
Hexagastrin	Y(SO_3H)GWMDF-NH_2	
Eugenin	pEQDY(SO_3H)VFMHPF-NH_2	*Macropus eugeni*

**Added in proof. Caerulein B1 [pEQDY(SO_3)GTGWMDF(NH_2)] and caerulein B2 [pEDY(SO_3)TGWMDF(NH_2)] have been isolated from the secretions of the African frogs* Xenopus borealis *and* Xenopus amieti. *[Zahid OK, Mechkarska M, Ojo OO, Abdel-Wahab YHA, Flatt PR, Meetani MA, Conlon JM. Caerulein and xenopsin-related peptides with insulin-releasing activities from skin secretions of the clawed frogs* Xenopus borealis *and* Xenopus amieti *(Pipidae). Gen Compar Endocrinol 2–11;172:314–320].*

Caerulein is similar in sequence to mammalian cholecystokinin 8 (CCK-8)[10] and hexagastrin[15] and the immunomodulator from the pouch of the Tammar wallaby *Macropus eugenii*[4] carrying young. The sequences of these mammalian peptides are listed in Table 1.

Caerulein is stored as inactive pro (spacer) caerulein in the skin glands and when the frog is attacked or is sick, a protease clips off the spacer peptide and the active caerulein is exuded onto the skin of the frog. Preprocaerulein has been sequenced using mRNA and cDNA methods,[20,21,28] and most recently from *Litoria peronii*.[14] Three caerulein clones of very similar amino acid sequence were identified from *L. peronii*; the amino acid sequence of one of these is shown in Table 2. This is compared with the sequence of signal-spacer-caerin 1.1 (preprocaerin 1.1),[24] whose pre (signal) sequence is conserved with those of a variety of other antimicrobial peptides from *Litoria*[22] together with a range of host-defense peptides from some American and European hylid and ranid frogs.[24] It is clear from the data shown in Table 2 that caerulein and caerin 1.1, although present in the secretions of the same frogs, arose via different ancestor genes.

During our initial studies, we assumed that the peptide profiles from the skin glands of tree frogs of the genus *Litoria* were essentially constant throughout the year. That this is not so was demonstrated during a monthly, 3-year study of male and female *L. splendida*, where it was shown that the male sex pheromone splendipherin [GLVSSIGKALGGLLADVVKSKGQPA-OH] was only present in the male secretion during the breeding season, and that the caerulein content varied, for both male and female, seasonally. In the reproductive (summer) season caerulein is the major

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TABLE 2 Amino Acid Sequences of the Signal-Spacer-Active Peptides (from cDNA Sequencing) of Caerulein (from *Litoria peronii*)[5,14] and Caerin 1.1 (from *Litoria caerulea*)[14,24]

MDKKGCVIMLLAMLATAAIC	Signal	Caerulein
MASLKKSLFVLLLGFVSVSIC	Signal	Caerin1.1
RPISEPEEARRDLVKKELAEL-HEAVRDMND	Spacer	Caerulein
EEEKKQEDEDEHEEEGESQEE-GSEEHR	Spacer	Caerin1.1
QQDYTGWMDF-NH_2		Caerulein
GLLSVLGSVAKHVLPHVVPVIA-EHL-NH_2		Caerin1.1

hypertensive, analgesic, and smooth muscle active component. In the winter (inactive season) at least half of the caerulein is converted into the less active desulfated caerulein [pEQDYTGWMDF-NH_2] and a new caerulein-type peptide (caerulein 1.2) is produced where the Met8 of caerulein is replaced with Phe8.[27]

The related *L. citropa* and *L. subglandulosa* have quite different antibiotic peptides compared with those of *L. splendida*. However, although the seasonal changes in caeruleins of these species are complex, they follow the same trend as that outlined above for *L. splendida*. Both species form many caeruleins in summer, namely, caerulein itself, together with caerulein 2.1, 3.1, and 4.1 (see Table 1). In winter, significant amounts of these four caeruleins are hydrolyzed to their desulfated forms, and four new caeruleins are produced (caeruleins 1.2, 2.2, 3.2, and 4.2). In all cases, Met8 has been replaced by Phe8. Their structures have been confirmed by negative ion electrospray mass spectrometry.[26,7] Why these two species of frog require such a complex cocktail of caerulein-type peptides is not clear.

The most complicated scenario for a species of the genus *Litoria* that has so far been studied occurs for *L. rothii.* In this case, both the antibiotic peptides and the smooth muscle active peptides vary seasonally.[22] Caerulein and caerulein 1.2 (Phe8 caerulein) are the major-smooth muscle active peptides in the summer. Caerulein is a very minor component in the winter: major "neuropeptide-type" components identified in the winter are caerulein 1.2 (Phe8 caerulein) and the unrelated immunomodulator rothein 1 (SVSNIPESIGF-OH).[22]

To complicate the situation even further, the related species *L. peronii*[5] varies its antibiotic peptides seasonally but keeps its "neuropeptide"—content constant. In this case, caerulein itself is present together with a range of small smooth muscle active peptides, for example, peroniin 1 (pEPWLPFG-NH_2), which shows moderate activity to 10^{-7} M.

The question here is "Why do some tree frogs markedly change the activity of their 'neuropeptides' from the reproductive season (summer) to the inactive season (winter)?" The answer presumably lies in the activities of these peptides, particularly the caeruleins. Caerulein is a primary host-defense peptide, and it also regulates dermal physiological action in the frog. It exhibits a spectrum of activity similar to those of the mammalian intestinal hormones gastrin and cholecystokinin (CCK). There is an extensive literature concerning the pharmacology of caerulein and CCK-8: for a detailed record, see the comprehensive and definitive review by Erspamer.[11] Both CCK-8 and caerulein contain a tyrosine sulfate residue; the bioactivity is significantly reduced if the sulfate group is removed. So hydrolysis of the sulfate is a method of reducing the activity of caerulein, particularly, if after exposure on the skin, caerulein shows some cytotoxicity toward the host anuran. Caerulein induces potent smooth muscle contraction at better than the nanomolar concentration; has gastrin-like activity; modifies satiety, sedation, and thermoregulation; and reduces blood pressure at less than nanograms per kilogram of body weight. Caerulein is recorded to be an analgesic 100–1000 times more potent than morphine: the particular figure depends on the biological system being tested. Caerulein has been used clinically before gall bladder surgery is performed.[11] During the last decade, there have been >300 references in the literature dealing with caerulein induced pancreatitis, mainly in mice and rats: this is beyond the scope of this article.

Of the caeruleins 1–4, apart from caerulein itself, only caerulein 1.2 has been tested for smooth muscle activity. The results indicate that changing Met8 of caerulein to Phe8 of caerulein 1.2 reduces the smooth muscle activity by some 50%.[22] No activity testing of the caeruleins 2, 3, and 4 has been undertaken. Frogs removing the sulfate of caerulein and converting caerulein to caerulein 1.2 in winter are reducing the caerulein smooth muscle activity seasonally from summer to winter. No comment can be made on possible changes in other activities.

CCK-8 and caerulein bind to G protein-coupled CCK receptors.[25] There are two types of CCK receptor: CCK_1 and CCK_2—differing in anatomical location and actions.[9] CCK_1 receptors are present on smooth muscle, and when activated, contract smooth muscle directly. In contrast, CCK_2 receptors act indirectly, causing release of acetylcholine from cholinergic nerves on the myenteric plexus, the acetylcholine then activates muscarinic receptors on smooth muscle. The sequences of the CCK receptors are known,[16] and models of their 3D structures have been reported.[16,17] Both NMR and other experimental data have been used to determine where the partial helix CCK-8 binds to these receptors,[12,13,16,17] but similar data are not available

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for caerulein. Caerulein and CCK-8 are able to act through both CCK_1 and CCK_2 receptors, whereas caerulein 1.2 (and rothein 1) act solely through CCK_2 receptors.[22]

ACKNOWLEDGMENTS

The Australian Research Grants Council funds our research on amphibian peptides and mass spectrometry. Associate Professor M.J. Tyler provided the glandular secretions of the *Litoria* species mentioned in this article.

REFERENCES

1. Anastasi A, Bertaccini C, Cei JM, DeCaro G, Erspamer V, Impicciatore M. Presence of caerulein in extracts of the skin of *Leptodactylus labyrinthicus labyrinthicus* and *Xenopus laevis*. *Br J Pharmacol* 1970;**38**:221–8.
2. Anastasi A, Erspamer V, Endean R. Isolation and amino acid sequence of caerulein, the active peptide of *Hyla caerulea*. *Arch Biochem Biophys* 1968;**125**:57–68.
3. Apponyi MA, Pukala TL, Brinkworth CS, Maselli VM, Bowie JH, Tyler MJ, et al. Host-defence peptides of Australian anurans: structure, mechanism of action and evolutionary significance. *Peptides* 2004;**25**:1035–54.
4. Baudinette RV, Boontheung P, Musgrave IF, Wabnitz PA, Maselli VM, Skinner J, et al. Eugenin: an immunomodulator used to protect young in the pouch of the Tammar wallaby, *Macropus eugenii*. *FEBS J* 2005;**272**:433–43.
5. Bilusich D, Jackway RJ, Musgrave IF, Tyler MJ, Bowie JH. The host defence skin peptide profiles of Peron's Tree Frog *Litoria peronii* in winter and summer. Sequence determination by electrospray mass spectrometry and activities of the peptides. *Rapid Commun Mass Spectrom* 2009;**23**:2628–36.
6. Boontheung P, Alewood PF, Brinkworth CS, Bowie JH, Wabnitz PA, Tyler MJ. The negative ion electrospray mass spectra of caerulein neuropeptides. An aid to structure determination. *Rapid Commun Mass Spectrom* 2002;**16**:282–6.
7. Brinkworth CS, Pukala TL, Bowie JH, Doyle JR, Tyler MJ, Llewellyn LE, et al. Host defence peptides from the skin glands of Australian amphibians. Caerulein neuropeptides and antimicrobial, anticancer and nNOS inhibiting citropins from the glandular extracts of *Litoria subglandulosa*. *Aust J Chem* 2004;**57**:693–701.
8. Conlon JM, Al-Ghaferi N, Ahmed E, Meetani MA, Leprince J, Nielsen PF. Orthologs of magainin, PGLa, procaerulein-derived, and proxenopsin-derived peptides from skin secretions of the octoploid frog *Xenopus amieti* (Pipidae). *Peptides* 2010;**31**:989–94, and references cited therein.
9. Cuq P, Gross A, Terraza A, Fourmy D, Clerk P, Dornand J, et al. mRNAs encoding CCKB but not CCKA receptors are expressed in human T lymphocytes abd Jurgat lymphoblastoid cells. *Life Sci* 1997;**61**:543–55.
10. Dockray GJ. Immunological evidence of CCK-like peptides in brain. *Nature* 1976;**264**:568–70.
11. Erspamer V. Bioactive secretions of the amphibian integument. In: Heatwole H, Barthalmus GT, editors. *Amphibian biology: the integument*, vol. 1. Norton-Chipping, NSW: Surry Beatty and Sons; 1994. p. 178–350.
12. Giragossian C, Mierke DF. Intermolecular interactions pf CCK-8 and the third extracellular loop of the CCKA receptor. *Biochem* 2001;**40**:3804–9.
13. Giragossian C, Mierke DF. Intramolecular interactions between CCK-8 and the third extracellular loop of CCKB. *Biochem* 2002;**41**:4560–6.
14. Jackway RJ, Pukala TL, Donnellan SC, Sherman PJ, Tyler MJ, Bowie JH. Skin peptide and cDNA profiling of Australian anurans: genus and species identification and evolutionary trends. *Peptides* 2011;**32**:161–72.
15. Matsumoto M, Park J, Sugano K, Yamada T. Biological activity of progastrin post-translational processing intermediates. *Am J Physiol* 1987;**252**:G315–9.
16. Noble F, Wank SA, Crawley JN, Bradwejn J, Seroogy KB, Hamon M, et al. Structure distribution and functions of cholecystokinin receptors. *Pharmacol Rev* 1999;**51**:745–81.
17. Pellegrini M, Mierke DF. The complex of CCK-8 and the N-terminus of CCKA receptor by NMR spectroscopy. *Biochem* 1999;**38**:14775–83.
18. Pukala TL, Bowie JH, Maselli VM, Musgrave IF, Tyler MJ. Host-defence peptides from the glandular secretions of amphibians: structure and activity. *Nat Prod Rev* 2006;**23**:368–93.
19. Resnick NM, Maloy WL, Guy HR, Zasloff M. A novel endoprotease from *Xenopus* that recognises a helical secondary structure. *Cell* 1991;**66**:41–544.
20. Richter K, Aschauer H, Kreil G. Biosynthesis of peptides from the skin of *Xenopus laevis*; isolation of novel peptides predicted from the sequence of cloned cDNAs. *Peptides* 1985;**6**(Suppl. 3):17–21.
21. Richter K, Egger R, Kreil G. Sequence of preprocaerulein from cDNAs cloned from the skin of *Xenopus laevis*: a small family of precursors containing one, three and four copies of the final product. *J Biol Chem* 1986;**261**:3676–80.
22. Sherman PJ, Jackway RJ, Nicholson EL, Musgrave IF, Boontheung P, Bowie JH. Activities of seasonably variable caerulein and rothein skin peptides from the tree frogs *Litoria splendida* and *Litoria rothii*. *Toxicon* 2009;**54**:828–35.
23. Tyler MJ, Stone DJM, Bowie JH. A novel method for the release and collection of dermal, glandular secretions from the skin of frogs. *J Pharm Toxicol Methods* 1992;**28**:199–200.
24. Vanhoye D, Brustion F, Nicolas P, Amiche M. Antimicrobial peptides from hylid and ranid frogs originated from a 150-million-year-old ancestral precursor with a conserved signal peptide but hypermutable antimicrobial domain. *Eur J Biochem* 2003;**270**:2068–81.
25. Varga G, Balint A, Burghardt B, D'Amarto M. Involvement of endhenous CCK and CCK1 receptors in colonic motor function. *Br J Pharmacol* 2004;**141**:1275–84.
26. Wabnitz PA, Bowie JH, Tyler MJ. Caerulein like peptides from the skin glands of the Australian Blue Mountains tree frog *Litoria citropa*. Sequence determination using electrospray mass spectrometry. *Rapid Commun Mass Spectrom* 1999;**13**:2498–502.
27. Wabnitz PA, Bowie JH, Tyler MJ, Wallace JC, Smith BP. Differences in skin peptides of the male and female Australian Magnificent Tree Frog *Litoria splendida*—a three year monitoring survey: the discovery of the aquatic male sex pheromone splendipherin, together with Phe^8 caerulein and the antibiotic caerin 1.10. *Eur J Biochem* 2000;**267**: 269–75.
28. Wakabayashi T, Kato H, Tachibana S. An unusual repetitive structure of caerulein mRNA in the skin of *Xenopus laevis*. *Gene* 1984;**31**:255–9.

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Chapter 49

Chromogranins/ Secretogranins

Maité Montero-Hadjadje, Charlène Delestre, Maité Courel and Youssef Anouar

ABSTRACT

Chromogranins/secretogranins (Cg/Sg) belong to a class of acidic, secretory proteins that occur in endocrine, neuroendocrine, and neuronal cells. Cloning and characterization of chromogranin A, chromogranin B, and secretogranin II revealed that selective sequences within these proteins, bounded by potential processing sites, have been remarkably conserved between amphibian and mammalian species. Peptides such as secretoneurin, vasostatin, and serpinin, which occur in these regions, exert several biological activities. Cg/Sg may also be involved in the formation of secretory granules in amphibians as in mammals. Recent results obtained on the function of Cg/Sg as propeptide precursors and as granulogenic factors in different vertebrate species are discussed.

DISCOVERY

The chromogranin story started with the biochemical studies performed in the midsixties on chromaffin granules that were aimed at understanding the physiology and the pharmacology of catecholamines. Thus, analysis of the secretion of catecholamines from chromaffin cells revealed the existence of a granule-specific, high-molecular-weight protein coreleased with the hormones on splanchnic nerve stimulation.[20] The term chromogranin A (CgA) was coined for this abundant secretory granule protein of chromaffin cells. Subsequently, hormone and neurotransmitter-containing organelles have been characterized in other tissues, and CgA was generally found in these organelles.[3,20] In fact, a large family of chromogranins corresponding to acidic, heat-stable, and soluble proteins has been characterized in the secretory granules of endocrine, neuroendocrine, and neuronal cells where these proteins are stored and released with hormones, peptides, and neurotransmitters in response to a variety of stimuli. Chromogranin B (CgB) was the second member of this family that was also identified in chromaffin cells, while the third chromogranin-like protein was first characterized in anterior pituitary cells and initially named chromogranin C. However, this protein is now known as secretogranin II (SgII) because it exhibits no sequence homologies with CgA and CgB, which are structurally related to each other.[20] This class of acidic, secreted proteins present in secretory granules is currently known as the granin family and has expanded to include other more recently described members such as secretogranins III to VI also called 1B1075, HISL-19, 7B2, and NESP55, respectively.[3]

STRUCTURE AND PROCESSING OF THE PRECURSORS

An important breakthrough in the field of the granins was the elucidation of their primary structures that were deduced from the cloning of the corresponding cDNAs in the mideighties.[20] It was noticed that CgA encompasses in its sequence numerous pairs of basic amino acids that are potential cleavage sites for prohormone convertases (Table 1). In addition, sequence comparisons showed that CgA may represent the precursor of a peptide named pancreastatin (PST) that has been previously isolated from the porcine pancreas and shown to inhibit glucose-induced insulin secretion.[20] These observations suggested that, akin to the precursors of hormones and neuropeptides, CgA could be a prohormone precursor that is processed to give rise to smaller bioactive peptides such as PST.[20] It was subsequently found that CgB, SgII, and all the granins identified to date contain multiple dibasic sites, further supporting the notion that granins may serve as precursors of biologically active peptides. Molecular cloning of granins in mammals also reveals the high content of acidic amino acids, which explains the acidic nature of these proteins (Table 1). This characteristic underlies the propensity of granins to aggregate in high calcium and acidic pH conditions, which are found in the intragranular milieu. It was thus proposed that granins may participate in the sorting and packaging of hormones and neuropeptide precursors into the secretory granules.[6]

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00049-X
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TABLE 1 Comparison of the Physicochemical Properties of Human and Frog Granins

	CgA		CgB		SgII	
	Human	Frog	Human	Frog	Human	Frog
Residues						
Precursor	457	399	677	599	617	601
Mature protein	439	381	657	580	590	574
Molecular mass (kD)*						
Calculated	49	44	76	70	68	67
Apparent	74	64	120	ND	86	98
Acidic residues (% E+D)	21 + 4	21 + 9	18 + 6	21 + 7	13 + 7	12 + 8
Isoelectric point (pHi)	4.37	4.31	4.83	4.95	4.47	4.57
Multibasic sites†	10	11	15	20	9	11
Disulfide bonded loop	17–38	17–38	16–37	16–37	No	No

CgA, chromogranin A; CgB, chromogranin B; SgII, secretogranin II; ND, not determined.
The molecular mass was deduced from the primary structure. The apparent molecular mass was determined by SDS-polyacrylamide gel electrophoresis.
†Multibasic sites refer to sites with two or more consecutive R or K residues.

Interest in granins in nonmammalian species started when a 33-amino-acid peptide that derives from the endoproteolytic cleavage of SgII was isolated from the frog brain.[29] The characterization of the first granin-derived peptide in a nonmammalian vertebrate, whose sequence has been highly conserved between frog and mammals, reinforced the idea that granins could be considered as propeptide precursors. A homologous peptide was subsequently characterized in mammalian species and named secretoneurin (SN) based on its abundance in the brain and its effects on neuronal activity.[10] It was then hypothesized that the molecular characterization of granins in nonmammalian species would allow the delineation of the sequences that have been preserved during evolution and that may represent the functional determinants of these proteins.

The cloning of the first nonmammalian granins CgA, CgB, and SgII was undertaken in the frog *Rana ridibunda*. Amphibians, which are the first vertebrates that have evolved to a terrestrial life, are a group of particular interest in which to investigate the neurochemical aspects of evolution. Because there was the possibility that the sequences of granins could considerably vary in frog, cloning of short DNA fragments corresponding to SN or the C-terminal regions of CgA and CgB that are strongly conserved in mammals was performed. These short sequences were amplified from the endocrine tissues of frog such as the pituitary or the adrenal and used as probes to isolate the full-length sequences encoding granins from a pituitary cDNA library.

The deduced frog CgA, CgB, and SgII amino acid sequences all contain signal peptides, which would be cleaved to generate mature proteins comprising 381, 580, and 574 amino acids, respectively.[1,2,27] As their mammalian homologs, frog granins are characterized by a high content of acidic amino acids and the occurrence of multiple dibasic cleavage sites, and the existence of two cysteine residues at the N-terminal part of CgA and CgB that have been shown to form a disulfide loop in mammals (Table 1). The abundance of acidic residues in frog granins largely explains the high apparent molecular weight observed for these proteins on SDS–polyacrylamide gel electrophoresis, as previously reported for their mammalian homologs.[20] However, the overall sequence identity for each of these proteins with the corresponding mammalian ortholog does not exceed 40–50% (Fig. 1). This is even more striking if the specific regions of these proteins are compared between mammals and frog. Thus, although certain discrete regions of frog granins display strong sequence conservation with the mammalian counterparts, the majority of the sequences of these proteins exhibit a rather loose homology between the two vertebrate groups. Interestingly, the sequences with high identity between amphibians and mammals are bounded by dibasic cleavage signals (Fig. 1), suggesting that these regions can be processed to generate peptides that may occur ubiquitously, at least in vertebrate species. The most highly conserved region in SgII is that encompassing SN and a flanking peptide (Fig. 1A) that was characterized in various mammalian species and named EM66 in consideration of its N- and C-terminal residues, and its size of 66 amino acids.[19] Cloning of SgII from another amphibian species, *Xenopus laevis*, from goldfish, from chicken,

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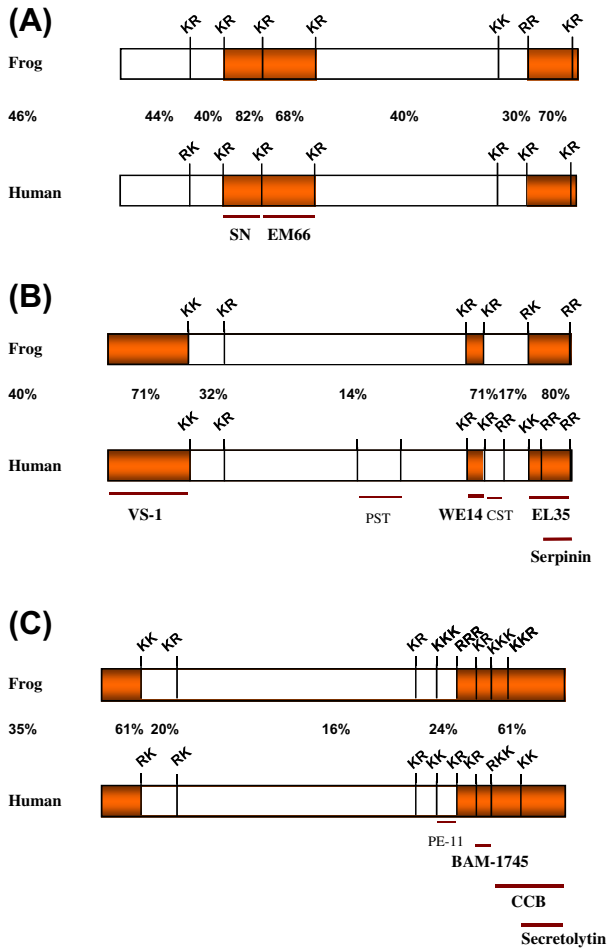

FIGURE 1 Comparison of the primary structures and the putative processing signals of human and frog SgII (A), CgA (B), and CgB (C). The shaded regions represent the highly conserved peptides that can be potentially generated by proteolytic cleavage at the dibasic sites. The percentages of identity between the amino acid sequences of frog and human granins are indicated. Conserved peptides are indicated in bold.

and from lizard[14,28] confirmed the sequence conservation of these peptides.

Within CgA and CgB, the most highly conserved regions occur at the N and C termini of the proteins, which show up to 80% sequence identity, whereas the rest of the two granins markedly diverge; the middle part of these proteins exhibits approximately 20% identity (Fig. 1B and C, see the supplemental disk). It has been shown that CgA is processed in a tissue-specific manner to yield several peptides, some of which are endowed with biological activities (see below). These include vasostatins (VS-1 and VS-2), which exert various effects in different model systems (see below), catestatin (CST), which has been shown to inhibit nicotine-evoked catecholamine release from chromaffin cells, and WE14, a peptide with no known function.[20] As depicted in Fig. 1B (see the supplemental disk), the sequences of VS, WE14 and additional peptides named EL35[20] and its C-terminal part serpinin[13] were preserved during evolution, whereas the region encoding PST in mammals has no homologous sequence in the frog protein and that encoding catestatin is not well conserved in the amphibian species. Several peptides that derive from the C-terminus of CgB have also been isolated and characterized (Fig. 1C). However, apart from a 13-amino-acid peptide purified from the adrenal medulla that exerts an antibacterial effect and named secretolytin,[17] no biological activity was attributed to these peptides.

Sequence comparisons confirm that CgA and CgB have probably evolved from an ancestral common precursor because the N- and C-terminal regions of these proteins display 36 and 45% sequence identity,[1] respectively, as previously reported for the mammalian proteins.[20]

DISTRIBUTION AND REGULATION OF THE mRNAs

The tissue distribution of granins has been extensively studied in several mammalian species. The majority of endocrine and neuroendocrine cells express at least one member of this family of acidic proteins. Pituitary cells, C cells of the thyroid, parathyroid cells, chromaffin cells of the adrenal medulla, and neuroendocrine cells of the gastrointestinal and pulmonary tracts produce and store granins in their secretory granules. In the central and peripheral nervous systems, granins are widely expressed with highest concentrations occurring in certain brain structures such as the hypothalamus, hippocampus, amygdala, and cerebellum.[3] The relative abundance of granins varies in a tissue- and species-specific manner. For instance, SgII is highly expressed in gonadotrophs compared with other pituitary cells or in glucagon-producing alpha cells compared with the other pancreatic endocrine cell types. Human CgA is predominantly expressed in the adrenal medulla, followed by the pituitary and the gastrointestinal tract.[3]

Although granins or granin-like molecules have been detected by immunocytochemistry in various nonmammalian species including birds, fish, flies, and even a unicellular eukaryotic organism, the paramecia, a precise mapping of the sites of synthesis of these proteins has awaited their cloning in frog.[20] Thus, northern blot, reverse transcription-polymerase chain reaction and in situ hybridization analyses provide the first overall mapping of granins in a nonmammalian species.[1,2,27] These studies show that granins are also widely expressed in endocrine, neuroendocrine, and neuronal cells in a nonmammalian vertebrate. Equivalent high levels of SgII mRNA are found in the frog brain, pituitary, and spinal cord, and moderate levels are detected in the adrenal gland. No expression could be seen in the pancreas, stomach, intestine, and kidney using northern blot analysis.

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The CgA gene is more strongly expressed in the frog pituitary than in the brain, spinal cord, or adrenal. Finally, the CgB transcript is abundantly expressed in various organs such as the brain, spinal cord, pituitary, adrenal, pancreas, and heart. In the frog brain, all three granins exhibit a widespread distribution (Fig. 2). In situ hybridization has shown that SgII expression is prominent in the pallium, the amygdaloid complex, and the hypothalamus. CgA mRNA is intensely expressed in thalamic and hypothalamic regions, whereas strong expression of CgB is observed in the lateral pallium, the dorsal hypothalamus, and the anteroventral tegmental area (Fig. 2A and B). Overall, the tissue-distribution studies in frog reveal that SgII is more highly expressed in neuronal tissues, that CgA is strongly expressed in the pituitary compared with all other tissues and that CgB exhibits a more diffuse expression.

Within the frog pituitary, CgA and SgII are present in both the distal and the neurointermediate lobes, whereas CgB only occurs in the pars distalis (Fig. 2B). In amphibians, the intermediate lobe of the pituitary is responsible for the synthesis of melanotropic peptides that induce darkening of the skin, allowing the animals to adapt to the color of the environment and therefore to hide from predators. Melanotrophs of the pars intermedia represent a valuable model in which the neuroendocrine regulation of hormone synthesis and release can be studied in physiological conditions. Thus, the biosynthesis of melanotropic peptides in these cells is tightly regulated through the control of the transcriptional activity of the proopiomelanocortin (POMC) gene, mainly by hypothalamic input.[30] Both SgII and CgA mRNA levels[28] are upregulated in parallel with those of POMC in the intermediate lobe after adaptation to a dark background, and conversely, the levels of these transcripts are downregulated when the animals are placed on a white background. We have observed that CgA and SgII are differentially expressed and processed in frog melanotrophs depending on the status of the secretory activity of these cells: although both SgII and CgA are elevated *in vivo* in dark background-adapted animals, the two proteins could play rather different roles in the regulated secretory pathway in melanotrophs. Thus, CgA expression and processing may be related to hormone storage in secretory granules, whereas SgII production and processing could be part of the active process of hormone release from melanotrophs. Further studies using this model are warranted to better understand the coordinate regulation of the SgII and CgA genes with the POMC gene during the neuroendocrine reflex of skin color adaptation in amphibians.

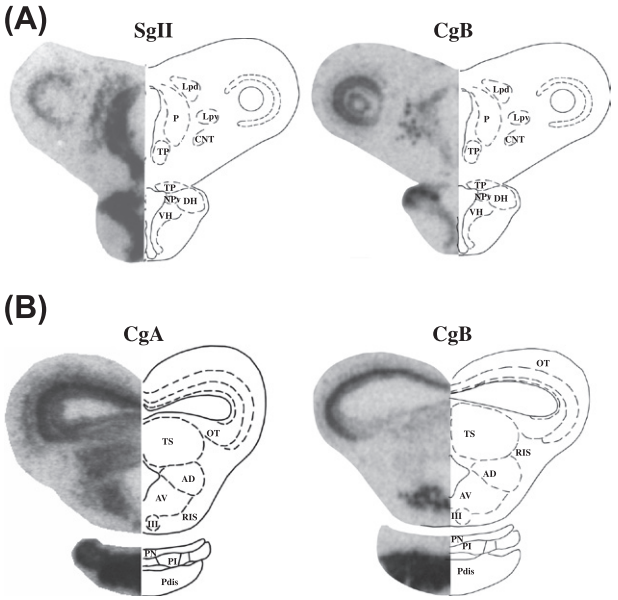

FIGURE 2 In situ hybridization histochemistry of granin mRNA in the brain and the pituitary of the frog *Rana ridibunda*. The left hemisections show autoradiograms obtained from frontal frog brain slices at the level of the diencephalon (A) and mesencephalon (B), hybridized with the CgA, CgB, and SgII antisense probes. The right hemisections designate the anatomical structures. Abbreviations: AD, anterodorsal tegmental nucleus; AV, anteroventral tegmental nucleus; CNT, central thalamic nucleus; DH, dorsal hypothalamic nucleus; Lpd, lateral thalamic nucleus, posterodorsal division; Lpv, lateral thalamic nucleus, posteroventral division; III, oculomotor and trochlear nuclei; NPv, nucleus of the periventricular organ; OT, optic tectum; P, posterior thalamic nucleus; Pdis, pars distalis; PI, pars intermedia; PN, pars nervosa; RIS, nucleus reticularis isthmi; TP, posterior tuberculum; TS, torus semicircularis; VH, ventral hypothalamic nucleus.

BIOLOGICAL ACTIONS

The search for the roles of granins in mammals has focused on their intracellular implication in the packaging of hormones and the formation of secretory granules, and on their extracellular activities as precursors of several peptides that could be released to exert hormonal effects in autocrine, paracrine, and endocrine manners.[3]

Intracellular Functions

Because granins are localized in secretory granules of virtually all neuroendocrine cells and because several studies have shown that these proteins aggregate in intravesicular conditions of acidic pH and high calcium, it has been proposed that they may play a fundamental role in granulogenesis.[6] Direct evidence in support of this hypothesis was lacking for several years until the demonstration by Kim et al.[12] of a pivotal role of CgA in the biogenesis of secretory vesicles in neuroendocrine cells. Inhibition of the expression of CgA in PC12 cells leads to a marked decrease in secretory granule formation and to impairment of the release of a transfected hormone. Subsequent studies revealed that CgB[11] and SgII[4] are also efficient in inducing the formation of secretory granule-like structures in non-neuroendocrine cells that are normally devoid of a regulated

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pathway of secretion, suggesting that granins may play a crucial role in the generation of secretory vesicles. In addition, stored SgII could be released in a regulated manner from transfected, constitutively secreting cells.[4] Interestingly, the expression of frog CgA also induced secretory granule-like structures in nonneuroendocrine COS7 cells and the deletion of the conserved terminal domains of fCgA strikingly reduced its granulogenic activity (Fig. 3),[21] suggesting that CgA, through its N- and C-terminal domains could play an intracellular role in secretory granule formation in different vertebrates. In addition, the high content of acidic amino acids in frog CgA, alone or in combination with the conserved N- and C-terminal regions, is probably also required to confer the aggregating properties of this granin. Because all granins contain a high proportion of acidic amino acids in all species studied to date, this structural feature may well represent a critical requirement for the granulogenic effect of these proteins.

Several studies have proposed additional intracellular roles for certain members of the granin family. For instance, CgA and CgB are involved in intracellular calcium (Ca^{2+}) mobilization because these granins bind to the inositol 1,4,5-trisphosphate (IP_3) receptor that is present on the membranes of secretory granules and facilitate Ca^{2+} release in response to IP_3.[33] Finally, a clear intracellular function has been established for the granin-like protein 7B2, which binds and activates PC2 through two domains that have been highly conserved in various species, including the toad *Xenopus laevis*.[16] Most of the functional characteristics of 7B2 have been deduced from initial studies performed in Xenopus melanotroph cells in which this protein is also regulated in concert with POMC during background color adaptation.[16] The introduction of 7B2-knockout mice models has allowed confirmation and extention of these earlier studies.[23] Knockout models for the granins CgA and CgB firmly show the involvement of these proteins in intracellular events associated with hormone and neurotransmitter release.[15,22] Homozygous double mutant CgA/CgB mice are still capable of generating secretory vesicles but with impaired capacity to concentrate catecholamines in chromaffin cells.[8] As SgII has also been shown to play a role in granulogenesis,[7] the generation of a knockout model for SgII will allow elucidation of whether this granin may compensate somehow for CgA and CgB function in secretory vesicle biogenesis.

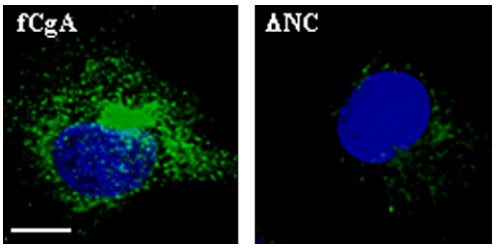

FIGURE 3 Granulogenic activity of frog CgA. COS7 cells transfected with the cDNA encoding full-length CgA (fCgA) or CgA deleted in its N- and C-terminal parts (ΔNC) were incubated with a rabbit antiserum directed against frog CgA ($CgA_{107–234}$) and examined for fCgA immunoreactivity using confocal microscopy. Scale bars, 10 μm. See color plate 14.

Extracellular Functions

The notion that granins may serve as precursors to biologically active peptides has received much attention since the first demonstration that the CgA-derived peptide PST exerts a potent inhibitory effect on insulin release from pancreatic cells.[20] Proteolytic processing of CgA, CgB, and SgII at both monobasic and dibasic residues gives rise to a wide spectrum of peptides, some of which exert biological actions, in particular inhibitory effects on hormone secretion from different endocrine glands.[3] In the context of this review, we will focus our attention only on peptides whose sequence has been conserved between frog and mammals (Table 2), which could be relevant to amphibian physiology. Among the peptides that could act in amphibians based on the remarkable preservation of their sequence, VS inhibits vasoconstriction of arteries in mammalian species.[20] Interestingly, VS and VS-derived peptides may exert cardioregulatory actions in frog.[20] VS-derived peptides promote fibroblast adhesion,[9] affect human colonic motility *in vitro*, trigger neuronal apoptosis, are involved in nociceptive responses in the rat,[3] and regulate tumor neovascularisation.[31] Further, the N-terminal peptides of CgA also exert potent antibacterial and antifungal activities.[17] Besides, a large polypeptide that derives from the highly conserved C-terminus of CgA has been previously isolated and named parastatin in consideration of its effect on parathyroid hormone secretion from parathyroid chief cells.[20] However, no results are currently available on the occurrence of this peptide and its actions in amphibian species. Nevertheless, a C-terminal peptide named EL35 is present in both frog and mammalian CgA and is produced in rat and human tissues,[18,27] suggesting that the processing of the C-terminal region of this granin may give rise to a ubiquitous peptide that may exert biological activities. A peptide encompassed in EL35 sequence (AL26) and named serpinin is secreted from endocrine cells to control granule biogenesis through the regulation of the protease nexin-1.[13]

Although peptides derived from the N and C termini of CgB have also been identified, there are few functional studies using these peptides. In particular, several C-terminal peptides have been isolated from various tissues such as GAWK and CCB, which have been purified from the human pituitary gland, a pyroglutamyl peptide named BAM-1745 that has been identified in bovine adrenochromaffin cells, and an 11-amino-acid peptide named PE-11 that has been characterized in the rat and human brain, but the activity of all these peptides remains unknown.[3] One exception is the

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TABLE 2 Functions of Granin-Derived Peptides Conserved between Frog and Mammals

Chromogranin A-derived peptides	Biological activity of the peptide
Vasostatin-1 (bCgA$_{1–76}$)	Inhibits vasoconstriction; inhibits cardiac performance; promotes fibroblast adhesion; inhibits parathyroid hormone secretion; triggers microglial-cell-mediated neuronal apoptosis; exerts bacteriolytic and antifungal effects; regulates tumor neovascularization.
Parastatin (pCgA$_{347–419}$)	Inhibits parathyroid hormone secretion from parathyroid chief cells.
Serpinin (bCgA$_{403–428}$)	Upregulates the expression of the protease nexin-1 and granule biogenesis in endocrine cells.
Chromogranin B-derived peptides	
Secretolytin (bCgB$_{614–626}$)	Exerts bacteriolytic effects.
Secretogranin II-derived peptides	
Secretoneurin (rSgII$_{154–186}$)	Stimulates luteinizing hormone release from gonadotroph cells and dopamine release from central striatal neurons; promotes chemotactic attraction of monocytes, eosinophils, and fibroblasts; stimulates proliferation and migration of vascular smooth muscle; stimulates migration and inhibits proliferation of endothelial cells; stimulates transendothelial migration of monocytes; activates endothelial cells for neutrophil adherence; facilitates endothelium-dependent relaxation; promotes neurite outgrowth in cerebellar granule cells and neuronal cell survival.

bCgA, bovine chromogranin A; pCgA, porcine chromogranin A; rSgII, rat secretogranin II; bCgB, bovine chromogranin B.

peptide secretolytin, which exerts antibacterial activity.[17] The sequence of the majority of these peptides is highly conserved in the frog protein, but their occurrence as free entities and their function in this species have not been studied.

The SgII-derived peptide SN, initially isolated from the frog brain,[29] has been extensively studied during the last decade.[10] SN has emerged mainly as a peptide that links the nervous and the immune systems. Thus, SN attracts monocytes, eosinophils, and endothelial cells at sites of injury, facilitates endothelium-dependent relaxation, and acts as an angiogenic factor.[5,24] In addition, SN stimulates dopamine release from rat striatal slices, influences neurite outgrowth in cerebellar granule cells,[10] and promotes neuronal survival and plasticity.[26] Signaling mechanisms involved in the effects of SN have been proposed and include activation of phosphoinositide 3-kinase, protein kinase C, and antiapoptotic proteins Bcl-2 and Bcl-xL through the Jak2/Stat3 pathway, as well as blockade of caspase-3 activation.[13,26] Finally, SN exerts stimulatory effects on pituitary luteinizing hormone release from goldfish pituitary and mouse LβT2 gonadotroph cells, which suggests an important role played by SN in reproduction.[34] Specific binding sites for SN have been found in human monocytes and may represent the putative functional plasma membrane receptor for SN, which has not been identified so far.[25] SN exhibits 82% sequence identity between frogs and humans and is the most highly conserved region in SgII, indicating that SN may represent an authentic neuropeptide in various species.

Flanking SN at its C-terminus, the peptide EM66, which occurs in all species studied to date, including the frog *R. ridibunda*[32] [and unpublished observations], is also of potential interest. The concentration of EM66 is modulated by food deprivation in the jerboa hypothalamus, suggesting that this peptide may be involved in the control of food intake and/or the stress associated with fasting.[20]

The functions of the neuroendocrine proteins chromogranins and secretogranins have been intensively investigated during the last 45 years, at least for CgA and CgB, which were the first members of this family that were discovered. Although their physicochemical properties, localization, processing, and regulation are now well known, their function still remains incompletely understood. An intracellular role for these proteins has received strong support during the last few years, and some fundamental notions are now emerging that will probably enhance our understanding of the intracellular intervention of these proteins in hormone storage and release. Besides, the role of granins as precursors to neuropeptides and hormones is an attractive hypothesis that still needs to be substantiated. Several questions related to the mode of action of the derived peptides often remain unanswered. SN,V, and serpinin whose sequences have been preserved during evolution, are highly potent at regulating different systems but the issues of the concentrations used, the existence of specific receptors and the effects of these peptides *in vivo* should be clarified. Other biologically active peptides such as PST and CST may be relevant only in mammals because their sequences are either lacking or poorly conserved in certain vertebrates such as frog, suggesting that these peptides may have emerged late during evolution and/or their sequence has evolved rapidly. Thus, elucidation of the sequences and the properties of granins in frog, and the study of their expression in this model have

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provided insights to help determine the physiological role of these proteins and to decipher the structure–activity relationships that may govern their function in all species.

ACKNOWLEDGMENTS

The work performed by the authors has been supported by INSERM (U982) and the Conseil Régional de Haute-Normandie.

REFERENCES

1. Ait-Ali D, Turquier V, Alexandre D, Grumolato L, Jegou S, Vaudry H, et al. Molecular characterization of frog chromogranin B reveals conservation of selective sequences encoding potential novel regulatory peptides. *FEBS Lett* 2002;**511**(1–3):127–32.
2. Anouar Y, Jegou S, Alexandre D, Lihrmann I, Conlon JM, Vaudry H. Molecular cloning of frog secretogranin II reveals the occurrence of several highly conserved potential regulatory peptides. *FEBS Lett* 1996;**394**(3):295–9.
3. Bartolomucci A, Possenti R, Mahata SK, Fischer-Colbrie R, Loh YP, Salton SR. The extended granin family: structure, function, and biomedical implications. *Endocr Rev* 2011;**32**:755–97.
4. Beuret N, Stettler H, Renold A, Rutishauser J, Spiess M. Expression of regulated secretory proteins is sufficient to generate granule-like structures in constitutively secreting cells. *J Biol Chem* 2004;**279**(19):20242–9.
5. Chan CK, Vanhoutte PM. Secretoneurin facilitates endothelium-dependent relaxations in porcine coronary arteries. *Am J Physiol Heart Circ Physiol* 2011;**300**(4):H1159–65.
6. Chanat E, Huttner WB. Milieu-induced, selective aggregation of regulated secretory proteins in the trans-Golgi network. *J Cell Biol* 1991;**115**(6):1505–19.
7. Courel M, Soler-Jover A, Rodriguez-Flores JL, Mahata SK, Elias S, Montero-Hadjadje M, et al. Pro-hormone secretogranin II regulates dense core secretory granule biogenesis in catecholaminergic cells. *J Biol Chem* 2010;**285**(13):10030–43.
8. Díaz-Vera J, Camacho M, Machado JD, Domínguez N, Montesinos MS, Hernández-Fernaud JR, et al. Chromogranins A and B are key proteins in amine accumulation, but the catecholamine secretory pathway is conserved without them. *FASEB J* 2012;**26**:430–8.
9. Dondossola E, Gasparri A, Bachi A, Longhi R, Metz-Boutigue MH, Tota B, et al. Role of vasostatin-1 C-terminal region in fibroblast cell adhesion. *Cell Mol Life Sci* 2010;**67**(12):2107–18.
10. Fischer-Colbrie R, Kirchmair R, Kähler CM, Wiedermann CJ, Saria A. Secretoneurin: a new player in angiogenesis and chemotaxis linking nerves, blood vessels and the immune system. *Curr Protein Pept Sci* 2005;**6**(4):373–85.
11. Huh YH, Jeon SH, Yoo SH. Chromogranin B-induced secretory granule biogenesis: comparison with the similar role of chromogranin A. *J Biol Chem* 2003;**278**(42):40581–9.
12. Kim T, Tao-Cheng JH, Eiden LE, Loh YP. Chromogranin A, an "on/off" switch controlling dense-core secretory granule biogenesis. *Cell* 2001;**106**(4):499–509.
13. Koshimizu H, Cawley NX, Kim T, Yergey AL, Loh YP. Serpinin: a novel chromogranin A-derived, secreted peptide up-regulates protease nexin-1 expression and granule biogenesis in endocrine cells. *Mol Endocrinol* 2011;**25**(5):732–44.
14. Leitner B, Schneitler C, Klocker H, Volknandt W, Zimmermann H, Winkler H, et al. Formation and sequence analysis of secretoneurin, a neuropeptide derived from secretogranin II, in mammalian, bird, reptile, amphibian and fish brains. *Neurosci Lett* 1998;**248**(2):105–8.
15. Mahapatra NR, O'Connor DT, Vaingankar SM, Hikim AP, Mahata M, Ray S, et al. Hypertension from targeted ablation of chromogranin A can be rescued by the human ortholog. *J Clin Invest* 2005;**115**(7):1942–52.
16. Mbikay M, Seidah NG, Chretien M. Neuroendocrine secretory protein 7B2: structure, expression and functions. *Biochem J* 2001;**357** (Pt 2):329–42.
17. Metz-Boutigue MH, Kieffer AE, Goumon Y, Aunis D. Innate immunity: involvement of new neuropeptides. *Trends Microbiol* 2003;**11**(12):585–92.
18. Montero-Hadjadje M, Vaudry H, Turquier V, Leprince J, Do Rego JL, Yon L, et al. Localization and characterization of evolutionarily conserved chromogranin A-derived peptides in the rat and human pituitary and adrenal glands. *Cell Tissue Res* 2002;**310**(2):223–36.
19. Montero-Hadjadje M, Pelletier G, Yon L, Li S, Guillemot J, Magoul R, et al. Biochemical characterization and immunocytochemical localization of EM66, a novel peptide derived from secretogranin II, in the rat pituitary and adrenal glands. *J Histochem Cytochem* 2003;**51**(8):1083–95.
20. Montero-Hadjadje M, Vaingankar S, Elias S, Tostivint H, Mahata SK, Anouar Y. Chromogranins A and B and secretogranin II: evolutionary and functional aspects. *Acta Physiol (Oxf)* 2008;**192**(2):309–24.
21. Montero-Hadjadje M, Elias S, Chevalier L, Benard M, Tanguy Y, Turquier V, et al. Chromogranin A promotes peptide hormone sorting to mobile granules in constitutively and regulated secreting cells: role of conserved N- and C-terminal peptides. *J Biol Chem* 2009;**284**(18):12420–31.
22. Obermüller S, Calegari F, King A, Lindqvist A, Lundquist I, Salehi A, et al. Defective secretion of islet hormones in chromogranin-B deficient mice. *PLoS One* 2010;**5**(1):e8936.
23. Peinado JR, Laurent V, Lee SN, Peng BW, Pintar JE, Steiner DF, et al. Strain-dependent influences on the hypothalamo-pituitary-adrenal axis profoundly affect the 7B2 and PC2 null phenotypes. *Endocrinology* 2005;**146**(8):3438–44.
24. Schgoer W, Theurl M, Jeschke J, Beer AG, Albrecht K, Gander R, et al. Gene therapy with the angiogenic cytokine secretoneurin induces therapeutic angiogenesis by a nitric oxide-dependent mechanism. *Circ Res* 2009;**105**(10):994–1002.
25. Schneitler C, Kahler C, Wiedermann CJ, Hogue-Angeletti R, Fischer-Colbrie R. Specific binding of a 125I-secretoneurin analogue to a human monocytic cell line. *J Neuroimmunol* 1998;**86**(1):87–91.
26. Shyu WC, Lin SZ, Chiang MF, Chen DC, Su CY, Wang HJ, et al. Secretoneurin promotes neuroprotection and neuronal plasticity via the Jak2/Stat3 pathway in murine models of stroke. *J Clin Invest* 2008;**118**(1):133–48.
27. Turquier V, Vaudry H, Jegou S, Anouar Y. Frog chromogranin A messenger ribonucleic acid encodes three highly conserved peptides. Coordinate regulation of proopiomelanocortin and chromogranin A gene expression in the pars intermedia of the pituitary during background color adaptation. *Endocrinology* 1999;**140**(9):4104–12.
28. Turquier V, Vaudry H, Montero M, Yon L, Anouar Y. Characterization of chromogranins in the frog *Rana ridibunda*. Structure, expression, and functional implications. *Adv Exp Med Biol* 2000;**482**:125–36.

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29. Vaudry H, Conlon JM. Identification of a peptide arising from the specific post-translation processing of secretogranin II. *FEBS Lett* 1991;**284**(1):31–3.
30. Vazquez-Martinez R, Castano JP, Tonon MC, Vaudry H, Gracia-Navarro F, Malagon MM. Melanotrope secretory cycle is regulated by physiological inputs via the hypothalamus. *Am J Physiol Endocrinol Metab* 2003;**285**(5):E1039–46.
31. Veschini L, Crippa L, Dondossola E, Doglioni C, Corti A, Ferrero E. The vasostatin-1 fragment of chromogranin A preserves a quiescent phenotype in hypoxia-driven endothelial cells and regulates tumor neovascularization. *FASEB J* 2011;**25**(11):3906–14.
32. Yon L, Guillemot J, Montero-Hadjadje M, Grumolato L, Leprince J, Lefebvre H, et al. Identification of the secretogranin II-derived peptide EM66 in pheochromocytomas as a potential marker for discriminating benign versus malignant tumors. *J Clin Endocrinol Metab* 2003;**88**(6):2579–85.
33. Yoo SH, So SH, Kweon HS, Lee JS, Kang MK, Jeon CJ. Coupling of the inositol 1,4,5-trisphosphate receptor and chromogranins A and B in secretory granules. *J Biol Chem* 2000;**275**(17):12553–9.
34. Zhao E, McNeilly JR, McNeilly AS, Fischer-Colbrie R, Basak A, Seong JY, et al. Secretoneurin stimulates the production and release of luteinizing hormone in mouse L{beta}T2 gonadotropin cells. *Am J Physiol Endocrinol Metab* 2011;**301**(2):E288–97.

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Chapter 50

Dermaseptins

Pierre Nicolas and Ali Ladram

ABSTRACT

The dermaseptins belong to a superfamily of host defense peptides that are made in the skin of Hylidae frogs. These peptides are genetically related, with a remarkable identity in signal sequences and intervening sequences of their preproforms, but they have markedly diverged to yield several families of peptides that are structurally and functionally distinct. These include the dermaseptins (*stricto sensu*), the phylloseptins, the plasticins, the dermatoxins, the phylloxins, the raniseptins, the caerin-related peptides, the caerins, the fallaxidins, the frenatins, and the aureins. Most of these peptides are cidal against wall-less bacteria, Gram-negative and Gram-positive bacteria, fungi, protozoa, yeast, and enveloped viruses but are weakly toxic against mammalian cells. Their antimicrobial activity results from their capacity to bind to the bacterial plasma membrane, thereby triggering transient wormhole formation or membrane disruption. The dermaseptin superfamily also includes D-amino acid–containing opioid peptides (dermorphins, deltorphins), bradykinin-related peptides, myoactive peptides (hyposins/sauvatide, tryptophyllins), and Bowman–Birk-like trypsin inhibitors.

THE DERMASEPTIN SUPERFAMILY: A LIBRARY OF STRUCTURALLY AND FUNCTIONALLY DISPARATE PEPTIDES ORIGINATING FROM A SINGLE GENE FAMILY

Hylidae (tree frogs) are one of the largest families of frogs. There are about 51 genera that are arranged in three subfamilies: Pelodryadinae, Phyllomedusinae, and Hylinae. The first member of the dermaseptin superfamily, named dermaseptin-S1, was isolated from an extract of dried pieces of skin of *Phyllomedusa sauvagii* (subfamily: Phyllomedusinae) in the early 1990s.[26] This 34-residue peptide has lytic activity against Gram-positive and Gram-negative bacteria, yeast, and protozoa, without harmful effects against mammalian cells. It was the first gene-encoded eukaryotic peptide to show lethal effect against *Leishmania* promastigotes[16] and filamentous fungi that are responsible for opportunistic infections resulting from the immunodeficiency syndrome or the use of immunosuppressive agents. This discovery was followed by the isolation of adenoregulin (also named dermaseptin-B2) from *P. bicolor* skin, a peptide that interacts with the adenosine receptor,[11] and dermaseptin-B1.[27] These 2 peptides were thought to be unrelated until attempts to clone their precursor polypeptides revealed the presence of a common preproregion and 5′- and 3′-UTRs as well as extensive sequence identities with precursors of opioid peptides dermorphin, dermenkephalin, and deltorphins from *Phyllomedusa* skin.[2,3] Since then, additional members of the dermaseptin superfamily were rapidly identified in various species of South American Hylidae frogs and grouped to form distinct families on the basis of their structural characteristics: (1) the dermaseptins (*stricto sensu*) from *Agalychnis annae*, *A. callidryas*, *A. dacnicolor*, *A. lemur*, *A. spurrelli* (*litodryas*), *Phasmahyla jandai*, *Phyllomedusa bicolor*, *P. burmeisteri*, *P. distincta*, *P. hypochondrialis*, *P. oreades*, *P. sauvagii*, *P. tarsius*, *P. tomopterna*, and *P. trinitatis* (Table 1). Most of these peptides share a signature pattern consisting of a conserved Trp residue at position 3 and an AA(A/G)KAAL(G/N)A consensus motif in the midregion; (2) the dermatoxins from *Agalychnis annae*, *A. dacnicolor*, *Phasmahyla jandaia*, *Phyllomedusa bicolor*, *P. sauvagii*, and *P. tarsius* (Table 2); (3) the phylloxins from *Phyllomedusa bicolor* and *P. sauvagii* (Table 3); (4) the phylloseptins from *Agalychnis lemur*, *Phasmahyla jandaia*, *Phyllomedusa bicolor*, *P. burmeisteri*, *P. hypochondrialis*, *P. palliata*, *P. sauvagii*, *P. tarsius*, and *P. tomopterna* (Table 4); (5) the plasticins from *Agalychnis dacnicolor*, *A. annae A. callidryas*, *Phyllomedusa bicolor*, and *P. sauvagii*, which are rich in Gly and Leu residues arranged in regular 5-mer motifs GXXXG (where X is any amino acid residue) (Table 5); (6) the raniseptins from *Hypsiboas raniceps* (Table 6); (7) the caerin-related peptides from *Agalychnis annae* and *A. callidryas* (Table 7); and (8) the orphan peptides from *Agalychnis dacnicolor*, *Phyllomedusa hypochondrialis*, and *P. sauvagii*, which do not resemble any members of the other peptide families (Table 7). As shown in Tables 1–7, different species of hylid frogs are equipped with different sets of peptides

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00050-6
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TABLE 1 The Dermaseptin (*stricto sensu*) family. Amino acid Sequences of Dermaseptins *stricto sensu* from South American Hylid Frogs (Subfamily Phyllomedusinae). X = Leu or Ile. *a* = C-Terminal Amidation. Names are Proposed According to the Frog Species Names Set Out in AMPHIBIAN Species of the World (http://research.amnh.org/vz/herpetology/amphibia) and Refs 4, 31. The Original Nomenclatures are in Brackets. ARP = adenoregulin-related peptide; DRS, DS = dermaseptin; DRP = dermaseptin-related peptide; DD = dermadistinctin; DRG = dermaseptin gene related. Accession number: http://www.uniprot.org/uniprot

Genus, species	Dermaseptin	Abbreviation	Sequence	Accession number/Ref.
Agalychnis				
A. annae	**A3**	**DRS-A3(DRP-AA-3-1)**	**SLWSKIKEMAATAGKAALNAVTGMVNQ*a***	O93223
	A4	**DRS-A4(DRP-AA-3-3)**	**GMFTNMLKGIGKLAGQAALGAVKTLA*a***	O93224
	A5	**DRS-A5(DRP-AA-3-6)**	**GMWSTIRNVGKSAAKAANLPAKAALGAISEAV*a***	O93226
A. callidryas	**C1**	**DRS-C1(ARP-AC1)**	**GMWSKIKEAGKAAAKAAAKAAGKAALDVVSGAI*a***	B6HY15
	C2	**DRS-C2(DRP-AC1)**	**SLLSTLGNMAKAAGRAALNAITGLVNQ*a***	B6YH16
	C3	**DRS-C3(DRP-AC3)**	**SVLSTITDMAKAAGRAALNAITGLVNQ*a***	Q800S0
A. dacnicolor				
(Pachymedusa dacnicolor)				
	DA2	**DRS-DA2(DRP-PD-2-2)**	**ALWKTLLKKVGKVAGKAVLNAVTNMANQNEQ**	O93452
	DA3	**DRS-DA3(DRP-PD-3-3)**	**GMWSKIKNAGKAAAKASKKAAGKAALGAVSEAL*a***	O93453
	DA4	**DRS-DA4**	**GMWSKIKNAGKAAKAAAKAAGKAALGAVSEAM**	7
	DA5	**DRS-DA5**	**GMWGKIKSTAKEAAKAAGKAALNAVSEAX*a***	25
	DA6	**DRS-DA6**	**GVWGIAKIAGKVLGNILPHVFSSNQS*a***	25
	DA7	**DRS-DA7**	**GXWNSXKEGGVGXAKQNVVVTGMVNQ*a***	25
	DA8	**DRS-DA8**	**GLWNSIKDMAAAAGRAALNAVTGMVNQ*a***	25
A. lemur	**L1**	**DRS-L1**	**GLWSKIKEAAKAAGKAALNAVTGLVNQGDQPS**	10
(Hylomantis lemur)				
A. spurrelli	**LI1**	**DRS-LI1(Peak-2.9)**	**AVWKDFLKNIGKAAGKAVLNSVTDMVNE**	24
(A. litodryas)				
Phasmahyla				
P. jandaia	**J1**	**DRS-J1**	**GLWKNMLSGIGKLAGQAALGAVKTLV*a***	P86635
	J2	**DRS-J2**	**GLWKNMLSGIGKLAGEAALGAVKTLV*a***	P86636
	J3	**DRS-J3**	**ALWKNMLSGIGKLAGQAALGAVKTLV*a***	P86637
	J4	**DRS-J4**	**ALWKDMLSGIGKLAGQAALGAVKTLV*a***	P86638
	J5	**DRS-J5**	**GLWSKIKEAGKAAVKAAGKAALGAVANSV*a***	P86683
	J6	**DRS-J6**	**GLWSKIKEAGKAAVKAAGKAALGAVADSV*a***	P86684
	J7	**DRS-J7**	**GLWSKIKAAGKEAAKAAAKAAGKAALNAVSEAV*a***	P86639
	J8	**DRS-J8**	**GLWKSLLKNVGKAAGKAALNAVTDMVNQA**	P86640
	J9	**DRS-J9**	**GLWKSLLKNVGKAAGKAALNAVTDMVNQS**	P86641
	J10	**DRS-J10**	**ALWKSLLKGAGQLVGGVVQHFMGSQGQPES**	P86642

(Continued)

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TABLE 1 The Dermaseptin (*stricto sensu*) family. Amino acid Sequences of Dermaseptins stricto sensu from South American Hylid Frogs (Subfamily Phyllomedusinae). X = Leu or Ile. a = C-Terminal Amidation. Names are Proposed According to the Frog Species Names Set Out in AMPHIBIAN Species of the World (http://research.amnh.org/vz/herpetology/amphibia) and Refs 4, 31. The Original Nomenclatures are in Brackets. ARP = adenoregulin-related peptide; DRS, DS = dermaseptin; DRP = dermaseptin-related peptide; DD = dermadistinctin; DRG = dermaseptin gene related. Accession number: http://www.uniprot.org/uniprot—Cont'd

Genus, species	Dermaseptin	Abbreviation	Sequence	Accession number/Ref.
Phyllomedusa				
P. bicolor	B1	DRS-B1	AMWKDVLKKIGTVALHAGKAALGAVADTISQ*a*	P80282
	B2	DRS-B2(adenoregulin)	GLWSKIKEVGKEAAKAAAKAAGKAALGAVSEAV*a*	P31107
	B3	DRS-B3	ALWKNMLKGIGKLAGQAALGAVKTLVGAE	P81485
	B4	DRS-B4	ALWKDILKNVGKAAGKAVLNTVTDMVNQ*a*	P81486
	B5	DRS-B5	GLWNKIKEAAKSAGKAALGFVNEMV*a*	P81487
	B6	DRS-B6	ALWKDILKNAGKAALNEINQLVNQ*a*	P81490
	B7	DRS-B7(DRG1)	GLWSNIKTAGKEAAKAALKAAGKAALGAVTDAV*a*	Q90ZK3
	B8	DRS-B8(DRG2)	GLWSKIKEAGKAVLTAAGKAALGAVSDAV*a*	Q90ZK5
	B9	DRS-B9(DRG3)	ALWKTIIKGAGKMIGSLAKNLLGSQAQPESEQ	P81488
P. burmeisteri	BU1	DRS-BU1(DS III-like)	ALWKNMLKGIGKLAGKAALGAVK	P86281
	BU2	DRS-BU2(DRSDI4-like)	ALWKNMLKGIGKLAGQAALGAVKTLVGA	P86279
	BU3	DRS-BU3(DS VIII-like)	ALWKTMLKKLGTVALHAGKAALGAAADTISQGA	P86280
P. distincta	DI1	DRS-DI1(DD K)	GLWSKIKAAGKEAAKAAAKAAGKAALNAVSEAV	P83638
	DI2	DRS-DI2(DD L)	ALWKTLLKNVGKAAGKAALNAVTDMVNQ	P83639
	DI3	DRS-DI3(DD M)	ALWKTMLKKLGTMALHAGKAAFGAAADTISQ	P83640
	DI4	DRS-DI4(DD Q1)	ALWKNMLKGIGKLAGQAALGAVKTLVGAES	P83641
	DI5	DRS-DI5(DD Q2)	GLWSKIKEAAKTAGLMAMGFVNDMV	P83642
P. hypochondrialis/azurea	H1	DRS-H1(DShypo 02)	GLWKSLLKNVGVAAGKAALNAVTDMVNQa	P84597
	H2	DRS-H2	ALWKSLLKNVGVAAGKAALNAVTDMVNQ*a*	Q0VZ36
	H3	DRS-H3	GLWSTIKNVAAAAGKAALGAL*a*	Q17UY8
	H4	DRS-H4(DShypo 01)	GLWSTIKNVGKEAAIAAGKAALGAL*a*	Q1EJP5
	H5	DRS-H5	GLWSTIKNVGKEAAIAAGKAVLGSL*a*	Q1EJP4
	H6	DRS-H6	GLWSTIKQKGKEAAIAAAKAAGQAALGAL*a*	P84936
	H7	DRS-H7	GLWSKIKDVAAAAGKAALGAVNEAL*a*	P84937
	H8	DRS-H8(DShypo03)	ALWKDVLKKIGTVALHAGKAAFGAAADTISQGGS	P84598
	H9	DRS-H9(DShypo 04)	GLWSTIKQKGKEAAIAAAKAAGKAVLNAASEAL*a*	P84599
	H10	DRS-H10(DS 01)	GLWSTIKQKGKEAAIAAAKAAGQAALGAL*a*	P84600
	H11	DRS-H11(DShypo 06)	GLWSTIKQKGKEAAIAAAKAAGQAVLNSASEAL*a*	P84601
	H12	DRS-H12(DShypo 07)	GLWSTIKQKGKEAAIAAAKAAGQAALNAASEAL*a*	P84880

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Genus, species	Dermaseptin	Abbreviation	Sequence	Accession number/Ref.
P. oreades	O1	DRS-O1(DS 01)	GLWSTIKQKGKEAAIAAAKAAGQAALGAL*a*	P83637
P. sauvagii	S1	DRS-S1(dermaseptin)	ALWKTMLKKLGTMALHAGKAALGAAADTISQGTQ	P24302
	S2	DRS-S2	ALWFTMLKKLGTMALHAGKAALGAAANTISQGTQ	P80278
	S3	DRS-S3	ALWKNMLKGIGKLAGKAALGAVKKLVGAES	P80279
	S4	DRS-S4	ALWMTLLKKVLKAAAKALNAVLVGANA	P80280
	S5	DRS-S5	GLWSKIKTAGKSVKAAAKAAVKAVTNAV	P80281
	S6	DRS-S6	GLWSKIKTAGKEAAKAAAKAAGKAALNAVSEAI*a*	Q7T3K9
	S7	DRS-S7	GLWKSLLKNVGKAAGKAALNAVTDMVNQ*a*	Q7T3K8
	S8	DRS-S8	ALWKTMLKKLGTVALHAGKAALGAAADTISQ*a*	Q7T3K7
	S11	DRS-S11	ALWKTLLKGAGKVFGHVAKQFLGSQGQPES	Q1EN13
	S12	DRS-S12	GLWSKIKEAAKTAGKMAMGFVNDMV*a*	Q1EN12
	S13	DRS-S13	GLRSKIKEAAKTAGKMALGFVNDMA*a*	Q1EN11
P. tarsius	TA1	DRS-TA1(DStar 01)	GLWSKIKETGKEAAKAAGKAALNKIAEAV*a*	P84921
	TA2	DRS-TA2(DStar 02)	ALWKDILKNVGKAAGKAVLNTVTDMVNQ*a*	P84922
	TA3	DRS-TA3(DStar 03)	GLFKTLIKGAGKMLGHVAKQFLGSQGQPES*a*	P84923
	TA4	DRS-TA4(DStar 04)	ALWKDILKNAGKAALNEINQIVQ*a*	P84924
	TA5	DRS-TA5(DStar 05)	GLWSKIKEAAKTAGKAAMGFVNEMV*a*	P84925
	TA6	DRS-TA6(DStar 06)	ALWKNMLKGIGKLAGQAALGAVKTLVGA	P84926
	TA7	DRS-TA7(DStar 07)	ALWKDVLKKIGTVALHAGKAALGAVADTISQ	P84927
P. tomopterna	TO1	DRS-TO1(DStomo01)	ALWKDLLKNVGIAAGKAVLNKVTDMVNQ*a*	P85523
P. trinitatis	TR1	DRS-TR1(DRS-B4)	ALWKDILKNVGKAAGKAVLNTVTDMVNQ*a*	P81486

belonging to different families. Orthologous peptides and paralogous peptides have different amino acid sequences in different species and most often display differential activity against a range of microorganisms. This impressive divergence between and within species is such that the evolutionary relationships of these peptides would never have been apparent without the strong conservation of their biosynthetic precursor preproregions (Section 2). Australian hylid frogs belonging to the Pelodryadinae subfamily also produce in their skin several families of antimicrobial peptides (caerins, fallaxidins, frenatins, aureins) (see the chapter by Bowie and Tyler, in this Section of the book) that have no structural similarity with South American hylid peptides, but the signal peptide and intervening sequences of the precursors of the caerins, fallaxidins, frenatins, and aureins are highly similar to the corresponding regions of the precursors of the South American hylid antimicrobial peptides (Table 9). Similarly, numerous antimicrobial peptides from Eurasian and North American frogs (families: Ranidae and Dicroglossidae) that belong to the brevinin, esculentin, nigrocin, palustrin, ranacyclin, ranalexin, ranatuerin,

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TABLE 2 The Dermatoxin family. Amino acid Sequences of Dermatoxins from South American Hylid Frogs (Subfamily Phyllomedusinae). *a* = C-Terminal Amidation. Names are Proposed According to the Frog Species Names Set out in Amphibian Species of the World (http://research.amnh.org/vz/ Herpetology/Amphibia) and Refs. 4, 31. The Original Nomenclatures are in Brackets. DRT = dermatoxin; DS = dermaseptin; DRP = dermaseptin-related peptide

Genus, species	Dermatoxin	Abbreviation	Sequence	Accession number
Agalychnis				
A. annae	**A1**	**DRT-A1(DRP-AA-1-1)**	**SLGSFMKGVGKGLATVGKIVADQFGKLLEAGQ*a***	O93221
A. dacnicolor				
(Pachymedusa dacnicolor)				
	DA1	**DRT-DA1(DRP-PD-1-5)**	**SLGSFMKGVGKGLATVGKIVADQFGKLLEAGKG**	O93451
Phasmahyla				
P. jandaia	**J1**	**DRT-J1**	**SLGGFLKGVGKALAGVGKVVADQFGNLLQAGQ*a***	P86621
	J2	**DRT-J2**	**SLGGFLKGVGKALAGVGKMVADQFGNLLQAGQ*a***	P86622
	J3	**DRT-J3**	**SLGGFLKGVGKVLAGVGKVVADQFGNLLEAGQ*a***	P86623
Phyllomedusa				
P. bicolor	**B1**	**DRT-B1(dermatoxin)**	**SLGSFLKGVGTTLASVGKVVSDQFGKLLQAGQ*a***	Q9PT75
P. sauvagii	**S1**	**DRT-S1(DRT-S)**	**ALGTLLKGVGSAVATVGKMVADQFGKLLQA*a***	Q5DVA5
P. tarsius	**TA1**	**DRT-TA1(DStar 08)**	**SLRGFLKGVGTALAGVGKVVADQFDKLLQAGQ*a***	P84928

TABLE 3 The Phylloxin family. Amino Acid Sequences of Phylloxins from South American Hylid Frogs (Subfamily Phyllomedusinae). *a* = C-Terminal Amidation. Names are Proposed According to the Frog Species Names Set out in Amphibian Species of the World (http://research.amnh.org/vz/herpetology/amphibia) and Refs. 4, 31. The Original Nomenclatures are in Brackets. PLX = phylloxin

Genus, species	Phylloxin	Abbreviation	Sequence	Accession number
Phyllomedusa				
P. bicolor	**B1**	**PLX-B1 (phylloxin)**	**GWMSKIASGIGTFLSGMQQ*a***	P81565
P. sauvagii	**S1**	**PLX-S1 (phylloxin S)**	**GWMSKIASGIGTFLSGVQQ*a***	Q5DVA6

amolopin, temporin, and limnonectin[34] families have precursors whose preproregions strikingly resemble those of preprodermaseptins (Table 9) (see the chapter by Conlon in this Section). Frog skin secretions are also a rich source of biologically active neuropeptides and hormones that are very similar to mammalian peptides that are produced in the brain and the gastrointestinal tract. Most interestingly, preprodermaseptin-encoded peptides also include D-amino acid-containing opioid peptides belonging to the dermorphin/deltorphin family (see the chapter by Negri and Melchiorri in this Section), bradykinin-related peptides, the myoactive peptides hyposins and tryptophyllins, and Bowman–Birk-like trypsin inhibitors[32] (Tables 8 and 9). The discovery of the considerable extent of sequence identity between the neuropeptide and antimicrobial peptide precursors is unprecedented. Thus, although the two groups of peptides are genetically related and belong to the same gene superfamily, they have strongly diverged to yield families of peptides that are both structurally and functionally distinct.

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TABLE 4 The Phylloseptin family. Amino Acid Sequences of Phylloseptins from South American Hylid frogs (Subfamily Phyllomedusinae). *a* = C-Terminal Amidation. Names are Proposed According to the Frog Species Names Set Out in Amphibian Species of the World (http://research.amnh.org/vz/herpetology/amphibia) and Refs. 4, 31. The Original Nomenclatures are in Brackets. PLS, PS, PSN = phylloseptin; PBN1 = *Phyllomedusa bicolor* new 1

Genus, species	Phylloseptin	Abbreviation	Sequence	Accession number/Ref.
Agalychnis				
A. lemur				
(Hylomantis lemur)	**L1**	**PLS-L1**	**LLGMIPLAISAISALSKL*a***	10
	L2	**PLS-L2**	**FLSLIPHVISALSSL*a***	1
Phasmahyla				
P. jandaia	**J1**	**PLS-J1**	**FLSLIPHAINAISAIANHF*a***	P86614
	J2	**PLS-J2**	**FLSLIPHAINAISAIADHF*a***	P86615
	J3	**PLS-J3**	**FLSLIPHAINAISAIANHL*a***	P86616
	J4	**PLS-J4**	**FLSLIPHAINAISAIAHHL*a***	P86617
	J5	**PLS-J5**	**FLSLIPHAISAISAIAHHL*a***	P86618
	J6	**PLS-J6**	**FLSLIPHAISAISAIANHL*a***	P86619
	J7	**PLS-J7**	**FLSLIPHAISAISAIADHL*a***	P86620
Phyllomedusa				
P. bicolor	**B1**	**PLS-B1(PBN1)**	**FLSLIPHIVSGVAALAKHL*a***	Q800R3
P. burmeisteri	**BU1**	**PLS-BU1(BU-1)**	**FLISIPYSASIGGTATLTGTA*a***	P86282
	BU2	**PLS-BU2(BU-2)**	**FLLSLPHLASGLASLVLSK*a***	P86283
P. hypochondrialis/azurea	**H1**	**PLS-H1(PS-1)**	**FLSLIPHAINAVSAIAKHN*a***	P84566
	H2	**PLS-H2(PS-2)**	**FLSLIPHAINAVSTLVHHF*a***	P84567
	H3	**PLS-H3(PS-3)**	**FLSLIPHAINAVSALANHG*a***	P84568
	H4	**PLS-H4(PS-6)**	**SLIPHAINAVSAIAKHF*a***	P84571
	H5	**PLS-H5(PS-7)**	**FLSLIPHAINAVSAIAKHF*a***	P85882
	H6	**PLS-H6(PS-8)**	**FLSLIPTAINAVSALAKHF*a***	Q0VZ41
	H7	**PLS-H7(PS-9)**	**FLGLLPSIVSGAVSLVKKL*a***	Q0VZ38
	H8	**PLS-H8(PS-10)**	**FLSLLPSLVSGAVSLVKKL*a***	Q0VZ39
	H9	**PLS-H9(PS-11)**	**FLSLLPSLVSGAVSLVKIL*a***	Q0VZ40
	H10	**PLS-H10(PS-12)**	**FLSLLPSIVSGAVSLAKKL*a***	Q17UY9
	H11	**PLS-H11(PS-13)**	**FLSLIPHAINAVGVHAKHF*a***	P84938
	H12	**PLS-H12(PS-14)**	**FLSLIPAAISAVSALADHF*a***	P84939
	H13	**PLS-H13(PS-15)**	**LLSLVPHAINAVSAIAKHF*a***	Q0VKG9
P. oreades	**O1**	**PLS-O1(PS-4)**	**FLSLIPHAINAVSTLVHHS*a***	P84569
	O2	**PLS-O2(PS-5)**	**FLSLIPHAINAVSAIAKHS*a***	P84570
P. palliata	**P1**	**PLS-P1**	**FLSLIPHAINAVSAIAKHF*a***	P86710
P. sauvagii	**S1**	**PLS-S1(PSN-1)**	**FLSLIPHIVSGVASIAKHF*a***	E3PQH3
P. tarsius	**TA1**	**PLS-TA1(PStar 01)**	**FLSLIPKIAGGIASLVKNL*a***	P84929
	TA2	**PLS-TA2(PStar 02)**	**FLSLIPHIATGIAALAKHL*a***	P84930
	TA3	**PLS-TA3(PStar 03)**	**FFSMIPKIATGIASLVKNL*a***	P84931
P. tomopterna	**TO1**	**PLS-TO1(PLS-8)**	**FLSLIPHAINAVSALAKHF*a***	P85447

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TABLE 5 The Plasticin family. Amino acid Sequences of Plasticins from South American Hylid Frogs (Subfamily Phyllomedusinae). *a* = C-Terminal Amidation. Names are Proposed According to the Frog Species Names Set out in Amphibian Species of the World (http://research.amnh.org/vz/herpetology/amphibia) and Refs. 4, 31. The Original Nomenclatures are in Brackets. DRP = dermaseptin-related peptide; PBN2 = *Phyllomedusa bicolor* new 2; DRS = dermaseptin; PTC = plasticin

Genus, species	Plasticin	Abbreviation	Sequence	Accession number
Agalychnis				
A. annae	**A1**	**PTC-A1(DRP-AA-2-5)**	**GLVSGLLNTAGGLLGDLLGSLGSLSG*a***	O93222
A. callidryas	**C1**	**PTC-C1(DRP-AC1)**	**GLLSGILNTAGGLLGNLIGSLSN*a***	Q800S2
	C2	**PTC-C2(DRP-AC2)**	**GLLSGILNSAGGLLGNLIGSLSN*a***	Q800S1
A. dacnicolor				
(Pachymedusa dacnicolor)				
	DA1	**PTC-DA2(DRP-PD-3-6)**	**GVVTDLLNTAGGLLGNLVGSLSG*a***	O93454
Phyllomedusa				
P. bicolor	**B1**	**PTC-B1(PBN2)**	**GLVTSLIKGAGKLLGGLFGSVTG*a***	Q800R4
P. sauvagii	**S1**	**PTC-S1(DRS-S10)**	**GLVSDLLSTVTGLLGNLGGGGLKKI**	Q1EN14

TABLE 6 The Raniseptin family. Amino Acid Sequences of Raniseptins from South American Hylid Frogs (Subfamily Hylinae). Names are Proposed According to the Frog Species Names Set out in Amphibian Species of the World (http://research.amnh.org/vz/herpetology/amphibia) and Refs. 4, 31. The Original Nomenclatures are in Brackets. RSP = raniseptin

Genus, species	Raniseptin	Abbreviation	Sequence	Accession number
Hypsiboas				
H. raniceps				
(Hyla roeschmanni)	**R1**	**RSP-R1(Rsp-1)**	**AWLDKLKSLGKVVGKVALGVAQNYLNPQQ**	P86037
	R2	**RSP-R2(Rsp-2)**	**AWLDKLKSLGKVVGKVAIGVAQHYLNPQQ**	P86185
	R3	**RSP-R3(Rsp-3)**	**AWLDKLKSIGKVVGKVAIGVAKNLLNPQ**	P86038
	R4	**RSP-R4(Rsp-4)**	**AWLDKLKSLGKVVGKVGLGVVQNYLNPRQ**	P86186
	R5	**RSP-R5(Rsp-5)**	**AWLDKLKNLGKVVGKVALGVVQNYLNPRQ**	P86187
	R6	**RSP-R6(Rsp-6)**	**ALLDKLKSLGKVVGKVALGVVQNYLNPRQ**	P86039
	R7	**RSP-R7(Rsp-7)**	**ALLDKLKSLGKVVGKVALGVAQHYLNPQQ**	P86040
	R8	**RSP-R8(Rsp-8)**	**ALLDKLKSLGKVVGKVAIGVAQHYLNPQQ**	P86188
	R9	**RSP-R9(Rsp-9)**	**ALLDKLKSLGKVVGKVAIGVAQHYLNPQ**	P86189

THE DERMASEPTIN SUPERFAMILY: GENE DUPLICATION, FOCAL HYPERMUTATION, AND DIVERSIFYING SELECTION

Peptides of the dermaseptin superfamily are synthesized in the multinucleated cells of the granular glands of the skin as prepropeptides that are processed by the removal of the signal peptide to the proform and then stored in the secretory granules of the glands. Glands may release their content onto the skin surface by a holocrine mechanism involving rupture of the plasma membrane and extrusion of the granules through a duct opening to the surface. Each peptide precursor results from transcription of a single genetic

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TABLE 7 The Caerin-Related Peptide Family and Orphan Antimicrobial Peptides. Amino Acid Sequences of Caerin-Related Peptides and Orphan Peptides from South American Hylid Frogs (Subfamily Phyllomedusinae). Names are Proposed According to the Frog Species Names Set out in Amphibian Species of the World (http://research.amnh.org/vz/herpetology/amphibia) and Refs. 4, 31. The Original Nomenclatures are in Brackets. CRP = caerin-related peptide; DRS = dermaseptin; DRP = dermaseptin-related peptide; ppdis = preprodistinctin

Genus, species	Peptide	Abbreviation	Sequence	Accession number
Caerin-related peptide				
Agalychnis				
A. annae	**A1**	**CRP-A1(DRP-AA-3-4)**	**GMWGSLLKGVATVVKHVLPHALSSQQS**	O93225
A. callidryas	**C1**	**CRP-C1(CRP-AC1)**	**GMWGTVFKGIKTVAKHLLPHVFSSQQS**	B6HY14
Orphan peptide				
Agalychnis				
A. dacnicolor				
(Pachymedusa dacnicolor)				
		DRP-PD-3-7	**LLGDLLGQTSKLVNDLTDTVGSIV**	O93455
Phyllomedusa				
P. sauvagii		**DRS-S9**	**GLRSKIWLWVLLMIWQESNKFKKM**	Q1EN15
P. hypochondrialis		**Distinctin-like (ppdis-H1)**	**NLVSALIEGRKYLKNVLKKLN-RLKEKNKAKNSKENN**	Q17UZ0

locus that encodes a single mature peptide at the C-terminus of the precursor sequence (Table 9). The canonical precursor architecture comprises a common N-terminal preprosequence of approximately 50 residues that is remarkably well conserved both within and between species, while the C-terminal sequence corresponding to antimicrobial peptide or neuropeptide varies markedly.[30] The conserved region comprises a 22-residue signal peptide followed by an acidic intervening sequence that ends in a typical prohormone processing signal Lys-Arg. This conservation also extends into the 5′- and 3′-untranslated regions of the corresponding mRNAs. Preprodermaseptin mRNAs thus show a unique pattern of very high sequence conservation surrounding a region of very high sequence variability. The contrast between the strikingly conserved preproregion and the hyperdivergent mature peptides is one of the most extreme examples observed to date for homologous gene products within a single order of organisms. Moreover, the pattern of conserved and variable regions in skin antimicrobial peptide precursors is the opposite of that of conventional secreted peptides, suggesting that the conserved preproregion is important for the biology of the expressing cell.

The remarkable similarity of preproregions of precursors that give rise to very different end products in distantly related frogs suggests that all the corresponding genes came from a common ancestor in the ancestor of South American and Australian hylids and North American and Eurasian ranids before their divergence in the Mezozoic age 150 million years ago. A combination of phylogenetic reconstructions, analysis of mutation rates, and geophysical models for the sequence of fragmentation of Gondwana suggest that the impressive diversity of these peptides and the number of peptides per species reflect the combination of multiple duplications of the ancestral gene before and during radiation of these species and within individual species, focal hypermutations of the mature peptide domain, and subsequent actions of diversifying (positive) selection.[18,33] Targeted hypermutation of the C-terminal antimicrobial-coding region of preprodermaseptin genes might have evolved as a way of increasing genetic diversity, thus accelerating the adaptation of frogs to noxious microbial fauna.

ANTIMICROBIAL ACTIVITIES OF THE PREPRODERMASEPTIN-DERIVED PEPTIDES FROM SOUTH AMERICAN HYLIDAE FROGS

Dermaseptins (*stricto sensu*): These peptides are prototypical members of a large class of membrane-damaging cationic peptides that undergo coil-to-helix transition on

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TABLE 8 Examples of Neuropeptides and Hormones of the Dermaseptin Superfamily from South American Hylid Frogs (subfamily Phyllomedusinae). X = Leu or Ile. hyP = hydroxyproline. *a* = C-terminal amidation. HPS = hyposin; PRP = Phe-rich peptide

Genus, species	Peptide	Sequence	Accession number
Bradykinin and related peptides			
Phyllomedusa			
P. hypochondrialis/azurea	**Phyllokinin**	**RPPGFSPFRIY(SO_3H)**	P84900
	[Thr^6, Val^{10}]-phyllokinin	**RPPGFTPFRVY**	AM283482
P. sauvagii	**[Thr^6]-phyllokinin**	**RPPGFTPFRIY(SO_3H)**	Q800F1
P. hypochondrialis/azurea	**[Val^1, Thr^6]-bradykinin-Leu-Thr**	**VPPGFTPFRLT**	Q0VTU0
	[Thr^6]-bradykinin	**RPPGFTPFR**	AM283482
	[Val^1, Hypro^2]-bradykinin	**VhyPPGFTPFR**	Q0VTT8
P. rohdei	**Phyllokinin**	**RPPGFSPFRIY**	Q7LZ52
P. sauvagii	**[Thr^6]-bradykinin**	**RPPGFTPFR**	Q800F1
Hyposin			
Phasmahyla			
P. jandaia	**HPS-J1**	**FRPAXIVRTGK*a***	P86613
Phyllomedusa			
P. hypochondrialis/azurea	**HPS-HA1**	**LRPAVIRPKGK*a***	P84954
	HPS-HA2	**LRPAFIRPKGK*a***	P84955
	HPS-HA3	**LRPAVIVRTKGK*a***	P84956
	HPS-HA4	**FRPALIVRTKGTRL*a***	P84957
	HPS-HA5	**LGPALITRKPLKGKP**	P84958
P. sauvagii	**HPS-S1 (sauvatide)**	**LRPAILVRTK*a***	C5J8E3
Tryptophyllin			
Agalychnis			
A. dacnicolor			
(Pachymedusa dacnicolor)	**PdT-1**	**KPhyPAWVP*a***	P83455
	PdT-2	**DMSPPWH*a***	C7C1L9
Phyllomedusa			
P. hypochondrialis/azurea	**Pha-T1**	**RPPSWIPK**	Q0VKG8
Phe-rich peptide			
Phyllomedusa			
P. hypochondrialis/azurea	**PRP-HA1**	**FLFFAFHPL*a***	Q0JRV5
Dermorphins/deltorphins			
Agalychnis			
A. dacnicolor	**Dermorphin**	**YAFGYPS*a***	O93456
(Pachymedusa dacnicolor)			
	[Ile^2]-deltorphin	**YIFHLMD**	O93456

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TABLE 8 Examples of Neuropeptides and Hormones of the Dermaseptin Superfamily from South American Hylid Frogs (subfamily Phyllomedusinae). X = Leu or Ile. hyP = hydroxyproline. a = C-terminal amidation. HPS = hyposin; PRP = Phe-rich peptide—Cont'd

Genus, species	Peptide	Sequence	Accession number
A. annae	Dermorphin	YAFGYPS*a*	O93227
Phasmahyla			
P. jandaia	Dermorphin	YAFGYPS*a*	P86633
	Deltorphin	YMFHLMD*a*	P86634
Phyllomedusa			
P. bicolor	[D-Ala^2]-deltorphin-1	YAFDVVG*a*	P21850
	[D-Ala^2]-deltorphin-2	YAFEVVGa	P21850
	[D-Ala2]-dermorphin-like	YAFGYP	P21850
	[D-Ala2]-dermorphin-like	YAFWYPN	P21850
P. burmeisteri	Deltorphin-like peptide	YMFHLMNa	P86285
P. hypochondrialis/azurea	Dermorphin	YAFGYhyPS*a*	P85887
P. sauvagii	Deltorphin (dermenkephalin)	YMFHLMD*a*	P05422
	Dermorphin	YAFGYPSa	P05422

binding to lipid bilayers.[21] They are cidal at micromolar doses against a wide spectrum of microorganisms, including wall-less bacteria (mollicutes), Gram-negative and Gram-positive bacteria, fungi, protozoa, yeast, and enveloped viruses, but each peptide has considerable variations in its antimicrobial activity.[28] All the dermaseptins, but DRS-S4, are not or weakly toxic against mammalian cells. The dermaseptins S exhibit synergy of action on combination, resulting in some cases in a 100-fold increase in antibiotic activity of the mixture over the activity of the peptides separately.[28] Shortening the peptide chain of DRS-S3 to DRS-S3-[1–16]–NH2 does not affect the potency of the peptide. Further reduction of the chain length yields derivatives gradually showing reduced activity, but analogs as short as 10–12 residues in length remain fully active against several bacterial strains.[29] [K^4]-DRS-S4-[1–16] has been investigated for antibacterial activity *in vivo*, using a peritonitis model of mice infected with *Pseudomonas aeruginosa*. Naive mice exhibited 75% mortality, compared with 18% mortality in mice that received a single i.p. injection (4.5mg/kg) of [K^4]-DRS-S4-.$^{1–16}$ Dermaseptins S1–S5 and DRS-H10 exhibit cidal activity against *Leishmania* in its promastigote form at micromolar doses,[9,16,28] and gold-labeled freeze-fracture microscopy shows that the peptides generate severe perturbations of the lipid bilayer, leading to the death of the parasites. DRS-S3 and derivatives of DRS-S4 selectively disrupt the plasma membrane of the intracellular parasite *Plasmodium falciparum* without harming that of the mammalian host cell.[12] The resulting antimalarial activity is allegedly exerted after the harmless peptide binding to the membrane of the host cell, followed by peptide translocation across a number of intracellular membrane systems and interaction with that of the intraerythrocytic parasite. Dermaseptin-O1, -DI1, and -DI2 show antiprotozoan activity against *Trypanosoma cruzi* in its tryptomastigote and epimastigote forms cultivated in both cell culture and blood media, without toxicity against mouse erythrocytes and white blood cells.[8] Dermaseptins S1–S5 display antiviral activity against herpes simplex virus type I and HIV-1 virus at micromolar doses.[22] The most potent peptide, DRS-S4, inhibits cell-free and cell-associated HIV-1 infection of D4-CCR5 indicator cells and human primary T lymphocytes. The peptide is effective against R5 and X4 primary isolates and laboratory-adapted strains of HIV-1. Its activity is directed against HIV-1 particles by disrupting the virion integrity.

Dermatoxins: The primary structures of dermatoxins are highly conserved exhibiting few chemically conservative amino acid substitutions (Table 2). DRT-B1 proved to be cidal toward mollicutes and Gram-positive bacteria and also, though to a lesser extent, toward Gram-negative bacteria. Measurement of the bacterial membrane potential revealed that the plasma membrane is the primary target of dermatoxin. Observation of bacterial cells by reflected light

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TABLE 9 Canonical Precursor Architecture of Antimicrobial Peptides and Neuropeptides from the Dermaseptin Superfamily. A/Diagram of Preprodermaseptin. The Coding Region, Including the Signal Peptide, the Acidic Propiece and the Peptide Progenitor Sequence is Drawn as a Rectangle. The Signal Peptide Includes the First 22 Amino Acid Residues, while the Acidic Propiece Comprises 19–26 Residues. B/Examples of Amino Acid Sequences of Preprodermaseptins Encoding Antimicrobial Peptides and Neuropeptides from South American (Subfamilies: Phyllomedusinae and Hylinae) and Australian Hylid Frogs (Subfamily: Pelodryadinae), and from North American, Asian, and European Frogs Belonging to the Ranidae and Dicroglossidae families. Identical Amino Acids have shaded backgrounds. Highly Similar Amino Acids are Colored Cyan. Accession number: http://www.uniprot.org/uniprot/Ref

A

B

Family: Hylidae

Subfamily: Phyllomedusinae

```
Dermaseptin-B2          MAFLKKSLFLVLFLGLVSLSIC EEEKRENEDEEEQEDDE---QSEMKR GLWSKIKEVGKEAAKAAAKAAGKAALGAVSEAVGEQ
Distinctin-like         ---LKKSLFLVTFLALVPLFLC EEEKREEENEERQDDD----QSEEKR NLVSALIEGRKYLKNVLKKLNRLKEKNKAKNSKENN
Dermatoxin-B1           MAFLKKSLFLVLFLGLVPLSLC ESEKREGENEEEQEDD----QSEEKR SLGSFLKGVGTTLASVGKVVSDQFGKLLQAGQG
Caerin-RP-A1            MAFLKKSLFLVLFLGLVSLSIC DEEKRENEDEEEQEDDE---QSEEKR GMWGSLLKGVATVVKHVLPHALSSQQS
Plasticin-C1            MAFLKKSLLLVLFLGLVSLSIC EEEKRENEDEEKQEDDD---QSENKR GLLSGILNTAGGLLGNLIGSLSNgES
Dermaseptin-S9          MAFLKKSLFLVLFLGLVSLSIC DEEKRENEDEENQEDDE---QSEMRR GLRSKIWLWVLLMIWQESNKFKKM
Phylloseptin-B1         MAFLKKSLFLVLFLGLVSLSIC EEEKRETEEKEYD-QGEDDK-SEEKR FLSLIPHIVSGVAALAKHLG
Phylloxin-B1            MVFLKKSLLLVLFVGLVSLSIC EENKRE-EHEEIEENKE----AEEKR GWMSKIASGIGTFLSGMQQG
[Thr6]-phyllokinin      MDILKKSLFLVLFLGLVSFSIC EEEKRDTEEEE--NDDEIEEESEEKK REAPERPPGFTPFRIY
[Thr6]-bradykinin       MDILKKSLFLVLFLGLVSFSIC EEEKRDTEEEE--NDDEIEEESEEKK REAPERPPGFTPFRIY
Hyposin-S1              MDILKKSLFLILFLGLVSISFC DGEKRQDDDEANESEEKKEIHEVEKR LRPAILVRTKGKGK
Dermorphin              MSFLKKSLLLILFLGLVSLSVC KEEKRETEEENENEENH-EEGSEMKR YAFGYPSG…
Deltorphin              MSFLKKSLLLVLFLGLVSHSVC KEEKRETEEENENEEENHEVGSEMKR YAFWYPNG…
```

Subfamily: Pelodryadinae

```
Caerin 1.12(Q800R8)     MAFLKKSLFLVLFLGLVSLSIC EEEKRQEDEDEEEEGENQEEGSEEKR GLFGILGSVAKHVLPHVVPVIAEHSG
Aurein 5.3(Q5K0E2)      MAFLKKSLFLVLFLGLVSLSIC EQEKREEENQE--EDEENEAASEEKR GLMSSIGKALGGLIVNVLKPKTPAS
Frenatin 3(P56249)      MHFLKKSIFLVLFLGLVSLSIC EKEKREDQNEE--EVDENEEESEEKR GLMSVLGHAVGNVLGGLFKPKS
Fallaxidin 4.1(B5LUQ4)  MASLKKSLFLVLFLGMVSLSIC DKEKREGENEE--EEEEHEEESEEKR GLLSFLPKVIGVIGHLIHPPS
```

Subfamily: Hylinae

```
Raniseptin-R1(P86037)   MAFLKKSLFLVLFLGIVSLSIC EEEKREGEEEEKQEEENEELSEEELRERR AWLDKLKSLGKVVGKVALGVAQNYLNPQQ
```

Family: Ranidae

```
Esculentin-2P(P82846)   MFTLKKSLLLLFFIGTISLSLC EEERNADEEEGGEVQEE-----EVKR GIFSLIKGAAKVVAKGLGKEVGKFGLDLMACKVTNQC
Palustrin-Ra2(D2K8I5    MFTLKKSLLLLFFLGTISLSLC EQE-RDADEDE-GETLE-----EVKR GLWDTIKQAGKKLFLNVLDKIRCKVAGGCRT
Nigrocin-2H(B0B2H4)     MFTLKKSLLLLFFLGMVSLALC EQERDANEEERRDELDERDVEA-IKR GLLSKVLGVGKKVLCGVSGLC
Brevinin-1Dyb(P0C5W7)   MFTLKKSLLLLFFLGTISLSLC EEE-RNAEEERRDYPEERDVEV-EKR FLSLALAALPKLFCLIFKKC
Ranalexin(P39084)       MFTLKKSLLLLFFLGTINLSLC EEE-RNAEEERRDNPDERDVEV-EKR FLGGLIKIVPAMICAVTKKC
B-B Inhibitor(B1VC43)   MFTLKKSLLLLFFLGTISLSLC EQE-RDSDDEDQGEVTEQVV----KR SVIGCWTKSIPPRPCFVKG
Temporin-B(P79874)      MFTLKKSLLLLFFLGTINLSLC EEE-RNAEEERRDEPDERDVQV-EKR LLPIVGNLLKSLLGK
```

Family: Dicroglossidae

Subfamily: Dicroglossinae

```
Limnonectin-1Fb[34]     MFTLKKSLLLLFFLGTINLSLC EEE-RNAEEEGRDEPDETDVEV-EKR SFHVFPPWMCKSLKKC
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fluorescence microscopy after DNA staining was consistent with a mechanism of cell killing based on the alteration of membrane permeability. In a liquid growth inhibition assay, DRT-B1 killed ≈50% of *Burkholderia cepacia*, a human pathogen that causes pneumonia in immunocompromised individuals with underlying lung disease (such as cystic fibrosis or chronic granulomatous disease). This showed that *B. cepacia* cell populations contain a mixture of dermatoxin-sensitive and dermatoxin-insensitive variants and that the liquid growth inhibition assay fails to reveal such a phenomenon.

Phylloxins: Two phylloxins from *Phyllomedusa* skin were identified, phylloxin-B1 from *P. bicolor* and phylloxin-S1 from *P. sauvagei* (Table 3). The primary structures of the two phylloxins are extremely conserved, exhibiting only one conservative amino acid substitution. Phylloxins have a narrow antimicrobial spectrum including the strains of the wall-less bacteria *Acholeplasma laidlawii* and *Spiroplasma melliferum*, the Gram-negative bacteria *Escherichia coli*, and the Gram-positive bacteria *Micrococcus luteus*. However, the Gram-negative bacteria *Pseudomonas aeruginosa*, *Sinorhizobium meliloti*, and *Salmonella typhimurium* and the Gram-positive bacteria *Staphylococcus aureus* and *Corynebacterium glutamicum* are resistant to PLX-B1. The antibiotic activity proved to be lethal for some of the sensitive strains, which were not capable of resuming growth on agar plates after a 2-h treatment with concentrations above the corresponding MICs.

Phylloseptins: The first member of the phylloseptin family, phylloseptin-B1, was discovered by cDNA cloning of preprodermaseptins from *P. bicolor*.[33] The common structural features of phylloseptins include a highly conserved sequence FLS[I,L]LI[L]P in the N-terminal region and C-terminal amidation[20] (Table 4). The antimicrobial activity spectrum of phylloseptins includes Gram-negative and Gram-positive bacteria, fungi, and protozoa: *Leishmania amazonensis* promastigotes; *Plasmodium falciparum* rings, trophozoites, and schizonts;[19] and *Trypanosoma cruzi* trypomastigotes.[20] PLS-H1 displays broad-spectrum activity against a range of planktonic organisms with a high potency (MIC = 5μM) against *Staphylococcus aureus*. In a specific bioassay with the same organism grown as a biofilm, the minimal biofilm eradication concentration was found to be of the same high potency.[35] No toxic effect was observed in an *in vitro* system and in an *in vivo* model with a concentration of PLS-H1 10-fold over that providing antimicrobial effect.

Plasticins: The plasticins, which are rich in Gly and Leu residues arranged in regular 5-mer motifs GXXXG (where X is any amino acid residue), have very similar amino acid sequences, hydrophobicities, and amphipathicities but differ markedly in their net charge, conformational plasticity, and activity spectra.[13,17] Plasticins are divided into two classes on the basis of their cytolytic activities. The strongly cationic peptides PTC-B1 and PTC-S1 that contain lysine residues are bactericidal against most of the tested microorganisms at micromolar concentrations and permeabilize the cytoplasmic membrane of *E. coli*, but the weakly cationic or neutral plasticins (PTC-A1, -C1, -C2, and -DA1) are hemolytic only. Plasticins display considerable conformational flexibility and polymorphism that modulate their ability to bind to and disrupt the bilayer membranes of prokaryotic and eukaryotic cells and/or to reach intracellular targets. For example, PTC-DA1 adopts a helical conformation when bound to anionic phospholipid vesicles but a β-sheet structure when bound to zwitterionic vesicles. All plasticins present a turn region that does not always result in folding into a β-hairpin-shaped conformation. However, a preformed β-hairpin in solution may act as a conformational lock that prevents the switch to α-helical structure. This lock lowers the antimicrobial efficiency and explains subtle differences in potencies of the most active antimicrobial plasticins.

Raniseptins: Raniseptins from *H. raniceps* display antimicrobial potential *in vitro* preferentially against Gram-negative bacteria and limited cytotoxicity against red blood cells. MALDI-IMS demonstrated that the mature raniseptins although stored inside the skin glands in their intact form within a defined glandular domain, undergo rapid proteolytic cleavage upon secretion. RSP-R1-(1–14) showed much lower or no antimicrobial action when compared with the intact RSP-R1. This contrasts with DRS-S1-$^{1–18}$ and shorter fragments of dermaseptin-S3 that still retain full potency against Gram-negative bacteria. Because RSP-R1-(1–14) possesses little or no antimicrobial activity, the raniseptin processing in Hylinae frogs presumably acts as a mechanism by which the protein and peptide content is altered to respond to different predatory attacks.

Caerin-related peptides: The 27-mer caerin-related peptides from *A. annae* and *A. callidryas* are most closely structurally related to caerins from Australian tree frogs—caerins 1.17 and 1.19 from *Litoria gracilenta*, and caerin 1.7 from *Litoria xanthomera* and *Litoria chloris*. This finding is not inconsistent with taxonomic suggestions and evidence that both Neotropical phyllomedusids and Australasian litorids are close phylogenetic relatives.[33]

Orphan peptides: Dermaseptin-S9 does not resemble any of the cationic and amphipathic antimicrobial peptides identified to date, having a highly hydrophobic core sequence flanked at either side by cationic termini. The peptide has broad-spectrum antibacterial activities and a low hemolytic activity, inhibiting the growth of Gram-positive and Gram-negative bacteria with minimal inhibitory concentrations in the micromolar range. DRS-S9 adopts a nonamphipathic α-helical conformation in trifluoroethanol, but it forms stable and ordered β-sheet aggregates in aqueous buffers or when bound to anionic or zwitterionic phospholipid vesicles. These structures slowly assemble into amyloid-like fibrils in aqueous environments via

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spherical intermediates, as revealed by electron microscopy and Congo red staining.[6] DRS-S9 induced the directional migration of neutrophils, T lymphocytes, and monocytes. The antimicrobial and chemotactic activities of DRS-S9 are modulated by its amyloid-like properties. Although spherical oligomers of DRS-S9 exhibit antimicrobial activity, the soluble, weakly self-associated forms of DRS-S9 act on human leukocytes to promote chemotaxis and/or immunological response activation in the same range of concentration as amyloidogenic peptides Aβ-(1–42) and the prion peptide PrP-(106–126).

MECHANISMS OF MICROBICIDAL ACTIVITY

The mode of antimicrobial action of most of the preprodermaseptin-derived peptides is believed to be the permeation/disruption of the lipid plasma membrane of the target cells through an interfacial location of amphipathic antimicrobial peptides inducing positive curvature of the bilayer and/or clustering of anionic lipids.[14,21] The high content of anionic lipids in prokaryotic membranes and their absence from the neutral matrix of erythrocytes account for the preferential binding of cationic peptides to the outer leaflet of bacterial bilayers through long-range electrostatic interactions. Accordingly, cationic peptides initially bind onto the negatively charged surface of the target membrane and cover it in a carpet-like manner. The four steps proposed to be involved in the model are as follows: (i) binding of the cationic peptide to the phospholipid head groups, with subsequent formation of an amphipathic peptide helix, (ii) alignment of the helical peptides on the surface of the membrane, (iii) rotation of the helical peptides leading to reorientation of the hydrophobic residues toward the hydrophobic core of the membrane. The resulting spatial segregation of polar and apolar amino acids on opposing faces along the long axis of the helix permits the insertion of a well-defined hydrophobic sector into the acyl core of the lipid bilayer, and (iv) disintegration of the peptide-coated membrane by disrupting the bilayer curvature. As peptides cover the cell surface, transient mixed peptide-lipid holes may be formed. They may enable the passage of low molecular weight molecules before membrane lysis.

THE DERMASEPTINS: MULTIFUNCTIONAL HOST DEFENSE PEPTIDES

Most of the peptides belonging to the dermaseptin superfamily have been identified primarily by their antimicrobial activity. However, additional biological functions have been recognized that may or may not be directly associated with pathogen clearance. For instance, adenoregulin (DRS-B2) was first isolated by Daly et al. as a peptide able to stimulate binding of agonists to A1 adenosine receptors.[11] Adenoregulin was further shown to enhance the binding of agonists to several G-protein–coupled receptors in rat brain membranes through an enhancement of guanyl nucleotide exchange at G-proteins. DRS-B4, PLS-L2, and FSIP, a DRS-B4 related peptide from *A. spurrelli,* significantly stimulate insulin release in acute incubations with glucose-responsive BRIN-BD11 cells or BRIN-BD11 clonal β-cell line.[1,23,24] In addition to their role as endogenous antibiotics, antimicrobial peptides participate in multiple aspects of immunity (inflammation, wound repair, and regulation of the adaptive immune system). For instance, DRS-S1 stimulates microbicidal activities of polymorphonuclear leukocytes.[5] Treatment of polymorphonuclear leukocytes with DRS-S1 (10–100nM) stimulates the production of reactive oxygen species and release of myeloperoxidase. In addition, low peptide concentrations (1–10nM) prime the stimulation of respiratory burst induced by zymosan particles. The induced burst is inhibited by selective protein kinase inhibitors, and is associated with early signaling events such as a rapid and transient elevation of cytosolic-free calcium concentration and phospholipase D activity. DRS-DA4 is a potent chemoattractant for human leukocytes and is devoid of hemolytic activity.[7] On the other hand, the orphan peptide dermaseptin DRP-PD-3-7 exhibits no antibacterial activity but has an inherent propensity to self-assembles into amyloid fibrils in a reversible pH-controlled fashion.[15] Above pH 5, the amyloid fibrils disassemble in a cooperative manner. At neutral pH, this process proceeds instantaneously to the soluble form. Within the transition interval (pH 5–6.5), however, "backward" granular aggregates, 10–500nm in size, are formed. Such metastable amorphous aggregates, which are quickly released from an amyloid depot by a shift in pH, can mediate a strong cytotoxic effect.

REFERENCES

1. Abdel-Wahab YH, Power GJ, Flatt PR, Woodhams DC, Rollins-Smith LA, Conlon JM. A peptide of the phylloseptin family from the skin of the frog *Hylomantis lemur* with potent in vitro and in vivo insulin-releasing activity. *Peptides* 2008;**29**:2136–43.
2. Amiche M, Ducancel F, Lajeunesse E, Boulain JC, Menez A, Nicolas P. Molecular cloning of a cDNA encoding the precursor of adenoregulin from frog skin. *Biochem Biophys Res Commun* 1993;**191**:983–90.
3. Amiche M, Ducancel F, Mor A, Boulain JC, Menez A, Nicolas P. Precursors of vertebrate peptide antibiotics dermaseptin b and adenoregulin have extensive sequence identities with precursors of opioid peptides dermorphin, dermenkephalin, and deltorphins. *J Biol Chem* 1994;**269**:17847–52.
4. Amiche M, Ladram A, Nicolas P. A consistent nomenclature of antimicrobial peptides isolated from frogs of the subfamily *Phyllomedusinae*. *Peptides* 2008;**47**:10513–25.
5. Ammar B, Perianin A, Mor A, Sarfati G, Tissot M, Nicolas P, et al. Dermaseptin, a peptide antibiotic, stimulates microbicidal activities of polymorphonuclear leukocytes. *Biochem Biophys Res Commun* 1998;**247**:870–5.

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6. Auvynet C, El Amri C, Lacombe C, Bruston F, Bourdais J, Nicolas P, Rosenstein Y. Structural requirements for antimicrobial versus chemoattractant activities for dermaseptin S9. *FEBS J* 2008;**275**: 4134–51.
7. Auvynet C, Joanne P, Bourdais J, Nicolas P, Lacombe C, Rosenstein Y. Dermaseptin DA4, although closely related to dermaseptin B2, presents chemotactic and Gram-negative bactericidal activities. *FEBS J* 2009;**276**:6773–86.
8. Brand GD, Leite JR, Silva LP, Albuquerque S, Prates MV, Azevedo RB, et al. Dermaseptins from *Phyllomedusa oreades* and *Phyllomedusa distincta*. Anti-*Trypanosoma cruzi* activity without cytotoxicity to mammalian cells. *J Biol Chem* 2002;**277**:49332–40.
9. Brand GD, Leite JR, de Sa Mandel SM, Mesquita DA, Silva LP, Prates MV, et al. Novel dermaseptins from *Phyllomedusa hypochondrialis*. *Biochem Biophys Res Commun* 2006;**347**:739–46.
10. Conlon JM, Woodhams DC, Raza H, Coquet L, Leprince J, Jouenne T, et al. Peptides with differential cytolytic activity from skin secretions of the lemur leaf frog *Hylomantis lemur* (Hylidae: Phyllomedusinae). *Toxicon* 2007;**50**:498–506.
11. Daly JW, Caceres J, Moni RW, Gusovsky F, Moos Jr M, Seamon KB, et al. Frog secretions and hunting magic in the upper Amazon: identification of a peptide that interacts with an adenosine receptor. *Proc Natl Acad Sci U S A* 1992;**89**:10960–3.
12. Efron L, Dagan A, Gaidukov L, Ginsburg H, Mor A. Direct interaction of dermaseptin S4 aminoheptanoyl derivative with intraerythrocytic malaria parasite leading to increased specific antiparasitic activity in culture. *J Biol Chem* 2002;**277**:24067–72.
13. El Amri C, Nicolas P. Plasticins: membrane-damaging peptides with 'chameleon-like' properties. *Cell Mol Life Sci* 2008;**65**:895–909.
14. Galanth C, Abbassi F, Lequin O, Ayala-Sanmartin J, Ladram A, Nicolas P, et al. Mechanism of antibacterial action of dermaseptin B2: interplay between helix–hinge–helix structure and membrane curvature strain. *Biochemistry* 2009;**48**:313–27.
15. Gossler-Schofberger R, Hesser G, Muik M, Wechselberger C, Jilek A. An orphan dermaseptin from frog skin reversibly assembles to amyloid-like aggregates in a pH-dependent fashion. *FEBS J* 2009;**276**:5849–59.
16. Hernandez C, Mor A, Dagger F, Nicolas P, Hernandez A, Benedetti EL, et al. Functional and structural damage in *Leishmania mexicana* exposed to the cationic peptide dermaseptin. *Eur J Cell Biol* 1992;**59**:414–24.
17. Joanne P, Falord M, Chesneau O, Lacombe C, Castano S, Desbat B, et al. Comparative study of two plasticins: specificity, interfacial behavior, and bactericidal activity. *Biochemistry* 2009;**48**:9372–83.
18. Konig E, Bininda-Emonds OR. Evidence for convergent evolution in the antimicrobial peptide system in anuran amphibians. *Peptides* 2011;**32**:20–5.
19. Kuckelhaus SA, Leite JR, Muniz-Junqueira MI, Sampaio RN, Bloch Jr C, Tosta CE. Antiplasmodial and antileishmanial activities of phylloseptin-1, an antimicrobial peptide from the skin secretion of *Phyllomedusa azurea* (Amphibia). *Exp Parasitol* 2009;**123**:11–6.
20. Leite JR, Silva LP, Rodrigues MI, Prates MV, Brand GD, Lacava BM, et al. Phylloseptins: a novel class of antibacterial and antiprotozoan peptides from the *Phyllomedusa* genus. *Peptides* 2005;**26**:565–73.
21. Lequin O, Bruston F, Convert O, Chassaing G, Nicolas P. Helical structure of dermaseptin B2 in a membrane-mimetic environment. *Biochemistry* 2003;**42**:10311–23.
22. Lorin C, Saidi H, Belaid A, Zairi A, Baleux F, Hocini H, et al. The antimicrobial peptide dermaseptin S4 inhibits HIV-1 infectivity in vitro. *Virology* 2005;**334**:264–75.
23. Marenah L, McClean S, Flatt PR, Orr DF, Shaw C, Abdel-Wahab YH. Novel insulin-releasing peptides in the skin of *Phyllomedusa trinitatis* frog include 28 amino acid peptide from dermaseptin BIV precursor. *Pancreas* 2004;**29**:110–5.
24. Marenah L, Shaw C, Orr DF, McClean S, Flatt PR, Abdel-Wahab YH. Isolation and characterisation of an unexpected class of insulinotropic peptides in the skin of the frog *Agalychnis litodryas*. *Regul Pept* 2004;**120**:33–8.
25. Meneses EP, Villa-Hernandez O, Hernandez-Orihuela L, Castro-Franco R, Pando V, Aguilar MB, et al. Peptidomic analysis of the skin secretions of the frog *Pachymedusa dacnicolor*. *Amino Acids* 2011;**40**:113–22.
26. Mor A, Nguyen VH, Delfour A, Migliore-Samour D, Nicolas P. Isolation, amino acid sequence, and synthesis of dermaseptin, a novel antimicrobial peptide of frog skin. *Biochemistry* 1991;**30**:8824–30.
27. Mor A, Amiche M, Nicolas P. Structure, synthesis, and activity of dermaseptin b, a novel vertebrate defensive peptide from frog skin: relationship with adenoregulin. *Biochemistry* 1994;**33**:6642–50.
28. Mor A, Hani K, Nicolas P. The vertebrate peptide antibiotics dermaseptins have overlapping structural features but target specific microorganisms. *J Biol Chem* 1994;**269**:31635–41.
29. Mor A, Nicolas P. The NH2-terminal alpha-helical domain 1–18 of dermaseptin is responsible for antimicrobial activity. *J Biol Chem* 1994;**269**:1934–9.
30. Nicolas P, Vanhoye D, Amiche M. Molecular strategies in biological evolution of antimicrobial peptides. *Peptides* 2003;**24**:1669–80.
31. Nicolas P, El Amri C. The dermaseptin superfamily: a gene-based combinatorial library of antimicrobial peptides. *Biochim Biophys Acta* 2009;**1788**:1537–50.
32. Song G, Zhou M, Chen W, Chen T, Walker B, Shaw C. HV-BBI, a novel amphibian skin Bowman–Birk-like trypsin inhibitor. *Biochem Biophys Res Commun* 2008;**372**:191–6.
33. Vanhoye D, Bruston F, Nicolas P, Amiche M. Antimicrobial peptides from hylid and ranin frogs originated from a 150-million-year-old ancestral precursor with a conserved signal peptide but a hypermutable antimicrobial domain. *Eur J Biochem* 2003;**270**:2068–81.
34. Wu Y, Wang L, Zhou M, Ma C, Chen X, Bai B, et al. Limnonectins: a new class of antimicrobial peptides from the skin secretion of the Fujian large-headed frog (*Limnonectes fujianensis*). *Biochimie* 2011;**93**:981–7.
35. Zhang R, Zhou M, Wang L, McGrath S, Chen T, Chen X, et al. Phylloseptin-1 (PSN-1) from *Phyllomedusa sauvagei* skin secretion: a novel broad-spectrum antimicrobial peptide with antibiofilm activity. *Mol Immunol* 2010;**47**:2030–7.SECTION | V Handbook of Biologically Active Peptides; Amphibian/Skin Peptides

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Chapter 51

Insulin-Releasing Peptides

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and J. Michael Conlon

ABSTRACT

Peptides with the ability to stimulate the release of insulin from BRIN-BD11 rat clonal β-cells at low concentrations that are not cytotoxic to the cells have been isolated from skin secretions and/or skin extracts from several frogs belonging to the families Bombinatoride, Dicroglossidae, Hylidae, Leptodactylidae, Pipidae, and Ranidae. In addition, tigerinin-1R from *Hoplobatrachus rugulosus*, brevinin-2GUb from *Hylarana güntheri*, phylloseptin-L2 from *Hylomantis lemur*, and brevinin-2-related peptide (B2RP) from *Lithobates septentrionalis* have been shown to be active *in vivo*, increasing plasma insulin concentrations and improving glucose tolerance in mice. Nontoxic analogs of B2RP and psuedin-2 from *Pseudis paradoxa* with more potent insulin-releasing activities than the naturally occurring peptides have been developed that show therapeutic potential for the treatment of type-2 diabetes.

INTRODUCTION

The incidence of type 2 diabetes mellitus is increasing dramatically in all regions of the world. The current pandemic affects approximately 285 million people worldwide, and this number is expected to double by 2025.[17] New approaches to treatment and management are urgently required. Natural products, including biologically active peptides from both vertebrate and invertebrate species and from plants, are being examined for their glucose-lowering properties. One such approach to treatment is the use of agents based on physiological incretins. An incretin is a factor released by the gut in response to nutrients that facilitates the uptake of glucose by peripheral tissues by stimulating secretion of insulin. Peptides based on the structure of the endogenous insulin-releasing peptide, glucagon-like peptide-1 (GLP-1), and on exendin-4, an agonist at the GLP-1 receptor that was isolated from the venom of a lizard, the Gila monster *Heloderma suspectum*, show promise as effective treatments to improve glycemic control in patients with type-2 diabetes.[15]

The skins of many, although by no means all, species of Anura (frogs and toads) synthesize peptides with the ability to inhibit the growth of pathogenic bacteria and fungi.[10] The peptides are released into skin secretions, often in very high concentrations, in response to stress or infection and constitute a component of the animal's system of innate immunity. However, studies have shown that several frog skin peptides that were first identified on the basis of their ability to inhibit the growth of bacteria also stimulate insulin release *in vitro* from BRIN-BD11 rat clonal β-cells at low concentrations that are not cytotoxic to the cells. The BRIN-BD11 cell line is a well-established and convenient model to study insulin secretion in response to a range of nutrients, hormones, neurotransmitters, and drugs.[26] This chapter summarizes the primary structures and properties of the insulinotropic peptides that have been isolated from species belonging to genera within the families Bombinatoride, Dicroglossidae, Hylidae, Leptodactylidae, Pipidae, and Ranidae. The species names and taxonomic recommendations of Frost[16] and recent guidelines for antimicrobial peptide nomenclature[6,8,9] are followed. The primary structures of these peptides are shown in Tables 1 and 2.

INSULINOTROPIC PEPTIDES FROM RANIDAE

The taxonomy of frogs belonging to the extensive family Ranidae has undergone a series of major revisions in recent years, but many issues remain to be resolved. Analyses based primarily on molecular criteria have resulted in the reclassification of many well-known species. Current recommendations by Frost[16] divide the 347 species in the family Ranidae into 16 genera with the genus *Rana* being retained for a more restricted group of 48 species from Eurasia and North America. It must be pointed out, however, that other established taxonomists have claimed that this assignment of species is both arbitrary and premature, and it has been proposed that the genus *Rana* should be retained for all North American members of the family Ranidae. A recent phylogenetic analysis based on maximum likelihood analysis of two mitochondrial and three nuclear genes has divided the Ranidae into four monophyletic genera: *Amolops*, *Huia*, *Rana*, and *Staurois* with all North American frogs placed in the genus *Rana*.[31]

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00051-8
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TABLE 1 Insulinotropic Peptides from Species in Genera Belonging to the Family Ranidae

Species	Peptide	Primary structure	Ref
Rana			
R. draytonii	Temporin-DRa	HFLGTLVNLAKKIL.NH_2	Abdel-Wahab et al.[1]
R. draytonii	Temporin-DRb	NFLGTLVNLAKKIL.NH_2	Abdel-Wahab et al.[1]
R. ornativentris	Temporin-Oe	ILPLLGNLLNGLL.NH_2	Abdel-Wahab et al.[1]
R. tagoi	Temporin-TGb	AVDLAKIANKVLSSLF.NH_2	Abdel-Wahab et al.[1]
Lithobates			
L. catesbeianus	Brevinin-1CBb	FLPFIARLAAKVFPSIICSVTKKC	Mechkarska et al.[27]
L. catesbeianus	Ranatuerin-1CBa	SMFSVLKNLGKVGLGF VACKVNKQC	Mechkarska et al.[27]
L. catesbeianus	Ranatuerin-2CBc	GFLDIIKNLGKTFAGH MLDKIKCTIGTCPPSP	Mechkarska et al.[27]
L. catesbeianus	Ranatuerin-2CBd	GFLDIIKNLGKTFAGHML DKIRCTIGTCPPSP	Mechkarska et al.[27]
L. catesbeianus	Palustrin-2CBa	GFLDIIKDTGKEFAV KILNNLKCKLAGGCPP	Mechkarska et al.[27]
L. catesbeianus	Temporin-CBa	FLPIASLLGKYL.NH_2	Mechkarska et al.[27]
L. catesbeianus	Temporin-CBf	FLPIASMLGKYL.NH_2	Mechkarska et al.[27]
L. pipiens	Brevinin-1Pa	FLPIIAGVAAKVFPKIFCAISKKC	Marenah et al.[21]
L. palustris	Palustrin-1c	ALSILRGLEKLAKMGIAL TNCKATKKC	Marenah et al.[19]
L. septentrionalis	Brevinin-2-related peptide	GIWDTIKSMGKV FAGKILQNL.NH2	Abdel-Wahab et al.[3]
L. virgatipes	Temporin-Va	FLSSIGKLIGNLL.NH_2	Abdel-Wahab et al.[1]
L. virgatipes	Temporin-Vb	FLSIIAKVLGSLF.NH_2	Abdel-Wahab et al.[1]
L. virgatipes	Temporin-Vc	FLPLVTMLLGKLF.NH_2	Abdel-Wahab et al.[1]
Hylarana			
H. guentheri	Brevinin-2GUb	GVIIDTLKGAAKT VAAELLRKAHCKLTNSC	Conlon et al.[13]
Glandirana			
G. emeljanovi	"Gaegurin-6"	FLPLLAGLAANFLPTIICKISYKC	Kim et al.[18]
Pelophylax			
P. saharicus	Esculentin-1	GIFSKFGRKKIKNLLISGLKNV GKEVGMDVVRTGIDIAGCKIKGEC	Marenah et al.[23]
P. saharicus	Esculentin-1b	GIFSKLAGKKLKNLLISGLKNVGK EVGMDVVRTGIDIAGCKIKGEC	Marenah et al.[23]
P. saharicus	Brevinin-1E	FLPLLAGLAANFLPKIFCKITRKC	Marenah et al.[23]
P. saharicus	Brevinin-2Ec	GILLDKLKNFAKTAGKGVLQSLLN TASCKLSGQC	Marenah et al.[23]
P. saharicus	Brevinin-2 fragment	KGAAKGLLEVASCKLSKSC	Marenah et al.[22]

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TABLE 2 Insulinotropic Peptides from Species in Genera Belonging to the Families Hylidae, Bombinatoridae, Leptodactylidae, Dicroglossidae, and Pipidae

Species	Peptide	Primary structure	Ref
Hylidae			
Hylomantis lemur	Phylloseptin-L2	FLSLIPHVISALSSL.NH_2	Abdel-Wahab et al.[4]
Phyllomedusa trinitatis	Dermaseptin B4	ALWKDILKNVGKAAGKAVLNT VTDMVNQ	Marenah et al.[24]
Agalychnis calcarifer	RK-13	RRKPLFPLIPRPK	Abdel-Wahab et al.[2]
Agalychnis litodryas	Dermaseptin-LI1	AVWKDFLKNIGKAAGKAVLNS VTDMVNE	Marenah et al.[25]
Pseudis paradoxa	Pseudin-2	GLNALKKVFQGIHEAIK LINNHVQ	Abdel-Wahab et al.[5]
Bombinatoridae			
Bombina variegata	GM-14	GKPFYPPPIYPEDM	Marenah et al.[20]
Bombina variegata	IN-21	IYNAICPCKHCNKCKPGLLAN	Marenah et al.[20]
Bombina variegata	Bombesin	<EQRLGHQWAVGHLM.NH_2	Marenah et al.[20]
Bombina variegata	Bombesin-related peptide	<EQRLGHQWAVGHLM.NH_2	Marenah et al.[20]
Bombina variegata	Bombesin-related peptide	<EDSFGNQWARGHFM.NH_2	Marenah et al.[20]
Leptodactylidae			
Leptodactylus laticeps	Ocellatin-L2	GVVDILKGAAKDLAGHLA TKVMDKL.NH_2	Conlon et al.[11]
Leptodactylus laticeps	Plasticin-L1	GLVNGLLSSV LGGGQGGGGLLGGIL	Conlon et al.[11]
Dicroglossidae			
Hoplobatrachus rugulosus	Tigerinin-1R	RVCSAIPLPICH.NH_2	Ojo et al.[28]
Pipidae			
Xenopus borealis	Caerulein-B1	<EQDY(SO_3)GTGWMDF.NH_2	Zahid et al.[32]
Xenopus amieti	Xenopsin	<EGKRPWIL	Zahid et al.[32]
Xenopus amieti	Xenopsin-AM2	<EGRRPWIL	Zahid et al.[32]

A 24 amino acid peptide with insulin-releasing activities belonging to the brevinin-1 family was purified from electrically stimulated skin secretions the Northern leopard frog *Lithobates pipiens* (formerly *Rana pipiens*).[21] This peptide, termed brevinin-1Pa, is identical to pipinin-1, previously identified in this species on the basis of its histamine-releasing activity, and it produces a 1.8-fold increase in insulin release from BRIN-BD11 cells compared with that of 5.6 mM glucose only. The insulinotropic effect of brevinin-1Pa is reminiscent of that of histamine-releasing peptide mastoparan, isolated from hornet and wasp venom (see the chapter on Hymenoptera Insect Peptides in the Venom Peptides section of the book). Mastoparan stimulates insulin release through the activation of Rho G proteins, specifically Rac.[7] Analysis of skin secretions from the pickerel frog *Lithobates palustris* (formerly *Rana palustris*) by reversed-phase HPLC led to the isolation and structural characterization of an insulinotropic peptide that is identical to the previously described palustrin-1c.[19] The potency and mechanism of action of this peptide were not determined. A peptide of the brevinin-1 family (termed "gaegurin-6" in the publication) from the Korean frog *Glandirana emeljanovi* (formerly *Rana rugosa*) stimulates insulin release from rat RINm5F insulinoma-derived cells and increases intracellular calcium concentrations.[18]

Multiple peptides with insulinotropic activities but low cytotoxicities are present in skin secretions of the Sahara frog *Pelophylax saharicus* (formerly *Rana saharica*).[23] Structural characterization showed that the peptides are

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identical to brevinin-1E, brevinin-2Ec, esculentin-1, and esculentin-1b previously isolated from *Rana esculenta* (now reclassified as a hybrid between *Pelophylax lessonae* and *Pelophylax ridibundus*). Preliminary mechanistic studies indicate that protein kinase A- and protein kinase C-dependent pathways may be involved in the insulinotropic effects of the esculentin-1 peptides. A previously undescribed insulinotropic peptide with structural similarity to the C-terminal domain of peptides of the brevinin-2 family was also isolated from *P. saharicus* secretions.[22]

The temporin family of antimicrobial peptides is widely distributed in both North American and Eurasian species belonging to genera *Rana* and *Lithobates* (see the chapter on this peptide in this section of the book). The insulin-releasing effects of synthetic replicates of seven temporins (temporin-Va, -Vb, and -Vc from *Lithobates virgatipes*, temporin-DRa and -DRb from *Rana draytonii*, temporin-Oe from *Rana ornativentris*, and temporin-TGb from *R. tagoi*) have been assessed.[1] Temporin-Oe, -Vb, -DRb, and -TGa stimulate *in vitro* insulin release from BRIN-BD11 in the concentration range 10^{-8}–10^{-6} M but do not stimulate the release of the cytosolic enzyme lactate dehydrogenase (LDH) indicating that the integrity of the plasma membrane is maintained. At a concentration of 10^{-6} M, temporin-Va and temporin-Vc also stimulate insulin release but are cytotoxic to the cells. Temporin-DRa was not insulinotropic. The component with maximum stimulatory ability (2.6-fold greater than the rate of release produced by 5.6 M glucose only) is temporin-Oe, which has the lowest cationicity of the peptides tested. Preliminary mechanistic studies show that temporins at 10^{-7} M do not increase intracellular calcium concentration suggesting that they may exert their effects via a K_{ATP} channel-independent pathway.

With a combination of reversed-phase HPLC and electrospray mass spectrometry, peptidomic analysis of norepinephrine-stimulated skin secretions of the American bullfrog *Lithobates catesbeianus* (formerly *Rana catesbeiana*) led to the identification and characterization of seven peptides with *in vitro* insulin-releasing activity (brevinin-1CBb, ranatuerin-1CBa, ranatuerin-2CBc, ranatuerin-2CBd, palustrin-2CBa, temporin-CBa, and temporin-CBf[27]). Ranatuerin-2CBd is the most potent peptide producing a significant stimulation of insulin release (119% of basal rate) from BRIN-BD11 cells at a concentration of 30 nM, with a maximum response (236% of basal rate) at a concentration of 3 μM. Ranatuerin-2CBd does not stimulate the release of LDH at concentrations up to 3 μM indicating low cytotoxicity. Brevinin-1CBb produces the maximum stimulation of insulin release (285% of basal rate at 3 μM) but the peptide is cytotoxic at this concentration.

The most potent insulin-releasing peptide identified in an extract of the skin of the Asian frog *Hylarana guentheri* (formerly *Rana guentheri*) belongs to the brevinin-2 family and is termed brevinin-2GUb.[13] The peptide lacks cytotoxic activity and produces stimulation of insulin release (139% of basal rate) at a concentration of 0.1 μM with a maximum response (373% of basal rate) at a concentration of 3 μM. Administration of brevinin-2GUb (75 nmol/kg of body weight) into healthy mice improves glucose tolerance following a intraperitoneal injection of glucose. The antimicrobial peptide B2RP (brevinin-2-related peptide), first isolated from skin secretions of the mink frog *Lithobates septentrionalis*, shows sequence similarity to antimicrobial peptides belonging to the brevinin-2 family but lacks the C-terminal cyclic heptapeptide domain (Cys-Lys-Xaa_4-Cys). Synthetic B2RP produces a stimulation of insulin release from BRIN-BD11 cells (148% of basal rate at a concentration of 1 μM with a maximum response of 222% of basal rate at a concentration of 3 μM) without increasing the rate of release of LDH.[3]

INSULINOTROPIC PEPTIDES FROM HYLIDAE

The extensive and widely distributed family Hylidae is divided into the subfamilies Phyllomedusinae (58 species), Pelodryadinae (197 species), and Hylinae (646 species).[16] The Central and South American tree frogs, particularly species in the genera *Agalychnis*, *Hylomantis*, *Pachymedusa*, and *Phyllomedusa* in the subfamily Phyllomedusinae, and the Australian tree frogs in the genus *Litoria* in the subfamily Pelodryadinae have proved to be a rich source of biological peptides (see the dermaseptins and the caeruleins chapters in this section of the book).

Peptides with significant insulinotropic effects have been isolated and characterized from four frog species in the subfamily Phyllomedusinae, notably *Phyllomedusa trinitatis*,[24] *Agalychnis calcarifer*,[2] *Agalychnis litodryas*,[25] and *Hylomantis lemur*.[4] Fractionation by HPLC of electrically stimulated skin secretions from *P. trinatatis* led to the isolation of a 28 amino acid peptide with insulin-releasing properties that were identical to dermaseptin B4 from *Phyllomedusa bicolor*.[24] A structurally similar insulin-releasing peptide belonging to the dermaseptin family was also isolated from the skin secretions of *A. litodryas*.[25] The potencies or mechanisms of action of these peptides were not determined. A 13 amino acid peptide, termed RK-13, with structural similarity to the N-terminal region of the proline–arginine-rich antimicrobial peptide PR-39 is present in skin secretions of *A. calcarifer*.[2] This peptide lacks antimicrobial activity but stimulates insulin release from BRIN-BD11 cells by a mechanism that may involve protein kinase A but is independent of pertussis-toxin-sensitive G proteins.

A peptide with the ability to release insulin from BRIN-BD11 cells was isolated from norepinephrine-stimulated skin secretions of the Lemur leaf frog *H. lemur*.[4] Structural characterization demonstrated that the peptide belongs to

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the phylloseptin family whose members have previously been identified in other Phyllomedusinae species (see the chapter on the dermaseptins in this section of the book). The 15 amino acid peptide, termed phylloseptin-L2, produces a stimulation of insulin release from BRIN-BD11 cells (134% of basal rate at a concentration of 30 nM, with a maximum response of 301% of basal rate at a concentration of 3 μM) without increasing the rate of release of LDH. The stimulatory action is maintained in the absence of extracellular Ca^{2+} and in the presence of verapamil (50 μM) and diazoxide (300 μM) suggesting that the mechanism of action of the peptide does not involve influx of Ca^{2+} or closure of ATP-sensitive K^+ channels. Administration of phylloseptin-L2 (50 nmol/kg of body weight) into healthy mice increases total release of insulin and improves glucose tolerance during the 60-min period after intraperitoneal injection of glucose.

Synthesis of antimicrobial and other biologically active peptides in the skin of species belonging to the subfamily Hylinae is sporadic.[10] Four structurally related peptides, termed pseudin 1–4, were isolated from an extract of the skin of the South American paradoxical frog *Pseudis paradoxa* (Hylinae) although these peptides were not detected in norepinephrine-stimulated skin secretions.[29] Pseudin-2 shows the highest antimicrobial potency particularly against Gram-negative bacteria and has very low hemolytic activity. Pseudin-2 at concentrations of 10^{-7} and 10^{-6} M stimulates insulin release from BRIN-BD11 and is without β-cell cytotoxicity.[5] Activity was retained in the absence of extracellular calcium and in the presence of known antagonist of insulin release (diazoxide and verapamil) suggesting that that the action of the peptide was mediated through a calcium-independent pathway.

INSULINOTROPIC PEPTIDES FROM BOMBINATORIDAE

The family Bombinatoridae family comprises two genera *Barbourula* and *Bombina*.[16] The six species of fire-bellied toads of the genus *Bombina* are widely distributed in Eurasia and have been a rich source of antimicrobial and other biologically active peptides (see the chapters on bombesin-like peptides, bombinins, and Bv8 peptides in this section of the book). Fractionation of electrically stimulated skin secretions of *B. variegata* by reversed-phase HPLC led to the identification of bombesin and two bombesin-related peptides with the ability to stimulate insulin release from BRIN-BD11 cells.[20] In addition, two novel noncytotoxic insulinotropic peptides termed GM-14 and IN-21 were isolated and characterized structurally, but their potencies were not determined. Preliminary results suggested the involvement of a protein kinase A-dependent pathway in the insulin-releasing action of these peptides.

INSULINOTROPIC PEPTIDES FROM LEPTODACTYLIDAE

The family Leptodactylidae (currently 100 species) has been divided into four genera: *Hydrolaetare*, *Leptodactylus*, *Paratelmatobius*, and *Scythrophrys*, but both morphological and molecular investigations have indicated that the Leptodactylidae probably is probably not monophyletic.[16] Antimicrobial peptides with relatively low potency against bacteria and low cytotoxicity against mammalian cells have been isolated from the skin secretions of several species belonging to the genus *Leptodactylus* (reviewed in Ref. 10). A glycine–leucine-rich peptide is present in the skin secretions of the Sante Frog *Leptodactylus laticeps* that contain the $(GXXXG)_3$ motif found in the plasticins, previously identified only in phyllomedusid frogs of the family Hylidae (see the dermaseptins chapter in this section of the book).[11] Like the plasticins, the peptide from *L. laticeps* (termed plasticin-L1) adopts a random coil conformation in water, a β-sheet structure in methanol, and an α-helical conformation in 50% trifluoroethanol water. Plasticin-L1 does not inhibit the growth of bacteria or lyse human erythrocytes but, at relatively high concentrations (≥1 μM), the peptide produces a modest (139% of basal rate at 3 μM), increase in the rate of glucose-induced release of insulin from BRIN-BD11 cells without increasing the rate of release of LDH. A peptide, termed ocellatin-L2 is also present in *L. laticeps* skin secretions, which is identical to the previously described ocellatin-L1 except for the substitution $Asn^{23} \rightarrow Asp$.[11] Ocellatin-L2 is devoid of antimicrobial and hemolytic actions but shows modest activity in stimulating insulin release from BRIN-BD11 cells (181% of basal rate at 3 μM).

INSULINOTROPIC PEPTIDES FROM DICROGLOSSIDAE

The Asian frog *Hoplobatrachus rugulosus* (formerly *Rana rugulosa*) is classified with three other species in the genus *Hoplobatrachus* in the extensive family Dicroglossidae (149 species).[16] A cyclic, C-terminally amidated dodecapeptide, termed tigerinin-1R, was isolated from an extract of the skin of *H. rugulosus*.[28] The tigerinin family of peptides was first identified in the Indian frog *Hoplobatrachus tigerinus* (formerly classified as *Rana tigerina*) on the basis of antimicrobial activity.[30] Tigerinin-1R lacks cytotoxic and hemolytic activities but stimulates the rate of release of insulin from BRIN-BD11 cells at concentrations ≥0.1 nM. The maximum response is 405% of the basal rate at 5.6 mM ambient glucose concentration and 290% of basal rate at 16.7 mM glucose. The magnitude of this effect is greater than that produced under the same experimental conditions by the antidiabetic drug, tolbutamide and the well-characterized insulinotropic peptides, GLP-1 and GIP. C-terminal α-amidation is necessary for high potency and a

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possible mechanism of action of the peptide involves membrane depolarization and an increase in intracellular Ca^{2+} concentration. Administration of tigerinin-1R (75 nmol/kg of body weight) to mice fed a high fat diet to induce insulin resistance and obesity enhances total insulin release and improves glucose tolerance during the 60-min period after an intraperitoneal glucose load.

INSULINOTROPIC PEPTIDES FROM PIPIDAE

The clawed frogs of the family Pipidae comprise five genera. *Pipa* is found in South America with the other four genera *Hymenochirus*, *Pseudhymenochirus*, *Silurana*, and *Xenopus* in sub-Saharan Africa.[16] S*ilurana* and *Xenopus* are united in the subfamily Xenopodinae but the cytogenetics and evolutionary history of this taxon is complex. A series of allopolyploidization events have generated tetraploid, octoploid, and dodecaploid species with only *Silurana tropicalis* retaining diploid status.[14]

Caerulein-B1 from the tetraploid frog *Xenopus borealis* stimulates the rate of insulin secretion from BRIN-BD11 cells at concentrations ≥30 nM compared with basal rate in the presence of 5.6 mM glucose only.[32] The maximum response (360% of basal rate, $P<0.05$) occurs with 3 μM. The peptide (1 μM) also produces a significant (128%, $P<0.05$) stimulation of the rate of insulin release in the presence of 16.7 mM glucose. Unfortunately, the therapeutic potential of caerulein-B1 may be limited by the observation that repeated intraperitoneal injections of caerulein into rats and mice produce acute edematous pancreatitis with cellular necrosis.

Xenopsin and xenopsin-AM2 from the octoploid frog *Xenopus amieti* stimulate the rate of insulin secretion from BRIN-BD11 cells at concentrations ≥30 nM compared with basal rate in the presence of 5.6 mM glucose only, but the peptides produce a significantly lower maximum response than caerulein-B1 (198% of basal rate for xenopsin and 207% of basal rate for xenopsin-AM2 at a concentration of 3 μM).[32] High dose infusions of xenopsin in dogs produce hyperglycemia resulting from a pronounced stimulation of glucagon and cortisol release, so that it seems improbable that either xenopsin or xenopsin-AM2 has potential for development into antidiabetic agents.

ANALOGS WITH INCREASED INSULIN-RELEASING ACTIVITY

The therapeutic potential of peptide-based pharmaceutical agents, particularly for systemic use, is limited by their short half lives in the circulation, possible immunogenicity, and toxicities toward human cells. Appreciable success has been achieved in transforming cytotoxic frog skin antimicrobial peptides with relatively low potency against microorganisms into promising antiinfective agents by designing analogs with substitutions of appropriate amino acids by either L- or D-lysine.[12] Increasing the cationicity of psuedin-2 from +2 to +3 by the substitution $Asn^{18} \rightarrow Lys$ increases the insulin-releasing potency and maximum response of the naturally occurring peptide. This analog produces a 46% increase in the rate of insulin release from BRIN-BD11 cells at 10^{-9} M and a 215% increase in insulin release at 10^{-6} M. However, increasing the molecular charge on the peptide to +6 in [N3K,Q10K, E14K]pseudin-2 and to +7 in [N3K,Q10K, E14K,N21K]pseudin-2 produces peptides that had no significant stimulatory effect on insulin release. Increasing the hydrophobicity of the peptide by the substitution $Ile^{16} \rightarrow Phe$ generates an analog that retains insulinotropic action but has increased hemolytic activity.[5]

Increasing cationicity of B2RP while maintaining amphipathicity by the substitution $Asp^{4} \rightarrow Lys$ enhances the insulin-releasing potency (137% of basal rate at a concentration of 0.3 μM) with no stimulation of LDH release. By contrast, the L18K and D4K,L18K analogs are toxic to the cells and the K16A analog, with increased amphipathicity and hydrophobicity, shows reduced potency. Administration of [D4K]B2RP (100 nmol/kg body weight) to mice fed a high fat diet to induce obesity and insulin resistance enhances total insulin release and improves glucose tolerance during the 60-min period after an intraperitoneal glucose load (18 mmol/kg of body weight).[3]

CONCLUSIONS

Naturally occurring nontoxic compounds with the ability to stimulate insulin release from pancreatic beta cells ("incretins") show promise as antidiabetic drugs. Many peptides present in the skin secretions of several frog species, including several that were first identified by their broad-spectrum antibacterial and antifungal activities, are able to stimulate the release of insulin from BRIN-BD11 clonal beta cells at concentrations that are not toxic to the cells and also improve glucose tolerance in mice. Such peptides represent candidates for development in drugs to treat patients with Type 2 diabetes.

REFERENCES

1. Abdel-Wahab Y, Marenah L, Flatt P, Conlon JM. Insulin releasing properties of the temporin family of antimicrobial peptides. *Prot Pept Lett* 2007;**14**:702–7.
2. Abdel-Wahab Y, Marenah L, Orr DF, Shaw C, Flatt PR. Isolation and structural characterisation of a novel 13 amino acid insulin-releasing peptide from the skin secretion of *Agalychnis calcarifer*. *Biol Chem* 2005;**386**:581–7.
3. Abdel-Wahab YH, Patterson S, Flatt PR, Conlon JM. Brevinin-2-related peptide and its [D4K] analogue stimulate insulin release in vitro and improve glucose tolerance in mice fed a high fat diet. *Horm Metab Res* 2010;**42**:652–6.

This book belongs to Central Library BADJI Mokhtar-ANNABA UNIVERSITY Copyright Elsevier 2024

4. Abdel-Wahab YHA, Power GJ, Flatt PR, Woodhams DC, Rollins-Smith LA, Conlon JM. A peptide of the phylloseptin family from the skin of the frog *Hylomantis lemur* (Phyllomedusinae) with potent *in vitro* and *in vivo* insulin-releasing activity. *Peptides* 2008;**29**:2136–43.
5. Abdel-Wahab YHA, Power GJ, Ng MT, Flatt PR, Conlon JM. Insulin-releasing properties of the frog skin peptide pseudin-2 and its [Lys18]-substituted analogue. *Biol Chem* 2008;**389**:143–8.
6. Amiche M, Ladram A, Nicolas P. A consistent nomenclature of antimicrobial peptides isolated from frogs of the subfamily Phyllomedusinae. *Peptides* 2008;**29**:2074–82.
7. Amin RH, Chen HQ, Veluthakal R, Silver RB, Li J, Li G, et al. Mastoparan-induced insulin secretion from insulin-secreting betaTC3 and INS-1 cells: evidence for its regulation by Rho subfamily of G proteins. *Endocrinology* 2003;**144**:4508–18.
8. Conlon JM. Reflections on a systematic nomenclature for antimicrobial peptides from the skins of frogs of the family Ranidae. *Peptides* 2008;**29**:1815–9.
9. Conlon JM. A proposed nomenclature for antimicrobial peptides from frogs of the genus *Leptodactylus*. *Peptides* 2008;**29**:1631–2.
10. Conlon JM. Structural diversity and species distribution of host-defense peptides in frog skin secretions. *Cell Mol Life Sci* 2011;**68**:2303–15.
11. Conlon JM, Abdel-Wahab Y, Flatt P, Leprince J, Vaudry H, Jouenne T, et al. A glycine–leucine-rich peptide structurally related to the plasticins from skin secretions of the frog *Leptodactylus laticeps* (Leptodactylidae). *Peptides* 2009;**30**:888–92.
12. Conlon JM, Al-Ghaferi N, Abraham B, Leprince J. Strategies for development of naturally occurring antimicrobial peptides into therapeutically valuable anti-infective agents. *Methods* 2007;**42**:349–57.
13. Conlon JM, Power GJ, Abdel-Wahab Y, Flatt P, Jiansheng H, Coquet L, et al. A potent, non-toxic insulin-releasing peptide isolated from an extract of the skin of the Asian frog, *Hylarana guntheri* (Anura:Ranidae). *Regul Pept* 2008;**151**:153–9.
14. Evans BJ. Genome evolution and speciation genetics of clawed frogs (*Xenopus* and *Silurana*). *Front Biosci* 2008;**13**:4687–706.
15. Flatt PR, Bailey CJ, Green BD. Recent advances in antidiabetic drug therapies targeting the enteroinsular axis. *Curr Drug Metab* 2009;**10**:125–37.
16. Frost DR. Amphibian species of the world: an online reference. Version 5.5. Electronic Database accessible at: New York, USA. *American Museum of Natural History*; 2011. <http://research.amnh.org/herpetology/amphibia/Index.php>.
17. *International Diabetes Federation*. *Diabetes Atlas*. Electronic Database accessible at: <http://www.diabetesatlas.org>; 2010.
18. Kim JH, Lee JO, Jung JH, Lee SK, You GY, Park SH, et al. Gaegurin-6 stimulates insulin secretion through calcium influx in pancreatic beta Rin5mf cells. *Regul Pept* 2010;**159**:123–8.
19. Marenah L, Flatt P, Orr DF, McClean S, Shaw C, Abdel-Wahab YHA. Brevinin-1 and multiple insulin-releasing peptides in the skin of the frog *Rana palustris*. *J Endocrinol* 2004;**181**:347–54.
20. Marenah L, Flatt P, Orr DF, McClean S, Shaw C, Abdel-Wahab Y. Skin secretion of the toad *Bombina variegata* contains multiple insulin-releasing peptides including bombesin and entirely novel insulinotropic structures. *Biol Chem* 2004;**385**:315–21.
21. Marenah L, Flatt P, Orr DF, Shaw C, Abdel-Wahab YHA. Characterization of naturally occurring peptides in the skin secretion of Rana pipiens frog reveal pipinin-1 as the novel insulin-releasing agent. *J Pept Res* 2005;**66**:204–10.
22. Marenah L, Flatt P, Orr DF, Shaw C, Abdel-Wahab YHA. Isolation and structural characterization of novel rugosin A-like insulinotropic peptide from the skin secretions of *Rana saharica* frog. *Peptides* 2005;**26**:2117–23.
23. Marenah L, Flatt P, Orr DF, Shaw C, Abdel-Wahab YH. Skin secretions of *Rana saharica* frogs reveal antimicrobial peptides esculentins-1 and-1B and brevinins-1E and-2EC with novel insulin releasing activity. *J Endocrinol* 2006;**188**:1–9.
24. Marenah L, McClean S, Flatt P, Orr DF, Shaw C, Abdel-Wahab Y. Novel insulin-releasing peptides in the skin of *Phyllomedusa trinitatis* frog include 28 amino acid peptide from dermaseptin BIV precursor. *Pancreas* 2004;**29**:110–5.
25. Marenah L, Shaw C, Orr DF, McClean S, Flatt PR, Abdel-Wahab YHA. Isolation and characterisation of an unexpected class of insulinotropic peptides in the skin of the frog *Agalychnis litodryas*. *Regul Pept* 2004;**120**:33–8.
26. McClenaghan NH, Flatt PR. Physiological and pharmacological regulation of insulin release: insights offered through exploitation of insulin-secreting cell lines. *Diabetes Obes Metab* 1999;**1**:137–50.
27. Mechkarska M, Ojo OO, Meetani MA, Coquet L, Jouenne T, Abdel-Wahab YHA, et al. Peptidomic analysis of skin secretions from the bullfrog *Lithobates catesbeianus* (Ranidae) identifies multiple peptides with potent insulin-releasing activity. *Peptides* 2011;**32**:203–8.
28. Ojo OO, Abdel-Wahab YH, Flatt PR, Mechkarska M, Conlon JM. Tigerinin-1R: a potent, non-toxic insulin-releasing peptide isolated-from the skin of the Asian frog, *Hoplobatrachus rugulosus*. *Diabetes Obes Metab* 2011;**13**:1114–22.
29. Olson L, Soto A, Knoop FC, Conlon JM. Pseudin-2: an antimicrobial peptide with low hemolytic activity from the skin of the paradoxical frog. *Biochem Biophys Res Commun* 2001;**288**:1001–5.
30. Sai KP, Jagannadham MV, Vairamani M, Raju MP, Devi SA, Nagaraj R. Tigerinins: novel antimicrobial peptides from the Indian frog *Rana tigerina*. *J Biol Chem* 2001;**276**:2701–7.
31. Wiens JJ, Sukumaran J, Pyron RA, Brown RM. Evolutionary and biogeographic origins of high tropical diversity in old world frogs (Ranidae). *Evolution* 2009;**63**:1217–31.
32. Zahid OK, Mechkarska M, Ojo OO, Abdel-Wahab YH, Flatt PR, Meetani MA, et al. Caerulein-and xenopsin-related peptides with insulin-releasing activities from skin secretions of the clawed frogs, *Xenopus borealis* and *Xenopus amieti* (Pipidae). *Gen Comp Endocrinol* 2011;**172**:314–20.

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Chapter 52

Neurohypophysial Peptides

Sunny K. Boyd

ABSTRACT

The amphibian neurohypophysis releases primarily two peptides—arginine vasotocin (AVT) and mesotocin (MT). Both are nonapeptides synthesized in multiple brain areas. Four specific receptor subtypes have been identified. The VT1aR is specific for AVT, found in the brain and kidney, and is coupled to a protein kinase C-mediated pathway. The VT1bR differs only in restricted expression exclusively in the brain and pituitary. The VT2R has a high affinity for AVT and hydrins and is found primarily in the skin and bladder, where it uses the cAMP signaling pathway. The MTR is specific for MT, couples to a protein kinase C–linked pathway, and is found in the brain and peripheral tissues. Biological actions of AVT include control of osmoregulation, behavior, reproductive tract contractions, and the cardiovascular system. Actions of MT are poorly understood. Finally, the amphibian neurohypophysis produces hydrins, which are extended forms of AVT with specific osmoregulatory effects on the skin and urinary bladder.

DISCOVERY

The class Amphibia contains three orders: the Anurans (frogs and toads), the Urodeles (salamanders and newts), and the Gymnophionans (caecilians). In the early 1900s, it was already recognized that the neurohypophysis (posterior pituitary) of frogs contained "active principles" that altered blood pressure. The structure of these principles was first elucidated in an anuran amphibian for arginine vasotocin (AVT) and mesotocin (MT) in the 1960s.[2] Characterization of AVT and MT structure in caecilians and urodeles, however, has only occurred within the last 15 years.[16,27] In all amphibians investigated, AVT and MT have been found, with the exception of *Bufo regularis* and *Plethodon shermani* in which mesotocin is replaced by [Ser5,Ile8]-oxytocin (seritocin) or [Val4]-mesotocin, respectively (Table 1[2,27]). Finally, anuran amphibians also possess unique closely related neurohypophysial peptides called hydrins.

PEPTIDE STRUCTURE AND PROCESSING OF PRECURSOR mRNAs

The nonapeptides AVT and MT are part of a conserved family of neurohypophysial hormones. Representative species from all vertebrate classes examined to date possess AVT, and it is thus considered the evolutionary precursor peptide in this family.[2] AVT is replaced by arginine vasopressin (AVP) in most mammals. Birds and reptiles also possess MT, in common with amphibians. Isotocin replaces MT in most fish and oxytocin (OT) is the mammalian homolog. All have a nine-amino acid peptide backbone with a disulfide bridge between conserved cysteine residues at positions 1 and 6. All possess an NH_2-blocked C-terminal glycine.

The nucleotide sequences coding for AVT and MT precursors have been reported for only four species of amphibians: the toad *Bufo japonicus*, two urodeles (*Taricha granulosa* and *Plethodon shermani*) and the caecilian, *Typhlonectes natans* (reviewed in Ref. 27). After a hydrophobic signal sequence, the amino acids of AVT or MT are represented. Next, a tripeptide is found which may facilitate processing or become incorporated into hydrin. This tripeptide is followed by a 93-amino acid neurophysin domain and a 36- or 37-amino acid glycoprotein domain. Although these domains result in the production of two peptides in mammals, this is not the case in amphibians where a single large neurophysin is produced.[2] In the MT precursor, a 93- or 94-amino acid peptide neurophysin also follows, but a glycoprotein is not present.

DISTRIBUTION OF AVT AND MT

Amphibian neurohypophysial peptides are synthesized in a variety of brain nuclei.[17,28] In all three amphibian orders, the most prominent cell group is in the magnocellular preoptic area. It is likely that these cells are the primary source of AVT and MT released from the neurohypophysis. AVT cell bodies can also be found in the pallium, bed nucleus of the stria terminalis, ventral thalamus, suprachiasmatic nucleus, hypothalamic nucleus, and midbrain tegmentum. On the other hand, MT cells are restricted primarily to diencephalic regions. Fibers are widespread, and these peptides likely function as neuromodulators. There can be significant variation in the location of AVT cell populations across species, especially outside the diencephalic regions. AVT concentrations in some brain areas are sexually dimorphic

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00052-X
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TABLE 1 Amphibian Neurohypophysial Peptides

	1	2	3	4	5	6	7	8	9	
Vasotocin	Cys-	Tyr-	Ile-	Gln-	Asn-	Cys-	Pro-	Arg-	Gly-	(NH_2)
Mesotocin	—	—	—	—	—	—	—	Ile-	—	(NH_2)
[Val[4]]-Mesotocin	—	—	—	Val-	—	—	—	Ile-	—	(NH_2)
Seritocin	—	—	—	—	Ser-	—	—	Ile-	—	(NH2)
Hydrin 1	—	—	—	—	—	—	—	—	—	Gly-Lys-Arg(OH)
Hydrin 1′	—	—	—	—	—	—	—	—	—	Gly–Lys(OH)
Hydrin 2	—	—	—	—	—	—	—	—	—	Gly(OH)

Dashes indicate residues identical to those of vasotocin.

and steroid sensitive.[6,7,28] Current evidence indicates this primarily results from the effects of androgens.

RECEPTOR STRUCTURE AND DISTRIBUTION

The MT receptor (MTR) and three subtypes of AVT receptor have been cloned in representative species of anuran and urodele amphibians.[1,4,15,18,26] All four belong to the G protein-coupled receptor family, with typical seven transmembrane domains. One AVT receptor subtype closely resembles the mammalian AVP receptor subtype V1a. The cDNA for this receptor (VT1aR) encodes a protein of 418 or 419 amino acids. The potency of ligands for this receptor is AVT>OT >AVP ≈ MT in an anuran and AVT>AVP>MT ≈ OT in a urodele amphibian. The VT1aR preferentially couples to the protein kinase C-mediated signaling pathway. The second AVT receptor subtype (VT2R) is more similar to the mammalian V2R. The receptor protein is 363 or 367 amino acids. The potency of ligands for this receptor is AVT=hydrin 1 > hydrin 2=AVP>MT=OT. The VT2R likely uses the adenylate cyclase-cAMP signaling pathway. The third subtype of AVT receptor has only been recently characterized in a newt.[15] This subtype VT1bR shares high sequence identity with the mammalian V1b receptor. Finally, the cDNA for the MTR encodes a receptor of 384–393 amino acids that shares significant homology with the OT receptor. The sensitivity of the MTR for ligands in this peptide family is MT>OT>AVT≥AVP or MT>AVT=OT>AVP > hydrin 1=isotocin=hydrin 2, depending on species. The frog MTR probably couples exclusively to the protein kinase C-linked signaling pathway.

The VT1aR mRNA is abundant in brain, anterior pituitary, heart, adrenal gland, kidney, and oviduct.[1] In the brain, the distribution of binding sites detected with 3H-AVP autoradiography is very similar to the distribution of the receptor mRNA.[1,6,15] Putative receptors are abundant in accumbens, pallium, striatum, lateral amygdala, preoptic area, magnocellular nucleus, hypothalamus, thalamus, and optic tectum. In the pituitary, VT1aR mRNA is found in the anterior pituitary and the intermediate lobe but not in the neurohypophysis.[1] Binding studies with 3H-AVP show receptors in the anterior pituitary but, in contrast, also show receptors in the neurohypophysis. This may reflect the presence of another subtype of AVT receptor in amphibians, not detected by the VT1aR probe. The presence of a VT1R subtype (either 1a or 1b) in amphibian kidney is also supported by autoradiographic and physiological evidence.[5,8,24] Binding sites for 3H-AVP or 125I-OVTA are found specifically located over the glomeruli and not renal tubules. These sites have ligand specificity more similar to mammalian V1 receptors than V2 receptors. The mRNA for the VT2R is expressed in the brain, heart, kidney, urinary bladder, and pelvic skin patch.[14,15,18] Lastly, the VT1b receptor is expressed almost exclusively in the brain and anterior pituitary in a newt.[15]

The MTR mRNA is abundant in the brain of anurans.[1,4,18] *In situ* hybridization shows highest levels in pallium, amygdala, ventral striatum, anterior preoptic area, nucleus of the periventricular organ, posterior tuberculum, ventral hypothalamic nucleus, thalamic nuclei, and optic tectum.[1] The MTR mRNA, like that for VT1aR, is found in the anterior pituitary and intermediate lobe but not in the neurohypophysis. The neural function of MT is unknown. The MTR mRNA is also found in several peripheral tissues, including the heart, adrenal gland, kidney, urinary bladder, skeletal muscle, fat body, and testis.[1,4,18] Importantly, the receptor is not found in the oviduct.[1] This suggests that, despite the structural similarities with the OT receptor, the MTR does not play a homologous role in reproductive tract smooth muscle contraction.

MAJOR BIOLOGICAL ACTIONS OF AVT AND MT

Osmoregulatory Effects

AVT control of osmoregulation in amphibians is complex and involves several different target organs—the kidneys,

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the urinary bladder, and the skin. At the renal level, AVT causes antidiuresis first by constricting preglomerular arteries and causing a reduction in glomerular filtration rate by as much as 85%.[33] The glomerular action is supported by the location of receptors over the glomeruli and presence of VT1aR mRNA. Whether AVT alters tubule reabsorption in amphibians is more controversial. AVT can cause antidiuresis by acting directly on tubules in a few species.[33] In addition, anuran amphibian kidney possesses an AVT-dependent aquaporin water channel protein.[30,32] Binding sites for radioactively labeled AVT analogs have not been located over tubules, however, and it is clear in some species that AVT has no tubular effects.[5,8] Mesotocin increases glomerular filtration rate and is diuretic in amphibians.[13,32,33]

The anuran urinary bladder served as the archetypal tissue for research on the water permeability of membranes for decades. Anurans can store fluid equivalent to 50% of body weight in the bladder. AVT stimulates the movement of water from the bladder back into the vasculature and this probably occurs via the VT2R.[33] AVT increases cAMP levels and the expression and translocation of aquaporin proteins.[30,32] Interestingly, although mammals possess a single AVP-dependent aquaporin in the kidney, anuran amphibians have three AVT-dependent proteins—aquaporin forms unique for the kidney, urinary bladder, and ventral pelvic skin. The anuran bladder also shows AVT-modulated sodium and urea reabsorption.[19,32] Mesotocin has a limited ability to stimulate water and sodium uptake across the anuran bladder, and the MTR has been located in this tissue.[32]

The permeable skin of amphibians allows the unique movement of water across that surface. AVT increases water permeability of ventral pelvic skin and facilitates rehydration of anurans and urodeles.[33] The VT2 receptor mRNA and protein is expressed in the pelvic skin and AVT regulates expression of water channel aquaporins in this tissue.[23,30] Mesotocin has been reported to increase cutaneous water permeability; however, MTR mRNA has not been found here.[1,4,18] Neither AVT nor MT alters water permeability of the skin of a caecilian amphibian.[29] Finally, active sodium transport across skin is also facilitated by AVT and MT in some, but not all, species.[31,33]

Behavioral Effects

In recent decades, the behavioral effects of neurohypophysial peptides in amphibians have received the most attention.[7] In many anurans, AVT modulates the display of vocal behavior.[21,34] The peptide facilitates the advertisement call used to attract mates, while it inhibits the release call given by unreceptive females. In addition, AVT stimulates the attraction of female anurans to the calls of males and alters locomotor behaviors. AVT also alters reproductive behaviors of urodeles. AVT stimulates amplectic clasping, pheromone release, and egg-laying behavior.[21,34] Effects of AVT are probably occurring via action in the central nervous system, and there is extensive interaction between AVT, androgens, and corticosterone in the modulation of amphibian reproductive behaviors.[7]

Reproductive Tract Effects

As in other vertebrate classes, the amphibian oviduct shows robust smooth muscle contractions after exposure to neurohypophysial peptides.[11] Unexpectedly, however, the oxytocin homolog, MT, is not the most potent peptide. Instead, AVT causes oviduct contractions. Sex steroids increase the responsiveness of the oviduct to AVT. In an analogous fashion, AVT stimulates contractions of the male urodele Wolffian duct.[35]

Cardiovascular Effects

It has been known since 1904 that pituitary extracts alter the circulatory system of the frog; however, this has received little attention. AVT is a vasopressor and causes the constriction of vessels *in vitro*.[9] Further, although AVT causes bradycardia *in vivo*, when autonomic receptors are blocked, AVT increases the heart rate and contractile force. On the other hand, MT acts as a vasodepressor in amphibians.[9] Three receptor subtypes (VT1R, VT2R, and MTR) are found in the heart, but it is unknown as to which receptors are present in the vasculature.[1,4,18] It has been proposed that the cardiovascular effects of AVT are the most primitive and that these gave rise to the osmotic effects of the peptide in amphibians and other tetrapods.

Other Effects

In amphibians, the adrenal gland homolog is the interrenal gland, where chromaffin cells are intermingled with steroidogenic cells. AVT immunoreactivity is found in virtually all chromaffin granules and may serve in a paracrine role to stimulate secretion of steroids.[20] AVT causes a rapid increase in corticosterone and aldosterone output from the interrenal gland.[12] The rank order potency of ligands suggests that VT2R is responsible.[20] However, the effect is via phosphoinositide-specific phospholipase C, and VT2R mRNA has not been found in the interrenal gland.[1,4,18,20]

AVT stimulates hepatic glycogenolysis and increases glycogen phosphorylase *a* activity in representative anuran and urodele amphibians.[3] This effect differs significantly from the stimulation of glycogenolysis in mammalian liver by AVP, however, because it seems to work via a VT2 subtype receptor and coupling to adenylate cyclase. VT2R mRNA has not been reported in the liver, but the liver receptor subtype may differ from the bladder VT2R.[14,18]

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STRUCTURE AND FUNCTION OF HYDRINS

Neurohypophysial extracts from anuran amphibians were first reported in 1961 to contain a third class of peptides. These peptides were sequenced and named "hydrins" in 1989 (Table 1[25]). Hydrin 2 is broadly distributed across anuran families. In *Xenopus laevis*, two variants of hydrin (termed hydrin 1 and 1′) are found instead. Hydrins have not been detected in any urodele or in any other vertebrate. Hydrins probably result from differential processing of the AVT preprohomone. The result is an extended peptide that is not amidated. Hydrins are as active as vasotocin in the anuran bladder, more active on anuran skin, but inactive in the kidney.[2,25] Given the high affinity of the VT2R for hydrins, it is probable that a receptor of this subtype is responsible for the biological actions of hydrins. Indeed, AVT, hydrin 1, and hydrin 2 have very similar effects on the translocation of aquaporin proteins in anuran ventral pelvic skin.[22] In addition to the effects on water reabsorption, hydrin 2 influences cutaneous ion transport.[10] Differences in physiological effects of AVT and hydrins on target tissues, however, suggest that two subtypes of VT2R may exist.

REFERENCES

1. Acharjee S, Do-Rego JL, Oh DY, Moon JS, Ahn RS, Lee K, et al. Molecular cloning, pharmacological characterization, and histochemical distribution of frog vasotocin and mesotocin receptors. *J Mol Endocrinol* 2004;**33**:293–313.
2. Acher R, Chauvet J, Rouille Y. Adaptive evolution of water homeostasis regulation in amphibians: vasotocin and hydrins. *Biol Cell* 1997;**89**:283–91.
3. Ade T, Segner H, Hanke W. Hormonal response of primary hepatocytes of the clawed toad, *Xenopus laevis*. *Exp Clin Endocrinol Diabet* 1995;**103**:21–7.
4. Akhundova A, Getmanova E, Gorbulev V, Carnazzi E, Eggena P, Fahrenholz F. Cloning and functional characterization of the amphibian mesotocin receptor, a member of the oxytocin/vasopressin receptor superfamily. *Eur J Biochem* 1996;**237**:759–67.
5. Ammar A, Rajerison RM, Roseau S, Blochfaure M, Butlen D. Frog glomerular vasotocin receptors resemble mammalian V-1b receptors. *Am J Physiol Regulat Integr Compar Physiol* 1994;**36**:R1198–208.
6. Boyd SK. Brain vasotocin pathways and the control of sexual behaviors in the bullfrog. *Brain Res Bull* 1997;**44**:345–50.
7. Boyd SK. Vasotocin modulation of social behaviors in amphibians. In: Choleris E, Pfaff D, Kavaliers M, editors. *Oxytocin, vasopressin and related peptides in the regulation of behavior*. Cambridge University Press, in press.
8. Boyd SK, Moore FL. Autoradiographic localization of putative arginine vasotocin receptors in the kidney of a urodele amphibian. *Gen Comp Endocrinol* 1990;**78**:344–50.
9. Chan DKO. Comparative physiology of the vasomotor effects of neurohypophysial peptides in the vertebrates. *Amer Zool* 1977;**17**:751–61.
10. Ford NA, Robinson GD. Effects of hydrin 2 on cutaneous electrical properties of *Rana pipiens*. *Gen Comp Endocrinol* 2003;**134**:103–8.
11. Guillette LJ, Norris DO, Norman MF. Response of amphibian (*Ambystoma tigrinum*) oviduct to arginine vasotocin and acetylcholine *in vitro* – influence of steroid hormone pretreatment *in vivo*. *Comp Biochem Physiol C Pharmacol Toxicol Endocrinol* 1985;**80**:151–4.
12. Gupta OP, Hanke W. Regulation of interrenal secretion in the axolotl, *Ambystoma mexicanum*. *Exp Clin Endocrinol* 1994;**102**:299–306.
13. Hartenstein HR, Stiffler DF. Renal responses to mesotocin in adult *Ambystoma tigrinum* and *Notophthalmus viridescens*. *Exp Biol* 1990;**48**:373–7.
14. Hasegawa T, Sugawara Y, Suzuki M, Tanaka S. Spatial and temporal expression of the ventral pelvic skin aquaporins during metamorphosis of the tree frog, *Hyla japonica*. *J Membr Biol* 2004;**199**:119–26.
15. Hasunuma I, Toyoda F, Kadono Y, Yamamoto K, Namiki H, Kikuyama S. Localization of three types of arginine vasotocin receptors in the brain and pituitary of the newt *Cynops pyrrhogaster*. *Cell Tissue Res* 2010;**342**:437–57.
16. Hilscher-Conklin C, Conlon JM, Boyd SK. Identification and localization of neurohypophysial peptides in the brain of a caecilian amphibian, *Typhlonectes natans* (Amphibia: Gymnophiona). *J Comp Neurol* 1998;**394**:139–51.
17. Hollis DM, Chu J, Walthers EA, Heppner BL, Searcy BT, Moore FL. Neuroanatomical distribution of vasotocin and mesotocin in two urodele amphibians (*Plethodon shermani* and *Taricha granulosa*) based on *in situ* hybridization histochemistry. *Brain Res* 2005;**1035**:1–2.
18. Kohno S, Kamishima Y, Iguchi T. Molecular cloning of an anuran V-2 type arg(8) vasotocin receptor and mesotocin receptor: functional characterization and tissue expression in the Japanese tree frog (*Hyla japonica*). *Gen Comp Endocrinol* 2003;**132**:485–98.
19. Konno N, Hyodo S, Matsuda K, Uchiyama M. Arginine vasotocin promotes urea permeability through urea transporter expressed in the toad urinary bladder cells. *Gen Comp Endocrinol* 2007;**152**:281–5.
20. Larcher A, Delarue C, Homodelarche F, Kikuyama S, Kupryszewski G, Vaudry H. Pharmacological characterization of vasotocin stimulation of phosphoinositide turnover in frog adrenal gland. *Endocrinol* 1992;**130**:475–83.
21. Moore FL, Boyd SK, Kelley DB. Historical perspective: hormonal regulation of behaviors in amphibians. *Horm Behav* 2005;**48**:373–83.
22. Ogushi Y, Kitagawa D, Hasegawa T, Suzuki M, Tanaka S. Correlation between aquaporin and water permeability in response to vasotocin, hydrin and beta-adrenergic effectors in the ventral pelvic skin of the tree frog *Hyla japonica*. *J Exp Biol* 2010;**213**:288–94.
23. Ogushi Y, Tsuzuki A, Sato M, Mochida H, Okada R, Suzuki M, et al. The water-absorption region of ventral skin of several semiterrestrial and aquatic anuran amphibians identified by aquaporins. *Am J Physiol Regul Integr Comp Physiol* 2010;**299**:R1150–62.
24. Prutskova NP. Renal filtration and reabsorption of GFP in *Rana temporaria*: effect of arginine vasotocin. *J Evol Biochem Physiol* 2011;**47**:59–68.
25. Rouille Y, Ouedraogo Y, Chauvet J, Acher R. Distinct hydro-osmotic receptors for the neurohypophyseal peptides vasotocin and hydrins in the frog *Rana esculenta*. *Neuropeptides* 1995;**29**:301–7.
26. Searcy BT, Bradford CS, Thompson RR, Filtz TM, Moore FL. Identification and characterization of mesotocin and V1a-like vasotocin receptors in a urodele amphibian, *Taricha granulosa*. *Gen Comp Endocrinol* 2011;**170**:131–43.
27. Searcy BT, Walthers EA, Heppner BL, Thompson RR, Moore FL. Identification of mesotocin and vasotocin nucleotide sequences in two species of urodele amphibian. *Gen Comp Endocrinol* 2009;**160**:111–6.

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28. Smeets W, Gonzalez A. Vasotocin and mesotocin in the brains of amphibians: state of the art. *Micros Res Tech* 2001;**54**:125–36.
29. Stiffler DF, Talbot CR. Exchanges of oxygen, carbon dioxide, nitrogen and water in the caecilian *Dermophis mexicanus*. *J Comp Physiol B Biochem Syst Environ Physiol* 2000;**170**:505–9.
30. Suzuki M, Tanaka S. Molecular diversity of vasotocin-dependent aquaporins closely associated with water adaptation strategy in anuran amphibians. *J Neuroendocrinol* 2010;**22**:407–12.
31. Takada M, Fujimaki-Aoba K, Hokari S. Vasotocin- and mesotocin-induced increases in short-circuit current across tree frog skin. *J Comp Physiol B Biochem Syst Environ Physiol* 2011;**181**:239–48.
32. Uchiyama M, Konno N. Hormonal regulation of ion and water transport in anuran amphibians. *Gen Comp Endocrinol* 2006;**147**: 54–61.
33. Warburg MR. Hormonal effect on the osmotic, electrolyte and nitrogen balance in terrestrial amphibia. *Zool Sci* 1995;**12**:1–1.
34. Wilczynski W, Lynch KS, O'Bryant EL. Current research in amphibians: studies integrating endocrinology, behavior, and neurobiology. *Horm Behav* 2005;**48**:440–50.
35. Zoeller RT, Lais LT, Moore FL. Contractions of amphibian wolffian duct in response to acetylcholine, norepinephrine, and arginine vasotocin. *J Exp Zool* 1983;**226**:53–7.

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Chapter 53

Opioid and Bv8 Peptides

Lucia Negri, Pietro Melchiorri and Roberta Lattanzi

ABSTRACT

Amphibian opioids are small peptides found in the skin of South American hylid frogs Phyllomedusinae. Two subfamilies of opioid peptides have been distinguished according to their selectivity for the μ- and δ-opioid receptors: the dermorphin and deltorphin families.

The distinctive feature of opioid peptides is the presence of a D-amino acid in the N-terminal sequence, Tyr-D-Xaa-Phe, which restricts peptide conformation and confers resistance against enzymatic degradation. Bv8 is a small protein isolated from the skin secretion of the frog *Bombina variegata*. Orthologs of Bv8 have been found in snakes (MIT) and in mammals (Prokineticins). The striking characteristics of these proteins are the identical amino terminal sequence AVITG and the presence of ten cysteines with identical spacing. Bv8/Prokineticin proteins have been shown to modulate several physiological and pathological conditions including neurogenesis, angiogenesis, cancer progression, pain perception, immune response, and circadian rhythms. Disruption of the prokineticin system has been implicated in human Kallmann's syndrome and Hirschprung's disease.

OPIOID PEPTIDES

Although Vittorio Erspamer and his colleagues discovered the opioid peptides in Amazonian frogs only in the1980s,[5] the Matses tribe of the upper Amazonian basin unveiled the pharmacological properties of amphibian skin opiates long ago. For centuries, they had habitually applied the dried skin secretions of *Phyllomedusa bicolor*, called "sapo" (the Spanish word for toad), to cuts in their skin during shamanistic hunting rituals. The abundance of the opioid peptides in "sapo" probably caused analgesia and behavioral excitation in hunters.

Classical amphibian opiate peptides have been found only in the skin of South American hylid frogs belonging to the subfamily Phyllomedusinae (*Phyllomedusa*, *Agalychnis*, and *Pachymedusa* species). They are small peptides codified by different genes and secreted by the syncytial cells forming the luminal wall of serous holocrine glands in the frog integument. Electron microscopy reveals that the serous glands of Phyllomedusinae have morphological features, especially the secretory granules, which differ from those of the glands in other frogs. The biosynthesis and secretion of opioid peptides occur together with dermaseptins, antibiotic peptides. Although these peptides differ in their structure and activity, they derived from precursors that have very similar preproregions.[11]

New peptides endowed with opioid activity have been recently identified in the skin secretion of an Australian frog and in the brain of the Xenopus. In the skin secretion of Australian frogs of the genus Litoria, two major peptides (FPWL-NH_2 and FPWP-NH_2) have been identified that display opiod activity at micromolar concentrations. They are analogs to peptides first isolated from *Phyllomedusa rohdei* and called tryptophyllin. The sequences of these peptides resemble those of the human brain endomorphins (e.g., YPWF-NH_2).[7] These peptides are released in the skin secretion in milligram amounts.

Nonmammalian prodynorphin-derived peptide was discovered by analysis of a Xenopus brain cDNA library. The prepropeptide contains five potential opioid-like peptides, collectively named xendorphins. One of these, xendorphin B1 (Xen)PDYN-B sequence 96-111: YGGFIRKPDKYKFLNA), is a hexadecapeptide that displays nanomolar affinity for κ-opioid receptors in rat brain membranes and produces a long-lasting and dose-dependent antinociceptive effect in the *Rana pipiens*.[24]

A Brief History of the Amphibian Opioid Peptides

The first to be discovered were two dermorphins (N. 1 and N. 2 in the Table 1) identified in the skin of *Ph. sauvagei*, *Ph. rohdei*, and *Ph. burmeisteri*. The very high dermorphin content in the skin of these Phyllomedusinae, about 50–80μg/g of fresh skin, made it easy to purify and sequence the peptides. Until the discovery of mammalian endomorphins,

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00053-1
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these peptides represented the most potent and selective μ-opiate receptor agonists identified in living organisms.[5]

The screening of a cDNA library prepared from the skin of *Ph. sauvagei* established the amino acid sequence of several dermorphin precursors. The sequence of one of these cDNAs indicated the existence of another peptide that contained methionine as the second amino acid. This peptide (N. 8 in Table 1) was subsequently isolated from the skin of *Ph. sauvagei* and proved to have higher affinity and selectivity for δ-opiate receptors than any other known natural compound. It has been given various names: deltorphin, D-Met-deltorphin, dermenkephalin, and deltorphin A. Subsequently, two additional peptides with an even higher affinity for the δ-opiate receptor were isolated from the skin of *Ph. bicolor*. Like dermorphin, these peptides contain D-alanine as the second amino acid. They have been termed D-Ala-deltorphin-I and D-Ala-deltorphin-II (N. 9 and 10 in Table 1). Screening of cDNA libraries from the skin of *Ph. bicolor* revealed the sequence of four precursors for D-Ala-deltorphin-I and -II. These precursors contain one copy of D-Ala-deltorphin-II and one, three, or no copies of D-Ala-deltorphin-I, respectively. The D-Ala-deltorphin precursors also contain three additional sequences related to dermorphins. As predicted by the absence of glycine at the carboxyl terminus of these peptides, the end products extracted from the skin contain a free α-carboxyl group at the C-terminus.[5,18] Alternative cleavage sites in the sequence of dermorphin can generate C-terminally elongated dermorphins: Two of these dermorphins have been isolated from *Ph. sauvage*i skin extracts (N. 6 and 7 in Table 1). In the Brazilian frog *Ph. burmeisteri*, a linear peptide of 17 amino acids with a D-Leu residue at position 2 has been identified and termed D-Leu-deltorphin-l7 (N. 11 in Table 1). During screening of cDNA libraries prepared from the skin of two additional species of Phyllomedusinae, the *Pachymedusa dacnicolor* and *Agalychnis annae*, using sequence information from cDNAs encoding dermorphin and deltorphin precursors from *Ph. sauvagei* and *Ph. bicolor*, we identified, in addition to four copies of dermorphin, a sequence that contains the genetic information for a novel peptide: Tyr-D-Ile-Phe-His-Leu-Met-Asp-NH_2 (N. 12 in Table 1). This peptide, an analog of D-Met-deltorphin, contains a D-Ile at the second position.[23]

Structure and Conformation

All amphibian opioid peptides contain the N-terminal sequence Tyr-D-Xaa-Phe, where the amino acids Tyr^1 and Phe^3 are of L-configuration, and D-Xaa^2 is a D-amino acid. The D-amino acid–containing opioid peptides arise from precursors showing a common preproregion (22-residue signal peptide and an 18–25-residue acidic prosequence) with precursors of the peptide antibiotics dermaseptins (24–34 residue polycationic and α-helical amphipathic peptides). The D-enantiomer is encoded, however, by the codon for the

TABLE 1 Natural Occurring Amphibian Opioid Peptides and Their Origin

Dermorphins			
1	Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH_2	Dermorphin	Ph. sauvagei, burmeisteri, rohdei, hypochondrialis, tarsius, Agalychnis
2	Tyr-D-Ala-Phe-Gly-Tyr-Hyp-Ser-NH_2	[Hyp^6]der	
3	Tyr-D-Ala-Phe-Gly-Tyr-Pro-Lys-OH	[Lys^7-OH]-der	Ph. bicolor
4	Tyr-D-Ala-Phe-Trp-Tyr-Pro-Asn-OH	[Trp^4,Asn^7-OH]-der	
5	Tyr-D-Ala-Phe-Trp-Asn-OH	[Trp^4,Asn^5-OH]-der	
6	Dermorphin-Gly-Glu-Ala-OH	Y10A	Ph. sauvagei
7	Dermorphin-Gly-Glu-Ala-Lys-Lys-Ile-OH	Y131	
Deltorphins			
8	Tyr-D-Met-Phe-His-Leu-Met-Asp-NH_2	D-Met-deltorphin	Ph. sauvagei, burmeisteri, rohdei, tarsius
9	Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH_2	D-Ala-deltorphin-I	Ph. bicolor
10	Tyr-D-Ala-Phe-Glu-Val-Val-Gly-NH_2	D-Ala-deltorphin-II	
11	Tyr-D-Leu-Phe-Ala-Asp-Val-Ala-Ser-Thr-Ile-Gly-Asp-Phe-Phe-His-Ser-Ile-NH_2	D-Leu-deltorphin-17	Ph. burmeisteri
12	Tyr-D-Ile-Phe-His-Leu-Met-Asp-NH_2	D-Ile-deltorphin	Pachymedusa dacnicolor, Agalychnis annae

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L-isomer in the precursor cDNA. Thus, L-Xaa^2 must be converted to D-Xaa^2 by an unusual posttranslational reaction that presumably takes place in the precursor itself in the secretory granules. The specificity of isomerization depends on the presence of structural and/or conformational determinants in the peptide N-terminus surrounding the isomerization site.[1]

Because [L-Xaa^2]-containing peptides have never been found in amphibian skin extracts, the epimerization mechanism probably involves a quantitative inversion of the chirality of the α-carbon of the amino acid residue, rather than a racemization, which would yield an equimolar mixture of L- and D-isomers. Enzymes catalyzing the formation of D-amino acids are so far known only in yeast. From Bombina skin secretions, Kreil[8] purified a 52-kDa glycoprotein that catalyzes the reaction Ile-Ile-Gly to Ile-D-allo-Ile-Gly. The partial conversion of Ile to D-allo-Ile in peptide linkage proceeds without the addition of cofactors.

Similar to mammalian prohormones, all opioid peptides in amphibian skin precursors are flanked by paired dibasic amino acids (Lys–Arg). Moreover, the precursor sequence contains an additional Gly residue at its carboxyl terminus; this extra residue is required for the carboxamidation of the mature heptapeptide.

Despite the common N-terminal tripeptide (Tyr-D-Xaa-Phe), the two families of amphibian opioids differ widely in receptor selectivity but bind to their own receptors with similar affinity. The N-terminal domain contains the minimum sequence essential for opioid receptor binding, whereas the C-terminal domain contains the address requisites for receptor selectivity. The presence within the putative transmembrane domains II and III of the μ-receptor protein of negatively charged amino acid residues (Asp [114], Asp [147]) that contribute to ligand binding may explain why positively charged dermorphins have high μ-opioid receptor selectivity and why the amidation of their terminal carboxyl group increases their affinity and potency.

Similarly, the negatively charged C-terminal tetrapeptide of deltorphins enhances deltorphin selectivity for the δ-opioid receptor by electrostatic attraction to the positively charged binding site of the δ-receptor (Arg [292]) and electrostatic repulsion from the negatively charged μ-receptor site. Unlike the positively charged C-terminus of dermorphins, the negatively charged C-terminal tail of deltorphins (especially in D-Ala^2-deltorphin-II) comes into close contact with the positively charged N-terminal tripeptide of the molecule and folds the backbone, thus placing the Tyr^1 and Phe^3 aromatic rings in specific orientations best suited for δ-receptor docking.

Biological Actions

The pharmacology of amphibian opiates has been extensively described.[5,16,18,22,23]

Dermorphin has a high affinity and selectivity for μ-opioid receptors: Its opioid potency in guinea pig ileum (GPI) preparations is 100 times higher than that of morphine. The amidated analogs of *Ph. bicolor* dermorphins display a similar or slightly higher μ-opioid receptor affinity but a significantly higher (15–20 times) κ-opioid receptor affinity than dermorphin does (Table 2). Like μ-opiate agonists, dermorphins produce antinociception and also catalepsy, respiratory depression, constipation, tolerance, and dependence, although at a lower degree than morphine does. Dermorphin-induced antinociception takes place at both the supraspinal and spinal levels. The analgesic potency of dermorphin is about 280 times higher than that of morphine after intracerebroventricular (icv) injection but comparable to that of morphine after subcutaneous (sc) injection because of its low CNS permeability and bioavailability. [Lys^7]dermorphin and some synthetic analogs bearing a hydrophilic group (a basic amino acid or a glycosyl residue) at the C-terminal end of the molecule enter the CNS at a 7–10 times higher rate than dermorphin does, suggesting facilitated transport across the blood brain barrier by a carrier or endocytosis. The amphibian opioid with the highest analgesic potency and efficacy is [Lys^7]dermorphin (AD_{50}: 30pmol per rat, by the icv route; 0.22μmol/kg, by the sc route). Its antinociceptive effect lasts more than 3h and is never accompanied by catalepsy. By contrast, [Trp^4,Asn^7]dermorphin induces catalepsy at icv doses that do not modify the reaction time in the tail-flick test: The ratio of antinociceptive to cataleptic ED_{50} (AD_{50}/CD_{50}) is 40, a value 500 times higher than that calculated for [Lys^7]dermorphin ($AD_{50}/CD_{50}=0.073$). The ratio of antinociceptive to respiratory-depressant ED_{50} doses is 17 times lower for [Lys^7]dermorphin than for morphine. Indeed, [Lys^7]dermorphin, in the range of the analgesic doses (36–120pmol, icv; 0.12–4.7μmol/kg, sc) significantly increases respiratory frequency and minute volume of rats breathing air or hypoxic inspirates. The early onset ventilatory stimulation produced by [Lys^7]dermorphin, and ascribed to the intact peptide, is mediated by serotonergic descending excitatory pathways that stimulate neurons of the brain stem respiratory network.[22] In rats and mice, central or peripheral administration of the dermorphin-like peptides induces a significantly slower development of tolerance to the antinociceptive effect than morphine does. Withdrawal symptoms precipitated by naloxone are less intense in peptide-dependent than in morphine-dependent rats. Like morphine, dermorphin inhibits gastric acid secretion, delays gastric emptying, and slows down intestinal and colonic propulsion. Compared with dermorphin, [Lys^7]dermorphin is slightly (3–5 times) more potent in both reducing acid gastric secretion and gastric emptying. By contrast [Trp^4,Asn^7]dermorphin is 20–50 times less potent than dermorphin in inhibiting gastric acid secretion but is 100–1000 times less potent in delaying gastric emptying. In spite of comparable μ-opioid receptor affinity, the two dermorphins from *Ph. bicolor*, [Lys^7]dermorphin and [Trp^4,Asn^7]dermorphin, differ in biological effects, underling distinct functional roles of the μ-opioid receptors.

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TABLE 2 Affinity and Selectivity for μ-, δ-, and κ-Opioid Receptors, Biological Activities on Guinea Pig Ileum (GPI) and Mouse Vas Deferens (MVD) Preparations and Analgesic Potency of Dermorphins and Deltorphins

	K_i, nM (mean ± S.E.)			IC_{50}, nM (mean ± S.E.)		Analgesia
Compounds	μ	δ	κ	GPI	MVD	AD_{50}, nmol per rat
Morphine	11 ± 1.5	500 ± 48		150 ± 18.5	1215 ± 115	8.7 ± 1.1
Dermorphin						
dermorphin	0.6 ± 0.02	929 ± 41	8162 ± 979	1.29 ± 0.11	16.5 ± 1.3	0.035 ± 0.01
[Hyp^6]der	0.7 ± 0.03	1200 ± 131	–	1.6 ± 0.12	18.1 ± 2.9	–
[Lys^7]der	0.09 ± 0.008	1105 ± 185	617 ± 66	1.15 ± 0.13	13.6 ± 1.5	0.026 ± 0.009
[Lys^7-OH]der	5.7 ± 0.51	1150 ± 172	–	3.82 ± 0.45	56.3 ± 7.8	–
[Trp^4,Asn^5]der	0.9 ± 0.052	480 ± 45	177 ± 12	5.00 ± 0.52	73.7 ± 9.1	0.43 ± 0.04
[Trp^4,Asn^5-OH]der	4.44 ± 0.39	715 ± 73	2396 ± 301	13.1 ± 1.2	205 ± 25	–
[Trp^4,Asn^7]der	0.32 ± 0.026	690 ± 57	427 ± 47	0.58 ± 0.06	6.6 ± 0.9	2.86 ± 0.31
[Trp^4,Asn^7-OH]der	2.9 ± 0.2	865 ± 69	905 ± 61	1.3 ± 0.2	10.4 ± 1.3	–
Deltorphin						
D-Ala^2-delt I	1985 ± 224	0.78 ± 0.08	>10,000	1239 ± 203	0.18 ± 0.02	15 ± 3.0
D-Ala^2-delt II	2222 ± 233	1.03 ± 1.09	>10,000	2500 ± 170	0.37 ± 0.03	54 ± 6.0
D-Met^2-delt	693 ± 37	1.18 ± 0.21	>10,000	1476 ± 185	0.97 ± 0.05	20 ± 5.0
D-Leu^2-delt	>10,000	>10,000	>10,000	>5000	2480 ± 378	–
D-Ile^2-delt	1021 ± 57	24 ± 3	>10,000	4200 ± 475	7.0 ± 0.9	6.7 ± 1.0
D-aIle^2-delt	452 ± 68	54 ± 65	>10,000	3200 ± 307	70 ± 8.2	9.8 ± 1.2

The discovery of deltorphins provided the tools for the functional characterization of the δ-opiate system. Although D-Ala^2-deltorphins have δ-binding affinity similar to D-Met^2-deltorphin, they consistently have the highest δ-opioid selectivity (Table 2). The rank order of selectivity ($K_{i\delta}/K_{i\mu}$) is D-Ala^2-deltorphin-I = D-Ala^2-deltorphin-II > D-Met^2-deltorphin >> D-Ile^2-deltorphin >> D-Leu^2-deltorphin heptadecapeptide or its N-terminal decapeptide fragment. Both D-Met^2-deltorphin and D-Ala^2-deltorphins are highly resistant to enzyme degradation. D-Ala^2-deltorphin-I and -II cross the blood–brain barrier *in vivo* and *in vitro*: D-Ala^2-deltorphin-II is a transport substrate of organic anion transporting polypeptides (Oatp/OATP), a family of polyspecific membrane transporters, strongly expressed in the rat and human blood–brain barrier.

In mice, D-Ala^2-deltorphin II, by the icv route (EC_{50} = 2.1 nmol per mouse) is half as potent as morphine and the δ-selective antagonist, naltrindole, antagonizes its analgesic effect. Repeated injections of D-Ala^2-deltorphin-II induce tolerance to the antinociceptive effect. Isobolographic analysis shows that the supraspinal antinociception induced by the δ-opioid agonist DPDPE and the spinal antinociception induced by D-Ala^2-deltorphin-II are synergistic in many nociceptive tests. In rats, intrathecal injections of D-Ala^2-deltorphin-II (from 0.6nmol per rat) produce a naltrindole-reversible inhibition of the tail-flick response by inhibiting the nociceptive neurons in the superficial and deeper dorsal horn of the medulla. Conversely, when injected icv in rats, D-Ala^2-deltorphin-II is a weak partial agonist (>30nmol per rat) and induces fleeting naloxone-sensitive antinociception. Both D-Ala^2-deltorphin-I and D-Met^2-deltorphin at doses between 6.5 and 52nmol per rat induce a naloxone-sensitive analgesia.

Results suggest that the δ-agonists play a predominantly modulatory role in antinociception rather than a primary role. In mice and in rats, the intensity of δ-opioid analgesia depends on the coactivation of μ-opiate receptors by endogenous or exogenous opioids. Accordingly, deltorphin-induced analgesia is weaker in homozygote mice with a disrupted μ-opiate receptor gene than in wild-type mice.

Injections of the deltorphins into the rat brain ventricles, ventral tegmental area, and nucleus accumbens at doses

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10–100 times lower than those inducing analgesia (0.06–3.8nmol per rat) invariably increase locomotor activity and induce stereotyped behavior. The δ-selective antagonist, naltrindole, antagonizes deltorphin-induced motor activity. Repeated icv injections of D-Ala^2-deltorphin-II in naive rats induce tolerance to the stimulant effects. High doses (10–50nmol per rat) of all the D-Met^2-deltorphin analogs and His^4-substituted D-Ala^2-deltorphins induce nonopioid motor dysfunction that is completely blocked by the non-competitive NMDA antagonist, dextrorphan. Deltorphin improves memory consolidation in a passive avoidance test in mice. D-Ala^2-deltorphin-I, at low doses, stimulates respiratory activity in fetal lambs. D-Ala^2-deltorphin-II inhibits diarrhea induced by castor oil and colonic bead expulsion, but it leaves the rate of transit through the small intestine unchanged. By the sc route, D-Ala^2-deltorphin-I inhibits acidified alcohol-induced gastric mucosal lesions. It is involved in the control of ingestive behavior, stimulating food intake and angiotensin-mediated water consumption in rats.

The discovery of the amphibian opioid peptides confirms that the amphibian skin and its secretions offer an inexhaustible supply of biologically active peptides. The amphibian peptides isolated from methanol extracts of amphibian skin are of a small or relatively small molecular mass (700–4600Da). Proteins, externally secreted by syncytial cells forming the wall of the integument glands, can be obtained upon electrical stimulation of the skin of the living frog. By repetitive electrical stimulation at weekly intervals, several milligrams of bioactive proteins can be collected from a single frog, yielding an amount of biologically active proteins that is enough for pharmacological research. This is the case of the new Bv8/prokineticin family.

BV8/PROKINETICIN FAMILY (SWISS-PROT: Q9PW66)

A basic small peptide, containing 77 amino acids of which ten are cysteine, has been isolated from the skin secretion of discoglossid frogs *Bombina variegata* and *Bombina bombina*[17] and named Bv8, to indicate its origin (*Bombina variegata*) and its molecular mass (about 8kDa). The amino acid sequence was established by automated Edman degradation and analysis of proteolytic fragments as well as by cDNA cloning. Homologs of Bv8 have been predicted (but not yet isolated) in skin secretions of *Rana temporaria* and *Rana esculenta* and have been demonstrated in the skin secretion of *Bombina orientalis* (Bo8) and *Bombina maxima* (Bm8a). These proteins are derived from simple precursors composed of a putative 19-residue signal peptide and the mature protein. Bo8 and Bm8a show 96% and 92% sequence identity with Bv8. Moreover, the putative signal peptides in all three proproteins are 100% identical (Fig. 1). The primary sequence of Bv8 is similar to MIT (mamba intestinal toxin), a constituent of the venom of the snake black Mamba. The amphibian and snake proteins share the same pattern of cysteine distribution, and their overall identity is 58%. cDNA cloning showed the existence of mouse (mBv8) and human (hBv8) homologs of amphibian Bv8.[21] The striking characteristics of these proteins are the identical amino terminal sequence, AVITG, and the presence of ten cysteines with identical spacing (Fig. 1). A similar Cys-motif is also present in mammalian colipase and in the carboxy-terminal region of members of the Dickkopf family of extracellular signaling proteins. However, the frog protein does not stimulate the activity of pancreatic lipase, and it is also inactive in an assay for Dickkopf functions. Starting from the 3D-structure of mammalian colipase, a model was built for frog Bv8 in which the hydrophobic amino terminal sequence AVITG forms a sort "beak" exposed at the surface of the tightly folded rest of the protein.[9]

Two forms of mBv8 and hBv8 have been characterized in the mouse and human testis. These forms differ in an exon coding for 21 amino acids, the majority of which are basic (Fig. 1). The predominant site of Bv8 expression in both humans and mice is primary spermatocytes in the seminiferus tubules of the testis. The genomic structure of these murine and human Bv8 genes has been determined, and the chromosomal localization was identified near a synteny breakpoint of mouse chromosome 6 and human 3p21.[21] By searching the EST database using the predicted coding of Bv8, Zhou[15] identified two human EST sequences, one encoding the human protein already described and the other encoding a slightly different Bv8-like protein. The two proteins were named prokineticin 2 (PK2) and prokineticin 1 (PK1) (Fig. 1). The name prokineticin refers to the ability of these peptides to contract GPI, a property shared with amphibian Bv8.[15] By screening a library of human-secreted proteins for the ability to induce proliferation in capillary endothelial cells, Ferrara[14] identified a protein that induced proliferation, migration, and fenestration in the endothelial cells of steroid synthesizing glands (ovary, testis, adrenals) and named it endocrine-gland-derived vascular endothelial growth factor (EG-VEGF). EG-VEGF and PK1 are the same protein and have an overall identity of 58% and homology of 76% with human PK2 and murine Bv8 and 43% identity with amphibian Bv8. PK1/EG-VEGF has been isolated and sequenced also from bovine milk. Rat and human mRNAs for PK1 and PK2 have been cloned and their expression patterns reported in peripheral tissues and the central nervous system.[15] The distribution of murine Bv8-like proteins, PK1 and PK2, and their mRNAs has been reported in the brain (olfactory bulb, cerebral cortex, hippocampus, some thalamic and hypothalamic nuclei, Purkinje cells of the cerebellum, many nuclei of the brain stem), spinal cord, gastrointestinal tract, endocrine glands, and other peripheral organs.[21] The PK2 mRNA expression pattern in the suprachiasmatic nucleus (SCN) of mice[2] and of rats[20] is rhythmic

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FIGURE 1 Amino acid sequences of Bv8-like proteins in the frog (Bv8, Bo8 and Bm8a) skin secretion compared with the snake venom protein (MIT) and the human prokineticins: PK1, PK2, and PK2L, the long form of PK2, containing a 21-amino acid insert.

(being the lowest in the dark phase) and is severely blunted in mutant mice deficient in Clock or cryptochrome genes. PK2/Bv8, constitutively expressed in cells and organs of myeloid origin, is dramatically upregulated by inflammatory processes.[6]

Receptors for PK1 and PK2 have been identified in humans, mice, and rats. These receptors, named prokineticin receptor 1 and 2 (PKR1 and PKR2), belong to the family of G protein-coupled receptors, share approximately 85% amino acid identity, and are about 80% identical to the previously described mouse orphan receptor gpr73. In a study aimed to map the expression of PKR1 and PKR2 in rat and mouse nervous system, we[21] and others[3] detected PKR2 mRNA in the brain (olfactory bulbs, SCN, hippocampus, paraventricular thalamic and hypothalamic nuclei, nucleus arcuatus, subfornical organ (SFO), amygdala, caudate putamen, and cortex) and in dorsal root ganglia (DRG). PKR1 is mainly expressed outside the mammalian central nervous system, with the highest density in the neurons of DRG. PKR1 is also expressed by murine macrophages. Mice lacking the PKR2 show a dramatic reduction in olfactory bulbs (OB) size, and they do not survive; mice lacking PKR1 are severely deficient in their responses to noxious thermal and chemical (capsaicin, protons) stimuli.[19,21]

Intensive investigation of the Bv8/prokineticin system over the past decade has revealed a dazzling array of physiological functions including the regulation of circadian rhythms, metabolism, angiogenesis, neurogenesis, muscle contractility, hematopoiesis, immune response, reproduction, and pain perception. In addition, the disruption of prokineticin system has been implicated in several pathological conditions, including cancer, immunological response, mood disorder (anxiety/depression),[10] cardiomyopathy, and persistent pain. Point mutations in the genes encoding for prokineticins and/or prokineticin receptors are involved in human Kallmann's syndrome and Hirschprung's disease.[19] The isolation of an adequate amount of the amphibian Bv8 protein has provided valuable research tools for characterizing the prokineticin pharmacology.

On cultured cerebellar granular cells, Bv8 reduces the extent of apoptotic death induced by switching the concentration of K^+ in the growing medium from 25 to 5mM. In cell-based assays, Bv8 and EG-VEGF/PK1 induce proliferation, migration, and survival of primary adrenal cortical capillary endothelial (ACE) cells. Delivery of Bv8 or EG-VEGF to the mouse testis results in a potent angiogenic response resulting from activation of PKRs on vascular endothelial cells within the interstitial space.[13,14]

Injections of Bv8 (from 0.25 to 60pmol) into the lateral ventricles of the rat brain suppress diurnal, nocturnal, deprivation-induced, and neuropeptide Y-stimulated feeding. Bv8 injected icv also stimulates drinking in a dose-dependent manner. We showed that Bv8 stimulates drinking through its receptors in the SFO and inhibits feeding after injection into the arcuate nucleus. Rat sensitivity to the Bv8-induced anorexogenic response increases significantly during the dark phase of the circadian cycle,[20] in accordance with the physiological role of Bv8/PK2 in controlling circadian rhythm from the SCN.[2]

In rats, icv and sc injections of Bv8 induce antidiuresis dependent on vasopressin release. Bv8 injected sc also induces the release of oxytocin and corticosterone.[21]

In rodents, subcutaneous, intrathecal, intraplantar (Fig. 2), and intrabrain administration of amphibian Bv8 induces hyperalgesia activating the prokineticin receptors that are present on peripheral nociceptors and in regions of the central nervous system associated with pain.[19,21]

We showed that amphibian Bv8 binding on PKR1 and PKR2 in dorsal root ganglion neurons produces $[Ca^{++}]_i$ mobilization, PKCε translocation, and sensitization of the vanilloid receptor TRPV1, hence decreasing the nociceptive threshold to mechanical and thermal stimuli.[19] Amphibian Bv8 injected into the periaqueductal gray (PAG) exerts a pronociceptive action by increasing the intrinsic GABAergic tone which, in turn, is responsible for the inhibition of PAG antinociceptive output neurons demonstrating that the Bv8/PKR system may also intervene in modulating central pain mechanisms.[4]

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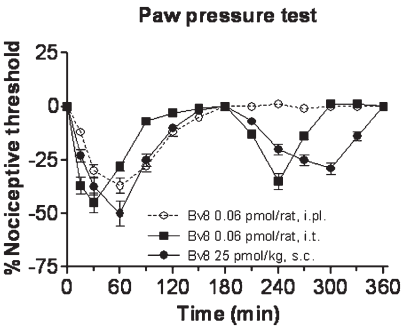

FIGURE 2 Time course of hyperalgesia induced in rats by subcutaneous (s.c.), intrathecal (i.t.), and intraplantar (i.pl.) injections of Bv8. Hyperalgesia is expressed as the percent decrease of the basal nociceptive threshold.

We have shown that amphibian Bv8 activates macrophages to migrate and to produce proinflammatory cytokines. It is also able to affect the T lymphocyte population, because the administration *in vitro* and *in vivo* of amphibian Bv8 skews the Th1/Th2 balance toward a Th1 response. All these effects depend on the activation of PKR1 because they lack in the lymphocytes and macrophages from PKR1-KO mice.[19]

Endogenous Bv8/PK2 is overexpressed in inflamed tissues and in animal and human neoplastic tissue, predominantly in infiltrating neutrophils. These cells can secrete it, since the sequence of the precursors contains the typical signal peptide for secretion.[19] Ferrara's group showed that Bv8 regulates myeloid-cell-dependent tumor angiogenesis. We showed that Bv8 has a crucial role in neutrophil-dependent inflammatory hypernociception.[6]

To develop PKR antagonists as novel analgesic and anti-inflammatory drugs, structural determinants required for the biological activity of Bv8 must be identified.

Studies from our group suggested that AVITGA family members could interact with PKR1/PKR2 by orienting the protein region that comprises the AVITGA sequence and the conserved tryptophan residue in position 24. The N-terminal deletion of the first two amino acids in amphibian Bv8 molecule (dAV-Bv8) yield an analog lacking any biological activity but still able to bind the receptors. dAV-Bv8 is a short acting antagonist *in vivo*. Substituting Trp with Ala in position 24 yields a molecule, A-24, that preferentially binds and activates PKR2. It displays weak affinity and efficacy on PKR, acting as an antagonist. A-24 has an extraordinary, long-lasting antihyperalgasic effect in animal models of postsurgical and inflammatory pain.[12]

Some nonpeptide molecules, triazine-guanidine derivatives, seem very promising. Local or systemic injection of the lead compound, PC1, abolishes the hyperalgesia in animal models of inflammatory[6] and postsurgical pain and speeds up the paw healing being, on a molar base, about a thousand times more potent than indomethacin (unpublished). Our results also show an important role for the Bv8/PKR in neuropathic pain at least in rodents. If this role is confirmed in humans, reducing Bv8 levels in damaged tissues or antagonizing PKRs might be an innovative strategy to control persistent, invalidating pain.

REFERENCES

1. Auvynet C, Seddiki N, Dunia I, Nicolas P, Amiche M, Lacombe C. Post-translational amino acid racemization in the frog skin peptide deltorphin I in the secretion granules of cutaneous serous glands. *Eur J Cell Biol* 2006;**85**:25–34.
2. Cheng MJ, Bullock CM, Li C, Lee AG, Bermak JC, Belluzzi J, et al. Prokineticin 2 transmits the behavioural circadian rhythm of the suprachiasmatic nucleus. *Nature* 2002;**417**:405–10.
3. Cheng MY, Leslie FM, Zhou QY. Expression of prokineticins and their receptors in the adult mouse brain. *J Comp Neurol* 2006;**498**:796–809.
4. De Novellis V, Negri L, Lattanzi R, Rossi F, Palazzo E, Marabese I, et al. The prokineticin receptor agonist Bv8 increases GABA release in the periaqueductal grey and modifies RVM cell activities and thermoceptive reflexes in the rat. *Eur J Neurosci* 2007;**26**:3068–78.
5. Erspamer V. Bioactive secretions of the amphibian integument. In: Heatwole H, editor. *Amphibian biology*. Surrey Beatty & Sons Publ; 1994. p. 178–350.
6. Giannini E, Lattanzi R, Nicotra A, Campese AF, Grazioli P, Screpanti I, et al. The chemokine Bv8/prokineticin 2 is up-regulated in inflammatory granulocytes and modulates inflammatory pain. *Proc Natl Acad Sci USA* 2009;**106**:14646–51.
7. Jackway RJ, Maselli VM, Musgrave IF, Maclean MJ, Tyler MJ, Bowie JH. Skin peptides from anurans of the *Litoria rubella* Group: sequence determination using electrospray mass spectrometry. Opioid activity of two major peptides. *Rapid Commun Mass Spectrom* 2009;**23**:1189–95.
8. Jilek A, Mollay C, Tippelt C, Grassi J, Mignogna G, Mullegger J, et al. Biosynthesis of a D-amino acid in peptide linkage by an enzyme from frog skin secretions. *Proc Natl Acad Sci USA* 2005;**102**:4235–9.
9. Kaser A, Winklmayr M, Lepperdinger G, Kreil G. The AVIT protein family. Secreted cysteine-rich vertebrate proteins with diverse functions. *EMBO Rep* 2003;**4**:469–73.
10. Kishi T, Kitajima T, Tsunoka T, Okumura T, Ikeda M, Okochi T, et al. Possible association of prokineticin 2 receptor gene (PROKR2) with mood disorders in the Japanese population. *Neuromolecular Med* 2009;**11**:114–22.
11. Lacombe C, Cifuentes-Diaz C, Dunia I, Auber-Thomay M, Nicolas P, Amiche M. Peptide secretion in the cutaneous glands of South American tree frog *Phyllomedusa bicolor*: an ultrastructural study. *Eur J Cell Biol* 2000;**79**:631–41.
12. Lattanzi R, Sacerdote P, Franchi S, Canestrelli M, Miele R, Barra D, et al. Pharmacological activity of a Bv8 analog modified in position 24. *Br J Pharmacol* 2012;**166**:950–63.
13. LeCouter J, Ferrara N. EG-VEGF and Bv8: a novel family of tissue-selective mediators of angiogenesis, endothelial phenotype and function. *Trends Cardiovasc Med* 2003;**13**:276–82.
14. LeCouter J, Kowalski J, Foster J, Hass P, Zhang Z, Dillard-Telm L, et al. Identification of an angiogenic mitogen selective for endocrine gland endothelium. *Nature* 2001;**412**:876–84.
15. Li M, Bullock CM, Knauer DJ, Ehlert FJ, Zhou QY. Identification of two prokineticin cDNAs: recombinant proteins potently contract gastrointestinal smooth muscle. *Mol Pharm* 2001;**59**:692–8.
16. Melchiorri P, Negri L. The dermorphin peptide family. *Gen Pharmacol* 1996;**7**:1099–107.

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17. Mollay C, Wechselberger C, Mignogna G, Negri L, Melchiorri P, Barra D, et al. Bv8, a small protein from frog skin and its homolog from snake venom induce hyperalgesia in rats. *Eur J Pharmacol* 1999;**374**:189–96.
18. Negri L, Giannini E. Deltorphins. In: Porreca F, Woods JH, Chang KJ, editors. *The delta receptors, molecule and effects of delta opioid compounds*. Marcel Dekker; 2003. p. 175–89.
19. Negri L, Lattanzi R, Giannini E, Canestrelli M, Nicotra A, Melchiorri P. Bv8/prokineticins and their receptors: a new pronociceptive system. *Int Rev Neurobiol* 2009;**85**:145–57.
20. Negri L, Lattanzi R, Giannini E, De Felice M, Colucci A, Melchiorri P. Bv8, the amphibian homologue of the mammalian prokineticins, modulates ingestive behaviour in rats. *Br J Pharmacol* 2004;**142**:181–91.
21. Negri L, Lattanzi R, Giannini E, Melchiorri P. Bv8/Prokineticin proteins and their receptors. *Life Sci* 2007;**81**:1103–16.
22. Negri L, Lattanzi R, Tabacco F, Melchiorri P. Respiratory and cardiovascular effects of the mu-opioid receptor agonist [Lys7]dermorphin in awake rats. *Br J Pharmacol* 1998;**124**:345–55.
23. Negri L, Melchiorri P, Lattanzi R. Pharmacology of amphibian opiate peptides. *Peptides* 2000;**21**:1639–47.
24. Stevens CW, Tóth G, Borsodi A, Benyhe S. Xendorphin B1, a novel opioid-like peptide determined from a Xenopus laevis brain cDNA library, produces opioid antinociception after spinal administration in amphibians. *Brain Res Bull* 2007;**71**:628–32.

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Chapter 54

Sodefrin and Related Pheromones

Sakae Kikuyama, Fumiyo Toyoda, Kazutoshi Yamamoto, Takeo Iwata, Tomoaki Nakada and Itaru Hasunuma

ABSTRACT

This chapter describes the current state of understanding of sodefrin, a peptide pheromone from the newt *Cynops pyrrhogaster*, and [Leu^3,Gln^8] sodefrin (silefrin) from the congeneric species, *C. ensicauda*. Both pheromones consist of 10 amino acids and were isolated from the abdominal glands of male newts. They exhibit a potent female-attracting activity only to the conspecific females, suggesting a contribution of the pheromones to maintaining the reproductive isolation of the species.

Both pheromones are generated from 20-kDa precursor molecules. They are emitted through the cloaca of the male and are directed toward the female partner's snout during courtship to act primarily on the lateral nasal sinus cells. Hormone dependency of secretion of and response to the pheromones has been observed.

DISCOVERY

It is well recognized that courting male red-bellied newts, *C. pyrrhogaster*, attract their partners by sending water from around the cloaca toward the female's snout by vibrating their tail and that during courtship they project numerous minute tubules that are connected to the abdominal gland, from the cloaca. This suggests that the male newts emit female-attracting pheromones through the cloaca.[8,16] We found that the water in which sexually active male newts are kept attracts conspecific females. The attractant was believed to be secreted by the abdominal gland of the cloaca because the water in which abdominal gland-ablated males had been kept did not attract females. Thus, an attempt was made to isolate and characterize the active component. Female-attracting activity was monitored by a preference test.[17]

An aqueous extract of the abdominal glands of sexually developed male red-bellied newts exhibits female-attracting activity. The minimum effective amount of the extract in a sponge block that attracts a female placed in a container filled with 3000ml of water is the equivalent of 0.1% of the abdominal gland content. When the water-soluble fraction of the abdominal gland is subjected to gel-filtration column chromatography, the most potent female-attracting activity elutes with a relative molecular mass <5000. Incubation of this fraction with pronase results in the loss of its female-attracting activity, indicating that the active substance is a peptide.

To isolate the active peptide from the crude fraction, two purification cycles of reversed-phase HPLC were used. The final product was subjected to direct sequencing by a pulsed gas-liquid phase protein sequencer, and the active substance was identified as a decapeptide with the amino acid sequence SIPSKDALLK. COOH-terminal analysis by carboxypeptidase-P digestion shows that a free (nonamidated) Lys residue is present at the COOH terminus. The relative molecular mass of 1071.2 estimated from fast atom bombardment mass spectrometry corresponds to that calculated from the amino acid sequence. Ten nanograms of the final product absorbed in a block of sponge is enough to attract female newts placed in a container filled with 3000ml of water. The synthetic version exhibits female-attracting activity similar to that of the natural material. The peptide shows no sequence similarity with any known peptide. Thus, the novel peptide is designated sodefrin (derived from the ancient Japanese word "sodefri," meaning "soliciting").[9]

To study the localization of sodefrin in the abdominal gland and to develop a specific radioimmunoassay (RIA) system for the measurement of the peptide, an antiserum against sodefrin was generated. Frozen sections of abdominal glands stained with the antiserum against sodefrin show that the apical region of the epithelial cells are positive for sodefrin[22] (Fig. 1). Immunoelectron microscopy of the abdominal gland reveals that the sodefrin-immunoreactive substance is localized mainly in the secretory granules[18] (Fig. 2). This clearly indicates that sodefrin is secreted by the epithelial cells of the abdominal gland.

To ascertain whether the abdominal gland of a congeneric species, *C. ensicauda* (the sword-tailed newt) synthesizes molecules that immunoreact with the sodefrin antiserum,

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00054-3
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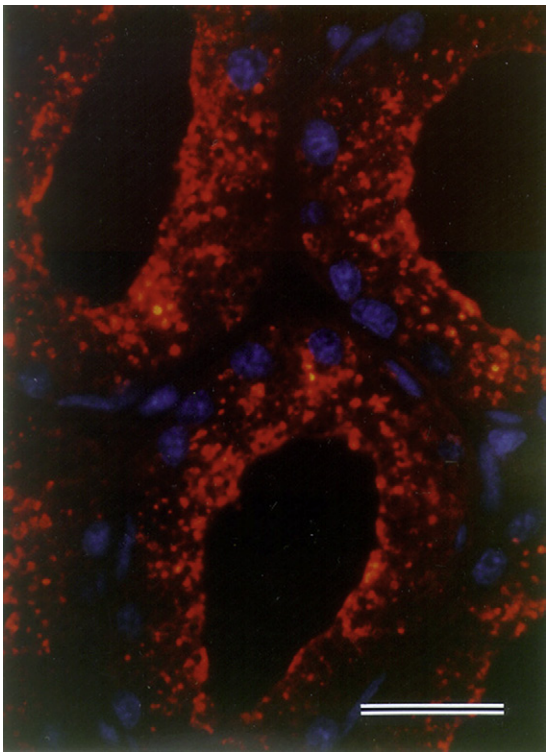

FIGURE 1 Immunofluorescence micrograph showing the localization of sodefrin in the abdominal gland of a red-bellied newt. The sodefrin antibody labels the epithelial cells red with rhodamine. Nuclear DNA is stained blue with 4′ 6-diamino-2-phenylindole. The bar represents 50μm. *From Ref.* 16 *Copyright 2000 by the Zoological Society of Japan.* See color plate 15.

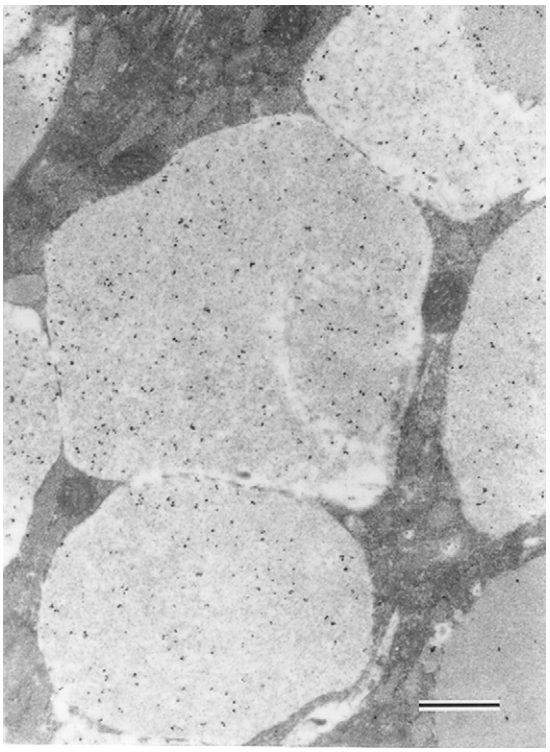

FIGURE 2 Immunoelectron micrograph showing the localization of sodefrin in the epithelial cells of the abdominal gland of a red-bellied newt. Immunogold particles indicating the presence of sodefrin are observed within the secretory granules. The bar represents 500nm. *From Ref.* 16 *Copyright 2000 by the Zoological Society of Japan.*

an aqueous extract of the abdominal gland was subjected to a sodefrin RIA. This extract showed no crossreactivity in this RIA system, whereas a water extract from the abdominal gland of *C. pyrrhogaster* showed an inhibition curve parallel to the sodefrin standard.[22] On the assumption that *C. ensicauda* abdominal glands contain a molecule possessing a female-attracting activity that is different from sodefrin, purification of putative *C. ensicauda* pheromone was performed. The pheromone was isolated from the abdominal glands by gel-filtration chromatography and reversed-phase HPLC. The final product consists of the 10 amino acid sequence SILSKDAQLK and a molecular mass of 1102.3 as estimated by electrospray mass spectrometry. This peptide is designated silefrin, a combination of the first three N-terminal amino acids, SIL and -efrin, derived from sodefrin.[23] The sequence of this peptide differs from that of sodefrin by two amino acids, with substitutions of Leu for Pro and Gln for Leu at positions 3 and 8, respectively. Both native and synthetic silefrin are equipotent in attracting conspecific females, 10ng being the minimum effective amount. Localization of silefrin in the abdominal gland of *C. ensicauda* shows an intense immunofluorescence on the apical side of the epithelial cells in the cryosections incubated with the antiserum.

STRUCTURE OF THE PRECURSOR mRNA/GENE

A sodefrin precursor cDNA, isolated from a cDNA library constructed from *C. pyrrhogaster* abdominal gland mRNA, contains 1364bp with an open reading frame of 567bp and encodes a sodefrin precursor polypeptide of 189 amino acids. The precursor polypeptide includes a predicted signal peptide and, in a region close to the C-terminus, the sodefrin molecule. The molecular mass of the sodefrin precursor deduced from its sequence is approximately 20kDa (Fig. 3A). Likewise, a cDNA clone encoding a silefrin precursor protein isolated from a *C. ensicauda* abdominal gland cDNA library consists of 1339bp; it contains a predicted open reading frame of 576bp encoding a precursor polypeptide of 192 amino acids, including a domain rich in hydrophobic amino acids that was presumed to be a signal peptide. Its nucleotide sequence shows 82% sequence identity with preprosodefrin from *C. pyrrhogaster*[3] (Fig. 3B).

A partial sodefrin precursor gene structure was identified by genomic PCR by the use of genome DNA purified from the liver of male newts captured in Niigata. At least one intron is inserted between nucleotide position 569 and 570 of sodefrin precursor cDNA. In addition, we

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(A)

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                    TGGCAGGTGAACAGGTGCAGAGACTCCATCACCCTATTCCTTACTCTCCTAGCACC
ATGAGGGCCATCCTTGCAGCTGTCGTCCTGCTCCAGGCACTGATAACTGGAGATTGCCTATTATGCGAGCAGTGT
 M  R  A  I  L  A  A  V  V  L  L  Q  A  L  I  T  G  D  C  L  L  C  E  Q  C
TTCGCTCTCCAAACCAGCAGCTGCTCGGGTATCTTCACGCAGTGCTCTCCTGACGTCACTCACTGCGTCGCAGGC
 F  A  L  Q  T  S  S  C  S  G  I  F  T  Q  C  S  P  D  V  T  H  C  V  A  G
CTAGAGAACTGCACACTGGGGACTCATGTTATTCTAACTGCGTTCAAGGACTGTCTGGATCCTTCCGAAAAAGCA
 L  E  N  C  T  L  G  T  H  V  I  L  T  A  F  K  D  C  L  D  P  S  E  K  A
GCCTGCGGTAGAGAGGTCTCCTTCACAGCTCCAGCGGCCTCTTTATGGACAAGCAGGACGTGCTGTGACTCTGAT
 A  C  G  R  E  V  S  F  T  A  P  A  A  S  L  W  T  S  R  T  C  C  D  S  D
TTCTGCAACGGTGGGGATGTGCAGGTGCCTCCTCCAGACGACACTCCCAGTGGTTGTGGCAGTGACCAGCCCTGC
 F  C  N  G  G  D  V  Q  V  P  P  P  D  D  T  P  S  G  C  G  S  D  Q  P  C
ACAGCGCCAGAACACCTAAGGGAAACAGTGCACTCTACAACATCGATTAGAGAGAAGAGAAGAAAGCGATTTTTT
 T  A  P  E  H  L  R  E  T  V  H  S  T  T  S  I  R  E  K  R  R  K  R  F  F
TGGTCATATTTTCCGATCAGAAGAACGCATGTGGCACCATCTATGGAACTGCCTCCAGGCCGGCTAAGACTGGGG
 W  S  Y  F  P  I  R  R  T  H  V  A  P  S  M  E  L  P  P  G  R  L  R  L  G
AGGAGTATACCTTCAAAGGATGCACTACTCAAGATTTCTGCATAGCTGGAATTTTCCACATGGCGGGGATGCAAG
 R  (S) (I) (P) (S) (K) (D) (A) (L) (L) (K) I  S  A  *
CCTACGATTATTATGTTTTAAAGTGTTCCCCTGCCCTAAAAGTTTGAGACTTTTGTTCATACCCCATAGGCACTC
CTACTCTAGCTTAGTAGTTGTCTGTAGAAACATTCATAAAGCGCTACAAGTATGTGGAATGCAGTGTCTGATCTT
GTGATGAGGAAGCATATGAACTCATGTCAGCCTCTCTGAGACACAGTGTACAGGTGGCCAATGTGCTTAGTACAA
TCTAGGCCGGCATGCTGTTTAACCACTGTCTTCTCTATTCAGCCATCTTAAGCGCCTGGGCATCTCAGAGGGTTA
TCTTGGATTCATGCATCGAGTGATCCAAGCACAGGCCAAGCAATCATGCAATGATGCTGTCTTATGGTTGTAGAA
GGTGCTTCTCCTGATGTGCTACTAATGCTGACTTCATGAGTAGCCATGAACAGCCATTCCTGCTTTTCTTCTGCT
TTTTGGTTGAATACCTCTTCTAACATAAAGTAATTGAGAATATCTGGCGCAGTTGTATTGATGCTGTCAAATATA
AGAGGACAGGGTTATTGGTTCATATCTCCAATATGAATGTGCCTTTTAATCCAGCAATAAGCATGCTTTGTGCCA
CAGCTATAACCCAAAATAGAACAAATATGTAGACCCGCTTTGTACTGCACATTGAAAAAATGAATAAACATTAAT
TTACACTGCTGCAAAAAAAAAAAAAAAAAAAAA
```

(B)

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                    TAGTTGAACAGGTGCAGAGACTCCATCACCCTACTCCTTACTCTCCTAGCACC
ATGAGGGCCATCATTGCAGCTGTCGTCCTGCTCCAGGCACTGATAACTGGAGATTGTCTATTATGCGAGCAGTGT
 M  R  A  I  I  A  A  V  V  L  L  Q  A  L  I  T  G  D  C  L  L  C  E  Q  C
TTCGCTCTCCACACCAGCAGCTGCTCGGGTATCTTCACGCAGTGCTCTCCTGACGTCACTCACTGCGCCGCAGCT
 F  A  L  H  T  S  S  C  S  G  I  F  T  Q  C  S  P  D  V  T  H  C  A  A  A
AAGAAGAACAACACAGCGGGGACTCATGTTATTCTAACTGCGTTCAAGGACTGTCTGGATCCTTCCCAAAAAGCA
 K  K  N  N  T  A  G  T  H  V  I  L  T  A  F  K  D  C  L  D  P  S  Q  K  A
GCCTGCGGTAGAGAGGTCTCCTTCACAGCTCCAGCGGCCTCTTTATGGACAAGCAGGACGTGCTGTGACTCTGAT
 A  C  G  R  E  V  S  F  T  A  P  A  A  S  L  W  T  S  R  T  C  C  D  S  D
TTCTGCAACGGCGGGGATGTGCAGGTGCCTCCTCCAGACGACACTCCCAATGGTTGTGGCAGTGACCAGTCCGCG
 F  C  N  G  G  D  V  Q  V  P  P  P  D  D  T  P  N  G  C  G  S  D  Q  S  A
AACGCCTGCACAGCGCCAGGACACCTGAGGGTAACAGTGCGCTCTACAACATCGATTAGAGAGAAGAGAAGAGAG
 N  A  C  T  A  P  G  H  L  R  V  T  V  R  S  T  T  S  I  R  E  K  R  R  E
CGACTGAATGTGTTCAGTGTTCTGGCAAGCAGAAGGCGTGTGGCACCTTCTAAGGAACTGCCTCTAGGCGTGATA
 R  L  N  V  F  S  V  L  A  S  R  R  R  V  A  P  S  K  E  L  P  L  G  V  I
AAACTGGGGAGGAGTATACTTTCAAAGGATGCACAACTCAAGATTTCTGCATAGATGGAATTTTCCACATGGCGG
 K  L  G  R  (S) (T) (L) (S) (K) (D) (A) (Q) (L) (K) I  S  A  *
GGACGCAAGCCTACAATTATGTTTTAAAGTGTTCCCCTGCCCTGAAAGTTTGAGACTTTTGTTCATACCCCATAG
GCACTCCTACTCTAGCTTAGTATTGTCTGTAGAAACATTCCTAAAGCTCTACAAGTATGTGGAATGCAGTGTCTG
ATCTTGTGATGAGGAAGCATATGAACTCATGTCAGCCTCTCTGAGACAAAGTGTACAGGTGGCCAATGTGCTCAG
TACAATCCAGGCCGGCATGCTGTTTAACCACTGTCTTCTCTATTCAGCCATCTTAAGCGCCTGGGCATCTCAGAG
GGTTATCTTGGATTCATGCATTGAGTGATCCGAGCACAGTCCAAGCAATCACCCAATGATGCTGTCTTATGGTTG
TAGAAGGTGCTTCTCCTGATGTGCTACTAATGCTGACTTCATGAGTAGCCATGAACAGCCATTCCTGCTTTTCTT
CTACTTTTTGGTTGAATACCTCTTCTAACATAAAGTAATTGAGAATATCTGGCGCAGTTGTGTTGATGCTTTCAA
ATATAAGAGGACAGGGTTATTGGTTCATATCTCCAATATGAATGTGCCTTTTAATCCAGCAATAAGCATGCTTTG
TGCTACAGCTATAACCCAAAATAAAAAAATATATAGACCCGCTTTGTACTGCACATTGAAAAAATGAATAAACAT
TAATTTACACA
```

FIGURE 3 Nucleotide and deduced amino acid sequences of the cDNA clone encoding sodefrin (A) and silefrin (B). The predicted amino acids are shown below the nucleotide sequence. The amino acids comprising sodefrin and silefrin molecules are circled. The asterisk indicates the termination codon, and the polyadenylation signal (AATAAA) is underlined. *From Ref.* 16 *Copyright 2000 by the Zoological Society of Japan.*

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confirmed the existence of the two kinds of genes that have different lengths of intron, one having a 2020-bp intron and the other having a 1535-bp intron. The sequence of the exon of the former encodes sodefrin and that of the latter encodes [Asn[10]] sodefrin (see a later section of this chapter).[2]

DISTRIBUTION OF THE mRNA

Northern blot analysis of sodefrin mRNA in the abdominal gland indicates a size of about 1.5kb and that sodefrin mRNA is expressed in the abdominal gland but not in other organs such as tail, liver, kidney, testis, and brain. Similarly, the expression of the silefrin precursor mRNA of *C. ensicauda* was also analyzed with silefrin precursor cDNA as a probe. A single band showing positive hybridization corresponding to approximately 1.5kb is found exclusively in the abdominal gland.[3] Localization of mRNA by in situ hybridization with ^{35}S-labeled silefrin precursor cDNA as a probe shows intense specific signals in the epithelial cells of the abdominal gland[5] (Fig. 4).

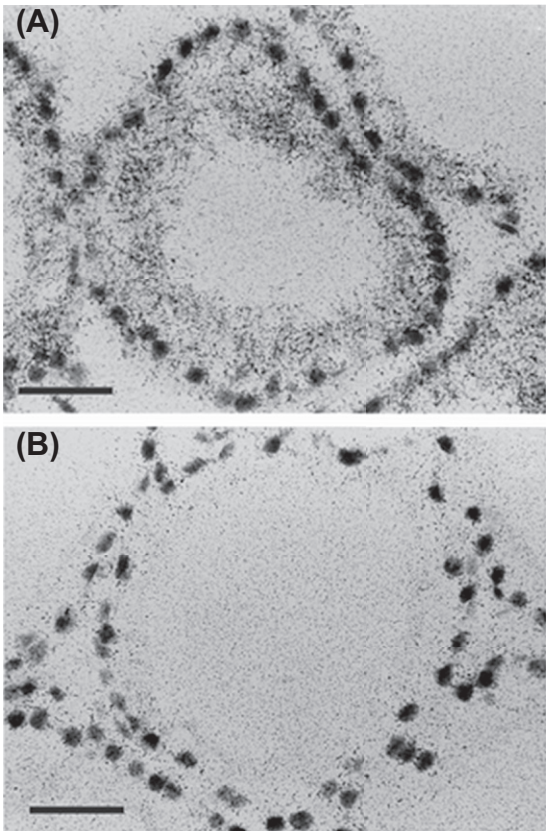

FIGURE 4 Micrographs of the signals for silefrin precursor mRNA in the abdominal gland of the newt, *C. ensicauda*. In situ hybridization was performed with a specific ^{35}S-labeled probe (A) and with a ^{35}S-labeled probe containing an excess of nonlabeled silefrin precursor cDNA (B). Bars represent 50 μm. *From Ref.* 5 *Copyright 2000 the Society for the Study of Reproduction.*

PROCESSING OF THE PRECURSORS

Sequence analysis of the cDNAs encoding sodefrin and silefrin reveals that both peptides are generated from precursor proteins. Both molecules are sandwiched between monobasic amino acids (Arg and Lys).[3] It is well known that most peptide hormones are derived from precursor proteins as a result of posttranslational processing (see Peptide Biosynthesis/Processing section of this book). The peptide hormone sequence is flanked by pairs of dibasic amino acids that are subjected to cleavage by enzymes such as the prohormone convertases PC1 and PC2. The absence of such dibasic amino acids flanking the sodefrin and silefrin molecules suggests that the mode of processing of these peptide pheromones are different from that of most peptide hormones.

Synthetic sodefrin C-terminally extended by Ile-Ser-Ala (the C-terminal portion of sodefrin precursor consisting of 13 amino acids) does not attract the females, indicating that these three amino acids must be removed from the C-terminus of this molecule for acquisition of biological activity.

To show the presence in the abdominal gland of enzymes that cleave the amino acids at the C- and N-termini of sodefrin, an assay for enzyme activity was developed with *t*-butoxycarbonyl (Boc)-LGR-methylcoumaryl-7-amide (MCA) and Boc-LLK-MCA as synthetic substrates. These substrates were designed on the basis of the predicted amino acid sequence of the C-terminal region of the sodefrin precursor protein (…LGRSIPSKDALLKISA). A *C. pyrrhogaster* abdominal gland extract hydrolyzed both Boc-LGR-MCA and Boc-LLK-MCA to liberate 7-amino-methyl-4-courain (AMC), suggesting that enzymes that generate sodefrin from its precursor molecule exist in the abdominal gland. The activity in the extract for cleaving Boc-LGK is optimal at PH 9.0 and 45°C and for Boc-LGK at PH 9.0 and 40 °C. The enzyme preparation loses activity when it is boiled or when proteinase inhibitors are present during incubation. The effects of a range of specific protease inhibitors on activities in the extract suggest an involvement of enzymes belonging to the serine protease family. The enzymatic activity in an extract of the abdominal glands of sexually developed males is significantly higher than that of sexually undeveloped ones.[13]

RECEPTORS

At present, no receptors for either sodefrin or silefrin have been identified. Bilateral, but not unilateral, nostril plugging in females with a cotton ball soaked in melted vaseline completely abolishes the female-attracting activity of sodefrin.[18] This strongly suggests that the action of sodefrin is mediated through the olfactory organ. This result concurs with the results of an electrophysiological study showing that sodefrin evokes a marked electro-olfactogram (EOG)

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response when applied to the lateral nasal sinus. The lateral nasal sinus region is considered to be a primitive vomeronasal organ. The EOG response to sodefrin is markedly enhanced in the females when they are treated with gonadotropin or estrogen in combination with prolactin. In the male, however, the effects of the similar hormonal treatments on the EOG response to sodefrin are not apparent. Thus, it was concluded that there is a sex difference in the responsiveness of lateral nasal sinus to the hormones and/or the pheromone.[19]

Sodefrin, but not silefrin, increases $[Ca^{2+}]_i$ in dissociated lateral nasal sinus cells from the female *C. pyrrhogaster*. Species specificity in terms of the responsiveness to the pheromone occurs at the cellular level. The number of cells that respond to sodefrin increase concentration dependently. Relatively few cells in the lateral nasal cells from female newts in the nonbreeding season or from male newts respond to sodefrin compared with cells from reproductive females. There are at least two cell types in the lateral nasal cells that respond to sodefrin by elevating $[Ca^{2+}]_i$. One increases $[Ca^{2+}]_i$ through the phospholipase C (PLC)-inositol 1, 4, 5-triphosphate pathway and the other increases $[Ca^{2+}]_i$ through the PLC-diacylglycerol-phosphokinase C pathway.[6]

STRUCTURE–ACTIVITY RELATIONSHIPS

In addition to sodefrin, several peptides related to sodefrin (SIPSKDALLK) have been identified. Minute amounts of peptides such as SKDALLK and DALLK were obtained during purification of sodefrin. These peptides, possibly degradation products of sodefrin, were subjected to the preference test. SKDALLK retains the ability to attract sexually developed female newts, whereas DALLK exhibits no activity. This indicates that the sequence Ser^4-Lys^5 in the sodefrin molecule is indispensable for mediating the female-attracting activity. From an extract of the abdominal glands of male red-bellied newts captured 5 months before the onset of the breeding season, a COOH-terminally extended form of sodefrin (SIPSKDALLKISA) was obtained. This peptide is biologically inactive, suggesting that activation of a protease that cleaves at the Lys–Ile bond to generate the active pheromone must occur by the time of onset of reproductive behavior. Another biologically inactive peptide isolated is a COOH-terminally extended form of [Asn^{10}] sodefrin (SIPSKDALLNISA). The presence of this sodefrin variant raises the possibility of the expression of multiple genes encoding preprosodefrin. Analyses of PCR products derived from total RNAs from the abdominal gland of individual newts collected from three different regions (Chiba, Niigata, and Nara) of Japan confirm the existence of multiple genes encoding sodefrin and its variants. Their expressions varied according to the individuals and the regions. Among them, mRNA encoding the sodefrin variant, SIPSKDAVLKICA ([Val^8] sodefrin. ICA) is expressed exclusively in the animals from Nara, whereas genes encoding sodefrin are expressed in all the specimens sampled.[7] [Val^8] sodefrin and sodefrin were isolated from the abdominal gland of the male newts inhabiting the Nara region. [Val^8] sodefrin is slightly more potent than sodefrin when tested with Nara females. A full-length cDNA encoding [Val^8] sodefrin precursor was cloned and characterized. The deduced amino acid sequence of prepro[Val^8] sodefrin shows 86% identity with that of the sodefrin precursor. The region-specific pheromone is designated aonirin ("aoni" derived from "aoni-yoshi," the conventional epithet of Nara and "rin" from sodefrin). Aonirin exhibits a potent female-attracting activity toward females belonging to the same population but no or much less activity to the females captured in different regions.[14] The occurrence of geographical variation in the courtship pheromone and of differences in the responsiveness toward the variant pheromone among the females inhabiting different regions might cause reproductive isolation and finally lead to the differentiation into separate species.

BIOLOGICAL ACTIONS

The biological test for female-attracting activity is performed according to the method established by Toyoda et al.[17] This preference test reveals that 10ng of both native and synthetic sodefrin adsorbed by a sponge block is enough to attract female newts and that the minimum effective concentration lies within the range 0.1–1.0pM.[9]

Both native and synthetic silefrin attract *C. ensicauda* females, the minimum effective amount absorbed in a sponge block being 10ng as in the case of sodefrin. Silefrin attracts only *C. ensicauda* females but not *C. pyrrhogaster* females. Likewise, sodefrin attracts only *C. pyrrhogaster* females.

A combination of prolactin and androgen produces the structural development of various male organs related to reproduction, including the abdominal gland.[10] The combination of both hormones elevates sodefrin precursor mRNA levels[4] and increases the content of immunoassayable sodefrin.[23] Likewise, silefrin precursor mRNA and silefrin content in the abdominal gland of *C. ensicauda* are increased by treatment with prolactin and androgen.[5] Both prolactin and androgen receptors exist in the epithelial cells of the abdominal gland.[11,12]

Arginine vasotocin (AVT) is considered to be a candidate hormone for regulating discharge of the pheromone. The water in which AVT-treated *C. pyrrhogaster* males are kept shows considerable female-attracting activity as compared with the water in which saline-injected males are kept. In addition, the sodefrin content in the abdominal gland is

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decreased by the hormonal treatment. The effect of another neurohypophyseal hormone, mesotocin, is less pronounced in this respect. AVT induces contraction of the excised gland concentration dependently. This AVT-induced contraction is blocked completely by a V1a receptor blocker. The expression of mRNA of V1a-type receptor has been confirmed in the abdominal gland of the newt *C. pyrrhogaster.*[1] Thus, AVT may serve as a hormone to induce the release of sodefrin, acting on a contractile structure of the abdominal gland through a V1a-type receptor.[20]

PHYSIOLOGICAL IMPLICATIONS

The existence of pheromones in the class Amphibia has long been postulated. Sodefrin is the first amphibian pheromone to be identified and the first peptide pheromone to be identified in a vertebrate. Since sexually mature newts lead an aquatic life, it is quite reasonable to expect a nonvolatile but water-soluble peptide as a pheromone in this animal.

A protein pheromone has been identified in a urodele *Plethodon jordani.*[15] In this species, courtship takes place on land, whereas courtship by *Cynops* is performed in water. In an anuran *Litoria splendida*, a female-attracting pheromone was shown to be a peptide consisting of 25 amino acids that is secreted by the male skin gland.[21] In this species mating, takes place in water. However, until the chemical nature of sex pheromones from various species of amphibians performing reproductive behavior in various environments has been elucidated, it may be premature to conclude that peptides are the dominant form of sex pheromone for amphibians in which courtship or mating takes place in water. Nevertheless, water-soluble peptide pheromones might be considered to be the ideal forms of species-specific reproductive pheromones for amphibians performing courtship or mating in aquatic environments where other species are liable to exist, since variant forms that may exert species-specific pheromonal activity can be generated by modification of the nucleotide sequence of the pheromone gene.

REFERENCES

1. Hasunuma I, Sakai T, Nakada T, Toyoda F, Namiki H, Kikuyama S. Molecular cloning of three types of arginine vasotocin receptor in the newt, *Cynops pyrrhogaster*. *Gen Comp Endcrinol* 2007;**151**:252–8.
2. Hasunuma I, Sakamoto S, Shimamori H, Toyoda F, Nakada T, Namiki H, et al. A multiplicity of genes encoding preprosodefrin of the newt *Cynops pyrrhogaster*. *Abstracts of 81st annual meeting of the Zoological Society of Japan, Tokyo*; 2010. p. 116.
3. Iwata T, Umezawa K, Toyoda F, Takahashi N, Matsukawa H, Yamamoto K, et al. Molecular cloning of newt sex pheromone precursor cDNAs: evidence for the existence of species-specific forms of pheromones. *FEBS Lett* 1999;**457**:400–4.
4. Iwata T, Toyoda F, Yamamoto K, Kikuyama S. Hormonal control of urodele reproductive behavior. *Comp Biochem Physiol* 2000;**B126**:221–9.
5. Iwata T, Yada T, Shioda S, Toyoda F, Kikuyama S. Effect of prolactin and androgen on the expression of the female-attracting pheromone silefrin in the abdominal gland of the newt, *Cynops ensicauda*. *Biol Reprod* 2000;**63**:1867–72.
6. Iwata T, Conlon JM, Nakada T, Toyoda F, Yamamoto K, Kikuyama S. Processing of multiple forms of preprosodefrin in the abdominal gland of the red-bellied newt *Cynops pyrrhogaster*: regional and individual differences in preprosodefrin gene expression. *Peptides* 2004;**25**:1537–43.
7. Iwata T, Kawahara G, Yamamoto K, Zhou CJ, Nakajo S, Shioda S, Kikuyama S. Signal-transduction pathway mediating the action of a newt pheromone, sodefrin. Abstracts 14th International Congress of Comparative Endocrinology, Sorrento; 2001. p. 68.
8. Kikuyama S, Tanaka S, Moore F. Endocrinology of reproduction. In: Sever DM, editor. *Reproductive biology and phylogeny of Urodela*. Einfield: Science Publishers, Inc; 2003. p. 275–321.
9. Kikuyama S, Toyoda F, Ohmiya Y, Matsuda K, Tanaka S, Hayashi H. Sodefrin: a female-attracting peptide pheromone in newt cloacal glands. *Science* 1995;**267**:1643–5.
10. Kikuyama S, Yazawa T, Abe S, Yamamoto K, Iwata T, Hoshi K, et al. Newt prolactin and its involvement in reproduction. *Can J Physiol Pharmacol* 2000;**78**:984–93.
11. Matsukawa H, Hasunuma I, Kato T, Yamamoto K, Miura S, Fujita T, et al. Expression of prolactin receptor mRNA in the abdominal gland of the newt *Cynops ensicauda*. *Comp Biochem Physiol A Mol Integr Physiol* 2004;**138**:79–88.
12. Matsumoto A, Arai Y, Toyoda F, Kikuyama S, Prins GS. Immunohistochemical analysis of androgen receptor in the abdominal glands of the cloaca of male red-bellied newts, *Cynops pyrrhogaster*. *Zool Sci* 1996;**13**:429–33.
13. Nakada T, Ishizuka Y, Iwata T, Toyoda F, Kato T, Conlon JM, et al. Evidence for processing enzymes in the abdominal gland of the newt, *Cynops pyrrhogaster*, that generate sodefrin from its biosynthetic precursor. *Zool Sci* 2007;**24**:521–4.
14. Nakada T, Toyoda F, Iwata T, Yamamoto K, Conlon JM, Kato T, et al. Isolation, characterization and bioactivity of a region-specific pheromone, [Val^{8}]sodefrin from the newt, *Cynops pyrrhogaster*. *Peptides* 2007;**28**:774–80.
15. Rollmann SM, Houck LD, Feldhoff RC. Proteinaceous pheromone affecting female receptivity in a terrestrial salamander. *Science* 1999;**285**:1907–9.
16. Toyoda F, Kikuyama S. Endocrine regulation of reproductive behavior in the newt *Cynops pyrrhogaster*. *Zool Sci* 2000;**17**:561–70.
17. Toyoda F, Tanaka S, Matsuda K, Kikuyama S. Hormonal control of response to and secretion of sex attractants in Japanese newts. *Physiol Behav* 1994;**55**:569–76.
18. Toyoda F, Hayashi H, Ohmiya Y, Tanaka S, Mochida H, Matsuda K, et al. Novel female-attractant in the abdominal gland of the male newt, *Cynops pyrrhogaster*. *C R Soc Biol* 1995;**189**:1143–8.
19. Toyoda F, Hayakawa Y, Ichikawa M, Kikuyama S. Olfactory responses to a female-attracting pheromone in the newt, *Cynops pyrrhogaster*. In: Johnston RE, Müller-Schwärz D, Sorensen PW, editors. *Advances in chemical signals in vertebrates*. New York: Kluwer Academic/Plenum Publishers; 1999. p. 607–15.
20. Toyoda F, Yamamoto K, Ito Y, Tanaka S, Yamashita M, Kikuyama S. Involvement of arginine vasotocin in reproductive events in the male newt, *Cynops pyrrhogaster*. *Horm Behav* 2003;**44**:346–53.

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21. Wabnitz PA, Bowie JH, Tyler MJ, Wallace JC, Smith BP. Aquatic sex pheromone from a male tree frog. *Nature* 1999;**401**:444–5.
22. Yamamoto K, Toyoda F, Tanaka S, Hayashi H, Kikuyama S. Radioimmunoassay of a newt sex pheromone, sodefrin, and the influence of hormone on its level in the abdominal gland. *Gen Comp Endocrinol* 1996;**104**:356–63.
23. Yamamoto K, Kawai Y, Hayashi T, Ohe Y, Hayashi H, Kawahara G, et al. Silefrin, a sodefrin-like pheromone in the abdominal gland of the sword-tailed newt, *Cynops ensicauda*. *FEBS Lett* 2000;**472**:267–70.

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Chapter 55

Tachykinins

Cinzia Severini and Giovanna Improta

ABSTRACT

As many as 20 tachykinins (TKs) have been isolated from amphibian skin and 11 from amphibian gut or brain. Amphibian TKs exert their biological actions in mammals through the activation of the three classical TK receptors and, in their species of origin, through activation of receptors that resemble the structure of the mammalian TK receptors. This suggests a high degree of conservation of the receptor sequence among different vertebrate species. As the potencies of the amphibian TK are comparable to, or can exceed the potencies of their mammalian counterparts, amphibian TKs represent a widely used tool in the characterization of the TK functional roles.

DISCOVERY

The history of both nonmammalian and amphibian TKs began in 1947. In that year, Vittorio Erspamer, while investigating the occurrence of biogenic amines, especially serotonin, in the extracts of the posterior salivary glands of a Mediterranean octopod (*Eledone moschata*), discovered an unidentified substance that lowered the blood pressure in rabbits and dogs, stimulated isolated intestinal smooth muscle, and caused profuse salivation in dogs and rats. The new substance, called eledoisin (ELS), was structurally characterized in 1962. In the same year, from the skin of the South American leptodactylid frog *Physalaemus biligonigerus* (formerly *fuscumaculatus*), a new peptide with eledoisin-like activity and called physalaemin (PHYS), was extracted and isolated. The structural characterization of this first amphibian TK in 1964 led to the prediction of the primary structure of substance P (SP), already identified by bioassay in 1931 but sequenced only 40 years later, in 1971. Therefore, it became evident that the short peptides of nonmammalian origin, named TKs by Vittorio Erspamer, represented the phylogenetic precursors of SP and of the other mammalian TKs (neurokinin-A/NKA and neurokinin-B/NKB).[4]

During the following years, a systematic screening of the peptide content in the skins of as many as 600 amphibian species from all over the world resulted in the discovery and isolation of numerous families of neuropeptides, including that of TKs.[5] Despite the large amount of TKs isolated from extracts of frog skin, there are relatively few studies describing the isolation of TKs from amphibian brain or gut.

Although with some exceptions, the great majority of amphibian TKs have, like all vertebrate TKs, the classical C-terminal pentapeptide sequence Phe-X-Gly-Leu-Met-NH_2 essential but not sufficient for their biological activity. The minimum chain length required for activity is six residues. In addition, the Phe residue at position 5 from the C-terminus and amidation at their C-terminus are crucial for their biological activity. On the basis of their chemical structure and receptor affinities, both amphibian skin TKs and amphibian brain/gut TKs can be grouped into two subfamilies: "Aromatic TKs" (with the C-terminal pentapeptide Phe-Tyr(Phe)-Gly-Leu-Met-NH_2) and "Aliphatic TKs" (with the C-terminal pentapeptide Phe-Val(Ile)-Gly-Leu-Met-NH_2).[28]

Parallel bioassays on a number of isolated and *in situ* test systems, using the natural peptides and selective synthetic analogs alone or in the presence of selective antagonists, together with radioactive ligand binding studies, allow the receptor selectivity of amphibian TKs to be functionally characterized, mostly in mammals and to a lesser degree in the species of origin. The main monoreceptor systems used are the rabbit vena cava for NK1-, endothelium-removed rabbit pulmonary artery and hamster urinary bladder for NK2-, and rat portal vein for NK3 receptors.

"Aromatic TKs" preferentially interact with the mammalian NK1 receptor, whereas "Aliphatic TKs" mainly interact with the mammalian NK2/NK3 receptors.[24,25,27]

Table 1 summarizes the sequence and source of the 20 TKs extracted from amphibian skin. All these peptides have a nonneuronal origin, as they are synthesized in the syncytial cells lining the wall of the granular glands. These cells are capable of cosynthesizing, costoring, and cosecreting not only peptides belonging to different families but also amines and alkaloids of various classes and families. The occurrence of such a variety of substances and neuropeptides in amphibian skin may perhaps be explained by the common embryogenic origin of the integument and the nervous system from the primitive ectoderm.

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00055-5
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TABLE 1 Amino Acid Sequence of Amphibian Skin TKs

Source/Peptide	Primary structure
Aromatic TKs	
Physalaemus biligonigerus (fuscumaculatus)	
PHYSALAEMIN (PHYS)	pGlu-Ala-Asp-Pro-Asn-Lys-Phe-Tyr-Gly-Leu-Met-NH_2
Uperoleia rugosa	
[Lys^5,Thr^6]PHYS	pGlu-Ala-Asp-Pro-Lys-Thr-Phe-Tyr-Gly-Leu-Met-NH_2
Uperoleia marmorata	
UPEROLEIN (UP)	pGlu-Pro-Asp-Pro-Asn-Ala-Phe-Tyr-Gly-Leu-Met-NH_2
Uperoleia inundata	
UPERIN	pGlu-Ala-Asp-Pro-Asn-Ala-Phe-Tyr-Gly-Leu-Met-NH_2
Kassina (Hylambates) maculata	
HYLAMBATIN (HYL)	Asp-Pro-Pro-Asp-Pro-Asn-Arg-Phe-Tyr-Gly-Met-Met-NH_2
Rana margaratae	
Odorrana grahami	
RANAMARGARIN	Asp-Asp-Ala-Ser-Asp-Arg-Ala-Lys-Lys-Phe-Tyr-Gly-Leu-Met-NH_2
Pseudophryne güntheri	
PG-SPI	pGlu-Pro-Asn-Pro-Asp-Glu-Phe-Tyr-Gly-Leu-Met-NH_2
PG-SPII	pGlu-Pro-Asn-Pro-Asn-Glu-Phe-Tyr-Gly-Leu-Met-NH_2
Agalychnis callidryas	
AC-AR1	Gly-Pro-Pro-Asp-Pro-Asp-Arg-Phe-Tyr-Pro-Gly-Met-NH_2
AC-AR2	Gly-Pro-Pro-Asp-Pro-Asp-Lys-Phe-Tyr-Pro-Gly-Met-NH_2
AC-AR3	pGlu-Pro-Asp-Pro-Asp-Lys-Phe-Tyr-Pro-Gly-Met-NH_2
AC-AR4	Gly-Pro-Pro-Asp-Pro-Asn-Lys-Phe-Tyr-Pro-Val-Met-NH_2
Aliphatic TKs	
Kassina senegalensis	
KASSININ (KASS)	Asp-Val-Pro-Lys-Ser-Asp-Gln-Phe-Val-Gly-Leu-Met-NH_2
Kassina (Hylambates) maculata	
[Glu^2,Pro^5]KASSININ	Asp-Glu-Pro-Lys-Pro-Asp-Gln-Phe-Val-Gly-Leu-Met-NH_2
Phyllomedusa bicolor	
PHYLLOMEDUSIN (PHYLL)	pGlu-Asn-Pro-Asn-Arg-Phe-Ile-Gly-Leu-Met-NH_2
Pseudophryne güntheri	
PG-KI	pGlu-Pro-His-Pro-Asp-Glu-Phe-Val-Gly-Leu-Met-NH_2
PG-KII	pGlu-Pro-Asn-Pro-Asp-Glu-Phe-Val-Gly-Leu-Met-NH_2
PG-KIII	pGlu-Pro-His-Pro-Asn-Glu-Phe-Val-Gly-Leu-Met-NH_2
Agalychnis callidryas	
AC-AL	Gly-Pro-Pro-Asp-Pro-Asn-Lys-Phe-Ile-Gly-Leu-Met-NH_2
Odorrana grahami	
OG1	Asp-Asp-Thr-Glu-Asp-Leu-Ala-Asn-Lys-Phe-Ile-Gly-Leu-Met-NH_2

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TABLE 2 Amino Acid Sequence of Amphibian Brain/Gut TKs

Source/Peptide		Primary structure
Aromatic TKs		
Rana catesbeiana		
RANATACHYKININS	**(RTK)**	
RTKA	Brain, Gut	Lys-Pro-Ser-Pro-Asp-Arg-Phe-Tyr-Gly-Leu-Met-NH_2
RTKB	Brain	Tyr-Lys-Ser-Asp-Ser-Phe-Tyr-Gly-Leu-Met-NH_2
RTKD	Gut	Lys-Pro-Asn-Pro-Glu-Arg-Phe-Tyr-Ala-Pro-Met-NH_2
Rana ridibunda		
RANAKININ (RNK)	Brain	Lys-Pro-Asn-Pro-Glu-Arg-Phe-Tyr-Gly-Leu-Met-NH_2
Bufo marinus		
BUFOKININ (BK)	Gut	Lys-Pro-Arg-Pro-Asp-Gln-Phe-Tyr-Gly-Leu-Met-NH_2
Amphiuma tridactylus		
	Gut	Asp-Asn-Pro-Ser-Val-Gly-Gln-Phe-Tyr-Gly-Leu-Met-NH_2
Xenopus laevis		
XENOPUS SP	Gut	Lys-Pro-Arg-Pro-Asp-Gln-Phe-Tyr-Gly-Leu-Met-NH_2
Aliphatic TKS		
Rana ridibunda		
RNK	Gut	His-Lys-Leu-Asp-Ser-Phe-Ile-Gly-Leu-Met-NH_2
Rana catesbeiana		
RTKC	Gut	His-Asn-Pro-Ala-Ser-Phe-Ile-Gly-Leu-Met-NH_2
Amphiuma tridactylus		
Gut		His-Lys-Asp-Ala-Phe-Ile-Gly-Leu-Met-NH_2
Xenopus laevis		
XENOPUS NKA	Gut	Thr-Leu-Thr-Thr-Gly-Lys-Asp-Phe-Val-Gly-Leu-Met-NH_2

The list of the 11 TKs extracted from frog gastrointestinal or nervous systems is shown in Table 2 and should be completed by authentic NKB found in the brain of *Rana esculenta*.

STRUCTURE OF THE PRECURSOR mRNA/GENE

The high degree of sequence similarity among amphibian and mammalian TKs, a C-terminal region completely identical in different species and crucial for TK biological activities, suggests that TK genes are phylogenetically ancient genes that have been highly conserved throughout the course of evolution. Therefore, our knowledge about the structure of the amphibian TK precursor mRNA/gene and its processing has been referred to information about mammalian TKs (for detailed information, see the Tachykinin chapter in the Brain Peptides section).

Only recently, the structures of amphibian preprotachykinins have been deduced from cDNAs cloned from the skin secretion library of two different amphibians, the Oriental ranid frog, *Odorrana grahami*[16] and the African hyperoliid frog, *Kassina senegalensis*.[34] From *O. grahami*, two cDNA sequences were characterized, encoding TK OG1 and ranamargarin, respectively. These precursors are composed of 61 amino acid (aa) residues including a signal peptide followed by an acidic spacer peptide and only one copy of mature TK-like peptide. Their overall structure is different from the structure of mammalian TK precursors such as preprotachykinin (TAC)-1 containing 143 aa including two kinds of TK peptides (SP and NKA), inserted at the middle of their precursor protein. Besides the obvious size difference, the only single copy of the mature TK-like peptides is at the end of the C-terminus of its precursor.[16]

Moreover, from *K. senegalensis* skin, two different cDNA sequences encoding kassinin and (Thr^2,Ile^9)-kassinin were determined. Each of these preprotachykinins consists of 80 aa residues encoding single copies of either kassinin or its analog and showed a different domain architecture from the *O. grahami* precursors. Indeed, a single TK-encoding domain, adjacent to the N-terminal putative signal peptide, characterizes each precursor. In conclusion, these findings suggest that the biosynthesis mode of TKs in amphibians is different from that of other animals.

PROCESSING OF THE PRECURSOR

Mammalian TKs, synthesized as large precursor proteins through alternative mRNA splicing, generate the distinct mature peptides (for detailed information, see the Tachykinin chapter in the Brain Peptides section). However, details of the biosynthesis of amphibian TKs remained unknown until the discovery of preprotachykinins from *Odorrana grahami* and *Kassina senegalensis* skin.

The *Odorrana grahami* precursors of OG1 and ranamargarin share an identical signal peptide and a highly conserved acidic peptide, while the regions of mature TK-like peptides are different. There is a dibasic processing site for trypsin-like proteases sited before the mature TK-like

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peptide. At the end of the mature TK-like peptide, a glycine provides the amide for the C-terminal methionamide.

In the *Kassina senegalensis* precursors, a typical propeptide convertase-processing site occurs flanking the C-terminus of the TK that is preceded by a glycyl residue amide donor constituting the cleavage/amidation motif. This motif occurs at two additional sites, implying that upstream peptides could likewise be cleaved at these sites and C-terminal amidated.

DISTRIBUTION

Studies on TK-mRNA distribution in nonmammalian vertebrates are very few, but the available information about final peptide localization could be a predictor of the corresponding mRNA expression sites. Evidence for the presence of TKs in amphibian skin, brain, and gut was obtained through techniques of isolation and structural characterization of the original peptides from extracts of these tissues. The use of radioimmunological and immunohistochemical techniques revealed the presence of TK-like substances in different amphibian tissues and organs.

In amphibians, as in mammals and other vertebrates, TK-like immunoreactivity (TK-LI) is widely distributed throughout both the central and peripheral amphibian nervous system.[10]

In *Rana pipiens*, SP-LI is found in the brainstem with a distribution similar to that detected in amniotes, thus emphasizing the phylogenetic conservation of large amounts of the structure and possibly of functional activities in the old reticular formation. TK-LI is also present in the hypothalamic–hypophysial system of *Rana esculenta*, and in visual systems of the frogs *Rana pipiens* and *Xenopus laevis*, especially in the retina (amacrine cells) and in the optic tectum, with a distribution of SP-related peptides remarkably similar to that seen in the homologous regions of mammals. In *Rana esculenta*, *Rana catesbeiana*, and *Xenopus* spinal cord, a dense TK-LI is observed in the dorsal horn where the TK functional organization seems to be similar to that of mammals, as well as the colocalization of TK-LI with calcitonin gene-related peptide (CGRP) immunoreactivity restricted to primary afferent fibers.[10,26]

In the peripheral nervous systems of mammals, the main source of TKs is the enteric nervous system. The TKs in amphibian gut have a distribution similar to that occurring in mammals, as shown by the dense SP-LI and bufokinin-LI (BFK-LI) in the myenteric plexus and in the fibers innervating submucosal and mucosal structures, and conversely, by the moderate immunoreactivity detected in the circular muscle of the intestine of the toad *Bufo marinus*.[20]

In *Rana catesbeiana*, TK-LI is also found in the nerve fibers innervating the frog palate, the carotid labyrinth, the respiratory system (pharynx and lung in colocalization with CGRP), and the vasculature. Recently, BFK-LI was mapped in varicose fibers in the wall of many peripheral blood vessels in the toad. In *Rana ridibunda*, TK-LI (SP-, NKA-, and ranakinin/RNK-LI) is observed in peptidergic fibers innervating the adrenal gland, supporting functional data relating to TK control of corticosteroid release in amphibians.[15]

As well as neuronal distribution, TKs are also localized in nonneuronal structures. In amphibians, the most complex and richest nonneuronal localization is certainly represented by the skin, which may be considered a huge workshop producing bioactive peptides and a true endocrine system.

RECEPTORS AND SIGNALING CASCADES

The biological activity of vertebrate TKs depends on their interaction with three main types of Gq/G_{11} protein-coupled receptors, NK1, NK2, and NK3, which mediate their functional roles by activating phospholipase C (PLC), inositol–phosphate formation, and increasing intracellular calcium. Although mammalian TK receptors have received great attention (for detailed information, see Tachykinin chapter in the Brain Peptides section), the nonmammalian counterparts of these receptors have been poorly studied. Because mammalian and nonmammlian TKs have broadly similar actions in both mammalian and nonmammalian species, it is probable that the sequences of the TK receptors have been conserved across different vertebrate species.

The first amphibian TK receptor cloned and characterized was an SP receptor complementary DNA from the sympathetic ganglion of bullfrog *Rana catesbeiana*. It has a protein sequence with 69% identity to the mammalian NK1 receptor, with the highest degree of conservation seen in the transmembrane and intracellular loop regions. The endogenous ligands of this receptor, the ranatachykinins (RTKs), act as full agonists at this site, inhibiting the potassium current that causes alterations in neuronal excitability. However, NK1 antagonists, at concentrations that block mammalian NK1 receptors, had little or no effect on the responses to the bullfrog NK1-like receptor.[31]

Recently, three NK1-like receptor isoforms (bNK1-A, bNK1-B, and bNK1-C), differing only at the intracellular COOH terminus, were cloned from cane toad brain and intestine by Liu et al., suggesting that the alternative splicing of the NK1 receptor is a mechanism that has been physiologically conserved across different species and that each isoform could play a distinct physiological role. Two of these bufokinin receptors (bNK1-A and bNK1-B) are similar to the truncated and full-length forms of the mammalian NK1 receptor, whereas bNK1-C does not correspond to any previously described receptor. The three isoform transcripts are strongly expressed in the gut, lung, and skeletal muscle and in the spinal cord and brain.[19] BFK receptors were pharmacologically characterized in the gastrointestinal tract of *Bufo marinus* and show functional similarities to the NK1

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mammalian receptor, although displaying very low affinity for the mammalian NK1 receptor–selective agonists and antagonists.[20] Moreover, in *X. laevis* gastrointestinal tract, two different TK receptors were functionally characterized, one an NK1-preferring form and the other an NK2-preferring receptor. Both may exist in the stomach, whereas the NK1-like receptor predominates in small intestine longitudinal muscle. However, no molecular evidence exists for the presence of either NK2 or NK3 receptor in amphibians. Additionally, as classical TK antagonists seem to interact differently with these amphibian receptors in comparison with mammalian receptors, the sequence of the "antagonist binding domain" of amphibian TK receptors is probably not well conserved.

Compared with the large amount of data about mammalian TK receptors, we have only limited knowledge about the signal transduction mechanisms of amphibian TK receptor activation.

Nevertheless, binding studies in toad intestinal smooth muscle membranes show the presence of a G protein–coupled TK receptor. Indeed, TK dose dependently increases the production of inositol phosphates in this tissue, suggesting that the mechanism of TKs in contracting toad intestine is via the inositol phospholipid hydrolysis pathway.[18] Similarly, in the frog adrenal gland, ranakinin enhances polyphosphoinositide hydrolysis, and this effect is mediated through the activation of a phospholipase-C positively coupled to a pertussis toxin-sensitive G protein.[14]

ACTIONS IN AMPHIBIANS

Amphibian TKs were available in pure form several years before the structures of mammalian TKs were elucidated and have been shown to have counterparts in mammalian tissues and to exert wide ranging biological actions mediated through the activation of the three classical TK receptors. Consequently, the earliest studies on the biological actions of TKs that involve the effects of amphibian TKs such as PHYS and KASS have contributed substantially to the explosive progress of the knowledge of the functional roles of TK system in mammalian species. Although the results obtained with KASS, an NK2/NK3 TK receptor agonist, do not exactly mimic results obtained with either NKA or NKB, the results obtained with PHYS, a selective NK1 agonist, are identical, with negligible quantitative differences, to those obtained later with SP, the mammalian selective NK1 receptor agonist. The availability in 1990 of PG-SPI and PG-KII, two highly selective and potent NK1 and NK3 TK receptor agonists, provided a further instrument to investigate the role of the TK system in mammals, in which amphibian TKs show a spectrum of biological actions that is usually identical with that of mammalian TKs.[30]

Conversely, although it has often been demonstrated that mammalian TKs are able to produce effects in nonmammalian species, the extent to which amphibian TKs mirror these effects in their species of origin is not clear, although some studies exist that indicate a number of similarities. In this chapter, we therefore summarize the main *in vitro* and *in vivo* actions of some amphibian TKs on different systems in both their species of origin and mammals.

ACTIONS OF AMPHIBIAN TKs IN THEIR SPECIES OF ORIGIN

PHYS exerts a strong excitatory action on motoneurons of bullfrog-isolated spinal cord. On a molar basis, PHYS was 1500 times more active than L-glutamate in depolarizing the motoneurones, suggesting that TKs are excitatory transmitters of primary sensory neurons in this animal species. PHYS and the other SP-like amphibian neuropeptides, RTKs and BFK, produce a potent and long-acting hypotensive effect without any reflex tachycardia in the anaesthetized cane toad *Bufo marinus*. The blood pressure depressant action of RTK is left unmodified by a nonpeptidic NK1 antagonist, indicating that the amphibian NK1 receptor mediating this effect differs from its mammalian counterpart. The occurrence of BFK-LI in varicose fibers of blood vessels of the toad suggests that the fall in systemic blood pressure is the consequence of a decrease in peripheral vascular resistance in this species. BFK may be released by antidromic stimulation of sensory nerves and may play a role in haemodynamic regulation in the toad. PHYS, KASS, RNK, and BFK are very potent and long-acting spasmogens in the toad intestine, and the presence of BFK-immunoreactive nerves in the myenteric plexus and *muscularis externa* supports this finding. The absence of BFK-LI in the intestinal mucosa suggests a minor role in regulating toad intestinal secretion.[21]

Among the *X. laevis* TK-like peptides, both the SP-like (structurally identical to BFK) and the NKA-like peptide induce an equipotent myotropic effect on isolated strips of circular smooth muscle from *Xenopus* stomach, whereas only the SP-like peptide acts as a spasmogen on intestinal musculature. The maximum response to *Xenopus* SP is lower than that to the NKA-like peptide, suggesting a more effective interaction of the NKA peptide with the TK receptors in *Xenopus* stomach. These findings confirm the presence of both NK1 and NK2 receptors in stomach and only of the NK1 subtype in intestine.[11]

Administration of PHYS in *Rana porosa porosa* has a positive effect on protein and mucous granule secretion from the lingual gland as detected by transmission electron microscopy. This reaction is very different from that observed in mammals, in which TKs stimulate only water secretions. In *Rana esculenta* skin, TKs stimulate ion transport (Na^+ absorption and Cl^- secretion, measured by short-circuit currents) by interacting with NK1-like receptors

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and by involving prostanoid synthesis. The rank order of TK potency is as follows: PG-KI > UP > HYL > KASS> PHYLL >RTKA >PHYS> RNK. The skin of this frog does not produce TKs, and it is probably a target for TKs produced in other tissues, which, by promoting fluid secretion, may regulate the pain response.

Amphibian TKs, which are detected in frog adrenal gland extracts, stimulate corticosteroid secretion by frog adrenal slices through a prostaglandin-dependent mechanism. The rank order of their potency in comparison with mammalian TKs is as follows: NKA >RNK >SP >KASS > PHYS > NKB suggesting that TKs released by nerve fibers control neuroendocrine secretions in amphibians. RNK, the most potent amphibian TK in stimulating steroid secretion, causes the mobilization of calcium from adrenochromaffin cells through the activation of an NK1-like receptor linked to phospholipase C and pertussis toxin-sensitive G protein, which is different from the mammalian NK1 subtype.[14]

ACTIONS IN MAMMALS

Cardiovascular System

TKs can relax or contract *in vitro* vascular preparations. Relaxation results from the activation of NK1 receptors located in the endothelium, whereas contraction is caused by the activation of NK2 receptors located on the arterial smooth muscle. Likewise, in the rat portal vein (with intact endothelium), contraction elicited by KASS is brought about by NK3 receptors, presumably located on the smooth muscle membrane. In *in vivo* studies, the response of the vasculature to TKs is complex, depending on the animal species, density in the smooth muscle cells and in the endothelium of the various receptor subtypes, as well as on the TK administered or released. The NK1 receptor-preferring TK, PHYS produces a marked dose-dependent hypotension in dogs and rabbits, whereas the NK2/NK3 agonist KASS is 10–20 times less potent. Conversely, PHYS produces moderate hypotension with tachyphylaxis, hypotension/hypertension, or frank hypertension in cats, sheep, rats, and pigeons. PHYS is a very potent vasodilator of the dog coronary, skeletal muscle, and hepatic vascular bed and after intradermal injection, increases rat, guinea pig, and human capillary permeability. These findings suggest that the striking amphibian TK hypotensive effect is the consequence of intense vasodilatation in several peripheral vascular beds due to direct TK action on the blood vessel wall promoting the release of endogenous relaxing factors from the endothelium.

Gastrointestinal Tract (GIT)

(a) Motor Effects

TKs, so-called because they evoke a fast contraction of intestinal smooth muscle, represent one of the most active substances inducing a clear-cut stimulation of motility in all sections of the gut (from esophagus to the rectum, and in all muscle layers, including longitudinal muscle, circular muscle and *muscularis mucosae*) of all mammalian and submammalian vertebrates examined to date. The actions of mammalian TKs in the gut have been the subject of extensive and thorough investigation (for detailed information, see the chapter on Substance P in the Gastrointestinal Peptides section of this book). As far as effects of amphibian TKs on the GIT are concerned, we can summarize by saying that all these peptides, with few exceptions, provoke a contractile response in *in vitro* gut preparations, although the intensity and reproducibility of the response may be considerably different depending on animal species, gut section, and agonist receptor selectivity. Moreover, *in vivo* studies have shown that peripherally administered amphibian TKs induce complex, mainly stimulatory and also inhibitory effects in the GIT propulsion in several mammalian species (dog, cat, guinea pig, rat, sheep).

(b) Secretory Effects

Amphibian TKs exert a potent stimulatory effect on salivary secretion, which was recognized several years before the sialagogic factor in a bovine hypothalamic extract was identified as SP. The most sensitive animal species are dog, ferret, rat, and guinea pig. The rank order of potency (PHYS=UP>SP=PG-SPI>PG-KII = KASS >> NKA >>> NKB) in causing salivary secretion in rats after intravenous injection indicates that this TK effect is preferentially mediated by the NK1 receptor. The greater potency of PHYS and UP in comparison to SP may be explained by the considerably higher resistance to enzyme attack offered in the two first peptides by their N-terminal pGlu residue. PHYS generates vomiting, diarrhea, and profuse salivation in dogs. Exogenous TKs cause secretion of fluid and electrolytes from the intestinal mucosa, and it has been suggested that also endogenous TKs may play a messenger role in the intestinal secretory pathway. Also, an increased permeability of the capillaries could contribute to increased secretions. Moreover, there is increasing evidence that TKs participate in hypersecretory, vascular, and immunological disorders associated with infection and inflammatory bowel disease.[28]

PHYS displays a moderate, short-lasting stimulatory action on exocrine pancreatic secretion in the dog. In dispersed acinar cells from guinea-pig pancreas, both NK1 (PHYS) and NK3 (PG-KII) TK receptor agonists increase amylase release, whereas in rat acinar cells, only the NK1 agonist is active. These functional findings indicate a species-related difference in the TK regulation of exocrine pancreatic secretion, which has been confirmed by immunocytochemical studies.[17] In a secretagogue-induced model of acute pancreatitis in rats, NK1 receptor expression in

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pancreatic acini is unchanged compared with normal pancreas, whereas NK3 receptors, which apparently have no physiological role in pancreatic secretion in rats, are overexpressed, become activated, alter cell function, and probably intervene in mediating acute pancreatitis.[2] PHYS is a potent stimulant of lachrymal secretion in the dog, while the rat is much less sensitive.

Airways System

In guinea pigs, KASS increases insufflation pressure, while PHYS, by activating receptors localized in the endothelial cells, increases the microvascular permeability to protein, indicating that both NK2 and NK1 receptors, respectively, are involved in the guinea-pig tracheobronchial tree response to TKs. In rats, KASS elicits a broncho-constrictor action that is much more potent than SP.[12]

Urinary Tract

TKs stimulate smooth muscle preparations of the urogenital tract, especially the urinary bladder, and display differences in their agonistic potency depending not only on the different animal species but also on the particular segment of the urinary tract, probably resulting from the involvement of different receptor types. Intravenous injection of TKs induces a phasic contraction of the urinary bladder (increase of internal pressure), and an activation of rhythmic contractions, with KASS being the most potent peptide. These different responses are mediated through different receptors and are brought about both by a direct effect on the bladder's smooth muscle and by an effect on intramural sensory nervous pathways ("micturition reflex").[22]

Central Nervous System

In the mammalian CNS, TKs occur in large amounts, particularly in areas involved in the central control of peripheral autonomic functions (blood pressure, respiration, micturition, gastrointestinal motility, and secretions), essential functions (e.g., drinking behavior), affective and emotional life (stereotyped behavior, motility, anxiety, aggressivity, pain), and higher cerebral functions (learning, memory). Therefore, systematic studies with intracerebroventricular administration of amphibian TKs that are selective for different receptor types allows the roles of these neuropeptides to be evaluated in the CNS control of several peripheral functions and the identification of the TK preferring receptor types involved.

(a) The NK2 receptor–preferring agonist KASS induces a dose-related delay in the gastric emptying of a liquid meal, whereas the activation of NK3 receptors by the highly selective NK3 agonist PG-KII mediates a colonic antipropulsive effect;[1]

(b) NK3 receptor activation by KASS and PG-KII mediates an inhibitory action on gastric acid secretion in pylorus-ligated rats;[7]

(c) NK2 receptors participate in the protection against gastric ulcers;[7]

(d) NK3 receptor activation via amphibian PG-KII exerts an antidipsogenic effect on angiotensin II-induced drinking behavior, an antidiuretic effect resulting from release of vasopressin, and a potent and long-lasting decrease in salt intake;[23]

(e) The NK3 receptor–preferring agonist PG-KII decreases ethanol intake in genetically alcohol-preferring rats;[23]

(f) The NK1 and NK3 receptor systems are involved in supraspinal analgesia in rats;[8]

(g) PHYS and other NK1 agonists do not affect the response of rat cerebellar granule neurons to the cytotoxic action of glutamate, whereas the NK2/NK3 receptor–preferring amphibian TKs, KASS, RKA, and PG-KII significantly increase glutamate excitotoxicity.[29]

PATHOLOGICAL IMPLICATIONS

Advances made during the past decade indicate that TKs, besides their physiological roles in both mammals and in amphibians, may play also significant roles under pathological conditions. An imbalance in the functionality of the mammalian TK system may contribute to several diseases, and so TK receptors could represent attractive target for novel agonist and antagonist therapeutic agents. In this regard, although results obtained with amphibian TKs are rather scanty, some representative studies both in experimental models and in human disorders show that these peptides, being more selective and more resistant to enzymatic degradation than mammalian TKs, may be helpful to clarify pathological implications.

PHYS administered through eye drops increases lachrymal secretion, ameliorates Sjögren's syndrome and other forms of keratoconjunctivitis sicca resulting from a deficit of lachrymal secretion.[3] In a screening study of new sialogogue agents that might be of use in the treatment of dry mouth in humans, PHYS is the most potent substance.[6] Central injection of PG-SPI (NK1 selective agonist) and PG-KII (NK3 selective agonist) in rats stimulate IL-1β production and increase granulocyte infiltration into the colon worsening experimental models of colitis, which are reminiscent of human inflammatory bowel disease (Crohn's disease and ulcerative colitis), thus providing further evidence of the central role of TKs in this gut pathology and of the potential usefulness of NK1 and NK3 TK receptor antagonists as therapeutic agents.[9] Fragment 25-35 of β-amyloid peptide, the primary constituent of senile plaques and cerebrovascular deposits in Alzheimer's disease and Down syndrome,

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shows important sequence similarity with the C-terminal region of TKs. Interestingly, the neurotoxic response to β-amyloid (1–40) is completely reversed by SP and PHYS and partially by NKB and KASS, raising the possibility that β-amyloid may interact with TK receptors.[32] Furthermore, in neuroblastoma cells, the toxic effect induced by β-amyloid (1–40) is inhibited by the concurrent treatment of the cells with PHYS.[33] In turn, β-amyloid(1-40), which alone weakly activates the TK receptors, in the presence of glutamate strongly activates both TK receptors (especially the NK1 receptors) and phosphatidylinositol turnover.[13] Taken together, all studies on amphibian TKs reported here cannot merely be considered of academic interest, as they have substantially contributed to the progress of research in the field of mammalian TKs. These studies may lead to the discovery of new TKs and elucidation of their structures and subsequently provide easily available models for studying structure–activity relationships and identifying physiopathological implications in mammals, including humans.

REFERENCES

1. Broccardo M, Improta G, Tabacco A. Central tachykinin NK3 receptors in the inhibitory action on the rat colonic propulsion of a new tachykinin, PG-KII. *Eur J Pharmacol* 1999;**376**:67–71.
2. Broccardo M, Linari G, Agostini S, Amadoro G, Carpino F, Ciotti MT, et al. Expression of NK-1 and NK-3 tachykinin receptors in pancreatic acinar cells after acute experimental pancreatitis in rats. *Am J Physiol Gastrointest Liver Physiol* 2006;**29**:G518–24.
3. De Caro G, Cordella M, Miani P. The treatment of Sjoegren's syndrome with physalaemin. *Ophthalmologica* 1969;**158**:284–7.
4. Erspamer V. The TK peptides family. *Trends Neurosci* 1981;**4**:267–9.
5. Erspamer V. Bioactive secretions of the integument. In: Heatwole H, Barthalmus GT, editors. *Amphibian biology. I. The integuments*. Chipping Norton NSW, Australia: Surrey Beatty & Sons; 1994. p. 178–350.
6. Gao C, Abe K. Structure–activity relationships of sialogogic heptapeptides analogous to physalaemin. *Oral Dis* 2000;**6**:180–6.
7. Improta G, Broccardo M, Severini C, Erspamer V. In vitro and in vivo biological activities of PG-KII, a novel kassinin-like peptide from the skin of the Australian frog, *Pseudophryne güntheri*. *Peptides* 1996;**17**:1003–8.
8. Improta G, Broccardo M. Effects of supraspinal administration of PG-SPI and PG-KII, two amphibian tachykinin peptides, on nociception in the rat. *Peptides* 2000;**21**:1611–6.
9. Improta G, Carpino F, Petrozza V, Guglietta A, Tabacco A, Broccardo M. Central effects of selective NK1 and NK3 tachykinin receptor agonists on two models of experimentally-induced colitis in rats. *Peptides* 2003;**24**:903–11.
10. Inagaki S, Senba E, Shiosaka S, Takagi H, Kawai Y, Takatsuki K, et al. Regional distribution of substance P-like immunoreactivity in the frog brain and spinal cord: immunohistochemical analysis. *J Comp Neurol* 1981;**201**:243–54.
11. Johansson A, Liu l, Holmgren S, Burcher E. Characterization of receptors for two *Xenopus* gastrointestinal tachykinin peptides in their species of origin. *Naunyn–Schmiedebergs Arch Pharmacol* 2004;**370**:35–45.
12. Joos GF, Pauwels RA, van der Straeten ME. The mechanism of tachykinin-induced bronchoconstriction in the rat. *Am Rev Respir Dis* 1988;**137**:1038–44.
13. Kimura H, Schubert D. Amyloid beta-protein activates tachykinin receptors and inositol trisphosphate accumulation by synergy with glutamate. *Proc Natl Acad Sci U S A* 1993;**90**:7508–12.
14. Kodjo MK, Desrues L, Lavagno L, Fasolo A, Conlon JM, Tonon MC, et al. Ranakinin, a naturally occurring tachykinin, stimulates phospholipase C activity in the frog adrenal gland. *Endocrinology* 1998;**139**:505–12.
15. Leboulenger F, Vaglini L, Conlon JM, Homo-Delarche F, Wang Y, Kerdelhue B, et al. Immunohistochemical distribution, biochemical characterization, and biological action of tachykinins in the frog adrenal gland. *Endocrinology* 1993;**133**:1999–2008.
16. Li J, Liu T, Xu X, Wang X, Wu M, Yang H, et al. Amphibian tachykinin precursor. *Biochem Biophys Res Commun* 2006;**350**:983–6.
17. Linari G, Improta G, Agostini S, Andreassi A, Broccardo M. Stimulatory effect of PG-KII, an NK3 tachykinin receptor agonist, on isolated pancreatic acini: species-related differences. *Peptides* 2004;**25**:45–51.
18. Liu L, Warner FJ, Conlon JM, Burcher E. Pharmacological and biochemical investigation of receptors for the toad and the gut TK peptide bufokinin, in its species of origin. *Naunyn Schmiedebergs Arch Pharmacol* 1999;**360**:187–95.
19. Liu L, Markus I, Vandeberg RJ, Neilan BA, Murray M, Burcher E. Molecular identification and characterization of three isoforms of tachykinin NK 1-like receptors in the cane toad *Bufo marinus*. *Am J Physiol Regul Integr Comp Physiol* 2004;**287**:575–85.
20. Liu L, Murray M, Conlon JM, Burcher E. Quantitative structure-activity analyses of bufokinin and other tachykinins at bufokinin (bNK1) receptors of the small intestine of the cane toad *Bufo marinus*. *Biochem Pharmacol* 2005;**69**:329–38.
21. Liu L, Burcher E. Tachykinin peptides and receptors: putting amphibians into perspective. *Peptides* 2005;**26**:1369–82.
22. Maggi CA, Giuliani S, Santicioli P, Abelli L, Regoli D, Meli A. Further studies on the mechanisms of the tachykinin-induced activation of micturition reflex in rats: evidence for the involvement of the capsaicin-sensitive bladder mechanoreceptors. *Eur J Pharmacol* 1987;**136**:189–205.
23. Polidori C, Panocka I, Ciccocioppo R, Broccardo M, Improta G, Regoli D, et al. Further evidence that the tachykinin PG-KII is a potent agonist at central NK-3, but not NK-1, receptors. *Peptides* 1997;**18**:825–33.
24. Quartara L, Maggi CA. The TK NK1 receptor. Part I: ligands and mechanisms of cellular activation. *Neuropeptides* 1997;**31**:537–63.
25. Quartara L, Maggi CA. The TK NK1 receptor. Part II: distribution and pathophysiological roles. *Neuropeptides* 1998;**32**:1–49.
26. Salio C, Fischer J, Wijkhuisen A, Franzoni MF, Conrath M. Distribution and ultrastructure of tachykinin-like immunoreactivity in the frog (*Rana esculenta*) spinal cord, notably, the dorsal horn. *J Comp Neurol* 2001;**433**:183–92.
27. Severini C, Salvadori S, Guerrini R, Falconieri-Erspamer G, Mignogna G, Erspamer V. Parallel bioassay of 39 TKs on 11 smooth muscle preparations. Structure and receptor selectivity/affinity relationship. *Peptides* 2000;**21**:1587–95.
28. Severini C, Improta G, Falconieri-Erspamer G, Salvadori S, Erspamer V. The tachykinin peptide family. *Pharmacol Rev* 2002;**54**:285–322.
29. Severini C, Ciotti MT, Mercanti D, Barbato C, Calissano P. A tachykinin-like factor increases glutamate toxicity in rat cerebellar granule cells. *Neuropharmacology* 2003;**44**:117–24.

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30. Simmaco M, Severini C, De Biase D, Barra D, Bossa F, Roberts JD, et al. Six novel tachykinin- and bombesin-related peptides from the skin of the Australian frog *Pseudophryne güntheri*. *Peptides* 1990;**11**:299–304.
31. Simmons MA, Brodbeck RM, Karpitskiy VV, Schneider CR, Neff DP, Krause JE. Molecular characterization and functional expression of a substance P receptor from the sympathetic ganglion of *Rana catesbeiana*. *Neuroscience* 1997;**79**:1219–29.
32. Yankner BA, Duffy LK, Kirschner DA. Neurotrophic and neurotoxic effects of amyloid beta protein: reversal by tachykinin neuropeptides. *Science* 1990;**250**:279–82.
33. Zhao X, Valantas JA, Vyas S, Duffy LK. Comparative toxicity of amyloid beta-peptide in neuroblastoma cell lines: effects of albumin and physalaemin. *Comp Biochem Physiol C* 1993;**103**: 165–70.
34. Wang L, Zhou M, Lynch L, Chen T, Walker B, Shaw C. *Kassina senegalensis* skin tachykinins: molecular cloning of kassinin and (Thr2, Ile9)-kassinin biosynthetic precursor cDNAs and comparative bioactivity of mature tachykinins on the smooth muscle of rat urinary bladder. *Biochimie* 2009;**91**:613–9.

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Chapter 56

Temporins

Andrea C. Rinaldi and J. Michael Conlon

ABSTRACT

The temporins belong to a family of short (8–17 amino acids), hydrophobic, C-terminally α-amidated peptides with antibacterial and antifungal properties that are synthesized in the skins of a wide range of North American and Eurasian frogs of the Ranidae family. Temporins adopt an α-helical conformation in hydrophobic environments and have the ability to perturb the integrity of target cell membranes. Not all temporins are cationic, but the number of positively charged amino acids correlates with antimicrobial potency. Temporins are mostly effective against Gram-positive bacteria, but some are also active against Gram-negative bacteria. Temporins show potential for development into therapeutically valuable anti-infective agents, particularly for use against antibiotic-resistant Gram-positive bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA) and *Enterococcus faecalis*, against anaerobic pathogens such as *Clostridium difficile*, and against the protozoan parasite *Leishmania* spp. Although the clinical usefulness of naturally occurring temporins is limited by high hemolytic activity, noncytotoxic analogs have been designed.

DISCOVERY AND DISTRIBUTION

Frogs of the family *Ranidae* synthesize in granular glands in the skin a remarkably diverse range of antimicrobial peptides that are released into skin secretions, often in very high concentrations, in a holocrine manner on stress or injury as a result of contraction of myocytes surrounding the glands.[6] These peptides represent a component of the animal's system of innate immunity and serve to protect the frog against invasion by a variety of pathogenic microorganisms (see the chapters on the Dermaseptins and Bombinins in this section of the book). Included in this arsenal of defensive peptides are members of the temporin family. Consisting of between 8 and 17 (typically 13) amino acids, they are among the smallest antimicrobial peptides to be found in nature. Temporins were first identified in the skin of the Asian frog *Rana erythraea*[34] and the European frog *Rana esculenta* (reclassified as a hybrid between *Pelophylax ridibundus* and *P. lessonae*)[31] on the basis of their hemolytic activity. These hydrophobic, C-terminally α-amidated peptides were originally described as "*Vespa*-like" because of their structural similarity to the short (13–14 amino acids) peptides with chemotactic and histamine-releasing properties isolated from the venom of wasps of the genus *Vespa*. The term "temporin" was first used by Simmaco and coworkers to describe a family of 10 structurally related peptides (temporins A-L) with antibacterial and antifungal properties identified in the electrically stimulated skin secretions of the European common frog *Rana temporaria*.[32]

Subsequent work has shown that the temporin family is widely, but not universally, distributed in ranid frogs of both North American and Eurasian origin (Figs 1 and 2). Adopting the classification of new world ranid frogs proposed by Frost,[12] temporins have been isolated from the skin secretions and/or extract of several North American species belonging to the genera *Rana* and *Lithobates* (reviewed in Ref. 9) (Fig. 1). Among the Eurasian ranid frogs, temporins have been identified in the skins of the Japanese brown frogs *R. dybowskii*, *R. japonica, R. ornativentris*, *R. pirica*, *R. sakuraii*, *R. tagoi tagoi*, *R. tagoi okiensis*, and *R. tsuschimensis*, but not the Ryukyu brown frog *R. okinavana*, and in several species belonging to the genera *Amolops*, *Hylarana*, and *Pelophylax* (reviewed in Ref. 6) (Fig. 2). Temporin-SHf (FFFLSRIF.NH_2) from skin secretions of specimens of *Pelophylax saharicus* collected in Tunisia is the smallest natural linear antimicrobial peptide found to date and, despite its small size and hydrophobicity, has broad-spectrum microbicidal activity against Gram-positive and Gram-negative bacteria and yeasts, with no hemolytic activity.[1]

BIOSYNTHESIS

Screening of a cDNA library prepared from the skin of *R. temporaria* with an oligonucleotide probe derived from the signal peptide region of preproesculentin-1 from *R. esculenta* led to the identification and characterization of the biosynthetic precursors of temporin B (LLPIVGNLLKSLL.NH_2), temporin G (FFPVIGRILNGIL.NH_2), and temporin H (LSPNLLKSLL.NH_2).[32] The structural organization of the three preprotemporins is the same and is illustrated

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00056-7
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Lithobates

L. catesbeianus
FLP*IASLLGKYL
FISAIASMLGKFL
FLSAIASMLGKFL
FISAIASFLGKFL
FLFPLITSFLSKVL

Rana clamitans
FLPFLAKILTGVL
FLPLFASLIGKLL
FLPFLASLLTKVL
FLPFLASLLSKVL
FLPFLATLLSKVL

L. grylio
SILPTIVSFLSKVF
SILPTIVSFLSKFL
SILPTIVSFLTKFL
FILPLIASFLSKFL

L. heckschieri
SIFPAIVSFLSKFL

L. okaloosae
FLPFLKSILGKIL

Rana septentrionalis
FLSAITSILGKFF
FLSAITSLLGKLL
FLSAITSILGKLF

Rana virgatipes
FLSSIGKILGNLL
FLSIIAKVLGSLF
FLPLVTMLLGKLF

L. aereolata
FLPIVGRLISGLL

L. capito
IPPFIKKVLTTVF
FLPIVGRLISGIL

L. onca
FLPTFGKILSGLF

L. palustris
FLPLVGKILSGLI

Rana

R. aurora
FLPIIGQLLSGLL

R. boylii
FLPIIAKVLSGLL

R. draytonii
HFLGTLVNLAKKIL
NFLGTLVNLAKKIL

R.luteiventris
VLPLISMALGKLL
NFLGTLINLAKKIM
FLPILINLIHKGLL

R. muscosa
FLPIVGKLLSGLL

R. pretiosa
FLPILGNLLSGLL
FLPIITNLLGKLL
NFLDTLINLAKKFI

FIGURE 1 Distribution and primary structures of the temporin peptides from North American frogs of the genera *Rana* and *Lithobates*. All peptides are C-terminally α-amidated. The classification of the species is according to Frost[12]; * denotes deletion of an amino acid.

schematically for preprotemporin B in Fig. 3. Molecular cloning studies have confirmed these findings for many other temporin biosynthetic precursors. A study of the expression of preprotemporin genes in the skin of Japanese mountain brown frog *R. ornativentris* found that although preprotemporin mRNAs were not detectable in the skins of tadpoles before the onset of metamorphosis, their levels increased markedly during metamorphosis, reaching a maximum at the stages of metamorphic climax.[25] This suggests a correlation with thyroid hormones, which "…may induce the expression of genes encoding preprotemporins in a coordinated fashion that is synchronized with the metamorphosis in tadpoles and with molting in adults to protect the skin surface from pathogens in the environment."[25]

The temporins conform to the general rule among the frog skin antimicrobial peptides that the signal peptide and acidic propeptide regions are very strongly conserved, even in the case of frogs belonging to different families, whereas the antimicrobial peptide domain at the C-terminus of the precursor is hypervariable (see the chapter on Dermaseptins

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Rana

R. temporaria
FLPLIGRVLSGIL
LLPIVGNLLKSLL
LLPILGNLLNGLL
LLPIVGNLLNSLL
VLPIIGNLLNSLL
FFPVIGRILNGIL
LSP***NLLKSLL
LLP***NLLKSLL
FVQWFSKFLGRIL

R. dybowskii
FIGPIISALASLFG

R. japonica
ILPLVGNLLNDLL

R. ornativentris
FLPLLASLFSRLL
FLPLIGKILGTIL
FLPLLASLFSRLF
FLPLLASLFSGLF
ILPLLGNLLNGLL

R. pirica
ILPILGNLLNGLL
ILPILGNLLNSLL

R. sakuraii
FLPVILPVIGKLLNGIL
FLPVILPVIGKLLSGIL

R. tagoi tagoi
FLPILGKLLSGIL

R. tagoi okiensis
FLPILGKLLSGFL
FLPILGKLLSGLL

R. tsuschimensis
FLGALAKIISGIF
FLPLLGNLLNGLL
FLPLLGNLLRGLL
FLPLLASLIGGML

Hylarana

H. erythraea
FLPILGKILGGLL
FLPLIIGALSSLLPKIF
FLPVIAGLLSKLF

H. guentheri
FLQHIIGALSHIF
FLPLLFGAISHIL
FFPLIFGALSSILPKIL

H. latouchii
FFPLVLGALGSILPKIF
FLPGLIAGIAKML
FLPIALKALGSIFPKIL
FIITGLVRGLTKLF
SLSRFLSFLKIVYPPAF

Amolops

A. lolenensis
FLPIVGKLLSGLSGLL
FLPIVGKLLSGLL
LLPIVGKGLSGKL
FLPIAGKLLSGLSGLL
FFPIVGKLLFGLSGLL
FFPIVGKLLSGLSGLL

Pelophylax

P. ridibundus/lessonae
FLPAIAGIKSQLF
FLPLIAGLLGKLF

P. saharica
FLSGIVGMLGKL
FLPIVTNLLSGLL
FLSHIAGFLSNLF
FFFLSRIF

FIGURE 2 Distribution and primary structures of the temporin peptides from Eurasian frogs of the family Ranidae. All peptides are C-terminally α-amidated. The classification of the species is according to Frost[12]; * denotes deletion of an amino acid.

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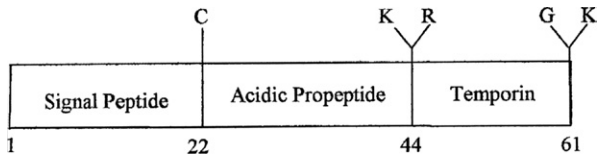

FIGURE 3 A schematic representation of the biosynthetic precursor of temporin B deduced from the nucleotide sequence of a cloned cDNA from the skin of *Rana temporaria*. Cys^{22} is the probable site of cleavage of the signal peptide, $Lys^{45}Arg^{46}$ is the site of cleavage of a prohormone convertase, and Gly^{60} acts as nitrogen donor for C-terminal α-amide formation.

in this section of the book). Thus, the Cys^{22} residue represents the site of cleavage of the signal peptide in the preprotemporins and in the preprodermaseptins and a range of other precursors. The mature temporin is generated by cleavage at a typical Lys-Arg prohormone processing site and the penultimate Gly residue in the propeptide acts as a substrate for peptidyl-glycine α-amidating monooxygenase to produce a C-terminal α-amidated residue in the secreted peptide (see the chapter on this enzyme in Peptide Biosynthesis/Processing section of the book).

Preprotemporin mRNAs are expressed at low levels in extradermal tissues of Tago's brown frog *R. tagoi tagoi*. cDNAs encoding temporin-TGa (FLPILGKLLSGIL.NH_2), previously isolated from an extract of the skin, were amplified and cloned from the stomach, liver, kidney, and skeletal muscle. However, a net insertion of 10 nucleotides results in the presence of a premature stop codon in the open reading frame that is not present in the corresponding region of preprotemporin-TGa from skin. The preprotemporin cDNA obtained from small intestine contains an additional 12 nucleotide insertion in the region that encodes the temporin sequence resulting in a novel peptide (FLPVILPVIG10KLLSGIL.NH_2), termed temporin-TGc. This cDNA also contains a premature stop codon in the open reading frame.[18] Nucleotide sequence analysis reveals marked polymorphism of preprotemporin genes among individual frogs. Twenty-seven distinct preprotemporin-Oe mRNAs have been identified in individual *R. ornativentris* specimens that contain synonymous substitutions in the antimicrobial peptide region and both synonymous and nonsynonymous substitutions in the signal peptide and intervening sequence regions. Up to eight preprotemporin-Oe mRNA variants are found within a single frog.[26]

The mRNA encoding temporin-CBa (FLPIASLLGKYL.NH_2) is detectable in the Harderian gland of the bullfrog *Lithobates catesbeianus*,[17] the first example of an AMP gene transcribed in such an organ. The Harderian gland is an orbital gland found in many vertebrates, where it is believed to play important roles such as lubrication of the eye and nictitating membrane, a source of pheromones and growth factors, as well as osmoregulatory, thermoregulatory, and immunoregulatory functions. By analogy with the situation in amphibian skin, the expression of AMP genes in the Harderian gland may be regulated by thyroid hormones.

MOLECULAR HETEROGENEITY

As shown in Figs 1 and 2, >90 members of the temporin family have been identified in the skins of Eurasian and North American ranids. The temporins are among the most highly variable of all antimicrobial peptides and no single residue in the peptide is invariant. Most temporins contain a single basic residue (generally Lys) giving a charge of +2 at physiological pH but several temporins, for example, temporin-Oe lack this structural feature. Conversely, temporin L from *R. temporaria*, temporin-Lb from *Rana luteiventris*, temporin-PRc from *Rana pretiosa*, temporin-CPa, from *Lithobates capito*, and temporins-DRa and -DRb from *Rana draytonii* contain two basic residues. All temporins isolated to date contain a preponderance of hydrophobic amino acids and are C-terminally α-amidated.

Structure–activity studies with temporin A have indicated that a hydrophobic N-terminal residue and bulky hydrophobic residues at positions 5 and 12 are important determinants of antibacterial activity.[33] Replacement of the Ile^5 and Ile^{12} residues by leucine resulted in an increase in antimicrobial potency but replacement by Ala abolished activity. The positively charged amino acid is usually located at position 7 or 11 to maintain the amphipathic character of the α-helix that is important for activity. Most temporins that lack a basic amino acid residue show very weak or no antimicrobial properties, the exception being temporin-1Od (FLPLLASLFSGLF.NH_2) from *R. ornativentris*, which is active against *Staphylococcus aureus*.

BIOPHYSICAL PROPERTIES AND INTERACTION WITH MEMBRANES

In common with the majority of frog skin antimicrobial peptides, the temporins exist in a random coil conformation in aqueous solution but have the propensity to form a stable amphiphathic α-helix in a membrane or when in hydrophobic environments, such as 50% trifluoroethanol/water. To gain deeper insight into conformation–activity relationships, temporin A and and temporin L from *R. temporaria* were selected to test the hypothesis that a significant correlation exists between the conformation adopted by the peptide in solution, that is, *before* its interaction with membranes, and its antimicrobial activity. Temporin A (FLPLIGRVLSGIL.NH_2) is preferentially active against Gram-positive bacteria and has a moderate hemolytic activity, whereas the strongly hemolytic temporin L (FVQWFSKFLGRIL.NH_2) displays the highest antimicrobial potency among temporins tested to date and has strong affinity for lipid membranes and for the lipopolysaccharide (LPS) component of the Gram-negative outer membrane.[15,16] A combination of molecular dynamics simulations, CD spectroscopy, and studies with model membranes revealed that temporin L possesses a higher, intrinsic propensity (negative free energy of folding)

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to form an α-helix in water, existing basically in a "semi-folded" state characterized by a 4(5)-residue helix.[11,28] Thus, the greater antimicrobial and hemolytic activity exhibited by temporin L may be ascribed to its higher propensity to assume a folded conformation in aqueous solution, which would facilitate, both thermodynamically and kinetically, the peptide folding in the microbial membrane.

The all-D enantiomer of temporin A is equipotent with the native peptide against bacteria, indicating that effects on the cell membrane are mediated through nonchiral interactions.[33] Studies involving the use of liposomes of different composition indicate that the lytic activities of the temporins are not greatly influenced by composition or surface charge of the lipid membrane, suggesting that hydrophobic rather than ionic interactions are of primary importance.[22] It is proposed that the temporins produce bacterial cell death by formation of transmembrane pores ("toroid pore" mechanism) rather than by causing a detergent-like disruption of the cell membrane into peptide-coated vesicles ("carpet" mechanism) (see the Cationic Antimicrobial Peptides chapter in the Bacterial/Antibiotic Peptide section). However, the possibility that at least some of these peptides could interact with intracellular targets is not excluded.

One of the most interesting and potentially important properties of selected temporins—shared with a number of other cationic antimicrobial peptides—is their affinity toward LPS, the endotoxin, responsible for the systemic inflammatory response syndrome (SIRS) and the often fatal septic shock that follow Gram-negative infections.[16,21] Temporin L penetrates efficiently into *Escherichia coli* LPS monolayers and binds with strong affinity to purified *E. coli* LPS *in vitro*, as shown by a fluorescent probe displacement assay.[15] The effect of several temporins, alone and when combined with each other, in inhibiting secretion of LPS-induced proinflammatory cytokines by LPS-activated macrophages have been examined.[21] The effect of a mixture of temporin L and temporin A or B on antiendotoxin and antibacterial activities is significantly greater than the corresponding additive effect of the single peptides, thus showing synergism. An NMR study of the interaction of temporins with LPS micelles reveals that temporins L and B are able to interact in a way that impacts the oligomerization status of peptides when bound to LPS, leading to changes in the structure of LPS itself and disruption of the outer membrane.[3] In a broader context, this contributes to the concept that the presence of multiple forms of structurally related peptides in the same frog specimen offers extended protection from infections.

BIOLOGICAL ACTIVITIES

In general, the temporins show greater potencies against Gram-positive bacteria such as *S. aureus*, *Staphylococcus epidermidis*, *Enterococcus faecalis*, and S*treptococcus* spp. than against Gram-negative bacteria.[22] The exceptions are temporin L,[20,29] temporin-CPa,[10] and temporin-DRa,[7] which bear a net positive charge of +3 and are active against clinically relevant Gram-negative species such as *E. coli* and *Pseudomonas aeruginosa* and against the opportunistic yeast pathogen *Candida albicans*. The therapeutic potential of the many of the temporins is limited by their toxicities against mammalian cells. Temporin L is strongly hemolytic against human erythrocytes and also cytolytic toward several human tumor cell lines.[29] Temporin A and B, however, have relatively weak hemolytic activity but have been shown to severely damage the plasma membrane of the human parasitic protozoan, *Leishmania donovani* leading to cell death.[23] Temporin A also shows antiviral activity, by reducing the infectivity of channel catfish virus and frog virus 3,[5] and the peptide inhibits the growth of the chytrid fungus *Batrachochytrium dendrobatidis* that has been implicated in declines in amphibian populations worldwide.[30]

The biological properties of the temporins are not confined to cidal activities. As well as their antiendotoxin properties, temporins B and L enhance the ability of secretory phospholipase A_2 from bee venom and in human lacrimal fluid to hydrolyze anionic liposomes.[35] In common with the structurally related peptides from wasp venom, temporin A and temporin-CBb (ranatuerin-6; FISAIASMLGKFL.NH_2) from *L. catesbeianus* induce migration of monocytes, neutrophils, and macrophages, both *in vitro* and *in vivo*, by a mechanism that involves interaction with the formyl peptide receptor-like 1.[4] Temporin-Gb (SILPTIVSFLSKFL.NH_2) and temporin-Gd (FILPLIASFLSKFL.NH_2), isolated from *Lithobates grylio* skin, elicit concentration-dependent relaxations of preconstricted vascular rings from the rat thoracic aorta with EC_{50} values of approximately 2 μM but the physiological significance, if any, of this observation is unclear.[19] Temporin-Vb from *Lithobates virgatipes*, -Oe from *R. ornativentris*, -DRb from *R. draytonii*, and -TGb from *R. tagoi tagoi* in the concentration range 10^{-8}–10^{-6} M, produce significant and dose-dependent stimulatory effects on insulin secretion from clonal rat BRIN-BD11 β cells without cytotoxicity.[2]

POTENTIAL CLINICAL AND COMMERCIAL APPLICATIONS

The emergence of strains of pathogenic microorganisms with resistance to commonly used antibiotics constitutes a serious threat to public health that has necessitated a search for new types of antimicrobial agents. The temporins are promising candidates for development into clinically useful anti-infectives, particularly with regard to topical applications, such as treatment of *acne vulgaris* and infected foot ulcers in diabetic patients. Several temporins show high potency *in vitro* against nosocomial Gram-positive pathogens such as MRSA[7,13] and antibiotic-resistant strains of *E. faecalis*.[14]

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The all D-isomer of temporin B is active against a range of anaerobic bacteria and is stable in a fecal milieu, suggesting the possibility of its use in the treatment of *Clostridium difficile* associated diarrhea.[24] Combination therapy with a conventional antibiotic, such as rifampicin, seems promising, as the temporin may increase the permeability of the bacterial plasma membrane facilitating the entry of the second bactericidal agent.[22] Infection of indwelling medical devices, such as central venous, urinary, and dialysis catheters and prosthetic heart valves, contributes significantly to mortality rates after surgery. In a subcutaneous pouch model in which rats are infected with drug-resistant clinical isolates of *S. epidermidis,* presoaking of a Dacron vascular graft in temporin A solution, either alone or in combination with vancomycin, markedly reduces the number of bacteria adhering to the graft.[13] Thus, temporins have potential as prophylactic agents in preventing vascular prosthetic graft infections.

The cytolytic properties of cationic α-helical peptides are determined by a complex interaction between cationicity, hydrophobicity, conformation, and amphipathicity[7,20] (Fig. 4). The dodecapeptide temporin-DRa (HFLGTLVNLAKKIL. NH_2) was first isolated from skin secretions of the California red-legged frog *R. draytonii* and shows potential for treatment of infections produced byMRSA.[7] Temporin-DRa shows high growth-inhibitory potency against clinical isolates of MRSA (MIC = 8 μM) and has the advantages of ease of synthesis and high solubility. Its therapeutic potency is limited by moderately high hemolytic activity (LC_{50} = 65 μM). However, the analog containing the amino acid substitution $Val^7 \rightarrow$ L-Lys and the analogs containing the helix-destabilizing substitutions $Thr^5 \rightarrow$ D-Lys and $Asn^8 \rightarrow$ D-Lys retain activity against MRSA (MIC in the range 8–16 μM) but have very low hemolytic activity (LC_{50} > 300 μM).[7] In addition to increasing cationicity, the substitution $Val^7 \rightarrow$ Lys decreases amphipathicity by increasing the polar angle θ (the angle subtended by the positively charged residues) from 100° to 140° thereby delocalizing the positive charge over a greater surface area of the molecule. Substitutions at Leu^9, Ile^{13}, and Leu^{14} by α-aminoisobutyric acid (Aib) produce analogs with decreased helicity and hydrophobicity that retain activity against microorganisms but show appreciably lower cytolytic activities against mammalian cells. In particular, the fourfold increase in therapeutic index [ratio of LC_{50} against erythrocytes to MIC against microorganisms] of [Aib^{13}]temporin-DRa identifies it as a compound with potential for development as a therapeutically valuable anti-infective agent.[8]

Related studies with temporin L show that the % helicity of analogs correlates directly with their hemolytic activities but not with their antimicrobial activities. The nature and position of basic residues (Lys and Arg) along the sequence significantly affects the biological properties of the analogs without changing their helicity, indicating different modes of interactions of positively charged side chains within the peptide's folded structure with anionic membranes. The analog [Pro^3,Orn^{11}]temporin L shows therapeutic potential displaying antimicrobial activity which is slightly lower than temporin L but the lowest hemolytic activity among the derivatives tested.[20]

The possibility of expressing genes that encode for an antimicrobial peptide to protect commercially important crops from bacterial and fungal diseases has excited interest within the agricultural biotechnology industry. Genetically engineered potatoes expressing the gene for temporin A have been grown successfully and are resistant to *Phytophthora infestans*, responsible for late blight, and to *Phytophthora erythroseptica*, responsible for pink rot. The transgenic tubers remained disease-free during prolonged storage.[27]

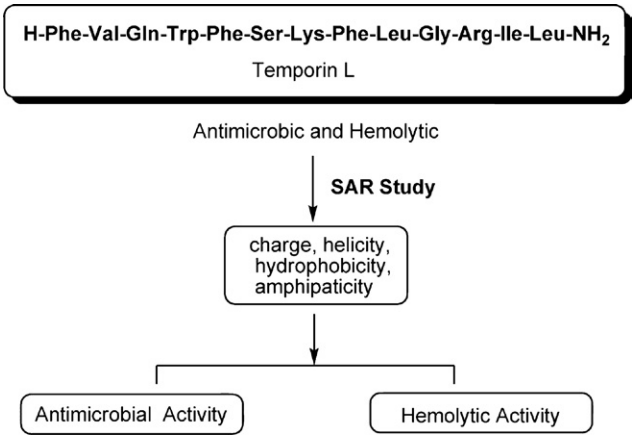

FIGURE 4 General strategy for the development of analogs of temporins (and other frog skin antimicrobial peptides) with a higher therapeutic index than the native peptide through structure-activity relationship (SAR) studies. Temporin L is presented as an example. Reproduced with permission from Ref. 20.

CONCLUSIONS

The small size and ease of synthesis of the temporins, especially temporin-L, -CPa, and -DRa that show broad-spectrum antibacterial and antifungal activities, make them promising candidates for development into therapeutically valuable anti-infective agents. The potent hemolytic activity of the naturally occurring temporins prevents their systemic use but analogs of temporin-L and -DRa have been designed that preserve high antimicrobial activity but show low cytotoxicity against mammalian cells.

REFERENCES

1. Abbassi F, Lequin O, Piesse C, Goasdoué N, Foulon T, Nicolas P, et al. Temporin-SHf, a new type of phe-rich and hydrophobic ultra-short antimicrobial peptide. *J Biol Chem* 2010;**285**:16880–92.
2. Abdel-Wahab YH, Marenah L, Flatt PR, Conlon JM. Insulin releasing properties of the temporin family of antimicrobial peptides. *Protein Pept Lett* 2007;**14**:702–7.

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3. Bhunia A, Saravanan R, Mohanram H, Mangoni ML, Bhattacharjya S. NMR structures and interactions of temporin-1Tl and temporin-1Tb with lipopolysaccharide micelles: mechanistic insights into outer membrane permeabilization and synergistic activity. *J Biol Chem* 2011;**286**:24394–406.
4. Chen Q, Wade D, Kurosaka K, Wang ZY, Oppenheim JJ, Yang D. Temporin A and related frog antimicrobial peptides use formyl peptide receptor-like 1 as a receptor to chemoattract phagocytes. *J Immunol* 2004;**173**:2652–9.
5. Chinchar VG, Bryan L, Silphadaung U, Noga E, Wade D, Rollins-Smith L. Inactivation of viruses infecting ectothermic animals by amphibian and piscine antimicrobial peptides. *Virology* 2004;**323**:268–75.
6. Conlon JM. Structural diversity and species distribution of host-defense peptides in frog skin secretions. *Cell Mol Life Sci* 2011;**68**:2303–15.
7. Conlon JM, Al-Ghaferi N, Abraham B, Leprince J. Strategies for transformation of naturally-occurring amphibian antimicrobial peptides into therapeutically valuable anti-infective agents. *Methods* 2007;**42**:349–57.
8. Conlon JM, Al-Kharrge R, Ahmed E, Raza H, Galadari S, Condamine E. Effect of aminoisobutyric acid (Aib) substitutions on the antimicrobial and cytolytic activities of the frog skin peptide, temporin-1DRa. *Peptides* 2007;**28**:2075–80.
9. Conlon JM, Kolodziejek J, Nowotny N. Antimicrobial peptides from the skins of North American frogs. *Biochim Biophys Acta* 2009;**1788**:1556–63.
10. Conlon JM, Meetani MA, Coquet L, Jouenne T, Leprince J, Vaudry H, et al. Antimicrobial peptides from the skin secretions of the New World frogs *Lithobates capito* and *Lithobates warszewitschii* (Ranidae). *Peptides* 2009;**30**:1775–81.
11. D'Abramo M, Rinaldi AC, Bozzi A, Amadei A, Mignogna G, Di Nola A, et al. Conformational behavior of temporin A and temporin L in aqueous solution: a computational/experimental study. *Biopolymers* 2006;**81**:215–24.
12. Frost DR. *Amphibian species of the world: an online reference. Version 5.5*. American Museum of Natural History. New York, USA; 2011. <http://research.amnh.org/vz/herpetology/amphibia/2011>.
13. Ghiselli R, Giacometti A, Cirioni O, Mocchegiani F, Orlando F, Kamysz W, et al. Temporin A as a prophylactic agent against methicillin sodium-susceptible and methicillin sodium-resistant *Staphylococcus epidermidis* vascular graft infection. *J Vasc Surg* 2002;**36**:1027–30.
14. Giacometti A, Cirioni O, Kamysz W, D'Amato G, Silvestri C, Del Prete MS, et al. *In vitro* activity and killing effect of temporin A on nosocomial isolates of *Enterococcus faecalis* and interactions with clinically used antibiotics. *J Antimicrob Chemother* 2005;**55**:272–4.
15. Giacometti A, Cirioni O, Ghiselli R, Mocchegiani F, Orlando F, Silvestri C, et al. Interaction of temporin L with lipopolysaccharide in vitro and in experimental rat models of septic shock caused by Gram-negative bacteria. *Antimicrob Agents Chemother* 2006;**50**:2478–86.
16. Giuliani A, Pirri G, Rinaldi AC. Antimicrobial peptides: the LPS connection. In: Giuliani A, Rinaldi AC, editors. *Antimicrobial peptides. Methods and protocols methods in molecular biology 618*. New York: Humana Press-Springer; 2010. p. 137–54.
17. Hasunuma I, Iwamuro S, Kobayashi T, Shirama K, Conlon JM, Kikuyama S. Expression of genes encoding antimicrobial peptides in the Harderian gland of the bullfrog *Lithobates catesbeianus*. *Comp Biochem Physiol C Toxicol Pharmacol* 2010;**152**:301–5.
18. Iwamuro S, Nakamura M, Ohnuma A, Conlon JM. Molecular cloning and sequence analyses of preprotemporin mRNAs containing premature stop codons from extradermal tissues of *Rana tagoi*. *Peptides* 2006;**27**:2124–8.
19. Kim JB, Halverson T, Basir YJ, Dulka J, Knoop FC, Abel PW, et al. Purification and characterization of antimicrobial and vasorelaxant peptides from skin extracts and skin secretions of the North American pig frog *Rana grylio*. *Regul Pept* 2000;**90**:53–60.
20. Mangoni ML, Carotenuto A, Auriemma L, Saviello MR, Campiglia P, Gomez-Monterrey I, et al. Structure-activity relationship, conformational and biological studies of temporin L analogues. *J Med Chem* 2011;**54**:1298–307.
21. Mangoni ML, Epand RF, Rosenfeld Y, Peleg A, Barra D, Epand RM, et al. Lipopolysaccharide, a key molecule involved in the synergism between temporins in inhibiting bacterial growth and in endotoxin neutralization. *J Biol Chem* 2008;**283**:22907–17.
22. Mangoni ML, Rinaldi AC, Di Giulio A, Mignogna G, Bozzi A, Barra D, et al. Structure–function relationships of temporins, small antimicrobial peptides from amphibian skin. *Eur J Biochem* 2000;**267**:1447–54.
23. Mangoni ML, Saugar JM, Dellisanti M, Barra D, Simmaco M, Rivas L. Temporins, small antimicrobial peptides with leishmanicidal activity. *J Biol Chem* 2005;**280**:984–90.
24. Oh H, Hedberg M, Wade D, Edlund C. Activities of synthetic hybrid peptides against anaerobic bacteria: aspects of methodology and stability. *Antimicrob Agents Chemother* 2000;**44**:68–72.
25. Ohnuma A, Conlon JM, Kawasaki H, Iwamuro S. Developmental and triiodothyronine-induced expression of genes encoding preprotemporins in the skin of Tago's brown frog *Rana tagoi*. *Gen Comp Endocrinol* 2006;**146**:242–50.
26. Ohnuma A, Conlon JM, Yamaguchi K, Kawasaki H, Coquet L, Leprince J, et al. Antimicrobial peptides from the skin of the Japanese mountain brown frog *Rana ornativentris*: evidence for polymorphism among preprotemporin mRNAs. *Peptides* 2007;**28**:524–32.
27. Osusky M, Osuska L, Hancock RE, Kay WW, Misra S. Transgenic potatoes expressing a novel cationic peptide are resistant to late blight and pink rot. *Transgenic Res* 2004;**13**:181–90.
28. Rinaldi AC, Di Giulio A, Liberi M, Gualtieri G, Oratore A, Bozzi A, et al. Effects of temporins on molecular dynamics and membrane permeabilization in lipid vesicles. *J Pept Res* 2001;**58**:213–20.
29. Rinaldi AC, Mangoni ML, Rufo A, Luzi C, Barra D, Zhao H, et al. Antimicrobial, haemolytic and cytotoxic activities, and effects on membrane permeabilization in lipid vesicles. *Biochem J* 2002;**368**:91–100.
30. Rollins-Smith LA, Conlon JM. Antimicrobial peptide defenses against chytridomycosis, an emerging infectious disease of amphibian populations. *Dev Comp Immunol* 2005;**29**:589–98.
31. Simmaco M, De Biase D, Severini C, Aita M, Erspamer GF, Barra D, et al. Purification and characterization of bioactive peptides from skin extracts of *Rana esculenta*. *Biochim Biophys Acta* 1990;**1033**:318–23.
32. Simmaco M, Mignogna G, Canofeni S, Miele R, Magoni ML, Barra D. Temporins, antimicrobial peptides from the European red frog *Rana temporaria*. *Eur J Biochem* 1996;**242**:788–92.
33. Wade D, Silberring J, Soliymani R, Heikkinen S, Kilpeläinen I, Lankinen H, et al. Antibacterial activities of temporin A analogs. *FEBS Lett* 2000;**11**(479):6–9.
34. Yashuhara T, Nakajima T, Erspamer V, Falconieri-Erspamer G, Tukamoto Y, Mori M. Isolation and sequential analysis of peptides in *Rana erythraea* skin. In: Kiso Y, editor. *Peptide chemistry 1985*. Osaka: Protein Research Foundation; 1986. p. 363–8.
35. Zhao H, Kinnunen PK. Modulation of the activity of secretory phospholipase A2 by antimicrobial peptides. *Antimicrob Agents Chemother* 2003;**47**:965–71.

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Chapter 57

Animal Toxins

Jean-Marc Sabatier and Michel De Waard

ABSTRACT

Animal toxins are renowned for their ability to regulate ion channel activity and hence cell function. Therefore, they have been investigated as lead compounds for the development of analogs with toxic activity or conversely with therapeutic potential. Besides the pharmacological usefulness of animal toxins, there is a second emerging field in which toxins may soon reveal all their potential. The advent of modern biology witnesses the development of novel biotechnological applications of interest for which toxins present tremendous advantages. In this chapter, we illustrate how toxins can be derived to carry on novel functions that make them become tools of choice for diagnostic, imaging, and therapeutic applications. Among the examples developed, the case of maurocalcine will be detailed. This toxin, discovered for its activity on the ryanodine receptor, a calcium channel, is now entering a new phase of development for *in vivo* drug or imaging agent cell delivery.

ANIMAL TOXINS AS CANDIDATE DRUGS

Animal venoms represent an extremely rich source of pharmacological peptides. These peptides are generally of a length comprising between 10 and 70 amino acids and come with a variety of folds. Proteomic profiling of animal venoms with MALDI-TOF mass spectrometry techniques is increasingly used and provides important information regarding peptide mass, disulfide bridging, and minimum number of toxins within given venom. According to many pioneering studies, venoms should contain no fewer than 100 peptides on average. More precise information on the identity of the peptides can be gained when proteomic analyses are combined to transcriptomic studies based on the isolation of venom gland mRNA. In one such study, it has been estimated that spider venoms may well contain 1,000 different peptides.[9] Of course, many of these peptides could differ by simply differences in posttranslational modifications, but these numbers remain representative of the important biodiversity encountered in various species. Considering for instance that there are about 80,000 spider species and assuming the idea that one spider venom contains not <200 unique peptides, then one may estimate the biodiversity in peptides to close to 16 millions. Adding to this diversity, the ones encountered in cone snails, scorpions, and snakes, to name a few, provide an estimate of the richness of these natural biolibraries.

Besides the numbers in venom peptides, these are three advantages to consider when deriving a drug from a natural toxin. First, the natural biolibraries made by animal venoms contain exclusively bioactive drugs in contrast to libraries of drugs of most pharmaceutical industries. Peptide toxins are tailored for efficient bioactivity *in vivo* by targeting crucial receptors (generally cell surface ion channels and G protein-coupled receptors). Although some toxins are evidently toxic *in vivo*, many others, present in the same venom, may have beneficial effects. Second, venoms are generally injected subcutaneously and reach the blood stream for efficient dissemination *in vivo*. Some deleterious effects can take hours to come into play showing an encouraging half-life of the active peptides *in vivo*. Third, gene duplication, extensive splicing, and posttranslational modifications have ensured that venoms contain toxins with exquisite affinity and selectivity for their pharmacological targets. There is therefore no doubt whatsoever that animal venom constitute an infinite source of biologically active peptides of pharmaceutical interest and therefore an excellent basis for screening programs. Although the initial motivation in studying animal venoms was to identify active principles (at best) or active HPLC fractions (at worst) involved in toxicity, combined with the production of therapeutic sera for treating envenoming, current efforts are also now devoted to identifying pharmacological drugs for therapeutics or pesticides for industrial use. Although identifying drugs of interest from animal venom is technically eased by combined proteomic and transcriptomic approaches, once a peptide of interest has been isolated, one also needs to overcome the difficulties inherent to its synthetic production. This can be performed by using

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00057-9
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recombinant techniques or by chemical synthesis, but this task is not always straightforward as the peptides need to undergo oxidative folding, a complex process in which the peptide acquires the correct disulfide bridge pairing pattern and adopts the right fold. Once this task is mastered however, access is gained to the possibility to create a large number of peptide analogs, overcoming the tedious task of toxin purification from natural sources that always come with limited availability and quantity. Analogs can be worked out to optimize certain characteristics, including greater affinity for a given target, improved selectivity or enhanced potency. In addition, the increasing development of bioinformatics has steadily improved the functional classification of the growing list of orphan toxins and their potential use in pharmacological applications.

With these considerations in mind, it is therefore not so surprising that several peptides, originating from animal venoms, made their way to the clinic and the drug market. Figure 1 illustrates a nonexhaustive list of peptides or analogs thereof that are in various clinical phases or already on the market. For instance, several *Conus* peptides, present in predatory cone snails, are now used for medication in clinics for pain (Ziconotide, the synthetic form of ω-conotoxin MVIIA, a Ca^{2+} channel N-type blocker, from Elan Pharmaceuticals), epilepsy, and other neuropathic disorders.[34] Natural peptides are therefore increasing the list of peptides (438 in total in 2009) being considered by the pharmaceutical industry in their development programs. Seventy-two of these 438 peptides reached phase III clinical trial and 48 were on the market. Four of them reached global sales over 500 millions $ each in 2007: Copaxane ($3.33 billion), Lupron ($1.88 billion), Byetta ($967 million), and Forteo ($709 million). The majority of these peptides target G protein–coupled receptors, although other targets are increasingly common such as ion channels. A complete report on the development of peptides as therapeutic drugs is available from http://www.peptidetherapeutics.org. Although the pharmacological potential of animal toxins is progressively unraveled and that exploitation of the natural resources for therapeutic aims is only in its infancy, this trend should not hide the fact that toxins are also increasingly used in biotechnological applications.

ANIMAL TOXINS IN BIOTECHNOLOGICAL APPLICATIONS

In 2004, the group of Wonnacott showed that the subcellular distribution of alpha7 nicotinic acetylcholine receptors can be investigated by coupling alpha bungarotoxin to gold nanoparticles.[13] In that respect, toxins seem as perfect tools to target anticancer agents directly to the site of tumor *in vivo* as far their targets are overexpressed in tumors. For instance, the G protein–coupled receptors (BBR1, BBR2, and BBR3) of bombesin, a 14 amino acid peptide from frog skin, are overexpressed in small cell carcinoma of lung, gastric cancer, neuroblastoma,[21] and human prostate cancer. This property has been used to prepare bombesin derivatives harboring lutetium-1777 for prostate cancer targeting, *in vivo* imaging and therapeutic intervention.[24] Another successful example for tumor applications includes chlorotoxin,[16,29] a 36-mer peptide with four disulfide bridges initially isolated from the venom of the Israeli scorpion *Leiurus quinquestriatus*. Although initially developed for the diagnosis and treatment of glioma, chlorotoxin specifically labels cancer cells from other solid tumors as well (melanoma, small cell lung carcinoma, neuroblastoma, medulloblastoma, Ewing's sarcoma, and pheochromacytoma). The identity of the biomarker on which it binds is still under debate (initially a chloride channel, then matrix metalloprotein 2, and now, which seems more probable, annexin 2A). Currently, a ^{131}iodinated version of the toxin from Eisai (TM601) has successfully ended clinical phase II for the treatment of recurrent glioma and has obtained FDA approval to go to phase III clinical trials. Besides, it has also obtained FDA approval to investigate the effect of TM601 on newly occurring glioma. TM601 is extremely stable, presents no immunogenicity and produces no toxicity in humans. Several

Name	Indication	Phase	Company
Prialt (ω-conotoxin MVIIA)	Pain	Market	Azur Pharma
Byetta (GLP 1)	Diabetes 2	Market	Eli Lilly
Capoten (mimetic)	Hypertension	Market	Bristol-Myers Squibb
Integrilin (mimetic)	Acute coronary syndrome	Market	Schering-Plough
Aggrastat (mimetic)	Acute coronary syndrome	Market	Merck
Chlorotoxin (TMI601)	Metastic tumor, glioma, solid tumor (prostate, lung, etc.)	Clinic III	Eisai
Viprinex (mimetic)	Ischemic stroke	Clinic III	Neurobiological Industries Inc.
Xen2174	Chronic pain/post operative pain	Clinic IIb	Xenome
OSK-1	Autoimmune disorders	Pre-clinical	Amgen
ShK	Autoimmune disorders	Pre-clinical	Airmid
Exanta (mimetic)	Atrial fibrillation	Withdrawal	Astra Zeneca

FIGURE 1 Examples of toxins or analogs thereof which are in various clinical phases. Notice the diversity of indications.

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derivative molecules (termed TM602, TM604, etc.) have been produced to facilitate phenotyping and histological staining, and patient treatment. The door is now open for the use of other toxins in cancer diagnosis and treatment. BmKCT, which presents 68% amino acid sequence identity with chlorotoxin, also targets glioma *in vivo* and prevent its progression. Similarly, the nontoxic B subunit of the pathogen-produced Shiga toxin, known to bind to the glycosphingolipid Gb3 which is overexpressed in some tumors, specifically labels human colorectal carcinoma in nude mice.[33] Other technological applications are possible with toxins. The 12 amino acid peptide Tet1, derived from tetanus toxin, is an efficient vector for the delivery of plasmid DNA in complex to polyethylenimine.[22] A similar fragment of tetanus toxin, when placed in fusion to the reporter protein GFP, allows mapping of synaptic connections of the mammalian central nervous system.[17]

Most animal toxins hit cell surface receptors. This is by far the most straightforward means to interfere with signaling pathways for peptides as these molecular entities are sometimes considered unable to cross the cell plasma membrane. There are many exceptions to this belief, as some animal toxins target intracellular ion channel receptors. This occurs with toxins targeting the ryanodine receptor, an intracellular calcium channel that is located in the membrane of the endoplasmic reticulum and that controls cytosolic Ca^{2+} release. These toxins present intriguing peptide sequences that efficiently favor their entry into the cytoplasm. Ion channel recognition and cell permeation can easily be dissociated to retain the latter property. Also, cell penetration can be derived for the cell entry of other compounds of pharmaceutical or imaging interests. We illustrate this point extensively by reviewing the case of maurocalcine, the first cell-penetrating peptide of toxin origin to be discovered.

MAUROCALCINE, AN ANIMAL TOXIN THAT TARGETS AN INTRACELLULAR RECEPTOR

Maurocalcine is a scorpion toxin from *Scorpio maurus palmatus* that was discovered in 2000.[19] Based on its sequence homology with imperatoxin A from the scorpion *Pandinus imperator*, it was presumed to be active on the ryanodine receptor. This turned out to be indeed the case as maurocalcine (i) triggers Ca^{2+} release from purified sarcoplasmic reticulum,[12] (ii) induces both an increase in channel opening and the appearance of a long-lasting subconductance state,[7] and (iii) stimulates the binding of [^{3}H]-ryanodine on its binding site.[11] All these effects occur with an EC_{50} of 10–20 nM. Maurocalcine interaction with the ryanodine receptor is direct as shown by skeletal muscle ryanodine receptor pulldown by a biotinylated derivative of maurocalcine bound to streptavidin-coated beads.[1] Maurocalcine obviously belongs to a larger family of toxins with high sequence homology comprising opicalcine 1 and 2 from *Opistophthalmus carinatus* scorpion, hemicalcin from *Hemiscorpius lepturus* scorpion and hadrucalcin from *Hadrurus gertschi* scorpion (Figure 2). All these toxins similarly act on the ryanodine receptor with only minor functional differences. At the structural level, maurocalcine is a 33-mer peptide containing three disulfide bridges. The pairing motif has been assigned to Cys^1-Cys^4, Cys^2–Cys^5 and Cys^3–Cys^6 and the peptide folds according to an inhibitor cystine knot (ICK motif). Although ICK motifs have been found on other toxins acting on cell surface receptors, maurocalcine and its analogs all present interesting distinguishing features. A close examination of the 3D structure of maurocalcine reveals that (i) the peptide is heavily charged (one-third of amino acids are basic, net charge of +12) and (ii) the charge distribution of the peptide is strongly asymmetric creating an important dipole moment. One of the most amazing properties of the peptide is that on external application to skeletal muscle myotubes, the peptide triggers Ca^{2+} release from internal stores within only a few seconds.[11] A similar observation was made in 2010 on cardiac myotubes with imperatoxin A. This observation points to the fact that the peptide is able to reach its pharmacological target by rapid translocation through the plasma membrane rather than by endocytosis. Also, two pieces of evidence indicate that maurocalcine should accumulate into the cytoplasm to regulate the ryanodine receptor. First, maurocalcine regulates channel activity

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                                                          Score (%)

Imperatoxin A  --GDCLPHLKRCKADNDCCGKKCKRRGTNAEKRCR 33     81.0
Hemicalcin     --GDCLPHLKLCKADKDCCSKKCKRRGTNPEKRCR 33     90.0
Maurocalcine   --GDCLPHLKLCKENKDCCSKKCKRRGTNIEKRCR 33     -
Opicalcine 1   --GDCLPHLKRCKENNDCCSKKCKRRGTNPEKRCR 33     90.0
Opicalcine 2   --GDCLPHLKRCKENNDCCSKKCKRRGANPEKRCR 33     87.0
Hadrucalcine   SEKDCIKHLQRCRENKDCCSKKCSRRGTNPEKRCR 35     75.0
                  **: **: *: ::***.***.***:* *****
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FIGURE 2 Sequence alignment of calcin toxins according to ClustalW2. Negatively charged residues are in blue, while positively charged amino acids are in purple. Sequence identities are given with maurocalcine as reference entry.

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from ryanodine receptors incorporated into artificial lipid bilayers only when applied to the cytoplasmic face of the channel.[12] Second, the maurocalcine binding site on the ryanodine receptor has been mapped to a domain that is localized within the cytoplasm according to the membrane topology of this receptor.[1]

Altogether, these findings led to the conclusion that maurocalcine rapidly crosses the plasma membrane and accumulates within the cytoplasm at concentrations >20 nM to effectively activate the ryanodine receptor. The possibility of indirectly imaging the cell penetration of maurocalcine by the release of internal Ca^{2+} is unique to the peptide world. Cell penetration of maurocalcine is not only limited to itself but also to cargoes attached to the peptide underscoring the huge technological potential of this uncommon toxin.

MAUROCALCINE IS A COMPETITIVE CELL-PENETRATING PEPTIDE

Investigation of the cell penetration properties of a peptide without labeling it is a tedious task. Therefore, the first evidence that maurocalcine may enter cells came from the use of a biotinylated analog. This analog coupled to fluorescent streptavidine enters into a variety of cell types.[10] This shows not only that maurocalcine enters into cells but also that it can be used as a peptide vector to facilitate the cell entry of a variety of cargoes, many of them being of a size in considerable excess than the vector itself. It was also the first report that a peptide toxin being a member of the growing family of cell-penetrating peptides (CPP). As expected, imperatoxin A was also shown later on to cross the plasma membrane along with a cargo. In parallel, crotamine, a 42 amino acid toxin from the rattlesnake *Crotalus durissus terrificus*, was shown to penetrate into dividing cells and to carry on plasmid DNA.[20] Reputed CPPs include Tat from the HIV-1 virus, the *Drosophila* transcription factor ANTP (encoded by the *antennapedia* gene), also called penetratin, and the herpes simplex virus type 1 (HSV-1) VP22 transcription vector. These peptides possess a number of common functional features in spite of impressive sequence divergences:

1. CPPs are generally small peptides that rarely exceed 20 amino acids. However, this issue is not mandatory because many CPP originate from larger proteins. The relationship between CPP size and cargo penetration efficacy is not known.
2. Many CPPs are enriched in basic amino acids. This led to the discovery that arginine-rich peptides are efficient CPPs.
3. CPPs lack clear cell selectivity although this issue is not well investigated. They can thus enter numerous cell types.
4. CPPs do not require specific membrane receptors for cell penetration. Indeed, optical CPP stereoisomers, made of D-amino acids instead of the natural L-amino acids, are at least as efficient as their L-counterparts in crossing the plasma membrane.[23] Nevertheless, CPPs do interact with cell surface components such as glycoaminoglycans (GAGs) and negatively charged lipids on the basis of electrostatic interactions.[8]
5. CPPs enter rapidly and efficiently into cells. Commonly, cell penetration occurs within a few minutes, although quantitatively saturation may take longer times. An important property hardly matched by other vectors is that CPPs can penetrate into 100% of a given cell type *in vitro*. We will see that *in vivo* this might not be the case.
6. True CPPs do not require metabolic energy for cell entry.[31] CCP cell entry should be preserved in cells maintained at 4°C or in the presence of metabolic inhibitors. This is however not always valid because for many peptides and depending of the cargo being transported, CPPs enter via endocytosis, which is an energy-consuming process.
7. CPPs trigger much interest for their ability to transport a great variety of cell-impermeable cargoes. The type of cargoes that have been coupled to CPPs and shown to enter into cells is astounding. So far, cell entry has been reported for DNA plasmids or mimics,[18] oligonucleotides, siRNA or shRNA,[14] peptide nucleic acids (PNA),[35] peptides, proteins,[5] drugs,[28] and nanoparticles.[32] The versatility in cargo that can be coupled to a given CPP opens an unprecedented number of applications that can't be matched by any other cell delivery system.

The discovery of new CPP sequences is steadily increasing, and there is enough choice now on the market to develop an application without worrying about the nature of the CPP. Why then would it be interesting to use maurocalcine as a CPP considering the fact that it is more complex (presence of disulfide bridges and difficulties for cargo grafting) and expensive (size) to synthesize than other CPPs? We summarize hereunder the advantage that maurocalcine has over other CPPs.

THE PHARMACOLOGICAL ACTIVITY OF MAUROCALCINE IS EASY TO NEUTRALIZE

Maurocalcine is notorious for its effects on the ryanodine receptor. Ca^{2+} release from the endoplasmic reticulum is a major signaling event that precludes the use of the original maurocalcine sequence as vector for the cell penetration of compounds of interest. Strategies had to be investigated to circumvent the bioactivity of the peptide. Four strategies turned out to be successful in that respect. They are all based on the observation that cell penetration of the peptide requires

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less stringent structural integrity than pharmacology. First, the peptide can be mutated to lose its ability to bind and stimulate the ryanodine receptor. Arg^{23} and Arg^{24} turn out to be two positions that are critical for maurocalcine activity. Analogs mutated on any one of these positions yield efficient CPP devoid of pharmacological activity.[15] The alanine scan of maurocalcine also reveals other interesting amino acid positions where mutations can improve the pharmacology and/or the cell penetration. Mutation of Glu^{12} is particularly interesting in that respect. Second, maurocalcine can be produced in its D-diastereomer conformation with D-amino acids rather than L-amino acids. Interestingly, the peptide folds normally and produces its normal disulfide bridging pattern. It is also lacking recognition of the ryanodine receptor but, as expected, preserves intact its cell penetration efficacy because its affinity for the plasma membrane is not based on a receptor/ligand type interaction.[23] Obviously, this peptide is a very interesting lead compound because it has all the desired advantages as a CPP (protease resistance and efficacy). In the future, mutated D-maurocalcine peptides will be designed that further enhance the efficacy of cell penetration. Third, maurocalcine can be mutated in such a way that it lacks disulfide bridges. Disulfide bridging contributes to the acquisition of the 3D structure of maurocalcine. Therefore, a disulfide-less peptide is unable to fold and loses its ability to recognize the ryanodine receptor.[27] Nevertheless, this disulfide-less maurocalcine preserves good cell penetration properties albeit with a reduced efficacy. This altered potency can nevertheless be partially overcome by point mutations in the amino acid sequence. Needless to say, a similar disulfide-less maurocalcine can be prepared in the future by incorporating D-amino acids that may render it protease resistant and increase the half-life *in vivo*. Since in its nonfolded version, maurocalcine still acts as a CPP, our group is presently designing a number of mini-maurocalcine analogs with reduced peptide length. The preliminary results show that several analogs can be designed with significantly reduced lengths (9 amino acids). The completion of these four strategies has led to three benefits: (i) the in depth understanding of the structural determinants of maurocalcine that preside to pharmacology and cell penetration, (ii) the identification of essential factors that contribute to an efficient cell penetration of maurocalcine, and (iii) the production of several new maurocalcine analogs dedicated only to cell penetration.

MAUROCALCINE PRODUCES CELL DELIVERY INTO THE CYTOPLASM

The list of discovered CPPs is getting impressive. However, the definition of a CPP remains too tolerant. A peptide is considered as a CPP if it enters into cells. The problem is that any kind of peptide that binds to the external face of the plasma membrane is susceptible to enter into the cell merely by a process of endocytosis. Once this has occurred, organizing the escape of this peptide from the endosome is far from an easy endeavor. For most applications however, the delivery of the cargo in the cytoplasm is simply mandatory. In this registry, maurocalcine seems to be a particularly competitive peptide. The native peptide (unmodified and uncoupled to any cargo) presents the following distinguishing features: (i) the ability to follow its cytoplasmic accumulation on line (by the release of internal Ca^{2+}), (ii) a minimal cytoplasmic accumulation of 10–20 nM (to observe Ca^{2+} release), and (iii) a cytoplasmic accumulation against the concentration gradient (owing to the net positive charge of the peptide). The natural tendency of maurocalcine to accumulate into the cytoplasm ensures high cytoplasmic concentrations of the peptide. This tendency is greatly helped by its structural characteristics. The predominant orientation of basic residues on one face of the peptide, the presence of a dipole moment and the existence of a hydrophobic face ensure that maurocalcine can accumulate against its concentration gradient by electrochemical attraction in normally polarized cells. This would not be feasible if the peptide first accumulated within endosomes as the cytoplasmic accumulation factor would be kinetically limited by the endocytosis process itself and second by the endosomal escape. This being said, it would be inexact to state that maurocalcine does not also enter into cells by endocytosis. In fact, this is even the preferred route of entry when maurocalcine or one of its analogs is coupled to some cargoes. Macropinocytosis is the main route of entry with streptavidine as cargo[10] or when maurocalcine is coupled to nanoparticles covered with streptavidine molecules.[26] The nature and (or) size of the cargo therefore are susceptible to inhibit the normal balance in cell entry modes (translocation *versus* endocytosis) probably by increasing the residency time of the peptide at or within the plasma membrane. Obviously, smaller cargoes still allow a normal translocation process to occur as witnessed in the case of a fluorescent indicator[23] or a small drug such as doxorubicin.[4] This is also the case for a maurocalcine-maleimide-cys-nanoparticle complex, suggesting that in nanotechnology, size is not necessarily a limitation for cytoplasmic targeting.[27] In conclusion, although it may seem desirable to get rid of the pharmacological activity of maurocalcine, it may in the first instance represent an asset when it comes to evaluating the correct cytoplasmic targeting of the cargo transported. This is rendered possible since it was observed that N-terminal modification of the native maurocalcine (e.g., biotinylation and coupling to streptavidine) does not change dramatically its ability to interact with the ryanodine receptor.[10] Upon completion of this evaluation, it is then easy to turn toward maurocalcine analogs that structurally resemble maurocalcine itself but are mutated for their interaction with the ryanodine receptor.

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MAUROCALCINE HAS AN EXCELLENT CELL ENTRY/TOXICITY RATIO

A drawback of many CPPs is their relative toxicity. This would not be a major problem if these CPPs entered into cells at doses much lower than their toxic concentration. Unfortunately, the concentration ratio between cell penetration efficiency and cell toxicity is often not that encouraging. For reasons that are unknown, maurocalcine is not toxic on intravenous injection in spite of its pharmacological activity. This is not the case for intracerebroventricular injections in mice. However, this issue is easily circumvented with the numerous pharmacologically inert maurocalcine analogs that are now available. These analogs have all been assessed for cell toxicity most often for long periods of time *in vitro* (24 hours) and on sensitive cells (neurons). It was systematically observed that there was an almost complete lack of toxicity at concentrations as high as 10 μM. By contrast, some efficient analogs show significant levels of cell penetration at 10 nM, suggesting that reaching a concentration ratio penetration/toxicity of 1,000 for maurocalcine analogs is feasible. Of course, these values are relevant for the maurocalcine vectors but ought to be reconsidered upon grafting a cargo on them. This favorable ratio stems from the observation that maurocalcine has been optimized by nature to invade cells with a minimal disturbance, which is not necessarily the case of other CPPs that originate most often as fragments of larger non–cell-penetrating proteins or are designed *de novo*. One additional feature that needs to be emphasized is the excellent concentration dependence of maurocalcine's cell penetration. In its folded conformation, the peptide enters at lower concentrations than the popular CPP (penetratin, Tat or poly-R). Further, optimizing these values seems as a reachable objective in spite of the fact that the cell membrane factors that contribute to the efficacy of cell penetration of CPP are ill defined. In an attempt to define these factors, it was found that maurocalcine binds with micromolar affinity onto cell surface glycoaminoglycans (GAG).[25] Interaction was evident with heparin, heparin sulfate, and chondroitin sulfate. Soluble GAG inhibits close to 80% of maurocalcine cell uptake because it screens maurocalcine positive charges required for cell entry and competes for its interaction with cell surface GAG. However, maurocalcine's cell penetration is well conserved (close to 50%) in GAG-deficient cells indicating that GAG mainly contributes to cell penetration at the quantitative level by acting as low-affinity reservoirs of CPPs. More importantly, maurocalcine interacts with several negatively charged lipids at suprananomolar affinities.[6] Maurocalcine interacts with gangliosides, such as GD3 (disialoganglioside NeuAcα2-8NeuAcα2-3 Galβ1-4Glcβ1-Cer), phosphatidylinositol (PtdIns)(3)P, PtdIns(4)P, and PtdIns(5)P), phosphatidic acid and sulfatide, and more weakly with lysophosphatidic acid, PtdIns(3,5)P2 and phosphatidylserine. It is difficult to envision that lipids are not directly concerned with the mechanism of membrane translocation of CPPs, while on the contrary GAG could be involved in endocytosis. The mutant maurocalcine analog Ala^{12} has improved cell penetration and lipid interaction. Conversely, the mutant analog Lys^{20} that has poor cell penetration has also limited lipid interaction. The problem is to identify which lipid is the most important for membrane translocation. Solving this question would tremendously help the design of better CPPs.

MAUROCALCINE IS A STABLE CPP VECTOR *IN VIVO*

Venom peptides have been designed by nature to be stable upon injection *in vivo*. The disulfide bridging and the compact fold is most likely also an advantage in that respect. We have investigated this question in the case of maurocalcine. A first analog was synthesized, tyr-maurocalcine, and iodinated with ^{125}I on the extra N-terminal tyrosine residue. Next, the peptide was incubated with mouse blood and its stability followed with time by HPLC. As shown in Figure 3, the folded maurocalcine analog with its disulfide bridges has excellent blood stability because one-third of the peptide is still not degraded after 24 hrs of incubation. A single metabolite is evident suggesting that maurocalcine can further be optimized in terms of *in vivo* stability. The same type of experiment was conducted with a similar analog but in which all cysteine residues were replaced by isosteric 2-aminobutyric acid residues. Such a peptide lacks disulfide bridges and also secondary structures. In that case, we found that the peptide has a two-fold reduced stability and also that more metabolites are present as well (not shown). This study illustrates that disulfide bridges bring in a significant competitive advantage in terms of peptide stability *in vivo*. For instance, Tat, a CPP that lacks disulfide bridges, degrades rapidly even in the extracellular culture medium of epithelial cells.

MAUROCALCINE PREFERENTIALLY TARGETS SOME CELL TYPES AND ORGANS *IN VIVO*

Although it is true that CPPs penetrate into all kinds of cell types, emerging evidence suggests that there are cell-specific differences in the efficiency of the penetration. We found for instance that maurocalcine penetrates better into glial cells than in neurons. Also, the snake venom CPP crotamine presents selective cell penetration into actively dividing cells. A close examination of FAM-D-maurocalcine distribution in human blood cells indicates that monocytes and lymphocytes NK are almost all invaded by maurocalcine, whereas a small

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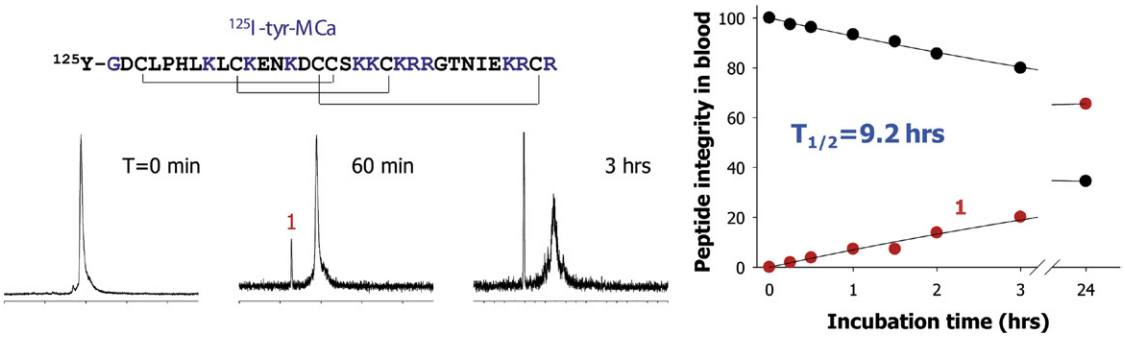

FIGURE 3 HPLC elution profile of iodinated Tyr-maurocalcine analog as a function of time of incubation in mice blood. The peptide was added at a concentration of 1mCi per milliliter of blood and incubated at 37°C. HPLC profile is from the protein-free plasma fraction. The half-life of maurocalcine exceeds 9 hours in these conditions.

percentage (<10%) of lymphocytes T and B take up maurocalcine. The reasons behind a selective cell-type cell penetration are unclear. One may assume that because CPPs have the ability to interact with different glycosaminoglycan species, changes in glycoaminoglycan patterns at the cell surface may explain differences in CPP cell selectivity.[8] It illustrates that CPP sequences may evolve in such a way to penetrate only into desired cell targets. After intravenous injection, 30% of iodinated Tyr-maurocalcine is associated to circulating cells, whereas 40% and 26% are associated to plasma and protein-free plasma, respectively. Combined nanoSPECT/CT imaging gives an indication of where the toxin accumulates *in vivo* (Figure 4). Additional organ quantification of the radioactively labeled peptide shows strong kidney accumulation and urine elimination. Other organs that light up quite well are stomach, spleen, skin, lung, liver, heart, and duodenum. No brain accumulation is evident even at longer periods of observation suggesting that the peptide does not cross the blood–brain barrier. These first observations give a hint to what organs would be best fitted for a therapy based on the use of maurocalcine sequence. It will be interesting to observe if cell and tissue selectivity can be altered by selective point mutations in maurocalcine sequence. If this could work, the door would be open for tissue-targeting cell-penetrating peptides for enhanced therapeutic potential.

CONCLUDING REMARKS

The existence of natural structural analogs of maurocalcine opens the route to new research programs aimed at discovering novel CPP molecules in the incredible rich sources of peptides that are venoms. The added benefit of increasing the number of known toxin-derived CPP is in their potential cell- and organ-specific targeting properties and in their usefulness for *in vivo* applications. Several discovery strategies may be pursued: (i) determining novel toxins active on intracellular ion channels by affinity column purification, (ii) screening for peptides that can enter into cells, and (iii) purifying peptides from venoms based on their basic or aliphatic nature. As for maurocalcine, these peptides will need optimization to remove any potential toxic or undesirable pharmacological effect. On the therapeutic side, what applications are best suited for maurocalcine or toxin-derived CPPs? The field of CPPs is blossoming with interesting and innovative applications at an exponential growth rate. Although most applications have focused on the cell entry of cargoes that are unable to enter into cells, there is also room for applications in which the CPP contributes not to the entry of a drug but to force it to stay into cells by limiting its passive diffusion or expulsion by multidrug resistance proteins for instance. Two such applications have been designed for maurocalcine. In one application, coupling maurocalcine to near-infrared emitting nanoparticles charged with MRI-detectable contrast agents allows for greater tissue retention time of the MRI signal in brain at the injection point, at least in much better proportions than the clinically used DOTAREM.[30] This kind of approach holds promises for theragnostic applications in which the therapeutic drug is combined to the imaging agent. In a second application, it was found that coupling maurocalcine to an antitumor agent, such as doxorubicin, a drug used for the treatment of solid tumors in clinics, finds useful applications for fighting chemoresistance.[2–4] Chemoresistance often occurs as the result of cancer cells that start overexpressing multidrug resistance proteins. Freely entering doxorubicin is readily expulsed by these cells, and tumors can then continue propagating. Increasing the therapeutic doses is not an option because of the secondary effects on healthy tissues, including neuropathies and cardiotoxicity. The coupling to maurocalcine turns a viable option, at least *in vitro* for the moment, as multidrug resistance proteins are no longer able to take into charge the complex molecule that keeps its antitumor activity. Coupled to cell-penetrating peptides that would be able to target cancer cells *in vivo*, such an application would hold great promises in the future. Now that maurocalcine is a proven and efficient CPP, it is on the track for biological, diagnostic, and technological applications.

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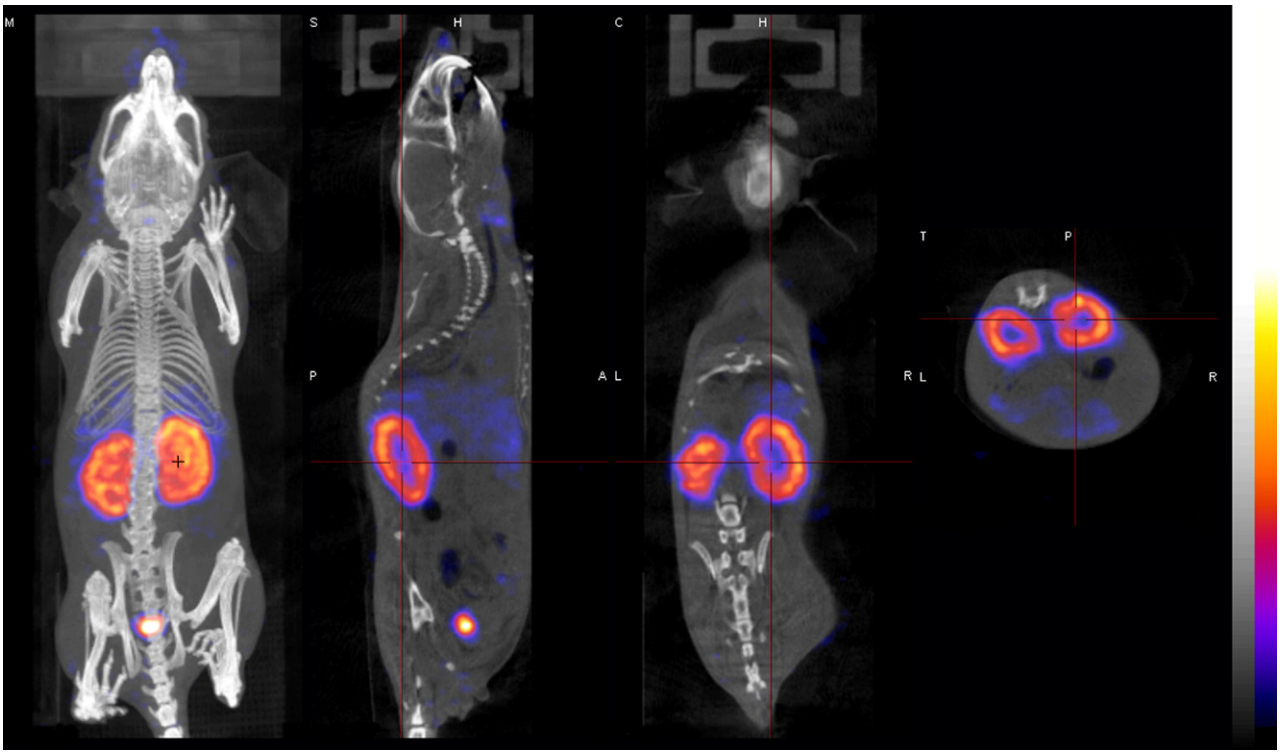

FIGURE 4 *In vivo* tomographic whole-body imaging of [^{125}I]-Tyr-maurocalcine biodistribution in CD-1 mice 15 min tail i.v. post-injection of 37 MBq. From the left to the right, 3D rendering, sagittal, coronal, and transverse views of tracer activity. See color plate 16.

REFERENCES

1. Altafaj X, Cheng W, Esteve E, Urbani J, Grunwald D, Sabatier JM, et al. Maurocalcine and domain A of the II-III loop of the dihydropyridine receptor Cav 1.1 subunit share common binding sites on the skeletal ryanodine receptor. *J Biol Chem* 2005;**280**:4013–6.
2. Aroui S, Brahim S, De Waard M, Breard J, Kenani A. Efficient induction of apoptosis by doxorubicin coupled to cell-penetrating peptides compared to unconjugated doxorubicin in the human breast cancer cell line MDA-MB 231. *Cancer Lett* 2009;**285**:28–38.
3. Aroui S, Brahim S, Hamelin J, De Waard M, Breard J, Kenani A. Conjugation of doxorubicin to cell penetrating peptides sensitizes human breast MDA-MB 231 cancer cells to endogenous TRAIL-induced apoptosis. *Apoptosis* 2009;**14**:1352–65.
4. Aroui S, Ram N, Appaix F, Ronjat M, Kenani A, Pirollet F, et al. Maurocalcine as a non toxic drug carrier overcomes doxorubicin resistance in the cancer cell line MDA-MB 231. *Pharm Res* 2009;**26**:836–45.
5. Bleifuss E, Kammertoens T, Hutloff A, Quarcoo D, Dorner M, Straub P, et al. The translocation motif of hepatitis B virus improves protein vaccination. *Cell Mol Life Sci* 2006;**63**:627–35.
6. Boisseau S, Mabrouk K, Ram N, Garmy N, Collin V, Tadmouri A, et al. Cell penetration properties of maurocalcine, a natural venom peptide active on the intracellular ryanodine receptor. *Biochim Biophys Acta* 2006;**1758**:308–19.
7. Chen L, Esteve E, Sabatier JM, Ronjat M, De Waard M, Allen PD, et al. Maurocalcine and peptide A stabilize distinct subconductance states of ryanodine receptor type 1, revealing a proportional gating mechanism. *J Biol Chem* 2003;**278**:16095–106.
8. Console S, Marty C, Garcia-Echeverria C, Schwendener R, Ballmer-Hofer K. Antennapedia and HIV transactivator of transcription (TAT) "protein transduction domains" promote endocytosis of high molecular weight cargo upon binding to cell surface glycosaminoglycans. *J Biol Chem* 2003;**278**:35109–14.
9. Escoubas P, Sollod B, King GF. Venom landscapes: mining the complexity of spider venoms via a combined cDNA and mass spectrometric approach. *Toxicon* 2006;**47**:650–63.
10. Esteve E, Mabrouk K, Dupuis A, Smida-Rezgui S, Altafaj X, Grunwald D, et al. Transduction of the scorpion toxin maurocalcine into cells. Evidence that the toxin crosses the plasma membrane. *J Biol Chem* 2005;**280**:12833–9.
11. Esteve E, Smida-Rezgui S, Sarkozi S, Szegedi C, Regaya I, Chen L, et al. Critical amino acid residues determine the binding affinity and the Ca^{2+} release efficacy of maurocalcine in skeletal muscle cells. *J Biol Chem* 2003;**278**:37822–31.
12. Fajloun Z, Kharrat R, Chen L, Lecomte C, Di Luccio E, Bichet D, et al. Chemical synthesis and characterization of maurocalcine, a scorpion toxin that activates Ca(2+) release channel/ryanodine receptors. *FEBS Lett* 2000;**469**:179–85.
13. Jones IW, Barik J, O'Neill MJ, Wonnacott S. Alpha bungarotoxin-1.4 nm gold: a novel conjugate for visualising the precise subcellular distribution of alpha 7* nicotinic acetylcholine receptors. *J Neurosci Methods* 2004;**134**:65–74.
14. Juliano RL. Intracellular delivery of oligonucleotide conjugates and dendrimer complexes. *Ann N Y Acad Sci* 2006;**1082**:18–26.
15. Mabrouk K, Ram N, Boisseau S, Strappazzon F, Rehaim A, Sadoul R, et al. Critical amino acid residues of maurocalcine involved in pharmacology, lipid interaction and cell penetration. *Biochim Biophys Acta* 2007;**1768**:2528–40.
16. Mamelak AN, Rosenfeld S, Bucholz R, Raubitschek A, Nabors LB, Fiveash JB, et al. Phase I single-dose study of intracavitary-adminis-

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tered iodine-131-TM-601 in adults with recurrent high-grade glioma. *J Clin Oncol* 2006;**24**:3644–50.
17. Maskos U, Kissa K, St Cloment C, Brulet P. Retrograde trans-synaptic transfer of green fluorescent protein allows the genetic mapping of neuronal circuits in transgenic mice. *Proc Natl Acad Sci U S A* 2002;**99**:10120–5.
18. Morris MC, Gros E, Aldrian-Herrada G, Choob M, Archdeacon J, Heitz F, et al. A non-covalent peptide-based carrier for in vivo delivery of DNA mimics. *Nucleic Acids Res* 2007;**35**:e49.
19. Mosbah A, Kharrat R, Fajloun Z, Renisio JG, Blanc E, Sabatier JM, et al. A new fold in the scorpion toxin family, associated with an activity on a ryanodine-sensitive calcium channel. *Proteins* 2000;**40**:436–42.
20. Nascimento FD, Hayashi MA, Kerkis A, Oliveira V, Oliveira EB, Radis-Baptista G, et al. Crotamine mediates gene delivery into cells through the binding to heparan sulfate proteoglycans. *J Biol Chem* 2007;**282**:21349–60.
21. Ohlsson B, Fredang N, Axelson J. The effect of bombesin, cholecystokinin, gastrin, and their antagonists on proliferation of pancreatic cancer cell lines. *Scand J Gastroenterol* 1999;**34**:1224–9.
22. Park IK, Lasiene J, Chou SH, Horner PJ, Pun SH. Neuron-specific delivery of nucleic acids mediated by Tet1-modified poly(ethylenimine). *J Gene Med* 2007;**9**:691–702.
23. Poillot C, Dridi K, Bichraoui H, Pecher J, Alphonse S, Douzi B, et al. D-Maurocalcine, a pharmacologically inert efficient cell-penetrating peptide analogue. *J Biol Chem* 2010;**285**:34168–80.
24. Pujatti PB, Santos JS, Couto RM, Melero LT, Suzuki MF, Soares CR, et al. Novel series of (177)Lu-labeled bombesin derivatives with amino acidic spacers for selective targeting of human PC-3 prostate tumor cells. *Q J Nucl Med Mol Imaging* 2011;**55**:310–23.
25. Ram N, Aroui S, Jaumain E, Sadoul R, Mabrouk K, Ronjat M, et al. Direct peptide interaction with surface glycosaminoglycans contribute to the cell penetration of maurocalcine. *J Biol Chem* 2008;**283**:24274–84.
26. Ram N, Texier-Nogues I, Pernet-Gallay K, Poillot C, Ronjat M, Andrieux A, et al. In vitro and in vivo cell delivery of quantum dots by the cell penetrating peptide maurocalcine. *Int J Biomed Nanosci Nanotechnol* 2011;**2**:12–32.
27. Ram N, Weiss N, Texier-Nogues I, Aroui S, Andreotti N, Pirollet F, et al. Design of a disulfide-less, pharmacologically-inert and chemically-competent analog of maurocalcine for the efficient transport of impermeant compounds into cells. *J Biol Chem* 2008;**283**:27048–56.
28. Sethuraman VA, Bae YH. TAT peptide-based micelle system for potential active targeting of anti-cancer agents to acidic solid tumors. *J Control Release* 2007;**118**:216–24.
29. Soroceanu L, Gillespie Y, Khazaeli MB, Sontheimer H. Use of chlorotoxin for targeting of primary brain tumors. *Cancer Res* 1998;**58**:4871–9.
30. Stasiuk GJ, Tamang S, Imbert D, Poillot C, Giardiello M, Tisseyre C, et al. Cell-permeable Ln(III) chelate-functionalized InP quantum dots as multimodal imaging agents. *ACS Nano* 2011;**5**:8193–201.
31. Thoren PE, Persson D, Karlsson M, Norden B. The antennapedia peptide penetratin translocates across lipid bilayers—the first direct observation. *FEBS Lett* 2000;**482**:265–8.
32. Tseng YL, Liu JJ, Hong RL. Translocation of liposomes into cancer cells by cell-penetrating peptides penetratin and tat: a kinetic and efficacy study. *Mol Pharmacol* 2002;**62**:864–72.
33. Viel T, Dransart E, Nemati F, Henry E, Theze B, Decaudin D, et al. In vivo tumor targeting by the B-subunit of shiga toxin. *Mol Imaging* 2008;**7**:239–47.
34. Wang CZ, Chi CW. Conus peptides—a rich pharmaceutical treasure. *Acta Biochim Biophys Sin (Shanghai)* 2004;**36**:713–23.
35. Zielinski J, Kilk K, Peritz T, Kannanayakal T, Miyashiro KY, Eiriksdottir E, et al. In vivo identification of ribonucleoprotein-RNA interactions. *Proc Natl Acad Sci U S A* 2006;**103**:1557–62.

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Chapter 58

Hymenoptera Insect Peptides

Mario Sergio Palma

ABSTRACT

The venoms of the social Hymenoptera evolved as defensive tools to protect the colonies of these insects from the attacks of predators. Generally, they are not lethal but cause mainly inflammatory and/or immunological reactions in the victims of their stings. However, sometimes systemic effects, such as respiratory and kidney failure, may occur. The peptide components of venoms from Hymenoptera represent up to 70% of the weight of freeze-dried venoms. Most of these toxins are linear polycationic amphipatic peptides, with a high content of α-helices in their secondary structures. These peptides generally account for cell lysis, hemolysis, and antibiosis, and they sometimes promote the delivery of cellular activators/mediators through interaction with the G-protein receptor. Some of these peptides are also neurotoxins that target Na^+ and/or Ca^{2+} channels or even the nicotinic ACh receptor. This review summarizes the current knowledge about the biologically active Hymenoptera venoms.

INTRODUCTION

The dominance of insects and other Arthropods on land can be mainly attributed to the extraordinary diversity of their defense mechanisms coupled to chemical weaponry. In this regard, the development of venoms and its injection apparatus among the Insecta represent evolutionary attributes that contribute to the adaptation of insects to the many different terrestrial environments.[13] Those species that evolved in the direction of social behavior developed the formation of castes and established a hierarchical relationship among nest mates of different castes. Thus, different orders of the Insecta developed their self chemical arsenal, particularly the social Hymenoptera (bees, wasps, and ants), which evolved into their venoms and stinging apparatuses according to their biology and behavior. Social behavior is found in ants, which use their sting for defense and for killing their prey, and in some bees and wasps, which use their venoms only for defending themselves and the colony against intruders and predators of their colonies.[31] Within these insects, a particular activity is their stinging action, causing pain and local damage or even death (multiple stings) in vertebrates. These species evolved their venoms to prevent the presence of predators, keeping them far from the colonies; the venom of these species is not used to promote lethal actions but to produce mnemonical actions on the victims of their stinging resulting from the uncomfortable effects of venoms such as pain, local burning, edema, swelling, bradycardia, tachycardia, headache, and, sometimes, systemic effects such as respiratory and kidney failure.[30] The venoms of the social Hymenoptera consist of a complex mixture of proteins, peptides, and low molecular mass compounds. The enzymes are responsible for the injury caused to tissues and are frequently immunoreactive to specific IgE and are therefore involved in the allergy caused by these venoms in the victims of wasp and/or bee stings.[15,35] The most abundant components of the venoms from social wasps and bees consist of peptide toxins, which play many different functional roles during the victim's envenoming.

This chapter will summarize the current knowledge concerning the biochemical and pharmacological properties of the most representative peptide toxins from the social insects of the order Hymenoptera, which are now thoroughly characterized. It is not easy to comprehensively review the extensive literature on all the major components of these venoms in the available space. Therefore, for the interest of readers, the focus will be on the most relevant groups of biologically active peptides from Hymenoptera venoms, and the references mentioned will enable a more extensive search of the literature.

PEPTIDES FROM THE VENOMS OF SOCIAL HYMENOPTERA

The peptide components of venoms from social Hymenoptera are spread over the molar mass range of 1400–7000kDa and together compose up to 70% of the weight of freeze-dried Hymenoptera venoms.[13] Most of these peptides are polycationic, amphipatic molecules, presenting a high content of α-helices in their secondary structures; these peptides

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00058-0
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generally account for cell lysis, hemolysis, and antibiosis, and they sometimes promote the delivery of cellular activators/mediators. In addition to this, the Hymenopteran venoms also may contain a few neurotoxic peptides.

PEPTIDE TOXINS FROM HONEY BEE VENOM

Honey bee (*Apis mellifera*) venom contains well-known peptides such as melittin, apamin, tertiapin, secapin, and MCD peptide. Some of these peptides present a detergent-like action on plasma membranes,[7] causing cell lysis, whereas others are neurotoxins. Some aspects of the primary sequences, secondary structures, and the biological actions will be emphasized.

Melittin

Melittin is the major component of honey bee venom, representing approximately 50% of the total honey bee venom. It consists of 26 amino acid residues, mostly with hydrophobic or at least uncharged side chains, except for the C-terminal region. Melittin can aggregate into a tetrameric form in the venom reservoir, being apparently inactive under this condition.[4] This peptide lowers the surface tension of water at the level of the plasma membrane, acting mainly by its natural detergent-like effect on the plasma membrane, causing cell lysis (specially of mast cells), followed by histamine delivery. Because of this lytic effect on cell membranes, this peptide may be considered a venom diffusion factor, facilitating the entry of venom into the blood stream of the stung victims. X-ray crystallography of mellitin indicates that residues 1–10 and residues 13–26 form α-helices aligned approximately 120° to each other, while the proline residue at the position 14 could be the cause of a bend in the middle of the rod structure;[5,16] thus, a single polypeptide chain has the conformation of a bent α-helical cylinder (Fig. 1A).[5,7] When packed, the tetramer does so in a double planar layer. To achieve tight hydrophobic interactions, the hydrophobic residues of all four chains extend toward the center of the tetramer. Because of its surfactant properties, melittin is considered a direct hemolytic factor, acting synergistically with phospholipase A_2, activating this enzyme.[2,4,5]

Apamin

The honey bee venom also contains apamin, a peptide of 18 amino acid residues. The CD spectroscopy study of apamin in solution is consistent with a peptide presenting as an alpha-helix an showing a high degree of stability over a wide range of pH values.[4,16] This stability is a consequence of the rigidity imposed by two disulfide bridges. H^1-NMR studies of apamin in solution indicates a rigid, folded structure stabilized not only by the two disulfide bridges but also by at least seven intrachain hydrogen bonds. The structure of apamin has been studied by using a number of spectroscopic techniques, such as H^1-NMR, CD, Raman, FT-IR, and structure prediction algorithms of energy minimization with NMR and CD data. At first, the most suitable interpretation of the experimental results was the proposal of the coexistence of both an alpha-helix and beta-turns.[5,19] However, until now, the best structure for apamin remains controversial, with several available structural models.

Apamin is permeable to the blood–brain barrier, causing its effects on the CNS by several routes of administration. It causes neurotoxic effects in the spinal cord of mammals, producing hyperactivity and convulsions in rats.[5,10] When peripherically applied, apamin seems to selectively and

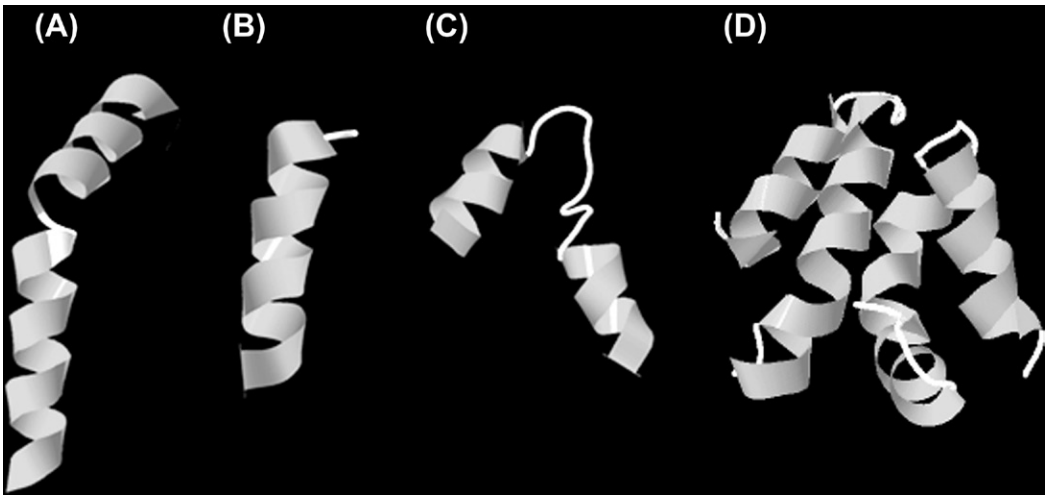

FIGURE 1 Secondary structures of some Hymenopteran venom peptide toxins: (A) Melittin from honeybees (*A. mellifera*); (B) Mastoparan X from the social wasp; (C) Poneratoxin from the ant *Paraponera clavata*, and (D) Ectatomin from the ant *Ectatomma tuberculatum.*

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potently affect the potassium permeability of certain membranes, such as the smooth muscle of the gut. At very low levels, it seems that apamin treatment can convert the normal hyperpolarizing response to epinephrine into a calcium-dependent depolarization.[8,19] The peripheral effects of apamin suggest that the central action may also result from decreased potassium fluxes, since this would broadly reduce inhibitory tone and increase the excitability.

Mast Cell Degranulating Peptide (MCD)

The MCD peptide is a 22 residue toxin. Its secondary structure by CD and ^{1}H-NMR,[14] indicate a close resemblance to α-helical peptides from positions 13 to 19; it is stable over the pH range 2–8, stabilized by two disulfide bridges and six intrachain hydrogen bonds, which form a 28-atom ring structure, also observed in apamin.[4,16] This peptide may present two closely related conformations in solution, possibly differing by *cis-trans* isomerism involving the residues Pro^{12}His^{13}.

MCD produces cytolysis, thus being considered a potent mast cell degranulator, causing the release of histamine.[13,30] This peptide is a facilitator of the response of honey bee venom, responsible for the reddening, swelling, and locally occurring pain at the site of honey bee stinging, typical of histamine-mediated responses.

Other Peptides

Secapin represents approximately 0.5% of total honey bee venom; it is a 25 amino acid residue peptide, containing a single disulfide bridge and high proline content. Apparently, this peptide has no known physiological and/or pharmacological activity, probably because it was not sufficiently tested so far.[4]

Tertiapin, which composes approximately 0.1% of total honey bee venom, is a 21 amino acid residue peptide containing a single disulfide bridge and a C-terminal residue in an amidated form. This peptide is not particularly toxic after intravenous injection; it degranulates mast cells with low potency. It has been compared pharmacologically to prostaglandins under the same conditions.[7] Tertiapin has been proposed to have both α-helical and β-sheet tendencies along the length of its sequence, raising the question of which conformational state the molecule is likely to be in. Considering the positions of both disulfide bridge and its CD spectrum that has no indication of a β-sheet, the structure of tertiapin is probably analogous to that of the MCD peptide.[5]

Adolapin, a basic polypeptide isolated from bee venom, shows potent anti-inflammatory and antinociceptive properties.[32] Its action involves the inhibition of both prostaglandin synthase and lipoxygenase in human platelets.[27] Like other nonsteroidal analgesics, this peptide also has antipyretic effects and inhibits the increase in mean body temperature.[11]

PEPTIDE TOXINS FROM BUMBLEBEE VENOMS

Bombolitins

The bombolitins are a family of heptadecapeptides isolated from the venom of the bumblebee *Megabombus pennsyluanicus*.[3,4] These peptides are able to form amphiphilic α-helical structures either by self-association or by interaction with amphiphilic matrices, such as micelles or vesicles. The biological activity of these peptides seems then to be directly correlated to their capability to form an amphiphilic order structure (α-helix) that allows them to associate and penetrate cellular membranes. They are capable of lysing erythrocytes and liposomes, in addition to increasing the activity of the enzyme phospholipase A_2. In more general terms, the bombolitins share common biological properties with other "toxin" peptides such as melittin, crabrolin, and mastoparans.

PEPTIDE TOXINS FROM SOCIAL WASP VENOMS

The venoms of social wasps contain a series of polycationic amphipathic peptides, such as mastoparans, chemotactic peptides, wasp kinins, and crabrolins, the group of compact peptides containing disulfide bridges, and the group of very short linear peptides. These toxins present a series of multifunctional pharmacological actions, which generally contribute to the occurrence of intense inflammatory processes.

Mastoparans

The most abundant peptide component from Vespid venoms is represented by the mastoparans, which involves a histamine-releasing principle. Mastoparans are generally polycationic, linear tetradecapeptide amides, rich in hydrophobic residues such as leucine, isoleucine, and alanine. The most representative molecules of species endemic from regions with a cold climate show characteristic lysine residues in positions 4, 11, and 12,[13] but novel peptide sequences showing a different pattern of lysine distribution have been identified recently in wasp species endemic from the tropical regions.[28,29] Mastoparans are randomly coiled in water, whereas in methanolic solution and/or in the presence of lipid vesicles, they are generally alpha helical peptides (Fig. 1B), causing their effects by interacting with plasma membranes because of their amphipaticity.[30] According to their mode of action, these peptides may be classified into two groups: (i) those acting by cell lysis from pore formation on cell membranes[1,13] (ii) and those acting by interaction with G-protein–coupled receptors, leading to the activation of degranulation mecha-

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nisms and resulting in many different types of secretions depending on the type of target cell.[20,21] In any situation, the final action will be the delivery of histamine from mast cells, serotonin from platelets, or even insulin from pancreatic β-cells. In addition to the interaction with membrane components, mastoparans also activate phospholipases A.[1] Antibacterial activities have been described for these peptides.[16,17]

The amphiphilic nature of these peptides make them interact with different types of cell membranes through different mechanisms, depending on their primary sequences. Nonlytic mastoparans may present the ability to interact with different types of cell strains, causing a minimal membrane deorganization but interact with cell membrane receptors, especially those coupled to G-proteins. The high affinity of these receptors make the mastoparans molecules very attractive lead compounds to develop a new generation of drugs to modulate the activity of G-proteins–coupled cell signaling system.[33]

Mastoparan stimulate insulin secretion from βTC3 and INS-1 cells. Preincubation of β-TC3 cells with either toxin B (from *Clostridium difficille*), which inactivates Rho, Cdc42 and Rac, or with *C. sordellii* toxin (which inactivates Ras, Rap, and Rac), attenuates mastoparan-induced insulin secretion, implicating Rac in this phenomenon. Mastoparan-stimulated insulin secretion is resistant to specific inhibitors of geranylation of Rho G-proteins (e.g., Rac), suggesting that these peptides induce the direct activation of Rac via GTP/GDP exchange. However, glucose-induced insulin secretion from these cells is abolished by toxin B, suggesting that the geranyl-geranylation step is critical for glucose-stimulated secretion. Mastoparan increases the translocation of cytosolic Rac and Cdc42 to the membrane fraction. Further, stable expression of a dominant negative (N17Rac) form of Rac into INS-1 cells resulted in a significant reduction in mastoparan-stimulated insulin secretion from these cells. Taken together, these findings implicate Rho G-proteins, specifically Rac, in mastoparan-induced insulin release.[1]

Mastoparans are capable of directly stimulating the guanine nucleotide exchange reaction of the α-subunit of animal heterotrimeric G-protein–coupled receptors, leading to a myriad of downstream events including the activation of mitogen-activated protein kinases (MAPKs). Because of these properties, mastoparans are considered as important peptide ligands of G-protein–coupled receptors and are being used as tools to dissect the signal transduction mechanisms for different cell strains, associated with different diseases.

In addition to be ligands of G-protein–coupled receptors, there are mastoparans that interact directly with cell membranes, perturbing the phospholipid organization and causing cell lysis or cell/vesicle fusion.[34] This type of peptide has attracted attention because of the possible biotechnological applications developed from them, as follows:

(a) These peptides are potent facilitators of mitochondrial permeability transition and may thus be used as an antitumor agents; for this purpose, they are encapsulated in a selective tumor delivery system. Transferrin-modified liposomes carrying pH-sensitive fusogenic mastoparans may be used to deliver chemotherapeutical drugs into the cytosol of sick cells for cancer therapy.[22,34]

(b) ome Mastoparans are potent antimicrobial peptides (AMPs) with the potential to be used in the treatment of multiple drug-resistant infections. These peptides exist in solution as unstructured monomers, folding into predominantly α-helical structures upon membrane binding with their long helical axis parallel to the bilayer surface. Studies with model membranes have shown that these peptides intercalate into the lipid bilayer just below the level of the phospholipids glycerol backbone, in a location requiring expansion of the outer leaflet of the bilayer. Evidence from a variety of experimental approaches indicates that expansion and thinning of the bilayer are common characteristics during the early stages of AMP–membrane interactions. Subsequent disruption of the membrane permeability barrier may occur through a variety of mechanisms, leading ultimately to the loss of cytoplasmic membrane integrity and cell death. These AMPs constitute part of the "innate" arm of host resistance, serving as the first line of defense against infection in the insects. Importantly, AMPs are believed to have a mechanism of action entirely distinct from those of current clinically used antibiotics, and so there is great interest in their development for treatment of drug-resistant infections.[17]

Crabrolin

This is a 13 amino acid residue polycationic peptide present in the venom of the hornet *Vespa crabro*, presenting a helical conformation.[29] This peptide produces potent hemolytic and antibacterial activity; however, it is a weak mast cell degranulator, with <20% the activity of mastoparan. Crabrolin is four times less active than mastoparan in guinea pig erythrocytes[2,3] and shows no effect in rat erythrocytes.

Chemotactic Peptides

The second most important group of peptides among the Vespid are the chemotactic peptides, which generally are trideca-polycationic peptides, with primary sequences resembling the mastoparans in regards to the richness of hydrophobic amino acid residues and the amidation of the C-terminal residue.[13] These peptides induce chemotaxis in polymorphonuclear leukocytes and macrophages[6] and

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sometimes also release histamine from mast cells. The result of such chemotactic activity is the development of a mild edema, accompanied by an inflammatory exudate around the site of stinging, containing mainly polymorphonuclear leucocytes. Thus, the chemotactic peptides do not cause pain directly, but they play an important role enhancing the inflammatory effect of wasp stings. A novel type of chemotactic peptide was reported in which the acetylation of the N-terminal residue seems to play a part in the modulation of polymorphonuclear leukocyte attraction.[13]

Kinin-Related Peptides (Wasp Kinins)

A third group of peptides biologically important among the Vespid toxins are the wasp kinins, which generally are known as pain-producing peptides. These peptides consist of bradykinin molecules elongated at the N-terminus with some extra amino acid residues. Sometimes, there is also an elongation from the original bradykinin C-terminal residue.[13] The general pharmacological properties are similar to those presented by human bradykinin; however, the effects of Vespidae kinins are much more pronounced and long lasting. These effects may be summarized as follows: When injected intravenously, they cause hypertension in rats, dogs rabbis, and cats; hypotension in chickens; brochoconstriction in guinea pigs; contraction of most isolated smooth muscle preparations; and relaxation of rat duodenum.[14,24]

Compact Peptides Presenting Disulfide Bridges

A fourth group of amphipathic polycationic peptides from Vespide venoms has been described, but few characterized are up to now; these peptides contain from 21 to 25 amino acid residues in their primary sequences, presenting a single disulfide bridge, constituting the first example of this type of structure among the Vespid peptide toxins. These peptides are mast cell degranulators, without known hemolytic activity, which apparently also inhibit the insulin secretion by β-cells.[14]

Very Short Peptides

The venoms of social Hymenoptera also contain a series of short peptides, not well studied up to now, which apparently induce chemotaxis of PMNLs and act as modulators of the classical chemotactic peptides.[6]

PEPTIDES FROM ANT VENOMS

Most of the ants only present traces of proteins/peptides in their venoms; however, in those species producing peptides as components of their venoms, these toxins follow the same general pattern already observed for peptides from wasps and bees venoms, that is, short and linear polycationic peptides, presenting a high content of α-helices, which are responsible for cell lysis, hemolysis, histamine release from mast cells, and antimicrobial actions. A few neurotoxins are also known.

"Myr p" Peptides and Pilosulin

The venom of the Australian ant *Myrmecia pilosula* seems to contain a complex mixture of allergenic peptides, some of them forming heterodimers, apparently maintained by disulfide bridges. From this complex was identified Myr p 1, the major expressed allergenic product being a 56 residue peptide, whereas Myr p 2 is a 27 residue peptide; these peptides may dissociate, or are cleaved to minor fragments, such as those previously identified as pilosulin-1 and pilosulin-2, characterized as the fragments 57–112 from Myr p1 and 49–75 from Myr p2.[5] Pilosulin-1 is a α-helical peptide with potent and broad spectrum of antimicrobial activity, both against standard and multidrug-resistant Gram-positive and Gram-negative bacteria, and *Candida albicans*.[29] Other pilosulins have been identified and characterized as hemolysins and histamine release peptides.[12,18,25]

Poneratoxins

Poneratoxin is a neuropeptide found in the venom of the ant *Paraponera clavata*. A peptide containing 25 amino acid residues, it affects voltage-dependent sodium channels and blocks synaptic transmission in the insect central nervous system in a concentration-dependent manner. Its NMR structure is in the form of two α-helices connected by a β-turn (Fig. 1C); the helices have quite different characteristics from each other: One of them is apolar, whereas the second contains polar and charged amino acids. This will result in different interactions with cell membranes. The extremely hydrophobic N-terminal helix may interact with uncharged lipid bilayers, while the C-terminal helix, slightly positively charged and terminating with arginine, will be able to attach to negatively charged cell surfaces as previously found for other membrane interacting peptides. Such a toxin can thus use two different complementary modes of interaction to attain its target, cellular membranes.[18,23]

Ponericins

Ponericins constitute a group of peptides isolated from the venom of the predatory ant *Pachycondyla goeldii*, exhibiting hemolysis, insecticidal activity against cricket larvae, and antimicrobial action against Gram-positive and Gram-negative bacteria. According to their primary structure similarities, they can be classified into three families: ponericin G, W, and L. Ponericins share high sequence similarities with

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known peptides: ponericins G with cecropin-like peptides, ponericins W with gaegurins and melittin, and ponericins L with dermaseptins. The comparison of the structural features of ponericins with those of well-studied peptides suggests that the ponericins may adopt an amphipathic α-helical structure in polar environments, such as cell membranes.[13]

Ectatomin

This peptide is a neurotoxin isolated from the venom of the *Ectatomma tuberculatum* ant and contains two highly homologous peptide chains (consisting of 37 and 34 amino acid residues) linked to each other by disulfide bonds.[25] Each chain consists of two alpha-helices and a hinge region of four residues forming a hairpin structure that is stabilized by disulfide bridges; the hinge regions of the two chains are connected together by a third disulfide bridge (Fig. 1D). Thus, ectatomin forms a four-alpha-helical bundle structure.[18] Ectatomin is a potent inhibitor of calcium currents after a latency of a few seconds in rat ventricular myocites.[25]

PATHOPHYSIOLOGY OF HYMENOPTERAN PEPTIDE TOXINS

The large group of Hymenopteran venom consists of biologically active peptides that are responsible for the main pathophysiological actions occurring during the envenoming processes. Among the different groups of social Hymenoptera (wasps, bees, and ants), there are a series of different families of peptides, characteristic of each group of insect: melittin, apamin, and MCD peptides are typical of honeybees, whereas mastoparans, chemotactic peptides, and kinins are characteristic of wasps; the group of ants is biologically much more diversified than wasps and bees, and consequently, ant peptides are also more diversified than the previous ones. It is important to emphasize that only a few groups of ants present venoms containing proteins and peptides; pilosulins are characteristic of Myrmicinae, Poneratoxins, and ponericins are typical of Ponerinae, whereas ectatomin is characteristic of the Ectatomminae ants.

Despite the large structural differences among the wide variety of Hymenopteran venom toxins, some of these families of peptides share common pathophysiological actions. Thus, as an example: mastoparans, crabrolins, bombolitins, MCD peptide, and melittin cause mast cell degranulation, cell lysis, and hemolysis, with the consequent occurrence of inflammation, pain, myonecrosis, and ischemia, which all together promote a cascade of actions that in turn result in hemoglobinuria, rhabdomyolysis, and renal failure. Neurotoxins such as apamin, poneratoxins, and ectatomin act at the level of the nervous system blocking different subtypes of glutamate receptors, which in turn may affect differently the functioning of calcium channels.[5,6,23,28] The excessive chemotaxis of polymorphonucleated leukocytes (PMNLs) induced by the chemotactic peptides may cause a massive delivery of oxygen peroxide around the site of stinging, causing hyperoxidation of the surrounding tissues, followed by their consequent cellular death. The kinin analogs cause hypotension and bronchoconstriction, whereas the short peptides apparently are involved with the chemotaxis of PMNLs and the inhibition of the release of catecholamines from chromaffin cells.[24] Figure 2 summarizes the general scheme of the pathophysiology of the Hymenopteran peptide toxins occurring during the envenomation process.

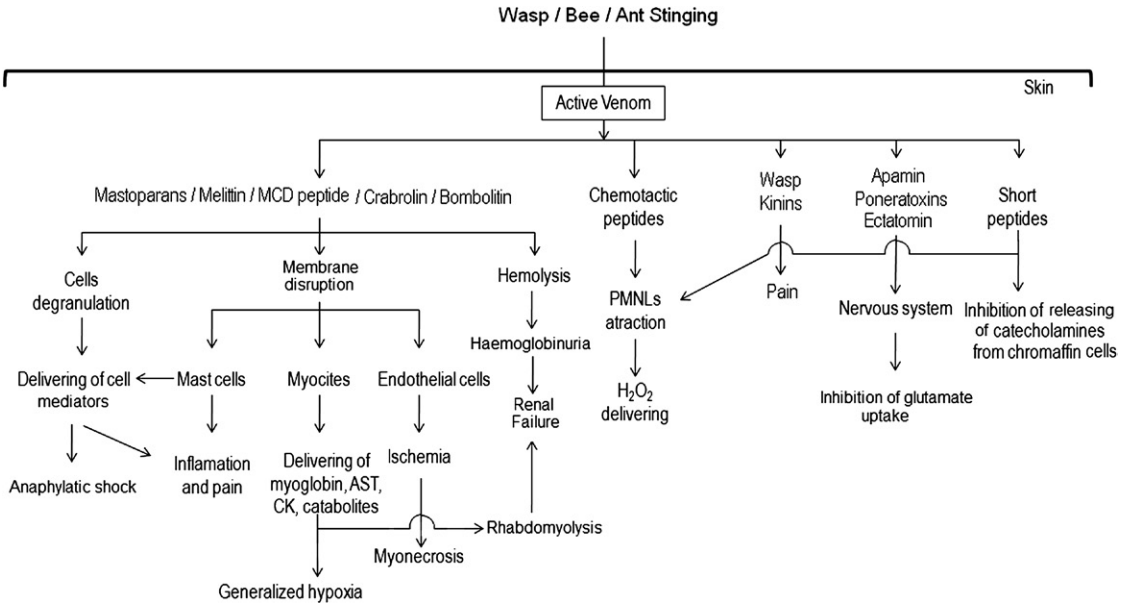

FIGURE 2 General mechanism of envenoming pathogenesis of the peptide toxins from the venoms of the social Hymenoptera.

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ACKNOWLEDGMENTS

The work of the authors of this review has been supported by a grant of the project: INCT-Immunology (iii)/MCT/CNPq and Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) through the program BIOprospecTA (06/57122-7). MSP is researcher of Brazilian National Council of Research and Technological Development (CNPq).

REFERENCES

1. Amin RH, Chen HQ, Veluthal R, Silver RB, Li J, Li G, et al. Mastoparan induced insulin secretion from insulin secreting beta TC3 and INS-1 cells: evidence for its regulation by Rho sub-family of G-proteins. *Endocrinology* 2003;**144**:4508–18.
2. Argaiolas A, Pisano JJ. Facilitation of phospholipase A2 activity by mastoparans, a new class of mast cell degranulation peptides from wasp venom. *J Biol Chem* 1983;**258**:13697–702.
3. Argiolas A, Pisano JJ. Isolation and characterization of 2 two new peptides, mastoparan-C and Crabrolin, from the venom of the European Hornet, *Vespa crabro*. *J Biol Chem* 1984;**259**:106–11.
4. Argiolas A, Pisano JJ. Bombolitins, a new class of mast-cell degranulating peptides from the venom of the bumblebee *Megabombus pennsylvanicus*. *J Biol Chem* 1985;**260**:1437–44.
5. Banks BEC, Shipolini RA. Chemistry and pharmacology of honeybee venoms. In: Piek T, editor. *Venoms of Hymenoptera: biochemical, pharmacological and behavioral aspects*. London, United Kingdom: Academic Press; 1986. p. 329–416.
6. Baptista-Saidemberg NB, Saidemberg DM, De Souza BM, César-Tognoli LM, Ferreira VMR, Mendes MA, et al. Protonectin (1–6): a novel chemotactic peptide from the venom of the social wasp *Agelaia pallipes pallipes*. *Toxicon* 2010;**56**:880–9.
7. Biló BM, Rueff F, Mosbech H, Bonifazi F, Oude-Elberink JNG. EAACI interest group on insect venom hypersensitivity. Diagnosis of hymenoptera venom allergy. *Allergy* 2005;**60**:1339–49.
8. Brand JM, Blum MS, Fales HM, McConnell JG. Fire ant venoms: comparative analyses of alkaloidal components. *Toxicon* 1972;**10**:259–231.
9. Brennan FM. Role of cytokines in experimental arthritis. *Clin Exp Immunol* 1994;**97**:1–3.
10. Chen J, Luo C, Li H, Chen H. Primary hyperalgesia to mechanical and heat stimuli following subcutaneous bee venom injection into the plantar surface of hindpaw in the conscious rat: a comparative study with the formalin test. *Pain* 1999;**83**:67–76.
11. De Groot H. Allergy to bumblebees. *Cur Opin Allergy Clin Immunol* 2006;**6**:294–7.
12. Den Hertog A. Calcium and the alpha-action of catechoalmines on guinea pig *Taenia caeca*. *J Physiol* 1981;**316**:109–25.
13. De Souza BM, Palma MS. Peptides from Hymenoptera venoms: biochemistry, pharmacology and potential applications in health and biotechnology. In: De Lima ME, Pimenta AMC, Martin-Euclaire MF, Zingali RB, editors. *Organizers, "Animal toxins: the state of art. perspectives on health and biotechnology."* Belo Horizonte, Brazil: UFMG; 2009, 800. p. 273–97.
14. Dohtsu K, Hagiwara K, Palma MS, Nakajima T. Isolation and sequence analysis of peptides from the venom of *Protonectarina sylveirae* (Hymenoptera, Vespidae) (I). *Natural Toxins* 1993;**1**:271–6.
15. Esher SH, Castro APB, Croce J, Palma MS, Malaspina O, Kalil JE, Castro FFM. Study of laboratorial methods for Hymenoptera allergy diagnosis: a critical analysis. *J Allerg Clin Immunol* 2001;**107**: Supplement 375.
16. Habermann E. Bee and wasp venom: the biochemistry and pharmacology of their peptides and enzymes are reviewed. *Science* 1972;**117**:314–22.
17. Hancock RE. Peptides antibiotics. *Lancet* 1997;**349**:418–22.
18. Hendrich AB, Mozrzymas JW, Konopinska D, Scuka M. The effect of poneratoxin on neuromuscular transmission in the rat diaphragm. *Cell Mol Biol Lett* 2002;**7**:195–202.
19. Hider RC, Ragnarson U. A proposal for the structure of apamin. *FEBS Lett* 1980;**111**:189–93.
20. Higashijima T, Wakamatsu K, Takemitsu M, Fujino M, Nakajima T, Miyazawa T. Conformational change of mastoparan from wasp venom on binding with phospholipid Membrane. *FEBS Lett* 1983;**152**:227–30.
21. Higashijima T, Burnier J, Ross EM. Regulation of G_i and G_0 by mastoparan related peptides and hydrophilic amines. *J Biol Chem* 1990;**265**:14176–86.
22. Hoffman AS, Stayton P, Oliver W, Tirrell D, Murthy N, Lackey C. Enhanced transport using membrane disruptive agents. United States Patent. 2004; 6,835,393.
23. Hue B, Piek T. Irreversible pre-synaptic activation-induced block mof the transmission in the insect CNS by hemi-cholinium-3 and threonine-6-bradykinin. *Comp Biochem Physiol* 1989;**93C**:87–9.
24. Ifrah D, Doisy X, Ryge TS, Hansen PR. Structure–activity relationship study of anoplin. *J Peptide Sci* 2005;**11**:113–21.
25. Inagaki H, Akagi M, Imai HT, Taylor RT, Kubo T. Molecular cloning and biological characterization of novel antimicrobial peptides, pilosulin 3 and pilosulin 4, from a species of the Australian ant genus *Myrmecia*. *Arch Biochem Biophys* 2004;**428**:170–8.
26. Kim JH, Kim Y, Lee SD, Lopez I, Arnold RS, Lambeth JD, et al. Selective activation of phospholipase D2 by unsaturated fatty acid. *FEBS Lett* 1999;**454**:42–6.
27. Koburova KL, Michailova SG, Shkenderov SV. Further investigation on the anti-inflammatory properties of adolapin-bee venom polypeptide. *Acta Physiol Pharmacol Bulg* 1985;**11**:50–5.
28. Konno K, Miwa A, Takayama H, Hisada M, Itagaki Y, Naoki H, et al. α-Pompilidotoxin (α - PMTX), a novel neurotoxin from the venom of a solitary wasp, facilitates transmission in the crustacean neuromuscular synapse. *Neurosci Lett* 1997;**238**:99–102.
29. Krishnakumari V, Nagaraj R. Antimicrobial and hemolytic activities of crabrolin, a 13-residue peptide from the venom of the European hornet, *Vespa crabro*, and its analogs. *J Peptide Res* 1997;**50**:88–93.
30. Nakajima T. In: Piek T, editor. *Venoms of the Hymenoptera: pharmacology and biochemistry of vespide venoms*. London, United Kingdom: Academic Press; 1986. p. 309–27.
31. Piek T. Neurotoxins from venoms of the Hymenoptera—twenty-five years of research in Amsterdam. *Comp Biochem Physiol* 1990;**96C**: 223–33.
32. Shkenderov S, Koburova K. Adolapin: a new isolated analgesic and anti-inflammatory polypeptide from bee venom. *Toxicon* 1982;**20**:317–21.
33. Shpakov AO, Pertseva MN. Molecular mechanisms for the effect of mastoparan on G proteins in tissues of vertebrates and invertebrates. *Bull Experim Biol Med* 2006;**141**:302–6.
34. Yamada Y, Shinohara S, Kakudo T, Chaki S, Futaki S, Kamiya H, et al. Mitochondrial delivery of mastoparan with transferrin liposomes equipped with a ph-sensitive fusogenic peptide for selective cancer therapy. *Int J Pharmacol* 2005;**303**:1–7.
35. Yee CJ, Palma MS, Malaspina O, Morato-Castro FM, Azevedo-Neto RS, Manso EC, et al. Acquired immunity to Africanized honeybee venom in Brazilian beekeepers. *J Invest Allergol Clin Imunol* 1997;**7**:583–7.

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Chapter 59

Scorpion Peptides

Ricardo C. Rodríguez de la Vega, Nicolas Vidal and Lourival D. Possani

ABSTRACT

Scorpion venoms are arsenal-like mixtures that provide the producing organism with the ability to kill their prey or deter possible competitors or predators. When the first edition of this book was published, most of the known scorpion venom components were encompassed into a single structural class dubbed as the Cystine-Stabilized α/β scaffold; their functions as ion channel modulators are now relatively well known and understood. However, mining on scorpion venoms using high-throughput techniques has revealed a large prevalence of noncanonical components that actually outnumber the typical toxin class. This chapter highlights the documented molecular diversity contained in scorpion venoms and reviews the known structural and functional classes. The biotechnological and pharmacological prospects of some scorpion venom peptides are also briefly covered.

INTRODUCTION

Scorpions rely on their venoms both for foraging and defending themselves against competitors or predators; they have been doing so over their 400 million years of evolution on land. Like many other toxic secretions from venomous animals, scorpion venoms are arsenal-like mixtures consisting of dozens of different compounds, including salts, organic molecules, and a wide diversity of proteins, ranging from short linear peptides to high molecular weight enzymes. Scorpion toxins are the venom components responsible for the envenomation caused by scorpion stings, whether in a trophic or a defensive context. Once injected, scorpion toxins gain access and bind directly to the victim's molecules prompting a cascade of physiological effects that can lead to death of susceptible animals; neurotoxic effects on mammals, for example, are triggered by the direct modulation of K^+ and Na^+ channels by scorpion toxins. Most of the research on scorpion venoms has been fueled by the medical interest in the toxins responsible for human envenomation and, later, by the use of some scorpion venom components as molecular tools for the study of ion channel function. By 2010, the sequences of >1000 scorpion venoms proteins were known.[12] Most of these components are peptidic ion channel modulators that belong to a single structural class built around the sequence signature of the Cystine-Stabilized α/β (CS-α/β) motif. Various studies aiming at cataloging the whole molecular diversity present in scorpion venoms have revealed, however, that within scorpion venoms CS-α/β proteins are actually outnumbered by proteins not belonging to this structural class, many of which display unprecedented biological functions.

This chapter deals with the molecular diversity documented in scorpion venoms and the molecular targets and mechanisms of action of well-known and not-so-well-known scorpion venom peptides. The biotechnological and pharmacological prospects of some of these are only briefly outlined as the chapters by Chandy and Beraud and by DeWaard and Sabatier present a deeper discussion. Important aspects of scorpion venom research, such as how venoms have attained their remarkable complexity through evolution and the puzzling effects that whole venoms exert on the immune response of affected mammals, are outside the scope of this chapter. The systematic characterization of scorpion venom components is expected to provide both a better understanding on how scorpion venoms accomplish their "native" functions and novel bioactive peptides that could be used to study many biological phenomena.

MINING ON SCORPION VENOMS

When the first edition of the Handbook of Biologically Active Peptides was published, sequence information of nearly 400 scorpion venom peptides and proteins was known, thanks to the painstaking job of dozens of laboratories around the world; the advent of high-throughput protein identification techniques by Mass Spectrometry and large-scale DNA sequencing have more than tripled that number in the last few years.

Comprehensive "venom proteomes" of twelve scorpion species have been published, including nine belonging to the Buthidae family (six *Tityus* species,[2] two *Androctonus* species,[4,20] and *Mesobuthus tamulus*[19]), two Liochelidae (*Liocheles australasie*[15] and *Opistacanthus cayaporum*[28]) and one Scorpionidae (*Heterometrus petersii*[14]). The number of identified components varies between 72 (*A. mauritanicus*

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00059-2
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mauritanicus) and >600 (*M. tamulus*). In general, the largest group corresponds to peptides with rather low molecular masses (≤1500 Da), for which virtually nothing is known about their function or structures (see below). Proteins with molecular weights >7501 Da are scarce among these scorpion venom proteomes, accounting for about 10% of all identified masses. The best-known scorpion venom peptides, the CS-α/β peptides active on K^+ or Na^+ channels, fall in the 3001- to 4500-Da and 6001- to 7500-Da ranges and collectively account for approximately 30% of all identified masses, with a significantly larger contribution of Buthidae scorpions (up to 78% of all identified masses in *A. mauritanicus mauritanicus*). A number of other studies reporting the overall composition of scorpion venoms determined by MS have been published, but the component lists are not available;[17] nonetheless, the composition of the venoms studied is in agreement for the most part with the trends outlined above.

More than two-thirds of unassigned molecular masses in all these scorpion venom proteomic studies demand alternative methods to establish their molecular identity; cDNA sequencing is helping to fill this gap. A handful of studies surveying the transcriptional profiles of scorpion venom glands have been published over the last few years, including the Caraboctonidae *Hadrurus gertschi*,[27] the Euscorpiidae *Scorpiops jendeki*,[13] the Liochelidae *O. cayaporum*,[29] the Scorpionidae *H. petersii*,[14] and three Buthidae (*T. discrepans*,[5] *Lychas mucronatus*,[35] and *Hottentota judaicus*[16]). Taken together, these studies provided sequence information for >600 previously unknown scorpion venom gland proteins. The largest sequence diversity (~50% of the unique sequences) reported in these studies could be assigned to comparatively low expressing proteins (<30% of the total reads), which would participate in the cellular processes of the venom gland. Somehow unexpectedly, transcripts encoding common toxin types, that is, CS-α/β peptides active on K^+ or Na^+ channels and Inhibitory Cystine Knot (ICK) peptides active on Ca^{2+} channels, constitute a relatively small proportion of both the total reads and the number of unique sequences in all but one of these transcriptomes: around 10% on average (*T. discrepans* not taken into consideration) with as low as 3% of the total unique sequences in *S. jendeki*. In comparison, secretory proteins rarely reported or altogether unprecedented in scorpion venoms constitute a twice as large proportion of the molecular diversity (averaging 20% of the unique sequences). On the other side of this spectrum, 50% of the total reads of a randomly sequenced *T. discrepans* cDNA library encode for typical toxin precursors. The overlap between the expected molecular weight of the translated sequences identified in these studies and the molecular masses determined by proteomic studies remains rather low (for instance only nine sequences *O. cayaporum* fall within the mass range 3001–4500 Da, from which 28 unique masses were identified in the proteomic study). The combined approach used to analyze *H. petersii* venom and venom gland is remarkable in that sense: the random sequencing of venom gland transcripts allowed the authors to assign to which toxin "family" belong >110 unique masses identified in their proteomic study of tryptic digested venom.[14]

Most of these analyses have been performed on pooled samples, without distinction of the ontological state of the specimens, the gender or the geographical source of the scorpions from which the venom has been extracted. It should be stressed that variations in venom composition or transcriptional profile have been documented for several scorpions,[18,19,35] thus posing a cautionary mark on the extent the compendia outlined above reflect the actual molecular diversity present in scorpion venoms. Further research should take into consideration this variability. In spite of these limitations, proteomic and transcriptomic analyses of scorpion venoms and venom glands are revealing the large contribution of peptides considered atypical or completely ignored for many years (see below).

DOCUMENTED MOLECULAR DIVERSITY

By the time the first edition of the Handbook of Biologically Active Peptides was in production, the search for all the sequence information about scorpion venom peptides was a rather laborious enterprise. Most data were not deposited in publicly available databases, and extensive literature search was mandatory. Fortunately, this panorama has changed thanks to current publication policies—which now ask for submission to public databases of virtually every piece of sequence data—and to the emergence of dedicated annotation initiatives.[12] The Animal Toxin Annotation Program of UniProt (www.uniprot.org/program/Toxins) provides manually curated protein entries for approximately 750 scorpion venom proteins, almost twice as many translated sequences are also available from the query form. Among the 91 scorpion species covered, Buthidae scorpions largely surpass all the rest with 74 species; nine Scorpionidae, four Liochelidae, two Caraboctonidae, and two Chactidae complete the list. The number of sequences from each family shows an even more biased pattern: Buthidae 88%, Liochelidae 5%, Scorpionidae 3%, and approximately 1% for the other two families. Although in recent years more articles on neglected scorpion families have been published, scorpion biodiversity is still poorly covered (<5% of the almost 2000 extant species). The gravity of the situation is even more striking for the estimated number of different molecular components of scorpion venoms; these sequences would represent not >1% of the estimated 300,000 different peptides in all scorpion venoms (taking the conservative number of 150 peptides per venom proteome).

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If the scorpion biodiversity is scantly sampled, the representation of the different types of venom components in sequence databases is only slightly better. For many years, CS-α/β peptides dominated the sequences available from scorpion venoms; by the time of the first edition of this book, almost 80% of the approximately 320 scorpion entries in UniProt with "evidence at the protein level" were classified as CS-α/β peptides. A small decrease of this proportion, to 70%, over the last few years has been prompted by the number of newly characterized peptides that do not belong to this group almost matching the number of novel CS-α/β peptides; although still moderately, these numbers speak about a shift in scorpion venom research toward the characterization of previously neglected venom components. Molecular diversity is even more obvious if translated sequences from scorpion venom glands are also taken into consideration;[23] the proportion of non CS-α/β scorpion venom peptides already reached 40% and is growing faster than CS-α/β peptides (see above).

STRUCTURAL AND FUNCTIONAL TYPES

According to proteomic studies, the largest group of scorpion venom peptides corresponds to the short peptides (<3001 Da), which dominate in most venom proteomes reported to date. The molecular identity of most of them remains to be uncovered and some could even be fragments of larger proteins, as suggested by the impressive finding that almost one-third of the masses within this range found in *T. serrulatus* are actually fragments of larger proteins;[24] remarkably, some fragments could be biologically active by themselves, thus adding to the functional complexity of scorpion venoms.[9] A wide range of linear peptides fall within this mass range, among the most abundant in sequence databases are α-helical peptides similar to the antimicrobial IsCT, which have been found in several species (Fig. 1): two Liochelidae (*Opisthacanthus madagascariensis*, where they were first reported, and *O. cayaporum*), the Scorpionidae *H. petersii* and four Buthidae (*H. judaica*, *Mesobuthus eupeus*, and *M. martensii*). Several other linear scorpion venom peptides have been reported in the literature, including some therapeutically promising ones highly active against antibiotic resistant bacteria (Fig. 1); a common membrane disrupting mechanism is assumed to explain both the antibiotic and cytolytic activities of these peptides.

Apart from the ubiquitous CS-α/β peptides from scorpion venoms, other types of disulfide rich peptides are also found in several species. Peptides folding into an ICK structural motif have been found in three Buthidae (*H. judaica*, *L. mucronatus*, and *M. martensii*), the Caraboctonidae *H. gertschi*, the Euscorpiidae *S. jendeki*, and four Scorpionidae (*H. petersii*, *Opistophtalmus carinatus*, *Pandinus imperator*, and *Scorpio maurus*). This structural motif is defined by a triple stranded beta sheet crosslinked by three disulfide bridges (although variations exist); it dominates among *Conus* and spider venom peptides, where it has been implicated in a true cornucopia of different biological actions (see the chapters by Nicholson and Olivera). In scorpions, the ICK peptides (Fig. 1), also called calcins or calcines, are invariably associated with agonistic activity on intracellular calcium release channels (the so-called Ryanodine Receptors, RyR); the ability of these peptide to crossmembranes without lysis of the cells, even with large cargoes, offers a promising perspective for intracellular drug delivery.[10,22] Interestingly, a peptide found in two *Liocheles* species displays the predicted two disulfide pattern and three-dimensional folding of the long forecasted Disulfide Directed Hairpin (Fig. 1), a putatively ancestral fold out of which ICK peptides would have evolved.[11,30] Other cysteine-rich peptides have been identified in scorpion venom gland transcriptomes, some of which show high similarity with biologically active peptides found in other venomous organisms (e.g., Kunitz protease inhibitors and Cysteine-Rich Secretory Proteins[8]), but their structures and functions remain to be determined. The most abundant component of *L. australasiae* venom illustrates our ignorance about disulfide rich peptides in scorpion venoms: La1 is a four disulfide bridged peptide[15] that makes up to 11% of total protein content in *L. australasiae* venom (Miyashita, personal communication) but no function for it or its homologs in *O. cayaporum*, *M. martensii*, and *H. judaica* has been reported yet. Other components with enzymatic activity have been patchily reported in scorpion venoms, including hyaluronidases (partial sequence information from *H. judaica*, *M. martensii*, and *T. serrulatus* as well as evidence for their presence in several others), phospholipases (three Buthidae; two Scorpionidae; and one each in Caraboctonidae, Chactidae, and Liochelidae), metalloproteases (in the Buthidae *H. judaica*, *M. eupeus*, and *T. serrulatus*[7]) and a sphingomyelinase homolog in the Liochelidae *Hemiscorpious lepturus*;[3] all of them are known to be important components of other venomous organisms.[8]

The CS-α/β peptides are the most thoroughly studied scorpion venom peptides and are still the ones receiving the largest attention (Fig. 1). The Cystine-Stabilized α/β scaffold consists of an α-helix joined by disulfide bridges to the last strand of a double- or triple-stranded β sheet. The CS-α/β scaffold is accompanied by a four-cysteine sequence signature ($C_i xxx C_{i+4} \ldots C_j x C_{j+2}$) forming two completely conserved disulfide bridges (C_i–C_j, C_{i+4}–C_{j+2}). Traditionally, scorpion venom CS-α/β peptides have been distinguished according to their length in short-chain (<45 residues long) and long-chain (55 residues long or more) peptides. This distinction is somehow mirrored by the pharmacological action of these peptides, with short-chain peptides being potassium channel blockers and long-chain peptides displaying different sodium channel modulation activities.

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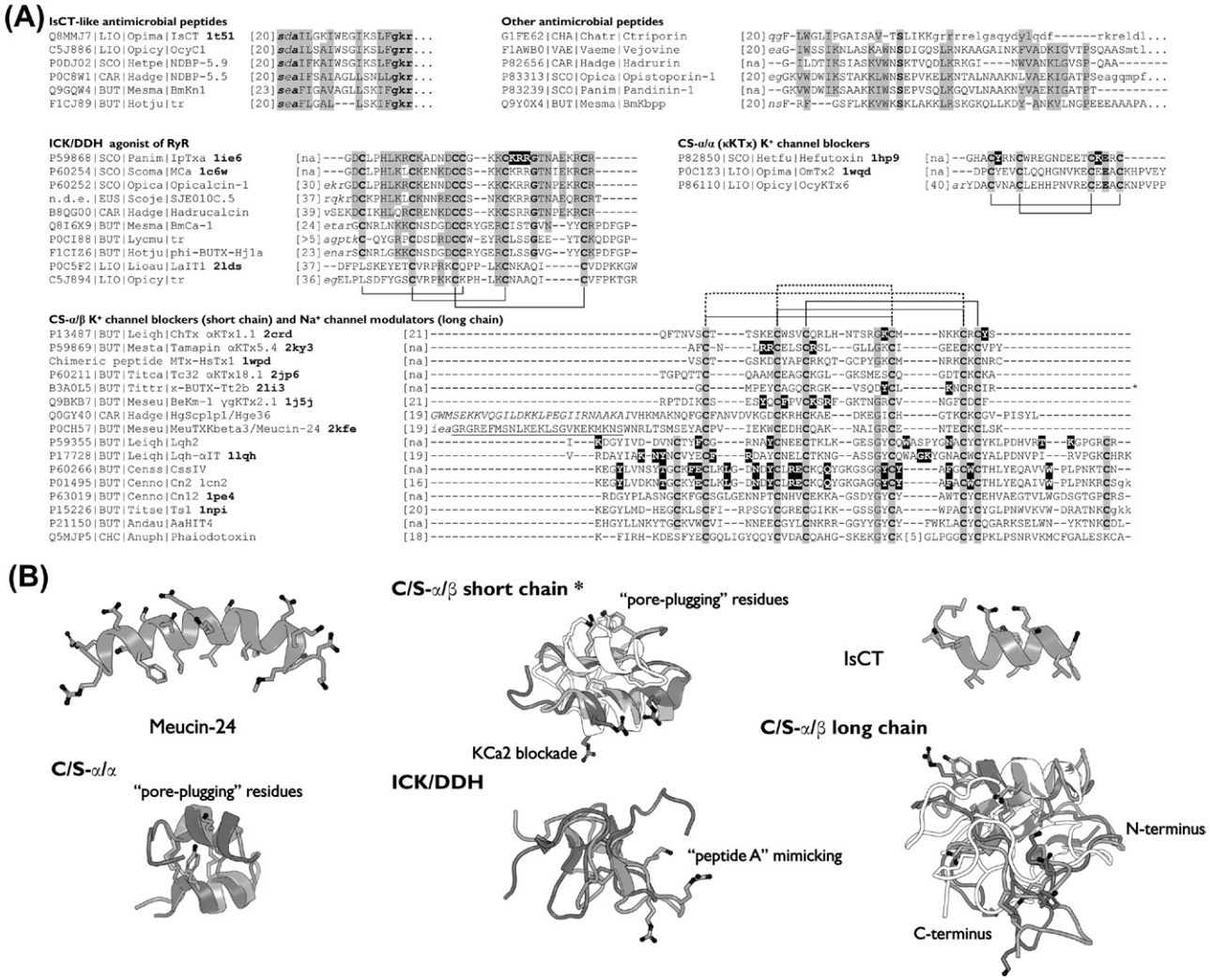

FIGURE 1 Structural and functional types of scorpion venom peptides. (A) Multiple sequence alignment of selected members of each class was performed with TCoffee (www.tcoffee.org/apps/tcoffee/play?name=regular). Sequences are labeled with UniProt accession numbers, followed by a two-letter code of the taxonomic family, a five-letter abbreviation of the species name, the name given in the original publications and PDB codes where available (bold). Conserved residues are highlighted in gray. Numbers between brackets denote N-terminal residues that are not shown. Lower case residues indicate that these are not present in the mature forms. Lines below the ICK/DDH and CSα/α and above CSα/β alignments specify the disulfide bridges. Broken lines indicate the two alternative disulfide bridges of the helix-loop-helix peptide κ-BUTX-Tt2b (*). The antimicrobial native fragment of MeuTXKbeta3, Meucin24, is underlined. The processed N-terminus of HgScplp1 not present in Hge36 is shown in italics (see Q0GY40). The residues important for the functions and highlighted in panel b and Fig. 2 are indicated on a black background. Family abbreviations: BUT, Buthidae; CAR, Caraboctonidae; CHA, Chaerelidae; CHC, Chactidae; EUS, Euscorpiidae; LIO, Liochelidae; SCO, Scorpionidae; VAE, Vaejovidae. (B) Three-dimensional structures and structural superimposition of selected scorpion venom peptides. Structural alignments were performed with TopMatch (topmatch.services.came.sbg.ac.at/TopMatchFlex.php) and the structures displayed with PyMOL (www.pymol.org). Residues considered important for the function are displayed as balls and sticks (see database entries for further reference): IsCT and Meucin24, amphipathic clustering of charged (top) and hydrophobic (bottom) residues; Hefutoxin, Kv channel "pore-plugging" residues Tyr5 and Lys21; IpTxa, basic loop (Lys22-Arg23-Arg24) mimicking the "peptide A" off the cognate RyR partner (the dihydropyridine receptor); ChTx, Kv channel "pore-plugging" residues Lys27 and Tyr36; Tamapin, basic residues in the region important for KCa2 channel blockade; κ-BUTX-Tt2b, putative "functional dyad" Tyr19 and Lys21; Cn2, conserved functional residues identified in CssIV; LqhαIT, residues important for toxicity and receptor binding (see P17728). Overlaid structures are CSα/α, Hefutoxin (light gray) and OmTx2 (dark gray); CSα/β short chain, ChTx (light gray), Tamapin (dark gray) and the helix-loop-helix κ-BUTX-Tt2b (white); CSα/β long-chain, αNaScTx LqhαIT (dark gray), βNaScTx Cn2 (light gray) and uNaScTx Cn12 (white); ICK/DDH, IpTxa (light gray) and LaIT1 (dark gray). Side chain heteroatoms are depicted in black. PDB codes are indicated in panel a.

The Protein Data Bank (www.rcsb.org/pdb/home/home.do) contains 135 structures for some 85 unique scorpion venom peptides from 22 species (67 from 17 Buthidae, 11 from 4 Scorpionidae, and 4 from 1 Liochelidae); 75 of them fold into the CS-α/β pattern. Although the permissiveness of the CS-α/β scaffold to different disulfide patterns has been known for some time, recently two peptides with helix-loop-helix structures and the same cysteine pattern

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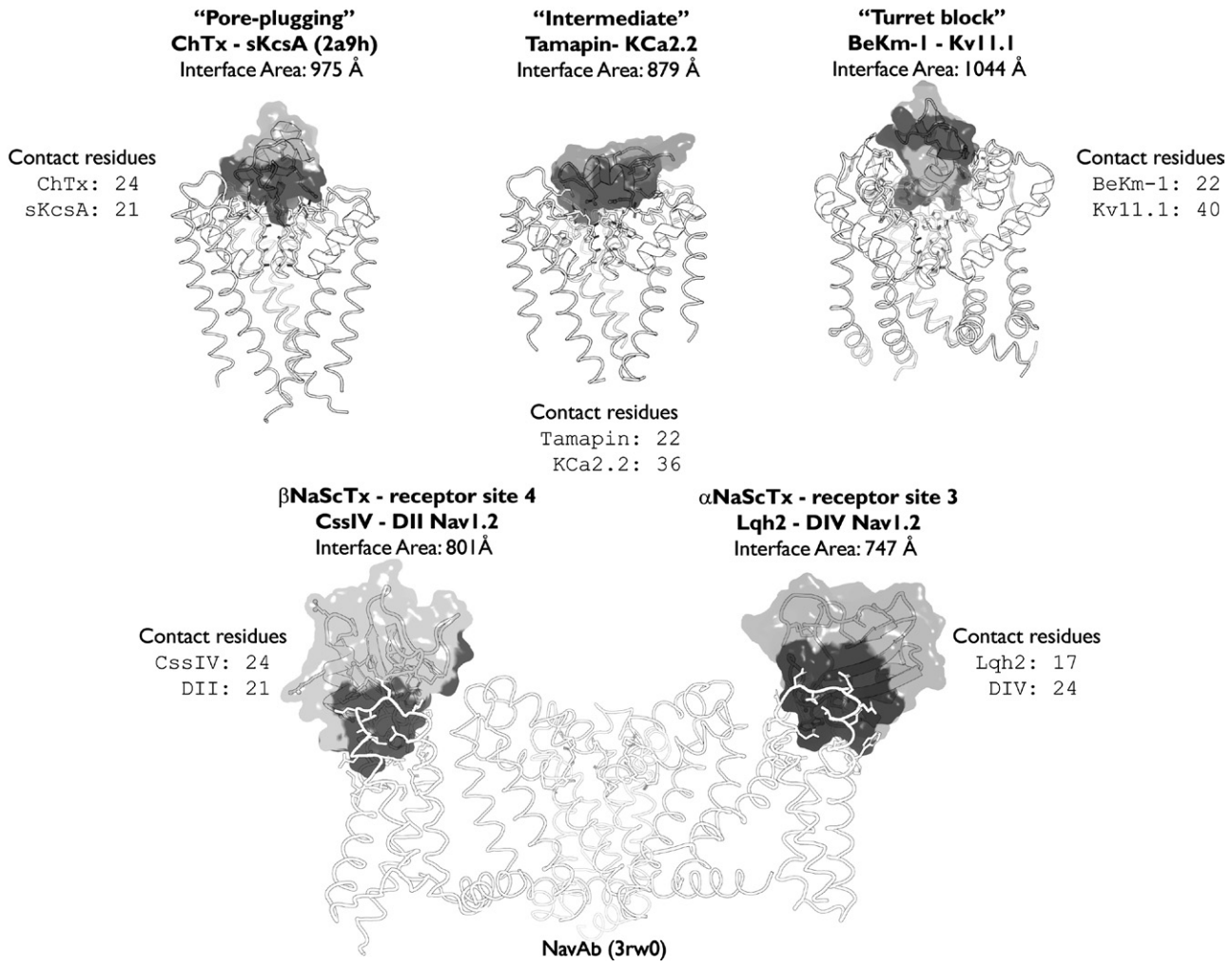

FIGURE 2 Molecular recognition between scorpion venom peptides and ion channels. Models taken from the original publications were displayed with PyMOL and analyzed with PISA (www.ebi.ac.uk/msd-srv/prot_int/pisastart.html). Channels are depicted as cartoon models, side chains are shown for residues making contacts with the scorpion venom peptides (any atom within 5 Å). The peptides are shown as semitransparent surface with embedded cartoon models; side chains of peptide residues important for the interaction are also displayed (Fig. 1), and the interacting area on surface is colored accordingly. Notice that several residues identified as important for NaScTx function are not in contact with the channel in the docking models. Interface details as evaluated in PISA are indicated for each model. ChTx–sKcsA structure was taken from the PDB (2a9h[33]), Tamapin– KCa2.2 model was rebuilt on the basis of the one published in Ref. 1, BeKm-1 – Kv11.1 from Ref. 31, Lqh2 – Domain IV voltage sensor of Nav1.2 from Ref. 32 and CssIV – Domain II voltage sensor of Nav1.2 from Ref. 34 The NaScTx – voltage sensor models were overlaid on NavAb channel structure.[21] The subunits closest to the viewer were removed for the sake of clarity. Templates for the channel modeling were KCa2.2, KcsA (1bl8); Kv11.1, KvAP in an open conformation (1ors); DII voltage sensor Nav1.2 model was based on a closed state model of Kv1.2[34]; DIV voltage sensor Nav1.2, Kv2.1-Kv1.2 chimera (3lut).

but shifted disulfides (Fig. 1) highlight an unforeseen structural versatility of this type of disulfide rich peptides.[26]

One of the main reasons for the large attention received by scorpion venom CS-α/β peptides is the exquisite specificity some could attain against closely related targets; thus, the great interest on defining the molecular basis of their target recognition.[6] For potassium channel blockers, also known as KTx, at least three different modes of interaction have been documented (Fig. 2): (a) in the "pore-plugging mode," ion conduction through the selectivity filter of Kv1, KCa1, and KCa3 channels is blocked by a pharmacophore consisting of a Lys residue assisted by an aromatic side chain located some 7 Å apart, other residues facing the channel vestibule also contribute to the binding[33]; (b) in KCa2 channel blockers, a patch of basic residues makes contacts with the vestibule in the so-called "intermediate" mode, although KTx binding is stabilized by contacts with a negatively charged extracellular loop of the channel,[1] and; (c) the unusual topology of Kv11 channels, the *ether-à-go-go* related gene homologs, are targeted by γKTx toward their binding to an extracellular α-helix, defining the interaction mode called "turret block."[31] Remarkably, a single KTx could bear more than one pharmacophore, as evidenced by the complete structural segregation of the molecular determinants

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for Kv1.1/Kv1.3 and KCa2.1 blockade in a chimeric peptide based on the Kv1.1/Kv1.3-blocking HsTx1 and the KCa2.1-blocking MTx.[25] In spite of the detailed knowledge about potassium channel recognition by KTx, the large structural diversity of scorpion venom peptides that are active on this type of channels suggests that novel interaction modes are yet to be discovered. On the other hand, recent years have witnessed a great advance in our understanding of the molecular basis of scorpion venom peptides active on sodium channels (NaScTx), which have been now finely dissected to the point of generating structural models of the interaction.[32,34] On the basis of their receptor sites and pharmacology, two classes of NaScTx have been described: the α-type binds to receptor site 3, mainly composed by domain IV voltage sensor, provoking an abnormally slow inactivation, and β-type binds to the receptor site 4, largely defined by domain II voltage sensor, which are trapped in their preactivated configuration, thus causing an hyperpolarization shift on the activation potential. The structural models shown in Fig. 2 represent a long waited improvement in our understanding of classical αNaScTx and βNaScTx. However, in these models, not all the residues identified as important for NaScTx function are in contact with the channel; thus, a complete picture of the molecular determinants of the interaction is still missing. Much less is known about the puzzling activities of NaScTx that cannot be classified in α or β classes such as, among several examples, AaHIT4 (binds to both receptor sites), Cn12 (sequence resembles βNaScTx but displays an α-like effect) and the only non Buthidae NaScTx characterized to date Phaiadotoxin (which slightly affects both activation and inactivation of insect channels). Further research will certainly benefit from the more recently solved three-dimensional structure of a voltage-gated sodium channel.[21]

CONCLUDING REMARKS

In spite of the great increase in the number of known scorpion venom peptides, the vast majority of those which have been functionally characterized at any detail fall into two large categories: ion channel modulators and/or membranolytic peptides. The large diversity of novel peptides revealed by proteomic and transcriptomic analyses should lead to the discovery of other interesting functions in the near future. Moreover, it is expected that a much needed larger sampling of scorpion biological diversity and the use of high-throughput functional assays will greatly expand the number and types of scorpion venom peptides for which some biological activity is known. However, it should be kept in mind that the search for biologically active peptides is seldom performed in ecologically relevant models, such as natural prey or predators. Forthcoming research would benefit from a deeper knowledge of the natural context in which the venom evolved and is now used, in such a way that potential targets could be suggested. A combination of large-scale screenings and biologically informed searching would prove critical if we are to understand how venoms have achieved their remarkable complexity.

ACKNOWLEDGMENTS

RC Rodríguez de la Vega's stay at the Muséum National d'Histoire Naturelle is sponsored by the Marie de Paris. Research at LD Possani laboratory is partially supported by a grant from DGAPA-IN24110 of the National Autonomous University of Mexico. The authors are indebted to Dr. V. Yarov-Yarovoy (University of California, Davis) and Prof. H.R. Guy (National Cancer Institute, Bethesda) for their kind sharing of the molecular models used here. Several primary references are available using the accession numbers in Fig. 1. Taxonomic families are the objects of debate; here, we followed the classification currently adopted at NCBI.

REFERENCES

1. Andreotti N, di Luccio E, Sampieri F, De Waard M, Sabatier JM. Molecular modeling and docking simulations of scorpion toxins and related analogs on human SKCa2 and SKCa3 channels. *Peptides* 2005;**26**:1095–108.
2. Batista CV, Román-González SA, Salas-Castillo SP, Zamudio FZ, Gómez-Lagunas F, Possani LD. Proteomic analysis of the venom from the scorpion *Tityus stigmurus*: biochemical and physiological comparison with other Tityus species. *Comp Biochem Physiol C Toxicol Pharmacol* 2007;**146**:147–57.
3. Borchani L, Sassi A, Shahbazzadeh D, Strub JM, Tounsi-Guetteti H, Boubaker MS, et al. Heminecrolysin, the first hemolytic dermonecrotic toxin purified from scorpion venom. *Toxicon* 2011;**58**:130–9.
4. Caliskan F, García BI, Coronas FI, Batista CV, Zamudio FZ, Possani LD. Characterization of venom components from the scorpion *Androctonus crassicauda* of Turkey: peptides and genes. *Toxicon* 2006;**48**:12–22.
5. D'Suze G, Schwartz EF, García-Gómez BI, Sevcik C, Possani LD. Molecular cloning and nucleotide sequence analysis of genes from a cDNA library of the scorpion *Tityus discrepans*. *Biochimie* 2009;**91**:1010–9.
6. Dutertre S, Lewis RJ. Use of venom peptides to probe ion channel structure and function. *J Biol Chem* 2010;**285**:13315–20.
7. Fletcher Jr PL, Fletcher MD, Weninger K, Anderson TE, Martin BM. Vesicle-associated membrane protein (VMAP) cleavage by a new metalloprotease from the Brazilian scorpion *Tityus serrulatus*. *J Biol Chem* 2010;**285**:7405–16.
8. Fry BG, Roelants K, Champagne DE, Scheib H, Tyndall JD, King GF, et al. The toxicogenomic multiverse: convergent recruitment of proteins into animal venoms. *Annu Rev Genomics Hum Genet* 2009;**10**:483–511.
9. Gao B, Xu J, Rodriguez Mdel C, Lanz-Mendoza H, Hernández-Rivas R, Du W, et al. Characterization of two linear cationic antimalarial peptides in the scorpion *Mesobuthus eupeus*. *Biochimie* 2010:92350–9.
10. Gurrola GB, Capes EM, Zamudio FZ, Possani LD, Valdivia HH. Imperatoxin A, a cell-penetrating peptide from scorpion venom, as a probe of ca-release channels/ryanodine receptors. *Pharmaceuticals (Basel)* 2010;**3**:1093–107.
11. Horita S, Matsushita N, Kawachi T, Ayabe R, Miyashita M, Miyakawa T, et al. Solution structure of a short-chain insecticidal

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toxin LaIT1 from the venom of scorpion Liocheles australasiae. *Biochem Biophys Res Commun* 2011;**411**:738–44.
12. Jungo F, Estreicher A, Bairoch A, Bougueleret L, Xenarios I. Animal toxins: how is complexity represented in databases? *Toxins* 2010;**2**:262–82.
13. Ma Y, Zhao R, He Y, Li S, Liu J, Wu Y, et al. Transcriptome analysis of the venom gland of the scorpion *Scorpiops jendeki*: implication for the evolution of the scorpion venom arsenal. *BMC Genomics* 2009;**10**:290.
14. Ma Y, Zhao Y, Zhao R, Zhang W, He Y, Wu Y, et al. Molecular diversity of toxic components from the scorpion *Heterometrus petersii* venom revealed by proteomic and transcriptome analysis. *Proteomics* 2010;**10**:2471–85.
15. Miyashita M, Otsuki J, Hanai Y, Nakagawa Y, Miyagawa H. Characterization of peptide components in the venom of the scorpion Liocheles australasiae (Hemiscorpiidae). *Toxicon* 2007;**50**:428–37.
16. Morgenstern D, Rohde BH, King GF, Tal T, Sher D, Zlotkin E. The tale of a resting gland: transcriptome of a replete venom gland from the scorpion *Hottentotta judaicus*. *Toxicon* 2011;**57**:695–703.
17. Nascimento DG, Rates B, Santos DM, Verano-Braga T, Barbosa-Silva A, Dutra AA, et al. Moving pieces in a taxonomic puzzle: venom 2D-LC/MS and data clustering analyses to infer phylogenetic relationships in some scorpions from the Buthidae family (Scorpiones). *Toxicon* 2006;**47**:628–39.
18. Nisani Z, Hayes WH. Defensive stinging by *Parabuthus transvaalicus* scorpions: risk assessment and venom metering. *Anim Behav* 2011;**81**:627–33.
19. Newton KA, Clench MR, Deshmukh R, Jeyaseelan K, Strong PN. Mass fingerprinting of toxic fractions from the venom of the Indian red scorpion, *Mesobuthus tamulus*: biotope-specific variation in the expression of venom peptides. *Rapid Commun Mass Spectrom* 2007;**21**:3467–76.
20. Oukkache N, Rosso JP, Alami M, Ghalim N, Saïle R, Hassar M, et al. New analysis of the toxic compounds from the *Androctonus mauretanicus* mauretanicus scorpion venom. *Toxicon* 2008;**51**: 835–52.
21. Payandeh J, Scheuer T, Zheng N, Catterall WA. The crystal structure of a voltage-gated sodium channel. *Nature* 2011;**475**:353–8.
22. Poillot C, Dridi K, Bichraoui H, Pêcher J, Alphonse S, Douzi B, et al. Maurocalcine, a pharmacologically inert efficient cell-penetrating peptide analogue. *J Biol Chem* 2010;**285**:34168–80.
23. Quintero-Hernández V, Ortiz E, Rendon MR, Schwartz EF, Becerril B, Corzo G, et al. Scorpion and spider venom peptides: gene cloning and peptide expression. *Toxicon* 2011;**58**:644–63.
24. Rates B, Ferraz KK, Borges MH, Richardson M, De Lima ME, Pimenta AM. Tityus serrulatus venom peptidomics: assessing venom peptide diversity. *Toxicon* 2008;**52**:611–8.
25. Regaya I, Beeton C, Ferrat G, Andreotti N, Darbon H, De Waard M, et al. Evidence for domain-specific recognition of SK and Kv channels by MTX and HsTx1 scorpion toxins. *J Biol Chem* 2004;**279**:55690–6.
26. Saucedo AL, Flores-Solis D, Rodríguez de la Vega RC, Ramírez-Cordero B, Hernández-López R, Cano-Sánchez P. et al.New tricks of an old fold: structural versatility of scorpion toxins with common cysteine spacing. *J Biol Chem* 2012;**287**:12321–30.
27. Schwartz EF, Diego-Garcia E, Rodríguez de la Vega RC, Possani LD. Transcriptome analysis of the venom gland of the Mexican scorpion *Hadrurus gertschi* (Arachnida: Scorpiones). *BMC Genomics* 2007;**8**:119.
28. Schwartz EF, Camargos TS, Zamudio FZ, Silva LP, Bloch Jr C, Caixeta F, et al. Mass spectrometry analysis, amino acid sequence and biological activity of venom components from the Brazilian scorpion *Opisthacanthus cayaporum*. *Toxicon* 2008;**51**:1499–508.
29. Silva EC, Camargos TS, Maranhão AQ, Silva-Pereira I, Silva LP, Possani LD, et al. Cloning and characterization of cDNA sequences encoding for new venom peptides of the Brazilian scorpion *Opisthacanthus cayaporum*. *Toxicon* 2009;**54**:252–61.
30. Smith JJ, Hill JM, Little MJ, Nicholson GM, King GF, Alewood PF. Unique scorpion toxin with a putative ancestral fold provides insight into evolution of the inhibitor cystine knot motif. *Proc Natl Acad Sci USA* 2011;**108**:10478–83.
31. Tseng GN, Sonawane KD, Korolkova YV, Zhqng M, Liu J, Ghrishin EV, et al. Probing the outer mouth structure of the HERG channel with peptide toxin fingerprinting and molecular modeling. *Biohys J* 2007;**92**:3524–40.
32. Wang J, Yarov-Yarovoy V, Kahn R, Gordon D, Gurevitz M, Scheuer T, et al. Mapping the receptor site for {alpha}-scorpion toxins on a Na+ channel voltage sensor. *Proc Natl Acad Sci USA* 2011;**108**:15426–31.
33. Yu L, Sun C, Song D, Shen J, Xu N, Gunasekera A, et al. Nuclear magnetic resonance structural studies of a potassium channel-charybdotoxin complex. *Biochemistry* 2005;**44**:15834–41.
34. Zhang JZ, Yarov-Yarovoy V, Scheuer T, Karbat I, Cohen L, Gordon D, et al. Structure–function map of the receptor site for {beta}-scorpion toxins in domain ii of voltage-gated sodium channels. *J Biol Chem* 2011;**286**:33641–51.
35. Zhao R, Ma Y, He Y, Di Z, Wu Y, Cao Z, et al. Comparative venom gland transcriptome analysis of the scorpion *Lychas mucronatus* reveals intraspecific toxic gene diversity and new venomous components. *BMC Genomics* 2010;**11**:452.

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Chapter 60

Sea Anemone Peptides

Raymond S. Norton

ABSTRACT

Sea anemone venoms continue to afford a range of interesting new peptides directed against a range of biological targets. The first complete genome of a sea anemone was described recently, and its complement of toxins has been subject to various analyses. Concurrently, expressed sequence tag analyses of other anemones have identified many putative peptide toxins, while transcriptomic analyses may be expected to identify even more in the near future. Venom proteomic analyses are also revealing novel peptide toxins. Of those peptides that have been isolated, synthesized, or expressed, and then characterized functionally, the majority interact with sodium or potassium channels, although peptides binding to other targets are beginning to emerge, including one that modulates TRPV1 activity. Some of these peptides, in particular those capable of selectively blocking certain potassium channels, are being developed as new therapeutic leads.

INTRODUCTION

Since the publication in 2006 of the first edition of the *Handbook of Biologically Active Peptides*, sea anemones have continued to yield interesting new peptides with a range of biological activities, while genomic analyses have identified an ever-expanding array of candidate peptides. This chapter documents these new findings and comments on other exciting developments relevant to sea anemone peptides.

Cytolysins of the actinoporin family were described in the corresponding chapter of the first edition[18] but will not be further elaborated upon here. Significant progress has been made in understanding the structure and function of these toxins[11] although the molecular details of the mechanism by which they so potently form pores in target membranes remain elusive. Homologs of the actinoporins have been found in other organisms, predominantly fish,[11] and another related protein was found more recently in the foot muscle of a brackish-water clam, where it is unlikely to function as a toxin for prey capture. An intriguing aspect of these cytolysins that has come to light is their similarity to components of some pore-forming complexes in higher organisms.

SEA ANEMONE GENOME

The first genome of a sea anemone, the starlet one *Nematostella vectensis*, published in 2007;[23] showed extensive conservation in gene content, structure, and organization between this eumetazoan and vertebrates and has further established it as a useful model organism for developmental and evolutionary biology. An analysis by Anctil[1] reveals numerous orthologs of enzymes and nicotinic receptors associated with cholinergic function, an even larger number of genes encoding enzymes, receptors and transporters for glutamatergic, GABAergic and aminergic transmission, two orthologs of purinergic receptors, four adenosine-like receptors and one nitric oxide synthase. No muscarinic acetylcholine receptors were identified and a large number of melatonin-related, but not serotonin, orthologs were found. Genes encoding prepropeptides and receptors related to RFamide, tachykinin, galanin, gonadotropin-releasing hormones, vasopressin/oxytocin, melanocortins, insulin-like peptides, glycoprotein hormones, and uniquely cnidarian peptide families were also identified. Matching the unique cnidarian peptides with their receptors will be a challenging but rewarding undertaking.

The genome of *N. vectensis* has also provided the opportunity to analyze gene families encoding peptide toxins. Surprisingly, this genome contains 13 putative genes encoding the same peptide, Nv1,[14] which belongs to the Type-1 sea anemone class of neurotoxins;[17] each of these genes consists of two exons separated by an intron, with the first exon encoding a leader sequence and a portion of the pro region and the second encoding the remaining pro region and the mature peptide. Nv1 was expressed in *Escherichia coli* and, as expected for this class of toxin, it inhibits the inactivation of voltage-gated sodium channels (VGSCs).[16] Nv1 shows high specificity for insect over mammalian VGSCs, suggesting that it may act on VGSCs in marine arthropods. Some residues functionally

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00060-9
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important in Av2 (aka ATX II) from *Anemonia viridis* (formerly known as *Anemonia sulcata*) are not present in Nv1,[16] although some of the key residues in Type-I anemone toxins[17] are conserved in Nv1.

Transcript analysis indicate that multiple Nv1 loci are transcribed at all developmental stages of *N. vectensis*, but transcripts are spliced only in the polyp stage,[16] making it unlikely that the embryo and planula stages produce Nv1 and providing a rare example of intron retention in the early developmental stages of this cnidarian.

The genome of *N. vectensis* also codes for four peptide domains with clear sequence similarity (28–41% identity) to the potassium channel blocker ShK toxin.[14] None of these sequences contains the KY functional dyad known to be important for K^+-channel blockade;[18,19] although similar elements are evident (QY, KW, TY, RF). The targets of these peptides have not yet been identified. Numerous ShK-like domains are found in nature, both as stand-alone peptides and domains within larger proteins,[24] and the functions of most of them remain to be clarified. As with the peptides encoded by the *N. vectensis* genome, many of the ShK-like domains found in other organisms lack the KY dyad. In common with many toxin folds, the ShK structure is clearly a robust scaffold on which a diverse range of functional epitopes can be displayed.

OTHER GENOMICS ANALYSES

Although *N. vectensis* is the only sea anemone for which the genome has been fully documented, useful insights are emerging from focused sequence comparisons in other species. For example, although the amino acid sequences of Av2 and AP-A (from *Anthopleura xanthogrammica*, and *A. elegantissima*) are highly similar,[17] Moran et al.[15] noted that their signal peptides differ, with that of AP-A being very similar to those of other Type-I toxins, whereas that of Av2 is closely related to the signal peptide of Av7, a Type-III toxin. They propose that the ancestor gene of Av2 and AP-A contains a signal peptide similar to those of AP-A and other Type-I toxins, and that subsequently the ancestor gene of Av2 lost this signal peptide sequence and acquired the signal peptide of Av7 in a lineage-specific manner. The fact that Av7 has a more complex gene structure than that of Av2 provides further support to the suggestion that a Type-III neurotoxin gene might have been the donor of the 5′-UTR and the region encoding the signal peptide of Type-1 neurotoxins in *A.viridis*.

Expressed sequence tags or ESTs are short segments of cDNA sequences that effectively provide a snapshot of the transcriptome of a particular tissue at a given time. Kozlov and Grishin[10] describe the use of single residue distribution analysis (SRDA) to identify polypeptide toxins in the EST database of the symbiotic anemone *Anemonia viridis*.[25] Searches of nearly 40,000 ESTs were undertaken using motifs based on key Cys residues and other motifs targeting the actinoporin family. New sequences and sequences with high homology to the already described toxins were identified and the precursor sequences were determined for two known toxins. Homologous peptides that differ by single or multiple amino acid substitutions were also found, as expected for venom components. In addition to 23 previously reported peptide toxins, 43 novel sequences were discovered, including short peptides with regulatory neuronal function and several groups of toxin-like polypeptides. As an example of the weaknesses and strengths of EST analyses, the known K^+-channel blocker kaliseptin (AsK) is not found in the library, although 11 similar peptides are.[10]

Shiomi et al.[31] used molecular cloning to elucidate the primary structures of Type-1 K^+-channel toxins, based on the sequences of cDNAs encoding representative K^+-channel toxins from the families Stichodactylidae and Actiniidae (Type-2 K^+-channel toxins are related to the Kunitz-type protease inhibitors and Type-3 toxins include the shorter BDS family,[8] as illustrated in Fig. 1). Twenty-one species of sea anemones were screened for Kv1 toxins by competitive inhibition of the binding of ^{125}I-α-dendrotoxin to rat synaptosomal membranes, with 11 species (two Actiniidae, one Hormathiidae, five Stichodactylidae, and three Thalassianthidae) found to be positive. Full-length cDNAs from three species of Stichodactylidae and three species of Thalassianthidae were cloned by a combination of RT-PCR and 3′- and 5′-RACE. The six toxins identified share > 90% sequence identity with one another and with ShK (Fig. 1). Putative signal and prosequences were also determined, the latter ending with a Lys-Arg pair.

By using a functional assay to guide the selection of species for cDNA analysis,[31] this study partially addressed one of the major issues associated with genomic analyses, namely, whether a peptide encoded by the genome is expressed at significant levels, and, if so, where and when in the life cycle of that species. An additional challenge is the identification of posttranslational modification, although this seems to be much less important for sea anemone peptides than for their counterparts in cone snails. For example, among the peptide sequences documented in Fig. 1, the only posttranslational modification identified is hydroxylation of Pro residues, and even that occurs in only three of those peptides, and only once in each. As a complement to genomic analyses, in-depth proteomic analyses of venoms are required to confirm the presence of particular peptides, while binding, electrophysiological, and other functional assays are required on naturally occurring, synthetic or recombinant peptides to characterize their targets and actions.

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Type 1 (K1)

Family	Toxin		
Stichodactylidae	ShK	P29187 (TXSHK_STOHE)	RSCIDTIPKSRC....TAFQCKHSMKYRLSFCRKTCGTC
	HmK	O16846 (TXHMK_RADMG)	RTCKDLIPVSEC....TDIRCRTSMKYRLNLCRKTCGSC
Thalassianthidae	CaK	E2S064 (E2S064_CRYAD)	RTCIDTIPKSRC....TAFQCKNSMKYRLSFCRKTCGTC
	TaK	E2S066 (E2S066_THAAS)	RTCIDTIPKSRC....TAFQCKHSMKYRLSFCRKTCGTC
Actiniidae	AETX K	Q0EAE5 (TXK_ANEER)	ACKDYLPKSEC....TQFRCRTSMKYKYTNCKKTCGTC
	AeK	P81897 (TXAEK_ACTEQ)	GCKDNFSANTCKHVKANNNCG.SQKYATN.CAKTCGKC
	BgK	P29186 (TXBGK_BUNGR)	VCRDWFKETACRHAKSLGNCRTSQKYRAN.CAKTCELC

Type 2 (K2)

AsKC 1 (kalicludine1) Q9TWG0 (KC1_ANESU) INKDCLLPMDVGRCRASHPRYYYNSSSKRCEKFIYGGCRGNANNFHTLEECEKVCGVR

APEKTx1 P86862 (APKT1_ANTEL) INSICLLPKKQGFCRARFPRFYYNSSTRRCEMFYYGGCGGNANNFNTLEECEKVCLGYGEAWKAP

Type 3 (K3)

BDS-I	P11494 (BDS1_ANESU)	AAPCFCSGKPGRGDLWILRGTCPGGYGYTSNCYKWP.NICCYPH
BDS-II	P59084 (BDS2_ANESU)	AAPCFCPGKPDRGDLWILRGTCPGGYGYTSNCYKWP.NICCYPH
APETx1	P61541 (APT1_ANTEL)	GTTCYC.GKT.IGIYWFGTKTCPSNRGYTGSCGYFLG.ICCYPVD
Am II	P69930 (TXAM2_ANTMC)	ALLSCRCEGKTEYGDKWLFHGGCONNYGYNYKCFMKPGAVCCYPQN

Type 4 (K4)

SHTX I	P0C7W7 (SHT12_STIHA)	XIIGAOCRRCYHSDGKGGCVRD.WSCGQQ
SHTX II	P0C7W7 (SHT12_STIHA)	XIIGAPCRRCYHSDGKGGCVRD.WSCGQQ
Bcg III	P86467 (TX234_BUNCN)	NIIDVPCRPCYYRDSSGNCVYDQLGCGA
Am I	P69929 (TXAM1_ANTMC)	NVAVPOCGDCYQQVGNT.CVRVPSLCPS

FIGURE 1 Representative K^+-channel toxin sequences from sea anemones.[4,19,29,31] UniProt accession numbers and entry names are given. Of the Type-1 toxins, ShK is from *Stichodactyla* (formerly *Stoichactis*) *helianthus*, HmK from *Heteractis* (formerly *Radianthus*) *magnifica*, CaK from *Cryptodendrum adhaesivum*, TaK from *Thalassianthus* aster, AETX K from *Anemonia erythraea*, AeK from *Actinia equina* and BgK from *Bunodosoma granulifera*. The KY functional dyad is underlined in the Type-1 toxins. The Type-2 toxin AsKC 1 is from *Anemonia sulcata* (now *Anemonia viridis*) and APEKTx1 from *Anthopleura elegantissima*. The Type-3 toxins illustrated are BDS-I and -II from *Anemonia sulcata*, APETx1 from *Anthopleura elegantissima* and Am II from *Antheopsis maculata*. Am II contains a Gly-Arg extension at the C-terminus that is believed to be removed from the precursor protein during processing. The entry for Am II is boxed to highlight the fact that the target channel for this toxin has not yet been identified,[29] even though its sequence (and presumably structural) similarity to the other Type 3 K^+-channel toxins shown here suggests that its target may be one of the specialist K^+-channels affected by those toxins. Of the Type-4 toxins, SHTX I and II are from *Stichodactyla haddoni*, Bcg III from *Bunodosoma cangicum* and Am I from *Antheopsis maculata*. Unidentified N-terminal residues for SHTX I and II and hydroxyPro for position 6 of SHTX I and Am I are denoted by 'X' and 'O', respectively.[7] The amino acid sequence shown for Bcg III is that deposited in UniProt (P86467; TX234_BUNCN); this differs slightly in the C-terminal region from the sequence for Bcg 23.41 shown in Figure 2 of Zaharenko et al.[33] The effects of Bcg 23.41 on crab leg nerve compound action potentials are similar to those of the Type-1 K^+-channel blocker ShK, although Bcg 23.41 is much less potent.[33] The entry for Am I is boxed to highlight that it probably acts on Na^+- rather than K^+-channels; it is lethal to crabs (LD_{50} 830 μg/kg) although much less potently than other anemone Na^+-channel toxins. The amino acid sequence shown here for Am I is taken from Figure 2 of Honma et al.[7]

NEW PEPTIDES FROM ANEMONES

The family tree of peptide toxins from sea anemones is dominated by those acting on Na^+-channels, including VGSC and ASIC channels.[3,8,19,30] Fewer K^+-channel toxins have been identified, although the number is increasing rapidly.[4,31] Honma and Shiomi[8] categorize the K^+-channel toxins as Type-1, exemplified by ShK (Fig. 1), Type-2, which are structural homologs of the Kunitz-type protease inhibitors and often retain protease inhibitory activity, and Type-3, represented by BDS-I and -II and APETx1, with 41 or 42 residues. To avoid confusion with sea anemone-derived Na^+-channel toxins and other anemone venom peptides, this nomenclature could perhaps be modified to Types K1, K2, and K3, but the original designations will be retained in this chapter. In any case, this classification has been extended already to Type-4[4] to accommodate two homologous peptides, SHTX I and II, from *S. haddoni*, which have 28 residues and two disulfide bridges (Fig. 1)[7] and one from *Bunodosoma cangicum*.[33] SHTX I and II are not lethal to crabs at 1000 μg/kg, although SHTX II inhibits the binding of ^{125}I-α-dendrotoxin to synaptosomal membranes, albeit weakly, and was therefore classified as a K^+-channel toxin.[7]

Shiomi et al. reported the isolation and cDNA cloning of a Type-1 K^+-channel toxin (AETX K) from *Anemonia erythraea*.[6] AETX K is an interesting member of the family Actiniidae, with a sequence much more similar to those of ShK and HmK (i.e., lacking the four-residue insert after the second Cys) than other representatives from this family, such as AeK and BgK (Fig. 1).

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As shown in Fig. 1, Type-1 K^+-channel toxins lacking the four-residue insert come mostly from anemones of the families Stichodactylidae and Thalassianthidae, while other representatives from the Actiniidae family include this insert.[6,31]

A new Type-2 K^+-channel blocker has been reported by Tytgat et al.[22] APEKTx1 from *Anthopleura elegantissima*, containing 63 residues and three disulfide bridges (Fig. 1), selectively blocks Kv1.1 channels with an IC_{50} of 0.9 nM, as well as inhibiting trypsin with a K_d of 124 nM. It is not clear whether the trypsin inhibitory activity of APEKTx1 (or any of the other Type-2 K^+-channel toxins) is physiologically important, but its selectivity for Kv1.1 over a wide range of other K^+-channels tested is intriguing.

APETx1, which acts on voltage-gated human ether-a-go-go-related gene (hERG) K^+-channels, was first isolated from *Anthopleura elegantissima* about a decade ago, but more recently, its mechanism of action and binding site have been characterized further.[35] Its effects are similar to those of known gating modifier toxins (e.g., hanatoxin) on the Kv2.1 channel, shifting the voltage-dependence of hERG activation in the positive direction and suppressing current amplitudes elicited by strong depolarizing pulses that maximally activate the channels. Its binding site is distinctly different from that of pore-blocking toxins; APETx1 seems to interact with the hERG channel in the vicinity of the S3b-S4 region of the channel.

BcIV, a new paralyzing peptide obtained from *Bunodosoma caissarum*,[21] contains six Cys residues that presumably form three disulfide bonds. BcIV has 45 and 48% identity to APETx1 and APETx2, respectively, from *Anthopleura elegantissima* and 42% with Am II and BDS-I and -II from *Antheopsis maculata* and *Anemonia sulcata*, respectively. The target is yet to be identified but is proposed to be a Na^+-channel. Despite its sequence similarity to K^+-channel toxins, BcIV does not seem to act via these channels although further assays are clearly warranted to resolve this.

A detailed investigation has been undertaken subsequently into the effects of Bc III, also from *Bunodosoma caissarum*, on sodium currents in rat dorsal root ganglia;[27] as expected, increases are observed in the sodium current inactivation time course and current peak amplitude. Evidence was also found for this and two related anemone toxins that the binding affinity may be reduced in a voltage-dependent manner, as observed for scorpion α-toxins.

Several new Na^+-channel toxins have also been characterized. Four Type-II Na^+-channel toxins have been described from three species of sea anemones (*Cryptodendrum adhaesivum*, *Heterodactyla hemprichii*, and *Thalassianthus aster*) belonging to the family Thalassianthidae.[12] Extracts of all three species exhibit potent lethality to crabs, and all of the isolated toxins show this activity. These toxins were named according to the systematic nomenclature proposed by King et al.,[9] as follows: δ-TLTX-Ca1a, δ-TLTX-Hh1a and δ-TLTX-Ta1a.

AP-C (ApC) is by no means a new peptide, having been described first in 1981,[17] but its effects on neuronal sodium currents have now been documented.[26] As expected, AP-C delays Na^+-channel inactivation, but with an IC_{50} of 1.25±0.9 μM on mammalian neurons, making it more potent than other Type-I Na^+-channel toxins (e.g., ATX II (AsII) 9.6 μM, BgII 4.1 μM and BgIII 11.9 μM).

Proteomics has been applied to the neurotoxic fraction from *Bunodosoma cangicum*, with peptides being fractionated by RP-HPLC, identified by ESI and MALDI-TOF mass spectrometry, and, in some cases, sequenced by Edman methods.[33] Of those sequenced partially or completely, three are Type-I Na^+-channel toxins, three are Type 3 K^+-channel toxins, and three have masses around 3kDa and show no sequence similarity to known anemone toxins. Although these peptides show clear sequence homology to one another, the numbers and locations of their Cys residues vary, with two of the peptides containing two putative disulfides and the third just one. Testing of one of these on crab leg nerve compound action potentials gives results suggestive of K^+-channel blockade, but further studies are clearly required to clarify the activity and classification of these peptides.

Oliveira et al.[20] investigated the selectivity of three Type-I Na^+-channel toxins (ATX II, AFT II from *Anthopleura fuscoviridis* and Bc III from *Bunodosoma caissarum*) on $Na_V1.1$ to $Na_V1.6$ channels expressed in HEK293 cells. The detailed electrophysiological characterization provides a useful guide for choosing which toxin to use to study a given Na_V1 subtype, and highlights the importance of individual residues in this family of toxins, with the substitution of Lys36 in ATX II for Ala in AFT II having significant effects on some aspects of the channel response but not others.

A similar approach was taken to characterize cangitoxin II (CGTX II) and two new Na^+-channel toxins, δ-AITX-Bcg1a and δ-AITX-Bcg1b, all from *Bunodosoma cangicum*.[34] The selectivity of these toxins is the highest for $Na_V1.5$, followed by that of $Na_V1.6$ and 1.1. The authors also speculate that the binding site for these toxins is not restricted to the putative site 3, which lies between segments S3 and S4 of domain IV of the Na^+-channel.

There are some discrepancies in the activities reported for the cangitoxin family. These are discussed in the report by Zaharenko et al.[32] that describes the isolation and characterization of cangitoxins II and III, which differ from one another in an Arg to His substitution at position 14, and from cangitoxin itself by an Asn to Asp substitution

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at position 16. In view of the nearly identical sequences of cangitoxin and cangitoxins II and III, it seems highly unlikely that cangitoxin could have a different biological activity profile from cangitoxins II and III.

Grishin et al.[2] identified a potentially interesting peptide in *Heteractis crispa* collected from the Seychelles islands. APHC1 is a 56-residue peptide with high sequence similarity to the Kunitz-type protease inhibitor family, which includes not only bovine pancreatic trypsin inhibitor and anemone-derived serine protease inhibitors but also dendrotoxin-α and Type-2 K^+-channel blockers from sea anemones such as kalicludine. APHC1 is a modulator of TRPV1 channels, causing partial antagonism of capsaicin-induced currents (32% inhibition) with an EC_{50} of 54 nM (capsaicin being a selective agonist of the TRPV1 receptor). *In vivo*, APHC1 at 0.1 mg/kg significantly prolongs tail-flick latency and reduces capsaicin-induced acute pain. Thus, APHC1 should be a useful tool for further studies of TRPV1, particularly if mutants could be found that blocked more of the current.

Finally, we draw attention to the two new peptide toxins from the acrorhagi of *Actinia equina*[8] These organs, which are found in anemones from the family Actiniidae, form a ring around the base of the tentacles and are used not for defense or the capture of prey but rather in intra-specific aggression. By following crab toxicity, Honma et al. isolated acrorhagins I and II (50 and 44 residues, respectively) and determined their amino acid sequences from their cDNA. Acrorhagin I shows no sequence homology with any known toxins, whereas acrorhagin II shows limited similarity with hainantoxin-I from the Chinese bird spider *Selenocosmia hainana*, ω-conotoxin MVIIB from the cone snail *Conus magus* and Tx 3-2 from the Brazilian armed spider *Phoneutria nigriventer*, although the locations of the six Cys residues are not fully conserved between acrorhagin II and the other three toxins. Thus, the acrorhagi may represent a new source of potentially interesting peptide toxins although it is clear already that not all species with acrorhagi contain toxins.[29] Several protease inhibitors have also been isolated from acrorhagi of *Actinia equina*, *Anthopleura xanthogrammica*, and *Anthopleura fuscoviridis*.[29]

STRUCTURES

The solution structure has been determined for CgNa, a 47 residue toxin from the giant Caribbean anemone *Condylactis gigantea*.[28] The structure displays significant homology with both Type-I and Type-II anemone toxins,[17,19] although several unique residues create negatively charged surface patches that disrupt a surface-exposed cluster of hydrophobic residues present in other anemone-derived toxins described to date. CgNa delays the inactivation of tetrodotoxin-sensitive VGSCs.

The Type-III Na^+-channel toxins PaTX, Da I and II (from *Dofleinia armata*) and Er I (from *Entacmaea ramsayi*) all have eight Cys residues, and presumably four disulfide bridges,[8] whereas ATX III (As III, Av3) has only three disulfides, even though there is clear sequence similarity between it and the other four peptides. There is ample precedent for the inclusion of a fourth disulfide without modifying the structural scaffold determined by the first three disulfides. The structure of one of the four disulfide members of this family is eagerly awaited for comparison with the structure already determined for ATX III.[19]

As noted previously,[19] the structures of BDS-I, APETx1, and APETx2 are similar to those of the Na^+-channel toxins such as AP-A but quite different from the ShK/BgK family of K^+-channel toxins. Sea anemones are capable of using a common structural scaffold to create blockers of distinct targets (AP-A, APETx1 and APETx2 act on VGSC, HERG, and ASIC channels, respectively), while also using different scaffolds (all-β in APETx1 vs all-α in ShK) to block similar channels (HERG and Kv1, respectively).

The recent crystal structure of a bacterial sodium channel provides hope that structures will follow for VGSCs from higher organisms, as has proven to be the case for potassium channels. The availability of these structures, or at the very least of improved models based on crystal structures from lower organisms, will pave the way for a detailed understanding of the sites of interaction of sea anemone toxins with mammalian VGSCs. Complementary mutagenesis studies, where the effects of concerted mutations of residues on both the toxin and the channel are quantified, will also clarify the energetic contributions of pairwise interactions to the free energy of binding. Studies of this sort will provide an excellent basis for understanding the vast body of knowledge on the roles of individual residues in the Type-I and Type-II Na^+-channel toxins that has accumulated over several decades from amino acid sequence comparisons, chemical modification studies, and, most recently, site-directed mutation of recombinantly expressed peptides.[3,13,17,30]

THERAPEUTIC APPLICATIONS

The exciting prospects for development of ShK and synthetic analogs as selective immunosuppressants for the treatment of autoimmune diseases such as multiple sclerosis, rheumatoid arthritis, and type-1 diabetes mellitus have been reviewed.[5] The basis for this activity is that $CCR7^-$ effector memory T (T_{EM}) cells express Kv1.3 channels that play a major role in their activation, and blocking these channels preferentially inhibits the activation of these cells, with little or no effects on $CCR7^+$ naïve and central memory T cells. ShK, a 35-residue peptide isolated from the Caribbean sea anemone *Stichodactyla*

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helianthus (Fig. 1), blocks Kv1.3 channels at pM concentrations, albeit with little selectivity for Kv1.3 over closely related Kv1 channels. Extensive mutagenesis studies combined with elucidation of the structure of ShK forms the basis for models of ShK docked with Kv1.3, which leads in turn to the development of new analogs with improved selectivity and increasing stability. One of these analogs, ShK-186, is scheduled to begin first-in-man phase-1 trials.

The actinoporins have been evaluated as the toxic warheads of immunotoxins directed against tumor cells and in various other biomedical applications.[11] The initial promise of the anthopleurins and related Type-I Na^+-channel toxins in the cardiovascular field has not been fulfilled, in part because of arhythmogenic activity in the heart.

To date, there is a paucity of examples of therapeutic or biomedical applications of sea anemone venom peptides. Moreover, the diversity of peptides found in these venoms does not match with those of cone snails or spiders. However, as pointed out by Honma and Shiomi,[8] only some 40 of the >800 recorded species of sea anemones have been investigated in any detail. The number of species investigated has increased since then, but there is every reason for optimism that the remaining 90% of species will provide interesting new peptides, some of which will no doubt have therapeutic potential. The application of state-of-the-art transcriptomics and the availability of complete genome sequences for other species of sea anemones will greatly accelerate the identification of such peptides, and continual improvements in the efficiency of peptide synthesis and recombinant expression will facilitate the production of such peptides for biological evaluation, although the challenge will remain to define the native disulfide connectivities for novel sequences containing multiple cysteine residues.

ACKNOWLEDGMENTS

The author dedicates this chapter to colleagues Laszlo Béress and Ted Norton, whose enthusiasm for sea anemone toxins helped stimulate the author's enduring interest in this field.

REFERENCES

1. Anctil M. Chemical transmission in the sea anemone *Nematostella vectensis*: a genomic perspective. *Comp Biochem Physiol Part D Genomics Proteomics* 2009;**4**:268–89.
2. Andreev YA, Kozlov SA, Koshelev SG, Ivanova EA, Monastyrnaya MM, Kozlovskaya EP, et al. Analgesic compound from sea anemone *Heteractis crispa* is the first polypeptide inhibitor of vanilloid receptor 1 (TRPV1). *J Biol Chem* 2008;**283**:23914–21.
3. Bosmans F, Tytgat J. Sea anemone venom as a source of insecticidal peptides acting on voltage-gated Na^+ channels. *Toxicon* 2007; **49**:550–60.
4. Castañeda O, Harvey AL. Discovery and characterization of cnidarian peptide toxins that affect neuronal potassium ion channels. *Toxicon* 2009;**54**:1119–24.
5. Chi V, Pennington MW, Norton RS, Tarcha E, Londono L, Sims-Fahey B, et al. Development of a sea anemone toxin as an immunomodulator for therapy of autoimmune diseases. *Toxicon* 2012;**59**:529–46.
6. Hasegawa Y, Honma T, Nagai H, Ishida M, Nagashima Y, Shiomi K. Isolation and cDNA cloning of a potassium channel peptide toxin from the sea anemone *Anemonia erythraea*. *Toxicon* 2006;**48**:536–42.
7. Honma T, Kawahata S, Ishida M, Nagai H, Nagashima Y, Shiomi K. Novel peptide toxins from the sea anemone *Stichodactyla haddoni*. *Peptides* 2008;**29**:536–44.
8. Honma T, Shiomi K. Peptide toxins in sea anemones: structural and functional aspects. *Marine Biotech* 2006;**8**:1–0.
9. King GF, Gentz MC, Escoubas P, Nicholson GM. A rational nomenclature for naming peptide toxins from spiders and other venomous animals. *Toxicon* 2008;**52**:264–76.
10. Kozlov S, Grishin E. The mining of toxin-like polypeptides from EST database by single residue distribution analysis. *BMC Genomics* 2011;**12**:88.
11. Kristan KC, Viero G, Dalla Serra M, Macek P, Anderluh G. Molecular mechanism of pore formation by actinoporins. *Toxicon* 2009;**54**:1125–34.
12. Maeda M, Honma T, Shiomi K. Isolation and cDNA cloning of type 2 sodium channel peptide toxins from three species of sea anemones (*Cryptodendrum adhaesivum*, *Heterodactyla hemprichii* and *Thalassianthus aster*) belonging to the family Thalassianthidae. *Comp Biochem Physiol B Biochem Mol Biol* 2010;**157**: 389–93.
13. Moran Y, Gordon D, Gurevitz M. Sea anemone toxins affecting voltage-gated sodium channels-molecular and evolutionary features. *Toxicon* 2009;**54**:1089–101.
14. Moran Y, Gurevitz M. When positive selection of neurotoxin genes is missing. The riddle of the sea anemone *Nematostella vectensis*. *FEBS J* 2006;**273**:3886–92.
15. Moran Y, Weinberger H, Lazarus N, Gur M, Kahn R, Gordon D, et al. Fusion and retrotransposition events in the evolution of the sea anemone *Anemonia viridis* neurotoxin genes. *J Mol Evol* 2009;**69**: 115–24.
16. Moran Y, Weinberger H, Reitzel AM, Sullivan JC, Kahn R, Gordon D, et al. Intron retention as a posttranscriptional regulatory mechanism of neurotoxin expression at early life stages of the starlet anemone *Nematostella vectensis*. *J Mol Biol* 2008;**380**:437–43.
17. Norton RS. Structure and structure-function relationships of sea anemone proteins that interact with the sodium channel. *Toxicon* 1991;**29**:1051–84.
18. Norton RS. Sea anemone venom peptides. In: Kastin AJ, editor. *Handbook of biologically active peptides*. San Diego: Elsevier (Academic); 2006. p. 363–7.
19. Norton RS. Structures of sea anemone toxins. *Toxicon* 2009;**54**: 1075–88.
20. Oliveira JS, Redaelli E, Zaharenko AJ, Cassulini RR, Konno K, Pimenta DC, et al. Binding specificity of sea anemone toxins to Nav 1.1–1.6 sodium channels: unexpected contributions from differences in the IV/S3–S4 outer loop. *J Biol Chem* 2004;**279**:33323–35.
21. Oliveira JS, Zaharenko AJ, Ferreira Jr WA, Konno K, Shida CS, Richardson M, et al. BcIV, a new paralyzing peptide obtained from the venom of the sea anemone *Bunodosoma caissarum*. A comparison with the Na^+ channel toxin BcIII. *Biochim Biophys Acta* 2006;**1764**:1592–600.

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22. Peigneur S, Billen B, Derua R, Waelkens E, Debaveye S, Beress L, et al. A bifunctional sea anemone peptide with Kunitz type protease and potassium channel inhibiting properties. *Biochem Pharmacol* 2011;**82**:81–90.
23. Putnam NH, Srivastava M, Hellsten U, Dirks B, Chapman J, Salamov A, et al. Sea anemone genome reveals ancestral eumetazoan gene repertoire and genomic organization. *Science* 2007;**317**:86–94.
24. Rangaraju S, Khoo KK, Feng ZP, Crossley G, Nugent D, Khaytin I, et al. Potassium channel modulation by a toxin domain in matrix metalloprotease 23. *J Biol Chem* 2010;**285**:9124–36.
25. Sabourault C, Ganot P, Deleury E, Allemand D, Furla P. Comprehensive EST analysis of the symbiotic sea anemone, *Anemonia viridis*. *BMC Genomics* 2009;**10**:333.
26. Salceda E, Garateix A, Aneiros A, Salazar H, Lopez O, Soto E. Effects of ApC, a sea anemone toxin, on sodium currents of mammalian neurons. *Brain Res* 2006;**1110**:136–43.
27. Salceda E, Lopez O, Zaharenko AJ, Garateix A, Soto E. The sea anemone *Bunodosoma caissarum* toxin BcIII modulates the sodium current kinetics of rat dorsal root ganglia neurons and is displaced in a voltage-dependent manner. *Peptides* 2010;**31**:412–8.
28. Salceda E, Perez-Castells J, Lopez-Mendez B, Garateix A, Salazar H, Lopez O, et al. CgNa, a type I toxin from the giant Caribbean sea anemone *Condylactis gigantea* shows structural similarities to both type I and II toxins, as well as distinctive structural and functional properties. *Biochem J* 2007;**406**:67–76.
29. Shiomi K. Novel peptide toxins recently isolated from sea anemones. *Toxicon* 2009;**54**:1112–8.
30. Wanke E, Zaharenko AJ, Redaelli E, Schiavon E. Actions of sea anemone type 1 neurotoxins on voltage-gated sodium channel isoforms. *Toxicon* 2009;**54**:1102–11.
31. Yamaguchi Y, Hasegawa Y, Honma T, Nagashima Y, Shiomi K. Screening and cDNA cloning of Kv1 potassium channel toxins in sea anemones. *Mar Drugs* 2010;**8**:2893–905.
32. Zaharenko AJ, Ferreira Jr WA, de Oliveira JS, Konno K, Richardson M, Schiavon E, et al. Revisiting cangitoxin, a sea anemone peptide: purification and characterization of cangitoxins II and III from the venom of *Bunodosoma cangicum*. *Toxicon* 2008;**51**:1303–7.
33. Zaharenko AJ, Ferreira Jr WA, Oliveira JS, Richardson M, Pimenta DC, Konno K, et al. Proteomics of the neurotoxic fraction from the sea anemone *Bunodosoma cangicum* venom: Novel peptides belonging to new classes of toxins. *Comp Biochem Physiol Part D Genomics Proteomics* 2008;**3**:219–25.
34. Zaharenko AJ, Schiavon E, Ferreira Jr WA, Lecchi M, Freitas JC, Richardson M, et al. Characterization of selectivity and pharmacophores of type 1 sea anemone toxins by screening seven Na_V sodium channel isoforms. *Peptides* 2011.
35. Zhang M, Liu XS, Diochot S, Lazdunski M, Tseng GN. APETx1 from sea anemone *Anthopleura elegantissima* is a gating modifier peptide toxin of the human ether-a-go-go- related potassium channel. *Mol Pharmacol* 2007;**72**:259–68.

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Chapter 61

Snail Peptides

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ABSTRACT

The venoms of the 500–700 species of cone snails (genus *Conus*) each contain approximately 100 relatively small (10–30 AA), disulfide-rich peptides, often with unusual posttranslationally modified amino acids (e.g. γ-carboxyglutamate, 6-bromotryptophan, D-phenylalanine). Most pharmacologically characterized *Conus* peptides selectively target a specific ion channel or receptor isoform in the nervous system. Conopeptides are widely used for basic neuroscience research; a few have reached human clinical trials, and one is an approved drug for intractable pain. Cone snails have only a minor fraction of venomous marine molluscan biodiversity. Biochemical characterization or transcriptome analysis of venoms from four other conoidean lineages shows the same remarkable complexity of peptides as in *Conus* venoms. Because an interdisciplinary approach ("concerted discovery") for the discovery of molluscan venom peptides with novel targeting selectivity has been established, these should become increasingly important pharmacological research tools in neuroscience.

BIODIVERSITY OF VENOMOUS MOLLUSCS AND DISCOVERY OF THEIR VENOM PEPTIDES

Marine snails are not the first group of venomous animals that come to mind when venoms are discussed. However, the total number of species of venomous marine gastropod snails is surprisingly large; in fact, the most biodiverse of all molluscan lineages is probably the superfamily Conoidea (or suborder Toxoglossa, "toxoglossates"). These are venomous predators comprising many thousands of described species (with probably an even larger number still undescribed). Toxoglossates are believed to have originated in the Cretaceous; several toxoglossan stem groups can be confidently identified from fossil beds of this geologic period. However, the major adaptive radiations of toxoglossates took place in the Cenozoic, giving rise to the major living lineages.

Traditionally, the superfamily Conoidea has been subdivided into three groups, the cone snails (family Conidae), augers (family Terebridae), and turrids (family Turridae, *sensu lato*). Of these, the turrids are the least well characterized and most heterogeneous. Present estimates suggest that there are 500–700 cone snail species, 300–500 auger species, and very probably >10,000 turrids (broadly defined). In a systematic biodiversity survey in New Caledonia, Bouchet and coworkers found only 51 *Conus* species but >1500 "turrid" species, most not yet named.[4] If this were the true ratio for locations in the tropics, because both terebrids and cone snails are largely tropical groups (only turrids have adapted to cold-water environments), the estimate of >10,000 turrid species may very well be a conservative one. In some respects, turrids were defined by exclusion, that is, a toxoglossate that is neither a cone snail nor a terebrid (the last two groups have shells with easily recognizable morphology, see Fig. 1). Primarily based on new molecular phylogenetic data, it has been proposed that the species assigned to the classical family Turridae be split into 12 newly designated family groups.[3]

The current information about peptides from molluscan venoms is highly skewed and not at all aligned to the actual number of species in each venomous snail lineage. The peptide toxins characterized from toxoglossan venoms to date are almost exclusively from cone snails; as a consequence, this review largely focuses on conopeptides ("conotoxins") from *Conus* venoms. However, peptide toxins from other molluscan venoms are beginning to be investigated, and because of their great pharmacological potential (which remains almost totally untapped at the present time), an overview of peptides from these other groups of venomous molluscans is presented for the very first time in this chapter. The neglect of other venomous molluscs except for cone snails in the past resulted, in part, from the inaccessibility of sufficient venom for conventional biochemical analysis. The larger size of *Conus* species, and the relative abundance of some cone snails in shallow water marine habitats made *Conus* venoms much more accessible for standard purification and peptide characterization. However, newer interdisciplinary methods for the discovery of peptides have made it more feasible to access the peptides from the neglected groups of venomous molluscs. In particular, the "concerted discovery" platform developed[23] for *Conus* peptide discovery can easily be adapted for other groups of venomous

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00061-0
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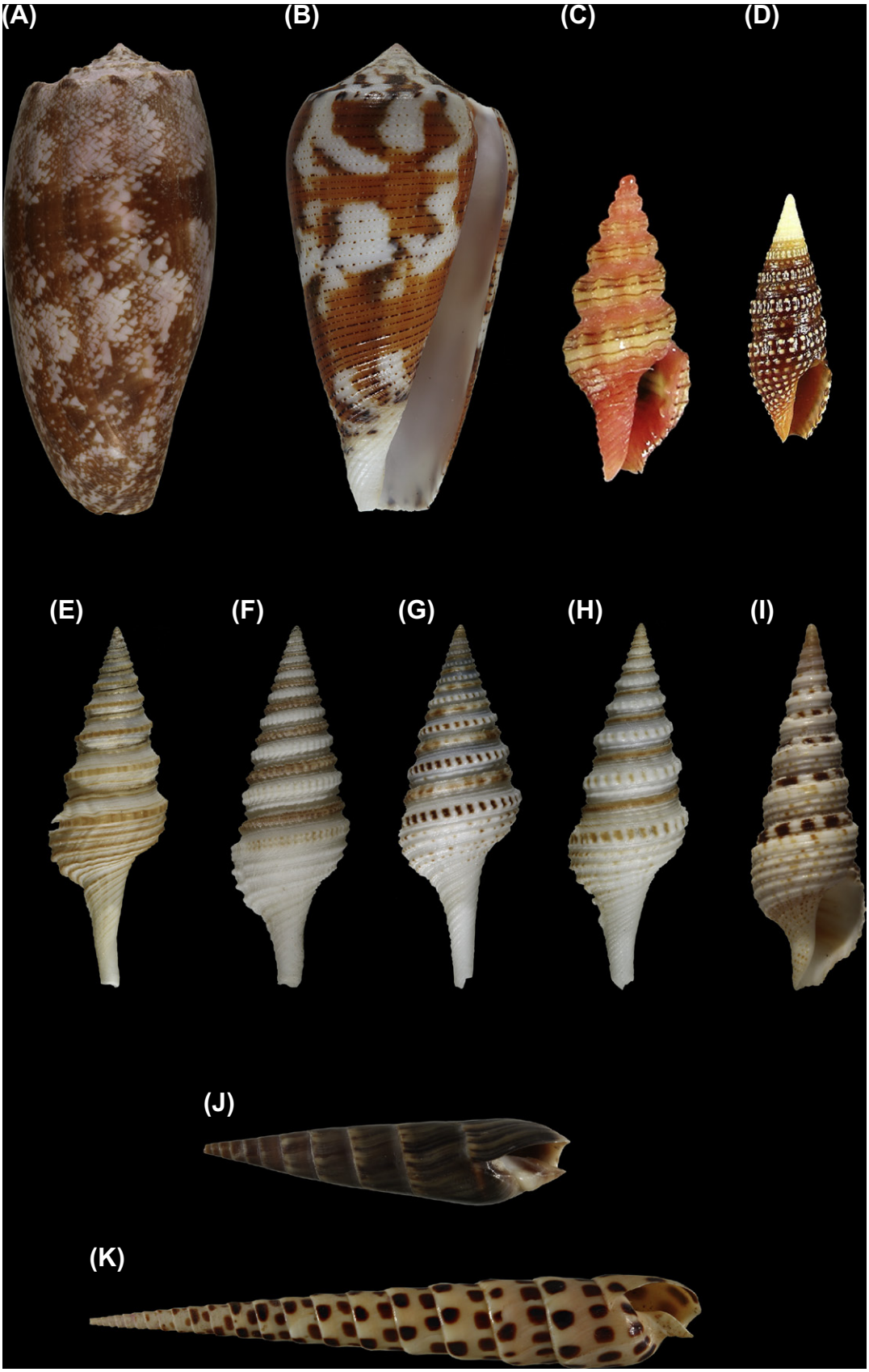

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molluscs. Instead of requiring venom from hundreds of specimens of a particular species, peptide discovery can, in principle, be performed using a single specimen of a venomous snail, even one that is tiny. This radically changes the range of venomous molluscan biodiversity that can be realistically investigated.

However, the efficient application of the concerted discovery platform requires knowledge of both the molecular phylogeny of the family of venomous snails being analyzed, and basic information regarding the peptide superfamilies expressed in the venom ducts of species in the family. With this framework, a transcriptomic analysis of venom ducts, coupled with chemical synthesis and/or expression of the predicted peptides makes it possible to produce the individual venom peptides for further functional characterization. As discussed in the following sections, a particular family of venom peptides expressed in a group of related molluscans targets a corresponding family of ion channels, receptors or transporters. Consequently, the successful characterization of any individual member of a widely expressed venom peptide family becomes a beacon for the further pharmacological characterization of all other peptides in the family. Thus, for effective discovery, molecular phylogeny, identification of venom peptide gene superfamilies, transcriptomics, peptides synthesis and the functional characterization of individual peptides need to be seamlessly integrated together.

DISCOVERY OF CONOTOXINS

The first conotoxins were purified and characterized from the venom of *Conus geographus*, a dangerous species of *Conus* that has caused human fatality. The first peptides investigated caused paralysis in either fish or mice and were shown to target ion channels that are important for neuromuscular transmission.[21] The discovery by Craig Clark, then a 19-year-old student at the University of Utah that the different components of *C. geographus* venom elicited different behavioral phenotypes when injected into the central nervous system of mice led to the characterization of a larger group of *Conus* peptides, mostly from the venoms of fish-hunting cone snails. These had biological activity on mammalian neurons,[22] and some were subsequently developed for direct therapeutic application. One peptide from *Conus magus*, ω-conotoxin MVIIA, was purified because it caused tremors in mice; it was approved as a drug for severe pain in December 2004 (commercial name Prialt).[19,34]

Initially, the discovery of each new conotoxin required biochemical purification from venom. Unusual posttranslationally modified amino acids were identified in the first group of conotoxins characterized.[7] As more cDNA clones encoding conotoxins were elucidated, a molecular cloning/PCR approach for identifying novel conotoxins became feasible; the first venom peptide characterized solely on the basis of the predicted sequence from a cDNA clone was ω-conotoxin MVIIC.[12] However, a problem with this approach is that if the conotoxin is posttranslationally modified, knowing the encoding DNA sequence does not necessarily permit an accurate prediction of which amino acids are posttranslationally modified.

As detailed below, it is highly probable that there are well >50,000 different peptides in the venoms of living cone snails, with an ever accelerating rate of discovery of new peptides. At this time, there are probably amino acid sequences of >2500 *Conus* peptides available, but of these, only a small minority have been biochemically characterized, and the mechanism of action of a miniscule fraction of those has been elucidated.

PRECURSOR STRUCTURE, EXPRESSION, AND PROCESSING: CONOTOXIN SUPERFAMILIES

The *Conus* peptides characterized over the last 25 years provide an overview framework for all conoidean venoms. The great majority of biologically active *Conus* venom components are peptides, initially synthesized through ribosomal translation as polypeptide precursors, and posttranslationally processed to yield the mature, biologically active venom peptides. *Conus* venoms are unusually complex; every *Conus* species can express its own repertoire of approximately 100–200 different peptide toxins in the venom duct. Because there is virtually no molecular overlap between the peptides found in the different *Conus* species, there are >50,000 different peptides in living cone snail venoms. The number of different venom peptides evolved by all species in the superfamily Conoidea is likely to greatly exceed 10^6.

The molecular genetic work done on *Conus* peptides provides a simplifying framework; the majority of the 100–200 peptides in an individual conoidean venom are generally encoded by a few gene superfamilies, with all peptides that belong to the same superfamily having a number of

FIGURE 1 Shells of venomous marine snails from which peptides have been characterized. As described in the text, four classes of venom peptides have been characterized. Best known are **conopeptides** ("conotoxins") that come from the venoms of cone snails such as the geography cone, *Conus geographus* (A) and the magician's cone, *Conus magus* (B). Other classes of venom peptides include **clathurellipeptides** from clathurellid conoideans such as the recently described *Lienardia totopotens*, Rosenberg, 2011(C); **crassipeptides** from crassispirid snails such as *Crassispira cerithina* (D); **turripeptides**, from the family Turridae such as *Gemmula speciosa* (E), *Gemmula diomedea* (F); *Gemmula kieneri* (G), *Gemmula sogodensis* (H), and *Lophiotoma olangoensis* (I), **augerpeptides** from species in the family Terebridae such as *Hastula impages* (J), and *Terebra subulata* (K). See color plate 17.

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shared characteristics [for a review, see Ref. 31]. is now well established that the genes encoding *Conus* peptide toxins are expressed in the epithelial cells of cone snail venom ducts and initially translated as prepropeptide precursors, with an N-terminal signal sequence, an intervening "pro" region and at the C-terminal end, the mature toxin in single copy. All members of a particular *Conus* peptide superfamily share a remarkable degree of sequence identity in their signal sequences. This is highly unusual, because in most gene families encoding secreted polypeptides, the signal sequences are the least conserved elements.

Like most peptidic toxins in animal venoms, *Conus* peptides are generally disulfide-rich. However, three characteristics differentiate conotoxins from other venom polypeptides. The mature toxins from *Conus* venoms are unusually small, typically 10–30 amino acids. The fully processed gene products of some major *Conus* peptide gene superfamilies are among the smallest biologically active gene products known; although many thousands of peptides in *Conus* venoms are probably between 10 and 20 amino acids, the great majority of these will be conformationally rigid because of the multiple disulfide crosslinks.

Another unusual feature is the presence, sometimes at high density, of posttranslationally modified amino acids. *Conus* peptides are first translated as longer precursors, and proteolytic processing and the formation of disulfide linkages are the required steps in maturation. Other posttranslational events that may occur at the ends of a mature *Conus* peptide toxin are C-terminal amidation and the cyclization of glutamine to pyroglutamate at the N-terminus, well-characterized modifications that occur in many other peptides.

However, many *Conus* peptides have nonstandard amino acids that are derived from a standard amino acid by the action of a modification enzyme. Table 1 shows the diverse set of posttranslationally modified amino acids discovered in *Conus* peptides so far; the presence of these unusual amino acids is one hallmark of peptide toxins from this group of venomous animals. The posttranslational modification of conotoxins has been reviewed.[5,7] The reader is referred to these more comprehensive reviews for a discussion of mechanisms of posttranslational modification, and the probable function of individual posttranslationally modified amino acids.

Another characteristic of *Conus* peptides is that a large diversity of "peptide folds" is found in each venom. In many animal venoms, polypeptide toxins have a dominant structural motif (e.g. the "three finger" motif of snake venom toxins). The characteristic arrangements of cysteine residues in the primary sequence of the mature *Conus* peptide toxins ("Cys patterns") reflect the patterns of the disulfide cross-links, and therefore an overall structural framework. All peptides belonging to the same conotoxin superfamily will typically have one major "Cys pattern"; additional minor Cys patterns may be observed in some superfamilies. These different Cys patterns reflect different structured folds in peptides encoded by the different conopeptide gene superfamilies. Thus, in *Conus* venoms, a greater diversity of peptide "folds" are found than is the case for most other animal venoms. The vast majority of *Conus* venom peptides are small, disulfide-rich and conformationally rigid. A small minority of *Conus* peptides lack disulfide crosslinks and are presumably much more flexible. There are preliminary indications that other conoidean groups may have a higher proportion of venom peptides that lack multiple disulfide crosslinks.

Examples of two different superfamilies, the O- and the A-conotoxin gene superfamilies, are shown in Tables 2 and 3. All peptides that belong to the A-superfamily have a highly conserved signal sequence different from that of the peptides of the O-superfamily. The arrangement of Cys residues differs between the two superfamilies: in the O-superfamily, only one major Cys pattern (-C-C-CC-C-C--) is found, reflecting an ICK (inhibitory cys knot) structural motif. A different major Cys pattern (-CC-C-C-) occurs in most peptides of the A-superfamily, but one with 3-disulfides (-CC-C-C--C-C-) is also found in a minority of the A-superfamily peptides.[26] The Cys patterns of the different conotoxin superfamilies were reviewed by Terlau and Olivera,[31] and an updated tally of all *Conus* peptide frameworks is maintained at the Conoserver website.[16]

There may be multiple peptides (>20) that belong to a single *Conus* peptide superfamily expressed in an individual venom duct. The major conotoxin gene superfamilies are widely distributed over the 500–700 different species in the genus. Some conotoxin superfamilies have been intensively investigated by molecular methods; thus, >500 different sequences are known that belong to the A-conotoxin superfamily at the present time, even though only a small minority of *Conus* species have been examined. A website with all conotoxin sequences that have been published, with appropriate references to the primary literature, is now available;[16] the reader is referred to this website to access the primary literature for individual peptide sequences discussed in this review.

A salient feature of *Conus* peptide gene superfamilies is that they undergo an unprecedented rate of accelerated evolution—as a result, there is essentially no molecular overlap between the venom peptides found in two different species of cone snails. The preliminary evidence is that this will be generally valid for all toxoglossan venom peptide genes.

It has been suggested that this rapid gene hypermutation can be anticipated for a wider class of genes, to which the genes that encode all toxoglossan venom peptides belong. This class of genes are the exogenes;[20] the products of exogenes act exogenously, not within the organism that produced

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TABLE 1 Posttranslationally Modified Amino Acids in *Conus* Peptides

Type of modification	Modified AA	Enzyme		*Conus* peptide
Hydroxylation	4-*trans*-Hydroxyproline	Proline hydroxylase	μ-GIIIA	RDCCT**OO**KKCKDRQCK**O**QRCCA*
	D-γ-Hydroxyvaline	hydroxylase with D-AA specificity	gld-V*	AOANS(**D-Hyv**)WS
	5-Hydroxylysine	Lysyl hydroxylase	de13a	DCOTSCOTTCANGWECC(**Hyk**) GYOCVN(**Hyk**)ACSGCTH*
Carboxylation	γ-Carboxyglutamate	γ-Glutayl carboxylase	Conantokin-G	GEγγLQγNQγLIRγKSN*
Bromination	6-Bromotryptophan	Bromo peroxidase	r7a/Light sleeper	**W**FGH γ γCTY**W**LGPC γVDDTCCSASC γSKFCGL**W**
Epimerization	D-Tryptophan	Epimerase	Contryphan-R	GCO(**D-W**)EPWC*
	D-Leucine	Epimerase	Leu-contryphan-P	GCV(**D-L**)LPWC*
	D-Phenylalanine	Epimerase	r11a	GOSFCKADEKOCEYHADCCNCCLSG ICAOSTNWILPGCSTSSF(**D-F**)KI
	D-Valine	Epimerase	mus-V	SOANS(**D-V**)WS
Cyclization	Pyroglutamate	Glutaminyl cyclase	Bromheptapeptide	**Z**CGQAWC*
O-Sulfation	O-Sulfated Tyr	Tyrosyl sulfotransferase	α-Epl	GCCSDPRCNMNNPD(**Y-SO$_3$H**)C
O-Glycosylation	O-Glycosylated Ser	Polypeptide HexNAc	κA-SIVA	ZKSLVP**S**$^+$VITTCCGYD(Hyp)GTM-COOCRCTNSC*
	O-Glycosylated Thr	Polypeptide HexNAc	Contulakin-G	ZSEEGGSNA**T**$^+$KKPYIL

*, C-terminal amidation; O, 4-trans-hydroxyproline; D-Hyv, D-γ-hydroxyvaline; W and W, 6-bromotryptophan; Hyk, 5-hydroxylysine; γ, γ-carboxyglutamate; D-X, D-amino acid; Z, pyroglutamate; Y-SO$_3$H, sulfotyrosine; $^+$, O-glycosylation.

them. Because the spectrum of biotic interactions changes as species diverge, it has been postulated that these are the genes that must encode gene products that diverge functionally at the species level.

DEFINITION OF CONOTOXIN FAMILIES, RECEPTOR TARGETS, AND STRUCTURES

As described in the previous section, peptides from a given conotoxin superfamily with the same Cys pattern are believed, to a first approximation, to have generally similar three-dimensional structures. Disulfide crosslinks are a dominant determinant of how the polypeptide backbones will fold; consequently, peptides sharing a Cys pattern will generally have similar folds. Thus, all peptides in a conotoxin superfamily probably have similar overall conformations. Several conotoxin structures have been published.[8,9]

Conus peptide superfamilies are subdivided into discrete families. The salient feature that distinguishes different conotoxin families belonging to the same superfamily is pharmacological: to which family of ion channels, receptors or transporters are the peptides targeted. In general, all the peptides in a particular family have the same class of receptor targets; peptides in different families, but in the same superfamily, will have divergent targeting specificity (even though they may be similar structurally). Thus, in the M-superfamily, Ψ-conotoxin family peptides inhibit nicotinic acetylcholine receptors, μ-conotoxin family peptides block voltage-gated sodium channels, and κM-conotoxin family peptides are voltage-gated potassium channel antagonists (Tables 2 and 3). Two different conotoxin families may target the same class of receptors; these invariably belong to different gene superfamilies. Thus, both the Ψ-conotoxin and the α-conotoxin families are nicotinic acetylcholine receptor antagonists[18] but belong to the M- and A-conotoxin superfamilies, respectively, and are structurally unrelated (in this particular case, peptides from the two families probably bind to different sites on the nicotinic acetylcholine receptor complex).

Some conotoxin families are widely distributed over the entire genus *Conus*; examples of these are the α-conotoxins that target nicotinic acetylcholine receptors and the δ-conotoxins that target voltage-gated Na channels. By contrast, other conotoxin families seem to be very restricted in their phylogenetic distribution within *Conus* and are found only in a small group of closely related *Conus* species; this

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TABLE 2 Conotoxins Targeted to Ligand-Gated Ion Channels

Nicotinic acetylcholine receptor; neuromuscular subtypes		
α-Conotoxin family (A-superfamily)		
α3/5 Subfamily	α-Conotoxin GI	E**CC**NPA**C**GRHYS**C**[*]
	α-Conotoxin MI	GR**CC**HPA**C**GKNYS**C**[*]
α4/7 Subfamily	α-Conotoxin EI	RDO**CC**YHPT**C**NMSNPQI**C**[*]
α-Conotoxin family (A-superfamily)		
Long αA-subfamily	αA-conotoxin PIVA	G**CC**GSYONAA**C**HO**C**KS**C**KDROSY**C**GQ[*]
	αA-conotoxin EIVA	G**CC**GPYONAA**C**HO**C**G**C**VGROOY**C**DROSGG[*]
Short αA-subfamily	αA-conotoxin OIVB[†]	**CC**GVONAA**C**PO**C**V**C**NKT**C**G[*]
	αA-conotoxin PeIVB[†]	**CC**GIONAA**C**HO**C**V**C**TGK**C**
Ψ-Conotoxin family (M-superfamily)		
	Ψ-Conotoxin PIIIE	HOO**CC**LYGK**C**RRYOG**C**SSAS**CC**QR[*]
Nicotinic acetylcholine receptor; neuronal subtypes		
α-Conotoxin family		
α4/7 Subfamily (A-superfamily)	α-Conotoxin MII[‡]	G**CC**SNPV**C**HLEHSNL**C**[*]
	α-Conotoxin PIA[§]	RDP**CC**SNPV**C**TVHNPQ**IC**[*]
Other α Subfamilies	α-Conotoxin ImII[¶]	A**CC**SDRR**C**RWR**C**[*]
	α-Conotoxin BuIA[‖]	G**CC**STPP**C**AVLY**C**[*]
5HT$_3$-receptor		
σ-Conotoxin family (S-superfamily)		
	σ-Conotoxin GVIIIA	G**C**TRT**C**GGOK**C**TGT**C**T**C**TNSSK**C**G**C**RYNVHPSG<u>B</u>G**C**G**C**A**C**S*
Glutamate receptors: NMDA subclass		
Conantokin family		
	Conantokin-G	GEγγLQγNQγLIRγKSN[*]
	Conantokin-L	GEγγVAKMAAγLARγDAVN[*]
Glutamate receptors: AMPA subclass		
	<u>Con-ikot-ikot</u>	SGPAD**CC**RMKE**CC**TDRVNE**C**LQRYSGREDKFVSF**C**YQEA TVT**C**GSFNEIVG**CC**YGYQM**C**MIRVVKPNSLS GAHEA**C**KTVS**C**GNP**C**A

[*]*Amidated C-terminus; <u>B</u>, 6-bromotryptophan.*
[†]*Preferentially targets the fetal subtype.*
[‡]*Preferred subtypes are α3β2- and α6β2-containing receptors.*
[§]*Preferred subtypes are α6β2-containing receptors.*
[¶]*Preferred subtypes are the α7 and α9/α10 receptors.*
[‖]*Very slowly dissociating from β4-containing neuronal subtypes.*

appears to be particularly true of those conotoxin families that target voltage-gated K channels.

Based on their major prey, the 500–700 species of cone snails are usually divided into fish-hunting, worm-hunting, and snail-hunting species.[25] However, it has become apparent that these are not monophyletic groups, with each derived from a single common ancestor. Rather, there are distinct groups of fish-hunting cone snails and an even greater number of divergent groups of worm-hunting *Conus* species.

The subgeneric group to which a cone snail species belongs is strongly predictive of the conotoxins to be found

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TABLE 3 Conotoxins Targeted to Voltage-Gated Ion Channels

Sodium channels		
Antagonists		
μ-Conotoxin family (M-superfamily)	μ-Conotoxin GIIIA	RD**CC**TOOKK**C**KDRQ**C**KOQR**CC**A*
	μ-Conotoxin PIIIA	ZRL**CC**GFOKS**C**RSRQ**C**KOHR**CC***
	μ-Conotoxin KIIIA	**CC**N**C**SSKW**C**RDHSR**CC***
μO-Conotoxin family (O-superfamily)	μO-conotoxin MrVIB	A**C**SKKWEY**C**IVPILGFVY**CC**PGLI**C**GPFV**C**V*
Inactivation inhibitors		
δ-Conotoxin family (O-superfamily)	δ-Conotoxin PVIA	EA**C**YAOGTF**C**GIKOGL**CC**SEF**C**LPGV**C**FG*
	δ-Conotoxin SVIE	DG**C**SSGGTF**C**GIHOGL**CC**SEF**C**FLW**C**ITFID
Facilitate activation		
ι Conotoxin family (I-Superfamily)	ι-RXIA	GOSF**C**KADEKO**C**EYHAD**CC**N**CC**LSGI**C**AOSTNWILPG**C**STSSFfKI
Calcium channels		
N- or -P-type Ca channels		
ω-Conotoxin family (O-superfamily)	ω-Conotoxin GVIA	**C**KSOGSS**C**SOTSYN**CC**RS**C**NOYTKR**C**Y*
	ω-Conotoxin MVIIA	**C**KGKGAK**C**SRLMYD**CC**TGS**C**RSGK**C***
	ω-Conotoxin MVIIC	**C**KGKGAP**C**RKTMYD**CC**SGS**C**GRRGK**C***
L-type Ca channels		
ω-Conotoxin family (O-superfamily)	ω-Conotoxin TxVIIA	**C**GGYSTY**C**γVDSγ**CC**SDN**C**VRSY**C**TLF*
Contryphan family	Contryphan-M	NγSγ**C**P<u>W</u>HPW**C***
Potassium Channels		
Antagonists		
κ-Conotoxin family (O-superfamily)	κ-Conotoxin PVIIA	**C**RIONQK**C**FQHLDD**CC**SRK**C**NRFNK**C**V*
κM-Conotoxin family (M-superfamily)	κM-Conotoxin RIIIK	LOS**CC**SLNLRL**C**OVOA**C**KRNO**CC**T*
κJ-Conotoxin family (J-superfamily)	κ-Conotoxin PlXIVA	FPRPRI**C**NLA**C**RAGIGHKYPF**C**H**C**R*
Tyrosine-rich peptides	CPY-Fe1	GTYLYPFSYYRLWRYFTRFLHKQPYYYVHI
	CPY-Pl1	ARFLHPFQYYTLYRYLTRHRYPVYYIRY
Conkunitzin family	Conkunitzin-S1	KDRPSL**C**DLPADSGSGTKAEKRIYYNSARKQ**C**LRFDYTGQGGNEN NFRRTYD**C**QRT**C**LYT
(Family unnamed—I superfamily)	ViTx	SR**C**FPPGIY**C**TPYLP**CC**WGI**CC**GT**C**RNV**C**HLRI
Modulation		
(Family unnamed—I superfamily)	BeTx	**C**RAEGTY**C**YNDSQ**CC**LNY**CC**WGG**C**GHO**C**RHPGKRSKLQEFFRQR

*, amidated C-terminus; γ, gamma-carboxyglutamate; <u>W</u>, D-tryptophan; f = D-phenylalanine.

in its venom. For example, different groups of fish-hunting cone snails appear to have different specialized toxin families to target the neuromuscular nicotinic acetylcholine receptor. Although predicting which conotoxins will be found in the venom of a particular *Conus* species is far from a precise science, the assignment of a species to a particular taxonomic subdivision within the genus *Conus* has proven to be the most reliable indicator of the peptide toxin families likely to be present. This has made the molecular phylogeny of *Conus* a powerful catalyst for accelerating discovery. However, individual species often have evolved idiosyncratic venom components not found at all even in very closely related *Conus* species. This is why purification and characterization of peptides from venoms should

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continue to reveal peptides with unexpected pharmacological targets.

BIOLOGICAL MECHANISMS: THERAPEUTIC APPLICATIONS

The biological effects of a particular conoidean venom are a function of the physiological targets of individual toxins expressed in that venom. The known receptor targets of molluscan venom peptides have been elucidated only for a small fraction of *Conus* peptides. Presently, these fall into three broad classes: (I) ligand-gated ion channels; (2) voltage-gated ion channels; and (3) targets other than those that belong to the two large groups of ion channels above. We discuss conotoxins specific for these target classes, and examples are provided for individual conotoxins with receptor targets in each of the three classes.

A large number of conotoxins that inhibit members of the ligand-gated ion channel superfamily have been identified; the largest group is nicotinic acetylcholine receptor antagonists. A surprisingly wide range of structural variation is seen for conotoxins that inhibit nicotinic receptors. The α-conotoxins comprise one well-characterized family;[2,26] these are competitive antagonists of various nicotinic acetylcholine receptor subtypes. Other classes of toxins that antagonize nicotinic receptors are the αA-conotoxins that have a different disulfide bonding framework (but also belong to the A-superfamily), and the αS-conotoxins, which are large, genetically unrelated toxins.[30] The last are more closely related to the σ-conotoxins, which target another class of ligand-gated ion channels, the serotonin (5HT3) receptor. In addition, many venom peptides target ion channels in the glutamate receptor superfamily. Peptides in the conantokin family target NMDA receptors, a major class of glutamate receptors. The structure of these unusual peptides is stabilized by a posttranslationally modified amino acid, γ-carboxyglutamate, rather than by disulfide crosslinks (which are generally absent in the conantokins). The presence of the γ-carboxyglutamate side chains in every three or four amino acids confers a stable helical structure to these peptides in the presence of Ca^{2+}. Some examples of these classes of *Conus* peptides are shown in Table 2.

Conotoxins that target various voltage-gated ion channels are shown in Table 3. The most widely used conotoxins for basic neurobiological research are the ω-conotoxins that block specific voltage-gated Ca channel subtypes. Conotoxins that target Na channels either inhibit conductance (such as the μ- and μO-conotoxins) or inhibit channel inactivation (the δ-conotoxins). Toxins with differential specificity for certain Na channel subtypes have been described.[35] Conotoxins that target voltage-gated K channels are particularly diverse structurally: three different groups of fish-hunting cone snails have different conotoxin families, all targeted to a single subfamily of K channels, the Shaker (or Kvl) channels.[13]

Although most established targets of conotoxins are either voltage-gated or ligand-gated ion channel, a spectrum of *Conus* peptides with other molecular targets have been identified; examples are given in Table 4. Several *Conus* peptides are known to affect the function of G-protein-coupled receptors (GPCRs). The *Conus* peptides shown to target GPCRS are from a diverse set of families and no major family of *Conus* peptides that targets diverse GPCRS has been identified so far. In addition, *Conus* peptides may target catecholamine transporters.[28]

TABLE 4 Miscellaneous Receptor Targets

G-Protein-Coupled Receptors		
Agonists		
p-Conotoxin family (A-superfamily)	p-Conotoxin TIA	FNWR**CC**LIPA**C**RRNHKKF**C**[*]
Antagonists		
Conopressin family	Conopressin-G	**C**FIRN**C**PKG[*]
	Conopressin-S	**C**IIRN**C**PRG[*]
(Unnamed family)	Contulakin-G	ZSEEGGSNAT[†]KKPYIL
(Conorfamide family)	Conorfamide	GPMGWVPVFYRF[*]
Transporter		
Norepinephrine transporter antagonists		
χ-Conotoxin family (T-superfamily)	χ-Conotoxin MrIA	NGV**CC**GYKL**C**HO**C**

*, amidated C-terminus; γ, γ-carboxyglutamate; †, O-glycosylated Thr.

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Mechanisms of individual conotoxins provide the most reductionist insights required to understand the physiological activity of the whole venom. It has become clear that groups of peptides in the venom act together to more efficiently achieve a desired physiological end point. Thus, cone snails have evolved a pharmacological strategy similar to the combination drug therapy favored by the modern pharmaceutical industry for treating AIDS or cancer. To paralyze the prey and interfere with neuromuscular transmission, cone snails simultaneously target presynaptic calcium channels, postsynaptic nicotinic receptors, and voltage-gated sodium channels on the muscle membrane of the prey. These combinations of *Conus* peptides have been termed "toxin cabals"; the combination interfering with neuromuscular transmission is called the "motor cabal." In the venoms of many fish-hunting cone snails, a different toxin combination causes an extremely rapid excitotoxic immobilization of prey,[32] resulting in tetanic paralysis (the "lightning-strike cabal"). An alternative strategy of a different set of fish-hunting cone snail species is to make the potential prey quiescent and sedated (the "nirvana cabal").

The conotoxins that belong to a toxin cabal typically have high potency and specificity for their individual receptor targets. As a consequence of their potency and target selectivity, an increasing number of conotoxins are finding both diagnostic and therapeutic application.[10,17] It should be noted that aquarium observations of how each particular species of fish-hunting *Conus* approaches and captures its prey reveals whether it uses the "lightning-strike cabal" or the "nirvana cabal"; thus, *in vivo* behavioral observations can facilitate peptide discovery. In effect, peptides evolved for a deadly purpose such as killing prey in the natural context may be benign and useful when applied in an entirely different biological context.

OVERVIEW OF PEPTIDES FROM OTHER VENOMOUS MARINE SNAILS

As described in the Introduction, there were traditionally three groups of venomous marine snails recognized, the cone snails (family Conidae), the auger snails (family Terebridae) and the turrids (family Turridae). It has been proposed that the >10,000 species in the last group, which has been shown to be polyphyletic, be assigned to 12 newly defined family groups.[3]

Venom peptides from the auger snails (family Terebridae) have been biochemically characterized. Of the 12 proposed family-level turrid groups, three have been investigated, the turrids (a term now restricted to the newly redefined, more narrowly delineated family Turridae), the crassispirids and the clathurellids. The peptides characterized to date are summarized in Table 5; most of these are known only by their sequences, obtained through molecular cloning.

Augerpeptides: Venom Peptides from the Family Terebridae

The auger snails (family Terebridae) are a distinctive group of highly specialized sand dwelling tropical molluscs believed to feed on polychaete worms. They are among the most well-known marine snails (Fig. 1), with distinctive long, narrow, multiple whorled shells, well adapted for the sand dwelling lifestyle. Several species of auger snails have secondarily lost their venom glands. The venom peptides from this conoidean lineage are known as augerpeptides.

Augerpeptides have been elucidated from two species of Terebridae. In the first study,[15] *Terebra subulata* venom peptides that were purified proved to have structural frameworks similar to well-known conopeptide superfamilies; two peptides had a typical O-superfamily cys pattern, with precursors having a typical conopeptide precursor organization. However, there was no detectable homology between the signal sequences of augerpeptide precursors and the O-superfamily peptides from *Conus*.

A much more extensive analysis using both direct purification from venom and cDNA cloning was carried out on a species in a different branch of the Terebridae, *Hastula hectica*.[14] Peptide sequences were determined both by direct purification from venom and through the analysis of cDNA clones. Even though some of the cysteine frameworks found in the *H. hectica* augerpeptide precursors predicted by cDNA clones were similar to the *Conus* O- and P-peptide superfamilies, no homologous signal sequences were found. Thus, there seem to be no overlapping superfamilies between the conopeptides and augerpeptides characterized to date. The absence of unusual posttranslationally modified amino acids in venom peptides purified from Terebridae was another notable feature.

Turripeptides: Evidence for Conopeptide-Like Gene Superfamilies

The most extensive group of non-*Conus* venom peptides elucidated from the superfamily Conoidea are the turripeptides. As noted above, the family Turridae used to encompass all conoidean species referred to as turrids (the term turrids is still widely used in this broad sense), but there is an emerging consensus to restrict the family Turridae to the monophyletic branch of Conoidea that includes the type species of the genus *Turris*, *Turris babylonia*. We define turripeptides using this narrower definition, as venom peptides from several well-known genera: *Turris*, *Gemmula*, *Lophiotoma*, *Unedogemmula*, *Polystira*, and *Turridrupa*. Presently, the sequences of venom peptides from several Turrids species have been elucidated, including: *Polystira albida*, *Gemmula diomedea*, *Gemmula kieneri*, *Gemmula sogodensis*, *Gemmula speciosa*, and *Lophiotoma* (*Xenuroturris*) *olangoensis* (Fig. 1, Table 5).

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TABLE 5 Venom Peptides from Other Toxoglossan Marine Snails

Augerpeptides			Ref.
Hastula hectica			
Two disulfides	hheTx1	D**C**LP**C**GHDV**CC***	14
	hhe1a	GE**CC**TD**C**AQTAAANY**C***	14
	Hhe9	ALSVLLQUS**C**TM**C**LF**CC**YL	14
	Hhe53	GLSQSG**C**QAFTGRW**C**VG**C**ERLRSRVVWE**C**SPKRVVNSI	14
P-like (three disulfides)	hhe9a	YEEN**C**GTEY**C**TSKIG**C**PGR**C**V**C**KEYNYNGEITRR**C**RA	14
	Hhe9.2	DEEVG**C**FPNV**C**KNDGN**C**SIETSTGMTR**C**Q**C**LEGYTGHV**C**ENPL	14
O-like (three disulfides)	hhe7a	AR**C**EQ**C**PSY**CC**QSDSPPE**C**DG**C**E	14
	Hhe6.2	ALP**C**PYG**C**PLR**CC**HMTDGV**C**LRNKQG**C**	14
	Hhe6.3	VLFTPPELLG**C**GNR**C**SDD**CC**KWGR**C**QPG**C**TD	14
	Hhe6.4	SSLH**C**GDDPW**C**PTG**CC**ENED**C**DIG**C**KRDWEKSRSQP	14
	Hhe6.1	GMGIGINLPP**C**IKNGEY**C**NPWTGSIILGGA**CC**GT**C**TDYE**C**H	14
Four disulfides	hheTx2	S**C**SSG**C**SD**C**NSDS**C**Q**C**TLNQFTNSDS**CCC**	14
	hheTx3	KQ**C**TSNM**C**SAD**C**SPG**CC**IIDKLEW**C**T**C**D**C**	14
	hheTx4	NEV**C**PPGE**C**QQY**CC**DLRK**C**K**C**INLSFYGLT**C**N**C**DS	14
	hheTx5	NEV**C**PPGR**C**EPY**CC**DPRK**C**K**C**LSIDFYGLV**C**N**C**DS	14
Terebra subulata			
O-like (three disulfides)	Agx-S6a	SLDEELKSND**C**PEY**C**PHGNE**CC**EHHE**C**RYDPWSRELK**C**LDS	15
	Agx-s7a	ATNRHQ**C**DTNDD**C**EEDE**CC**VLVGGNVNNPGVQTRI**C**LA**C**S	15
Four disulfides	Agx—Slla	D**C**EQHTD**C**SAASGPVY**CC**QDSD**CC**GGVDYI**C**TNYGQ**C**VRHF	15
Clathurellapeptide			
Clathurella cincta			
Two disulfides	Clone 1	DNFV**C**PFYYVYRRLSS**C**LF**C**LFFLL**C**IKILR	28
Crassipeptide			
Crassispira cerithina			
P-like (three disulfides)	cce9a	GS**C**GLP**C**HENRR**C**GWA**C**Y**C**DDGI**C**KPLRV	6
Turripeptides			
Lophiotoma olangoensis			
Two disulfides	OL172	QEE**C**DDPDGLM**CC**TISEMPT**C**	34
	OL57	LT**C**LTKMVE**C**IQLPLDVEDSSDTL**CC**	34
P-like (three disulfides)	OL08	QHD**C**T**C**VEEGQP**C**QGQA**C**AAE**C**EYNQ**C**LFDGR	34
	OL142	QY**C**PSNT**C**NERDE**C**PGN**C**ND**C**EYVPGTGPNKR**C**VKK	34
	OL11	**C**LSV**C**SAEYEPV**C**GSDGKTYANK**C**HLMTEA**C**WSPTSITLVHEGK**C**	34
	OL135	VE**C**NET**C**EEF**C**TY**C**DDNNAEETEN**C**RTDQTDHSR**C**VDFYTANNLPT	34
O-like (three disulfides)	OL38	SEVLE**C**YFE**C**GNWEGT**CC**DTGI**C**VGIHN**C**KIPEN	34
	OL25	SLV**C**DLE**C**SAEVTT**CC**ETGT**C**HGITTYN**C**VGGTEPET	34

Continued

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TABLE 5 Venom Peptides from Other Toxoglossan Marine Snails—*Cont'd*

Four disulfides	OL127	YYI**C**ES**C**WT**C**ES**C**AGSTESS**C**VSA**C**NA**C**DL**C**PNK	34
	OL47	TS**C**NAATGRSPG**C**F**C**NNDNN**C**RDT**CC**PRSDTEKK**C**TGGPDP**C**PPRQWPD	34
	OL105	FNT**C**PGQYDE**C**GNGPEEGE**CC**GTHNY**CC**KNA**C**TTYHH**C**HGGRDAGKLLRSLKKLKLTTH	34
	OL67	SNT**C**PGYFGE**C**GDGPEEGE**CC**GMYNY**CC**KGR**C**LMLAS**C**QKRRDAGRLLRSLKKLKLTTH	34
Gemmula speciosa			
P-like (three disulfides)	gsp9a	IDOORY**C**NHII**C**YγDSγ**C**SQW**C**TAG**C**NSITSK**C**DT	11
	Gsp9.1	VDOORF**C**NHKI**C**YγDSγ**C**SQW**C**TAG**C**NSITSK**C**DTL	11
	gsp9b	GDOORF**C**RDKL**C**SGDGD**C**SVW**C**TAG**C**NHDMGK**C**DTL	11
	Gsp9.2	GDOORF**C**RDYL**C**SGDGD**C**SVW**C**TAG**C**NHDMGK**C**DTL	11
Gemmula sogodensis			
	Gsg9.1	LDOOGY**C**THKI**C**YγDGγ**C**NQW**C**TAG**C**NODTGK**C**DTT	11
Gemmula diomedea			
	Gdm9.1	GDOORF**C**VHKI**C**YγDSγ**C**NQW**C**TGG**C**NOTQGK**C**DTA	11
Gemmula kieneri			
	Gkn9.1	GVOORF**C**RDKN**C**NγDSγ**C**NQW**C**TGG**C**SSVKGN**C**γS	1
Lophiotoma albina			
	Clone 1	**C**MTI**C**TMEYWPV**C**GSDGKTYPNK**C**HLTSTA**C**TSQKDITVLHEGK**C**	34
Lophiotoma cingulifera			
	Clone 1	**C**LSV**C**SMEYWPV**C**GSDGKTYPNE**C**HLTSEA**C**MSNTDITVAHVGK**C**DQ	34
Polystira albida			
	pal9a	NV**C**DGDA**C**PDGV**C**RSG**C**T**C**DFNVAQRKDT**C**FYPQ*	1

*Amidated C-terminus.

Two peptides are purified directly from *G. speciosa* venom (gsp9a and gsp9b). These peptides are notable in that they contain two posttranslationally modified amino acids also found in conopeptides, 4-hydroxyproline and γ-carboxyglutamate. Further, the two purified peptides were homologous to each other, with significant sequence similarity, and the same framework of cys residues. This framework was similar to that of the P-conopeptide superfamily, but cDNA cloning from *G. speciosa* venom demonstrated that there is no apparent sequence homology between the signal sequences of the *G. speciosa* peptide precursors and the P-conopeptide superfamily. It was suggested that this superfamily of turripeptides be called the Pg-superfamily (G for the genus *Gemmula*). When standard PCR was carried out on other *Gemmula* species, additional homologous peptides were obtained from three other *Gemmula* species, *G. sogodensis*, *G. diomedea*, and *G. kieneri*. The Pg-superfamily sequences fell into two groups, suggesting that these may act on two different but homologous molecular targets.

The analysis of venom peptides using cDNA cloning from *Lophiotoma* (*Xenuroturris*) *olangoensis* also suggested turripeptide superfamilies; one superfamily encoded peptides with 16 cysteine residues, a set of venom components significantly larger than encoded by any *Conus* peptide superfamily. Another unusual aspect of the venoms of Turridae is venom peptides with no cysteine residues and very unusual amino acid compositions. Thus, both methionine-rich and leucine-rich peptides (see López-Vera et al. *Toxicon* 2004), quite unlike any conopeptides characterized, have been identified from turrid venoms (Table 5).

Crassipeptides: Venom Peptides from Crassispirid Snails

The crassispirids belong to a large group of species once assigned to the family Turridae. Recent molecular phylogenetic data[6,24] have established that these are a large biodiverse group of venomous snails that comprise a different

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branch from the turrids; the phylogenetic evidence suggests that they are closer to the family Drillidae. The correct taxonomic designation of this group (family Crassispiridae or Pseudomelatomidae) is still unsettled; we will refer to this branch of Conoidea as the crassispirids, and the venom peptides as crassipeptides. So far, the venom of only one species of crassispirid has been investigated, *Crassispira cerithina*.

A recent report described one crassipeptide, Cce9a, which has been chemically synthesized in a biologically active form.[6] The peptide, elicited novel symptomatology on injection into the mouse CNS that is developmentally dependent, suggesting that this crassipeptide may have a novel molecular target in the mouse CNS. More recently, an analysis of cDNA clones from *C. cerithina* has defined 7 different venom gene superfamilies, encoding peptides with between 1 and 4 disulfide linkages (Cabang et al., 2012, in preparation).

Clathurellipeptides: from Minute Venomous Conoideans

The clathurellids belong to one of the major groups of turrids recently raised to the family level (Clathurellidae) by Bouchet et al.[3] These are believed to be one of the more closely related of the turrid groups to *Conus*, and indeed, in one prior taxonomic scheme,[29] it was proposed that the clathurellids be included with *Conus* in the family Conidae. The emerging consensus is that this an extremely biodiverse group of small, deep-water venomous molluscs that is a well-defined monophyletic branch of Conoidea using conventional molecular phylogenetic data, and we therefore, refer to any peptides in venoms of the family Clathurellidae, H. Adams and A. Adams, 1858, as clathurellipeptides.

Enormous progress can be anticipated in elucidating clathurellipeptide sequences; the reason is that these previously inaccessible molluscs are now collected by a highly-specialized fishery called *lumun–lumun*[27] and using this technology (which was not developed for research purposes, but to supply the commercial need for very small seashells for the Japanese microscope hobbyist market), several dozen different species can be recovered in a single net, with entirely different species occurring at different localities. Because of the extremely small size of most clathurellids recovered through the *lumun-lumun* methods, relatively few have been analyzed to date. However, three sequences have been published, one of them a conventional conopeptide-like precursor, encoding a clathurellid peptide with two disulfide linkages. Many additional sequences have been determined in addition to the one already published (Seronay and Watkins, unpublished results). It is anticipated that the *lumun–lumun* fishery will provide a steady stream of new Clathurellidae and that as techniques improve for getting access to venom peptide sequences in micromolluscs, we can expect to uncover novel pharmacology from this extremely biodiverse group.

PERSPECTIVES

There are many major developments with respect to molluscan peptides that can be anticipated in the coming years. We conclude this chapter by presenting what are probably the most important of these. The rate of discovery of novel peptides from cone snail and other conoidean venoms is likely to be greatly accelerated, particularly for peptides highly selective for specific ion channel and receptor isoforms. The elucidation of new gene superfamilies that encode these peptides makes access to new sequences much more efficient; once the physiological function of a specific venom peptide in a gene superfamily has been elucidated, identifying other peptides that have a related molecular target becomes a much more organized and productive effort. The combination of knowing both the gene superfamily and the molecular phylogeny of each family of venomous snails makes a focused search for peptides with related molecular targets feasible, once the selectivity and specificity of the first peptide is established. The combination of having a framework for understanding gene superfamilies expressed in a lineage of venomous snails, as well as molecular phylogeny of the species that express venom peptides in these gene superfamilies is highly synergistic and makes the discovery process much more facile.

A second important development that can be anticipated is the increasing use of conopeptides (and other conoidean venom peptides) for distinguishing between closely related molecular isoforms of ion channel and receptor families expressed in nervous systems. As noted above, there has already been significant progress in characterizing peptides that can differentiate between subtypes of nicotinic acetylcholine receptors and sodium channels; it is becoming increasingly important in the understanding of the nervous system function to be able to distinguish between closely related subtypes of signaling molecules such as ion channels and receptors, at both the cellular and circuit levels. Thus, establishing an effective neuropharmacology for other ion channel and receptor families depends on discovering novel subtype-selective peptides. Molluscan venoms have proven to be a primary source of such target-specific peptides in the past, and there seems to be a good probability that these venoms will provide a major fraction of the novel pharmacological tools required for investigating the more complex ion channel/receptor families for which a functional characterization of individual molecular isoforms is lacking.

Finally, as mentioned in the Introduction to this chapter, venom peptides from other lineages in the superfamily Conoidea can now be accessed, at least in principle. For

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most of the non-*Conus* toxoglossan species, because these are almost all very small and relatively rare,[4] there is additional expertise required to apply the "concerted discovery" platform described above in an effective manner. To effectively carry out a transcriptomic analysis, considerable anatomical dissection skill is required. Thus, the range of interdisciplinary expertise that is necessary encompasses an impressive range of fields: taxonomy and phylogeny, peptide biochemistry, neuroscience, molecular genetics, anatomy, and field biology (for collecting the biodiversity). However, the peptides present in these unexplored molluscan venoms represent a new frontier that can increasingly be productively mined to provide unique pharmacological tools in the years to come.

ACKNOWLEDGMENTS

Most of the work included in this review has been supported by grant GM 48677 from the U.S. National Institute of General Medical Sciences. GP Concepcion acknowledges the support of Pharmaseas from the Department of Science and Technology, Republic of the Philippines. Some of the collaborative research of the authors has been supported by ICBG grant # 1U01TW008163 from the Fogarty International Center, National Institutes of Health.

REFERENCES

1. Aguilar MB, de la Rosa RA, Falcon A, Olivera BM, Heimer de la Cotera EP. Peptide pal9a from the venom of the turrid snail Polystira albida from the Gulf of Mexico: purification, characterization, and comparison with P-conotoxin-like (framework IX) conoidean peptides. *Peptides* 2009;**30**:467–76.
2. Azam L, McIntosh JM. Alpha-conotoxins as pharmacological probes of nicotinic acetylcholine receptors. *Acta Pharmacologica Sinica* 2009;**30**:771–83.
3. Bouchet P, Kantor Y, Sysoev AV, Puillandre N. A new operational classification of the Conoidea (Gastropoda). *Journal of Molluscan Studies* 2011:273–308.
4. Bouchet P, Lozouet P, Maestrati P, Heros V. Assessing the magnitude of species richness in tropical marine environments: high numbers of molluscs at a New Caledonia site. *Biological Journal of the Linnean Society* 2002;**75**:421–36.
5. Buczek O, Bulaj G, Olivera BM. Conotoxins and the posttranslational modification of secreted gene products. *Cellular and Molecular Life Sciences* 2005;**62**:3067–79.
6. Cabang AB, Imperial JS, Gajewiak J, Watkins M, Corneli PS, Olivera BM, et al. Characterization of a venom peptide from a crassispirid gastropod. *Toxicon* 2011;**58**:672–80.
7. Craig AG, Bandyopadhyay P, Olivera BM. Post-translationally modified peptides from *Conus* venoms. *European Journal of Biochemistry* 1999;**264**:271–5.
8. Daly NL, Craik DJ. Structural studies of conotoxins. *IUBMB Life* 2009;**61**:144–50.
9. Grant MA, Morelli XJ, Rigby AC. Conotoxins and structural biology: a prospective paradigm for drug discovery. *Current Protein and Peptide Science* 2004;**5**:235–48.
10. Han TS, Teichert RW, Olivera BM, Bulaj G. Conus venoms—a rich source of peptide-based therapeutics. *Current Pharmaceutical Design* 2008;**14**:2462–79.
11. Heralde 3rd FM, Imperial J, Bandyopadhyay PK, Olivera BM, Concepcion GP, Santos AD. A rapidly diverging superfamily of peptide toxins in venomous Gemmula species. *Toxicon* 2008;**51**:890–7.
12. Hillyard DR, Monje VD, Mintz IM, Bean BP, Nadasdi L, Ramachandran J, et al. A new *Conus* peptide ligand for mammalian presynaptic Ca^{2+} channels. *Neuron* 1992;**9**:69–77.
13. Imperial J, Silverton N, Olivera BM, Bandyopadhyay P, Sporning A, Ferber M, et al. Using chemistry to reconstruct evolution: on the origins of fish-hunting in venomous cone snails. *Proceedings of the American Philosophical Society* 2007;**151**:185–200.
14. Imperial JS, Kantor Y, Watkins M, Heralde 3rd FM, Stevenson B, Chen P, et al. Venomous auger snail Hastula (Impages) hectica (Linnaeus, 1758): molecular phylogeny, foregut anatomy and comparative toxinology. *Journal of Experimental Zoology Part B: Molecular and Developmental Evolution* 2007;**308**:744–56.
15. Imperial JS, Watkins M, Chen P, Hillyard DR, Cruz LJ, Olivera BM. The augertoxins: biochemical characterization of venom components from the toxoglossate gastropod Terebra subulata. *Toxicon* 2003;**41**:391–8.
16. Kaas Q, Westermann JC, Halai R, Wang CK, Craik DJ. ConoServer, a database for conopeptide sequences and structures. *Bioinformatics* 2008;**24**:445–6.
17. Livett BG, Gayler KR, Khalil Z. Drugs from the sea: conopeptides as potential therapeutics. *Current Medical Chemistry* 2004;**11**: 1715–23.
18. McIntosh JM, Santos AD, Olivera BM. *Conus* peptides targeted to specific nicotinic acetylcholine receptor subtypes. *Annual Review of Biochemistry* 1999;**68**:59–88.
19. Miljanich GP. Ziconotide: neuronal calcium channel blocker for treating severe chronic pain. *Current Medicinal Chemistry* 2004;**11**:3029–40.
20. Olivera BM. Conus peptides: biodiversity-based discovery and exogenomics. *Journal of Biological Chemistry* 2006;**281**:31173–7.
21. Olivera BM, Gray WR, Zeikus R, McIntosh JM, Varga J, Rivier J, et al. Peptide neurotoxins from fish-hunting cone snails. *Science* 1985;**230**:1338–43.
22. Olivera BM, Rivier J, Clark C, Ramilo CA, Corpuz GP, Abogadie FC, et al. Diversity of *Conus* neuropeptides. *Science* 1990;**249**: 257–63.
23. Olivera BM, Teichert RW. Diversity of the neurotoxic Conus peptides: a model for concerted pharmacological discovery. *Molecular Interventions* 2007;**7**:251–60.
24. Puillandre N, Meyer CP, Bouchet P, Olivera B. Genetic divergence and geographic variation in the deep-water *Conus Orbignyi* complex (mollusca: Conoidea). *Zoological Scripta* 2011;**40**:350–63.
25. Röckel D, Korn W, Kohn AJ. *Manual of the living Conidae*. Wiesbaden, Germany: Verlag Christa Hemmen; 1995.
26. Santos AD, McIntosh JM, Hillyard DR, Cruz LJ, Olivera BM. The A-superfamily of conotoxins: structural and functional divergence. *Journal of Biological Chemistry* 2004;**279**:17596–606.
27. Seronay RA, Fedosov AE, Astilla MA, Watkins M, Saguil N, Heralde 3rd FM, et al. Accessing novel conoidean venoms: biodiverse lumun-lumun marine communities, an untapped biological and toxinological resource. *Toxicon* 2010;**56**:1257–66.

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28. Sharpe IA, Gehrmann J, Loughnan ML, Thomas L, Adams DA, Atkins A, et al. Two new classes of conopeptides inhibit the α1-adrenoceptor and noradrenaline transporter. *Nature Neuroscience* 2001;**4**:902–7.
29. Taylor JD, Kantor Y, Sysoev AV. Foregut anatomy, feeding mechanisms, relationships and classification of the Conoidea (=Toxoglossa) (Gastropoda). *London Bulletin, Natural History Museum* 1993;**59**:125–70.
30. Teichert RW, Jimenez EC, Olivera BM. αS-conotoxin RVIIIA: a structurally unique conotoxin that broadly targets nicotinic acetylcholine receptors. *Biochemistry* 2005;**44**:7897–902.
31. Terlau H, Olivera BM. *Conus* venoms: a rich source of novel ion channel-targeted peptides. *Physiological Reviews* 2004;**84**:41–68.
32. Terlau H, Shon K, Grilley M, Stocker M, Stühmer W, Olivera BM. Strategy for rapid immobilization of prey by a fish-hunting cone snail. *Nature* 1996;**381**:148–51.
33. Watkins M, Hillyard DR, Olivera BM. Genes expressed in a turrid venom duct: divergence and similarity to conotoxins. *Journal of Molecular Evolution* 2006;**62**:247–56.
34. Webster LR, Fakata KL. Ziconotide for chronic severe pain. *Practical Pain Management* 2005;**5**:47–51:5–6.
35. Wilson MJ, Yoshikami D, Azam L, Gajewiak J, Olivera BM, Bulaj G, et al. Block of sodium channels NaV1.1-1.8 by a panel of μ-conotoxins: identity of channels responsible for action potentials in sciatic nerve. *PNAS* 2011;**338**:689–93.

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Chapter 62

Snake Peptides

Alan L. Harvey

ABSTRACT

Snake venoms are complex mixtures of small molecules, peptides, and proteins. Most of the biologically active toxins are peptides or enzymes. This chapter covers the nonenzymatically active peptides and toxins. The peptides belong to several structural classes, and they have many different biological actions. The best characterized peptides are the so-called three-finger toxins that have 60–70 amino acid residues in three peptide loops stabilized by four disulfide bridges. Despite their common 3D shape, these peptides can interfere selectively with different biological targets, including nicotinic and muscarinic acetylcholine receptors, acetylcholinesterase, ion channels, and cell membranes. Other small peptides can block K^+ or Ca^{2+} channels and are based on Kunitz serine proteinase inhibitors (~60 amino acid residues and three disulfide bonds), whereas other toxins have different molecular scaffolds and different pharmacological activities. Many snake venom peptides are useful pharmacological tools, whereas others are leads for drug development.

INTRODUCTION

Snake venoms have been long known to have potent biologically acting molecules that are proteins or peptides. Although much early work focused on enzymes, the fashion changed in the mid-1960s with the advent of gel filtration and ion-exchange chromatography to look at the chemical and biological properties of smaller peptides. This research has been incredibly productive in terms of understanding how peptide structure contributes to biological activity and also in revealing molecules with highly selective pharmacological actions. More recent research on venom peptides has been aided by the application of molecular biology techniques and proteomics.[1] New classes of peptides with novel pharmacological effects are still being discovered, with three new examples being included since the first edition of this publication. This chapter concentrates on nonenzymatically active peptides.

THREE-FINGER TOXINS

The first members of this group ("α-neurotoxins") were isolated from the venoms of cobras and sea snakes because of their potent muscle paralyzing activity. They have 60–74 amino acid residues in a single chain crosslinked by four or five disulfide bonds. They contain a central core stabilized by four S–S bridges and three protruding peptide loops (like three fingers of a hand). It was long apparent that another group of cobra venom toxins with a completely different biological activity (the "cardiotoxins" or "cytotoxins") had a molecular architecture similar to that of the α-neurotoxins. Since then, nearly 300 different examples of snake peptides with the three-finger motif have been described, and they are known to have several quite different pharmacological actions. This makes them extremely interesting in terms of structure–activity relationships and for an understanding of molecular evolution[8]

α-Neurotoxins

(1) Discovery: These are generally found in the venoms of snakes of the Elapidae (cobras, kraits, mambas, etc.) and Hydrophidae (sea snakes) families, although similar molecules occur in venoms of some Colubridae. The α-neurotoxins can be subdivided into "short" (60–62 residues; four disulfides), and "long" (66–74 residues; five disulfides), including the κ-neurotoxins (66 residues; five disufides) and "weak" neurotoxins and other homologs (63–66 residues; five disulfides).

(2) Structure of the precursor/gene; Distribution of the mRNA; Processing: The nucleotide sequence of a cDNA encoding erabutoxin a was derived from the material extracted from venom glands, and subsequently, the relevant genes encoding erabutoxins were cloned from a genomic library prepared from the sea snake *Laticauda semifasciata.* The gene for cobrotoxins from *Naja naja atra* has a similar organization to those for the erabutoxins, with three exons separated by two introns. However, the gene for denmotoxin from the colubrid snake *Boiga dendrophila* has four exons and three introns.[19] For a review on work on the gene structures of α-neurotoxins, see Ref. 34.

Clearly, the toxins are found in venom that is synthesized and stored in the venom glands of the snake. The control of toxin synthesis is not yet understood, although transcriptional regulation of toxin genes may be involved.

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00062-2
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(3) Receptors: The α-neurotoxins bind to nicotinic acetylcholine receptors. Most early work concentrated on the receptors at the neuromuscular junction or on electroplaques of electric fish. These "muscle-type" receptors are pentameric proteins with two α subunits and one each of β, γ, and ε(or δ), and such receptors are powerfully blocked by the high affinity binding of both short and long α-neurotoxins.

Venoms of *Bungarus multicinctus* and other kraits also contain κ-bungarotoxins that have little affinity for muscle-type nicotinic receptors but which bind to some neuronal isoforms (particularly the α3β2 subtype). More recently, long α-neurotoxins were found to bind not only to muscle-type nicotinic receptors but also to homo-oligomeric nicotinic receptors (comprising five α subunits) that are found on neurons.The so-called "weak" toxins can also bind with relatively high affinity to such neuronal nicotinic receptors.

(4) Conformation: The three-dimensional structures of the short chain toxin erabutoxin b, the long-chain α-bungarotoxin, and κ-bungarotoxin have been described. The overall shape is formed by a central core that is stabilized by four S–S bonds and three peptide loops protruding like fingers from a hand. More recently, a covalently linked dimeric form with α-neurotoxin properties has been characterized from the venom of the colubrid *Boiga irregularis*.[20]

(5) Biological activity: The short and long α-neurotoxins paralyze the skeletal muscle by blocking the ability of the neurotransmitter acetylcholine to bind to and activate the nicotinic receptors at the neuromuscular junction. These α-neurotoxins are among the most potent of venom constituents and presumably evolved to help elapid and hydrophid snakes catch their prey. The biological activities of the neurotoxins that bind to neuronal acetylcholine receptors are not clearly understood. A structurally homologous toxin named γ-bungarotoxin was isolated from the venom of *Bungarus multicinctus*. It is lethal on injection, presumably because it binds weakly to nicotinic cholinoceptors. γ-Bungarotoxin also inhibits platelet aggregation.

Muscarinic Toxins

(1) Discovery: The venom of mamba snakes had been reported to contain acetylcholine or a cholinomimetic compound. However, competition-binding studies with synaptosomal membranes led to the discovery of three-finger-type peptides that bound to some central muscarinic receptors (see Ref. 24 for a review). The first peptides to be characterized were named muscarinic toxin 1 (MTx1) and toxin-2, although their toxicity has not been established. These toxins are relatively selective for the m1 subtype of muscarinic receptor, and other toxins from mamba venoms have different selectivities. More recently, homologous peptides have been isolated from cobra venoms, and a larger peptide BM14 (82 amino acid residues including 10 cysteines) was found in *Bungarus multicinctus* venom that bound to m2 muscarinic receptors. γ-Bungarotoxin also binds to m2 receptors.

(2) Structure of the precursor/gene; Distribution of the mRNA; Processing: The cDNA encoding muscarinic toxin-2 was cloned, including that of m1-toxin 1. Although not a classical muscarinic toxin, the muscarinic receptor binding peptide BM14 from *Bungarus multicinctus* venom is coded by a gene that has a similar organization as α-neurotoxin and cardiotoxin genes.

(3) Receptors: Different muscarinic toxins have different binding affinities for different subtypes of muscarinic receptors (see Ref. 24 for a review). From green mamba (*Dendroaspis angusticeps*) venom, MTx1 is relatively selective for the m1 subtype; MTx3 is highly selective for the m4 subtype; and MTx7 (or m1-toxin) is extremely selective for the m1 subtype. MTα from black mamba (*Dendroaspis polylepis*) venom has nanomolar affinity for α_{2B}-adrenoceptors,[9] whereas MTx1 and 2 binds to α_1-adrenoceptors.

(4) Conformation: The solution structure of MTx2 was solved by NMR and shown to be three finger-like.

(5) Biological activity: MTx 1 and 2 from green mamba venoms seem to be very unusual for toxins in that they cause an allosteric activation of their receptor target rather than a block. When injected into the hippocampus of mice, these peptides can enhance learning and memory processes, whereas MTx3 (which blocks m4 muscarinic receptors) is inhibitory. MTx7 (or m1-toxin) blocks m1 muscarinic receptors virtually irreversibly. The contribution of the muscarinic toxins to the action of mamba venoms is unknown, although they may aid absorption of venom through actions on lymphatic vessels.

Anticholinesterases

(1) Discovery: The anticholinesterase activity of green mamba venom was revealed in the pharmacological experiments by Osman et al. in 1973 and predicted from the muscle fasciculations caused by the injection of venom fractions into mice. The isolated peptides with powerful inhibitory effects on acetylcholinesterase are called "fasciculins."

(2) Structure of the precursor/gene; Distribution of the mRNA; Processing: cDNA encoding fasciculin 1 has been cloned. The cDNA is organized similarly to that of α-neurotoxins, although the signal peptide sequence for fasciculin 1 lacks a cysteine residue generally encoded in structurally related toxin genes.

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(3) Receptors: Fasciculins bind tightly to a peripheral anionic site on acetylcholinesterases from most species but not from birds. They also have very little affinity for butyrylcholinesterases. The interaction of fasciculin with enzyme has been studied in great detail, and the complexity of enzyme and inhibitor has also been studied.
(4) Conformation: The structure of fasciculin 1 is similar to that of the other three-finger toxins.
(5) Biological activity: Fasciculins produce a long-lasting inhibition of acetylcholinesterase activity, thereby prolonging the action of the neurotransmitter acetylcholine. *In vivo*, this can lead to visible trembling ("fasciculations") of muscles under the skin. When combined with other toxins that are present in mamba venoms, the fasciculins can cause muscle paralysis by inducing a depolarization-type blockade resulting from prolonged activation of acetylcholine receptors at the neuromuscular junction.

Three-Finger, Disintegrin-Like Peptides from Mamba Venoms

(1) Discovery: Many components of snake venoms inhibit platelet aggregation. Most are enzymes, but some peptides that are in the three-finger structural class have also been found, first in mamba venoms (mambin) and then in krait venom (γ-bungarotoxin).
(2) Structure of the precursor/gene; Distribution of the mRNA; Processing: There have been no reports on the cloning of these antiplatelet peptides from venoms of mambas, but gene organization for γ-bungarotoxin is similar to those of α-neurotoxin and cardiotoxin genes.
(3) Receptors: Mambin (also known as dendroaspin) and γ-bungarotoxin contain the sequence RGD that is common to proteins that bind to the glycoprotein receptor IIb-IIIa on platelets. Homologous sequences were noted in thrombostatin and peptide S5C1, which are also from mamba venoms and assumed to be platelet inhibitors.
(4) Conformation: The conformation of γ-bungarotoxin and mambin is similar to that of other three-finger toxins in being predominantly β-sheet.
(5) Biological activity: These peptides prevent the aggregation of blood platelets.

Ca^{2+}-Channel Blockers

(1) Discovery: From the screening of components of black mamba venom for activity on isolated smooth muscle, a 60-residue peptide was isolated and named calciseptine. Earlier, a similar molecule named FS2 had been isolated, although its biological activity was not known. They are in the general three-finger class of toxins.
(2) Structure of the precursor/gene; Distribution of the mRNA; Processing: The genes for these peptides do not seem to have been cloned.
(3) Receptors: The peptides bind to L-type Ca^{2+} channels, probably via the recognition site for 1, 4-dihydropyridines. Calciseptine does not affect N-type or T-type Ca^{2+} channels.
(4) Conformation: The solution structure of FS2 by NMR experiments is similar to those of other three-finger toxins.
(5) Biological activity: Both calciseptine and FS2 block Ca^{2+} currents through L-type Ca^{2+} channels in cardiac and smooth muscle cells, although calciseptine acts as an agonist on L-type channels in skeletal muscles.[5] Both *in vivo* and *in vitro*, calciseptine and FS2 inhibit contractions of smooth muscle; because of this, the peptides have hypotensive effects.

Cardiotoxins

(1) Discovery: Cobra venoms were long known to have cardiovascular effects *in vivo* and to cause direct damage to isolated cardiac preparations. Protein fractions showing such activity were called "cardiotoxins." Such fractions could lyse cells, particularly erythrocytes, and they also became known as "cytotoxins." Purified cardiotoxins were shown to be homologous in size and structure to the short chain α-neurotoxins.
(2) Structure of the precursor/gene; Distribution of the mRNA; Processing: Genomic DNAs encoding several cardiotoxins were isolated from the liver of the Taiwan cobra *Naja naja atra.* Similarities to the genes for α-neurotoxins were noted. Several cDNAs for cardiotoxins have also been obtained from RNA isolated from cobra venom glands.
(3) Receptors: The mechanism of action of cardiotoxins remains obscure despite decades of research. They are generally thought to disrupt cell membranes, but whether or not through binding to a specific receptor is unknown. Cardiotoxins are very basic peptides, and they have been suggested to bind to negatively charged lipids on membranes. Certainly, Lys residues on the peptides are important for activity, but cardiotoxins affect some cells much more than others, implying some selective binding. More recently, evidence was found for sulfo-containing receptors on the membranes of neuronal and cardiac cells.[30]
(4) Conformation: The structure of a cardiotoxin has been determined by X-ray crystallography and by NMR spectroscopy. The differences in a projection from the tip of the third loop may correlate with activity.
(5) Biological activity: Cardiotoxins depolarize electrically excitable cells (neurones and muscles) in an irreversible manner, leading to sustained contraction of muscles.

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Some cardiotoxins have hemolytic activity, although this is difficult to ascribe directly to the cardiotoxins because they act in a highly synergistic manner with very low amounts of venom phospholipase A_2. Cardiotoxins have been shown to induce apoptosis in various tumor cell lines. A noncytolytic homolog of cardiotoxins binds to αvβ3 integrin and can inhibit bone resorption *in vitro*.[31]

Synergistic Toxins

(1) Discovery: Fractions from mamba venoms were noted to have little lethality in mice until combined, leading to the isolation of several peptides with a three-finger structure that were named as "synergistic toxins." Some of these were subsequently shown to have anticholinesterase properties, but others act by still unknown mechanisms.
(2) Structure of the precursor/gene; Distribution of the mRNA; Processing: The cDNA coding for a likely synergistic toxin was cloned from RNA from the venom glands of a green mamba *Dendroaspis angusticeps.*
(3) Receptors: The binding sites are unknown.
(4) Conformation: It is assumed that the synergistic toxins have a conformation broadly similar to the other members of the three-finger class.
(5) Biological activity: Little is known about the biological activity, other than *in vivo* toxicity.

Adrenoceptor Antagonists

(1) Discovery: Screening of green mamba (*Dendroaspis angusticeps*) venom in binding assays with various G-protein–coupled receptors revealed components with affinity for α-adrenoceptors.[13] Two three-finger-type peptides were isolated: ρ-Da1a (or AdTx1) and ρ-Da1b, specific for α_{1A} and α_2 adrenoceptors, respectively. These peptides had 65 and 66 amino acid residues and four disulfide bridges.
(2) Receptors: ρ-Da1a has a Kd at α_{1A} adrenoceptors of 0.6nM. ρ-Da1b binds to all three subtypes of α_2 adrenoceptor, being most potent at α_{2A}.
(3) Biological activity: Both peptides act as antagonists at their target receptors. When tested on isolated rabbit prostatic smooth muscle preparations, ρ-Da1a acts as an irreversible antagonist. In functional assays on cells expressing cloned α_{2A} adrenoceptors, ρ-Da1b behaves as a competitive antagonist.

Homologs of Unknown Activity

Protein isolation and cDNA cloning have provided examples of several peptides that are homologous to the three-finger toxins but whose activities are not yet known. These so-called "orphan toxins" have been studied from a phylogenetic perspective, but this does not point to pharmacological classifications. Further work is required.

PROTEASE INHIBITOR HOMOLOGS

Enzyme Inhibitors

(1) Discovery: Proteinase inhibitors are very widespread throughout nature, so it is not surprising that they have been isolated from various snake venoms, including viperids and elapids. Most are in the Kunitz class of serine proteinase inhibitors. Serum of the snake *Trimeresurus mucrosquamatus* has a novel glycoprotein that inhibits the activity of zinc-dependent metalloproteinases but not serine proteases.
(2) Structure of the precursor/gene; Distribution of the mRNA; Processing: cDNA for trypsin and chymotrypsin inhibitors has been cloned from the venom glands of *Vipera ammodytes*, *Naja naja atra*, and *Bungarus multicinctus.* There is a shared gene structure across a wide range of homologs, including some with markedly different biological activities (see the following sections on dendrotoxins and Ca^{2+}-channel blocking toxins).
(3) Receptors: The peptides bind to serine proteinases, particularly to trypsin and chymotrypsin. However, their physiological targets are not known.
(4) Conformation: The peptides adopt the same conformation as bovine pancreatic trypsin inhibitor.
(5) Biological activity: The peptides inhibit the activity of various proteinases, but their purpose in venoms is obscure, although they may interfere with blood clotting by inhibiting enzymes involved in the clotting cascade. Protease inhibitor-like protein-1 (PILP-1) from *Bungarus multicinctus* venom downregulates a disintegrin and metalloprotease 17 (ADAM 17) and induces apoptosis of leukemia cells.[11]

Dendrotoxins

(1) Discovery: The Venom from the Eastern green mamba *Dendroaspis angusticeps* increases acetylcholine release from motor nerve endings, an effect subsequently correlated with block of some neuronal K^+ channels.[6] The peptides responsible (dendrotoxins) had been isolated and sequenced before their biological activity was known.
(2) Structure of the precursor/gene; Distribution of the mRNA; Processing: The gene for dendrotoxin K was cloned from a cDNA library made from the venom glands of black mamba *Dendroaspis polylepis.* Most dendrotoxins have a pyroglutamic acid residue at their N-terminus.

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(3) Receptors: α-Dendrotoxin from green mamba and dendrotoxin I from the black mamba block some cloned voltage-dependent K^+ channels (Kv1.1, Kv1.2 and Kv1.6) in the low nanomolar range; dendrotoxin K from the black mamba selectively blocks Kv1.1 channels at picomolar concentrations.[6] Interactions with channel proteins have been studied.
(4) Conformation: The solution structures of dendrotoxin I and K were determined by NMR, and the X-ray crystal structure of α-dendrotoxin was also solved. All are similar to bovine pancreatic trypsin inhibitor (BPTI).
(5) Biological activity: The first biological activity of the dendrotoxins to be noticed was the augmentation of twitch responses of skeletal muscle preparations in response to nerve stimulation. Although not very toxic on peripheral injection, dendrotoxins are potent toxins when injected centrally in mice, causing convulsions and death. These effects result from the peptides blocking some of the voltage-activated K^+ channels in neurons, leading to increased neuronal firing and excitability. Dendrotoxin K, which is highly selective for Kv1.1 potassium ion channels, reduces tumor formation in a mouse xenograft model.

Ca^{2+}-Channel Blockers

(1) Discovery: A BPTI homolog with blocking activity at L-type Ca^{2+} channels was isolated from green mamba venom and named calcicludine.
(2) Structure of the precursor/gene; Distribution of the mRNA; Processing: The gene for calcicludine has not been characterized.
(3) Receptors: Calcicludine blocks L-type Ca^{2+} channels in cerebellar granular cells and cardiac myocytes. The block of the α1C form of the L-type channels is partial but irreversible, while calcicludine can increase the activity of the α1A, B, and E subtypes.[25]
(4) Conformation: The NMR structure of calcicludine is similar to that of dendrotoxins.
(5) Biological activity: Calcicludine can reduce contractions of cardiac muscle.

CROTAMINE-LIKE MYOTOXINS

(1) Discovery: Rattlesnake venoms are known to cause damage to skeletal muscle, and several small peptides (42 residues with three disulfides) associated with that activity have been isolated. These include myotoxin A from the prairie rattlesnake *Crotalus viridis viridis* and crotamine from the South American rattlesnake *C. durissus terrificus*.
(2) Structure of the precursor/gene; Distribution of the mRNA; Processing: A 1.8kb gene Crt-p1 coding for crotamine was found in specimens of *C. durissus terrificus*.
(3) Receptors: Crotamine and homologous myotoxins can bind to Na^+ channels on skeletal muscle membranes and also to lipid membranes.
(4) Conformation: The NMR-derived structure of crotamine has a short N-terminal α-helical region and a small antiparallel triple stranded β-sheet area; overall, the folding has a resemblance to that of β-defensins, and crotamine has some antimicrobial properties.
(5) Biological activity: The crotamine-like myotoxins can depolarize skeletal muscles and cause myotoxic damage. Crotamine also has analgesic activity that is reversed by naloxone. Crotamine penetrates into dividing cells and localizes in the nucleus.[7]

SARAFOTOXINS

(1) Discovery: The venom of the burrowing asp *Atractaspis engaddensis* causes cardiac arrest. A family of toxins, the sarafotoxins, has been characterized. They have 21 amino acid residues in a single chain stabilized by two disulfide bonds.[4] A similar toxin, bibrotoxin, was found in the venom of *Atractaspis bibroni*. Longer homologs, with 24 residues, were discovered in the venom of *Atractaspis microlepidota microlepidota* and 25-residue homologs are found in another species, *Atractaspis irregularis*. All are homologs of the mammalian endothelins (see endothelin chapters in several sections of the book).
(2) Structure of the precursor/gene; Distribution of the mRNA; Processing: The gene encoding sarafotoxins from the burrowing asp is unusual in coding for five different isotoxins in a "rosary-type" organization. The mechanisms for processing the mature toxin peptides are unknown.
(3) Receptors: The sarafotoxins bind to endothelin receptors. There may be a different, as yet uncharacterized subtype that recognizes the "long" sarafotoxins.
(4) Conformation: The solution structure of a sarafotoxin has been solved using NMR techniques. The molecule is largely α-helical, with a very flexible C-terminal region.
(5) Biological activity: The principal activity is vasoconstriction mediated by actions on endothelin receptors. *In vivo*, death is caused by cardiac arrest.

CRISP FAMILY TOXINS

(1) Discovery: Cysteine-rich secretory proteins (CRISPs) are found in mammalian epididymis and the immune system, though their functions are unknown. They are widely distributed in snake venoms, with examples

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being known from colubrid, elapid and viperid snakes.[33] Pseudechetoxin was discovered by screening a wide variety of venoms for activity on a particular ion channel.

(2) Structure of the precursor/gene; Distribution of the mRNA; Processing: cDNAs encoding ablomin, catrin, latisemin, ophanin, piscivorin, tigrin, and triflin have been cloned. They code for mature proteins of 221 residues including 16 cysteines.[33] Similar molecules with different pharmacological actions were cloned from Australian elapids: pseudechetoxin from *Pseudechis australis* and pseudecin from *Pseudechis porphyriacus*.[32] These gene products have 238 amino acid residues, including a 19-residue signal peptide. A further eight residues are thought to be cleaved enzymatically to give the mature peptide.

(3) Receptors: It is suggested that ablomin, catrin, latisemin, ophanin, piscivorin, and triflin may be L-type Ca^{2+}-channel blockers (they reduce contractions of arterial smooth muscle stimulated by K^{+}-induced depolarization with no effect on contractions stimulated by release of intracellular Ca^{2+} by caffeine).[33] Pseudechetoxin and pseudecin block channels in olfactory and retinal cells that are gated by cyclic nucleotides.[32]

(4) Conformation: A crystal structure of triflin has been obtained. It has a large globular portion linked to a rod-like cysteine-rich domain containing five disulfide bridges.

(5) Biological activity: The function of the CRISP proteins in snake venoms is unknown. Because they are so widely distributed in snakes of different families and in different geographical regions, perhaps they serve a common purpose such as aiding the spread of venom after a bite. A CRISP protein from *Bothrops alternatus* venom can induce angiogenesis and also improve healing in an experimental wound model.

NATRIURETIC PEPTIDES

(1) Discovery: A vasorelaxant peptide of 38 amino acid residues and with homology to atrial natriuretic peptide (ANP) was isolated from green mamba venom and named dendroaspis natriuretic peptide, DNP. Homologous peptides have been isolated from the venom of the inland taipan *Oxyuranus microlepidotus*.[27]

(2) Structure of the precursor/gene; Distribution of the mRNA; Processing: DNP is expressed in venom glands, but it is not known if it plays a signaling role elsewhere in the snake. DNP has been found in human tissues.[10] Several Asian and South American snakes contain genes for C-type natriuretic peptides, and cDNA for a natriuretic peptide precursor has been found from a venom gland cDNA library from king cobra, *Ophiophagus hannah.*

(3) Receptors: DNP activates ANP-A receptors rather than ANP-B receptors.

(4) Conformation: The overall conformation of DNP has not been described. It is assumed to have a disulphide bridge between residues 7 and 23.

(5) Biological activity: DNP acts on ANP-A receptors to activate guanylate cyclase and increase the levels of cyclic GMP, which cause vascular relaxation. DNP also causes natriuresis. It has been suggested that DNP may have a use in treating patients with congestive heart failure, either as the native peptide or as a chimeric molecule with C-type natriuretic peptide.

DISINTEGRINS

(1) Discovery: Many components of snake venoms interfere with blood coagulation through actions on platelets.[2] Disintegrins have a variety of peptide structures (including some three-finger-shaped molecules described earlier) but generally share a three-residue section of RGD. They have been divided into five groups according to the size and disulfide bonds. The largest also has enzymatic activity.

(2) Structure of the precursor/gene; Distribution of the mRNA; Processing: Several cDNA sequences have been cloned and found to be quite variable in length, although a common ancestor is likely.[2]

(3) Receptors: The peptides bind to various integrins, including $\alpha IIb\beta 3$, $\alpha 2\beta 3$, and $\alpha 5\beta 1$. A cardiotoxin homolog binds to $\alpha v\beta 3$ integrin.[31]

(4) Conformation: A crystal structure of schistatin from the saw-scaled viper *Echis carinatus* has been solved. This is a homomeric dimer formed by two disulfide bonds between monomers. Each monomer has three sets of antiparallel β-strands and four intramolecular disulfide bonds. The conformation of albolabrin has been defined by NMR measurements.

(5) Biological activity: The disintegrins prevent platelet aggregation. They may also have potential in reducing metastatic spread of cancer cells.

C-TYPE LECTINS AND LECTIN-LIKE PROTEINS

(1) Discovery: Lectins are complex proteins that bind to various sugars and have a variety of biological effects. C-type lectin-like proteins lack carbohydrate binding activity and have only been found in various snake venoms. Over 80 are known.[18] They affect platelet functions and/or coagulation factors and therefore disturb blood coagulation.

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(2) Structure of the precursor/gene; Distribution of the mRNA; Processing: Phylogenetic analysis of the A and B chains of the C-type lectins and the lectin-like proteins indicate that the C-type lectin-like proteins evolved separately from the lectins.[18]

(3) Receptors: Different C-type lectin-like proteins seem to bind to different receptors on platelets, including the integrins α2β1 and αIIβ3, glycoproteins in the leucine-rich repeat family GPIb-IX-V and the immunoglobulin superfamily GPIV, causing either inhibition or activation.[12,30] They can also bind to von Willebrand factor, collagen, and fibrinogen. Other C-type lectin-like proteins have anticoagulant activity through binding to different coagulation factors in blood.[16,18]

(4) Conformation: The C-type lectin-like proteins are made up of various combinations of homologous subunits (A and B chains, each of ~14kDa). The structures and structure–activity relationships are being actively studied.[12,14,16,18]

(5) Biological activity: Many of the proteins can both inhibit and activate platelets.[12] Overall, most seem to produce thrombocytopenia. Others have anticoagulant properties.[16]

WAGLERINS

(1) Discovery: Waglerins are small peptides from the venom of Wagler's pit viper, *Tropidolaemus (Trimeresurus) wagleri* that are lethal to mice.

(2) Structure of the precursor/gene; Distribution of the mRNA; Processing: The peptides have 22 or 24 amino acid residues, including seven proline residues and one disulfide bond. The genes for waglerins do not seem to have been cloned, but the peptides can be readily synthesized.

(3) Receptors: Waglerins bind to muscle-type nicotinic acetylcholine receptors containing the ε-, but not the γ-subunit.[15] Waglerin-1 also binds to sites on different forms of $GABA_A$ receptors.

(4) Conformation: The 3D conformation of waglerin-1 has been resolved by NMR studies. There is a short, rigid loop formed by the S–S bond and two extended and flexible tails.

(5) Biological activity: When injected into mice, waglerins cause respiratory failure because of blockade of acetylcholine receptors at the neuromuscular junction. Rats are insensitive to the toxins.

BRADYKININ-POTENTIATING PEPTIDES

(1) Discovery: The venom of the Brazilian snake *Bothrops jararaca* was found to potentiate the effects of bradykinin *in vitro* and *in vivo*.[3] Several small peptides of 5–14 residues with that activity were isolated, and other examples have been found in other venoms of *Bothrops* and *Crotalus* snakes. Because these act by inhibiting the activity of angiotensin-converting enzyme (ACE), they led the way to the discovery and development of clinically useful ACE inhibitor drugs.[3]

(2) Structure of the precursor/gene; Distribution of the mRNA; Processing: Several genes have been cloned from a cDNA library of venom glands of *Bothrops jararaca* that code for the precursor of bradykinin-potentiating peptides along with C-type natriuretic peptide. Similar genes were found in the venom glands of three Asian snakes: *Trimeresurus flavoviridis*, *Trimeresurus gramineus*, and *Agkistrodon halys blomhoffi.* The control of expression and the modification to give the pyroglutamic residue found in the active peptides are not known. However, similar mRNA was detected in various brain regions in the snakes, suggesting that the peptides may have a signaling role within the snake.

(3) Receptors: Bradykinin-potentiating peptides interact with the active sites on ACE. Some also can activate argininosuccinate synthase to lead to increased production of nitric oxide.[3] A homologous peptide, blomhotin from *Agkistrodon halys blomhoffii*, causes contractions of rat stomach preparations in a manner consistent with there being specific receptors for the peptide; these have not yet been identified. More recent work indicates that bradykinin-potentiating peptides from *Bothrops jararaca* can activate m1 muscarinic receptors and B2 bradykinin receptors.

(4) Conformation: The conformation of bradykinin-potentiating peptide F from *Agkistrodon piscivorus piscivorus* was studied by NMR. Two major forms were found (stretched and folded), and it was speculated that one was associated with the activity on ACE and the other with potentiating responses to bradykinin.

(5) Biological activity: The peptides enhance the contractile activity of bradykinin on smooth muscle, although potency does not necessarily correlate with the ability to inhibit ACE activity.[3]

AVIT PEPTIDES

(1) Discovery: Among several peptides isolated from black mamba venom and then sequenced were protein A and A′ that had 81 amino acid residues, including 10 cysteines and which were not homologous to any known peptide group. A very similar peptide was later found to cause contractions of intestinal smooth muscle preparations and was called "mamba intestinal toxin" (MIT1).

(2) Structure of the precursor/gene; Distribution of the mRNA; Processing: Although MIT1 does not seem to have been cloned, mammalian homologs have been found. These are known as prokineticins, and they are expressed in the gastrointestinal tract, brain, and other tissues.

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(3) Receptors: Schweitz et al. noted that radiolabeled MIT1 bound to ileal and brain membranes with very high affinity, and there was pharmacological evidence that prokineticins act on G-protein–coupled receptors. Two such receptors have been cloned.
(4) Conformation: MIT1 is unusual in the arrangement of its five disulfide bonds, and, from NMR experiments, it was found to have an overall homology with colipase. In addition to the mammalian prokineticins, there are homologs in amphibian skin. All share a conserved N-terminal sequence of AVIT, hence the proposed name of "AVIT protein family."
(5) Biological activity: MIT1 powerfully contracts smooth muscle in sections of the gastrointestinal tract. The peptide also can sensitize pain receptors. However, its contribution to the overall effects of mamba venom is not known.

CYSTATINS

(1) Discovery: These were originally found in several different snake venoms (*Bitis arietans*, *Naja naja atra*, *Notechis scutatus*) because of their ability to inhibit cysteine protease activity. Recent studies indicate that cystatins are widely distributed in snake venoms.[23]
(2) Structure of the precursor/gene; Distribution of the mRNA; Processing: The cDNA for a cystatin from *Bitis gabonica* has been reported, and 12 cystatin cDNA sequences were obtained from venom glands of several species of elapid snakes.[23] There is a common sequence for a 26 amino acid residue propeptide.
(3) Receptors: Cystatins bind to and inhibit the activity of the C1 family of cysteine proteases, including cathepsin L and papain.
(4) Conformation: The snake venom cystatins have about 140 amino acid residues and two intrachain disulfide bridges. Their three-dimensional structure has not been directly determined, but homology modeling indicates structural similarities with ovocystatin.[23]
(5) Biological activity: The biological function of cystatins in snake venoms is unknown. However, one of them has been shown to reduce metastatic spread of cancer cells in an experimental animal model.

CATHELICIDINS

(1) Discovery: Peptides with antimicrobial properties are widespread in animals, plants, and microbes. Some snake venom peptides are structurally related to defensins. Another family of antimicrobial peptide, the cathelicidins, has been found in venoms of some cobras and kraits.[35]
(2) Structure of the precursor/gene; Distribution of the mRNA; Processing: Three cDNA sequences coding for elapid cathelicidins have been studied. They code for a 22-amino acid residue signal peptide, a 135-amino acid residue cathelin domain, and a mature antimicrobial peptide of 34 residues.
(3) Receptors: The molecular mechanism of action is unknown, but they may disrupt membranes in susceptible cells.
(4) Conformation: They are linear peptides of 34 residues. CD experiments indicate a random coil conformation with the possibility of forming α-helices.
(5) Biological activity: The peptide that has been tested, cathelicidin from *Bungarus fasciatus*, has a broad spectrum of antibacterial activity with minimum inhibitory concentrations (MICs) of <1μM, although strains of *Staphylococcus aureus* were more resistant. The peptide was also active against different species of fungus but had little cytotoxicity against mammalian cells and was not hemolytic.[29]

WAPRINS

(1) Discovery: By fractionating venom of the cobra *Naja nigricollis* and using mass spectrometry to detect peptides with masses different from known classes of toxin, a novel 51 amino acid residue peptide was isolated and characterized.[26] It is homologous to whey acidic proteins (WAPs), and the cobra-derived peptide was named nawaprin. A similar peptide named omwaprin has been isolated from the venom of the inland taipan *Oxyuranus microlepidotus*.[17]
(2) Structure of the precursor/gene; Distribution of the mRNA; Processing: This is not known, although a synthetic gene for omwaprin has been expressed in *E. coli*.[17]
(3) Receptors: Nawprin is homologous to the elastase inhibitor elafin, but it does not inhibit elastase. Omwaprin is able to disrupt membranes of some Gram-positive bacterial cells.[17]
(4) Biological activity: This is not known for nawaprin. Omwaprin has selective antibacterial effects of some species of Gram-positive bacteria, and it is not hemolytic against human erythrocytes.[17]

MISCELLANEOUS PEPTIDES

In the late 1960s, the advances in protein purification and analysis techniques gave rise to information about the structures of many venom components whose activity was unknown. Often, it took many years before a biological activity could be ascribed to such peptides. Recently, a similar phenomenon is appearing because of the use of

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proteomics to reveal the presence of more and more peptides in venoms (see Ref. 1 for a review). Many of these peptides may have biological activity, but that remains to be determined. Other peptides have been ascribed a biological activity because of similarities to other better characterized molecules, but their significance in snake venom is still unknown. Some current examples follow.

Vascular endothelial growth factors are proteins that stimulate angiogenesis. Several examples have been found in snake venoms (e.g. *Bothrops* and *Vipera* species), and it is suggested that they may act to enhance distribution of venom after a bite by increasing vascular permeability.

A small protein of 107 residues was isolated from the venom of the king cobra (*Ophiophagus hannah*) and named ohanin.[21] It is different from other snake venom proteins, but it has homology with SPRY domain proteins. It is not lethal in mice, but it decreases locomotor activity and sensitizes responses to painful stimuli. Its mechanism of action is unknown.

Low molecular weight (1.3–2.4kDa) peptides have been isolated from *Dendroaspis angusticeps* venom.[22] They are unusual because of their high number of proline residues and because they are glycosylated. No biological activity is known.

REFERENCES

1. Calvete JJ, Juárez P, Sanz L. Snake venomics. Strategy and applications. *J Mass Spectrom* 2007;**42**:1405–14.
2. Calvete JJ, Marcinkiewicz C, Monleon D, Esteve V, Celda B, Juarez P, et al. Snake venom disintegrins: evolution of structure and function. *Toxicon* 2005;**45**:1063–74.
3. Camargo AC, Ianzer D, Guerreiro JR, Serrano SM. Bradykinin-potentiating peptides: beyond captopril. *Toxicon* 2011 Aug 2:[Epub ahead of print].
4. Ducancel F. Endothelin-like peptides. *Cell Mol Life Sci* 2005;**62**: 2828–39.
5. Garcia MC, Hernandez-Gallegos Z, Escamilla J, Sanchez JA. Calciseptine, a Ca^{2+} channel blocker, has agonist actions on L-type Ca^{2+} currents of frog and mammalian skeletal muscle. *J Membr Biol* 2001;**184**:121–9.
6. Harvey AL, Robertson B. Dendrotoxins: structure-activity relationships and effects on potassium ion channels. *Curr Med Chem* 2004;**11**:3065–72.
7. Kerkis A, Kerkis I, Radis-Baptista G, Oliveira EB, Vianna-Morgante AM, Pereira LV, et al. Crotamine is a novel cell-penetrating protein from the venom of rattlesnake *Crotalus durissus terrificus*. *FASEB J* 2004;**18**:1407–9.
8. Kini RM, Doley R. Structure, function and evolution of three-finger toxins: mini proteins with multiple targets. *Toxicon* 2010;**56**:855–67.
9. Koivula K, Rondinelli S, Näsman J. The three-finger toxin MTα is a selective α_{2B}-adrenoceptor antagonist. *Toxicon* 2010;**56**:440–7.
10. Lee S, Park SK, Kang KP, Kang SK, Kim SK, Kim W. Relationship of plasma *Dendroaspis* natriuretic peptide-like immunoreactivity and echocardiographic parameters in chronic haemodialysis patients. *Nephrology* 2004;**9**:171–5.
11. Liu WH, Chang LS. Suppression of ADAM17-mediated Lyn/Akt pathways induces apoptosis of human leukemia U937 cells: *Bungarus multicinctus* protease inhibitor-like protein-1 uncovers the cytotoxic mechanism. *J Biol Chem* 2010;**285**:30506–15.
12. Lu Q, Navdaev A, Clemetson JM, Clemetson KJ. Snake venom C-type lectins interacting with platelet receptors. Structure-function relationships and effects on haemostatis. *Toxicon* 2005;**45**:1089–98.
13. Maïga A, Mourier G, Quinton L, Rouget C, Gales C, Denis C, et al. G protein-coupled receptors, an unexploited animal toxin targets: exploration of green mamba venom for novel drug candidates active against adrenoceptors. *Toxicon* 2011:[Epub ahead of print].
14. Matsui T, Hamako J. Structure and function of snake venom toxins interacting with human von Willebrand factor. *Toxicon* 2005;**45**: 1075–87.
15. Molles BE, Tsigelny I, Nguyen PD, Gao SX, Sine SM, Taylor P. Residues in the epsilon subunit of the nicotinic acetylcholine receptor interact to confer selectivity of waglerin-1 for the alpha-epsilon subunit interface site. *Biochemistry* 2002;**41**:7895–906.
16. Morita T. Structures and functions of snake venom CLPs (C-type lectin-like proteins) with antocoagulant-, procoagulant-, and platelet-modulating activities. *Toxicon* 2005;**45**:1099–114.
17. Nair DG, Fry BG, Alewood P, Kumar PP, Kini RM. Antimicrobial activity of omwaprin, a new member of the waprin family of snake venom proteins. *Biochem J* 2007;**402**:93–104.
18. Ogawa T, Chijiaw T, Oda-Ueda N, Ohno M. Molecular diversity and accelerated evolution of C-type lectin-like proteins from snake venom. *Toxicon* 2005;**45**:1–4.
19. Pawlak J, Kini RM. Unique gene organization of colubrid three-finger toxins: complete cDNA and gene sequences of denmotoxin, a bird-specific toxin from colubrid snake *Boiga dendrophila* (mangrove catsnake). *Biochimie* 2008;**90**:868–77.
20. Pawlak J, Mackessy SP, Sixberry NM, Stura EA, Le Du MH, Ménez R, et al. Irditoxin, a novel covalently linked heterodimeric three-finger toxin with high taxon-specific neurotoxicity. *FASEB J* 2009;**23**:534–45.
21. Pung YF, Wong PT, Kumar PP, Hodgson WC, Kini RM. Ohanin, a novel protein from king cobra venom, induces hypolocomotion and hyperalgesia in mice. *J Biol Chem* 2005;**280**:13137–47.
22. Quinton L, Gilles N, Smargiasso N, Kiehne A, De Pauw E. An unusual family of glycosylated peptides isolated from *Dendroaspis angusticeps* venom and characterized by combination of collision induced and electron transfer dissociation. *J Am Soc Mass Spectrom* 2011:[Epub ahead of print].
23. Richards R, St Pierre L, Trabi M, Johnson LA, de Jersey J, Masci PP, et al. Cloning and characterisation of novel cystatins from elapid snake venom glands. *Biochimie* 2011;**93**:659–68.
24. Servent D, Blanchet G, Mourier G, Marquer C, Marcon E, Fruchart-Gaillard C. Muscarinic toxins. *Toxicon* 2011 Aug 30:[Epub ahead of print].
25. Stotz SC, Spaetgens RL, Zamponi GW. Block of voltage-dependent calcium channel by the green mamba toxin calcicludine. *J Memb Biol* 2000;**174**:157–65.
26. Torres AM, Wong HY, Desai M, Moochhala S, Kuchel PW, Kini RM. Identification of a novel family of proteins in snake venoms. Purification and structural characterization of nawaprin from *Naja nigricollis* snake venom. *J Biol Chem* 2003;**278**:40097–4104.
27. Vink S, Jin AH, Poth KJ, Head GA, Alewood PF. Natriuretic peptide drug leads from snake venom. *Toxicon* 2010 Dec 10:[Epub ahead of print].

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28. Wang CH, Monette R, Lee SC, Morley P, Wu WG. Cobra cardiotoxin-induced cell death in fetal rat cardiomyocytes and cortical neurons: different pathway but similar cell surface target. *Toxicon* 2005;**46**: 430–40.
29. Wang Y, Hong J, Liu X, Yang H, Liu R, Wu J, et al. Snake cathelicidin from *Bungarus fasciatus* is a potent peptide antibiotics. *PLoS One* 2008;**3**:e3217.
30. Wijeyewickrema LC, Berndt MC, Andrews RK. Snake venom probes of platelet adhesion receptors and their ligands. *Toxicon* 2005;**45**: 1051–61.
31. Wu PL, Lee SC, Chuang CC, Mori S, Akakura N, Wu WG, et al. n-cytotoxic cobra cardiotoxin A5 binds to alpha(v)beta3 integrin and inhibits bone resorption. Identification of cardiotoxins as non-RGD integrin-binding proteins of the Ly-6 family. *J Biol Chem* 2006;**281**:7937–45.
32. Yamazaki Y, Brown RL, Morita T. Purification and cloning of toxins from elapid venoms that target cyclic nucleotide-gated ion channels. *Biochemistry* 2002;**41**:11331–7.
33. Yamazaki Y, Hyodo F, Morita T. Wide distribution of cysteine-rich secretory proteins in snake venoms: isolation and cloning of novel snake venom cysteine-rich secretory proteins. *Arch Biochem Biophys* 2003;**412**:133–41.
34. Yee JSP, Nanling G, Afifiyan F, Donghui M, Lay PS, Armugam A, et al. Snake postsynaptic neurotoxins: gene structure, phylogeny and applications in research and therapy. *Biochimie* 2004;**86**:37–149.
35. Zhao H, Gan TX, Liu XD, Jin Y, Lee WH, Shen JH, et al. Identification and characterization of novel reptile cathelicidins from elapid snakes. *Peptides* 2008;**29**:1685–91.

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Chapter 63

Spider Peptides

Graham M. Nicholson

ABSTRACT

Spider peptide and protein toxins are recognized as highly potent and specific molecular tools that modulate signaling in excitable cells via interaction with a variety of ion channels, receptors, and transporters in vertebrates and invertebrates. By a process of natural selection, spider venoms have evolved as complex preoptimized combinatorial peptide libraries, displaying extremely diverse pharmacology with a wide range of molecular targets, designed to subdue or kill their prey or predators. The discovery that these disulfide-rich proteins and peptides bind to their cognate receptors with high affinity, and in many cases with outstanding selectivity, means that spider venoms are now being investigated as sources of molecular tools for probing ion channel and synaptic neurotransmission, for validating novel insecticide targets, and as lead compounds in therapeutic and biopesticide discovery pipelines.

INTRODUCTION

Spiders are the most speciose venomous animals and the most successful terrestrial predators, with >42,000 extant species described to date.[22] Because they rely completely on predation as a trophic strategy for survival, spiders have developed during evolution a complex preoptimized combinatorial library of enzymes, neurotoxins, and antimicrobial and cytolytic peptides in their venom glands.[27] These comprise mainly of three groups delineated by their molecular mass: (i) low molecular mass acylpolyamines (<1 kDa), (ii) disulfide-rich neurotoxins and linear cytolytic peptides (1–10 kDa), and (iii) high molecular mass proteins (>30 kDa) – mainly enzymes and neurotoxins. Given the focus of this book, this review will center on the peptide neurotoxins and cytolytic peptides, particularly as high molecular mass spider venom neurotoxins and enzymes were already described in the first edition of this book.

Since the publication of the first edition of the *Handbook of Biologically Active Peptides* in 2006, some 380 additional new spider peptides have been formally reported. To date, around 800 peptide toxins from 78 spider species have been described. These are from the venom of 20 of the 110 extant spider families, including representative families from the two major suborders—aranemorph ("modern") and mygalomorph ("primitive") spiders (Fig. 1A). These venoms comprise mainly of 3 to 9 kDa peptide toxins, mainly focused on 3 to 5 kDa peptides and also with some larger proteins in the 30- to 40 kDa (sphingomyelinases D involved in loxoscelism; Fig. 5) and 130 kDa range (latrotoxins, latroinsectotoxins and latrocrustatoxins). In the recent years, it has become clear that these spider venom libraries are considerably more complex than previously appreciated, comprising >1000 distinct peptides.[8] If one uses a conservative estimate of 100,000 species and 200 peptides per venom, then spider venoms probably contain at least 10 million bioactive peptides. Consequently, <0.01% of the potential proteomic diversity of spider venoms has been tapped to date.

There has been an exponential increase in the number of spider toxins that have been reported in the literature.[15] This has resulted from the advent of high-throughput modern analytical techniques especially involving proteomic, transcriptomic and genomic approaches. As a result of the explosion in the number of new peptide toxins being discovered and characterized a rational nomenclature system based on a Greek letter 'activity prefix' together with a toxin name based on the family, genus and species of the spider has been proposed and adopted by UniProtKB.[15] In addition, a curated spider toxin peptide and protein database (ArachnoServer Spider Toxin Database; www.arachnoserver.org) has also been developed.[12] This review will use this new nomenclature to facilitate the identification of orthologs and paralogs and will also provide original names of the toxin. In addition, the readers are directed to relevant entries in the ArachnoServer database for original literature references, biological activity, molecular targets, sequence and 3D structure (where known).

Spiders use their venoms to paralyze and/or kill prey or predators as rapidly as possible. Therefore, their venoms are particularly rich in neurotoxins that rapidly modify ion conductance (ion channel toxins) and to a lesser extent affect neurotransmitter exocytosis (presynaptic toxins). Like scorpion toxins, however, they lack significant numbers of toxins that block the action of neurotransmitters (postsynaptic toxins) particularly common in snakes and,

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00063-4
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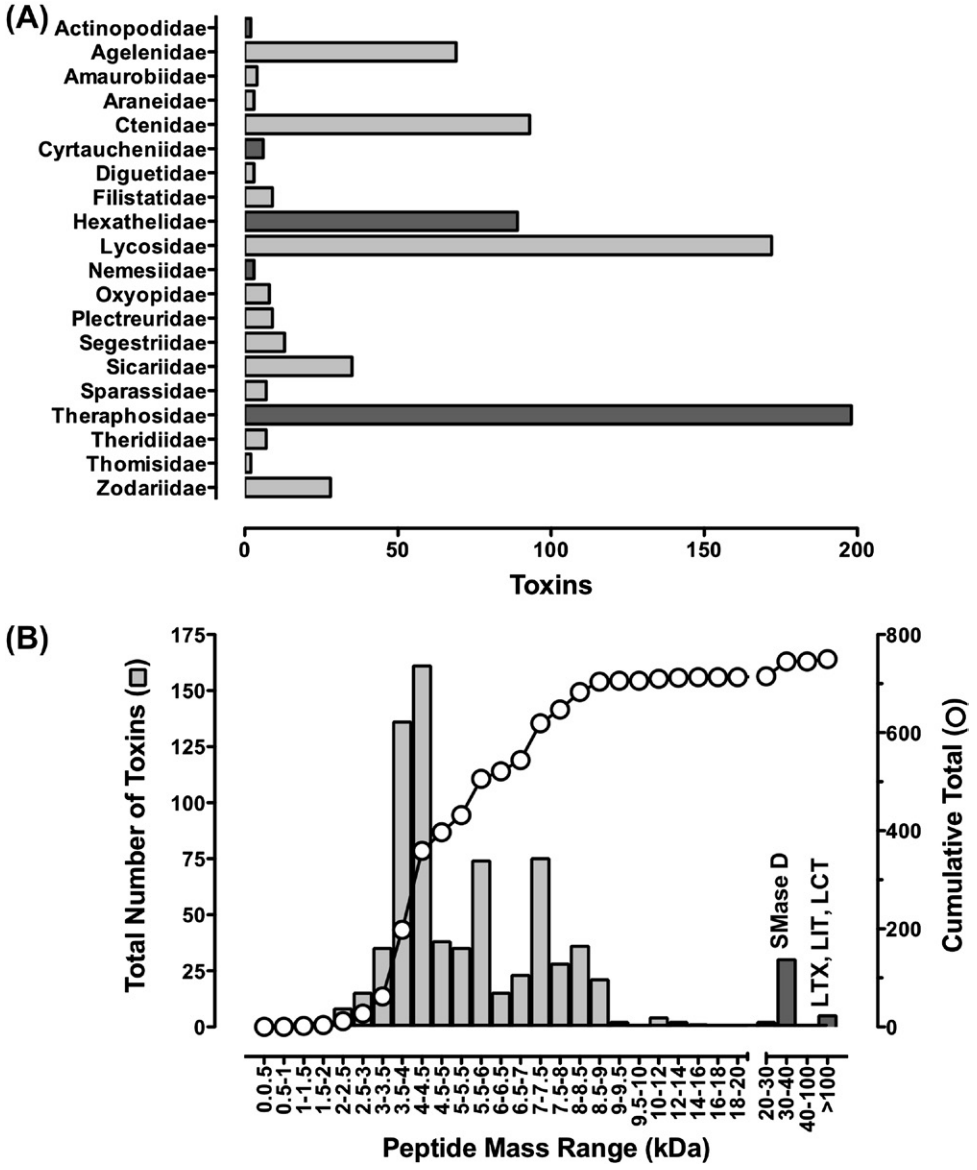

FIGURE 1 Distribution of spider venom peptide toxins. (A) Distribution of extant spider toxins organized by spider family. Bars in dark gray indicate peptide toxins from mygalomorph spiders, whereas light gray bars are from araneomorph spiders. (B) Mass distribution of extant spider toxins. Masses represent the monoisotopic oxidized mass sorted into 500 Da bins. The overlaid curve shows the cumulative total number of peptides. Dark gray columns show mass range dominated by sphingomyelinase D (SMase D), latrotoxins (LTX), latroinsectoxins (LIT), and latrocrustatoxins (LCT). *Data were collated from the ArachnoServer Spider Toxin Database (*www.arachnoserver.org [12]*) on November 25, 2011.*

to a lesser extent, marine cone snail venoms. Many of these spider peptide toxins are selectively insecticidal or modulate the activity of various targets in vertebrates, including humans. In particular, insect-selective toxins have been patented for their possible use as bioinsecticidal agents for the control of phytophagous pests or insect vectors of new and re-emerging diseases.[13] Other neurotoxins have been invaluable molecular tools for the identification and characterization of ion channel subtypes and for unraveling the process of neurotransmitter exocytosis. Yet others, have already been patented as antiarrhythmic and analgesics.[17,18] Indeed, in addition to their well known neurotoxic actions spider peptide toxins have been identified with an extensive range of other biological activities including antimicrobial, antiparasitic, cytolytic/haemolytic, as well as enzyme activity and protease inhibition (Fig. 2A). The focus of this review is the discovery, processing, structure, and function of spider venom peptides. In particular, it will detail the site and mechanism of their action, the molecular determinants for their pharmacology, and discuss the application of these peptides in the fields of pharmacology and neuroscience and the development of novel insecticides.

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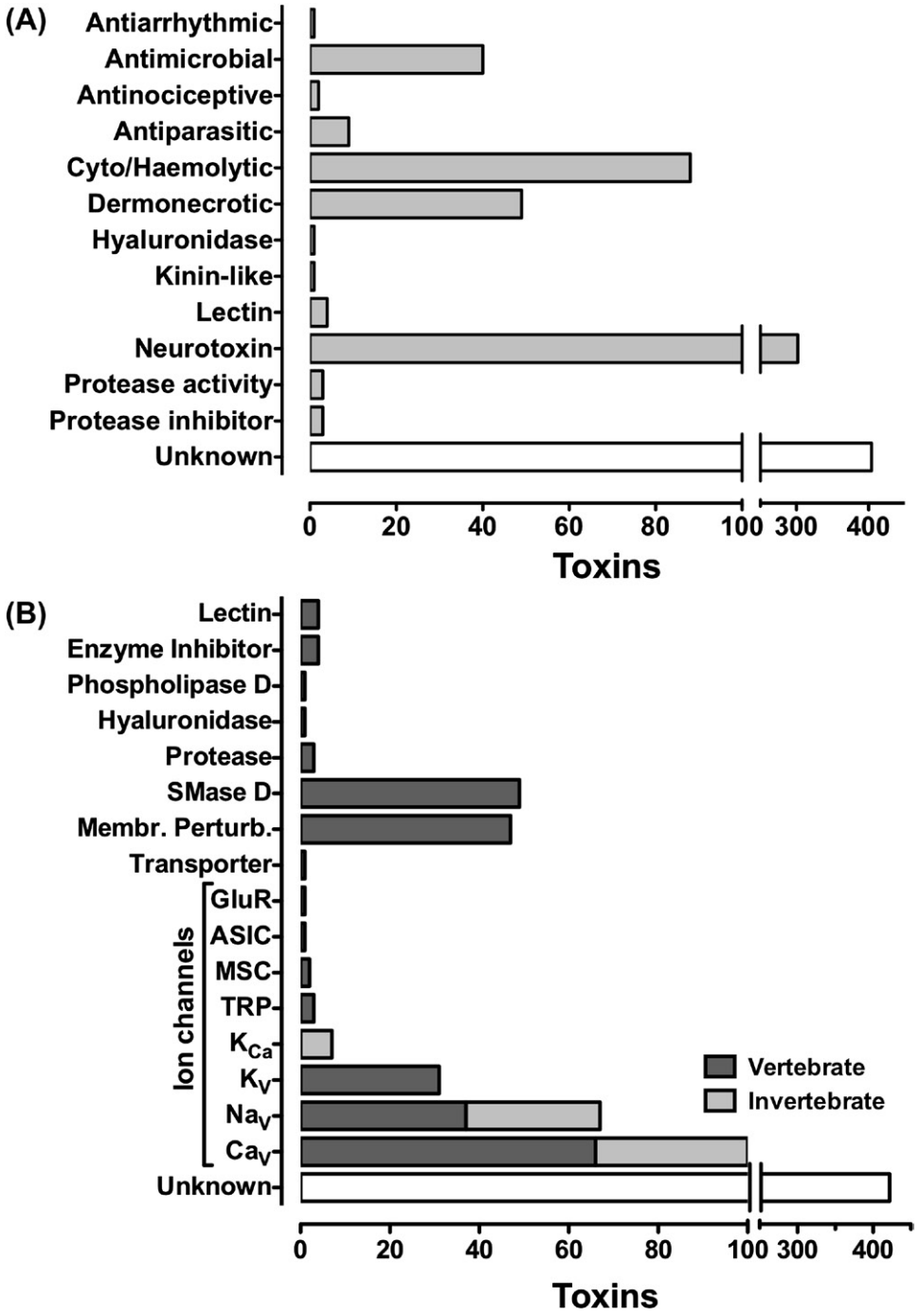

FIGURE 2 Biological activity and targets of spider toxins. Range of biological activities (A) and molecular targets (B) of spider toxins. Dark gray bars represent vertebrate targets, whereas light gray bars represent invertebrate targets. Toxins may have more than one activity and or target and may be phyla-selective or nonselective. Ion channel abbreviations: GluR, glutamate receptor; ASIC, acid-sensing ion channel; MSC, mechanosensitive channel; TRP, transient receptor potential; K_{Ca}, calcium-activated potassium channel. Note the discontinuous *x*-axis. *Data were collated from the ArachnoServer Spider Toxin Database (*www.arachnoserver.org [12]*) on November 25, 2011.*

METHODOLOGICAL APPROACHES IN THE ISOLATION OF SPIDER VENOM PEPTIDES

Several different methodological approaches have led to the isolation of novel spider peptide toxins. The most commonly used approach has been the use of insect and mammalian bioassays to drive venom fractionation using acute toxicity testing via injection assays or isolated organ bath screening. The weakness with this approach is that numerous toxins remain uncharacterized in terms of target

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and mode of action. Indeed in the ArachnoServer Spider Toxin Database (www.arachnoserver.org), there are currently >400 peptides with an unknown target (those designated with a 'U' prefix), representing a little over 50% of all spider peptides toxins so far reported (Fig. 2B). Also the potential array of targets for a novel toxin is too vast to be comprehensively screened. Another approach has been to study all the components of a single venom, or venoms of closely related species, and characterize their mode of action in target-oriented bioassays. This has been the approach taken with the hexatoxins (HXTXs; formerly atracotoxins) and theraphotoxins (TRTXs) where several families of toxins that modulate voltage-activated Na^+ (Na_V), Ca^{2+} (Ca_V), and K^+ (K_V) channels have been described. The limitation of this approach is that it does not always yield the most active toxin for a specific target and again many toxins remain uncharacterized. Currently, a number of groups are using a systematic approach employing screening assays using cloned ion channels expressed in *Xenopus* oocytes or other cells, or well-characterized channels in clonal cell lines or native cells. Such electrophysiological, ELISA, radioligand binding or absorbance/fluorescence-based methods have revealed high-affinity and often highly selective ligands that block or modulate ion channel subtypes for which there were no previous pharmacological tools (e.g. π-TRTX-Pc1a, τ-TRTX-Pc1c and purotoxin-1; see below). Unfortunately, this approach is limited if a potential therapeutic or insecticidal target has not been previously validated or if the target has not yet been cloned. This restricts the discovery of ligands to only those with affinity for known target subtypes. Accordingly, methodologies centered on a combination of the last two approaches are likely to be the most successful.

STRUCTURE OF THE PRECURSOR SPIDER VENOM PEPTIDE AND POSTTRANSLATIONAL PROCESSING

The expression of spider venom peptides, like those from marine cone snails and sea anemones, are translated mostly as prepropeptides that undergo posttranslational modification to yield the mature toxin.[27] These prepropeptide precursors typically are composed of an N-terminal signal peptide that precedes a propeptide region rich in acidic residues and of highly variable length, which is followed by the mature toxin sequence. It seems that during evolution, families of toxins within spider venoms underwent hypermutation in the mature peptide region while conserving the basic disulfide framework. This cystine framework seems to be associated with a specific signal sequence. However, the signal peptide was conserved because its role was, most likely, to direct the precursor to a specific secretory pathway thereby to ensure correct peptide folding. The specific role(s) of the propeptide region, however, is still not understood but most likely involved in the proteolytic processing and posttranslational modifications such as N-terminal pyroglutamine formation, isomerization, palmitoylation, and C-terminal trimming and amidation (Fig. 3B). In the case of the MIT-like U_1-HXTX-Hv1a (formerly ACTX-Hvf17) and high molecular mass latrotoxins, this N-terminal propeptide is absent.

LINEAR PEPTIDES

Membrane-acting Antimicrobial Peptides and Cytolytic Peptide Toxins

To date, several groups of cytolytic peptides with antimicrobial activity have been discovered in araneomorph spider venoms, especially from the members of the araneomorph spider families Ctenidae (ctenitoxins, CNTXs), Lycosidae (lycotoxins, LCTX), Oxyopidae (oxotoxins, OXTXs), and Zodariidae (zodatoxins, ZDTXs). These have been classified as membrane-acting antimicrobial peptides (MAMPs with the activity prefix 'M'). For a review of the structure and biological activities of cytolytic peptides from spider venom see Ref. 28.

These cytolytic peptides are short (<50 residues) linear, highly cationic, amphipathic peptides lacking cysteine residues and have isoelectric points >10 (Fig. 5). They are characterized by being highly basic peptides because of the high proportion of lysine residues, often with few arginine residues. In addition, they often possess an amidated C-terminus. In solution, these peptides are disordered; however, their secondary structure acquires an α-helical conformation, typical of antimicrobial pore-forming peptides, upon contact with membranes. The amphipathic motif is characterized by a right-handed ribbon of positively charged lysine and polar residues winding around the α-helix. The cytoplasmic membrane therefore serves as a target for MAMP action. The positive charge of MAMPs provides for electrostatic binding to the membrane surface, while their amphiphilic nature allows the peptides to incorporate into the bilayer resulting in the disturbance of the bilayer integrity and subsequent cell death. Several models have been proposed to explain the mechanism of pore formation including 'barrel stave,' 'carpet,' and 'toroidal' models or a combination of these, but no one model fits all spider venom peptide actions.[28] These peptides are highly active against Gram-negative and Gram-positive bacteria with minimal inhibitory concentrations in the low micromolar range. Furthermore, many MAMPs display fungicidal (against *Candida* spp.) and antitrypanosomal activity. The insecticidal effect of MAMPs on insects has been reported but is negligible, with very high LD_{50} values (5–10 nmol/g). However, coinjection of the neurotoxin ω-OXTX-Ol1a (formerly oxytoxin) with the MAMP M-OXTX-Ot1a (formerly oxyopinin 1)

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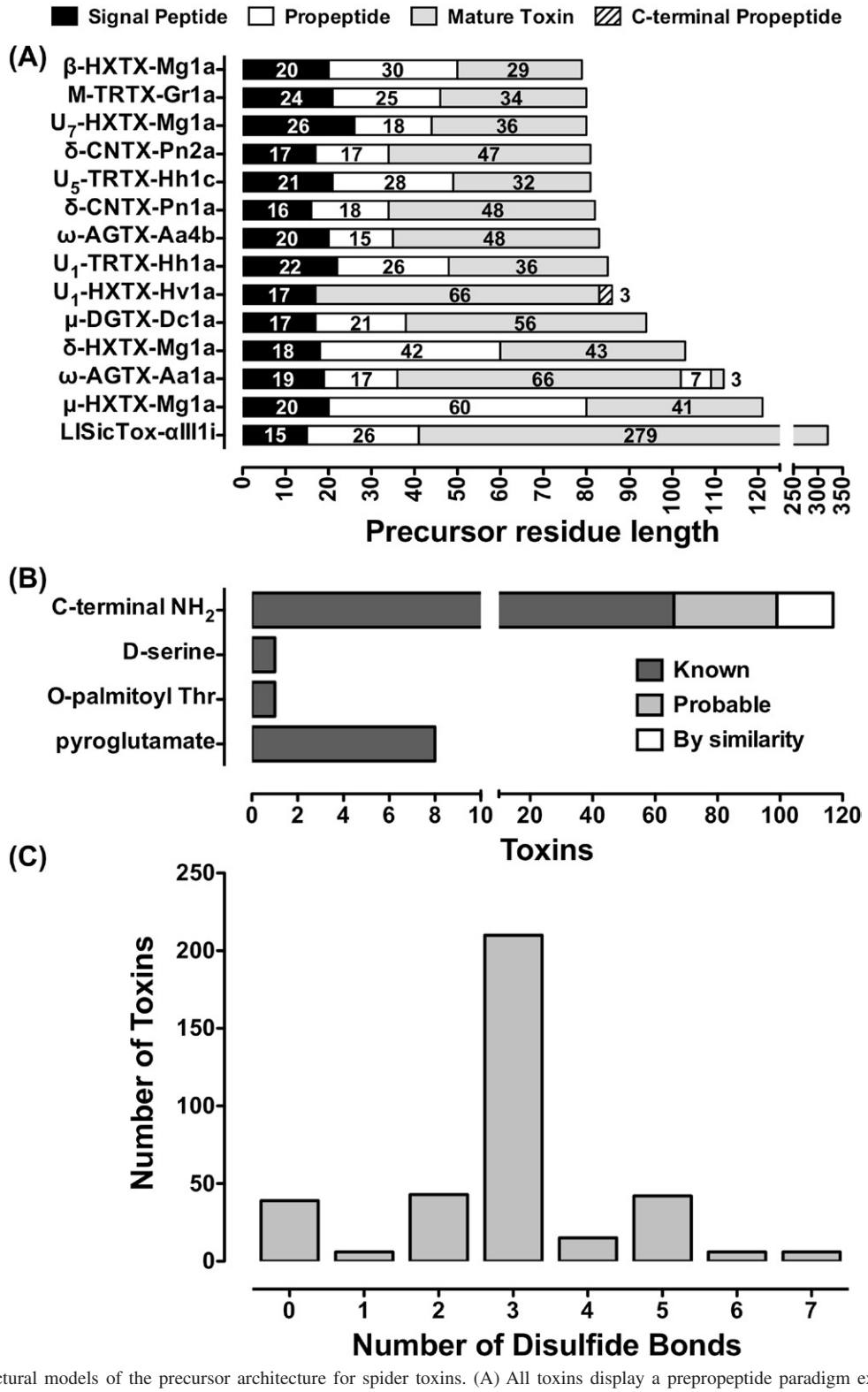

FIGURE 3 Structural models of the precursor architecture for spider toxins. (A) All toxins display a prepropeptide paradigm except for MIT-like U_1-HXTX-Hv1a and latrotoxins (not shown). ω-AGTX-Aa1a is a heterodimer consisting of a 66-residue major chain that is coupled via a disulfide bond to a 3-residue minor chain. (B) Known spider toxin posttranslational modifications (dark gray bars) as well as probable (light gray bars) and those predicted from sequence homology (white bars). (C) Distribution of the number of disulfide bonds found in spider toxins. Toxins with unknown disulfide connectivity are not shown. *Data were collated from the ArachnoServer Spider Toxin Database (*www.arachnoserver.org [12]*) on November 25, 2011.*

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into insect larvae resulted in a significant increase in the paralytic and lethal activity of the neurotoxin. Electrophysiological studies showed that M-oxotoxins reduce cell membrane resistance by opening nonselective ion channels. Unfortunately, these peptides also exhibit a hemolytic effect on human erythrocytes that is dependent on the content of phosphatidylcholine. Synthetic N- and C-terminal truncates of M-CNTX-Cs1d (formerly cupiennin 1d) showed that the residues involved in the cytolytic activity are located in the hydrophobic N-terminal chain segment. Removal of the first six N-terminal residues totally abrogated cytolytic activity, while the polar C-terminal region seems to modulate the peptide association at negatively charged cell surfaces to reduce activity. This may be because many MAMPs are amidated at the C-terminus to protect the peptide against proteolytic cleavage.

In addition to these short MAMPs, the venom of *Lachesana tarabaevi* also contains a family of long, linear M-ZDTX-Lt toxins (cyto-insectotoxins) with insecticidal, cytolytic, and antimicrobial activity. These are more than twice the length of typical MAMPs and possess very high charge at neutral pH. They still retain the α-helical motif but seem to be composed of two short MAMPs joined together in a 'head-to-tail' configuration by a short four-residue linker. The major difference between cyto-insectotoxins and conventional MAMPs is that the former seem to possess much high insecticidal activity.[29]

Several groups have speculated as to the role of these antimicrobial and cytolytic peptides in the venom of various spiders. They may play several roles; first to protect the spider from potentially infectious organisms arising from prey ingestion, second to assist in external digestion via cytolytic degradation of prey membranes, or, more probable, functioning as membrane disrupting agent in a cooperative fashion with disulfide-rich neurotoxins in the prey capture strategy by facilitating absorption and distribution of the neurotoxins.

DISULFIDE-RICH PEPTIDES

Structural Organization of Disulfide-Rich Peptides: Variations on an Ancestral Fold

Around 90% of spider venom peptides are compact globular proteins possessing several disulfide bridges. The number of disulfide bonds range from one to seven, but nearly 60% of all toxins have three disulfides bridges (Fig. 3C). In addition, several have modified N-termini, such as formation of pyroglutamate, or most commonly C-terminal amidation (Fig. 3B) that increase their *in vivo* stability. These peptides, predominantly targeting voltage-activated ion channels, often contain a 'disulfide pseudoknot' which places them in a class of toxins and inhibitory polypeptides with an 'inhibitory cystine-knot' (ICK) motif.[20] This structural motif is normally exemplified by a triple-stranded, antiparallel β-sheet stabilized by disulfide bridges. Because not all ICK peptides exhibit the N-terminal β-sheet (β1 in Fig. 4Cb), a modified definition composed of 'an antiparallel β-hairpin stabilized by a cystine-knot' without a mandatory third β-sheet has been proposed. The three disulfide bridges and intervening backbone form a pseudoknot consisting of a ring (C_I–C_{IV}, C_{II}–C_V) penetrated by a third disulfide bridge (C_{III}–C_{VI}) (Fig. 4Db). However, within this fold-class, the biological activities of spider ICK toxins are quite diverse with activity at Na_v, Ca_v, K_V, acid-sensing channels, transient receptor potential, and mechanosensitive channels (see below). This highlights the observation that different biological functions are often grafted onto the same, or similar, structural scaffolds. Another structural fold has been defined for spider toxins. The disulfide-directed β-hairpin (DDH) fold lacks the disulfide knot and is composed of a double-stranded antiparallel β-hairpin stabilized by two mandatory disulfide bridges with a current consensus sequence of $–CX_{4–19}–CX_2$[G or P]$X_2–CX_{4–19}$–C– where X is any amino acid (Fig. 4A–B). The ICK motif seems to have evolved from this simpler canonical ancestral fold.[32] This DDH fold has been observed in a range of toxins with unknown targets such as MIT-like U_1-HXTX-Hv1a,[33] U_1-TRTX-Hh1 toxins, U_1-TRTX-Asp1f and -Asp1g, and U_1-TRTX-Lp1a and -Lp1b.[7] The ICK fold creates hyperstable miniproteins with tremendous chemical, thermal, and biological stability. ICK toxins are typically resistant to extremes of pH, organic solvents, and high temperatures.[4] However, from a therapeutic perspective, their most important property is their resistance to proteases. Specific differences in the DDH and ICK structural folds, determined by the spacing between cysteine residues and their connectivity, is critical for the presentation of key functional residues to the target, thus generating a vast array of peptides acting on distinct biological systems. This together with their protease resistance and compact nature provides an effective scaffold for the design of molecular tools and therapeutics.[20]

Voltage-Activated Sodium Channels: Blockers and Gating Modifiers

Na_v channels are responsible for the initiation and propagation of the action potential in excitable cells. To date, nine mammalian Na_v channels (Na_V1.1–1.9) have been cloned, and in all cases, except for Na_V1.9, they are functionally expressed and extensively characterized. Interestingly, much of their structure and function has been elucidated by the use of various plant and animal toxins. These molecular probes bind with seven identified targets, referred to as neurotoxin receptor sites 1–7 of which four sites bind spider peptide toxins.[2] Spider venoms are becoming an interesting source of peptide toxins that modulate Na_V channel conductance and/or gating.

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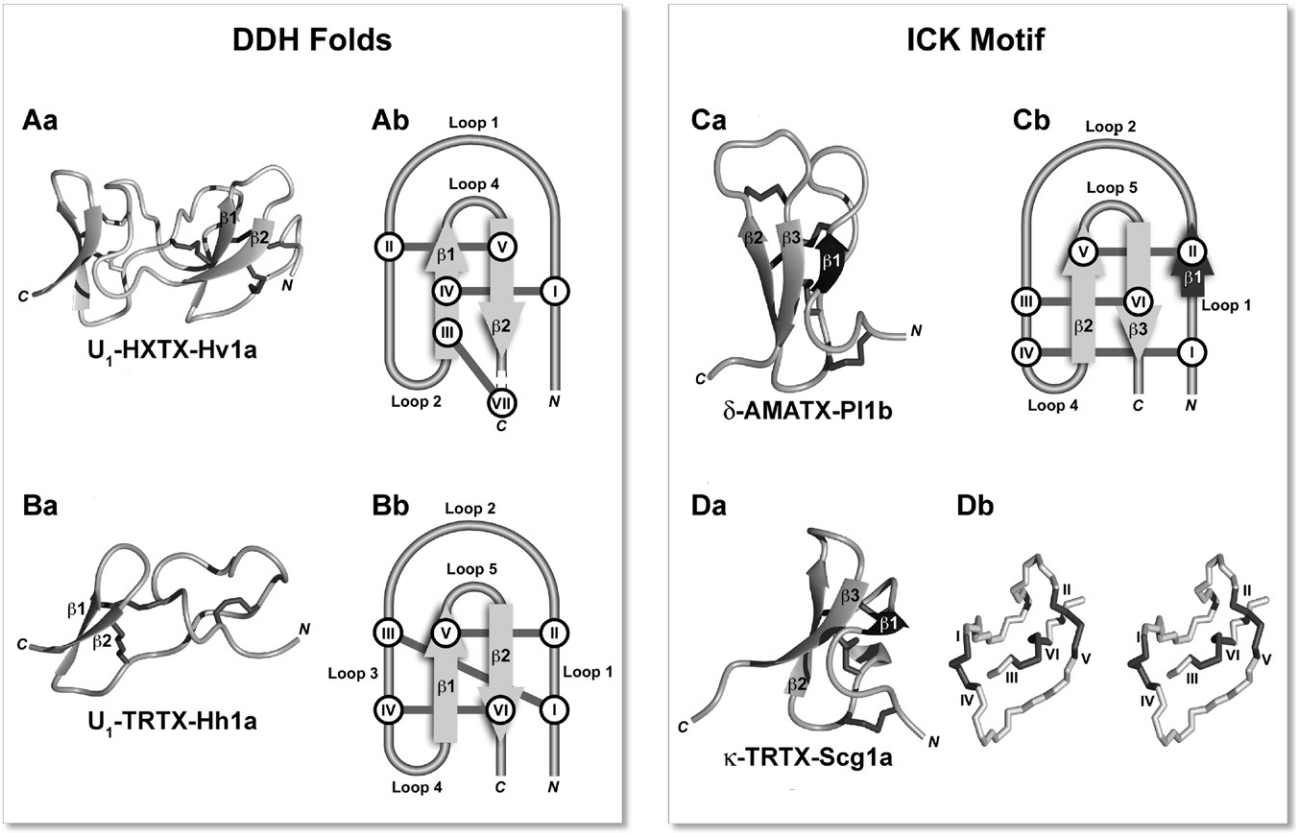

FIGURE 4 Evolution of DDH-related folds (A,B) into the ICK structural motif (C,D). Left-hand panels (Aa-Da) show a schematic view of the 3D structures of typical representatives of the two structural motifs. (Aa) MIT-like U_1-HXTX-Hv1a from *Hadronyche versuta* modeled on the structure of MIT1 from the black mamba snake *Dendroaspis p. polylepis*, (Ba) U_1-TRTX-Hh1a (formerly HWTX-II; PDB 1I25), (Ca) δ-AMATX-Pl1b (PDB 1V91) and (Da) κ-TRTX-Scg1a (formerly SGTx1; PDB 1LA4). Right-hand panels (Ab-Cb) show schematic representations of the structural motifs depicting the formation of the cystine knot and possible addition of the third β-sheet. The dark arrow (β1) in panel Cb represents the additional β-sheet not always present in ICK spider toxins. (Db) Stereoview of the pseudoknot of κ-TRTX-Scg1a. In all panels, β-sheets are shown as gray arrows, and disulfide bridges connecting cysteine residues are shown as dark gray lines with roman numerals.

Several spider toxins have been found to block Na^+ conductance such as μ-CNTX-Pn1a (formerly PNTx1) from *Phoneutria nigriventer*, μ-TRTX-Hh2a (formerly huwentoxin-4) from the venom of *Haplopelma huwenum* and the structurally related μ-TRTX-Hhn1a, -Hhn1b, and -Hhn2a (formerly hainantoxin III–V) from *Haplopelma hainanum*. This action most probably occurs via an interaction with site 1 of the Na_V channel. Interestingly, an ortholog, μ-TRTX-Hhn2b (formerly hainantoxin-I), also has a similar action but displays a 15-fold increase in selectivity for insect vs. mammalian Na_v channels. Therefore, these peptides represent the first family of spider toxins to selectively block Na^+ conductance and, in the case of μ-TRTX-Hhn2b, the first insect-selective toxin to block the Na_v channel.

Spider δ-toxins from funnel-web (*Hadronyche* and *Atrax* spp.) and eastern mouse spiders (*Missulena bradleyi*) are lethal toxins, responsible for the major symptoms of human envenomation (e.g. δ-HXTX-Ar1a; Fig. 5). Together with δ-HXTX-Mg1a (formerly Magi-4) from *Macrothele gigas*, δ-TRTX-Cj1a (formerly jingzhaotoxin-I) from *Chilobrachys jingzhao* and δ-CNTX-Pn2a (formerly PnTx2-6) from *Phoneutria nigriventer*, this family of 33–43 residue peptides has been shown to target the Na_V channel.[14,35] Electrophysiological studies have identified that the lethal δ-HXTXs alter neuronal excitability in both insect and mammalian neurons by causing a prolongation of action potential duration resulting in the appearance of plateau potentials, accompanied by spontaneous repetitive firing. These actions result mainly from a slowing of Na_V channel inactivation. Radiolabel binding experiments reveal that this results from an interaction with site 3 on both mammalian and insect Na_V channels in a comparable manner with the scorpion α-toxins.[2]

Other excitatory toxins interact with Na_V channel site 4 by acting as 'gating modifiers' of activation. δ-Amaurobitoxins (δ-AMATX-Pl1 toxins; formerly δ-paluITs) from the spider *Pireneitega luctuosus* are a family of four insect-selective peptides that act by slowing down insect Na_v channel inactivation, similar to site 3 scorpion neurotoxins such as LqhαIT. Despite this, δ-AMATX-Pl1a (Fig. 4Ca) has been shown to

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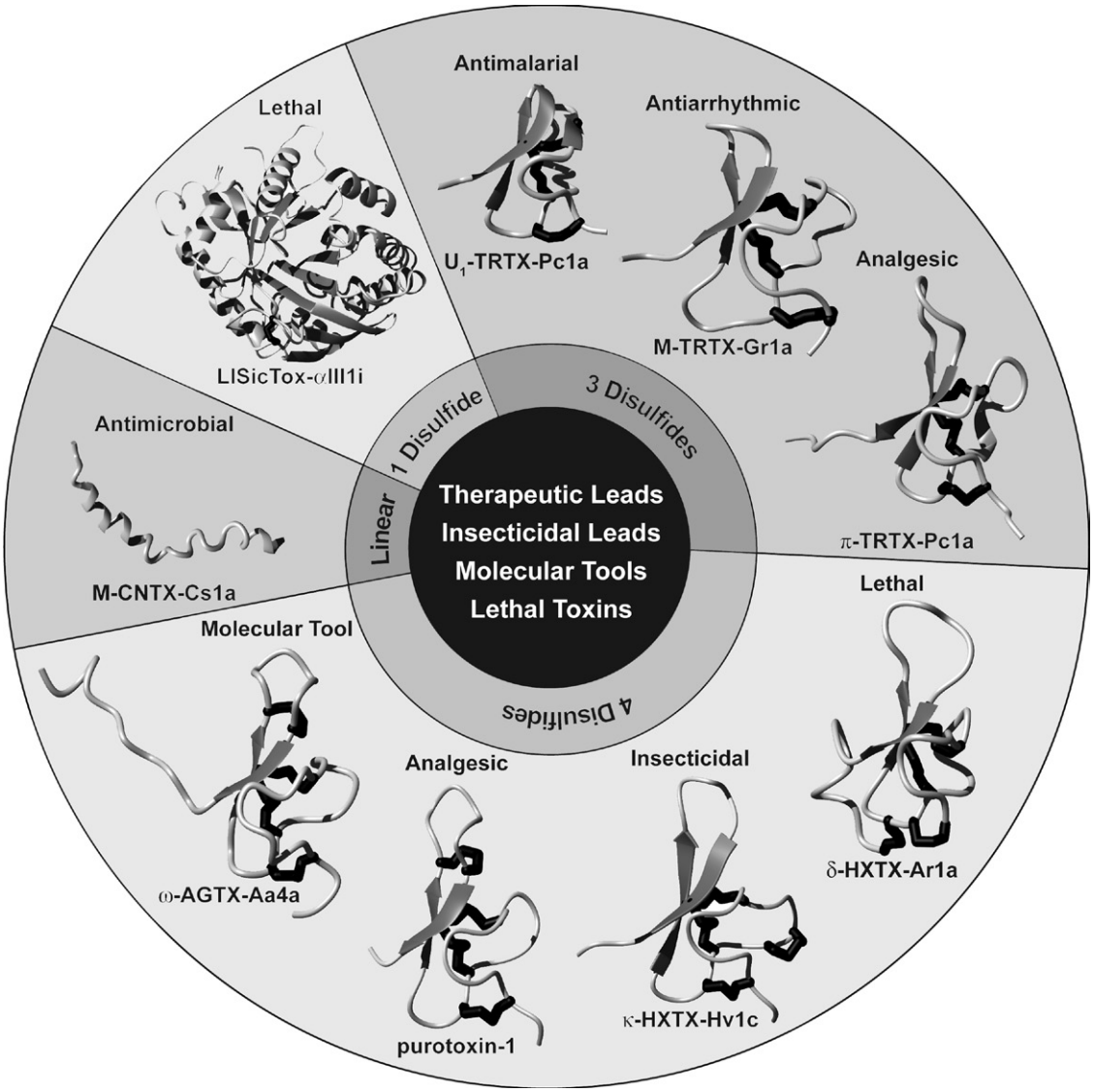

FIGURE 5 Spider venom peptides being investigated as therapeutic or insecticidal leads, together with toxins that are used as molecular tools or have been identified as the causative agent in lethal envenomation. Schematic view of NMR 3D structures showing the location of β-strands (dark gray arrows) and α-helices (light and dark gray helices). Disulfide bridges are shown as thick black tubes. LlSicTox-aIII1i is the X-ray crystal structure of the sphingomyelinase D from *Loxosceles laeta* (PDB 1XX1) responsible for clinical cases of loxoscelism. This toxin has a single disulfide bond Cys^{51}–Cys^{57}.

displace the site 4 excitatory scorpion β-toxin, Bj-xtrIT, from cockroach neuronal membranes and fail to displace LqhαIT binding. Thus, δ-AMATX-Pl1 toxins reveal that the modulation of Na_v channel inactivation can be achieved by binding to a site thought to be associated with the effects on channel activation. In support, the μ-agatoxins from *Agelenopsis* and *Hololena* spp. and β-HXTX-Mg1a (formerly Magi-5) also seem to share this activity.[1] This provides further support for the hypothesis that the insect site 4 is a macrosite, which may be allosterically linked to channel inactivation.

Perhaps the most important discovery in this area is that spider venoms may be excellent sources of Na_V channel subtype specific toxins that could be developed as novel analgesics. Previously, $Na_V1.3$, $Na_V1.7$ or $Na_V1.8$ channels have been identified to be involved in pain signaling, particularly the $Na_V1.7$ subtype. Presently, three spider toxins show $Na_V1.7$ channel subtype selectivity. Of these, the most potent and selective was β-TRTX-Tp2a (formerly protoxin II) a 30-residue ICK peptide from the Green velvet tarantula *Thrixopelma pruriens*. Unfortunately, despite its 100-fold selectivity for $Na_V1.7$ compared with the other Na_V channel subtypes tested, β-TRTX-Tp2a still has relatively high potency against $Na_V1.2$ and $Na_V1.5$ resulting in severe toxicity in rats.[25] In contrast, a highly homologous toxin, β-TRTX-Gr1b (formerly GsAF I), from the Chilean rose tarantula

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Grammostola rosea failed to produce signs of toxicity at analgesic doses.[17] At present, the Na_V subtype selectivity of β-TRTX-Gr1b is unknown, but structure function studies using β-TRTX-Gr1b and β-TRTX-Tp2a should provide key information for the rational design of novel analgesics.

Voltage-Activated Potassium Channels

Voltage-activated K^+ channels are the most abundant ion channels and play important roles in excitable cells such as controlling neuronal excitability, resting membrane potential, and neurotransmitter release. Although there are many voltage-activated K^+ channel families, two are of particular importance to this discussion. First, the outward-rectifying K^+ (K_V) channels are classified into four groups K_V1-K_V4 and, second, Ca^{2+}-activated K_V (K_{Ca}) channels. Spider toxins have been instrumental in the structural and pharmacological characterization of the K_v2 and K_v4 channels particularly those isolated from "tarantula" spider venoms.[7] κ-Sparatoxins-1 toxins (κ-SPRTX-1; formerly heteropodatoxins 1–3) from *Heteropoda venatoria* (Sparassidae) are a family of toxins that prolong the duration of the cardiac action potential via a voltage-dependent block of $K_V4.2$, but not $K_V1.4$, channels. Moreover, the toxins slowed the time course of channel activation and inactivation and caused a depolarizing shift in the voltage-dependence of activation. Thus, κ-SPRTX-Hv1 toxins represent pharmacological tools with which to study transient outward K_V currents in cardiac tissue. Other $K_v4.2$ channel blockers include κ-TRTX-Ps1a and -Ps1b (formerly phrixotoxin-1 and -2) from the venom of *Paraphysa scrofa* and κ-TRTX-Gr1a and -1b (formerly hanatoxins-1 and -2), from *Grammostola rosea*. κ-TRTX-Sc1a (formerly stromatoxin-1), from *Stromatopelma calceatum* blocks $K_V4.2$, but in addition it inhibits $K_V2.2$, and to a lesser extent, $K_V2.1$ and $K_V2.1/K_V9.3$ subtypes. By contrast, κ-TRTX-Hm1a and -Hm2a (formerly heteroscodratoxin-1 and -2), from *Heteroscodra maculata* venom, bind to the voltage sensor and inhibit K_V channels. κ-TRTX-Hm2a seems to be specific for K_V2 channels, whereas κ-TRTX-Hm1a also inhibits $K_V4.1$.[7]

Of interest has been the discovery of a family of insect-selective neurotoxins from the Australian funnel-web spider *Hadronyche versuta* and *Eucratoscelus constrictus*. κ-HXTX-Hv1c (formerly Janus-faced atracotoxin-Hv1c) and κ-TRTX-Ec1 toxins both target K_{Ca} channels in cockroach neurons.[11,34] The most remarkable feature of κ-HXTX-Hv1c is a vicinal disulfide bridge between the sidechains of Cys^{13} and Cys^{14} (Fig. 5). Vicinal disulfide bridges are exceptionally rare in peptides and proteins. By alanine-scanning mutagenesis, the vicinal disulfide as well as Arg^8, Pro,[9] and Tyr^{31}, were determined to form the binding epitope (pharmacophore). Intriguingly, the functionally critical Arg^8 and Tyr^{31} residues in κ-HXTX-Hv1c align extremely well with the Lys–Phe/Tyr diad that is conserved across structurally dissimilar K_{Ca} channel blockers such as BgK, agitoxin2, and charybdotoxin.[11]

Voltage-Activated Calcium Channels

A variety of Ca_V channels are classified into low-voltage-activated (LVA) channels that inactivate rapidly, and high-voltage-activated (HVA) channels that show no inactivation. HVA Ca_V channels are further subdivided according to their pharmacological and biophysical characteristics into $Ca_V1.1$–1.4 (L-type), and $Ca_V2.1$ (P/Q-type), $Ca_V2.2$ (N-type), and $Ca_V2.3$ (R-type). LVA Ca_V channels comprise $Ca_V3.1$–3.3 subtypes (T-type). In the heart and CNS, the $Ca_V2.x$ and $Ca_V3.x$ channels (non-L type) are inhibited by many peptide toxins from animal venoms including a number of spider toxins. Given the role of these Ca_V channel subtypes in neurotransmission, selective toxins could therefore have potential as lead compounds for the development of neuroprotective and analgesic agents. Undoubtedly, the best characterized Ca_V channel blockers are the ω-agatoxins (ω-AGTXs) from *Agelenopsis* spp. spider venom that inhibit release of a variety of neurotransmitters.[1] ω-Agatoxins vary from 5–10 kDa in length and are classified according to their sequence homology and spectrum of activity against insect and vertebrate Ca_v channels. Type- I and Type- II ω-AGTXs are selective blockers of insect Ca_V channels. Type- III ω-AGTXs are inactive in insects but block the binding of the $Ca_V2.2$ channel ligand [^{125}I]ω-conotoxin GVIA to vertebrate membranes, as do toxins from group I. They bind in a voltage-dependent manner to all Ca_V1 and Ca_V2, but not Ca_V3, channels in mammalian neurons. Type- IV toxins such as ω-agatoxin-Aa4a (Fig. 5; formerly ω-Aga IVA) are not toxic at the fly neuromuscular junction but do block Ca_V channels in insect central neurons and block $Ca_V2.1$ channels in mammals. This results from a depolarizing shift in the voltage dependence of activation. ω-Agatoxin-Aa4a has therefore become the defining pharmacology for $Ca_V2.1$ channels. Recently, ω-LCTX-Gsp2671a (formerly ω-Lsp-IA) from a *Geolycosa sp*. has been found to also inhibit $Ca_V2.1$ channels. This toxin decelerates activation kinetics and partially inhibits the amplitude of P-currents in rat cerebellar Purkinje neurons. It also competes with ω-agatoxin-Aa4a for binding to $Ca_V2.1$ channels in hippocampal neurons.[23] A selective $Ca_V2.2$ blocker ω-TRTX-Hh1a (formerly huwentoxin-10) from *Haplopelma huwenum* has also been described, which is likely to be useful as a therapeutic lead for the treatment of pain.[19]

Phoneutria nigriventer and *reidyi* venom also contain several toxins targeting a variety of Ca_v channel subtypes including: ω-CNTX-Pn2a ($Ca_V2.1$ and $Ca_V2.3$), ω-CNTX-Pn3a (Ca_V1 and $Ca_V2.2$), ω-CNTX-Pn1a (Ca_V1), ω-CNTX-Pn4a ($Ca_V2.1$), and ω-CNTX-Pr1a (Ca_V1, $Ca_V2.1$ and $Ca_V2.3$).[9,30] Other spider toxins that target various Ca_V channel subtypes have also been isolated

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and characterized from mygalomorph (ω-TRTX-Gr1a and ω-TRTX-Hg1a) and araneomorph (ω-CNTX-Cs1a, ω-SGTX-Sf1a, ω-FLTX-Kh1a, ω-PLTX-Pt1a) spiders. Unfortunately, Ca_V subtype selectivity was not always clearly established for many of these latter toxins.

Several insect-selective Ca_v channel blockers have been discovered in various spider venoms. ω-Hexatoxins from the venom Australian funnel-web spiders from *Atrax* and *Hadronyche* spp. in particular have undergone extensive characterization. These peptides are lethal neurotoxins with ω-HXTX-Hv1a (Fig. 5; formerly ω-atracotoxin-Hv1a) one of the most potent insecticidal peptide toxins discovered to date. ω-HXTX-Hv1a binds to orthopteran Ca_V channels at low nanomolar concentrations, whereas it has no effect on cloned human $Ca_V2.1$, $Ca_V2.2$, or $Ca_V1.2$ channels. ω-HXTX-Hv2a, a 45 residue insect-selective toxin, inhibits insect Ca_v channels but with a vastly different 3D structure.[14] Recently, several toxins have been partially characterized from other spider venoms. Perhaps the best characterized is ω-TRTX-Hh2a (formerly huwentoxin-5) from *Haplopelma huwenum*. This peptide is an insecticidal toxin that blocks HVA but not LVA Ca_V channels in adult cockroach DUM neurons but lacks activity on vertebrate neurons. It also lacks activity on insect Na_V or K_V channels. The toxin reversibly paralyzes locusts and cockroaches and causes death at high doses, but it has no effect on mice by intraabdominal or intracerebroventicular injection.[6] Other toxins that have been less well characterized in terms of their target or activity include the ω-oxotoxins (formerly oxytoxin 1 and 2) from *Oxyopes* spp. that are insecticidal but with weak activity on vertebrate HVA Ca_V1 and Ca_V2 channels. ω-oxotoxin-Ol1a was shown to be lethal at high dose to lepidopteran larvae, while being nontoxic to mice at modest doses.[31] ω-TRTX-Ba1a is another insecticidal toxin that is lethal to crickets but causes no adverse effects when injected either intracranially or intraperitoneally into mice at modest doses. The molecular target of the toxin has not been experimentally determined, but it has no effect on insect Para/tipE Na_V channels or mammalian $Na_V1.2$ and $Na_V1.5$ channels. The toxin shows strong sequence homologies to ω-TRTX-Asp1g from the related spider *Aphonopelma sp.* that is known to be insecticidal and which is assumed to inhibit Ca_V channels.[5] Finally, ω-PLTX-Pt1a is assumed to be insecticidal as it seems to block presynaptic Ca_V2 channel currents in *Drosophila* nerve terminals but not synaptic transmission at the frog neuromuscular junction. Interestingly, it contains a unique posttranslational modification, namely a C-terminal *O*-palmitoyl threonine amide residue, which is essential for toxin activity.[16]

Acid-Sensing Ion Channels

An exciting development has been the discovery of a spider toxin targeting novel acid-sensitive ion channels (ASIC) belonging to the epithelial sodium channel/degenerin (ENaC/DEG) superfamily. These proton-gated sodium channels are associated with nociception, taste transduction, and perception of extracellular pH changes in the brain and sensory ganglia and may also contribute to synaptic plasticity, learning, and memory. The ASIC1a subunit, the most abundant ASIC subunit in the CNS, has been implicated as a novel therapeutic target in pain, ischemic stroke, depression, and autoimmune and neurodegenerative diseases such as multiple sclerosis, and Huntington's and Parkinson's Disease. π-TRTX-Pc1a (Fig. 5; formerly psalmotoxin 1), isolated from the venom of *Psalmopoeus cambridgei*, has been found to selectively inhibit homomultimeric ASIC1a channels, but not other ASIC subtypes. The mechanism of ASIC1a inhibition by π-TRTX-Pc1a is not yet understood, however, given that gating of ASIC channels by protons is likely to be different from that of the voltage-activated channel family, this may be via a novel mechanism. So far this is the only selective ligand with which to study the functional role and pathophysiology of ASIC channels. π-TRTX-Pc1a is efficacious as an analgesic in rat models of acute pain and as a neuroprotectant in a model of ischemic stroke. Docking models of the π-TRTX-Pc1a–ASIC1a complex are now offering new insights into the mechanism by which π-TRTX-Pc1a modulates the activity of ASIC1a.[24]

Mechanosensitive Ion Channels

Mechanosensitive channels (MSCs) potentially transduce many external (sound, vibration, and touch) and internal stimuli (local blood flow control, cell volume regulation, and dilation-induced heart rate changes). The only well-characterized MSCs cloned from eukaryotes are a subset of the K^+ selective 2P family. However, the physiological role of these channels is only just starting to be explored after the discovery of M-TRTX-Gr1a (Fig. 5; formerly GsMTx4) as a specific ligand.[21] This peptide was isolated from the venom of *Grammostola rosea* and produces a complete and specific block of MSCs in astrocytes and kidney cells. However, this seems to result from a perturbation of the channel-bilayer boundary, which no doubt underlies its antimicrobial activity (hence 'M' prefix). M-TRTX-Gr1a also blocks MSCs in atrial tissue, suggesting it could also be a new lead compound for the development of antiarrhythmics and drugs to treat atrial fibrillation, spinal cord damage, muscular dystrophy, and gliomas. Several spider toxins, which block cation-selective MSCs with additional actions on voltage-activated ion channels, have been isolated from *G. rosea* venom. These include κ-TRTX-Gr2c (formerly GsAF II) that has been patented as an antiarrhythmic and analgesic peptide[17,18] as well as κ-TRTX-Gr2a (formerly GsMTx-2) and the identical peptide κ-TRTX-Ps1b from *Paraphysa scrofa* that have only weak affinity with MSCs.

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Purinergic (P2X) Receptors

$P2X_1$–$P2X_7$ purinergic receptors are ATP-gated cation-selective ion channels, of which some subtypes are associated with the transmission of nociception. In particular, $P2X_3$ receptors are involved in a wide range of pain states. Purotoxin-1 from *Geolycosa* sp. is the first potent and selective modulator of $P2X_3$ receptors from spider venom (Fig. 5). This 35-residue ICK peptide produces potent analgesic effects in the low nanomolar range in a variety of inflammatory pain models. This involves a complex mechanism of action to prolong the rate of recovery from $P2X_3$ channel desensitization.[10] Thus, purotoxin-1 represents a promising lead compound for the development of analgesics targeting $P2X_3$ receptors.

Transient Receptor Potential (TRP) Channels

Transient receptor potential (TRP) channels are a superfamily of cation channels that are also the target of spider peptides. TRPV1 is a nonselective cation channel that is preferentially expressed on Aδ and C pain fibers and in sensory neurons in pain pathways. τ-TRTX-Pc1c (formerly vanillotoxin 3), and to a lesser extent τ-TRTX-Pc1b (formerly vanillotoxin 2), are potent and selective members of a family of toxins from *Psalmopoeus cambridgei* that activate TRPV1 channels.[26] Another toxin τ/κ-TRTX-Pc1a (formerly vanillotoxin 1) also inhibits $K_V2.1$ channels, supporting potential similarities between TRP and voltage-gated channel structures. TRP channels can now be included among the targets of peptide toxins, showing that spiders, like plants (e.g. capsaicin from chilli plants), deter predators by activating TRP channels on sensory nerve fibers to elicit pain and inflammation.

Glutamate Receptors or Transporters

Arguably, the most important excitatory neurotransmitter in the mammalian CNS and insect peripheral nervous system is L-glutamate. Although there have been a wide range of glutamate receptor antagonists found in spider venoms, these are mostly acylpolyamines (in particular α-agatoxins [1]). The only peptide toxin from spider venom known to interact with glutamate receptors is Γ-CNTX-Pn1a (formerly PnTx4(5–5)) from *Phoneutria nigriventer*, an insecticidal toxin that also reversibly inhibits ionic currents mediated through NMDA receptors in rat hippocampal neurons.[9] However, δ-CNTX-Pn1b (formerly PnTx4-3) from the same venom also inhibits glutamate uptake in rat cortical synaptosomes. ω-CNTX-Pn3a (formerly PnTx3-4) decreases glutamate release from synaptosomes via a block of glutamate transporters in synaptosomes in addition to its activity to block $Ca_V2.x$ channels. These toxins therefore provide *P. nigriventer* spiders with the means to disrupt specific aspects of insect glutamate neurotransmission.

Antimalarial Toxins

The ability of spider venom peptides to produce selective toxicity against various microorganisms also encompasses the malaria parasite *Plasmodium falciparum*. Two unrelated ICK peptides, U_1-TRTX-Pc1a (Fig. 5; formerly psalmopeotoxin I) and U_2-TRTX-Pc1a (formerly psalmopeotoxin II) both from *Psalmopoeus cambridgei* inhibit the intraerythrocyte stage of *Plasmodium falciparum*.[3] Importantly, neither peptide has significant hemolytic, antifungal or antibacterial activity or displayed overt neurotoxicity. Presently, the mechanism underlying the antimalarial action is unknown but may involve new permeability pathways that are established in the erythrocyte membrane following parasite invasion. Thus, in addition to being useful therapeutic leads, these peptide toxins might help validate a new antimalarial drug target.

CONCLUSIONS AND FUTURE DIRECTIONS

Using several molecular mechanisms, spiders have developed a combinatorial peptide library strategy to vary their toxin pool.[27] New pharmacologies have been produced by hypermutation of the mature toxin sequence resulting in a rich diversity of neurotoxins with high potency and selectivity for multiple cellular targets built onto a stable protease-resistant peptide backbone. This confers an evolutionary advantage for the spider, enabling them to efficiently immobilize a wide variety of prey. As pharmacologists, we can take advantage of this preoptimized peptide library as a diversified source of molecular probes for identifying subtype differences in specific targets and as leads for novel therapeutics such as analgesics, antimalarials, antiarrhythmics, neuroprotectants, and antimicrobials.

Given that spiders rely upon their venom to immobilize or kill their prey, it is not surprising that they also contain a wide variety of insect-selective toxins that may provide the basis for the development of biopesticides. Interestingly, a large number of these phyla-specific toxins target voltage-activated ion channels. These represent suitable targets for the future development of insecticides because they are ubiquitous among insects. This may lead to the development of more efficacious and more selective insecticidal toxins.

To date, very few studies have investigated the actions of spider toxins on transmitter-gated ion channels, G protein-coupled receptors or transporters. Given the important role these structures play in homeostasis in invertebrates and vertebrates, and the increasing use of target-based high-throughput assays, future research should lead to discovery of additional spider toxin pharmacologies beyond those already discussed in this review. This should also provide leads for the development of new therapeutics and bioinsecticides and validate novel drug and insecticide targets.

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REFERENCES

1. Adams ME. Agatoxins: ion channel specific toxins from the American funnel web spider, *Agelenopsis aperta*. *Toxicon* 2004;**43**:509–25.
2. Cestèle S, Catterall WA. Molecular mechanisms of neurotoxin action on voltage-gated sodium channels. *Biochimie* 2000;**82**:883–92.
3. Choi S-J, Parent R, Guillaume C, Deregnaucourt C, Delarbre C, Ojcius DM, et al. Isolation and characterization of Psalmopeotoxin I and II: two novel antimalarial peptides from the venom of the tarantula *Psalmopoeus cambridgei*. *FEBS Lett* 2004;**572**:109–17.
4. Colgrave ML, Craik DJ. Thermal, chemical, and enzymatic stability of the cyclotide kalata B1: the importance of the cyclic cystine knot. *Biochemistry* 2004;**43**:5965–75.
5. Corzo G, Bernard C, Clement H, Villegas E, Bosmans F, Tytgat J, et al. Insecticidal peptides from the theraposid spider *Brachypelma albiceps*: an NMR-based model of Ba2. *Biochim Biophys Acta* 2009;**1794**:1190–6.
6. Deng M, Luo X, Meng E, Xiao Y, Liang S. Inhibition of insect calcium channels by huwentoxin-V, a neurotoxin from Chinese tarantula *Ornithoctonus huwena* venom. *Eur J Pharmacol* 2008;**582**:12–6.
7. Escoubas P, Rash LD. Tarantulas: eight-legged pharmacists and combinatorial chemists. *Toxicon* 2004;**43**:555–74.
8. Escoubas P, Sollod B, King GF. Venom landscapes: mining the complexity of spider venoms via a combined cDNA and mass spectrometric approach. *Toxicon* 2006;**47**:650–63.
9. Gomez MV, Kalapothakis E, Guatimosim C, Prado MA. *Phoneutria nigriventer* venom: a cocktail of toxins that affect ion channels. *Cell Mol Neurobiol* 2002;**22**:579–88.
10. Grishin EV, Savchenko GA, Vassilevski AA, Korolkova YV, Boychuk YA, Viatchenko-Karpinski VY, et al. Novel peptide from spider venom inhibits $P2X_3$ receptors and inflammatory pain. *Ann Neurol* 2010;**67**:680–3.
11. Gunning SJ, Maggio F, Windley MJ, Valenzuela SM, King GF, Nicholson GM. The Janus-faced atracotoxins are specific blockers of invertebrate K_{Ca} channels. *FEBS J* 2008;**275**:4045–59.
12. Herzig V, Wood DLA, Newell F, Chaumeil P-A, Kaas Q, Binford GJ, et al. ArachnoServer 2.0, an updated online resource for spider toxin sequences and structures. *Nucleic Acids Res* 2011;**39**:D653–7.
13. King G, Sollod McFarland B, Nicholson G, Gunning S. Insecticidal polypeptides and methods of use thereof. United States Provisional Application Serial No 11/267,815 2005.
14. King GF, Escoubas P, Nicholson GM. Peptide toxins that selectively target insect Na_V and Ca_V channels. *Channels (Austin, Tex)* 2008;**2**:100–16.
15. King GF, Gentz MC, Escoubas P, Nicholson GM. A rational nomenclature for naming peptide toxins from spiders and other venomous animals. *Toxicon* 2008;**52**:264–76.
16. Kuromi H, Honda A, Kidokoro Y. Ca^{2+} influx through distinct routes controls exocytosis and endocytosis at *Drosophila* presynaptic terminals. *Neuron* 2004;**41**:101–11.
17. Lampe R. Analgesic peptides from the venom of *Grammostola spatulata* and use thereof. 1999; US Patent 5,877,026.
18. Lampe R, Sachs F. Antiarrhythmic peptide from the venom of the spider *Grammostola spatulata*. 1999; US Patent 5,968,838.
19. Liu Z, Dai J, Dai L, Deng M, Hu Z, Hu W, et al. Function and solution structure of Huwentoxin-X, a specific blocker of N-type calcium channels, from the Chinese bird spider *Ornithoctonus huwena*. *J Biol Chem* 2006;**281**:8628–35.
20. Norton R, Pallaghy P. The cystine knot structure of ion channel toxins and related polypeptides. *Toxicon* 1998;**36**:1573–83.
21. Ostrow KL, Mammoser A, Suchyna T, Sachs F, Oswald R, Kubo S, et al. cDNA sequence and *in vitro* folding of GsMTx4, a specific peptide inhibitor of mechanosensitive channels. *Toxicon* 2003;**42**:263–74.
22. Platnick NI. *The world spider catalog, version 12.0*. American Museum of Natural History; 2011. Online at http://research.amnh.org/iz/spiders/catalog. http://dx.doi.org/0.5531/db.iz.0001.
23. Pluzhnikov K, Vassilevski A, Korolkova Y, Fisyunov A, Iegorova O, Krishtal O, et al. Omega-Lsp-IA, a novel modulator of P-type Ca^{2+} channels. *Toxicon* 2007;**50**:993–1004.
24. Saez NJ, Mobli M, Bieri M, Chassagnon IR, Malde AK, Gamsjaeger R, et al. A dynamic pharmacophore drives the interaction between psalmotoxin-1 and the putative drug target acid-sensing ion channel 1a. *Mol Pharmacol* 2011.
25. Schmalhofer WA, Calhoun J, Burrows R, Bailey T, Kohler MG, Weinglass AB, et al. ProTx-II, a selective inhibitor of $Na_V1.7$ sodium channels, blocks action potential propagation in nociceptors. *Mol Pharmacol* 2008;**74**:1476–84.
26. Siemens J, Zhou S, Piskorowski R, Nikai T, Lumpkin EA, Basbaum AI, et al. Spider toxins activate the capsaicin receptor to produce inflammatory pain. *Nature* 2006;**444**:208–12.
27. Sollod BL, Wilson DT, Zhaxybayeva O, Gogarten JP, Drinkwater R, King GF. Were arachnids the first to use combinatorial peptide libraries? *Peptides* 2005;**26**:131–9.
28. Vassilevski AA, Kozlov SA, Grishin EV. Molecular diversity of spider venom. *Biochem Biokhim* 2009;**74**:1505–34.
29. Vassilevski AA, Kozlov SA, Samsonova OV, Egorova NS, Karpunin DV, Pluzhnikov KA, et al. Cyto-insectotoxins, a novel class of cytolytic and insecticidal peptides from spider venom. *Biochem J* 2008;**411**:687–96.
30. Vieira LB, Pimenta AM, Richardson M, Bemquerer MP, Reis HJ, Cruz JS, et al. Leftward shift in the voltage-dependence for Ca^{2+} currents activation induced by a new toxin from *Phoneutria reidyi* (Aranae, Ctenidae) venom. *Cell Mol Neurobiol* 2007;**27**:129–46.
31. Villegas E, Adachi-Akahane S, Bosmans F, Tytgat J, Nakajima T, Corzo G. Biochemical characterization of cysteine-rich peptides from *Oxyopes sp*. venom that block calcium ion channels. *Toxicon* 2008;**52**:228–36.
32. Wang X, Connor M, Smith R, Maciejewski MW, Howden ME, Nicholson GM, et al. Discovery and characterization of a family of insecticidal neurotoxins with a rare vicinal disulfide bridge. *Nature Struct Biol* 2000;**7**:505–13.
33. Wen S, Wilson DC, Kuruppu S, Korsinczky ML, Hedrick J, Pang L, et al. Discovery of an MIT-like atracotoxin family: spider venom peptides that share sequence homology but not pharmacological properties with AVIT family proteins. *Peptides* 2005;**26**:2412–26.
34. Windley MJ, Escoubas P, Valenzuela SM, Nicholson GM. A novel family of insect-selective peptide neurotoxins targeting insect large-conductance calcium-activated K^+ channels isolated from the venom of the theraphosid spider *Eucratoscelus constrictus*. *Mol Pharmacol* 2011;**80**:1–3.
35. Yamaji N, Little MJ, Nishio H, Billen B, Villegas E, Nishiuchi Y, et al. Synthesis, solution structure, and phylum selectivity of a spider δ-toxin that slows inactivation of specific voltage-gated sodium channel subtypes. *J Biol Chem* 2009;**284**:24568–82.

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Chapter 64

Targets and Therapeutic Properties

Christine Beeton

ABSTRACT

A large number of animals produce venoms to defend themselves from predators or to catch, eat, and digest prey. When injected into humans, these venoms can induce unpleasant effects, from mild itching to death within minutes. However, these venoms also have the potential for development into drugs useful for human health. Each venom contains as many potential drugs as the compound library of a medium to large pharmaceutical company. Some of these peptides may even have more than one potential therapeutic use. Such peptides are identified almost daily, but most are yet to be studied in detail for their potential usefulness. Venoms therefore still remain a largely untapped source for therapeutics. This chapter reviews the relatively few venom peptides that have been identified to date and are at different stages of development into potential therapeutics.

The ability of animals to kill with tiny amounts of powerful venom has inspired both fascination and fear in humans around the world since the dawn of civilization. Snakes and scorpions were worshipped as deities in many ancient religions in Egypt, Greece, India, China, and Mesoamerica. In the Biblical Garden of Eden, the snake tempted Eve into tasting the forbidden fruit, forcing her and Adam into exile in an impermanent world. The Aztec deity Quetzalcoatl was a feathered snake and in Greek mythology, Medusa's head of writhing snakes inspired terror. More recently, French author Antoine de Saint-Exupéry used the venomous bite of a snake "fast as lightening" to send the Little Prince back to his planet and his very special rose.

In spite of all this bad press, venomous animals have also long been associated with healing and in early Babylon the symbol of two intertwined snakes represented fertility, wisdom, and healing. Asclepius, the Greek god of medicine, carried a staff with a sacred snake coiled around it, which has become and remained the Caduceus, the symbol of Western medical science. Ancient and modern medicine both use venom as a rich source of compounds with a wide range of useful pharmacological activities. For example, traditional Chinese medicine uses venom, extracts, or whole scorpions to treat a variety of conditions, including rheumatic diseases, pain, meningitis, epilepsy, or stroke.

This chapter focuses on recent scientific advances in the exploitation of venom, an extensive and still largely untapped pharmacopoeia, for the development of therapeutics. It was organized by disease targets, and for each target, peptides of interest are grouped by their animal source. Some peptides, such as mellitin from the venom of the European honey bee, *Apis Mellifera*, have >1 potential therapeutic target and are therefore listed more than once in this chapter.

CARDIOVASCULAR DISEASES

Anticoagulants and Thrombolytic Agents

Venomous creatures are an abundant source of anticoagulants and thrombolytic agents that are widely used in the management and diagnosis of cardiovascular diseases. These include disintegrins, direct thrombin inhibitors, fibrinolytic compounds and plasminogen activators.[27]

Snake Venoms

Snake venoms contain a variety of disintegrins that affect both the platelet aggregation and adhesion. Two of these have already been approved for preventing and treating thrombotic complications in patients undergoing percutaneous coronary intervention (such as stenting or angioplasty) and in patients with acute coronary syndrome.[27] These drugs are Eptifibatide/Integrilin® (barbourin, eptifibatide), a cyclic heptapeptide from the venom of the Pygmy rattlesnake (Sistrurus miliarus barbouri), and Tirofiban/Aggrastat®, a peptidomimetic of echistatin from the venom of *Echis carinatus*. They inhibit platelet aggregation by binding with high affinity to the fibrinogen receptor (a.k.a integrin αIIbβ3) via a Lys-Gly-Asp recognition sequence.

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00064-6
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Other disintegrins isolated from snake venom use the more common Arg-Gly-Asp recognition sequence to interact with their target receptor.[27] The list is extensive—rhodostomin and Ancrod/Viprinex™ from the Malaysian pit viper (*Calloselasma rhodostoma*) developed to treat acute ischemic stroke, Batroxobin/Defibrase® from *Botrox atrox moojeni*, salmosin from the Siberian pit viper (*Agkistrodon halys brevicaudus*), echistatin from the African saw-scaled viper (*E. carinatus*), dendroaspin (mambin) from Jameson's Mamba (*Dendroaspis jamesoni kaimose*), and crotavirin from the Western Rattlesnake (*Crotalus viridis*). Although disintegrins have similar functions, they have different structures.

Fibrolase from the Southern Copperhead snake is an enzyme that cleaves fibrin. It consists of 203 amino acids, has a molecular weight of approximately 23kDa, is held together by three disulfide bonds, and belongs to the class of nonglycosylated metalloproteinases known as metzincins or alpha-fibrinogenases.[32] Alfimeprase, a recombinant fibrinolytic enzyme derived from fibrolase, was evaluated in clinical trials. It unfortunately failed to meet the primary endpoint in phase-III clinical trials and was therefore suspended.

Ximelagatran/Exanta®, from cobra venom, was the first oral anticoagulant to follow warfarin, a vitamin K antagonist. However, it causes severe hepatotoxicity and was thus removed from the market.[27]

Medicinal Leech Venom

Hirudin exemplifies antithrombin anticoagulants.[5] It is a peptide of 65 amino acids with three disulfide bonds and is derived from the saliva of the medicinal leech. Hirudin forms a biomolecular complex with thrombin via its acidic C-terminus and thereby prevents the activity of thrombin. Hirudin has been produced synthetically and used in the treatment of deep vein thrombosis and for coronary angioplasty reparation. A topical cream manufactured from hirudin is used to shrink hematomas caused by musculoskeletal injuries. Desirudin, a recombinant form of hirudin, is effective in the treatment of heparin-induced thrombocytopenia. A number of derivatives of hirudin are available, including hirugen, a synthetic C-terminal peptide fragment of hirudin, hirulog (bivalirudin; 20 amino acid peptide), a derivative of hirugen, and Argatroban (heterocyclic peptidomimetic).

Vampire Bat Venom

Desmoteplase, a salivary plasminogen activator from the vampire bat *Desmodus rotundus* (DSPA alpha 1) exhibits 85% sequence similarity with human tissue plasminogen activator. It was shown >40 years ago to be a potent plasminogen activator and is currently undergoing clinical trials for use in acute ischemic stroke with encouraging results.[9]

Tick Venoms

Contact phase inhibitor, a protein from the tick *Ixodes ricinus* saliva, inhibits human kallikrein and factors Xia and XIIa in the coagulation cascade. It reduces venous and arterial thrombi and protects against embolic events in rodents.[5]

Ixolaris, from the saliva of the tick *Ixodes scapularis*, blocks coagulation factors X and Xa and decreases the formation of venous thrombi in rodents.[5]

A protein isolated from the tick *Ornithodoros moubata* that inhibits coagulation factor Xa has been used in experimental femoral artery balloon angioplasty in rabbits.[5]

Procoagulant Drugs

Haemocoagulase® is a mixture of two proteinases isolated from the venom of the snake *Botrox atrox*.[27] They have a thromboplastin-like and a thrombin-like activity. One activates factor X in the coagulation cascade, whereas the other cleaves off fibrinopeptide A, leading to coagulation of fibrinogen. Haemocoagulase® is indicated for the prevention and treatment of hemorrhages by accelerating platelet aggregation and shortening coagulation time and thus loss of blood.

Diagnosis of Coagulopathies

Many snake venom-derived peptides are used as diagnostic tools for coagulation disorders.[27]

ACC-C/Protac® is a serine proteinase from *Agkistrodon contortix* venom that potently and rapidly activates protein C through a thrombomodulin-independent mechanism.[27] It is used to diagnose protein C and protein S deficiencies or defects in the protein C anticoagulant pathway.

RVV-V is a serine proteinase from *Daboia russelli* venom that activates factor V by cleaving it at Arg1545 and is not inhibited by antithrombin.[27] It is used to test for blood levels of factor V and for identifying the resistance of factor V to inactivation by activated protein C caused by the coagulation factor V Leiden mutation. One of the commercial tests (Pefakit®) also includes a group D prothrombin activator, noscarin from *Notechis scutatus*.

RVV-X is a factor X activator from *D. russelli* venom used in several clotting tests for assaying blood levels and deficiencies in factor X in the coagulation cascade, and in lupus anticoagulant assays.[27]

Ecarin is a selective group A prothrombin activator from *E. carinatus* venom.[27] The Ecarin clotting time is a laboratory test for monitoring prothrombin levels in patients undergoing anticoagulant therapy with Hirudin (described above in section Anticoagulants and Thrombolytic Agents). It is also used for diagnosing disseminated intravascular coagulation and for preparing meizothrombin.

Botrocetin® is a C-type lectin-like protein isolated from the venom of *Bothropps jararaca*. It aggregates platelets by

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inducing the von Willebrand factor to bind to the platelet GPIb receptor. It is used to determine the concentration of von Willebrand factor in plasma and for detecting disorders such as von Willebrand and Bernard–Soulier diseases.

Alboaggregin-B, another C-type lectin-like protein, from *Trimeresurus albolabris* venom competes with the von Willebrand factor for binding to platelet GPIb receptor and has been used to quantitate von Willebrand factor on platelets.[27]

Batroxobin, a snake venom thrombin-like enzyme isolated from *Bothrops atrox* venom, plays a role in the Reptilase time test.[27] This assay is an alternative to the thrombin time clotting assay for samples containing heparin, a thrombin inhibitor. Ancrod/Viprinex™ can also be used in this assay in place of Batroxobin. The Reptilase time test is used to diagnose various fibrinogen polymerization diseases, low levels of fibrinogen, and the presence of fibrin degradation products. Batroxobin is also used for the preparation of desAA-fibrinogen (fibrinogen lacking fibrinopeptide A) for the assay of tissue plasminogen activator.

Antihypertensive Agents

Antihypertensive drugs have important and increasing medical uses because of the increase in chronic hypertension in developed countries.

Snake Venoms

The Brazilian arrowhead viper (*Bothrops jararaca*) produces peptide inhibitors (e.g. nonapeptide SQ 20,881 or teprotide) of angiotensin-converting enzyme (ACE), a hormone that cleaves angiotensin I to generate angiotensin II and causes vasoconstriction and increased blood pressure. These snake peptide inhibitors bind to the active site of ACE in the same manner as natural substrates and reduce blood pressure. Captopril was the first drug based on a protein derived from snake venom >30 years ago.[14]

Snake venoms, such as those from *Dendroaspis angusticeps*, *Micrurus corallines*, *B. jararaca*, *Trimeresurus flavoviridis*, *Trimeresurus gramineus*, or *Agkistrodon halys blomhoffii* (and many others), represent one of the major sources of exogenous natriuretic peptides. These peptides are potent body fluid volume regulators and therefore reduce blood pressure.

Frog Venoms

A number of neurotensin-like peptides identified from frog skin (e.g. margaratensin and ranatensin) are hypotensive in rats and have the potential for development into novel therapeutics.

Antiarrhythmic Agents

Atrial fibrillation can be acute or chronic and has potentially serious squeals, including stroke and death.

Spider Venom

The Venom from the South American tarantula (*Grammostola spatulata*) contains GsMtx-4, a small peptide belonging to the 'cysteine-knot' family that blocks cardiac stretch-activated ion channels in the mid-nanomolar range and suppresses atrial fibrillation in rabbits. Efforts are underway to develop therapeutics for atrial fibrillation based on GsMtx-4. This peptide also holds promise as an analgesic and for the treatment of Duchenne muscular dystrophy, as described later in this chapter.[3,5]

Heart Failure

Human natriuretic peptides, such as Neseritide and Carperitide, are used in the management of congestive heart failure, but several of their exogenous reptilian counterparts hold therapeutic value too.

Snake Venoms

A chimeric peptide (CD-NP) was formed by fusing the 15 residue C-terminal tail of the dendroaspis natruiretic peptide (isolated from the green mamba, *Dendroaspis angusticeps*) with the taipan natruiretic peptide c (isolated from the inland taipan, *Oxyuranus microlepidotus*). This chimeric peptide was well tolerated with minor side effects in healthy humans and is currently in phase-II clinical trials in acute decompensated heart failure and stable chronic heart failure.[34]

INFECTION, IMMUNITY, AND ONCOLOGY

Antimicrobial Peptides

The increasing resistance of pathogenic or opportunistic bacteria to antibiotics is a major health concern, especially with the emergence of nosocomial infections, and new antibacterial compounds need to be developed. Many venomous creatures produce one or more antimicrobial peptides, which could be developed into new classes of antibiotics or antimicotics.

Frog and Toad Venoms (see the Amphibian/Skin Peptides Section of the Book)

The first peptide with antimicrobial activity was isolated from the skin secretions of the African clawed frog, *Xenopus laevis* (Pipidae). Since then, several hundred more such antimicrobials have been identified in skin secretions or skin extracts from frogs and toads. Although not all frogs and toads produce antimicrobial peptides in their skin secretions, a large number across families do produce interesting antimicrobial compounds, from ancient Archaeobactrachia

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to the more recent Neobatrachia.[8] These peptides can be grouped into families based on limited similarities in amino acid sequence, as listed below.

Within the Leiopelmatidae family, *Ascaphus truei* produce at least eight peptides (ascaphin 1–8) with broad-spectrum antimicrobial properties but greatest activity against Gram-negative bacteria. Ascaphin-8 is particularly interesting for development into an anti-infective agent.[8]

In the Alytidae family, *A. obstetricians* produces two families of structurally related antimicrobial peptides. Alyteserin-1 peptides display selective antibacterial effects against Gram-negative bacteria and also have low hemolytic activity against human erythrocytes.[8] Alyteserin-1c is especially interesting for its potency against multidrug-resistant strains of the nosocomial pathogen *Acinetobacter baumannii*. Alyteserin-2 peptides in contrast preferentially target Gram-positive bacteria, such as *Staphylococcus aureus* and also exhibit low hemolytic activity.[8]

Antimicrobial peptides have been discovered in the skin secretions of fire-bellied toads from Europe (*Bombina bombina* and *B. variegata*) and from Asia (*B. orientalis* and *B. maxima*), all members of the Bombinatoridae family. Bombinin has a strong hemolytic activity. Its analogs BLP-1 and BLP-3 have antimicrobial activity against both Gram-positive and Gram-negative bacteria, as well as against *Candida albicans*. Both have low hemolytic activity, making them interesting lead compounds for further clinical use. In contrast, bombinin H2 and H4 are more hemolytic and less potent against microorganisms and therefore less interesting as lead compounds for the development of new therapeutics.[8]

In the Pipidae family, *Xenopus laevis* produces magainin-1 and -2, peptide glycine-leucine amide (PGLa), and additional antimicrobial peptides with varying potencies and specificities.[8] A lysine-substituted analog of magainin-2 (Pexiganan) is a potent broad-spectrum antimicrobial with low toxicity against mammalian cells. It is being developed as a topical therapy for infected foot ulcers in patients with diabetes. Orthologs of magainin-1 and -2 were identified in the skin secretions of *X. borealis* and *X. amieti*. The skin secretion of *Silurana tropicalis* contains at least seven host-defense peptides.

Within the Myobatrachidae family, antimicrobial peptides have been found in the extracts of skin glands of species belonging to the *Uperoleia* and *Crinia* genera.[8] Uperins from the Australian frogs *U. mjoberii* and *U. inundata* are active against Gram-positive bacteria. *C. signifera* produces two highly homologous peptides, signiferin 2.1 and 2.2, also active against Gram-positive bacteria. A structurally related peptide, riparian 2.1, was isolated from *C. riparia*.[8]

The large Hylidae family is a rich source of antimicrobial peptides grouped into five families based on amino acid sequence similarities (dermaseptins, phylloseptins, plasticins, dermatoxins, and phylloxins) and a number of orphan peptides.[8]

Most frogs from the Leptodactylidae family and the genus *Leptodactylus* produce one or more structurally related peptides with antimicrobial activity.[8]

Within the African frogs of the Hyperoliidae family, antimicrobial peptides have to date only been identified in the skins of the running frogs from the genus *Kassina*. Kassinatuerin-1, from *K. senegalensis*, displays potent growth inhibitory activity against *Escherichia coli* and *Staphylococcus aureus*. However, it also displays strong hemolytic activity, which limits its attractiveness as a potential drug for clinical use.[8]

Four small peptides (11–12 amino acids) with broad-spectrum antimicrobial activity were identified in the skin of the Indian *Hoplobatrachus tigerinus*, from the Dicroglossidae family, and named tigerinins.[8]

Frogs from the Ranidae family produce very diverse antimicrobial peptides in their skin secretions. Temporins are small (8–21 residues) and easy to synthesize, they have therefore received attention for development into therapeutics.[8] Most temporins affect specifically Gram-positive bacteria, but temporin-L from *Rana temporaria* and temporin-1DRa from *R. darytoniii* are active against clinically relevant Gram-negative bacteria and *C. albicans*. Other antimicrobial peptides produced by Ranidae frogs include the following families: brevinin-1 and -2, esculentin-1 and -2, ranatuerin-1, palustrin-2, and nigrocin-2.

Ant Venoms

The jumper ant *Myrmecia pilosula* produces broad-spectrum antibacterial and antifungal peptides (the pilosulins) but, like many frog-derived antimicrobials, these peptides also exhibit significant hemolytic activity.[11] Modification of pilosulin-1 increased its antimicrobial activity while reducing its hemolytic activity, making it far attractive as a potential therapeutic.

Scorpion Venoms

Antimicrobial peptides have also been isolated from both the venoms and the hemolymph of scorpions. Most are small (15–22 amino acids), amphipathic, and positively charged. These peptides can be grouped into three different families, as detailed below.[26]

The first family consists of homologs of insect defensins.[26] These can eliminate both Gram-positive and Gram-negative bacteria, and also fungi, protozoans, and viruses.[4] Both insect and scorpion defensins are receiving great interest as lead compounds for the development of therapeutics.

The second group includes cysteine-free antimicrobial scorpion peptides. These include parabutoporine from *Parabuthus schlechteri*, pandinins 1 and 2 from *Pandinus imperator*, hadrurin from *Hadrurus aztecus*, Bmkbpp3 from *Buthus martensii*, and opistoporins 1 and 2 from *Opistophtalmus carinatus*.[26]

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The third group consists of peptides that combine the properties of the first two families. It includes scorpine from *Pandinus imperator* and IsCT from *Opisthacanthus madagascariensis*.[26]

Spider Venoms

Antimicrobial peptides called oxyopinins (composed of 48 amino acid residues) were isolated from the wolf spider (*Oxyopes kitabensis*) and the lynx spider (*O. takobius*) and share sequence similarity with the frog dermaseptins.

GsMTX-4, isolated from the venom of the tarantula *Grammostola spatulata*, affects the growth of both Gram-positive and Gram-negative bacteria.[3]

Wasp, Hornet, and Bee Venoms

A 10-residue antimicrobial peptide called anoplin found in the venom of the solitary wasp *Anoplius samariensis*, and its synthetic analogs, exhibit broad-spectrum antimicrobial activity against both Gram-positive and Gram-negative bacteria, including *E. coli* and *S. aureus*.[19] Other antimicrobial peptides from wasp, hornet, and bee venoms include two peptides from the venom of the neotropical social wasp *Agelaia pallipes pallipes* called protonectin and agelaia, crabrolin, a 13-residue peptide present in the venom of the hornet *Vespa crabro*, mellitin, a 26-amino acid peptide from European honey bee venom, and bombolitins from the bumble bee (*Megabombus pennsylvanicus)*.

Snake Venoms

Peptides derived from a snake venom phospholipase A2 are effective as broad-spectrum bactericidal agents and also as antiendotoxic compounds.[28] Antifungal peptides have also been isolated from the venom of the Brazilian arrowhead viper (*Bothrops jararaca*). Crotamin, from the venom of *Crotalus durissus terrificus*, is a potent antimicrobial.[13]

Sea Squirt

Halocidin, a heterodimer antimicrobial peptide from the sea squirt tunicate (also known as sea peach) *Halocynthia aurantium*, consists of two subunits containing 18 and 15 amino acids, respectively. It is effective against methicillin-resistant *Staphylococcus aureus* and multidrug-resistant *Pseudomonas aeruginosa* and is therefore of special interest for the development of treatments against nosocomial infections.[12]

Centipede

Two peptides isolated from the Chinese red-headed centipede (*Scolopendra subspinipes mutilans*), scolopin 1 and 2, are antibacterial *in vitro*.[5]

Immunomodulatory Peptides

Venom-derived peptides from a number of sources are being evaluated as immunomodulators for the treatment of autoimmune or allergic diseases and the prevention of graft rejection.

Ant Venom

Venom from the tropical ant *Pseudomyrmex triplarinus* contains heterodimeric peptides called myrmexins that relieve pain and inflammation in patients with rheumatoid arthritis and inhibit inflammatory carragenin-induced edema in mice.[24]

Scorpion Venoms

The venoms of scorpions represent a rich source of peptides that block the Kv1.3 and KCa3.1 channel in human T and B lymphocytes. The most potent scorpion Kv1.3 channel blockers include OSK1 (from *Orthochirus scrobiculosus*), its analog OSK1-$K^{16}D^{20}$, margatoxin (from *Centruroides margaritatus*), kaliotoxin (from *Androctonus mauretanicus mauretanicus*), agitoxin-2 (from *Leiurus quinquestriatus*), hongotoxin-1 (from *Centruroides limbatus*), noxiustoxin (from *Centruroides noxius*), Pi1 (from *Pandinus imperator*), and anuroctoxin (from *Anuroctonus phaiodactylus*). Many more are discovered every year, and a large number are still probably unknown.[33] Several of these peptides showed efficacy in treating animal models of inflammation in rodents and pigs, such as delayed-type hypersensitivity, experimental autoimmune encephalomyelitis (an animal model for multiple sclerosis), and inflammatory bone resorption in experimental periodontal disease. Maurotoxin, from *Scorpio maurus*, is the most potent inhibitor of the human T and B lymphocyte KCa3.1 channel, whereas charybdotoxin, from *Leiurus quinquestriatus*, blocks both Kv1.3 and KCa3.1 channels at low nanomolar concentrations.[33]

Sea Anemone Venoms

Two peptides with 35 amino acids, ShK and BgK, were isolated from the Caribbean sea anemones *Stichodactyla heliantus* and *Bunadosoma granulifer*a, respectively.[6] These were later shown to block Kv1.3 channels. ShK blocks T and B lymphocyte Kv1.3 channels with an IC_{50} value of 11pM, and it also blocks the neuronal Kv1.1 channel at similar concentrations. Modification of ShK generated an analog, ShK-186, with high affinity and selectivity for Kv1.3 channels. It is an extremely stable compound, suppresses the proliferation of autoimmune associated disease-relevant effector memory cells at picomolar concentrations, is effective *in vivo* in preventing and treating animal models of delayed-type hypersensitivity, multiple sclerosis, and rheumatoid arthritis, and exhibits an excellent safety index

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in rodents and nonhuman primates. ShK-186 is being developed as an injectable therapeutic for the treatment of autoimmune diseases.[6]

Snake Venoms

A derivative of cobratoxin from the Thailand cobra (*crotalus dirissus terrificus*), called CAM-NTX, prevents disease in a guinea pig model of the chronic inflammatory disease multiple sclerosis by reducing lymphocytic infiltration into the brain. Antibody titers to myelin basic protein (MBP), a major component of central nervous system myelin, are also reduced in treated animals. Its mechanism of action is yet to be defined.

Bee Venoms

Apitherapy (the use of bee products) has long been used in patients with the chronic inflammatory diseases such as rheumatoid arthritis and multiple sclerosis. Venom from the honey bee (*Apis mellifera*) is often injected at a traditional acupuncture point (Zusanli), and its analgesic and anti-inflammatory actions have been shown both in animal models and human trials of autoimmune arthritis and multiple sclerosis.[16] The anti-inflammatory effects of bee venom are at least in part mediated by melittin through the inhibition of the cyclooxygenase COX-2 and of the production of superoxide by neutrophils, both major players in the maintenance and amplification of inflammation in the joints during rheumatoid arthritis. However, mellitin also exhibits hemolytic and cytotoxic activities against mammalian cells and needs to be modified before it can be safely developed into a drug.

Bombolitins from the bumble bee *Megabombus pennsylvanicus* degranulate mast cells and induce the release of histamine and other bioactive compounds. It may therefore have therapeutic use.[1]

Frog Venoms

Tachykinins and their receptors are widely recognized as therapeutic targets for inflammatory diseases. Ranamargarin is one of more than 20 tachykinins isolated from frog skin and could serve as a template for a novel anti-inflammatory drugs.[17]

Antitumor Peptides

Venomous creatures are an abundant source of antiproliferative peptides, many of which are in clinical trials for the treatment of various types of cancer.

Scorpion Venoms

Chlorotoxin, a 36-amino acid basic peptide from the venom of the scorpion *Leiurus quinquestriatus*, blocks chloride channels expressed by glioma cells but not by normal glial cells.[31] Chlorotoxin binds *in vitro* to medulloblastomas, neuroblastomas, ganglioneuromas, melanomas, pheochromocytomas, and small cell lung carcinomas and to mice gliomas *in vivo*. ^{131}I-labeled chlorotoxin (^{131}I-TM-601) has been effective in targeted radiotherapy of gliomas in mice. ^{131}I-TM-601 was also evaluated in phase-I clinical trials and was deemed safe and tumor specific. Further clinical trials are under way.[31]

Bengalin, isolated from the Indian black scorpion (*Heterometrus Bengalis Koch*), is a large protein of 72kDa.[5] It induces apoptosis in human leukemic cells *in vitro*. Bengalin also shows efficacy in a rat model of osteoporosis but exhibits subacute cardiotoxicity.

Toad Venoms

Bombesin, a tetradecapeptide neurohormone isolated from the skin of the fire-bellied toad (*Bombina bombina*), binds to Bombesin receptors. These receptors are frequently expressed at high levels by a variety of tumors (such as breast, colon, lung, ovarian, urinary bladder, skin, and prostate). Bombesin is used to target cytotoxic drugs directly to these tumors. AN-152, a cytotoxic analog of bombesin containing doxorubicin, exhibited disease stabilization during phase-I clinical trials in ovarian and endometrial cancers. It is now undergoing phase-I or phase-II clinical trials in various cancers.[29]

Snake Venoms

Disintegrins from snake venom may have therapeutic use in suppressing tumor growth by inhibiting angiogenesis. Contortrostatin from the Broad Banded Copperhead snake (*Agkistrodon contortrix*) is a homodimeric disintegrin, each subunit having a molecular mass of 6750Da. Contortrostatin inhibits β1-integrin-mediated cell adhesion of human melanoma and blocks experimental metastasis of these tumors and breast cancers. Vicrostatin, a chimeric recombinant disintegrin generated through the fusion of the C-termini of echistatin and contortrostatin reduces breast cancer tumor growth in rodents and is well tolerated.[20] Jerdonin, another disintegrin from the venom of the Oriental pit viper (*Trimeresurus jerdonii*), improves the survival time of B16 tumor-bearing mice.

Crotamin, a neurotoxin isolated from the venom of *Crotalus durissus terrificus*, exerts antitumor activity against human lung adenocarcinoma through apoptosis induction.[13]

Venoms from Marine Sources

Didemnins, isolated from the Caribbean tunicate *Trididemnum solidum*, are a family of cyclic depsipeptides.[30] Didemnin B, the most potent didemnin in an antitumor screening assay, was tested in phase-I and phase-II clinical

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studies but showed poor efficacy and was abandoned. Aplidin® (dehydrodidemnin B), a second generation didemnin isolated from the Mediterranean tunicate *Aplidium albicans*, was more effective than Didemnin B in preclinical trials. Phase-I clinical trials show that it is generally well tolerated, and it is currently being evaluated in phase-II clinical studies in solid tumors. It was also granted "orphan drug status" in the European Union for acute myeloblastic leukemia.

Dolastatins are antimitotic peptides originally identified in the sea hare (*Dolabella auricularia*).[30] The two most promising peptides of this family, dolastatin 10 and dolastatin 15 were selected for development and are currently undergoing phase-II clinical trials. These peptides also exhibit antifungal activity. However, the complexity of their synthesis and their poor water solubility are significant obstacles for their development in to marketable drugs. LU103793, a stable and water-soluble analog of dolastatin 15, showed moderate and dose-dependent toxicity in phase-I clinical studies. It is currently being evaluated in phase-II clinical studies for breast, lung, ovarian, prostate, and colon cancer patients.

Thiocoraline, isolated from Micromonospora marine, a marine microorganism located in the Mozambique Strait inhibits RNA synthesis.[30] It is currently being evaluated in clinical trials.

Kahalalides are peptides isolated from the Hawaiian mollusk (*Elysia rufescens*).[10] Seven of these are cyclic depsipeptides (kahalalides A-F and O) and three are linear (kahalalides G, H, and J). Kahalide F is in clinical trials in patients with advanced androgen refractory prostate cancer.[30]

NERVOUS SYSTEM

Analgesia

A number of antinociceptive agents have been derived from venomous sources and are at various stages of development for therapeutic use.

Marine Cone Snail Venoms

Ziconotide (Prialt®) was approved for the treatment of severe chronic pain by intrathecal infusion by the US Food and Drug Administration in 2004.[15] With morphine, it is the only treatment for intractable pain approved for intrethecal injection. It is a synthetic analog of ω-conopeptide MVIIA, isolated from the fish-hunting marine snail *Conus magus*. It blocks N-type voltage-sensitive calcium channels in mammalian pain-sensing neurons and is therefore potently antinociceptive in conditions where morphine is poorly or not active, making it the best studied intrathecal analgesic drug to date. Ziconotide has an excellent safety profile with no neurotoxicity, inflammation, teratogenicity, mutagenicity, or carcinogenicity, and low immunogenicity.[15]

Scorpion Venoms

Extracts from the scorpion *Buthus martensis Karsh* have long been used in China to treat neurological diseases such as apoplexy, epilepsy, and paralysis.[25] These effects have been partly attributed to the peptides *Bm*K dIT-AP, *Bm*K dIT-AP3, and *Bm*K AS1 that exhibit analgesic effects in mice and rats.[25] These are short 31 amino acid peptides with three disulfide bonds, but their tertiary fold differs from that of other scorpion toxins.

Frog Venoms

Caerulein a decapeptide from the skin of the Tree frog *Hyla caerula*, shares sequence similarity to the C-terminal octapeptide of cholecystokinin. Peptides related to Caerulein have been isolated from the skin of the Australian Blue Mountains tree frog *Litoria citropa*. Caerulin and its analogs exhibit antinociceptive activity and cause sedation, inhibition of water intake, and anticonvulsive effects.[35] However, caerulein induces acute pancreatitis.

Dermorphins and deltorphins are peptides isolated from the skin of South American frogs belonging to the subfamily Phyllomedusinae.[22] Dermorphins act like μ-opiate agonists and induce antinociception, catalepsy, rigidity, and sedation. Within this peptide family, [Lys^7] dermophin exhibits the highest analgesic potency. Ala-deltorphin-I is the most potent γ-opiate agonist within the family.

Snake Venoms

Snake toxins have antinociceptive effects in rodents. Crotamine (molecular mass ~5kDa) is a mild toxin derived from the venom of the Northeastern Brazilian rattlesnake (*Crotalus durissus terrificus*). It is about 30-fold more potent than morphine as an analgesic. Hannalgesin (a.k.a. Oh9-1) from the venom of the King Cobra (*Ophiophagus hannah*) and Cobrotoxin from the venom of the Chinese cobra (*Naja naja atra*) are also potent analgesics and do not cause neurological or muscular deficits. However, in a clinical trial, a modified version of cobrotoxin with oral bioavailability did not produce significant pain relief to patients suffering from the demyelinating disorder adrenomyeloneuropathy.[21] Interestingly, cobrotoxin has a similar structural fold as the disintegrin dendroaspin although they have very different activities.

Spider Venoms

Psalmotoxin 1, a 40 amino acid peptide from the South American tarantula (*Psalmopoeus Cambridgei*), inhibits the acid-sensing ion channel and may have use in reducing acid-induced pain.[5] Psalmotoxin 1 has an inhibitor cysteine knot (ICK) fold and is related to gating modifier toxins of voltage-gated ion channels.

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GsMTx-4, a peptide isolated from the Chinese rose tarantula (*Grammostola spatulata*) blocks pain reactions in rat experimental pain models.[3,5]

Spinal Cord Injury

Spinal cord injury is common after road or sports accidents. It most often leads to permanent disability. GsMTx-4 showed efficacy in stimulating *in vitro* neurite growth through a reduction of local calcium influx. This peptide may therefore have potential for the treatment of spinal cord injury.[3]

Anticonvulsants

Many conditions can cause seizures, from epilepsia to kidney or liver failure, brain injuries, high fevers, infections, or drug use. BmK AS, a sodium channel site 4-selective modulator isolated from the venom of the scorpion *Buthus martensi Karsh*, exhibits anticonvulsant activity in rodents and has the potential for being developed into a useful drug for human clinical use.[5]

METABOLIC AND OTHER DISEASES

Obesity

Obesity is a major and growing health problem. Centipede acidic protein, isolated from the Chinese red-headed centipede (*Scolopendra subspinipes mutilans*), lowers the levels of cholesterol, triglycerides, and low-density lipoprotein (LDL) in rodents and may help manage some aspects of obesity.[5]

Diabetes Mellitus

Diabetes mellitus wreaks a deadly toll on the human population.

Gila Monster Venom

The venom of the gila monster (*Heloderma suspectus*) contains exendin-3 and exendin-4, two peptides that stimulate insulin secretion in response to increases in glycemia and modulate gastric emptying to slow the entry of ingested sugars in the blood. The 39 amino acid peptide exendin-4 has been developed into an injectable drug (Exenatide), which was approved for the treatment of type-2 diabetes by the US Food and Drug Administration in 2005.[2]

Frog Venoms

Peptides with insulin-releasing activity have been isolated from the skin secretions of the frogs *Agalychnis litodryas* and *Hoplobatrachus rugulosus*. They may serve as templates for a novel class of insulin secretagogues.[18,23]

Liver Diseases

Liver failure leads to death rapidly, unless a liver transplant donor is available. New treatments for various liver disorders have the potential of reducing the need for liver transplants or at least increasing the waiting time for patients.

Snake Venom

A preparation of snake venom from the Chinese water mocassin (*Agkistrodon halys pallas*) has long been used in Chinese medicine to treat liver diseases. Its beneficial effect in a rat model of fibrotic/cirrhotic liver disease results from a combination of increased bile flow, improved hepatic microcirculation, altered expression of bile salt transporter, and fibrinolytic and antithrombotic effects. The active ingredient in the venom remains to be identified and could lead to a new class of therapeutics for liver diseases.

Bee Venom

Mellitin, a cationic peptide from the venom of the honey bee (*Apis mellifera*) protects hepatocytes against injury from transforming growth factor β1 (TGF-β1).

Duchenne Muscular Dystrophy

Duchenne muscular dystrophy is the most common form of dystrophies. It is also the first genetic disease for which the responsible gene was identified through positional cloning. However, there still is no cure for this devastating disease. By inhibiting the elevation in calcium concentrations in stretched muscle fibers, GsMTx-4, from the venom of the South American tarantula (*Grammostola spatulata*) may provide a new strategy to treat Duchenne muscular dystrophy.[3]

Cell-Penetrating Peptides for Drug Delivery

A number of cell-penetrating peptides have been identified in the last few years. These are short cationic peptides (also called nanomolecules) that can traverse cell membranes through covalent or noncovalent binding. Most are nontoxic and do not interfere with the function of the macromolecules they bring through the cell membranes. They include crotamine, from the venom of the snake *Crotalus durissus terrificus*[13] and dermaseptins from the skin of various frogs.[7]

Antioxidants

Oxidative stress has been involved in a large number of pathologies, including Alzheimer's disease and multiple sclerosis, and also in the normal but often disliked process of skin aging.

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Scorpion Venom

Parabutoporin, a peptide from the venom of the scorpion Parabuthus schlechteri, has antioxidant properties through the inhibition of superoxide production.[26]

Bee Venom

Mellitin, from the venom of the honey bee *Apis mellifera*, also displays antioxidant properties by inhibiting the production of superoxide.

CONCLUSIONS

Peptides derived from venoms have already proven effective in treating several human diseases and many more are on the way to be developed into drugs. Venoms from insects, snakes, arachnids, amphibians, marine cones, and sea anemones are widely recognized sources of peptides with potential medical applications and new peptides are identified on an almost daily basis. However, only a very small number of venoms have been studied for potential therapeutic effects, and from these venoms only a few peptides have been studied in detail. Most certainly there are useful hidden venom peptides, as well as venomous animals, still awaiting discovery on dry land and in our oceans, lakes, and rivers.

ACKNOWLEDGMENTS

Grants from the National Institutes of Health (AI084981, AR059838, and NS073712) and the Department of defense (BC096018) have allowed the author to prepare this review.

REFERENCES

1. Argiolas A, Pisano JJ. Bombolitins, a new class of mast cell degranulating peptides from the venom of the bumblebee *Megabombus pennsylvanicus*. *Biol Chem* 1985;**260**:1437–44.
2. Aroda VR, Deyoung MB. Clinical implications of exenatide as a twice-daily or once-weekly therapy for type 2 diabetes. *Postgrad Med* 2011;**123**:228–38.
3. Bowman CL, Gottlieb PA, Suchyna TM, Murphy YK, Sachs F. Mechanosensitive ion channels and the peptide inhibitor GsMTX-4: history, properties, mechanisms and pharmacology. *Toxicon* 2007;**49**: 249–70.
4. Cederlund A, Gudmundsson GH, Agerberth B. Antimicrobial peptides important in innate immunity. *FEBS J* 2011;**278**:3942–51.
5. Cherniack EP. Bugs as drugs, part two: worms, leeches, scorpions, snails, ticks, centipedes, and spiders. *Altern Med Rev* 2010;**16**:50–8.
6. Chi V, Pennington MW, Norton RS, Tarcha EJ, Londono LM, Sims-Fahey B, et al. Development of a sea anemone toxin as an immunomodulator for therapy of autoimmune diseases. *Toxicon* 2012;**59**:529–46.
7. Chugh A, Eudes F, Shim YS. Cell-penetrating peptides: nanocarrier for macromolecule delivery in living cells. *IUBMB Life* 2010;**62**:183–93.
8. Conlon JM. Structural diversity and species distribution of host-defense peptides in frog skin secretions. *Cell Mol Life Sci* 2011;**68**:2303–15.
9. Frendl A, Csiba L. Pharmacological and non-pharmacological recanalization strategies in acute ischemic stroke. *Front Neurol* 2011;**2**:32.
10. Hamann MT, Otto CS, Scheuer PJ, Dunbar DC. Kahalalides: bioactive peptides from a marine mollusk *Elysia rufescens* and its algal diet *Bryopsis* sp. (1). *J Org Chem* 1996;**61**:6594–600.
11. Inagaki H, Akagi M, Imai HT, Taylor RW, Kubo T. Molecular cloning and biological characterization of novel antimicrobial peptides, pilosulin 3 and pilosulin 4, from a species of the Australian ant genus *Myrmecia*. *Arch Biochem Biophys* 2004;**428**:170–8.
12. Jang WS, Kim KN, Lee YS, Nam MH, Lee IH. Halocidin: a new antimicrobial peptide from hemocytes of the solitary tunicate, *Halocynthia auranti*. *FEBS Lett* 2002;**521**:81–6.
13. Kerkis I, Silva Fde S, Pereira A, Kerkis A, Rádis-Baptista G. Biological versatility of crotamine—a cationic peptide from the venom of a South American rattlesnake. *Expert Opin Investig Drugs* 2010;**19**: 1515–25.
14. Koh CY, Kini RM. From snake venom toxins to therapeutics—cardiovascular examples. *Toxicon* 2012;**59**:497–506.
15. Kress HG, Simpson KH, Marchettini P, Ver Donck A, Varrassi G. Intrathecal therapy: what has changed with the introduction of ziconotide. *Pain Practice* 2009;**9**:338–47.
16. Lee MS, Pittler MH, Shin BC, Kong JC, Ernst E. Bee venom acupuncture for musculoskeletal pain: a review. *J Pain* 2008;**9**:289–97.
17. Li J, Liu T, Xu X, Wang X, Wu M, Yang H, et al. Amphibian tachykinin precursors. *Biochem Biophys Res Commun* 2006;**350**:983–6.
18. Marenah L, Shaw C, Orr DF, McClean S, Flatt PR, Abdel-Wahab YH. Isolation and characterisation of an unexpected class of insulinotropic peptides in the skin of the frog *Agalychnis litodryas*. *Regul Pept* 2004;**120**:33–8.
19. Meinike K, Hansen PR. Peptoid analogues of anoplin show antibacterial activity. *Protein Pept Lett* 2009;**16**:1006–11.
20. Minea R, Helchowski C, Rubino B, Brodmann K, Swenson S, Markland FJ. Development of a chimeric recombinant disintegrin as a cost-effective anti-cancer agent with promising translational potential. *Toxicon* 2012;**59**:472–86.
21. Mundy HR, Jones SJ, Hobart JC, Hanna MG, Lee PJ. A randomized controlled study of modified cobratoxin in adrenomyeloneuropathy. *Neurology* 2003;**61**:528–30.
22. Negri L, Melchiorri P, Lattanzi R. Pharmacology of amphibian opiate peptides. *Peptides* 2000;**21**:1637–47.
23. Ojo OO, Abdel-Wahab YH, Flatt PR, Mechkarska M, Conlon JM. Tigerinin-1R: a potent, non-toxic insulin-releasing peptide isolated from the skin of the Asian frog, *Hoplobatrachus rugulosus*. *Diabetes Obes Metab* 2011;**13**:1114–22.
24. Pan J, Hink WF. Isolation and characterization of myrmexins, six isoforms of venom proteins with anti-inflammatory activity from the tropical ant, *Pseudomyrmex triplarinus*. *Toxicon* 2000;**38**:1403–13.
25. Rajendra W, Armugam A, Jeyaseelan K. Toxins in anti-nociception and anti-inflammation. *Toxicon* 2004;**44**:1–7.
26. Remijsen Q, Verdonck F, Willems J. Parabutoporin, a cationic amphipathic peptide from scorpion venom: much more than an antibiotic. *Toxicon* 2010;**55**:180–5.
27. Sajevic T, Leonardi A, Krizaj I. Haemostatically active proteins in snake venoms. *Toxicon* 2011;**57**:627–45.
28. Santamaria C, Larios S, Quiros S, Pizarro-Cerda J, Gorvel J-P, Lomonte B, et al. Bactericidal and antiendotoxic properties of short cationic peptides derived from a snake venom Lys49 phospholipase A2. *Antimicrob Agents Chemother* 2005;**49**:1340–5.

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29. Schally AV, Engel JB, Emons G, Block NL, Pinski J. Use of analogs of peptide hormones conjugated to cytotoxic radicals for chemotherapy targeted to receptors on tumors. *Curr Drug Deliv* 2011;**8**:11–25.
30. Singh R, Sharma M, Joshi P, Rawat DS. Clinical status of anti-cancer agents derived from marine sources. *Anticancer Agents Med Chem* 2008;**8**:603–17.
31. Sontheimer H. An unexpected role for ion channels in bran tumor metastasis. *Exp Biol Med (Maywood)* 2008;**233**:779–91.
32. Stocker W, Bode W. Structural features of a superfamily of zinc-endopeptidases: the metzincins. *Curr Opin Struct Biol* 1995;**5**:383–90.
33. Varga Z, Hajdu P, Panyi G. Ion channels in T lymphocytes: an update on facts, mechanisms and therapeutic targeting in autoimmune diseases. *Immunol Lett* 2010;**130**:19–25.
34. Vink S, Jin AH, Poth KJ, Head GA, Alewood PF. Natriuretic peptide drug leads from snake venom. *Toxicon* 2012;**59**:434–45.
35. Zetler G. Caerulein and its analogues: neuropharmacological properties. *Peptides* 1985(Suppl. 6):33–46.

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Chapter 65

Worm Peptides

William R. Kem

ABSTRACT

There are at least 12 animal phyla that are worms. They have received little scientific attention except as they influence human affairs. Nevertheless, an investigation of the substances that worms use to attack or defend against other organisms provides useful probes for examining receptors, ion channels and even mechanisms of innate immunity toward infectious viruses, microbes, and parasites. Nemertines are carnivorous marine worms that produce a plethora of peptidic neurotoxins and cytolysins. The venom of the blood worm *Glycera*, a marine annelid, contains a very large protein that stimulates neurotransmitter release by targeting voltage-gated calcium channels in nerve terminals. Another marine annelid (*Arenicola*) and nematodes produce antimicrobial peptides with different structures. Finally, parasitic worms (liver flukes) secrete cytolytic peptides to aid in the digestion of host tissues. This chapter summarizes the current knowledge of the structures and modes of action of these worm peptides.

INTRODUCTION

Worms have relatively simple body plans that allow growth without having to undergo metamorphosis. Along their length, a basic anatomical–functional unit is repeated, whether the worm is externally segmented (as in annelids) or not. Worms within a given phylum vary in size from tiny meiofauna living between grains of sand to long (but thin) species whose length can exceed 30m (the nemertine *Lineus longissimus*). What a worm may lack in speed of movement is often made up for by an uncanny ability to hide in cracks between rocks, burrow in soil, or produce a toxic chemical defense against predators. These include venoms that can also efficiently immobilize their prey. Worms, like most living animals, also possess antimicrobial peptides that represent an innate immune mechanism against infectious organisms such as bacteria, fungi, and viruses. Examples of these different types of peptides will be considered in this chapter.

NEMERTINE NEUROTOXINS

Nemertines are a phylum of marine worms that use a large, eversible proboscis to capture their prey.[12] Most nemertines are relatively small, inconspicuous inhabitants of marine coastal waters. Thus, it is not surprising that fewer than a thousand species are known. The phylum has been subdivided into three main groups: hoplonemertines possessing a proboscis stylet (barb) capable of puncturing the skin of a prey animal, paleonemertines which are considered to represent the most primitive nemertines, and heteronemertines. Paleonemertines and heteronemertines apparently lack a means of injecting a venom into their prey. Although hoplonemertine toxins are used for offensive and defensive purposes, it is believed that the other two groups use their toxins to repel predators.

In 1936, the Belgian pharmacologist Bacq reported his serendipitous discovery of two different types of toxic activity in nemertines.[1] Homogenates of a hoplonemertine (*Amphiporus lactifloreus*) potently contracted isolated frog skeletal muscle and stimulated the cat cervical autonomic ganglion in a manner similar to nicotine. Because this biological activity survived highly alkaline conditions, it could not result from acetylcholine, the neurotransmitter that normally stimulates these synapses. Extracts of paleonemertines and heteronemertines contained a different type of neurotoxic activity that, rather than affecting nicotine-responsive cells, generated spiking (action potential) activity in crustacean nerves. These are, respectively, called "amphiporine" and "la nemertine," and both types of homogenates caused convulsions, paralysis, and death when injected into shore crabs. Although "amphiporine" activity readily passed across a dialysis membrane, "la nemertine" activity only passed across very slowly.

About 30 years had elapsed before nemertine toxins were reinvestigated. Fortunately, many new separation and analytical methods became available, facilitating the isolation of these natural products even in relatively small amounts. These methods included thin-layer and column chromatography (and later HPLC), as well as amino acid analysis and Edman sequencing methods for the analysis of peptides. The first toxin isolated was the hoplonemertine alkaloid anabaseine, a nicotinoid compound possessing a biological and chemical profile similar to Bacq's "amphiporine."[22] Related compounds, described elsewhere, were found in other hoplonemertines.[16,19] The pharmacological

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00065-8
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properties of anabaseine and a variety of anabaseine derivatives have been assessed.[25] One of these derivatives, GTS-21 (also called DMXBA), selectively stimulates alpha7 nicotinic acetylcholine receptors that are involved in cognitive brain function and is currently undergoing clinical tests.[21,26] Heteronemertines, though lacking alkaloid toxins, possess peptide neurotoxins resembling the activity profile of Bacq's "la nemertine," as well as cytolytic peptides.[16,17]

Many heteronemertines possess peptide neurotoxins, as assayed by their ability to rapidly paralyze shore crabs or freshwater crayfish.[16] However, the only heteronemertine toxins that have been purified and chemically characterized to date were obtained from the mucous secretions of two large (>1-m length) marine species. Although *Cerebratulus lacteus* belongs to a very large heteronemertine family (Lineidae) and is widely distributed in the colder latitudes of both the Atlantic and Pacific oceans, the largest species, *Parborlasia corrugatus*, is confined to Antarctic and nearby cold waters of the southern hemisphere.

Cerebratulus neurotoxins have molecular sizes of about 6000Da and are crosslinked by three disulfide bonds.[18] These were originally designated as "B" toxins because they eluted after a cytolytic peptide "A" fraction during gel chromatography. The very similar sequences of the two most abundant and active toxins, B-II and B-IV, are known.[5,8] Both are very basic peptides containing a single residue of 4-hydroxyproline near the N-terminus. Unlike the scorpion and sea anemone peptide sodium channel neurotoxins whose secondary structures mostly consist of antiparallel B-strands, the B toxins are devoid of B-sheet structure but rich in alpha-helix. The secondary structure of B-IV calculated from circular dichroism measurements was similar to that predicted from the sequence.[24] The solution structure of B-IV was eventually determined by NMR methods.[13] Two long stretches of α-helix occurring at positions 11–23 and 34–49 are connected by a loop consisting of two inverse gamma-turns and a beta-turn. The region encompassing residues 11–49 constitutes a rather unique helical hairpin structure.[2]

Considerable results are available implicating some amino acid side chains in the toxic action of toxin B-IV. The initial studies used a chemical modification approach and focused on the few aromatic residues (2 tyrosyls, 2 tryptophanyls). By manipulation of the conditions of the reactions, it was possible to differentially label these residues, the former by nitration[6] and the latter by alkylation.[4] By bioassay of the toxin samples at different degrees of modification, it was deduced that Tyr 9 and Trp 30 are probably involved in receptor binding, because toxicity was significantly reduced without changes in the secondary structure. Blumenthal's laboratory subsequently used molecular biological methods to express B-IV in *Escherichia coli* and to obtain mutants for structure–activity studies.[14,15] Initial experiments showed that replacement of the hydroxyproline at position 10 with Pro did not affect crayfish paralytic activity, nor did replacement of Ala residues at either position 3 or 8 with serine. In fact, the toxicity of B-IV was enhanced by the simultaneous substitution of serine at positions 3 and 8. Arg17 was implicated in receptor binding as toxicity was undetectable, although the toxin CD spectrum was unchanged when Glutamine, Ala or Lys was substituted at this position. Replacement of Arg25 with Lys reduced crayfish toxicity 400-fold.[35] Some of the side chains implicated in the toxicity of B-IV include the guanidinyl side chains of Args 17, 25, and 34 and the aromatic side chains of Tyr 9 and Trp 30. It was rather surprising that these residues are found along the entire length of one surface of the toxin.

Cerebratulus B-IV is not toxic when injected intravenously into mice, insects, and snails. However, it is extremely toxic to crustaceans, especially freshwater crayfish.[18] Initially, a crustacean displays tremors and flipping of the tail but then convulses with a massive contracture of the limbs and tail. Within a few minutes, the contractural paralysis is followed by flaccid paralysis and eventually leads to death. Toxicity of the *Cerebratulus* B toxins seems to be mediated through its action on crustacean nerves as tetrodotoxin effectively blocks the action of B-IV on isolated crayfish nerve-muscle preparations. At relatively high (micromolar) concentrations, toxin B-IV also causes some repetitive spiking in isolated crab nerves. It is probable that this toxin activates a small population of sodium channels sufficient to generate spontaneous nerve action potentials that cause a massive release of excitatory neurotransmitter at the neuromuscular synapse.

Lieberman and Blumenthal[29] measured the binding of iodinated toxin B-IV to membranes prepared from lobster (*Homarus vulgaris*) nerves. The specific binding could not be displaced by a scorpion alpha-toxin. SDS gel electrophoresis of radioactively labeled toxin crosslinked to solubilized lobster nerve membrane proteins revealed a 40,000-Da band, of a smaller molecular size than would be predicted for the interaction with the sodium channel alpha-subunit. This suggests that the toxin may bind to a smaller (β?) subunit of the sodium channel or that it interacts with some other membrane protein.

Assays of extracts from other heteronemertines belonging to the genus *Lineus* indicate that they are actually more toxic than the *Cerebratulus* extracts.[16] The molecular sizes of these toxic peptides are 3000–6000Da.[17] Although they paralyze crustaceans in a manner that is superficially indistinguishable from the *Cerebratulus* toxins, the *Lineus* toxins primarily prolong action potential duration in crustacean neurons, whereas *Cerebratulus* toxin B-IV causes repetitive spiking without significantly delaying repolarization of each action potential (Kem, unpublished results).

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NEMERTINE PEPTIDE CYTOLYSINS

Lytic toxins are practically ubiquitous among all living organisms, including bacteria, plants, and animals. Almost all animal venoms include lytic substances that enhance the penetration of toxins into the circulation of the affected organism and possibly potentiate the actions of certain toxins. So far, cytolytic proteins have only been found in the anoplan class of nemertines, which includes both heternomertines (to be described below) and paleonemertines (Kem, unpublished results).

Cerebratulus contains at least four homologous integumentary protein lysins (MW ~10,000Da) called A toxins that contain many basic amino acids.[23,20] The most abundant isotoxin, A-III, was sequenced and shown to contain three disulfide bonds.[7] CD and Raman spectroscopic analyses of this toxin reveal the presence of approximately 60% alpha-helix and 10% B-sheet (Kem et al., unpublished results). The C-terminal portion, which is not crosslinked by a disulfide bond, is thought to exist as a helical hairpin structure. Because of its amphipathic nature, this part of the toxin sequence may interact with membrane lipids and contribute to membrane insertion.

Similar cytolysins have been isolated from the gigantic Antarctic heteronemertine *Parbolasia corrugatus* (Fig. 1). This species feeds on dead organic materials and live animals, and the only known predator is a jellyfish. Despite many attempts with a variety of chromatography supports, it was not possible to resolve the very similar isotoxins from each other. Nevertheless, Edman sequencing revealed an N-terminal sequence similar to that of the *Cerebratulus* A toxins and circular dichroism spectral analysis revealed a very similar secondary structure.[3]

The *Cerebratulus* A toxins are relatively potent cytolysins for a variety of cells. Although hemolysis assays are technically straightforward for identifying this type of toxin, hemolytic toxins presumably attack other sites under natural conditions, such as respiratory (gill) and digestive systems of potential predators. *Cerebratulus* toxin A-III blocks squid axon sodium channels and also affects the kinetics of opening and closing of voltage-gated potassium channels. It also permeabilizes membranes, causing loss of cellular potassium and depolarization of the membrane potential.[20] It is currently thought that the A toxins primarily act as natural detergents, disrupting lipid organization of cell membranes; this does not preclude channel forming or modifying mechanisms on certain cells at low concentrations. A-III inhibits brain phospholipid sensitive Ca^{2+}-dependent protein kinase *in vitro*, as do other peptide toxins that possess a detergent-like action.[27]

FIGURE 1 The gigantic Antarctic heteronemertine *Parbolasia corrugatus* and several adult starfish with diameters of approximately 30cm. See color plate 18.

In maritime Canada, *Cerebratulus lacteus* is a nuisance in aquaculture beds of bivalve mollusks where it is a dominant predator.[10] It inserts its proboscis between the two shells and may secrete the peptidic A and B toxins into this enclosed space of its prey.

ANNELID (*GLYCERA*) NEUROTOXIN

Bloodworms belonging to the genus *Glycera* are active intertidal predators that attack and paralyze their prey by means of a muscular pharynx that possesses four venom-injecting fangs, each attached to its own venom gland. Only very minute amounts of the venom peptide toxins have been partially purified.[9,30] The major neurotoxin possesses a molecular size of approximately 320,000Da by SDS-PAGE. This toxin causes a massive release of neurotransmitter at certain synapses. This action has been primarily studied electrophysiologically at amphibian neuromuscular synapses, as the toxin is not active at most or all mammalian neuromuscular synapses. The reason for this selectivity is that glycerotoxin targets a particular voltage-gated calcium channel, the so-called N-type, which is expressed to a greater degree at amphibian neuromuscular synapses.[30] Although its action is superficially similar to that of α-latrotoxin from the black widow spider, besides stimulating exocytotic release of neurotransmitter it also enhances the recycling of these vesicles so that nerve terminals do not become depleted of vesicles for neurotransmitter release.[31]

ANNELID ANTIBACTERIAL PEPTIDE TOXINS

The lugworm (genus *Arenicola*) was found to contain two potent antibacterial peptides that have been aptly named arenicins.[32,33] These amphipathic basic peptides contain 21 amino acid residues and a single disulfide bond that connects Cys3 to Cys20. A β-sheet secondary structure occurs between the first and second halves of each monomer sequence. In solution, two molecules form a twisted dimer that contains 9 hydrogen bonds between the two monomers.

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Solid-state NMR and other spectroscopic studies show that this dimeric structure becomes less twisted, exposing a highly cationic surface that can readily interact with negatively charged phospholipid headgroups of acidic lipids present in bacterial membranes. Although relatively small conductance pores develop on artificial bilayers formed with lipids from Gram-negative bacteria, membranes formed with the more acidic lipids of Gram-positive bacteria develop much larger conductance pores indicative of the formation of a "torus" containing many arenicin dimers oriented perpendicular to the plane of the membrane, that are essentially arranged like the staves of a barrel.[34] Such pores cause the destruction of bacteria and fungi.

NEMATODE AND PLATYHELMINTH PEPTIDE CYTOLYSINS

Cytolytic peptides occur in parasitic helminth (liver fluke, phylum Platyhelminthes) and hookworms (phylum Nematoda). These pore-forming peptides are thought to be important for the initial breakdown of the host cells on which the parasite feeds. The helminth (schistosome) peptide clonorin (9000Da molecular size) possesses a sequence with six half cystines located at essentially the same positions as in amoebapore. Although the overall sequence similarity with amoebapore is quite low,[28] these peptides may be homologous. A hookworm hemolytic peptide has a molecular size of 60,000–65,000Da.[11]

CONCLUDING COMMENTS

More systematic screening of these organisms will probably reveal a plethora of peptide toxins used in either controlling other metazoans (predator–prey interactions) or defense against infectious organisms such as bacteria, fungi and viruses. Certain substances such as the arenicins may serve as useful lead compounds for the design of new antibiotics.

REFERENCES

1. Bacq ZM. Les poisons des Nemertiens. *Bull Acad Roy Belg Cl Sci* 1936;**22**(5):1072–9.
2. Barnham KJ, Dyke TR, Kem WR, Norton RS. Structure of neurotoxin B-IV from the marine worm *Cerebratulus lacteus*: a helical hairpin cross-linked by disulphide bonding. *J Mol Biol* 1997;**268**:886–902.
3. Berne S, Sepcic K, Krizaj I, Kem WR, McClintock JB, Turk T. Isolation and characterization of a cytolytic protein from mucus secretions of the Antarctic heteronemertine *Parborlasia corrugatus*. *Toxicon* 2003;**41**:483–91.
4. Blumenthal KM. Inactivation of *Cerebratulus lacteus* toxin B-IV concomitant with tryptophan alkylation. *Arch Biochem Biophys* 1980;**203**:822–6.
5. Blumenthal KM, Kem WR. Primary structure of *Cerebratulus lacteus* toxin B-IV. *J Biol Chem* 1976;**251**:6025–9.
6. Blumenthal KM, Kem WR. Inactivation of *Cerebratulus lacteus* toxin B-IV by tyrosine nitration. *Arch Biochem Biophys* 1980;**203**:816–21.
7. Blumenthal KM, Kem WR. Primary structure of *Cerebratulus lacteus* toxin A-III. *J Biol Chem* 1980;**255**:8266–72.
8. Blumenthal KM, Keim PS, Heinrikson RL, Kem WR. Structure and action of heteronemertine polypeptide toxins. Amino acid sequence of *Cerebratulus lacteus* toxin B-II and revised structure of toxin B-IV. *J Biol Chem* 1981;**256**:9063–7.
9. Bon C, Saliou B, Thieffry M, Manaranche R. Partial purification of alpha-glycerotoxin, a presynaptic neurotoxin from the venom glands of the polychaete annelid *Glycera convoluta*. *Neurochem Int* 1985;**7**:63–75.
10. Bourque D, Miron G, Landry T. Predation on soft-shell clams (*Mya arenaria*) by the nermertean *Cerebratulus lacteus* in Atlantic Canada: implications for control measures. *Hydrobiologia* 2001;**456**:33–44.
11. Don TA, Jones MK, Smyth D, O'Donoghue P, Hotez P, Loukas A. A pore-forming haemolysin from the hookworm, *Ancylostoma caninum*. *Int J Parasitol* 2004;**34**:1029–35.
12. Gibson R. *Nemerteans*. London: Hutchinson University Library; 1972.
13. Hansen PE, Kem WR, Bieber AL, Norton RS. ^{1}H-NMR study of neurotoxin B-IV from the marine worm *Cerebratulus lacteus*. Solution properties, sequence-specific resonance assignments, secondary structure and global fold. *Eur J Biochem* 1992;**210**:231–40.
14. Howell ML, Blumenthal KM. Cloning and expression of a synthetic gene for *Cerebratulus lacteus* neurotoxin B-IV. *J Biol Chem* 1989;**264**:15268–73.
15. Howell ML, Blumenthal KM. Mutagenesis of *Cerebratulus lacteus* neurotoxin B-IV identifies NH_2-terminal sequences important for biological activity. *J Biol Chem* 1991;**266**:12884–8.
16. Kem WR. A study of the occurrence of anabaseine in *Paranemertes* and other nemertines. *Toxicon* 1971;**9**:23–32.
17. Kem WR. Biochemistry of nemertine toxins. In: Martin DF, Padilla GM, editors. *Marine pharmacognosy: marine biotoxins as probes of cellular function*. New York, NY: Academic Press; 1973. p. 37–84.
18. Kem WR. Purification and characterization of a new family of polypeptide neurotoxins from the heteronemertine *Cerebratulus lacteus* (Leidy). *J Biol Chem* 1976;**251**:4184–92.
19. Kem WR. Pyridine alkaloid distribution in the hoplonemertinea. *Hydrobiologia* 1988;**156**:145–53.
20. Kem WR. Structure and membrane actions of a marine worm cytolysin, *Cerebratulus* toxin A-III. *Toxicology* 1994;**87**:189–203.
21. Kem WR. The brain alpha7 nicotinic receptor may be an important therapeutic target for the treatment of Alzheimer's disease: studies with DMXBA (GTS-21). *Behav Brain Res* 2000;**113**:169–83.
22. Kem WR, Abbott BC, Coates RM. Isolation and structure of a hoplonemertine toxin. *Toxicon* 1971;**9**:15–22.
23. Kem WR, Blumenthal KM. Purification and characterization of the cytolytic *Cerebratulus* A toxins. *J Biol Chem* 1978;**253**:5752–7.
24. Kem WR, Tu CK, Williams RW, Toumadje A, Johnson Jr WC. Circular dichroism and laser Raman spectroscopic analysis of the secondary structure of *Cerebratulus lacteus* toxin B-IV. *J Protein Chem* 1990;**9**:433–43.
25. Kem WR, Mahnir VM, Papke R, Lingle C. Anabaseine is a potent agonist upon muscle and neuronal alpha-bungarotoxin sensitive nicotinic receptors. *J Pharmacol Exp Ther* 1997;**283**:979–92.
26. Kem WR, Mahnir VM, Prokai L, Papke RM, Cao XF, LeFrancois S, et al. Hydroxy metabolites of the Alzheimer's drug candidate DMXBA (GTS-21): their interactions with brain nicotinic receptors, and brain penetration. *Mol Pharmacol* 2004;**65**:56–67.

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27. Kuo JF, Raynor RL, Mazzei GJ, Schatzman RC, Turner RS, Kem WR. Cobra polypeptide cytotoxin I and marine worm polypeptide cytotoxin A-IV are potent and selective inhibitors of phospholipid sensitive Ca^{2+}-dependent protein kinase. *FEBS Lett* 1983;**153**:183–6.
28. Lee JY, Cho PY, Kim TY, Kang SY, Song KY, Hong SJ. Hemolytic activity and developmental expression of pore-forming peptide, clonorin. *Biochem Biophys Res Commun* 2002;**296**:1238–44.
29. Lieberman DL, Blumenthal KM. Structure and action of heteronemertine polypeptide toxins. Specific cross-linking of *Cerebratulus lacteus* toxin B-IV to lobster axon membrane vesicles. *Biochim Biophys Acta* 1986;**855**:41–8.
30. Meunier FA, Feng ZP, Molgo J, Zamponi GW, Schiavo G. Glycerotoxin from *Glycera convoluta* stimulates neurosecretion by up-regulating N-type Ca^{2+} channel activity. *EMBO J* 2002;**21**:6733–43.
31. Meunier FA, Nguyen TH, Colasante C, Luo F, Sullivan RKP, Lavidis NA, et al. Sustained synaptic-vesicle recycling by bulk endocytosis contributes to the maintenance of high-rate neurotransmitter release stimulated by glyceratoxin. *J Cell Sci* 2010;**123**:1131–40.
32. Ovchinnikova TV, Aleshina GM, Balandin SV, Krasnosdembskaya AD, Markelov ML, Frolova EI, et al. Purification and primary structure of two isoforms of arenicin, a novel antimicrobial peptide from marine polychaeta *Arenicola marina*. *FEBS Lett* 2004;**577**:209–14.
33. Ovchinnikova TV, Shenkarev ZO, Nadezhdin KD, Balandin SV, Zhmak MN, Kudelina IA, et al. *Biochem Biophys Res Comm* 2007;**360**:156–62.
34. Shenkarev ZO, Balandin SV, Trunov KI, Paramonov AS, Sukhanov SV, Barsukov LI, et al. Molecular mechanism of action of β-hairpin antimicrobial peptide arenicin: oligomeric structure in dodecylphosphocholine micelles and pore formation in planar lipid bilayers. *Biochemistry* 2011;**50**:6255–65.
35. Wen PH, Blumenthal KM. Role of electrostatic interactions in defining the potency of neurotoxin B-IV from *Cerebratulus lacteus*. *J Biol Chem* 1996;**271**:29752–8.

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Chapter 66

Adrenomedullin

Frank Cuttitta and Alfredo Martínez

ABSTRACT

Adrenomedullin (AM), initially discovered almost two decades ago, is highly conserved in nonvertebrate and vertebrate evolution, essential for species survival, regulates diverse physiology, and underpins a variety of human diseases.[5] In this chapter, we review the role of AM in carcinogenesis, encompassing its regulatory effects on proliferation, migration, invasion, angiogenesis, and lymphangiogenesis. We also describe the complex cell/cell interactions occurring in the tumor microenvironment regarding anatomical sources of AM, modulation of component partner cells function in the stroma and their effects on augmenting the neoplastic process. Finally, we identify several therapeutic approaches to target AM for the potential clinical management of malignant disorders.

INTRODUCTION

Adrenomedullin (AM) was first isolated from a human pheochromocytoma and demonstrated to function as a hypotensive peptide.[20] Cumulative research findings over the past 18 years have revealed AM to play a critical role in diverse physiology and modulate cell growth associated with embryogenesis, and wound repair.[5] In addition, AM has been associated with a multitude of human diseases including: diabetes, asthma, cardiovascular disorders, renal anomalies, Alzheimer's, and cancer.[8,13,16,21,29] A variety of epithelial and neural tumors overexpress AM; these encompass neoplasms of the liver, kidney, lung, breast, pancreas, ovary, prostate, brain, and skin.[5] Elevated AM tumor mRNA or plasma levels correlate with a worsened prognosis for patients with ovarian, prostate, renal, and neuroendocrine cancers.[5,7,27] The process of epithelial to mesenchymal transition (EMT) can result in establishing a cancer stem cell niche, which in turn is known to be responsible for the initiation of malignant cell conversion and maintenance of a neoplastic phenotype.[2] Hypoxia and inflammation are critical pathways that drive EMT and also augment AM expression in normal and cancer cells.[2,5,31] Interestingly, AM, Wnt, hypoxia, and inflammation all seem to track with tumor cell EMT and survival.[4,5,10,17,22]

In the remaining text of this chapter, we outline the major anatomical sources of AM, the peptide's role in tumor progression and metastasis, potential therapeutic approaches for targeting AM and its receptor as modulators of malignant disease, and finally end with a discussion on using an established drug for type 2 diabetes as a therapy for cancer with an underlying focus on AM.

ANATOMICAL SOURCES OF AM

AM is produced in every major organ of the body including brain, heart, lung, intestine, liver, kidney, spleen, pancreas, skin, testes, and ovary and is present in specialized cells such as smooth muscle, macrophage, mast cells, adipocyte, fibroblast, and trophoblast, as well as epithelial and neural tumor cells.[3,5,13] Hence, there are multiple anatomical sources of AM expression that could serve to augment the carcinogenesis process under the right circumstances. In the next section, we discuss the possible biological events that could lead to AM overexpression.

REGULATORY PATHWAY THAT STIMULATE AM EXPRESSION

Hypoxia and chronic inflammation are major contributory pathways that enhance AM expression in normal cells and in cancer.[5,31] Hypoxia-inducible factor-1 (HIF-1) is a strong inducer of AM mRNA transduction via its interaction with several hypoxia response elements (HRE) on the AM gene.[5] Proinflammatory factors such as interleukin-1 (IL-1), tumor necrosis factor-alpha (TNF-α), and lipopolysaccharide (LPS) enhance AM expression.[5] Inflammatory infiltrate cells (macrophages and mast cells) of human tumors can produce AM which in turn can amplify multiple aggressive aspects of the neoplastic lesion as will be described in detail later on in this book chapter. Other alternative regulatory mechanisms of AM expression also exist. Exposure to environmental toxins augments AM levels in airway epithelium.[4] Given this observation, one obvious question is

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what would be the effect of tobacco smoke on pulmonary expression of AM and does this have any possible link to the know correlation between heavy cigarette smoking and lung cancer?[15] Finally, there is a single obscure 2008 report showing that the saliva of the soft tick contains AM which suppresses the host immune response and ameliorates the feeding process.[9] Although not a true pathway of AM induction, the soft tick infection sequence does represent an external source of AM which may parallel the known role of *Schistosoma* biological products in the development of human squamous cell bladder cancer.[1]

AM INDUCED CELL GROWTH

AM has been previously reported as a critical growth factor involved in cell proliferation during embryogenesis, wound repair and carcinogenesis.[5] We reported that AM is responsible for the autocrine/paracrine growth of multiple human tumor cell lines that include cancers of the lung, colon, breast, ovary and prostate.[5] More recently, human glioblastoma, pancreatic and endometrial cancer cells were also shown to be dependent on AM for proliferation.[5] Finally, Park et al. have reported that AM drives hepatocellular carcinoma growth, enhances tumor vascular density, and tracks with worsen prognosis.[26]

AM-MEDIATED ANTIAPOPTOSIS

In vitro serum starvation of both normal and cancer cells induces programmed cell death, which is circumvented by the exogenous addition of AM.[5] This peptide induced antiapoptotic protection event involves Max bHLH and Stat3 driven mechanisms and the downregulation of Bax, Bid, caspase 6–8, MEKK3, and TRADD.[5] AM suppressed apoptosis can be specifically blocked by either antiligand or antireceptor neutralizing antibodies validating the importance of this peptide in augmenting cell growth under stringent environmental conditions.[5,18]

AM-REGULATED CELL MIGRATION/ INVASION

We were one of the earliest laboratories to report that AM could induce human cancer cells migration after a rise/fall dose-dependent response curve.[5] We have also shown that tumor cell derived AM could enhance mast cell chemotaxis toward the neoplastic lesion, mediate a localized proinflammatory response, and in turn augment the malignant phenotype of the tumor.[5] Interestingly, AM induces the *in vitro* migration and invasion of clear-cell renal cancer cells.[7] An article by Chen et al. shows that AM stimulates the migration of human microvascular endothelial cells, and this peptide source could be generated from the macrophage infiltrates associated with human melanomas which track with increased tumor vascular density and worsened disease outcome.[3] Finally, AM enhances matrix metalloproteinase-2 (MMP-2) expression in fibroblast and induces stromal matrix degradation, a prerequisite event that leads to metastatic spread of cancer cells.[5]

AM EFFECTS ON THE IMMUNE RESPONSE

AM is both a positive and negative regulator of the immune response.[30] We have previously discussed the autocrine effects AM has on macrophages and mast cells, which include the stimulation of pro-angiogenesis factor release (AM, IL-1B, IL-6, MIF, histamine, VEGF-A, bFGF, and MCP-1), chemotaxis, and suppression of TNF-α release.[5] The abundance of macrophages or mast cells in tumor infiltrates are known to track with enhanced neoplastic vessel density, increased metastatic involvement, and worsened clinical outcome.[32] AM has now been implicated as a major macrophage factor released by tumor infiltrate cells that enhances neoplastic growth, local angiogenesis and metastasis.[3]

AM STIMULATES ANGIOGENESIS AND LYMPHANGIOGENESIS

Angiogenesis and lymphangiogenesis are known vascular pathways that increase tumor growth and the dissemination of primary cancer cells to distal anatomical sites.[35] AM is a critical regulator of embryonic angiogenesis and lymphangiogenesis.[11,34] In addition, AM has been linked to increased tumor growth attributed to its role in enhancing vessel density for cancers of the breast, brain, pancreas, endometrium and liver.[5,26] Given the current tread in using antiangiogenic drugs for the treatment of cancer, developing reagents that block AM ligand/receptor interaction would be a plausible approach to suppress tumor induced angiogenesis/lymphangiogenesis, thus potentially inhibiting cancer cell growth and metastasis.

AM-TARGETED THERAPIES FOR CANCER

Given that AM is known to drive the carcinogenesis process by enhancing tumor cell proliferation, migration, invasion, EMT, angiogenesis, and lymphangiogenesis as previously discussed in this book chapter, it becomes intuitively logical that drugs which disrupt AM bioactivity would in turn be candidate compounds for use in anticancer therapy. There are actually several approaches that have been applied or could be applied to cancer management by disrupting AM biology which include the use of peptide antagonists, anti-peptide antibodies, and anti-AM receptor antibodies.[5,18] In

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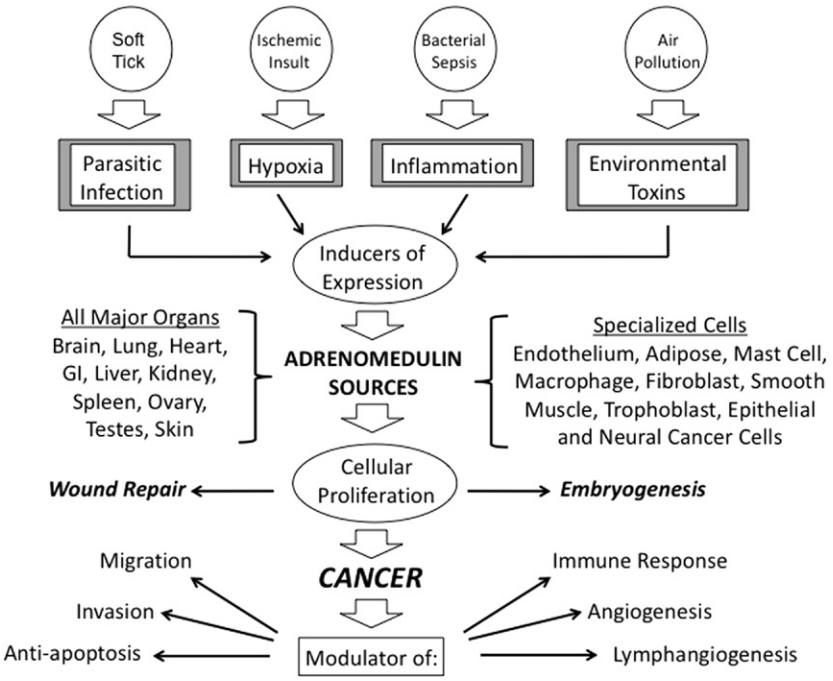

FIGURE 1 The adrenomedullin/cancer axial link.

addition, matrix metalloproteinase-2 (MMP2 or gelatinase A) selectively degrades intact $AM_{1–52}$, destroying its vasodilatory ability and generating the vasoconstrictor peptide $AM_{11–22}$ via enzymatic processing.[5] It would be extremely interesting to see what effect $AM_{11–22}$ has on the involvement of $AM_{1–52}$ in the carcinogenesis process.

Another promising line of research pursues the identification and iterative design of small molecules targeting the AM system. The first set of such molecules was identified with the help of a neutralizing antibody-based screening strategy.[5] The description of the three-dimensional structure of AM[28] and the resolution of the extracellular portion of the CGRP receptor complex[33] open the possibility of a rational design of novel small molecules based on their interaction with either the ligand or the receptor.

AM, OBESITY, DIABETES, AND CANCER—AXIAL LINK

As an extension of AM drug development, we would like to discuss a possible AM/obesity/diabetes/cancer axis and the potential use of an established type 2 diabetic therapy for cancer treatment.

Obesity increases the risk for at least twenty different anatomically distinct human cancers.[12] AM is produced by adipocytes and AM plasma levels are elevated in obese patients compared with that of normal volunteers.[23,25] Obesity and type 2 diabetes have a strong clinical correlative relationship underscored by low-grade chronic inflammation of adipose tissue.[14,19] Inflammation is a key modulator of AM expression.[5,31] Interestingly, AM is known to suppress insulin secretion increasing blood sugar and is elevated in the plasma of type 2 diabetic, whose peptide concentration parallels disease severity.[24] Finally, elevated plasma glucose levels preferentially augment hypoxia-inducible factor-1alpha (HIF-1α) expression in cancer cells while selectively suppressing production of this pro-AM transcription factor in normal cells.[6] Given the evidence presented above, it can be hypothesized that the cancer risk associated with obesity might potentially be related with enhanced AM expression generated in the inflamed adipose tissue or produced by HIF-1α stimulated transformed cells, thus augmenting the entire carcinogenesis pathway. Supporting this type of "connecting-the-dots" logic is the fact that metformin, a preferred drug for the treatment of type 2 diabetes, which lowers blood glucose and suppresses adipose function, is now being used in phase III clinical trials as an anticancer compound and has been shown to suppress angiogenesis.[12] What effect metformin has on AM and its role in diabetes and cancer may be a worthwhile research avenue to pursue to unravel the yet-to-be answered questions in the adrenomedullinergic field.

CONCLUSIONS

We have reviewed our current knowledge on the role of AM in the carcinogenesis pathway and have defined several areas of interest that are summarized in Fig. 1. Multiple environmental mechanisms can lead to the induction of AM expression in a given target cell, which primarily

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involve hypoxia and inflammation but also include exposure to air pollutants and parasitic infections. Every major organ of the body can produce AM with alternative sources coming from specialized cells that include: endothelia, adipocytes, mast cells, macrophages, fibroblasts, smooth muscle, trophoblasts, and cancers of epithelial and neural origin. AM underpins a variety of fundamental characteristics of transformed cells that encompass growth, migration, invasion, circumventing immune surveillance, resistance to apoptosis, EMT conversion, angiogenesis, and lymphangiogenesis. We have identified several possible therapeutic approaches that negate AM biology and have potential anticancer application in the clinical management of malignant disease. Finally, we have introduced anecdotal evidence that implicates a new neoplastic axis based on the existence of an AM/obesity/diabetes/cancer relationship and have revealed that metformin is an interesting antidiabetic drug now being used in cancer therapy as a plausible candidate compound to be evaluated for negative modulatory effects on AM biology.

ACKNOWLEDGMENTS

A. Martínez's laboratory is supported by grants from Spain's Ministry of Science and Innovation (SAF2009-13240-C02-01) and Ministry of Health (DIN-021).

REFERENCES

1. Botelho MC, Machado JC, Brindley PJ, Correla de Costa JM. Targeting molecular signaling pathways of Schistosoma haemotobium infection in bladder cancer. *Virulence* 2011;**2**:267–79.
2. Cabarcas SM, Mathews LA, Farrar WL. The cancer stem cell niche—there goes the neighborhood? *Int J Cancer* 2011;**129**:2315–27.
3. Chen P, Huang Y, Bong R, Ding Y, Song N, Wang X, et al. Tumor-associated macrophages promote angiogensis and melanoma growth via adrenomedullin in both paracrine and autocrine manners. *Clin Cancer Res* 2011 October 12. [Epub ahead of print].
4. Cuesta N, Martínez A, Cuttitta F, Zudaire E. Identification of adrenomedullin in avian type II pneumocytes: increased expression after exposure to air pollutants. *J Histochem Cytochem* 2005;**53**:773–80.
5. Cuttitta F, Portal-Núñez S, Falco C, Garayoa M, Pío R, Montuenga LM, et al. Adrenomedullin: an esoteric juggernaut of human cancer. In: Abba J Kastin, editor. *Handbook of biologically active peptides*. Boston: Elsevier Academic Press; 2006.
6. Dehne N, Hintereder G, Brüne B. High glucose concentrations attenuate hypoxia-inducible factor-1alpha expression and signaling in non-tumor cells. *Exp Cell Res* 2010;**316**:1179–89.
7. Deville JL, Batoli C, Berenguer C, Fernandez-Sauze S, Kaafarani I, Delfino C, et al. Expression and role of adrenomedullin in renal tumors and value of its mRNA levels as prognostic factor in clear-cell renal carcinoma. *Int J Cancer* 2009;**125**:2307–15.
8. Ewers M, Mielke NM, Hampel H. Blood-based biomarkers of microvascular pathology in Alzheimer's disease. *Exp Gerontol* 2010;**45**:75–9.
9. Francischetti IM, Meng Z, Mans BJ, Gudderra N, Hall M, Veenstra TD, et al. An insight into the salivary transcriptome and proteome of the soft tick and vector of epizootic bovine abortion. *Ornithodoros coriaceus*. *J Proteomics* 2008;**71**:493–512.
10. Franco-Montoya ML, Boucherat O, Thibault C, Chailley-Heu B, Incitti R, Delacourt C, et al. Profiling target genes of FGF18 in the postnatal mouse lung: possible relevance for alveolar development. *Physiol Genomics* 2011;**43**:1226–40.
11. Fritz-Six KL, Dunworth WP, Li M, Carson KM. Adrenomedullin signaling is necessary for murine lymphatic vascular development. *J Clin Invest* 2008;**118**:40–50.
12. Guppy A, Jamal-Hanjani M, Pickering L. Anticancer effects of metformin and its potential use as a therapeutic agent for breast cancer. *Future Oncol* 2011;**7**:727–36.
13. Hay DL, Walker CS, Poyner CR. Adrenomedullin and calcitonin gene-related receptors in endocrine-related cancers: opportunities and challenges. *Endocr Relat Cancer* 2010;**18**:C1–14.
14. Hemminki K, Li X, Sundquist J, Sundquist K. Obesity and familial obesity and risk of cancer. *Eur J Cancer Prev* 2011;**20**:438–43.
15. Hymowitz N. Smoking and cancer: a review of public health and clinical implications. *J Natl Med Assoc* 2011;**103**:695–700.
16. Ishimitsu T, Ono H, Minami J, Matsuoka H. Pathophysiologic and therapeutic implications of adrenomedullin in cardiovascular disorders. *Pharmacol Ther* 2006;**111**:909–27.
17. Jing Y, Han Z, Zhang S, Liu Y, Wei L. Epithelial–mesenchymal transition in tumor microenvironment. *Cell Biosci* 2011;**1**:29.
18. Kaafarani I, Fernandez-Sauze S, Berenguer C, Chinot O, Delfinho C, Dussert C, et al. Targeting adrenomedullin receptors with systemic delivery of neutralizing antibodies inhibits tumor angiogenesis and suppresses growth of human tumor xenografts in mice. *FASEB J* 2009;**23**:3424–35.
19. Kaplupahana NS, Moustaid-Moussa N, Claycombe KJ. Immunity as a link between obesity and insulin resistance. *Mol Aspects Med* 2011 October 21. [Epud ahead of print].
20. Kitamura K, Kangawa K, Kawamoto M, Ichiki Y, Nakamura S, Matsuo H, et al. Adrenomedullin: a novel hypotensive peptide isolated from human pheochromocytoma. *Biochem Biophys Res Commun* 1993;**192**:553–60.
21. Kucukosmanoglu E, Keskin O, Karcin M, Cekmen M, Balat A. Plasma adrenomedullin levels in children with asthma: any relation with atopic dermatitis? *Allergol Immunopathol (Madr)* 2011 September 1. [Epub ahead of print].
22. Lausson S, Cressent M. Signal transduction pathways mediating the effects of adrenomedullin on osteoblast survival. *J Cell Biochem* 2011;**112**:3807–15.
23. Linscheid P, Seboek D, Zulewski H, Keller U, Müller B. Autocrine/paracrine role of inflammation-mediated calcitonin gene-related peptide and adrenomedullin expression in human adipose tissue. *Endocrinology* 2005;**146**:2699–708.
24. Martínez A, Elsasser TH, Bhathena SJ, Pío R, Buchanan TA, Macri CJ, et al. Is adrenomedullin a causal agent in some cases of type 2 diabetes? *Peptides* 1999;**20**:1471–8.
25. Nomura I, Kato J, Tokashiki M, Kitamura K. Increased plasma levels of the mature and intermediate forms of adrenomedullin in obesity. *Regul Pept* 2009;**158**:127–31.
26. Park SC, Yoon JH, Lee JH, Yu SJ, Myung SJ, Kim W, et al. Hypoxia-inducible adrenomedullin accelerates hepatocellular carcinoma cell growth. *Cancer Lett* 2008;**271**:314–22.

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27. Pavel ME, Hoppe S, Papdopoulos T, Linder V, Mohr B, Hahn EG, et al. Adrenomedullin is a novel marker of tumor progression in neuroendocrine carcinomas. *Horm Metab Res* 2006;**38**:112–8.
28. Péez-Castells J, Martín-Santamaria S, Nieto L, Ramos A, Martínez A, Pascual-Teresa B, et al. Structure of micelle-bound adrenomedullin: a first step toward the analysis of its interactions with receptors and small molecules. *Biopolymers* 2012;**97**:45–53.
29. Ruzicska E, Toth M, Tulassay Z, Somogyi A. Adrenomedullin and diabetes mellitus. *Diabetes Metab Res Rev* 2001;**17**:321–9.
30. Souza-Moreira L, Campos-Salinas J, Caro M, Gonzalez-Rey E. Neuropeptides as pleiotrophic modulators of the immune response. *Neuroendocrinology* 2011;**94**:89–100.
31. Talero E, Sánchez-Fidalgo S, Villegas I, de la Lastra CA, Illanes M, Motilva V. Role of different inflammatory and tumor biomarkers in the development of ulcerative colitis-associated carcinogenesis. *Inflamm Bowel Dis* 2011;**17**:696–710.
32. Talmadge JE. Immune cell infiltration of primary and metastatic lesions: mechanisms and clinical impact. *Semin Cancer Biol* 2011;**21**: 131–8.
33. Ter Haar E, Koth CM, Abdul-Manan N, Swenson L, Coli JT, Lippke JA, et al. Crystal structure of the ectodomain of the CGRP receptor, a class-B GPCR, reveals the site of drug antagonism. *Structure* 2010;**18**:1083–93.
34. Witlin AG, Li ZY, Wimalawansa SJ, Grady JJ, Grafe MR, Yallampalli C. Placental and fetal growth and development in late rat gestation is dependent on adrenomedullin. *Biol Reprod* 2002;**67**:1025–31.
35. Witte MH, Dellinger MT, McDonald DM, Nathanson SD, Boccardo FM, Campisi CC, et al. Lymphangiogenesis and hemangiogenesis: potential targets for therapy. *J Sur Oncol* 2011;**103**:489–500.

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Chapter 67

Angiotensin II/ Angiotensin-(1–7)

Patricia E. Gallagher, Alison L. Arter, Bhavani Krishnan, Maria A. Garcia-Espinosa and E. Ann Tallant

ABSTRACT

The renin–angiotensin system (RAS) has classically been studied based on its role in the regulation of blood pressure and homeostasis. However, increasing evidence suggests that the angiotensin peptides have pleiotrophic cellular actions, such that the RAS may be involved in diverse pathologies. Two biological molecules of the RAS—the octapeptide angiotensin II (Ang II) and the heptapeptide angiotensin-(1–7) [Ang (1–7)]—have opposing actions, to either stimulate (Ang II) or inhibit [Ang-(1–7)] cell proliferation, angiogenesis, fibrosis, and inflammation. This chapter explores the more recent relevant information directly assessing the two angiotensin peptides and their role in the regulation of malignant cell growth. An increase in Ang II or a reduction in Ang-(1–7) may be involved in the initiation and progression of cancer, suggesting that therapeutics that regulate the circulating and tissue levels of the peptide hormones, their selective receptors, or synthesizing enzymes may serve as targets for intervention.

INTRODUCTION

Decades of research established the renin–angiotensin system (RAS) as a primary, physiological regulator of blood pressure, sodium balance, and fluid volume. However, it is the effects of the RAS on angiogenesis, fibrosis, inflammation, cell migration, and proliferation that play a role in diverse pathologies such as diabetes, pancreatitis, Alzheimer's disease, and cancer. The biological actions of the RAS are mediated by peptide hormones, and this chapter will focus on the octapeptide angiotensin II (Ang II) and the heptapeptide angiotensin-(1–7) [Ang-(1–7)]. In the RAS cascade (Fig. 1), angiotensinogen, predominately produced by the liver and secreted into the circulation, is cleaved by the enzyme renin, producing the decapeptide angiotensin I (Ang I). Proteolytic processing of Ang I by angiotensin-converting enzyme (ACE) yields Ang II; attenuation of ACE actions by selective inhibitors is commonly used to treat patients with high blood pressure. Ang-(1–7) is normally present in the circulation at concentrations similar to Ang II and is derived from Ang I by tissue peptidases, including neprilysin, thimet oligopeptidase, and prolyl endopeptidase (Fig. 1). The octapeptide hormone can be further metabolized by angiotensin-converting enzyme 2 (ACE2) to Ang-(1–7), whereas the heptapeptide hormone is degraded to the inactive metabolite Ang-(1–5) by ACE. Treatment of animals or patients with ACE inhibitors results in an increase in both circulating and urinary Ang-(1–7), by preventing the conversion of Ang I to Ang II and the degradation of the heptapeptide hormone.

Although differing by only a single amino acid, Ang II and Ang-(1–7) often have opposing biological actions. Ang II is a potent vasoconstrictor that also promotes fibrosis, oxidative stress, angiogenesis, cellular proliferation and migration, inflammation, hypertrophy, and atherogenesis. Conversely, Ang-(1–7) is a modest vasodilator with antiproliferative, antifibrotic, anti-inflammatory, antiangiogenic, antihypertrophic, and antithrombotic properties. These two peptide hormones mediate contrasting physiological responses through the activation of distinct, high-affinity cellular receptors. Ang II is an endogenous ligand for two pharmacological classes of seven transmembrane, G protein-coupled receptors—the angiotensin type 1 (AT_1) and angiotensin type II (AT_2) receptors. The majority of physiological effects associated with Ang II, including vasoconstriction, natriuresis, diuresis, and mitogenesis, are mediated by the AT_1 receptor and antagonists selective for blockade of AT_1 receptor function (angiotensin receptor blockers or ARBs) are commonly used to treat hypertension. The AT_2 receptor has a more limited tissue distribution but is present in increased amounts in prenatal tissues and after tissue injury. Ang-(1–7) binds and activates the seven transmembrane G protein-coupled receptor *mas* and counterregulates cellular signaling stimulated by a variety of mitogens, including Ang II.

Although the RAS was initially characterized as an endocrine system, ample evidence shows the presence of tissue RAS that are involved in local synthesis, release, and action

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00067-1
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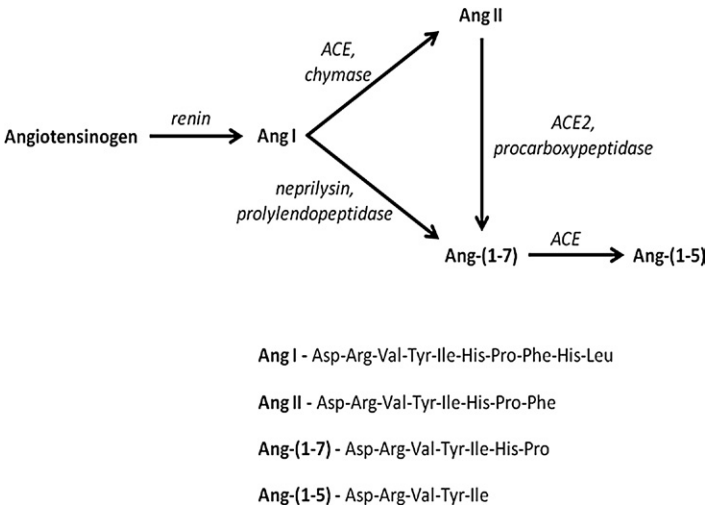

FIGURE 1 Proteolytic processing of angiotensinogen to angiotensin peptides. Ang I, angiotensin I; Ang II, angiotensin II; Ang-(1–7), angiotensin-(1–7); ACE, angiotensin-converting enzyme; ACE2, angiotensin-converting enzyme 2.

of angiotensin peptides. A functional tissue RAS was identified in every organ and tissue investigated, although some components of the pathway, particularly renin, are acquired from the endocrine RAS. In addition to normal tissues, RAS components are expressed in a variety of tumor cells, including carcinomas of the bladder, brain, cervix, colon, kidney, liver, lung, pancreas, prostate, skin, and stomach. Excellent reviews highlight the major studies on the role of the RAS in specific cancer types, the molecular mechanisms of RAS regulation of cancer growth, polymorphisms of RAS components, as well as the use of ACE inhibitors and ARBs as potential cancer therapeutics.[9,30] This chapter summarizes the major findings since 2005, specifically focusing on studies directly examining the effects of the angiotensin peptide hormones Ang II and Ang-(1–7) on cancer.

DETECTION OF ANG II IN TUMOR TISSUE AND CANCER CELL LINES

Positive immunostaining of Ang II and the AT_1 receptor occurs in both estrogen receptor positive and negative breast tumors as well as in human MDA-MB-468 and MCF-7 breast cancer cells.[10] Further, mRNA for angiotensinogen, renin, ACE, AT_1 receptor, and AT_2 receptor have been detected in the two breast cancer cell lines, suggesting the presence of a tissue RAS. Rhodes et al.[23] found that the AT_1 receptor is overexpressed in a subset of estrogen receptor positive tumors with a >20-fold increase in 7 of 45 tumors (15.5%) and >100-fold increase in 2 primary tumors and 1 metastatic tumor. Elevated numbers of AT_1 receptors are present in tumor cells with a gain in copy number and in tumor cells with the normal gene complement, indicating that multiple mechanisms are involved in the receptor overexpression. Zhou et al.[35] showed that the concentration of Ang II is greater in tissue homogenates of pancreatic ductal adenocarcinoma as compared with the surrounding normal tissue. Similarly, the Ang II levels in the tissue homogenates of twelve non-small-cell lung cancers are elevated significantly as compared with homogenates from matching nonmalignant lung tissue.[6] Taken together, these studies establish the presence of Ang II in premalignant and malignant cancer cells and suggest that the octapeptide hormone may be involved in tumorigenesis.

Ang II and AT_1 receptor immunostaining also was detected in 81.9% and 59.6% (94 cases), respectively, of primary endometrial carcinoma and correlates with reduced prognosis, suggesting that the assessment of RAS components may be a viable determinate of clinicopathological status.[26] Although there was no positive correlation between Ang II concentration and histopathological grades of gastric tumors, the octapeptide hormone is increased in the cancer tissue as compared with normal regions.[13] AT_1 receptor protein was also detected in 16 of 20 gastric tumors and >50% of gastric cancer cell lines examined. AT_1 receptor immunostaining is more robust in gastric tumors as compared with normal gastric mucosa. The basal epithelial cell layer of benign prostatic hyperplasias and all 28 human prostate cancers examined also showed positive immunoreactive Ang II with no significant difference in staining between cancer grades.[16] In 15 high-grade prostatic intraepithelial neoplasias, more intense Ang II staining occurred in the basal cells as compared with prostate cancer cells. Additionally, the androgen-dependent LNCaP as well as the androgen-independent DU145 and PC3 cell lines stained positive for immunoreactive Ang II. The observed increase in the AT_1 receptor probably results from Ang II-mediated upregulation in the cancer tissues. The elevated levels of the receptor coupled with increased Ang II may play a prominent role in

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promoting cancer cell proliferation and tumor angiogenesis. If so, it would be expected that Ang II and/or AT_1 receptor concentrations would correlate with histological grade, prognosis or survival. Although this positive correlation was observed in patients with primary endometrial carcinoma,[26] no association was found for gastric or prostate cancer.[13,16] Obviously, these three studies are not sufficient to allow any conclusions. Further analyses are certainly warranted as this is an important question not only for the potential use of the RAS as a diagnostic predictor but also for an understanding of the role of the RAS in the carcinogenesis process.

Ang II is increased and ACE2 decreased in pancreatic ductal adenocarcinoma as compared with normal pancreatic tissue.[35] Further, Ang II treatment of human BxPC3 and SW1990 ductal carcinoma cells causes a significant downregulation of ACE2, and reduction of ACE2 by RNA interference enhances the proliferation of cultured pancreatic cancer cells. ACE2 is also decreased significantly in 11 of 15 non-small-cell lung cancers as compared with adjacent lung tissue, and this variation correlates with reduced Ang II in homogenates from the tumors.[6] These findings suggest that Ang II downregulates ACE2 in tumor tissue to promote Ang II-mediated mitogenic effects. As a corollary, it would be of interest to quantify the concentration of Ang-(1–7), the product of the Ang II catalysis by ACE2, to determine whether this antiproliferative hormone is reduced in tumor tissue. A reduction in Ang-(1–7) would implicate this peptide hormone in the progression of carcinogenesis. In addition, it would be valuable to know whether ACE is elevated in tumor cells and tissue, as this enzyme converts Ang I to Ang II and therefore would further increase the concentration of the mitogenic octapeptide hormone and reduce the antiproliferative Ang-(1–7), since ACE catalyzes the conversion of the heptapeptide hormone to Ang-(1–5).

ANG II STIMULATES CANCER CELL PROLIFERATION

Ang II induces cancer cell growth by activating the AT_1 receptor. The increased proliferation of OCUM2MD3 gastric cancer cells by the octapeptide hormone is effectively inhibited by a specific AT_1 receptor antagonist, candesartan.[13] Candesartan also significantly prolongs survival time in a mouse model of peritoneal carcinomatosis compared with the control group. Ang II activates ERK1/2, nuclear transcript factor-κB (NF-kB) and survivin, showing that cellular proliferation and survival pathways are involved in the response. The transcription factor PAX2 is also increased significantly in prostate epithelial cells and DU145 prostate cancer cells after incubation with Ang II.[3] The ARB losartan but not the AT_2 receptor antagonist attenuates the Ang II-mediated elevation in PAX2. In addition, Ang II stimulates the phosphorylation of PAX2 through activation of ERK1/2, JAK II, and STAT3 growth-promoting pathways, suggesting that regulation of PAX2 may be a potential chemotherapeutic target for prostate cancer. Ang II also induces DNA synthesis in human LNCaP prostate cancer cells; the ARB losartan inhibits the Ang II-stimulated incorporation of [^{3}H]-thymidine into DNA, whereas the AT_2 receptor agonist, CGP42112A or antagonist PD123,319 has no effect, indicating an AT_1 receptor-mediated process.[4] In human PC3 prostate cancer cells, Ang II does not stimulate DNA synthesis, and the authors suggest that PC3 cells do not have a functional AT_1 receptor, as previously reported by Uemura et al.[33] Ang II increases the proliferation of prostate cancer cells with an associated upregulation of prostate-specific antigen, NF-kB, c-myc, and the androgen receptor;[11] the effect is blocked by an siRNA to the AT_1 receptor. Further, AT_1 receptor KO mice have reduced androgen receptor expression in the prostate, suggesting a role for Ang II in the regulation of the androgen receptor in prostate cancer. Finally, Feng et al.[6] showed that overexpression of ACE2 reduces the proliferation of human A549 lung cancer cells and Ang II-induced VEGF production, suggesting that either reduction of Ang II, an increase in Ang-(1–7) or dysregulation of both peptide hormones effectively attenuates lung tumor growth and angiogenesis. These studies show that the Ang II axis of the RAS may promote tumorigenesis through enhanced proliferation of cancer cells by multiple molecular mechanisms, providing further support for therapies that target the AT_1 receptor signaling pathways.

ANG II PROMOTES TUMOR ANGIOGENESIS

Ang II regulates the production of angiogenic factors to stimulate blood vessel formation and tumor growth. The octapeptide hormone causes a significant increase in VEGF production by pancreatic ductal adenocarcinoma cells that involves tyrosine phosphorylation.[2] Preincubation of the cells with the MEK1/2 selective inhibitor PD098059 or U0126 prevents the elevation of VEGF by the octapeptide hormone, suggesting that MAP kinase activation is required. Incubation of human SKOV-3 ovarian cancer cells with Ang II causes a significant increase in VEGF mRNA and VEGF secretion.[28] Candesartan blocks the enhanced production of VEGF in SKOV-3 cells treated with Ang II and in SKOV-3 tumor xenografts in mice. In addition, AT_1 receptor expression, detected in 85% of the ovarian tumors examined, is positively correlated with VEGF and tumoral vessel density but not with histological subtype.[26] Ang II also significantly increases gene expression of HIF-2alpha, TIMP-1, and VEGF in human MDA-MB-468 cells. This effect is attenuated by the ARB candesartan,[10] suggesting that the octapeptide hormone may activate the AT_1 receptor to elevate VEGF concentrations through a HIF/TIMP-1 cascade to

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enhance tumor angiogenesis. By contrast, no alteration in the expression of these target genes is observed after Ang II incubation with MCF-7 cells, even though these cells show positive immunostaining for the AT_1 receptor. The molecular mechanism for the lack of response by these breast cancer cells is not known. Candesartan blocks the increased secretion of IL-8 and VEGF secretion after incubation of human KU-19-19 bladder cancer cells with Ang II as well as attenuates microvessel density in KU-19-19 bladder tumor xenografts with an associated reduction in VEGF.[15] Ang II also induces VEGF production in *cis*-dichlorodiammineplatinum (CDDP)-treated human T24, KU-1, and KU-19-19 bladder cancer cells,[29] and the effect is blocked by an AT_1 receptor antagonist. More importantly, tumors from patients who underwent cystectomy after neoadjuvant CDDP-based chemotherapy have markedly increased concentrations of the AT_1 receptor and VEGF as compared with control transurethral resection specimens. Feng et al.[6] also showed that overexpression of ACE2 reduces the proliferation of human A549 lung cancer cells and Ang II-induced VEGF production, suggesting that either reduction of Ang II, an increase in Ang-(1–7) or dysregulation of both peptide hormones effectively attenuates lung tumor growth and angiogenesis. The current cardiovascular pathologies associated with antiangiogenic drugs and the short time to onset of resistance suggest a need for new therapeutic regimens with effective antiangiogenic activity and enhanced safety profiles. The blockade of the proangiogenic effects of Ang II with ACE inhibitors or angiotensin receptor blockers suggests that these drugs may serve as novel therapeutics to reduce angiogenesis and decrease tumor size.

EFFECTS OF ANG II ON THE TUMOR MICROENVIRONMENT

Ang II has a robust effect on cells of the tumor microenvironment. Tumor angiogenesis with an associated reduction in VEGF production is markedly inhibited in AT_{1a} receptor knockout mice harboring S-180 sarcoma xenografts.[7] This reduction in VEGF is not inhibited by an ARB, suggesting that Ang II regulates VEGF production in stromal cells to inhibit blood vessel formation and tumor growth. Similarly, a dose-dependent increase in COX-2 and prostaglandin E2 occurs in normal lung fibroblasts after incubation with Ang II; conversely no change occurs in human A549 lung adenocarcinoma cells.[17] Further, Ang II enhances the upregulation of COX-2 by the proinflammatory cytokines IL-1beta or TNF-alpha in normal lung fibroblasts, suggesting that the octapeptide hormone may promote lung tumorigenesis by activating inflammatory pathways in cells of the tumor microenvironment. Incubation of prostate stromal cells with Ang II results in a significant increase in cell proliferation with an associated activation of MAPK and secretion of IL-6.[32] Treatment of PC3 prostate cancer cells with the conditioned media from the prostate stromal cells incubated with Ang II enhances phosphorylation of MAPK. More importantly, the tumor volume of xenografts produced from PC3 prostate cancer cells and prostate stromal cells is markedly reduced after administration of an AT_1 receptor blocker, whereas PC3 xenografts are not affected. Because PC3 cells have reduced AT_1 receptors and do not respond to Ang II,[33] these studies suggest that Ang II indirectly promotes tumor growth by stimulating the production of secreted mitogenic factors from prostate stromal cells. The octapeptide hormone similarly increases the proliferation of hepatic stellate cells,[20] and the effect is blocked by an AT_1 receptor antagonist. Activation of pancreatic stellate cells by Ang II results in an upregulation of the AT_1 receptor as well as the fibrotic proteins, alpha-smooth muscle actin, collagen, and TGF-β.[24] These studies suggest Ang II may play a role in the conversion of stromal cells to activated myofibroblasts, which promote tumorigenesis through the secretion of growth factors and cytokines and provide structural support for tumor growth. Crosstalk between cancer cells and surrounding tissue is essential for the development and progression of tumors. The interaction between cancer cells and the adjacent microenvironment transforms the stroma into an abnormal phenotype, altering tissue architecture, cell morphology, and extracellular matrix–cell interactions that directly contribute to tumor formation. The study of Ang II effects on the tumor microenvironment is in its infancy and should provide a fruitful area of research with years of new discoveries that advance our understanding of the RAS and mitogenesis.

ANG II STIMULATES CANCER CELL MIGRATION AND INVASION

Ang II increases migration of human SKOV-3 ovarian cancer cells in a modified Boyden's chamber and peritoneal dissemination from ovarian tumor xenografts in a mouse model.[28] Ang II also reduces adhesion as well as invasion of MCF-7 and T47D breast cancer cells to collagen-, fibronectin- and laminin-coated wells and membranes, respectively, with an associated reduction in integrin alpha3 and beta1 through protein kinase C signaling.[22] This action is inhibited by cotreatment with the ARB losartan, indicating a functional AT_1 receptor in MCF-7 cells. Ang II induces the activation of matrix metalloproteinase (MMP) 2 and MMP-9 protein and mRNA in a dose-dependent manner in MKN-28 gastric cancer cells.[12] The stimulatory effect of Ang II could be blocked by an AT_1 receptor antagonist. Incubation of the gastric cancer cells with AG490, a selective JAK inhibitor, markedly reduces the MMP-2 concentrations induced by the octapeptide hormone, suggesting that Stat3 signaling may be involved in Ang II regulation of tumor metastasis and

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invasion. Overexpression of ACE2 also inhibits the migration of human pancreatic cells[35] and decreased MMP-2 and MMP-9 to attenuate migration of human A549 lung cancer cells, suggesting that reduction in Ang II and an increase in Ang-(1–7) by elevated ACE2 may inhibit cancer cell migration and invasion.[6] Finally, cell invasion using a reconstituted basement membrane chamber assay is significantly enhanced after Ang II incubation of human epithelial or breast cancer cell lines overexpressing the AT_1 receptor; the effect is blocked by the AT_1 receptor antagonist losartan.[23] Ang II stimulation of breast cancer cells with low levels of the AT_1 receptor does not exhibit increased invasive properties. The initial treatment for many different types of cancer consists of resection of the primary tumor followed by chemotherapy to reduce metastasis. Because two important steps in the metastatic process include the migration of the tumor cells from the primary tumor before intravasation into the circulation as well as migration of the tumor cells into distant organs after extravasation, drugs that block tumor cell migration may reduce metastatic growth. Since Ang II increases migration, ACE inhibitors, ARBs or drugs that prevent the Ang II-mediated stimulation of migration may serve as novel chemotherapeutics to block migration and tumor metastasis.

ANG II INDUCED CANCER CELL SIGNALING

Multiple signaling pathways are activated by the octapeptide hormone to mediate the regulation of cancer cell proliferation, angiogenesis, migration, and invasion. Ang II stimulates the phosphorylation of protein kinase B (Akt) in human LNCaP cells through the activation of the AT_1 receptor.[31] Incubation of C4-2AT6 androgen positive, castration-resistant prostate cancer cells with phosphoinositide 3-kinase (PI3K)/Akt inhibitor Ly294002 causes a marked decrease in the AT_1 receptor, whereas the elevation of phosphorylated Akt by serum starvation enhances AT_1 receptor expression in C4-2 prostate cancer cells, suggesting that the activation of Akt is involved in the regulation of the AT_1 receptor.[14] AT_1 receptor activation by Ang II in MCF-7 breast cancer cells also induces the phosphorylation of Akt at threonine 308 and serine 473 as well as attenuates cleavage of the Akt-downstream effector caspase 9 to inhibit apoptosis.[34] In addition, the octapeptide hormone stimulates MCF-7 breast cancer cell proliferation with an associated activation of Akt, an increase in cyclin D1, an upregulation of GSK3beta serine/threonine kinase, and a reduction in the cyclin-dependent kinase inhibitor p27. Taken together, these studies indicate that Ang II may play a critical role in cancer cell growth, adhesion, invasion, and inhibition of apoptosis in part through activation of the Akt signaling pathway.

Ang II also upregulates p47phox, superoxide dismutase, and glutathione peroxidase in human LNCaP prostate cancer cells; the increase in these oxidative stress proteins is attenuated by the ARB candesartan or catalase.[31] In addition, incubation of human LNCaP prostate cancer cells with Ang II causes a significant increase in superoxide, which is prevented by candesartan or catalase treatment as well as LY294002, an inhibitor of PI3K. The octapeptide hormone also induces the phosphorylation of p53 at serine 15, lipid peroxidation, 8-hydroxyguanine, and inducible nitric oxide synthase, an effect blocked by candesartan or catalase. These studies show that Ang II induces oxidative stress in human prostate cancer cells and that an ARB, an antioxidant enzyme or an inhibitor of PI3K can attenuate the effect. Ang II treatment of human promyeloctic HL-60 cells also causes a significant increase in DNA damage production by reactive oxygen species.[25] The effect is prevented by the ARB candesartan or the antioxidants *N*-acetylcysteine and α-tocopheral but not by the AT_2-receptor antagonist PD123319. Taken together, these studies suggest that Ang II activation of the AT_1 receptor promotes oxidative stress in human cancer cells.

Studies have only begun to explore cell signaling pathways activated by Ang II in malignant cells or the supporting stroma that contribute to tumor growth and metastasis. With increased understanding of the molecular mechanisms by which Ang II stimulates cell growth, drugs that target Ang II synthesis, the Ang II receptor, or the signaling pathways that are activated by Ang II in either the tumor cells or the stromal cells that support tumor growth (tissue fibroblasts, vascular smooth muscle cells, or endothelial cells) may provide novel treatments for cancer, either alone or in combination with drugs that target other growth stimulatory pathways.

ANG-(1–7) ATTENUATES TUMOR GROWTH

The number of studies on the effect of the heptapeptide hormone Ang-(1–7) on cancer is limited. There are no publications that identify the presence of Ang-(1–7) in tumor cells, assess the concentration of the Ang-(1–7) receptor *mas* in cancer cells and tumors, or determine whether the heptapeptide hormone regulates tumor cell migration and invasion. However, there are several publications that describe the inhibition of tumor growth by Ang-(1–7).

We observed that the tumor volume of human A549 lung cancer xenografts is significantly reduced after infusion with Ang-(1–7) as compared with tumors from control animals.[18] The tumor size in the saline-treated animals increases 2.5-fold, whereas tumor volume is reduced by 30% after Ang-(1–7) administration as compared with the size before treatment. Similar results are obtained if the heptapeptide hormone is injected daily into tumor-bearing

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mice.[27] Ang-(1–7) does not cause any observable adverse side effects, including changes in heart and body weight, heart rate, or blood pressure. The tumor inhibition correlates with a reduction in the proliferation marker Ki67 and cyclooxygenase-2 (COX-2) mRNA and protein in tumors from the Ang-(1–7)-treated animals as compared with the saline control tumor tissue. By contrast, no change in COX-1 mRNA is observed in xenograft tumors from animals infused with Ang-(1–7). Similar results for COX regulation are observed with the parent A549 human lung cancer cells in tissue culture. These results suggest that Ang-(1–7) may decrease COX-2 activity and proinflammatory prostaglandins to inhibit lung tumor growth.

We also found that Ang-(1–7) markedly decreases tumor volume and wet weight of orthotopic human breast tumors positive for the estrogen receptor (BT-474 or ZR-75-1) and overexpressed HER2 (BT-474).[5] Both types of breast tumors increase significantly over time after subcutaneous infusion with saline, while Ang-(1–7) significantly inhibits tumor growth. In addition, tumor burden of colorectal cancer cell metastases was markedly reduced in the liver after infusion of Ang-(1–7) as compared with untreated controls.[1] The ARB irbesartan alone or in combination with the heptapeptide hormone does not decrease liver metastasis. The mechanism for the lack of effect is not known. In a previous study, irbesartan alone did significantly reduce liver metastasis in the same mouse model.[19] Taken together, these studies suggest that Ang-(1–7) may serve as a first-in-class, targeted therapy for cancer.

ANG-(1–7) INHIBITS TUMOR ANGIOGENESIS

Infusion or subcutaneous injection of Ang-(1–7) not only causes a significant reduction in human A549 lung tumor xenograft growth in mice but it also markedly decreases vessel density.[27] Similarly, the heptapeptide hormone attenuates angiogenesis in orthotopic BT-474 and ZR-75-1 breast tumor xenograft,[5] suggesting that Ang-(1–7) inhibits angiogenesis to reduce tumor size. VEGF-A protein and mRNA are reduced in lung tumor xenografts from mice infused or injected with Ang-(1–7) as compared with tumors from saline-treated animals.[8,27] A similar decrease in VEGF-A is observed in parent A549 human lung cancer cells after incubation with Ang-(1–7). D-alanine-(7)-Ang-(1–7) [D-Ala^7-Ang-(1–7)], a specific antagonist for the Ang-(1–7) receptor, completely blocks the reduction in VEGF-A by the heptapeptide hormone. These results suggest that Ang-(1–7) attenuates tumor angiogenesis by reducing VEGF-A. In support, the heptapeptide hormone causes a significant decrease in human endothelial cell tubule formation in Matrigel.[8,27] The Ang-(1–7)-mediated effects are blocked by the specific Ang-(1–7) receptor antagonist [D-proline(7)]-Ang-(1–7). The chorioallantoic membrane (CAM) assay also was used to assess the effect of Ang-(1–7) on vascularization *in vivo*.[8,27] A 2-day incubation with Ang-(1–7) significantly inhibits blood vessel formation and vessel branching as compared with saline-treated control, effects that are blocked by an Ang-(1–7) receptor antagonist. These studies suggest that Ang-(1–7) activates the $AT_{(1–7)}$ receptor to attenuate blood vessel formation to reduce tumor growth.

EFFECTS OF ANG-(1–7) ON THE TUMOR MICROENVIRONMENT

We showed that Ang-(1–7) reduces interstitial fibrosis in human BT-474 and ZR-75-1 orthotopic breast tumors in association with a significant decrease in collagen I deposition, a similar reduction in perivascular fibrosis is observed in the ZR-75-1 tumors.[5] The heptapeptide hormone markedly attenuates *in vitro* growth of cancer associated fibroblasts (CAFs) isolated from orthotopic ZR-75-1 breast tumors, as well as reduces fibronectin, TGF-β, and ERK1/2 kinase activity. An associated increase in the MAPK phosphatase DUSP1 (dual-specificity phosphatase 1) after treatment with Ang-(1–7) suggests that the heptapeptide hormone reduces MAPK phosphorylation and activation by upregulating a MAP kinase phosphastase. Consistent with these *in vitro* observations, immunohistochemical analysis of Ang-(1–7)-treated orthotopic breast tumors reveals reduced TGF-β and increased DUSP1. Together, our findings indicate that Ang-(1–7) not only inhibits the proliferation of breast tumor cells and reduces tumor angiogenesis but also targets the tumor microenvironment to inhibit CAF growth and tumor fibrosis. Thus, Ang-(1–7) may serve as a first-in-class, antifibrotic agent that targets the tumor microenvironment to reduce breast cancer tumor growth and fibrosis.

ANG-(1–7) CLINICAL TRIALS

We conducted a Phase I Clinical Trial to determine the toxicity of Ang-(1–7) in patients with solid tumors.[21] Of 15 evaluable patients, 4 showed clinical benefit; three patients were treated for >3 months without tumor progression and one patient was treated for >10 months, with a 19% reduction in overall tumor measures. Clinical benefit was associated with a reduction in the proangiogenic factor placental growth factor (PlGF), in agreement with preclinical studies showing that Ang-(1–7) inhibits angiogenesis.[5,27] The drug was well tolerated with no hypertension, bleeding disorders, or drug-related deaths. A Phase II Clinical Trial, for the treatment of sarcoma patients with Ang-(1–7), is currently recruiting.

Ang-(1–7) is distinctive as a targeted therapy for cancer. The inhibition of cell growth by Ang-(1–7) is mediated by a novel, G protein-coupled receptor, *mas*, indicating

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that the heptapeptide hormone targets a unique receptor. Ang-(1–7) does not affect a single molecule or pathway; the heptapeptide hormone has pleiotropic effects, including inhibition of proliferation, fibrosis, and angiogenesis. Our results suggest that Ang-(1–7) inhibits both tumor and fibroblast cell growth. Many targeted therapies in clinical use or being tested in clinical trials inhibit the activity of protein kinases to reduce cell growth. Because many of these kinases are critically involved in normal homeostasis, their inhibition causes the adverse quality-of-life side effects associated with their clinical use. By contrast, Ang-(1–7) reduces MAPK activity to control levels, such that the activated enzyme is available for normal cellular functions. Further, we showed that Ang-(1–7) decreases fibrosis in human orthotopic breast tumors.[5] Because increasing evidence suggests that interaction between tumor cells and stromal cells is essential to promote tumor growth, a drug such as Ang-(1–7) that targets both the primary tumor cells as well as the tumor microenvironment will have additive effects on tumor growth. Finally, Ang-(1–7) reduces tumor angiogenesis with a reduction in the proangiogenic factors.[5,21,27] Other antiangiogenic drugs target the growth factor receptor(s) or the signaling pathways that are stimulated by receptor activation. Thus, the molecular mechanisms for the antiangiogenic effects of the heptapeptide hormone are unique, suggesting that Ang-(1–7) may be used in combination with other antiangiogenic drugs to target multiple mechanisms and inhibit angiogenesis.

CONCLUSIONS

Although the RAS is a classic peptide hormone system and its role in the regulation of blood pressure, heart rate, and renal function is established, studies indicate that the angiotensin peptides may be involved in carcinogenesis. The study of the RAS and cancer is in its infancy, and there are many remaining questions about the role of the RAS in cancer. For example, is the RAS involved in the initiation or progression of carcinogenesis or is activation of the RAS in tumor tissue a consequence of tumorigenesis? Can components of the RAS—angiotensin peptides, angiotensin receptors, or processing enzymes—be used as effective diagnostic predictors of cancer? Does Ang II have true oncogenic properties and is Ang-(1–7) a tumor suppressor? Do alterations in the ratio of the angiotensin peptides play a role in cancer initiation? What are the molecular signaling pathways that are activated by Ang II to induce tumor cell proliferation and angiogenesis and by Ang-(1–7) to inhibit these processes? Is the RAS a viable target for drug intervention? These are but a few of the questions that need to be answered.

Drugs that regulate either the production of Ang II—ACE inhibitors—or the interaction of Ang II with its receptors—ARBs—are efficacious treatments for high blood pressure and heart disease with strong safety profiles. Unfortunately, the use of ACE inhibitors and ARBs as cancer therapeutics has had mixed success. The variability in success may be because of the tumor type assessed, tumor stage at treatment, or the particular ACE inhibitor or ARB used for treatment. The development of drugs that increase ACE2 activity or the use of recombinant human ACE2 may provide an additional avenue for the treatment of malignancies by regulating circulating or tissue Ang II and Ang-(1–7). Recent studies—both preclinical and our Phase I clinical trial—suggest that Ang-(1–7) may be useful as a novel, cancer therapeutic by inhibiting tumor cell growth and angiogenesis. Because Ang-(1–7) also inhibits fibrosis, the heptapeptide hormone may reduce tumor fibrosis and the growth of stromal cells that provide support and stimulate cytokines for tumor growth. In addition, many of the classical chemotherapeutic drugs that are effective at reducing tumor size are limited in their usefulness based on their cardiotoxicity. Because Ang-(1–7) reduces not only tumor fibrosis but also interstitial fibrosis in the heart and perivascular fibrosis surrounding coronary vessels, the use of the heptapeptide hormone as an adjuvant therapy to reduce cardiac toxicity should also be explored.

REFERENCES

1. Ager EI, Wen SW, Chan J, Chong WW, Neo JH, Christophi C. Altered efficacy of AT1R-targeted treatment after spontaneous cancer cell-AT1R upregulation. *BMC Cancer* 2011;**11**:11.
2. Anandanadesan R, Gong Q, Chipitsyna G, Witkiewicz A, Yeo CJ, Arafat HA. Angiotensin II induces vascular endothelial growth factor in pancreatic cancer cells through an angiotensin II type 1 receptor and ERK1/2 signaling. *J Gastrointest Surg* 2008 Jan;**12**(1):57–66.
3. Bose SK, Gibson W, Giri S, Nath N, Donald CD. Angiotensin II upregulates PAX2 oncogene expression and activity in prostate cancer via the angiotensin II type I receptor. *Prostate* 2009 Sep;**69**(12):1334–42.
4. Chow L, Rezmann L, Imamura K, Wang L, Catt K, Tikellis C, et al. Functional angiotensin II type 2 receptors inhibit growth factor signaling in LNCaP and PC3 prostate cancer cell lines. *Prostate* 2008 May;**68**(6):651–60.
5. Cook KL, Metheny-Barlow LJ, Tallant EA, Gallagher PE. Angiotensin-(1–7) reduces fibrosis in orthotopic breast tumors. *Cancer Res* 2010;**70**(21):8319–28.
6. Feng Y, Wan H, Liu J, Zhang R, Ma Q, Han B, et al. The angiotensin-converting enzyme 2 in tumor growth and tumor-associated angiogenesis in non-small cell lung cancer. *Oncol Rep* 2010 Apr;**23**(4):941–8.
7. Fujita M, Hayashi I, Yamashina S, Fukamizu A, Itoman M, Majima M. Angiotensin type 1a receptor signaling-dependent induction of vascular endothelial growth factor in stroma is relevant to tumor-associated angiogenesis and tumor growth. *Carcinogenesis* 2005 Feb;**26**(2):271–9.
8. Gallagher PE, Cook K, Soto-Pantoja D, Menon J, Tallant EA. Angiotensin peptides and lung cancer. *Curr Cancer Drug Targets* 2011 May;**11**(4):394–404.
9. George AJ, Thomas WG, Hannan RD. The renin-angiotensin system and cancer: old dog, new tricks. *Nat Rev Cancer* 2010 Nov;**10**(11):745–59.

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10. Herr D, Rodewald M, Fraser HM, Hack G, Konrad R, Kreienberg R, et al. Potential role of rennin-angiotensin-system for tumor angiogenesis in receptor negative breast cancer. *Gynecol Oncol* 2008 Jun;**109**(3):418–25.
11. Hoshino K, Ishiguro H, Teranishi J, Yoshida S, Umemura S, Kubota Y, et al. Regulation of androgen receptor expression through angiotensin II type 1 receptor in prostate cancer cells. *Prostate* 2011 Jun;**71**(9):964–75.
12. Huang W, Wu YL, Zhong J, Jiang FX, Tian XL, Yu LF. Angiotensin II type 1 receptor antagonist suppress angiogenesis and growth of gastric cancer xenografts. *Dig Dis Sci* 2008 May;**53**(5):1206–10.
13. Kinoshita J, Fushida S, Harada S, Yagi Y, Fujita H, Kinami S, et al. Local angiotensin II-generation in human gastric cancer: correlation with tumor progression through the activation of ERK1/2, NF-kappaB and survivin. *Int J Oncol* 2009 Jun;**34**(6):1573–82.
14. Kosaka T, Miyajima A, Shirotake S, Kikuchi E, Oya M. Phosphorylated Akt up-regulates angiotensin II type-1 receptor expression in castration resistant prostate cancer. *Prostate* 2011 Feb:[Epub].
15. Kosugi M, Miyajima A, Kikuchi E, Horiguchi Y, Murai M. Angiotensin II type 1 receptor antagonist candesartan as an angiogenic inhibitor in a xenograft model of bladder cancer. *Clin Cancer Res* 2006 May;**12**(9):2888–93.
16. Louis SN, Wang L, Chow L, Rezmann LA, Imamura K, MacGregor DP, et al. Appearance of angiotensin II expression in non-basal epithelial cells is an early feature of malignant change in human prostate. *Cancer Detect Prev* 2007;**31**(5):391–5.
17. Matsuzuka T, Miller K, Pickel L, Doi C, Ayuzawa R, Tamura M. The synergistic induction of cyclooxygenase-2 in lung fibroblasts by angiotensin II and pro-inflammatory cytokines. *Mol Cell Biochem* 2009 Jan;**320**(1–2):163–71.
18. Menon J, Soto-Pantoja DR, Callahan MF, Cline JM, Ferrario CM, Tallant EA, et al. Angiotensin-(1–7) inhibits growth of human lung adenocarcinoma xenografts in nude mice through a reduction in cyclooxygenase-2. *Cancer Res* 2007;**67**:2809–15.
19. Neo JH, Malcontenti-Wilson C, Muralidharan V, Christophi C. Effect of ACE inhibitors and angiotensin II receptor antagonists in a mouse model of colorectal cancer liver metastases. *J Gastroenterol Hepatol* 2007 Apr;**22**(4):577–84.
20. Okamoto K, Tajima H, Ohta T, Nakanuma S, Hayashi H, Nakagawara H, et al. Angiotensin II induces tumor progression and fibrosis in intrahepatic cholangiocarcinoma through an interaction with hepatic stellate cells. *Int J Oncol* 2010 Nov;**37**(5):1251–9.
21. Petty WJ, Miller AA, McCoy TP, Gallagher PE, Tallant EA, Torti FM. Phase I and pharmacokinetic study of angiotensin-(1–7), an endogenous antiangiogenic hormone. *Clin Cancer Res* 2009;**15**:7398–404.
22. Puddefoot JR, Udeozo UK, Barker S, Vinson GP. The role of angiotensin II in the regulation of breast cancer cell adhesion and invasion. *Endocr Relat Cancer* 2006 Sep;**13**(3):895–903.
23. Rhodes DR, Ateeq B, Cao Q, Tomlins SA, Mehra R, Laxman B, et al. AGTR1 overexpression defines a subset of breast cancer and confers sensitivity to losartan, an AGTR1 antagonist. *Proc Natl Acad Sci USA* 2009 Jun;**106**(25):10284–9.
24. Sakurai T, Kudo M, Fukuta N, Nakatani T, Kimura M, Park AM, et al. Involvement of angiotensin II and reactive oxygen species in pancreatic fibrosis. *Pancreatology* 2011;**11**(Suppl. 2):7–13.
25. Schupp N, Schmid U, Rutkowski P, Lakner U, Kanase N, Heidland A, et al. Angiotensin II-induced genomic damage in renal cells can be prevented by angiotensin II type 1 receptor blockage or radical scavenging. *Am J Physiol Renal Physiol* 2007 May;**292**(5):F1427–34.
26. Shibata K, Kikkawa F, Mizokami Y, Kajiyama H, Ino K, Nomura S, et al. Possible involvement of adipocyte-derived leucine aminopeptidase via angiotensin II in endometrial carcinoma. *Tumour Biol* 2005 Jan;**26**(1):9–16.
27. Soto-Pantoja DR, Menon J, Gallagher PE, Tallant EA. Angiotensin-(1–7) inhibits tumor angiogenesis in human lung cancer xenografts with a reduction in vascular endothelial growth factor. *Mol Cancer Ther* 2009 Jun;**8**(6):1676–83.
28. Suganuma T, Ino K, Shibata K, Kajiyama H, Nagasaka T, Mizutani S, et al. Functional expression of the angiotensin II type 1 receptor in human ovarian carcinoma cells and its blockade therapy resulting in suppression of tumor invasion, angiogenesis, and peritoneal dissemination. *Clin Cancer Res* 2005;**11**:2686–94.
29. Tanaka N, Miyajima A, Kosaka T, Shirotake S, Hasegawa M, Kikuchi E, et al. Cis-dichlorodiammineplatinum upregulates angiotensin II type 1 receptors through reactive oxygen species generation and enhances VEGF production in bladder cancer. *Mol Cancer Ther* 2010 Nov;**9**(11):2982–92.
30. Uemura H. Role of renin-angiotensin system (RAS) in cancer. *Curr Cancer Drug Targets* 2011 May;**11**(4):393–450: [a series of reviews edited by Dr. H. Uemura].
31. Uemura H, Ishiguro H, Ishiguro Y, Hoshino K, Takahashi S, Kubota Y. Angiotensin II induces oxidative stress in prostate cancer. *Mol Cancer Res* 2008 Feb;**6**(2):250–8.
32. Uemura H, Ishiguro H, Nagashima Y, Sasaki T, Nakaigawa N, Hasumi H, et al. Antiproliferative activity of angiotensin II receptor blocker through cross-talk between stromal and epithelial prostate cancer cells. *Mol Cancer Ther* 2005 Nov;**4**(11):1699–709.
33. Uemura H, Ishiguro H, Nakaigawa N, Nagashima Y, Miyoshi Y, Fujinami K, et al. Angiotensin II receptor blocker shows antiproliferative activity in prostate cancer cells: a possibility of tyrosine kinase inhibitor of growth factor. *Molecular Cancer Therapeutics* 2003;**2**:1139–47.
34. Zhao Y, Chen X, Cai L, Yang Y, Sui G, Fu S. Angiotensin II/angiotensin II type I receptor (AT1R) signaling promotes MCF-7 breast cancer cells survival via PI3-kinase/Akt pathway. *J Cell Physiol* 2010 Oct;**225**(1):168–73.
35. Zhou L, Zhang R, Yao W, Wang J, Qian A, Qiao M, et al. Decreased expression of angiotensin-converting enzyme 2 in pancreatic ductal adenocarcinoma is associated with tumor progression. *Tohoku J Exp Med* 2009 Feb;**217**(2):123–31.

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Chapter 68

Apelin

Frank Cuttitta and David S. Salomon

ABSTRACT

Apelin, a 13-amino-acid peptide (APL-13), was initially identified as a bovine GI hormone that regulates acid production and represented the cognate ligand for the orphan G-protein-coupled receptor, APJ. It is highly conserved in vertebrates, regulates diverse physiological activities, and underpins multiple disease states in humans. Recent advancements in the field have shown APL-13 to function as a major growth factor in tumor progression via its proliferative role in angiogenesis and lymphangiogenesis pathways. In the following text, we review the current literature on APL-13 in cancer and discuss its potential value as a prognostic tool or targeted therapy in the clinical management of malignant disorders.

INTRODUCTION

Apelin (APL) was initially isolated from cow's stomach and shown to modulate acid production and interact with the APJ receptor; its corresponding bovine/*Homo sapiens* cDNAs were determined.[37] In mammals, the APL gene codes for a 77-amino-acid prepro precursor molecule that is processed into apelin-36 (APL-36), apelin-31 (APL-31), apelin-28 (APL-28), and apelin-13 (APL-13) through a cascade of proteolytic cleavage events.[17] APL-13 is highly conserved in vertebrate evolution with a single amino acid substitution existing between the human derivative and that of bony fish, representing 500,000 years of genetic adaptation.[30] Given this fact, APL-13 is believed to play a critical role in species survival and was shown to be required for normal vascular development in the frog embryo.[5] Interestingly, APL gene knockout mice are not embryonic lethal but are phenotypically linked to aging-associated reduced cardiac contractility and increased susceptibility to pressure overload-induced heart failure.[21] Over the past decade, APL has been proven to be a pluripotent regulatory peptide involved in normal physiology and pathological conditions that include neurotransmission, water uptake, food consumption, gut motility, hormone release, glucose use, diuresis, immune response, embryogenesis, diabetes, liver cirrhosis, HIV susceptibility, cardiovascular disorders, angiogenesis, lymphangiogenesis, and carcinogenesis.[2,10,23]

The process of carcinogenesis (initiation, progression, and metastasis) involves complex pathways encompassing an accumulation of mutational insults that transforms normal cells to a neoplastic phenotype. This is followed by spatial/temporal influences of component cells of the surrounding tumor microenvironment, development of angiogenic/lymphangiogenic vascular networks in and around the tumor body, culminating in secondary site involvement that leads to organ failure and patient death via malignant expansion of cancer cells.[14] For cancer cells to advance through the carcinogenesis pathway completely, they must acquire the following characteristics: (1) replicative immortality, (2) sustained proliferative state, (3) resistance to cell death (apoptosis), (4) circumvent immune surveillance, (5) induce angiogenesis/lymphangiogensis, and (6) migrate to distal anatomic sites (invasion/metastasis).[14] For the remainder of this chapter, we will discuss the role of APL in the majority of these tumor cell characteristics, reveal cellular mechanisms that regulate APL expression and propose possible drug developmental avenues that target APL for anticancer therapies.

ROLE OF APELIN IN CANCER

APL induces growth and protection against apoptosis in a variety of anatomically distinct cell types.[33,35] Given this capability, APL was proven to be a positive influence in normal embryogenesis or wound repair.[20,32] Unfortunately, similar positive repercussions have been assigned to APL in carcinogenesis by selectively allowing the tumor cell to survive harsh microenvironmental conditions such as hypoxia and inflammation.[1,13] Epithelial to mesenchymal transition (EMT) plays a critical role in tumor formation/invasion process that is modulated by hypoxia/inflammatory insults and initiated through the Wnt/β-catenin, cripto-1/nodal, notch, and APL/APJ signaling pathways.[6,12,19,25] Hence, the APL/APJ system seems to be involved (directly or indirectly) in several complex mechanisms that influence tumor progress and metastasis, thereby mimicking a similar role it plays during normal embryo development and in stem cell function.[6,20]

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00068-3
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Angiogenesis and lymphangiogenesis are important support infrastructures for cancer cell growth and metastasis.[16] As tumors exceed a 2-mm diameter, size they no longer can maintain proliferative self-sufficiency and undergo growth reduction that ultimately leads to a dormant state.[11] If however these tumor colonies begin to produce angiogenic or lymphangiogenic factors that induce endothelial cell chemotaxis and vessel sprouting, then newly formed vascular networks will supply the neoplastic lesion with additional nutrients from an enriched blood supply leading to expanded growth and establishing alternative routes for distal cancer cell dissemination.[11,16] Tumor metastasis is a multipronged process that involves the migration of the cancer cell through surrounding stromal tissue. These tumor cells then enter vascular networks (blood vessel or lymphatics) or traverse vascular/neural highways externally via angio-, lymphangio-, or neruotrophic mechanisms and eventually establishing foothold colonies at distal anatomical sites.[9,16,26,31] APL is a potent angiogenic/lymphangiogenic factor produced by human cancers and is thus considered a rational target for the development of anticancer therapies.[23] Interestingly, endothelial cells and neurons are an endogenous source of APL production that in turn can mediate tumor cell chemotaxis and stimulate the expression of MMP-3/MMP-9, which fosters cell migration through the extracellular matrix and increases the metastatic potential of cancer cells.[7,18,34] Berta et al. have shown that in nonsmall cell lung cancer patients, elevated APL tumor expression tracks with increased vascular density in the neoplastic lesion and worsens clinical outcome.[2]

Cancer patient fatalities stem from multiple organ failures caused by the displacement of normal cells with invading cancer cells.[26] Therefore, drugs that prevent the spread of cancer cells to distal tissue sites should have a major influence on the resulting mortality rates for a given neoplasm. Neutralizing antibody to APL decreases the peptide's ability to induced angiogenesis/lymphangiogenesis.[23] In addition, the peptide antagonist APL-13(P13A) suppresses APL regulated hypotension and neovascularization.[22,28] Finally, the carboxypeptidase, angiotensin-converting enzyme (AEC2), rapidly degrades APL by removing the peptide's carboxy-terminal phenylalanine making it biologically inert.[29] By applying these findings collectively, there seem to be a variety of compounds that could be evaluated in preclinical/clinical trails to inhibit APL bioactivity and potentially prove to be effective therapeutics in cancer management.

We have previously discussed the role of hypoxia and inflammation as direct mediators of APL expression and the influence of this peptide can have on cancer progression and invasion. There are several other physiological conditions that can lead to APL overexpression and indirectly contribute to the carcinogenesis pathway. Obesity, for example, is known to increase the risk of cancer development and has been associated with 20 different anatomical tumors.[15] APL is a product of adipocytes (a member of the adipokine family), blocks glucose-stimulated insulin secretion, and its plasma concentrations are increased in obese/type 2 diabetic patients.[10] Given these findings, APL may prove to be a regulatory link between obesity and cancer that propagates normal to malignant cell conversion. There exists another alternative possibility involving APL expression that might indirectly effect cancer cell progression as reflected in neural biology. APL was recently identified in proopiomelanocortin (POMC) neurons and functions to regulate the release of alpha-melanocyte-stimulating hormone (α-MSH) in an autocrine fashion.[24] Interestingly, Wnt/β-catenin signaling is activated by α-MSH in melanoma cells which in turn could lead to the initiation of a Cripto-1/Nodal, Notch, and APL/APJ cascade inducing EMT shifts and stem cell conversion that ultimately would stimulate cancer progression/invasion.[3,4,6,8,12,19,25] We have therefore defined multiple regulatory mechanisms whereby the apelinergic system may influence human carcinogenesis and serve as potential therapeutic targets for anticancer drug development.

CONCLUSIONS

In this chapter, we have presented an overview of the APL/APJ system's involvement in human carcinogenesis, stressing its role in cancer growth, angiogenesis, lymphangiogenesis, and dissemination to distal secondary sites. We have also discussed potential anticancer drug development that targets APL or the APJ receptor and prevents its promalignant bioactivities that encompass a neutralizing antibody, a peptide antagonist (APL-13[P13A]) and the catabolic enzyme ACE2 that selectively degrades the peptide. Finally, we have implicated APL as a regulator

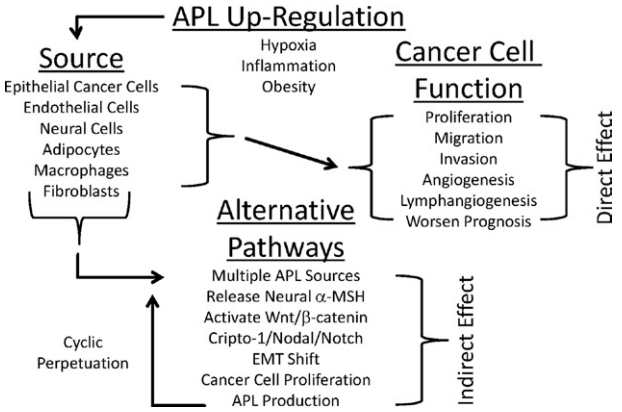

FIGURE 1 The role of apelin in cancer.

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of complex interconnecting pathways involving Wnt/β-catenin, Cripto-1/Nodal, Notch, EMT pathways, and stem cell conversion that underlie cancer progression and invasion, thus making it a good candidate for targeted therapy in the clinical management of human cancer. As summarized in Fig. 1, there are multiple sources of APL production capable of driving tumor cells through the carcinogenesis pathway and both direct and indirect mechanisms on how APL can influence cancers. Future research along these lines will help confirm the critical role APL plays in tumor development and hopefully identify intervention strategies that will effectively prevent or suppress human cancers. As a closing note, we propose the following question: Are APL-36, APL-31, APL-28, APL-17, and APL-13 the only bioactive peptide products of the apelinegic family or are there potentially other gene-related factors that may prove to be even more important in human carcinogenesis.

REFERENCES

1. Ameri K, Luong R, Zhang H, Powell AA, Montgomery KD, Espinosa I, et al. Circulating tumour cells demonstrate an altered response to hypoxia and an aggressive phenotype. *Br J Cancer* 2010;**102**:561–9.
2. Berta J, Kenessey I, Dobos J, Tovari J, Klepetko W, Jan Ankersmit H, et al. Apelin expression in human non-small cell lung cancer: role in angiogenesis and prognosis. *J Thorac Oncol* 2010;**5**:1120–9.
3. Bianco C, Rangel MC, Castro NP, Nagaoka T, Rollman K, Gonzales M, et al. Role of Cripto-1 in stem cell maintenance and malignant progression. *Am J Pathol* 2010;**177**:532–40.
4. Bianco C, Salomon D. Targeting the embryonic gene Cripto-1 in cancer and beyond. *Expert Opin Ther Pat* 2010;**20**:1739–49.
5. Cox CM, D'Agostino SL, Miller MK, Heimark RL, Krieg PA. Apelin, the ligand for the endothelial G-protein-coupled receptor, APJ, is a potent angiogenic factor required for normal vascular development in the frog embryo. *Dev Biol* 2006;**296**:177–89.
6. D'Aniello C, Lonardo E, Iaconis S, Guardiola O, Liguoro AM, Liguori GL, et al. G protein-coupled receptor APJ and Its ligand apelin act downstream of cripto to specify embryonic stem cells toward the cardiac lineage through extracellular signal-regulated Kinase/p70S6 kinase singaling pathway. *Cir Res* 2009;**105**:231–8.
7. del Toro R, Prahst C, Mathivet T, Siegfried G, Kaminer JS, Larrivee B, et al. Identification and functional analysis of endothelial tip cell-enriched genes. *Blood* 2010;**116**:4025–33.
8. De Luca A, Lamura L, Strizzi L, Roma C, D'Antonio A, Margaryan N, et al. Expression and functional role of CRIPTO-1 in cutaneous melanoma. *Br J Cancer* 2011;**105**:1030–8.
9. Entschladen Pam D, Drell IV TL, Lang K, Zaenker KS. Connecting a tumor to the environment. *Curr Pharm Des* 2007;**13**:3440–4.
10. Falcãp-Pires I, Ladeiras-Lopes R, Leite-Moreira AF. The apelinergic system: a promising therapeutic target. *Expert Opin Ther Target* 2010;**14**:633–45.
11. Folkman J. Tumor angiogenesis: therapeutic implications. *New Engl J Med* 1971;**285**:1182–6.
12. Hamada S, Watanabe K, Hirota M, Bianco C, Strizzi L, Mancino M, et al. Beta-Catenin/TCF/LEF regulates expression of the short form of human Cripto-1. *Biochem Biophys Res Commun* 2007;**355**:240–4.
13. Han S, Wang G, Qi X, Englander EW, Greeley Jr GH. Involvement of a Stat3 binding site in the inflammation-induced enteric apelin expression. *Am J Physiol Gastrointest Liver Physiol* 2008;**295**:G1968–78.
14. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell* 2011;**144**:647–74.
15. Hemminki K, Li X, Sundquist J, Sundquist K. Obesity and familial obesity and risk of cancer. *Eur J Cancer Prev* 2011;**20**:438–43.
16. Holopainen T, Bry M, Alitalo K, Saaristo A. Perspectives on lymphangiogenesis and angiogenesis in cancer. *J Surg Oncol* 2011;**103**:484–8.
17. Hosoya M, Kawamata Y, Fukusumi S, Fujii R, Habata Y, Hinuma S, et al. Molecular and functional characterisitics of APJ. Tissue distribution of mRNA and interaction with endogenous ligand apelin. *J Biol Chem* 2000;**275**:21,061–7.
18. Hu PF, Chen WP, Tang JL, Bao JP, We LD. Apelin plays a catabolic role on articular cartilage: in vivo and in vitro studies. *Int J Mol Med* 2010;**26**:357–63.
19. Jing Y, Han Z, Zhang S, Liu Y, Wei L. Epithelial-mesenchymal transition in tumor microenvironment. *Cell Biosci* 2011;**1**:29.
20. Kälin R, Kretz MP, Meyer AM, Kispert A, Heppner FL, Brändli AW. Paracrine and autocrine mechanisms of apelin signaling govern embryogenesis and tumor angiogenesis. *Dev Biol* 2007;**305**:599–614.
21. Kuba K, Zhang L, Imai Y, Arab S, Chen M, Maekawa Y, et al. Impaired heart contractility in apelin gene-deficient mice associated with aging and pressure overload. *Circ Res* 2007;**101**:e32–42.
22. Lee DK, Saldivia VR, Nguyen T, Cheng R, George SR, O'Dowd BF. Modification of the terminal residue of apelin-13 antagonizes its hypotensive action. *Endocrinology* 2004;**146**:231–6.
23. Rayalam S, Della-Fera MA, Kasser T, Warren W, Baile CA. Emerging role of apelin as a therapeutic target in cancer: a patent review. *Recent Pat Anticancer Drug Discov* 2011;**6**:367–72.
24. Reaux-Le Goazigo A, Bodineau L, Picco-De Mota N, Jeandel L, Chartrel N, Knauf C, et al. Apelin and the proopiomelanocortin system: A new regulatory pathway of hypothalamic [alpha]—MSH release. *Am J Physiol Endocrinol Metab* 2011 Aug 16. [Epub ahead of print].
25. Schell C, Eaton EN, Li SH, Chaffer CL, Reinhardt F, Kah KJ, et al. Paracrine and autocrine signals induce and maintain mesenchymal and stem cell states in the breast. *Cell* 2011;**145**:926–40.
26. Sleeman JP, Nazarenko I, Thiele W. Do all roads lead to Rome? Routes to metastasis development. *Int J Cancer* 2011;**128**:2511–26.
27. Tatemoto K, Hosoya M, Habata Y, Fujii R, Kakegawa T, Zuo MX, et al. Isolation and characterization of a novel endogenous peptide ligand for the human APJ receptor. *Biochem Biophys Res Commun* 1998;**251**:471–6.
28. Tiani C, Garcia-Pras E, Mejias M, de Gottardi A, Berzigotti A, Bosch J, et al. Apelin signaling modulates splanchnic angiogenesis and portosystemic collateral vessel formation in rats with portal hypertension. *J Hepatol* 2009;**50**:296–305.
29. Vickers C, Hales P, Kaushik V, Dick L, Gavin J, Tang J, et al. Hydrolysis of biological peptides by human angiotensin-converting enzyme-related carboxypeptidase. *J Biol Chem* 2002;**277**:14,838–43.
30. Volkoff H, Wyatt JL. Apelin in goldfish (*Carassius auratus*): cloning, distribution and role in appetite regulation. *Peptides* 2009;**30**:1434–40.
31. Wong SY, Hynes RO. Lymphatic or hematogenous dissemination: How does a metastatic tumor cell decide? *Cell Cycle* 2006;**5**:812–7.

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32. Zeng XJ, Zhang LK, Wang HX, Lu LQ, Ma LQ, Tang CS. Apelin protects heart against ischemia/reperfusion injury in rat. *Peptides* 2009;**30**:1144–52.
33. Zeng XJ, Yu SP, Zhang L, Wei L. Neuroprotective effects of the endogenous neural peptide apelin in cultured mouse cortical neruons. *Exp Cell Res* 2010;**316**:1773–83.
34. Zhang Q, Yao F, Raizada MK, O'Rourke ST, Sun C. Apelin gene transfer into the rostral ventrolateral medulla induces chronic blood pressure elevation in normotensive rats. *Circ Res* 2009;**104**:1421–8.
35. Zhang Z, Yu B, Tao GZ. Apelin protects against cardiomyocyte apoptosis induced by glucose deprivation. *Chin Med J* 2009;**122**: 2360–5.

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Chapter 69

Bombesin Peptides

Robert T. Jensen and Terry W. Moody

ABSTRACT

Mammalian bombesin-related peptides and their receptors are frequently expressed by human cancers and have important effects on tumoral growth, in some cases in an autocrine fashion. Their receptors are one of the groups most frequently ectopically/overexpressed by human cancers, and this is increasingly being investigated for possible tumor localization and for receptor-mediated cytotoxicity in which ligands for these receptors are used to target cytotoxic agents to the tumors. In this chapter, their effects/role in human cancer are briefly updated from the previous addition of this book with particular attention to advances in their cellular signaling cascades in cancer, roles in imaging and possible cytotoxicity, and their potential roles in cancer treatment.

INTRODUCTION

Bombesin (Bn)-related peptides [Gastrin-releasing peptide (GRP) and neuromedin B (NMB)] are regulatory peptides that are widely distributed in the central nervous system and gastrointestinal (GI) tract, where they primarily function as neurotransmitters/neuromodulators.[6] In the previous edition of this book[7] their role in cancer current to 2006 was reviewed including their expression and their receptor's frequent ectopic/overexpression by human cancers, their proliferative effects on tumors, their signaling cascades, and their roles in treatment. A few important points from this previous 2006 chapter will briefly be summarized below and the rest of this chapter will review the important advances of their role in cancer, especially their signaling cascades and use in tumor localization and targeted tumoral therapies.

Bn-RELATED PEPTIDES (GRP, NMB)

General

The 27-amino acid peptide, GRP is derived from a 148-amino acid precursor, whereas NMB is a 10-amino acid peptide derived from a 32-amino acid precursor originating from a larger 76-amino acid precursor.[6] Their cellular actions are mediated by two G protein-coupled receptors (GPCR): a GRP-R (384 amino acids) and NMB-R (390 amino acids).[6] A third orphan GPCR, bombesin receptor subtype-3 (BRS-3)(399 amino acids)[6] has 46–49% amino acid identities with GRP-R/NMB-R, but the endogenous ligand for it is unknown; however it is included in the Bn-related receptor class because of its high homology to GRP-R/NMB-R. Each mammalian Bn receptor is coupled to phospholipase C (PLC) as well as tyrosine phosphorylation and MAP kinase cascades.[6]

Expression of Bn-Peptides and Their Receptors in Cancer

As summarized in the 2006 chapter,[7] a wide range of cancers synthesize Bn-related peptides and many ectopically and/or overexpress GRP-R, NMB-R, and/or BRS-3.[7] Either Bn receptors or receptor mRNA was detected frequently in 70–100% of small-cell lung cancer (SCLC), non-small-cell lung cancer (NSCLC), pancreatic cancer cell lines, prostate cancer, head/neck squamous cell cancer, glioblastomas, intestinal carcinoids, and neuroblastomas.[7] A study[19] examining 32 different types of human cancers (bladder, colon, renal, ovarian, uterus, sarcoma) shows that GRP-Rs are expressed in vessels in all the tumors and that they are localized with particularly high expression in a subgroup of vessels, which also have high VEGF expression. This study suggested this localization might be used to deliver targeted antiangiogenic therapy or angiodestructive targeted radiotherapy with radioactively labeled Bn analogs.[19]

Effect of Bn-Receptor Activation on Cancer Growth/Proliferation

Bn-like peptides alter growth and/or differentiation of a wide range of cancers frequently in an autocrine fashion.[5,7] They stimulate growth of cancers of the pancreas, lung, head/neck, glioblastomas, neuroblastomas, kidney, prostate, breast, colon, ovary, and intestine.[5,7,14,35] Bn-R receptor agonists directly stimulate the growth both *in vitro* and *in vivo* for many of these different tumors and/or Bn-R antagonists

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00069-5
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or silencing of the GRP-R by receptor knockdown studies suppress tumorigenesis, metastatic potential or growth in various studies.[5,7,11,18,35] Furthermore, in some tumors such as head/neck squamous cell tumors, the addition of a GRP-R antagonist to an EGFR tyrosine kinase inhibitor (erlotinib) results in synergistic inhibition of growth and tumor invasion, increased apoptosis, cell cycle arrest of the tumor cells, and increased survival.[35] Furthermore, a similar result occurs in lung cancer cells where stimulation of the GRP-R results in increased resistance to the cytotoxic effects of the EGFR tyrosine kinase inhibitor, gefitinib, coupled with other findings to lead to the conclusion that GRP release can rescue the lung cancer cells exposed to gefitinib, leading the authors to propose that GRP-R blockade should be combined with EGFR inhibition to better modulate therapeutic effects in lung cancer.[11] Similarly, NMB-R can show similar growth synergistic effects with EGFR activation as shown in a study in lung cancer cells where the addition of an NMB-R antagonist results in an increased sensitivity to the cytotoxic effects of the EGFR tyrosine kinase inhibitor, gefitinib.[13] In colonic cancer cells, Bn-related peptides are weak mitogens, and extensive clinical and laboratory studies support the conclusion that the ectopic overexpression of GRP-R is functioning as a morphogen and is of benefit to the patient.[20,28,30] The clinical evidence includes the observation that the mean survival of patients with colon cancers expressing GRP-R is significantly longer than those without GRP-R expression; that only the better differentiated tumor cells express GRP/GRP-R, that expression of GRP or GRP/GRP-R by the tumor is associated with a delayed recurrence of the tumor and increased survival ($p=0.0124$) as well as fewer lymph node metastases.[20] These studies suggest that ectopic/overexpression of Bn-Rs by different tumors may have differing effects on growth/differentiation. Bn-related peptides also function as proangiogenic factors in various tumors.[7] In one study[17] NMB-R inhibition by PD168368, a specific NMB-R peptoid antagonist,[4] inhibits *in vivo* and *in vitro* angiogenesis; it inhibits MDA-MB-231 breast cancer cell growth by inducing cell cycle arrest and apoptosis and suppresses tumor growth in a xenograft model of breast cancer. This leads to the conclusion that NMB-R blockade has significant antitumor effects in breast cancer cells by simultaneously inhibiting neovascularization and cancer growth.[17]

Mechanism of Bn-Related Peptide Induced Growth Effects on Cancer Cells

Activation of all mammalian Bn-Rs stimulates phospholipase C (PLC) activity and causes activation of tyrosine and MAP kinase cascades.[5–7,31] In some tumors the growth stimulatory effects are mediated directly by the Bn-R activated cascades, whereas in other cases, transactivation of the EGF receptor (EGF-R) is required, and in still other cases by stimulation of the release of growth factors.[5–7,31] The growth stimulatory signaling cascades of the GRP-R have been investigated in the most detail.[7] GRP-R stimulates EGFR-independent growth of the hepatocellular carcinoma HepC2 cells, which is mediated by activation of ERK1/2.[9] In lung cancer cells GRP-R activation of PLC causing increased cytosolic calcium, but not the activation of PKC, is critical to mediating anchorage-independent growth and decreased survival. This requires the activation of Src family kinases, coupled with activation of the focal adhesion kinase, proline-rich tyrosine kinases (Pyk2), which result in GTP-loading of Ras and increased ERK1/2 activation.[21] Protein kinase D (PKD)[26] plays an important role in mediating GRP-R stimulated growth in Swiss 3T3 cells, with the early phase of PKD activation (<15min) resulting from PKC activation; however the later phase of PKD activation (>60 mins) is PKC-independent, but requires activation of EGFR. The ability of PKD activation to stimulate growth and DNA synthesis is partially mediated by its ability to increase the duration of ERK1/2 signaling. Prostate cancer cells very frequently overexpress the GRP-R and GRP-R activation can stimulate their growth.[7] In the androgen-insensitive cells line, PC-3, the activation of GRP-R results in increases in COX-2 mRNA and protein expression and the release of PGE2, which is mediated by the activation of the MAP kinase, p38 as well as requiring the activation of the PI3K/AKT pathway.[32] In the aggressive neuroblastoma cell line, BE (2)-C, the knockdown of GRP-R results in decreased proliferation, DNA synthesis and a cell cycle arrest at G2/M, which is associated with upregulation of PTEN, an inhibitor of the PI3K/AKT pathway, and a marked decrease in activated AKT, as well as phosph-p70S6K and its downstream target molecule S6, which are key regulators of protein synthesis and cell metabolism. Similarly, in various neuroblastoma cells (LAN-1, SK-N-SH, BE (2-C) in which GRP acts in an autocrine growth fashion, activation of the GRP-R stimulates AKT activity as well as pGSK-3B and an inhibitor of AKT, LY294002, abrogates GRP stimulated increases in phospho-AKT, and reverses GRP's effect on the cell cycle targets as well as inhibits GRP stimulated growth. The increase in p38 results in an increase in COX-2 mRNA by retarding its degradation.[32] The activation of the androgen receptor (AR) may play an important role in the growth of castration-resistant prostate cancer.[12] Activation of GRP-R can stimulate prostate cancer cells growth in an androgen-free environment, and this is partially mediated by the ability of GRP-R to stimulate androgen receptor phosphorylation at Tyr-534, which is dependent on Src activation.[12] Androgen receptor phosphorylation at Tyr-534 is linked to enhanced AR recruitment and expression of AR target genes at sub-optimal androgen concentrations.[12] GRP-R mediated transactivation of EGFR in head/neck squamous cell cancer cells (HNSCC cells) is mediated by Src-dependent cleavage of EGFR proligands, which result in MAPK activation.[35] The

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combination of a GRP-R antagonist and an EGFR inhibitor (erlotinib) has synergistic effects on growth inhibition of HNSCC cells[35] and it was associated with decreased phospho-EGFR, phospho-MAPK, phospho-c-Jun, phospho-p70S6K, and phospho-p38 kinase.[35] Similarly, in lung cancer cells[11] GRP-R stimulates growth by transactivating EGFR, which is associated with AKT activation.[11] The AKT activation is mediated by c-Src-mediated transactivation of the EGFR resulting in the activation of matrix metalloproteinases, release of amphiregulin, and then through a PI3K mediated mechanism.[11] The cytotoxic effect of the EGFR inhibitor, gefitinib on lung cancer cells, is inhibited by GRP administration and this protective effect of GRP is mimicked by the pretreatment of lung cancer cells with amphiregulin and activation of the AKT pathway, suggesting that GRP-R and/or EGFR autocrine pathways in lung cancer cells may modulate therapeutic responses to EGFR inhibitors, by rescuing the cancer cells from its cytotoxic effects.[11]

In colonic cancer cells, where activation of ectopic, overexpressed GRP-R has a weak growth effect and it is proposed that GRP-R is functioning as a morphogen and is of benefit to the patient, a number of studies into the possible mechanisms involved are reported.[20,22,28,30] In a study to identify targets activated in response to GRP-R signaling a proteomic approach was used to analyze two colonic cell lines, Caco-2 and HT-29, which express GRP-R as a function of confluence.[22] With GRP-R expression/activation five proteins were specifically downregulated (Bach2, creatine kinase B, p47, 2-not identified) and six were upregulated (gephrin, heat shock protein 70(HSP70), HP1, ICAM-1, ACAT, 1-not identified).[22] In colon cancer cells.[28] GRP-R activation causes heat shock protein 72(Hsp72) to be expressed via activation of $p125^{FAK}$, which promotes the binding of CD16+ and CD94+ natural killer cell lymphocytes resulting in tumor cell cytolysis and thus providing a novel mechanism for tumor cell destruction. An additional study[30] attempted to provide an insight into the possible effects of overexpression of Hetochromatin protein 1 by the ectopic activation of GRP-R in colon cancer cells.[22] The Heterochromatin 1 family (HP1) are epigenetic modifiers of gene transcription consisting of three members ($HP1^{Hs}\alpha$, $HP1^{Hs}\beta$, $HP1^{Hs}\gamma$), which protect against tumor cell aggressiveness and metastases in other cancers.[30] In this study,[30] inhibiting GRP-R signaling decreases $HP1^{Hs}\beta$ expression and increases colon cancer cell invasiveness, providing a possible mechanism whereby GRP-R overexpression is improving the outcome of patients with colon cancer, a result which is seen in clinical studies.[20,22,28,30]

Two other studies provide evidence that activation of ectopically expressed/overexpressed NMB-R's or BRS-3 by tumors may have similar mechanisms causing tumor growth in some cases, like that reported above with the GRP-R.[13,14] In two studies of lung cancer cells, which frequently overexpress NMB-R's or BRS-3,[6,7] activation of either NMB-R or BRS-3 stimulates growth by transactivating EGFR. For both NMB-R and BRS-3 the transactivation of EGFR is inhibited by a matrix metalloproteinase inhibitor (GM6001), by a universal SFK inhibitor (PP2), and by agents that inhibited the generation of reactive oxygen species, N-acetylcysteine (antioxidant), Tiron (superoxide scavenger), DPI (NADPH oxidase inhibitor).[13] For the NMB-R, EGFR transactivation is mediated by the generation of transforming growth factor alpha.[13]

Role of Bn-Related Peptides in Treatment of Human Tumors

The presence of Bn-related peptides/receptors on human tumors and their effects on growth are not at present specifically used in the treatment/management of any tumors. However, there is much interest and many recent studies on their ability to play potentially important roles as prognostic factors; as molecular targets to inhibit tumor growth by inhibition of their growth promoting activities; as molecular targets to image tumor extent/location, and as molecular targets to deliver Bn-R mediated cytotoxicity through radioactively labeled compounds and/or cytotoxic agents coupled to Bn-R ligands.[5–7,15,23] Each of these areas will be briefly dealt with.

Bn Peptides as Prognostic Agents

In several studies, plasma levels of GRP precursors (i.e. such as proGRP) in patients with non-small-cell lung cancer or small-cell lung cancer gives important prognostic information and is useful in some studies to assess response to therapy.[3,16,29,34]

Bn Peptides as Molecular Targets to Inhibit Tumor Growth by Inhibition of Their Growth Promoting Activities

Studies continue to support the proposal the inhibition of the autocrine growth effect of Bn-like peptides on tumor growth using Bn-receptor antagonists, antisense constructs, or other inhibitors, may be a novel approach for antitumor treatment.[7,18] Potentially particularly important is the observation in both lung tumors and head/neck squamous cell tumors that the blockade of Bn-R receptor activation (GRP-R, NMB-R, BRS-3) potentiates the ability of various EGFR antagonists such as gefitinib to inhibit tumor growth.[11,13,14,35] With non-small-cell lung cancer, one of the main treatments is the use of EGFR tyrosine kinase inhibitors such as gefitinib, however many patients do no respond or develop resistance to these with continued treatment, therefore the finding that Bn-R blockade potentiates

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EGFR inhibitors action may be of particular importance in the treatment of this tumor.[13,14]

Bn-Rs as Molecular Targets to Image Tumor Extent/Location

The potential usefulness of using the ectopic expression/ overexpression G protein-coupled receptors for tumor localization/imaging such as with Bn-Rs is best seen with the use of this approach with somatostatin receptor overexpression by neuroendocrine tumors.[23] With neuroendocrine tumors, which >80% overexpress somatostatin receptors, the use of various radioactively labeled somatostatin analogs is now one of the most clinically widely used and sensitive methods to both localize the primary tumor as well as to stage the disease.[23] However, many of the common malignancies, such as breast, pancreatic, colon, gastric, head/neck squamous tumors, and prostate cancers do not overexpress somatostatin receptors; however >70% overexpress Bn-Rs, and therefore there is a increasing interest in trying to apply this approach with radioactively labeled Bn analogs to these tumors.[7,23] The results from >200 studies have been summarized in one review[23] and other reviews cover various aspects of this approach.[1] Many synthetic analogs of Bn-related peptides are used in these studies including primarily COOH terminal analogs of GRP, Bn, or litorin and particularly analogs of Bn (2–14), Bn (6–14), and Bn (7–14).[23] These analogs are labeled with different isotopes depending on whether they are primarily going to be used for imaging, the type of imaging, or for Bn receptor-mediated cytotoxicity (next section), with the isotopes used including ^{99m}Technetium, ^{111}Indium, ^{64}Copper, ^{125}Iodine, $^{185/187}$Rhenium, ^{18}Fluorine, ^{68}Gallium, ^{90}Yttrium or ^{177}Lutetium.[23] Both *in vitro* studies and *in vivo* studies using conventional gamma radiation detection or PET imaging (positron emission tomography), most of the various Bn-R analogs examined were able to specifically identify tumors containing Bn-R-bearing cells.[23] In all of the original studies radioactively labeled Bn-receptor agonists were used, based on analogies to results with somatostatin analogs and numerous studies that showed only agonists are internalized and thus thought likely to give better imaging.[2,6,23] However, radioactively labeled Bn-receptor antagonists may be superior targeting agents to agonists.[2] One study compared the agonist, desmobesin 4 [Tetramine^0, Pro^1, Tyr^4, NLeu^{14}] Bn and the GRP-R antagonist, desmobesin 1 [Tetramine^0, D-Phe^6-Leu-NHEt^{13}, desMet^{14}] Bn (6–14)], which had similar affinities for the GRP-R, and found almost a 8-fold greater tumor to kidney ratio with the antagonist, ^{99m}Tc-Desmobesin 1, four hours after injection in prostate cancer (PC-3) bearing mice.[2] At present the difference in the molecular basis of the greater binding and superior imaging properties of Bn-R antagonists over agonists is unknown. There is particular interest in imaging prostate cancers which frequently (>70%) overexpress/ ectopically express GRP-R.[6,7,25] In *in vitro* studies of various prostate cancer cells and *in vivo* studies both in humans and in prostate cancer-bearing animal, imaging results with various GRP-R agonists and antagonists, are promising.[1,23,25] One important finding that will be particularly important for imaging with GRP-R analogs in patients with prostate cancer is that the expression of human GRP-R in human androgen-dependent prostate cancer xenografts is markedly reduced by androgen ablation. Some studies[4,24,31] for the first time report systematic evaluations of the absolute and relative affinities/potencies for various human Bn-R subtypes (GRP-R, NMB-R, BRS-3) for a large number of Bn-related peptides that are reported to function as agonists or antagonists, primarily in nonhuman cells. These studies show a number of important differences in the pharmacophore of human Bn-Rs compared with that of rat Bn-Rs that are commonly used in experimental studies, and besides providing possible templates for further studies, they also make the point that the pharmacology of any proposed ligands for human studies should be performed in human Bn-R containing cells.

Bn-Rs as Molecular Targets to Deliver Bn-R-Mediated Cytotoxicity Through Radiolabeled Compounds and/or Cytotoxic Agents Coupled to Bn-R Ligands

Because many common tumors frequently (>70%) overexpress Bn-Rs,[7] not only can labeled analogs of various Bn-related peptides possibly be used to image the tumors, as discussed in the preceding paragraph, but there is also increased interest in using them to target cytotoxic agents (radioactively labeled other cytotoxic agents) to the tumor.[23] Results from >100 studies have been summarized and reviewed.[23] This approach is given support by the very promising results with radioactively labeled analogs of somatostatin [^{111}In-, ^{90}Y-, ^{177}Lu-labeled somatostatin analogs], which are being widely used to treat advanced malignant neuroendocrine tumors.[8,23] Similar to the imaging studies discussed above, numerous synthetic agonist analogs of Bn-related peptides [i.e. especially analogs of GRP, Bn, litorin equivalent to Bn (2–14), Bn (6–14), and Bn (7–14)[23]] were used in these cytotoxic studies.[23] For Bn receptor-mediated radio-cytotoxicity, the isotopes primarily used including ^{68}Gallium, ^{90}Yttrium, or ^{177}Lutetium,[23] which were coupled to various Bn analogs using various linkers and chemical groups that would avidly bind the isotopes (DOTA, DOTA analogs, DO3A, NOTA, or NOTA-Aca).[23] A number of these describe radioactively labeled Bn analogs that retain high affinity for the Bn-R bearing tumors *in vivo* have reduced renal toxicity and tumoricidal effects *in vivo*.[23] In human prostate cancer cells, Bn analogs coupled

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to alpha particle emitters (such as ^{213}Bismuth) have greater cytotoxicity than do the β-emitter ^{177}Lu–Bn analog,[33] suggesting that this approach should be consider in the future.

In addition to coupling Bn analogs to radioactively labeled Bn-related analogs that have cytotoxic effects, there is increasing interest in targeting other cytotoxic agents to tumors overexpressing/ectopically expressing Bn receptors.[23] Cytotoxic Bn analogs formed by coupling Bn-related peptides to chemotherapeutic agents (doxorubicin) (AN-215) inhibit the growth of pancreatic, lung, prostate, and gastric cancers[7,23] and GRP analogs coupled to camptothecin inhibit lung cancer growth.[10,15,23] The camptothecin-coupled Bn analogs mediate their cytotoxic effects by interacting with tumoral Bn-Rs.[15] Bn analogs have also been coupled to the marine toxins, hemisasterlin and dolastin, and the conjugates have cytotoxic effects on the lung cancer cell line, NCI-H1299, which contains Bn-Rs.[27] Similarly, conjugation of Bn agonist analogs to magainin, an antimicrobial peptide, markedly enhances (10-fold) its cytotoxicity for tumor cells.[10]

At present, the usefulness of using Bn-like peptides or the presence of Bn-R overexpression/ectopic expression by tumors in any of the areas of investigation reviewed above for tumor treatment/diagnosis is not established.

ACKNOWLEDGMENTS

This study was partially funded by intramural money from NIDDK and NCI, NIH.

REFERENCES

1. Ananias HJ, de Jong I, Dierck RA, Van de Wiele C, Helfrich W, Elsinga PH. Nuclear imaging of prostate cancer with gastrin-releasing-peptide-receptor targeted radiopharmaceuticals. *Curr Pharm Des* 2008;**14**:3033–47.
2. Cescato R, Maina T, Nock B, Nikolopoulou A, Charalambidis D, Piccand V, et al. Bombesin receptor antagonists may be preferable to agonists for tumor targeting. *J Nucl Med* 2008;**49**:318–26.
3. Gandhi L, Camidge DR, Ribeiro de Oliveira M, Bonomi P, Gandara D, Khaira D, et al. Phase I study of Navitoclax (ABT-263), a novel Bcl-2 family inhibitor, in patients with small-cell lung cancer and other solid tumors. *J Clin Oncol* 2011;**29**:909–16.
4. Gonzalez N, Mantey SA, Pradhan TK, Sancho V, Moody TW, Coy DH, et al. Characterization of putative GRP- and NMB-receptor antagonist's interaction with human receptors. *Peptides* 2009;**30**: 1473–86.
5. Gonzalez N, Moody TW, Igarashi H, Ito T, Jensen RT. Bombesin-related peptides and their receptors: recent advances in their role in physiology and disease states. *Curr Opin Endocrinol Diabetes Obes* 2008;**15**:58–64.
6. Jensen RT, Battey JF, Spindel ER, Benya RV. International Union of Pharmacology. LVIII. Mammalian Bombesin receptors: nomenclature, distribution, pharmacology, signaling and functions in normal and disease states. *Pharmacol Rev* 2008;**60**:1–42.
7. Jensen RT, Moody TW. Bombesin-related peptides and neurotensin: effects on cancer growth/proliferation and cellular signaling in cancer. In: Kastin AJ, editor. *Handbook of biologically Active Peptides*. Amsterdam: Elsevier; 2006. p. 429–34.
8. Kwekkeboom DJ, de Herder WW, Krenning EP. Somatostatin receptor-targeted radionuclide therapy in patients with gastroentero-pancreatic neuroendocrine tumors. *Endocrinol Metab Clin North Am* 2011;**40**:173–85.
9. Li X, Lv Y, Yuan A, Yi S, Ma Y, Li Z. Gastrin-releasing peptide promotes the growth of HepG2 cells via EGFR-independent ERK1/2 activation. *Oncol Rep* 2010;**24**:441–8.
10. Liu S, Yang H, Wan L, Cai HW, Li SF, Li YP, et al. Enhancement of cytotoxicity of antimicrobial peptide magainin II in tumor cells by bombesin-targeted delivery. *Acta Pharmacol Sin* 2011;**32**:79–88.
11. Liu X, Carlisle DL, Swick MC, Gaither-Davis A, Grandis JR, Siegfried JM. Gastrin-releasing peptide activates Akt through the epidermal growth factor receptor pathway and abrogates the effect of gefitinib. *Exp Cell Res* 2007;**313**:1361–72.
12. Liu Y, Karaca M, Zhang Z, Gioeli D, Earp HS, Whang YE. Dasatinib inhibits site-specific tyrosine phosphorylation of androgen receptor by Ack1 and Src kinases. *Oncogene* 2010;**29**:3208–16.
13. Moody TW, Berna MJ, Mantey S, Sancho V, Ridnour L, Wink DA, et al. Neuromedin B receptors regulate EGF receptor tyrosine phosphorylation in lung cancer cells. *Eur J Pharmacol* 2010;**637**:38–45.
14. Moody TW, Sancho V, Di Florio A, Nuche-Berenguer B, Mantey S, Jensen RT. Bombesin receptor subtype-3 agonists stimulate the growth of lung cancer cells and increase EGF receptor tyrosine phosphorylation. *Peptides* 2011;**32**:1677–84.
15. Moody TW, Sun LC, Mantey SA, Pradhan T, Mackey LV, Gonzales N, et al. In vitro and in vivo antitumor effects of cytotoxic camptothecin-bombesin conjugates are mediated by specific interaction with cellular bombesin receptors. *J Pharmacol Exp Ther* 2006;**318**:1265–72.
16. Nisman B, Heching N, Biran H, Barak V, Peretz T. The prognostic significance of circulating neuroendocrine markers chromogranin a, pro-gastrin-releasing peptide and neuron-specific enolase in patients with advanced non-small-cell lung cancer. *Tumour Biol* 2006;**27**:8–16.
17. Park HJ, Kim SR, Bae SK, Choi YK, Bae YH, Kim EC, et al. Neuromedin B induces angiogenesis via activation of ERK and Akt in endothelial cells. *Exp Cell Res* 2009;**315**:3359–69.
18. Qiao J, Kang J, Ishola TA, Rychahou PG, Evers BM, Chung DH. Gastrin-releasing peptide receptor silencing suppresses the tumorigenesis and metastatic potential of neuroblastoma. *Proc Natl Acad Sci USA* 2008;**105**:12891–6.
19. Reubi JC, Fleischmann A, Waser B, Rehmann R. Concomitant vascular GRP-receptor and VEGF-receptor expression in human tumors: molecular basis for dual targeting of tumoral vasculature. *Peptides* 2011;**32**:1457–62.
20. Rivera CA, Ahlberg NC, Taglia L, Kumar M, Blunier A, Benya RV. Expression of GRP and its receptor is associated with improved survival in patients with colon cancer. *Clin Exp Metastasis* 2009;**26**: 663–71.
21. Roelle S, Grosse R, Buech T, Chubanov V, Gudermann T. Essential role of Pyk2 and Src kinase activation in neuropeptide-induced proliferation of small cell lung cancer cells. *Oncogene* 2008;**27**: 1737–48.
22. Ruginis T, Taglia L, Matusiak D, Lee BS, Benya RV. Consequence of gastrin-releasing peptide receptor activation in a human colon cancer cell line: a proteomic approach. *J Proteome Res* 2006;**5**:1460–8.

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23. Sancho V, Di Florio A, Moody TW, Jensen RT. Bombesin receptor-mediated imaging and cytotoxicity: review and current status. *Curr Drug Deliv* 2011;**8**:79–134.
24. Sancho V, Moody TW, Mantey SA, Di Florio A, Uehara H, Coy DH, et al. Pharmacology of putative selective hBRS-3 receptor agonists for human bombesin receptors (Bn-R): affinities, potencies and selectivity in multiple native and Bn-R transfected cells. *Peptides* 2010;**31**:1569–78.
25. Schroeder RP, van Weerden WM, Krenning EP, Bangma CH, Berndsen S, Grievink-de Ligt CH, et al. Gastrin-releasing peptide receptor-based targeting using bombesin analogues is superior to metabolism-based targeting using choline for in vivo imaging of human prostate cancer xenografts. *Eur J Nucl Med Mol Imaging* 2011;**38**:1257–66.
26. Sinnett-Smith J, Jacamo R, Kui R, Wang YM, Young SH, Rey O, et al. Protein kinase D mediates mitogenic signaling by Gq-coupled receptors through protein kinase C-independent regulation of activation loop Ser744 and Ser748 phosphorylation. *J Biol Chem* 2009; **284**:13434–45.
27. Sun LC, Luo J, Mackey VL, Fuselier JA, Coy DH. Effects of camptothecin on tumor cell proliferation and angiogenesis when coupled to a bombesin analog used as a targeted delivery vector. *Anticancer Drugs* 2007;**18**:341–8.
28. Taglia L, Matusiak D, Benya RV. GRP-induced up-regulation of Hsp72 promotes CD16+/94+ natural killer cell binding to colon cancer cells causing tumor cell cytolysis. *Clin Exp Metastasis* 2008;**25**:451–63.
29. Tang JH, Zhang XL, Zhang ZH, Wang R, Zhang HM, Zhang ZL, et al. Diagnostic value of tumor marker pro-gastrin-releasing peptide in patients with small cell lung cancer: a systematic review. *Chin Med J (Engl)* 2011;**124**:1563–8.
30. Tell R, Rivera CA, Eskra J, Taglia LN, Blunier A, Wang QT, et al. Gastrin-releasing peptide signaling alters colon cancer invasiveness via heterochromatin protein 1Hsbeta. *Am J Pathol* 2011;**178**:672–8.
31. Uehara H, Gonzalez N, Sancho V, Mantey SA, Nuche-Berenguer B, Pradhan T, et al. Pharmacology and selectivity of various natural and synthetic bombesin related peptide agonists for human and rat bombesin receptors differs. *Peptides* 2011;**32**:1685–99.
32. Wen X, Chao C, Ives K, Hellmich MR. Regulation of bombesin-stimulated cyclooxygenase-2 expression in prostate cancer cells. *BMC Mol Biol* 2011;**12**:29.
33. Wild D, Frischknecht M, Zhang H, Morgenstern A, Bruchertseifer F, Boisclair J, et al. Alpha- versus beta-particle radiopeptide therapy in a human prostate cancer model (213Bi-DOTA-PESIN and 213Bi-AMBA versus 177Lu-DOTA-PESIN). *Cancer Res* 2011;**71**:1009–18.
34. Wojcik E, Kulpa JK, Sas-Korczynska B, Korzeniowski S, Jakubowicz J. ProGRP and NSE in therapy monitoring in patients with small cell lung cancer. *Anticancer Res* 2008;**28**:3027–33.
35. Zhang Q, Bhola NE, Lui VW, Siwak DR, Thomas SM, Gubish CT, et al. Antitumor mechanisms of combined gastrin-releasing peptide receptor and epidermal growth factor receptor targeting in head and neck cancer. *Mol Cancer Ther* 2007;**6**:1414–24.

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Chapter 70

Endothelin-1

Samer-ul Haque, Hazel Welch and Marilena Loizidou

ABSTRACT

Endothelin-1 (ET-1) is a small vasoactive peptide that was first identified in 1988. It is one of a family of three endothelins that exert their action through two G-protein coupled receptors, ET_A and ET_B. This chapter reviews the evidence implicating ET-1 in tumorigenesis. In particular, we concentrate on the role of ET-1 in mitogenesis, apoptosis, angiogenesis, tumor invasion, and metastasis. Evidence relating to downstream effectors is discussed, and trials relating to the potential for endothelin-system modulation as an adjuvant therapeutic strategy are reviewed.

ENDOTHELIN-1

The potent vasoconstrictor peptide endothelin-1 (ET-1) is one of a family of three multifunctional peptides (ET-1, 2, and 3). They comprise a 21-amino acid structure characterized by a single α-helix and 2 disulfide bridges, encoded on chromosomes 6, 1, and 20 respectively. Initially described in 1988 as a vasoconstrictive substance isolated from bovine aortic endothelial cells, ET-1 is the most extensively studied of the three peptides and the one most implicated in tumorigenesis.

The ET physiological effect is exerted via ET_A and ET_B receptors, which are G-protein coupled transmembrane receptors (GPCR) found in both vascular and non-vascular tissues. The ET_A receptor has varying affinities for each isoform (higher for ET-1 than for ET-2, with a two-fold lower affinity for ET-3), whereas the ET_B receptor shows no selective affinity for any of the ET subtypes.[29]

The endothelins have been implicated in numerous pathological conditions including hypertension and cardiac failure. Interest in the role of ET-1 in cancer has grown over the last decades, and currently there is evidence that ET-1 can modulate mitogenesis, apoptosis, angiogenesis, tumor invasion, and development of metastases.

ET-1 ASSOCIATED SIGNAL TRANSDUCTION PATHWAYS

Translation of the ET-1 gene produces a number of precursors that eventually result in the production of Big-ET-1. This has negligible biological activity and is converted by the metalloproteinase endothelin-converting enzyme 1 (ECE-1) into the active ET-1 peptide. Production of ET-1 is stimulated by Interleukin (IL-1ß), tumor necrosis factor (TNFα), transforming growth factor (TGFß), platelet derived growth factor (PDGF), vasopressin, hypoxia, and shear stress. ET-1 inhibitory factors include nitric oxide, prostacyclins, and atrial natriuretic peptide (ANP).[24]

The consequences of ET-1-induced receptor stimulation are complex with many intracellular pathways being activated (Fig. 1). ET-1 binding to its receptor results in dissociation of the α and βγ subunits of one of several possible associated G-proteins. The activated G-protein then phosphorylates one of several upstream pathway initiators, which include phospholipase C and D, phospholipase A_2, adenylate cyclase, and guanylate cyclase.[31] The former two initiators activate PI3K and Protein Kinase C (PKC) which ultimately result in mitogen activating protein kinase (MAPK) pathway activation. This is achieved either directly by Ras activation through PI3K or indirectly via increased intracellular Ca2+ through PKC. Raf-1 (downstream in the MAPK pathway) is activated directly via PKC.[6] Ultimately, MAPK results in early response gene transcription of fos and jun. Resultant cellular effects include mitogenesis and motility.

More recently, transactivation of other cell surface receptors as a result of ET receptor stimulation has been shown in Rat-1 fibroblasts,[9] ovarian cancer and colorectal cancer (CRC) cells, the Tyrosine Kinase Epidermal Growth Factor Receptor (EGFR) is transactivated and induces MAPK activity.[12] ET-1 has also been shown to potentiate the effects of PDGF on human smooth muscle cells, suggesting interactions with other tyrosine kinase receptors.[35]

As ET_B receptor-binding affinity is equal for all isoforms, this suggests a role in the clearance pathway via lysosomal degradation after receptor uptake.[7] A second clearance pathway has been suggested involving catabolism by the extracellular neutral peptidase nepilysin (NEP).

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00070-1
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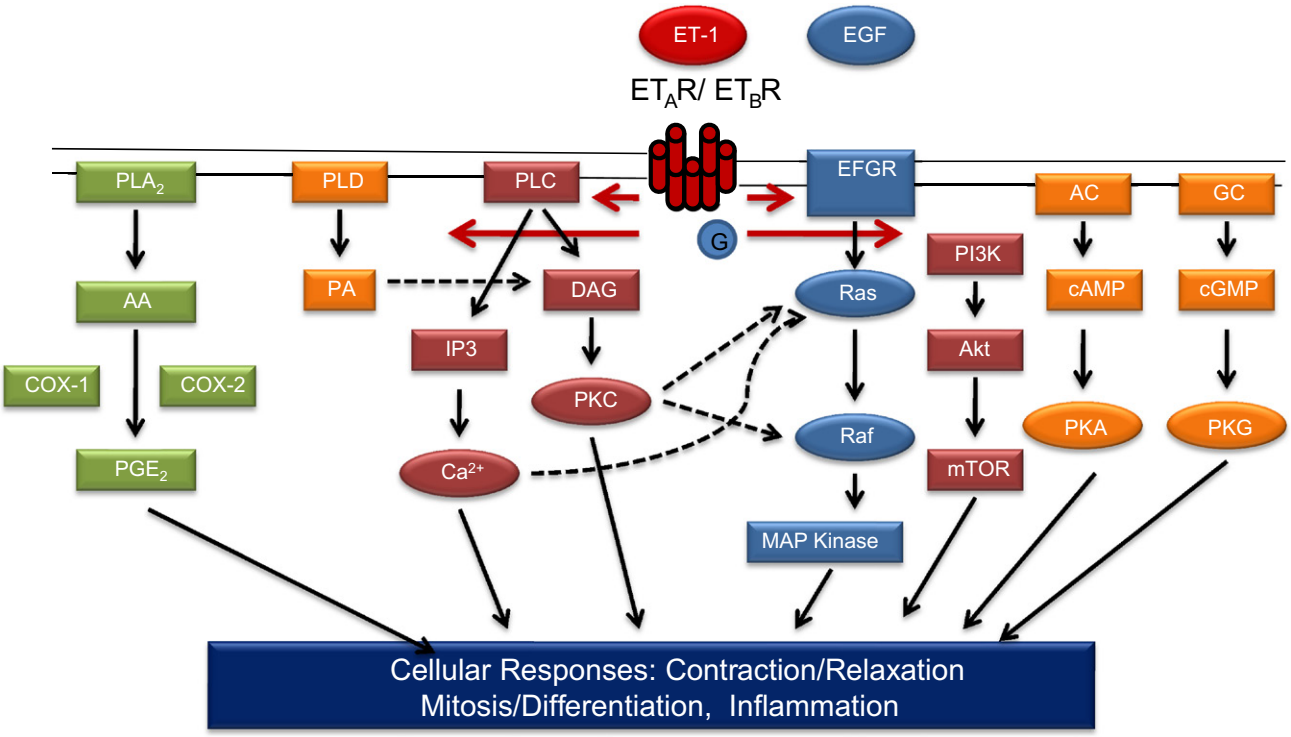

FIGURE 1 Schematic representation of the signal transduction pathways associated with stimulation of the Endothelin receptors. Upstream transduction molecules activated (phosphorylated) by the G-protein are shown at the top of each pathway and include the Epidermal Growth Factor Receptor (EGFR), Phospholipase A_2, C, D (PLA_2, PLC, PLD), Adenylate Cyclase (AC), Guanylate Cyclase (GC), and Phosphoinositide 3-Kinase (PI3K). Downstream effectors include phosphatidyl inositol 3 kinase (IP_3), diacylglycerol (DAG), phospholipase A (PA), cyclic GMP (cGMP), protein kinase A, C, G (PKA, PKC, PKG), Akt, mTOR, cyclooxygenase 1, 2 (COX-1, COX-2), and prostaglandin E_2 (PGE_2). See color plate 19.

ET-1 EXPRESSION IN CANCER

Many human cancer cell lines have been shown to synthesize ET-1, including colonic, breast, stomach, prostate, and glioblastoma cells.[2,20] Similarly, *in vivo*, increased tissue immunoreactivity for ET-1 has been seen in several cancer types, including ovarian, breast, and colorectal tumors.[5]

In normal colorectal tissue, low expression of ET-1 and ECE-1 was reported. However, these molecules are significantly upregulated in 80% of primary CRCs and in the majority of metastatic disease. Furthermore, ET-1 expression levels within tumor-adjacent stromal and endothelial cells are raised, in keeping with its known paracrine function.[4,30] Our group showed that plasma levels of ET-1 are increased in patients with both primary colorectal cancers with or without liver metastasis.[4,20] Other studies have found that both preoperative and intraoperative portal plasma levels are significantly higher in metastatic disease. Elevated plasma concentrations of ET-1 have also been detected in patients with other various solid tumors, including gastric and prostate cancer, where levels are greatest in patients with metastastic, hormone refractory disease.[22]

ET-1 dysregulation as an early event in colorectal tumorigenesis was suggested by the observation that premalignant colorectal adenomas show increased expression of Big-ET-1 and ECE-1 mRNA when compared with normal colon. ET-1 immunoreactivity in breast ductal carcinoma *in situ* (DCIS) specimens is also significantly higher ($P<0.005$) than that of normal breast tissue, suggesting that the modulation of the endothelin system may be an early phenomenon in tumorigenesis.

ENDOTHELIN RECEPTOR EXPRESSION IN CANCER

Varied expression levels of ET receptor subtypes in different cancers have been investigated by use of immunohistochemistry, autoradiography, and mRNA studies. Expression of the ET_A receptor is upregulated predominately in colorectal, ovarian, renal, and prostate cancers,[1,5,22] whereas ET_B receptors are downregulated. On the other hand, not only are ET_A receptors upregulated, but ET_B receptors are also upregulated or have varied expression levels in lung and breast carcinomas. The question remains whether the variety of techniques used to examine levels may give conflicting or false results for ET_B expression.

Specifically within CRCs, we showed that pharmacologically functional ET_A receptors are over-expressed in

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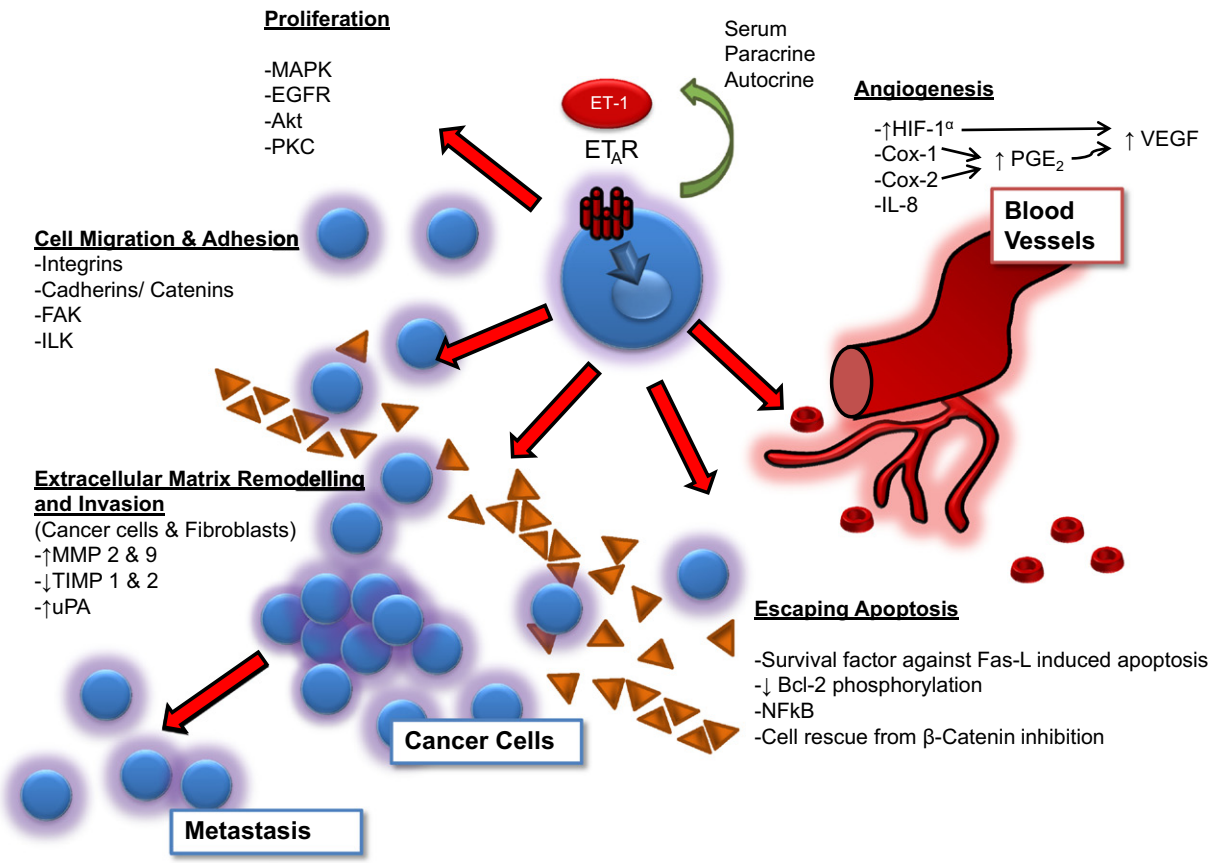

FIGURE 2 Summary of the role of ET-1 in the spread of cancer. See color plate 20.

all colorectal cancer tissues, compared with normal. Most of this localization is around blood vessels and fibroblasts, with less localization to epithelial cancer cells. By contrast, ET_B receptors are prominent in normal tissue with a marked down-regulation in cancer associated blood vessels, fibroblasts, and cancer epithelial cells.[15] In prostate tumors, levels of receptor expression have been found to correlate with the presence of metastases, similar to the findings in CRCs.

Interestingly, relative hypermethylation of the ET_B gene has been reported in prostate, bladder, and colon cancer cell lines. Furthermore, this correlates with transcriptional down-regulation, providing a plausible mechanism for reduced ET_B receptor expression in malignant tissue.

ET-1 AS A MITOGEN

Effects on mitogenesis are the most intensely studied among all the different tumorigenic actions of ET-1 (Fig. 2); they are driven through a complex network of multiple pathways, presented earlier, and involve the MAPK cascade. For example, in ovarian cancer cell lines, ET_A receptor antagonism reduces EGFR transactivation, implicating this receptor in mitogenesis. ET-1 stimulates the growth of several human cancer cell lines. In CRC experimental models, ET_A receptor antagonism significantly reduces proliferation not only in cancer cell lines but also in colonic fibroblasts.[2,19] In ovarian cancers, the use of ET_A receptor antagonists ABT-627 and ZD4054 inhibits cell proliferation and protects against paclitaxel induced apoptosis.[27] The same receptor is associated with prostate and cervical cancer proliferation.[24,25] In the latter atrasentan, an ET_A receptor antagonist, inhibits growth in xenografts. Other tumors with ET-1 mitogenic activity via the ET_A receptor include lung, bladder, and nasopharyngeal carcinomas. In breast and Kaposi's sarcomas, both ET_A and ET_B receptors are over-expressed. The use of dual receptor antagonists, bosentan in breast and A-182086 in Kaposi's sarcomas, significantly inhibits tumor growth and vascularity.[10] Studies on human melanoma cells show that the mitogenic effect of ET-1 is purely ET_B receptor dependent. This has also been confirmed *in vivo*, where the specific ET_B antagonist (BQ788) significantly slows melanoma tumor growth in nude mice.[21]

The role of ET-1 as an autocrine growth factor has been shown in human ovarian and colon cancer cell lines.[1] A paracrine role for ET-1 has also been elucidated in ovarian cancer, where ET-1 production by human ovarian cancer cells stimulates growth of carcinoma associated fibroblasts

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in co-culture, an effect which is partially inhibited by both ET_A and ET_B antagonism.

ET-1 AND APOPTOSIS

ET-1 also modulates apoptosis. Within rat colon carcinomas, Peduto-Eberl et al. showed that ET-1 acts as a survival factor for rat colon carcinoma cells against FasL-induced apoptosis.[26] They also showed that dual endothelial receptor antagonism with bosentan results in human CRC sensitization to FasL-induced caspase mediated apoptosis. Protection from FasL-induced apoptosis, through suppressed Bcl-2 phosphorylation and PI3K mediated Akt activation, also occurs in ovarian cancers.[26] Furthermore in CRC, ET-1 (which is a direct target of β-Catenin) rescues cells from growth arrest and apoptosis, which were originally caused by β-Catenin inhibition.[18] This suggests that ET-1 has an oncogenic effect by interfering with the β-Catenin pathway.

In both melanocytes and melanoma cell lines, ET-1 decreases basic apoptotic rates. Like the proliferative action of ET-1 in these cells, the effect on apoptosis is ET_B dependent.

ET-1 AND ANGIOGENESIS

In addition to mitogenic actions on epithelial cancer cells, ET-1 stimulates growth of endothelial cells, vascular smooth muscle cells (VSMC), fibroblasts, and pericytes. Furthermore, ET-1 potentiates the effect of several pro-angiogenic factors *in vitro*, including PDGF and VEGF. ET-1 promotes cancer neovascularization via ET_A receptor stimulation on vascular smooth muscle cells and ET_B receptor stimulation on endothelial cells. ET-1 also stimulates various stages of neovascularisation, including protease production, tubular formation, endothelial cell proliferation, migration, and invasion.

There is a significant correlation between microvascular density, ET-1 and VEGF expression in ovarian cancer specimens. From *in vitro* studies, in both an ovarian cancer cell line and vascular smooth muscle cells, ET-1 promotes transcription and protein expression of VEGF to a level similar to that stimulated after hypoxia. This effect is mediated through ET-1 binding to ET_A receptors, stimulation of hypoxia inducible factor (HIF-1) production (or its stabilization in normoxic conditions), which then binds to hypoxia responsive elements and in turn increases VEGF production.[32] A reciprocal relationship exists where HIF-1 itself also stimulates ET-1 production. Furthermore, ET-1 acting via the ET_A receptor increases the expression of COX-1 and COX-2 in ovarian cancer cells. This results in increased prostaglandin signaling, particularly PGE_2, and stimulation of several MAPK dependent signaling pathways, including p38 MAPK and p42/22 MAPK. This results in the production of VEGF, MMP activation and angiogenic cellular invasion.[33]

Invasive tumor cells, including melanomas, prostate, breast, and ovarian carcinomas form *de novo* extracellular matrix rich vascular channels expressing vascular associated molecules. Both MMP2 and MT1-MMP, which are expressed by invasive ovarian cancer cells, play a key role in developing vasculogenic-like networks and matrix remodeling. Interestingly, ET_A receptor antagonism of these cells inhibits the formation of tumor-like vascular channels.

In vivo studies using subcutaneous implants of matrigel impregnated with either ET-1 or VEGF confirm increased vascular effects and vascularity above that of other angiogenic factors, for example, bFGF.

In addition to an effect on tumor cells, ET-1 protects human endothelial cells and VSMCs from serum deprivation induced apoptosis *in vitro*. This suggests that ET-1 could have a pro-angiogenic effect by acting as a survival factor for newly formed blood vessels.

ET-1 AND TUMOR PROGRESSION/ METASTASES

Further to its pro-angiogenic role, ET-1 may also influence tumor invasion and metastases by stimulating secretion of matrix remodeling proteins. In colonic fibroblasts, we reported that ET-1 increases TIMP-1 and MMP-2 protein expression, which are known to be involved in key tumorigenic processes: matrix degradation, activation of growth factors and degrading enzymes, and modulation of angiogenesis.[19] Expression of both these molecules is inhibited by predominantly ET_A and also ET_B receptor antagonism. Furthermore, we showed that ET-1 stimulates expression of CTGF and Collagen Type XI mainly via the ET_A receptor. CTGF activates intracellular pathways resulting in increased migration, proliferation, and MMP production, whereas Collagen Type XI is associated with colonic diseases. We also conducted proliferation and desmoplastic activation studies in colonic fibroblast strains using the specific ET_A receptor antagonist zibotentan: ET_A receptor blockade inhibits growth, contraction, migration, and MMP production by these cells.[13,14]

In ovarian cancer, ET-1 stimulates the expression of several MMPs, particularly MMP-2 and MMP-9, and down-regulates tissue inhibitors of matrix metalloproteinases, TIMP1 and 2. Similar up-regulation of MMPs has also been shown in Kaposi's sarcoma cells. Furthermore, ET-1 can contribute to the creation of a "reactive" tumor stroma, by stimulating myofibroblast induction (via ET_A), and the expression of matrix remodeling genes by these cells.[34] Interestingly, tumor adherence to collagen is enhanced by integrin-linked kinase (ILK), a multidomain focal adhesion protein. Blockade of ET-1/ET_A receptor induced ILK results in the inhibition of MMP activation, as well as cell motility

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and invasiveness, indicating that the ET-1 axis is involved in aggressive cellular behavior.

In prostate cancer, ET-1 may contribute to the growth of bony metastases. *In vitro*, ET-1 production by prostate cancer cells is enhanced by bone contact, which in turn blocks osteoclastic bone reabsorption. Similarly, in an *in vivo* osteoblastic tumor model, tumors transfected to overexpress ET-1 produced significantly more bone growth in nude mice compared with vector only controls.[23]

Furthermore, we have shown increased ET-1 immunoreactivity in endothelial cells within colorectal liver metastases compared with surrounding vessels,[13] suggesting that ET-1 may be involved in modulation of tumor blood flow.

ENDOTHELIN ANTAGONISM *IN VIVO*

Several *in vivo* models have been used to assess the role of endothelin antagonism in tumorigenesis. Work originating from our department using intra-portally injected syngeneic MC28 cells in rats showed that ET_A antagonism with BQ123 significantly reduces hepatic tumor load compared with controls[4]

The effect of bosentan, a dual receptor antagonist, on growth of peritoneal tumors derived from a syngeneic rat colonic adenocarcinoma cell line has been investigated. Although bosentan was not able to control tumor progression, tumors are generally of lower grade, and there are fewer spontaneous deaths in the treated versus the untreated groups. Egidy et al. used the same tumor model to assess histological differences between tumors of bosentan treated animals and controls.[11] They showed that tumor cells are less densely packed, and there is less collagen matrix around tumor nodules in the treated compared with the untreated group.

Using an osteoblastic tumor model in nude mice Nelson et al. have shown that ET_A antagonism with A127722 significantly reduces the growth of new bone compared with vehicle treated controls.[23]

Finally, Rosano et al. have combined targeting of the ET_A and EGF receptors with promising results. They showed that co-administration of ZD4054 and gefitinib led to a partial (82%) or complete tumor regression on HEY ovarian carcinoma xenografts. This also results in decreased vascularization, MMP2, VEGF, MAPK and EGFR, and enhances E-Cadherin expression.[28]

To date, results from *in vivo* models are encouraging and warrant further investigation.

CLINICAL TRIALS

The availability of orally active bioavailable endothelin receptor antagonists offers potential opportunities in adjuvant cancer treatment. One example is the drug atrasentan, which has a 1000-fold greater affinity for the ET_A receptor than the ET_B receptor. A phase II randomized, placebo-controlled trial in 288 patients with asymptomatic hormone-resistant metastatic prostate cancer evaluated three groups; placebo, 2.5mg, or 10mg atrasentan. Delayed time to progression (TTP) was observed in the 10-mg group, and stabilization of biochemical markers, including prostate specific antigen and lactate dehydrogenase compared with controls. In phase III clinical trials involving 809 prostate cancer patients, 10mg of atrasentan daily was shown to delay time to disease progression when compared with placebo, albeit statistically non-significant, in men with metastatic hormone-resistant prostate cancer.[8] However, secondary end-point analysis showed significantly delayed progression of bone acid phosphatase levels, and preserved prostate cancer-specific quality of life, particularly in terms of pain-related symptoms. Another phase III trial in non-metastatic prostate cancer once again showed statistically non-significant delay in disease progression (93 days) when compared with placebo with the same dosing of this drug.[25] It has been suggested that the future use of atrasentan may lie in combining it with other drugs. This was shown by a phase I–II trial in patients with resistant prostate cancer, where results from this drug combined with docetaxel were comparable to results produced by docetaxel and prednisolone.[3]

Another orally active ET receptor antagonist, currently used in pulmonary hypertension, is Bosentan. This is a non-selective, dual ET_A and ET_B receptor antagonist. Promising preclinical results showing inhibition of tumor progression occurred in *in vitro* and *in vivo* models of melanoma.[21] However, a phase II clinical trial reported that patients with stage 4 metastatic melanoma developed progressive disease despite receiving 500mg BD of Bosentan.[17]

The novel specific ET_A receptor antagonist Zibotentan (trans,trans-2(4-methoxydhenyl)-4-(1-3-benzodiazol-5-yl)-1-(dibutylaminocarbonylmethyl)-pyrrolidine-3-carboxylic acid (ZD4054)), has been investigated in a number of clinical trials. In the clinical arena, the original focus was within the field of prostate cancer. A phase II double blinded clinical trial allocated a total of 312 patients with pain free or mildly symptomatic hormone-resistant prostate cancer patients with bony metastases to either receive daily doses of ZD4054 (10–15mg) or placebo. There was no significant difference seen for progression-free survival although there was a difference in the overall survival of these patients compared with the placebo group. Importantly this drug has an acceptable safety and tolerability profile.[16] A Phase III clinical trial looked at ZD4054 in non-metastatic hormone-resistant prostate cancer. This was terminated early in 2011 as it was unlikely that the trial would meet its primary efficacy end-point of progression-free survival and overall survival benefits. Because of the disappointment of monotherapy, interest has grown in its use as adjuvant

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therapy, for example the latest ongoing phase II clinical trial (FOLFERA), which combines ZD4054 with chemotherapy agents irinotecan, fluorouracil, and folinic acid (FOLFIRI) in patients with advanced colorectal cancer.

The endothelin axis is altered in cancer, aiding tumor growth and progression. It seems that selective ET_A antagonism provides the most effective method of endothelin-system inhibition in cancer. With generally acceptable side effects, and suggested anti-tumor activity, further clinical evaluation of these agents is warranted to determine possible therapeutic potential as an adjuvant anti-cancer strategy.

REFERENCES

1. Ali H, Dawas K, Loizidou M, Savage F, Taylor I. Endothelin receptor expression in colorectal cancer. *J Cardiovasc Pharmacol* 2000;**36**:S69–71.
2. Ali H, Loizidou M, Dashwood M, Savage F, Sheard C, Taylor I. Stimulation of colorectal cancer cell line growth by ET-1 and its inhibition by ET(A) antagonists. *Gut* 2000;**47**:685–8.
3. Armstrong AJ, Creel P, Turnbull J, Moore C, Jaffe TA, Haley S, et al. A phase I-II study of docetaxel and atrasentan in men with castration-resistant metastatic prostate cancer. *Clin Cancer Res* 2008;**14**:6270–6.
4. Asham E, Shankar A, Loizidou M, Fredericks S, Miller K, Boulos PB, et al. Increased endothelin-1 in colorectal cancer and reduction of tumour growth by ET(A) receptor antagonism. *Br J Cancer* 2001;**85**: 1759–63.
5. Bagnato A, Salani D, Di CV, Wu Wong JR, Tecce R, Nicotra MR, et al. Expression of endothelin 1 and endothelin A receptor in ovarian carcinoma: evidence for an autocrine role in tumour growth. *Cancer Res* 1999;**59**:720–7.
6. Blaukat A, Barak A, Cross M, Offermanns S, Dikic I. G protein coupled receptor mediated mitogen-activated protein kinase activation through co-operation of G alpha-q and G alpha-i signals. *Mol Cell Biol* 2000;**20**:6837–48.
7. Bremnes T, Paasche JD, Mehlum A, Sandberg C, Bremnes B, Attramadal H. Regulation and intracellular trafficking pathways of the endothelin receptors. *J Biol Chem* 2000;**275**:17596–604.
8. Carducci MA, Saad F, Abrahamsson PA, Dearnaley DP, Schulman CC, North SA, et al. Atrasentan Phase III Study Group Institutions. A phase 3 randomized controlled trial of the efficacy and safety of atrasentan in men with metastatic hormone-refractory prostate cancer. *Cancer* 2007;**110**:1959–66.
9. Daub H, Weiss U, Wallasch C, Ullrich A. Role of transactivation of the EGF receptor in signaling by G-protein-coupled receptors. *Nature* 1996;**379**(557):460.
10. Dréau D, Karaa A, Culberson C, Wyan H, McKillop IH, Clemens MG. Bosentan inhibits tumor vascularization and bone metastasis in an immunocompetent skin-fold chamber model of breast carcinoma cell metastasis. *Clin Exp Metastasis* 2006;**23**(1):41–53.
11. Egidy G, Juillerat-Jeanneret L, Jeannin J, Korth P, Bosman F, Pinet F. Modulation of human colon tumour-stromal interactions by the endothelial system. *Am J Pathol* 2000;**157**:1863–74.
12. Grant K, Knowles J, Dawas K, Burnstock G, Taylor I, Loizidou M. Mechanisms of endothelin 1-stimulated proliferation in colorectal cancer cell lines. *Br J Surg* 2007;**94**:106–12.
13. Haque S, Heetun M, Farooqui N, Loizidou M, Dashwood M, Shiwen X, et al. Efficacy of the Specific ET_A Receptor antagonist Zibotentan (ZD4054) in cancer cells and fibroblasts from colorectal cancer. *Mol Cancer Ther* 2009;**8**(Suppl. 12):B190.
14. Haque S, Heetun M, Taylor I, Shiwen X, Winslet M, Loizidou M. Efficacy of the specific ET_A receptor antagonist zibotentan in colorectal cancer cells and colorectal fibroblasts. *Eur J Surg Oncol* 2009; **35**(11):1206.
15. Hoosein MM, Dashwood MR, Dawas K, Ali HM, Grant K, Savage F, et al. Altered endothelin receptor subtypes in colorectal cancer. *Eur J Gastroenterol Hepatol* 2007;**19**(9):775–82.
16. James ND, Caty A, Borre M, Zonnenberg BA, Beuzeboc P, Morris T, et al. Safety and efficacy of the specific endothelin-A receptor antagonist ZD4054 in patients with hormone-resistant prostate cancer and bone metastases who were pain free or mildly symptomatic: a double-blind, placebo-controlled, randomised, phase 2 trial. *Eur Urol* 2009;**55**(5):1112–23.
17. Kefford R, Beith JM, Van Hazel GA, Millward M, Trotter JM, Wyld DK, et al. A phase II study of bosentan, a dual endothelin receptor antagonist, as monotherapy in patients with stage IV metastatic melanoma. *Invest New Drugs* 2007;**25**:247–52.
18. Kim TH, Xiong H, Zhang Z, Ren B. beta-Catenin activates the growth factor endothelin-1 in colon cancer cells. *Oncogene* 2005;**24**:597–604.
19. Knowles JP, Shiwen X, Haque S, Bhalla A, Dashwood MR, Yang S, et al. Endothelin-1 stimulates colon cancer adjacent fibroblasts. *Int J Cancer* 2011 Mar 28; doi:10.1002/ijc.26090.
20. Kusuhara M, Yamaguchi K, Nagasaki K, Hayashi C, Hori S, Handa S, et al. Production of endothelin in human cancer cell lines. *Cancer Res* 1990;**50**:3257–61.
21. Lahav R, Heffner G, Patterson PH. An endothelin receptor B antagonist inhibits growth and induces cell death in vitro and in vivo. *Proc Natl Acad Sci U S A* 1999;**96**:11496–500.
22. Nelson JB, Chan-Tack K, Hedican SP, Magnuson S, Opgenorth JT, Bora G. Endothelin-1 production and decreased endothelin B receptor expression in advanced prostate cancer. *Cancer Res* 1996;**56**:663–8.
23. Nelson JB, Nguyen SH, Wu Wong JR, Opgenorth JT, Dixon BD, Chung LW, et al. New bone formation in an osteoblastic tumour model is increased by endothelin-1 overexpression and decreased by ET_A receptor blockade. *Urology* 1999;**53**:1063–9.
24. Nelson J, Bagnato A, Battistini B, Nisen P. The endothelin axis: emerging role in cancer. *Nat Rev Cancer* 2003;**3**:110–6.
25. Nelson JB, Love W, Chin JL, Saad F, Schulman CC, Sleep DJ, et al. Atrasentan phase 3 study group. Phase 3, randomized, controlled trial of atrasentan in patients with nonmetastatic, hormone-refractory prostate cancer. *Cancer* 2008;**113**:2478–87.
26. Peduto Eberl L, Bovey R, Juillerat-Jeanneret L. Endothelin receptor antagonists are pro-apoptotic and antiproliferative in human colon cancer cells. *British Journal of Cancer* 2003;**88**:788–95.
27. Rosanò L, Di Castro V, Spinella F, Nicotra MR, Natali PG, Bagnato A. ZD4054, a specific antagonist of the endothelin A receptor, inhibits tumor growth and enhances paclitaxel activity in human ovarian carcinoma in vitro and in vivo. *Mol Cancer Ther* 2007;**6**: 2003–11.
28. Rosano L, Di Castro V, Spinella F, Tortora G, Nicotra MR, Natali PG, et al. Combined targeting of endothelin a receptor and epidermal growth factor receptor in ovarian cancer shows enhanced antitumor activity. *Cancer Res.* 2007;**67**(13):6351–9.

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29. Sakamoto A, Yanagisawa M, Takuwa Y, Masaki T. Cloning and functional expression of human cDNA for the ET_B endothelin receptor. *Biochemical and Biophysical Research Communications* 2001;**178**:656–63.
30. Shankar A, Loizidou M, Aliev G, Fredericks S, Holt D, Boulos PB, et al. Raised endothelin 1 levels in patients with colorectal liver metastases. *Br J Surg* 1998;**85**:502–6.
31. Shome K, Rizzo MA, Vasudevan C, Andreson B, Romero G. The activation of phospholipase D by endothelin-1, angiotensin II, and platelet derived growth factor in vascular smooth muscle A10 cells is mediated by small G proteins of the ADP-ribosylation factor family. *Endocrinology* 2000;**141**:2200–8.
32. Spinella F, Rosano L, Di Castro V, et al. Endothelin-1 induces vascular endothelial growth factor by increasing hypoxia-inducible factor-1alpha in ovarian carcinoma cells. *J Biol Chem* 2002;**277**:27850–5.
33. Spinella F, Rosano L, Di Castro V, Nicotra MR, Natali PG, Bagnato A. Inhibition of cyclo-oxygenase-1 and -2 expression by targeting the endothelin A receptor in human ovarian carcinoma cells. *Clinical Cancer Research* 2004;**10**:4670–9.
34. Xu SW, Howat SL, Renzoni EA, Holmes A, Pearson JD, Dashwood M, et al. Endothelin-1 induces expression of matrix-associated genes in lung fibroblasts through MEK/ERK. *Journal of Biological Chemistry* 2004;**279**:23098–103.
35. Yang Z, Kraznici N, Luscher TF. Endothelin-1 potentiates human smooth muscle cell growth to PDGF: effects of ET_A and ET_B receptor blockade. *Circulation* 1999;**100**:5–8.

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Chapter 71

Gastrin

Jens F. Rehfeld and Jens P. Goetze

ABSTRACT

The gastrin and gastrin receptor genes are frequently expressed in common carcinomas in the esophagus, stomach, pancreas, colorectal mucosa, bronchial mucosa, ovary, and thyroid gland. In addition, rare neuroendocrine tumors also express gastrin and its receptor. The expression is often modified at the posttranscriptional and posttranslational levels. As a result of less complete processing, progastrin and processing intermediates are therefore often released from cancer cells in increased fractions. Moreover, the receptor gene may be expressed as splice variants and in larger or truncated forms due to abnormal posttranslational modifications. The growth-promoting effect of gastrin peptides suggests that the gastrin system (ligands and receptor) has broad carcinogenic significance. Consequently, attempts to target different components of the gastrin system in cancer therapy by receptor antagonists or vaccination are under way.

INTRODUCTION

Gastrin was discovered in extracts of the gastric antrum in 1905 and identified as a pair of heptadecapeptides in 1964.[10] Until the 1970s, gastrin was believed to be produced only in antroduodenal cells and its function only to be the regulation of gastric acid secretion.

Now, the structure of gastrin and gastrin receptor genes as well as their widespread expression also outside the stomach are well known (for a review, see Ref. 23). Moreover, gastrin is also a growth factor. Thus, gastrin controls the growth of the fundic mucosa cells and seems to influence also the growth of epithelial mucosal cells elsewhere (for a review, see Ref. 29).

Growth factors are often involved in cancer. But the oncogenic role of gastrin is still not well defined. A large number of studies, however, implicate gastrin peptides and their receptors in cancer development.[5-7,28] This chapter discusses the carcinogenetic role of gastrin critically.

EXPRESSION OF GASTRINS IN NORMAL TISSUE

By far the most gastrin in the normal organism is produced in antroduodenal G-cells (for a review, see Ref. 23). Consequently, gastrin biosynthesis studies have focused on antral tissue[4,12,16] and shown that the cellular gastrin synthesis is quite complex.

As a result of the elaborate biosynthetic pathway, antral G-cells release a mixture of progastrin products from the secretory granules. A small percentage are nonamidated processing intermediates, of which glycine-extended gastrins have attracted some interest.[12,30] But in man, >98% are α-amidated bioactive gastrins, of which nearly 85% are gastrin-17, 5–10% are gastrin-34, and the rest is a mixture of gastrin-71, -52, -14, and -6.[16,23,24]

Because of major differences of metabolic clearance rates, the larger gastrins with long half lives predominate over gastrin-17 and shorter gastrins in plasma.[16] In achlorhydria and gastrinomas, the translational activity in the gastrin-producing cells increases considerably, and apparently the processing enzymes cannot keep up with such rate of synthesis. Consequently, the fraction of immature precursors and processing intermediates increases markedly in tissue and plasma.[2,16]

The gastrin gene[35] is expressed also in extraantral cells that normally, however, contribute only little to the gastrin measured in the blood. So far, we have encountered extraantral gastrin expression in the small intestinal and in the colorectal mucosa,[19] in pancreatic islet cells,[3,18] in pituitary corticotrophs and melanotrophs, hypothalamic–pituitary, cerebellar, and vagal neurons, in bronchial mucosa,[26] and in spermatogenic cells (for a review, see Ref. 25 and Table 1).

The significance of extraantral gastrin synthesis is largely unknown, but it may have carcinogenetic relevance. Thus, gastrin-producing cancers seem to originate from cells that normally express the gastrin gene at low levels at some stage of the development (for a review, see Ref. 25).

THE GASTRIN RECEPTOR(S)

The gastrin receptor was cloned and identified as a protein of 453-amino acid residues. It belongs to the superfamily of G protein–coupled receptors with seven transmembrane α-helical domains. The gastrin receptor binds acid-stimulatory peptides containing the C-terminal sequence -Gly-Trp-Met-Asp-PhNH_2.

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00071-3
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TABLE 1 Gastrin Expression at the Peptide Level in Normal Mammalian Tissue*

Tissue	Total translation product (pmol per gram tissue)	Precursor percentage
Gastrointestinal tract		
Antral mucosa	10,000	5
Duodenal musoca	400	20
Jejunal mucosa	40	30
Ileal mucosa	20	85
Colonic mucosa	0.2	100
Neuroendocrine tissue		
Cerebellum	5	20
Vagal nerve	8	10
Adenohypophysis	200	98
Neurohypophysis	30	5
Adrenal medulla	2	100
Pancreas	2	95
Genital tract		
Ovaries	0.5	100
Testicles	6	100
Spermatozoa	2	55
Respiratory tract		
Bronchial mucosa	0.3	100

**Orders of magnitude based on examination of different mammalian species.*

Hence, the receptor binds all carboxyamidated gastrins and the homologous cholecystokinins (CCK). The gastrin and CCK peptides are bound with similar affinity regardless of length and degree of tyrosyl sulfation. But in terms of hormonal regulation, the CCK peptides are of marginal significance as gastrin receptor ligands because they circulate in 10- to 20-fold lower concentrations than the gastrins in plasma. The gastrin receptor is structurally closely related to the CCK receptor. The amino acid sequences of the two receptors display nearly 50% homology. But the CCK receptor is more selective as it binds only tyrosyl sulfated CCK peptides with high affinity (for a review, see Ref. 34).

The naming of the gastrin and CCK receptors has been confusing because the gastrin receptor also binds CCK peptides. It has therefore been proposed[22] that the CCK receptor should be named the "CCK-A or CCK_1" receptor and the gastrin receptor the "CCK-B or CCK_2" receptor. In this review, however, the original names, gastrin and CCK receptors, will be used.

The gastrin receptor in normal mammals is expressed primarily on ECL and parietal cells in the stomach and on neurons in most regions of the brain. In the central nervous system, CCK-8 seems to be the principal ligand, whereas gastrin-17 and -34 are major ligands in the periphery. The gastrin receptor is promiscuous in the sense that it is expressed — although with some species differences — at a lower level on other cells and in other tissues during ontogenetic development and during malignant transformation (for a review, see Ref. 25).

A number of gastrin receptor variants have been discovered. One isoform is the result of a splicing variation of the fourth exon, and it encodes a receptor with five additional amino acid residues near the third intracellular loop.[14,32] Another form is truncated without the N-terminal extracellular domain.[21] Moreover, both high (74kDa) and low (40kDa) molecular weight forms have been described. The differences are assumed to reflect different degrees of glycosylation and other posttranslational modifications. Finally, a gastrin receptor variant of particular oncogenetic relevance has been found in pancreatic[31] and colorectal carcinomas.[11] This variant has retained the fourth intron of the receptor gene and encodes accordingly an extra sequence of 69 amino acid residues in the third intracellular loop.[11,31] The prolongation changes the function of G-proteins associated with calcium signaling. The splicing variation is caused by reduced activity of the U2AF35 ribonudeoprotein particle splicing factor.[8] The intron-4 containing splice variant is of major interest for pancreatic and colorectal carcinogenesis and has led to the suggestion that it deserves the name of a "CCK-C" receptor.[31] At our present state of knowledge, and considering that other variants exist, such a name may, however, be premature.

EXPRESSION OF GASTRIN IN TUMORS

In 1955, Zollinger and Ellison described a syndrome consisting of severe duodenal ulcer disease, gastric acid hypersecretion, and neuroendocrine tumors in the pancreas. The syndrome was subsequently shown to result from hypersecretion of gastrin from pancreatic G-cell tumors, gastrinomas. Although many gastrinomas originate in the pancreas, small gastrinomas have also been found with increasing frequency in the duodenum, ovaries, and a few other locations. Some pancreatic gastrinomas also contain cells that produce other pancreatic hormones. The gastrinoma syndrome occurs with a frequency of one new case per million inhabitants per year.[15] It is important to diagnose and localize gastrinomas at an early stage because, in spite of slow growth, gastrinomas are malignant adenocaracinomas.[2,27]

The gastrin gene, however, is more frequently expressed at the peptide level in some brain tumors, pituitary adenomas,

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TABLE 2 Gastrin Expression at the Peptide Level in Tumors (Range)

Tumor*	Total translation product (pmol per gram tissue)	Precursor percentage
Neuroendocrine tumors		
Acustic neuromas† (14/19)	0.2–10	10–10
Pituitary adenomas (12/87)	0.5–200	20–90
Pancreatico-duodenal gastrinomas (27/27)	400–2,500,000	15–19
Pheochromocytomas† (13/13)	0.2–400	90–100
Common carcinomas		
Bronchogenic carcinomas† (17/17)	0.2–20	25–100
Colorectal carcinomas (12/12)	0.5–2.0	85–100
Gastric carcinomas (19/20)	0.1–400	10–100
Ovarian carcinomas† (16/16)	0.8–25	10–50

**Numbers in parentheses indicate the number of tumors with detectable gastrin expression in relation to total number of tumors examined.*
†Tumor type in which carboxyamidated, bioactive gastrin occurs only as gastrin-17.

pheochromocytomas, and bronchogenic,[26] pancreatic,[9,31] and colorectal[11,13,33] carcinomas. The level of expression is often so low that gastrins from these tumors do not contribute significantly to the concentration of bioactive gastrin in plasma (Table 2). Consequently, these neoplasias do not elicit symptoms associated with the gastrinoma syndrome. Often, only a minor fraction of the progastrin is processed to bioactive gastrins although the processing varies considerably in each individual tumor. However, even though the secretion of gastrin from brain, lung, pancreatic, and colonic tumors is too small to increase the concentration in plasma, the expression of gastrin may have carcinogenic significance. Local secretion may stimulate growth of tumor cells equipped with receptors for gastrin, variants or not.[13] Moreover, it is possible that the release of progastrin and processing intermediates may contribute to the precursor concentrations in plasma and make progastrin a tumor marker.[2,26,33] Progastrin in plasma may even be an indicator of the degree of malignancy.[2]

GASTRIN AS A TUMOR GROWTH FACTOR

Gastrin has growth-promoting effects on the normal gastric and colorectal mucosa,[17] and several normal gastric, pancreatic, and colorectal epithelial cells are equipped with gastrin receptors (for a review, see Ref. 25). Moreover, in countless studies of cancer cell lines, gastrin peptides stimulate growth. Consequently, it is probable that *in vivo* growth of human bronchial, gastric, colorectal ovarian, esophageal, and pancreatic carcinomas is stimulated by autocrine, endocrine, neurocrine, or paracrine release of gastrin.

There is, however, confusion about the molecular nature of the growth-promoting gastrins. It is generally agreed that carboxyamidated and acid-stimulatory gastrins (mainly gastrin-17 and gastrin-34) can stimulate growth of normal and transformed epithelial cells that express the gastrin receptor. But the observation that the progastrin products in colorectal carcinomas are incompletely processed and not carboxyamidated has led to the suggestion that also processing intermediates such as glycine-extended gastrins via specific receptors stimulate the growth of carcinoma cells.[30] The suggestion is interesting, but two essential pieces of evidence are missing. First, 18 years after the initial suggestion and in spite of subsequent intensive search in many laboratories, the proposed receptor or receptor mechanism has still not materialized. Second, half of all known bioactive peptide systems in vertebrates are carboxyamidated and often affect, as does gastrin, growth of their target cells. Because the glycine residue is the obligatory amide donor for carboxyamidated peptides, glycine-extended precursors for all amidated bioactive peptides are necessarily produced during hormone and neuropeptide biosynthesis. Nevertheless, it is striking that independent activity via specific receptors of glycine-extended intermediates has been proposed only for the gastrin system. Taken together, the idea about a growth-promoting carcinogenetic role of glycine-extended gastrins is therefore still short of decisive support.

Another confusing issue is the progastrin molecule as such. Several authors have suggested that the progastrin molecule in itself stimulates growth (for a review, see Ref. 1). Because the concentration of progastrin in normal, adult tissue, and plasma is negligible,[2,24] progastrin as such is not available for promotion of growth of normal cells. But in bronchogenic, colorectal, and pancreatic cancer cells is an accumulation of progastrin.[9,26,33] Hence, the cellular concentration of progastrin and cellular growth may correlate in certain carcinomas. Such correlation, however, constitutes no proof. Moreover, in most reports on this issue, progastrin is rarely or poorly defined.[1] The expression often refers to immunoreactivity without precise epitope or sequence definition. Consequently, the studies may have dealt with undefined progastrin products and not with the progastrin molecule itself. Again, as for glycine-extended gastrins, progastrin as such is not a ligand for the gastrin receptor, and therefore, a separate progastrin receptor mechanism remains to be found. Finally, specific bioactivity of the primary precursor (i.e. the proper prohormone) has so far not been observed for other prohormones.

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In view of the pronounced molecular heterogeneity of carboxyamidated gastrins and the many processing intermediates produced along the biosynthetic pathway in cells that express the gastrin gene, there is a need for precise definition and naming of the progastrin products. Hopefully, systematic definitions will reduce the confusion about gastrins as tumor growth factors, especially because of the many variants of the gastrin receptor.

CONCLUSIONS

The evidence for a role of gastrin in the carcinogenesis of common cancers within and outside the gastrointestinal tract has grown markedly in the last decades. The poor prognosis and limited success in treatment of, for instance, ovarian, pancreatic, colorectal, and lung carcinomas have so far brought gastrin peptides and their receptors (in their multiple molecular variants) into focus as possible targets for new therapeutic approaches. These include specific receptor antagonists and programs for vaccination against gastrin peptides (for a review, see Ref. 20). Rational and effective therapy, however, requires precise molecular understanding and definition of the system and an unequivocal naming of the targets. The literature published so far abounds with inconsistencies in these respects. When the necessary ambiguity has been resolved, the gastrin system may open new avenues for rational attacks on widespread cancers.

REFERENCES

1. Aly A, Shulkes A, Baldwin GS. Gastrins, cholecystokinins and gastrointestinal cancer. *Biochim Biophys Acta* 2004;**1704**:1–10.
2. Bardram L. Progastrin in serum from Zollinger–Ellison patients: an indicator of malignancy. *Gastroenterology* 1990;**98**:1420–6.
3. Bardram L, Hilsted L, Rehfeld JF. Progastrin expression in mammalian pancreas. *Proc Natl Acad Sci U S A* 1990;**87**:298–302.
4. Brand SJ, Klarlund J, Schwartz TW, Rehfeld JF. Biosynthesis of tyrosine 0-sulfated gastrins in rat antral mucosa. *J Biol Chem* 1984;**59**:13246–52.
5. Burkitt MD, Varro A, Pritchard DM. Importance of gastrin in the pathogenesis and treatment of gastric tumors. *World J Gastroenterol* 2009;**15**:1–6.
6. Chao C, Hellmich MR. Gastrin, inflammation and carcinogenesis. *Curr Opin Endocrinol Diabetes Obes* 2010;**17**:33–9.
7. Copps J, Murphy RF, Lovas S. The production and role of gastrin-17 and gastrin-17 Gly in gastrointestinal cancers. *Protein Pept Lett* 2009;**16**:1504–18.
8. Ding WQ, Kuntz SM, Miller LJ. A missspliced form of the cholecystokinin-B/gastrin receptor in pancreatic carcinoma: role of reduced cellular U2A F35 and a suboptimal 3′-splicing site leading to retention of the fourth intron. *Cancer Res* 2002;**62**:947–52.
9. Goetze JP, Nielsen FC, Burcharth F, Rehfeld JF. Closing the gastrin loop in pancreatic carcinomas: coexpression of gastrin and its receptor in solid human pancreatic adenocarcinoma. *Cancer* 2000;**88**: 2487–94.
10. Gregory H, Hardy PM, Jones DS, Kenner GW, Sheppard RC. The antral hormone gastrin. I. Structure of gastrin. *Nature* 1964;**204**:931–3.
11. Hellmich MR, Rui XL, Hellmich HL, Fleming RY, Evers BM, Townsend CM. Human colorectal cancers express a constitutively active cholecystokinin-B/gastrin receptor that stimulates growth. *J Biol Chem* 2000;**275**:32122–8.
12. Hilsted L, Rehfeld JF. Alpha-carboxyamidation of antral progastrin: relation to other post-translational modifications. *J Biol Chem* 1987;**262**:16953–7.
13. Hoosein NM, Kiener PA, Curry RL, Brattain MG. Evidence for autocrine growth stimulation of cultured colon tumor cells by a gastrin/cholecystokinin-like peptide. *Exp Cell Res* 1990;**186**:15–21.
14. Ito M, Iwata N, Taniguchi T, Murayama T, Chihara K, Matsui T. Functional characterization of two cholecystokinin-B/gastrin receptor isoforms: a preferential splice donor site in the human receptor gene. *Cell Growth Differ* 1994;**5**:1127–35.
15. Jacobsen O, Bardram L, Rehfeld JF. The requirement for gastrin analysis. *Scand J Clin Lab Invest* 1986;**46**:272–4.
16. Jensen S, Borch K, Hilsted L, Rehfeld JF. Progastrin processing during antral G-cell—hypersecretion in humans. *Gastroenterology* 1989;**96**:1063–70.
17. Johnson LR. The trophic action of gastrointestinal hormones. *Gastroenterology* 1976;**70**:278–88.
18. Larsson LI, Rehfeld JF, Hakanson R, Sundler F. Pancreatic gastrin in foetal and neonatal rats. *Nature* 1976;**262**:609–11.
19. Lüttichau HR, van Solinge WW, Nielsen FC, Rehfeld JF. Developmental expression of the gastrin and cholecystokinin genes in rat colon. *Gastroenterology* 1993;**104**:1092–8.
20. Morton M, Prendergast C, Barrett TD. Targeting gastrin for the treatment of gastric acid related disorders and pancreatic cancer. *Trends Pharmacol Sci* 2011;**32**:201–5.
21. Miyake A. A truncated isoform of human CCK-B/gastrin receptor generated by alternative usage of a novel exon. *Biochem Biophys Res Comm* 1995;**208**:230–7.
22. Noble F, Wank SA, Crawley JN, Bradwejn J, Seroogy KB, Hamon M, et al. Structure, distribution and functions of cholecystokinin receptors. *Pharmacol Rev* 1999;**51**:745–81.
23. Rehfeld JF. The new biology of gastrointestinal hormones. *Physiol Rev* 1998;**78**:1087–108.
24. Rehfeld JF, Johnsen AH. Identification of component I as gastrin-71, the largest possible bioactive progastrin product. *Eur J Biochem* 1994;**223**:765–73.
25. Rehfeld JF, van Solinge WW. The tumor biology of gastrin and cholecystokinin. *Adv Cancer Res* 1994;**63**:295–347.
26. Rehfeld JF, Bardram L, Hilsted L. Gastrin in bronchogenic carcinomas: constant expression but variable processing of progastrin. *Cancer Res* 1989;**49**:2840–3.
27. Rehfeld JF, Gingras MH, Bardram L, Hilsted L, Goetze JP, Poitras P. The Zollinger–Ellison syndrome and mismeasurement of gastrin. *Gastroenterology* 2011;**140**:1444–53.
28. Reubi JC, Schaer JC, Waser B. Cholecystokinin (CCK)-A and CCK-B/gastrin receptors in human tumors. *Cancer Res* 1997;**57**:1377–86.
29. Rozengurt E, Walsh JH. Gastrin, CCK, signaling and cancer. *Ann Rev Physiol* 2001;**63**:49–76.
30. Seva C, Dickinson CJ, Yamada T. Growth-promoting effects of glycine-extended progastrin. *Science* 1994;**265**:410–2.
31. Smith JP, Stanley WB, Verderame MF, Zagon IS. The functional significance of the cholecystokinin-C (CCK-C) receptor in human pancreatic cancer. *Pancreas* 2004;**29**:271–7.

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32. Song I, Brown DR, Wiltshire RN, Gantz I, Trent JM, Yamada T. The human gastrin/cholecystokinin-B receptor gene: alternative splice donor site in exon 4 generates two variant mRNAs. *Proc Natl Acad Sci USA* 1993;**90**:9085–9.
33. van Solinge WW, Nielsen FC, Friis-Hansen L, Falkner UG, Rehfeld JF. Expression but incomplete maturation of pro-gastrin in colorectal carcinomas. *Gastroenterology* 1993;**104**:1099–107.
34. Wank SA. Cholecystokinin receptors. *Am J Physiol* 1995;**269**:G628–46.
35. Wiborg O, Berglund L, Boel E, Norris F, Rehfeld JF, Marcker KA, et al. Structure of a human gastrin gene. *Proc Natl Acad Sci USA* 1984;**81**:1067–9.

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Chapter 72

Leptin/Adiponectin

Hong Tu

ABSTRACT

Obesity is associated with an increased risk of many types of cancers. Leptin and adiponectin, both mainly produced by adipose tissue, were originally described as circulating hormones involved in food intake and energy homeostasis. Growing evidence has shown that leptin and adiponectin not only have a relevance in obesity and insulin resistance, but they may be also involved in tumorigenesis, especially in the development of obesity-associated cancers such as breast, colorectal, and prostate cancers. *In vitro* studies have shown that leptin promotes human cancer cell proliferation, whereas adiponectin exhibits antiproliferative effects. This chapter summarizes our recent understanding of leptin and adiponectin as two potential molecular targets for cancer prediction, prevention, and therapy.

INTRODUCTION

Recently, adipose tissue has been considered as a major endocrine organ that secretes a wide range of biologically active molecules including leptin, adiponectin, vascular endothelial growth factor (VEGF), tumor necrosis factor-alpha (TNF-α), and interleukin-6 (IL-6). These factors are collectively termed 'adipocytokines,' and are thought to be key mediators in the obesity-related disorders.

Epidemiological results have shown that obesity is closely associated with increased risk of some types of cancers.[23] Moreover, an excess of adipose tissue favors metastasis and recurrence of cancer and is associated with a higher mortality. Adipokines, particularly leptin and adiponectin, have been postulated to be a potential link between obesity and cancer. Recent studies have revealed that the functions of leptin and adiponectin extend far beyond metabolism. Most of the *in vitro* studies indicate that leptin promotes cell proliferation, whereas adiponectin exhibits antiproliferative actions. Levels of leptin and adiponectin in the circulation and in tumor microenvironments are frequently altered in cancer patients. In addition, animal studies also suggest a possible role of leptin and adiponectin in cancer progression.

LEPTIN AND CANCER

Leptin, a 16-kDa protein encoded by the ob gene, is predominantly produced by adipocytes. Leptin was originally described as a circulating hormone involved in feeding behavior and energy homeostasis through its actions on the hypothalamus where leptin receptor (ObR) is highly expressed. Later, ObRs were found to have a wide distribution in different tissues, suggesting that leptin may have numerous peripheral effects. Indeed, leptin has since been found to act as a mitogen, survival factor, metabolic regulator, or angiogenic factor depending on the target tissue.

CLINICAL EVIDENCE OF LEPTIN RELATED TO CANCER[10]

In humans, obesity is not related to leptin deficiency but to the development of leptin resistance. Thus, leptin concentrations are usually elevated in obese individuals. Leptin is generally present in human serum in the range of 5–50 ng/ml. However, in obese individuals, concentrations >100 ng/ml are common. Whether elevated concentrations of leptin are associated with an increased risk of cancer is not conclusive. The inconsistent data obtained on circulating leptin in cancer patients might result from the different analytic method used, or different sample preparation where some influencing factors, such as food intake and circadian rhythm, were not properly controlled. It is also possible that the carcinogenic effect of leptin is induced by an overabundance of local rather than systemic leptin.

Leptin and Breast Cancer

Expression of leptin and its receptors in breast cancer cells has been addressed by the use of immunohistochemistry or RT-PCR. Studies have documented the overexpression of leptin and ObR in breast cancer tissues versus noncancer breast epithelium. In addition, there is an increased incidence of hematogenous metastasis or cancer recurrence in

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00072-5
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distant organs in patients with increased ObRb expression in primary breast tumors.[12]

The association between circulating leptin levels and breast cancer or breast cancer risk has not been clarified.[28] Some authors have observed that serum leptin is associated with breast cancer regardless of the menopausal status, whereas others describe a negative correlation between leptin and breast cancer in the premenopausal but not in the postmenopausal group. Other reports claim that circulating leptin is not associated with breast cancer risk in premenopausal or postmenopausal women. Clearly, better controlled studies are needed to unequivocally establish whether serum leptin is associated with breast cancer etiology.

Leptin and Colorectal Cancer

Increased plasma leptin concentrations are associated with a 2- to 3-fold increased risk of colorectal cancer or adenoma in men, but not in women, in northern Europe and America.[5] These associations did not result from differences in body mass index (BMI) because adjustment for BMI only caused a modest attenuation in risk. More recently, high circulating levels of soluble leptin receptor were detected in patients with advanced stage of colon cancer, further suggesting a role for leptin in cancer progression and aggressiveness.[29] On the other hand, other authors note that leptin levels are lower in colon cancer patients.

Leptin and Prostate Cancer

Among four prospective and three case-control published studies, two report a significant positive association of leptin with prostate cancer. Interestingly, those two studies report higher leptin concentrations associated with more advanced tumors, characterized by larger size and higher grade. However, more studies, including a 25-year prospective cohort study,[16] conclude that circulating leptin is unrelated to either prostate cancer risk or mortality.

Leptin and Hepatocellular Carcinoma (HCC)

Several studies have attempted to elucidate the possible effects of leptin in HCC development.[32] In a report that evaluated the expression of leptin and its receptor in HCC and adjacent nontumor tissues, leptin was involved in the carcinogenesis of HCC. High leptin expression was also found to be associated with an increased intratumor microvessel density. However, several studies have reported contradictory effects of leptin in HCC growth. One report showed the expression of ObR in HCC specimens inversely correlates with vascular invasion of HCC. Furthermore, high leptin expression was associated with better survival of HCC patients who treated postoperatively with medroxyprogesterone acetate.[31]

IN VITRO RESULTS OF LEPTIN AS A MITOGENIC FACTOR

In vitro, leptin can stimulate the proliferation and counteract apoptosis in many types of cancer cell lines. It can also promote the invasiveness of human cancer cells in collagen gel. The proliferative activity of leptin is mediated through different signaling pathways;[13] (1) Leptin induces the phosphoinositide 3-kinase (PI3 K)/Akt survival pathway by activating the phosphorylation of Akt Thr308 or Akt Ser473 and by stimulating the protein expression of protein kinase C-alpha (PKCα), which is controlled by PI3 K. (2) Leptin activates the mitogen-activated protein kinase (MAPK) pathway by inducing extracellular signal-regulated kinase 1/2 (ERK1/2) phosphorylation. (3) Leptin directly stimulates the signal transducer and activator of transcription 3 (STAT3) pathway through the crossphosphorylation and activation of Janus-family tyrosine kinase (JAK). STAT3 protein is an important mediator for leptin to upregulate the expression of the c-myc gene. (4) Leptin accelerates cell cycle progression by upregulating the expression of cdk2 and cyclin D1 genes.

The procarcinogenic effect of leptin results not only from an enhanced activity of proliferation process but also from a downregulation of apoptosis.[13] Leptin can upregulate the expression of antiapoptotic genes Bcl2 and survivin and downregulate the expression of apoptosis inducer genes p53 and BAX. Leptin has been shown to suppress docetaxel-induced apoptosis by inhibiting caspase-9 activity.[14] Interestingly, although leptin alone shows proliferative effects on the breast cancer cell line MDA-MB-231, leptin cotreatment with cAMP elevating agents shows a strong apoptotic action. Thus, at least in some cases, leptin can potentiate the action of antiproliferative compounds and may exert opposite actions related to the apoptosis process.

Leptin can promote tumor angiogenesis by itself or with vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF).[6,26] Leptin enhances vascular endothelial cell proliferation *in vitro* with a potency similar to that of VEGF. In addition, leptin increases the expression of VEGF and its receptor VEGFR-2 in mouse mammary cancer cells. Blockade of leptin receptor signaling slows down breast cancer growth and results in reduced expression of VEGF, VEGFR-2, and cyclin D1 *in vivo*. Inhibition of JAK, PI3 K, and MEK-1/MAPK signaling prevents the leptin-induced VEGF secretion and VEGFR-2 expression. The role of leptin in neovascularization is supported additionally by the observation that it can increase the levels and activity of enzymes involved in angiogenesis, for example, matrix metalloproteinases (MMPs) 2 and 9.

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SYSTEMIC REGULATION OF LEPTIN ON CANCER

Several reports indicate that ObR are highly abundant in many tumor tissues compared with nontumor tissues. ObR-overexpressed tumors include breast cancer, pancreatic cancer, gastrointestinal tumors (such as esophageal, gastric, and colon cancer).[22] Meanwhile, leptin has also been detected in many types of cancer tissues. In breast cancer, the expression level of leptin is be positively correlated with ObR, and both molecules are most abundant in less differentiated G3 tumors. Importantly, distant metastases are more frequently seen in ObR positive cancers with strong immunoreactivity for leptin.[12] These results suggest the regulation of leptin on cancer may act in three ways:[13] in an endocrine manner (via external adipose depots), in a paracrine pathway (via local adipose tissue and non adipose sources including stromal cells and inflammatory cells) and in an autocrine action (via tumor cell itself). Leptin produced by adipose tissue or local sources binds to ObRb expressing cells within the tumor microenvironment and activates the downstream kinases, such as PI3 K, ERK1/2, and Jak2/STAT3. These pathways contribute to various steps of tumor progression, from cancer stem cell survival and proliferation to metastatic tumor growth.[22]

IN VIVO RESULTS OF LEPTIN AND CANCER

Obese mice with congenital leptin deficiency resulting from a mutation in leptin gene ob (ob/ob mice) and obese mice with leptin resistance resulting from a spontaneous mutation on the leptin receptor gene db (db/db mice) are useful models to assess the potential of leptin on cancer development and metastasis. Most of the existing reports support the positive association between leptin and cancer *in vivo*. Obese mice have both an increase incidence of spontaneous tumors and burden of transplanted tumors. MMTV-Wnt-1 mammary tumors transplanted into db/db mice grow to eight times the volume of tumors transplanted into lean wild-type mice. However, such tumors are reduced in leptin-deficient ob/ob mice.[35] Along the same line of evidence, genetically obese MMTV-TGF-alpha/Lep −/− female mice fail to develop oncogene-induced breast tumor, whereas mice Lep +/+ and Lep +/− developed tumors in 50% and 67% of cases, respectively.[7] In addition, homozygous db/db mice promote the cell growth of gastric and colon carcinomas and enhance the metastasis of B16-BL6 melanoma and Lewis lung carcinoma with elevated serum concentrations of leptin.

On the other hand, there are also several lines of evidence suggesting that leptin deficiency is unlikely to have contributed significantly to the enhancement of carcinogenesis. In carcinogen-induced skin papilloma and p53 deficiency animal models, ob/ob mice developed malignancies similarly to their lean littermates. The volume of transplanted murine prostate cancer in ob/ob mice are significantly larger, whereas in db/db mice, they are significantly smaller compared with controls. In the same study, an inverse correlation is observed between leptin plasma concentrations and tumor weight and tumor proliferative markers.[24] The inverse relationship between leptin and cancer is also supported by the observation that experimental pulmonary metastasis of melanoma B16-BL6 is markedly promoted in leptin-deficient ob/ob mice.[18]

Results from mice with normal weight concerning the action of leptin on cell growth are contradictory. Leptin injection stimulates,[17] or has no effect,[2] or even inhibits the growth of cancer xenografts.[1] In rats, the promoting effect of leptin on cell proliferation was shown on the right, but not the left, colonic mucosa.

Taken together, leptin exerts a more complex action on the tumor cells than first suspected. It has not been conclusive *in vivo* whether leptin is a hazard or protective factor for cancer. The controversial results could be related to the different tumor models. Meanwhile, it is worth noting that ob/ob or db/db mice diverge not only in leptin levels but also in other circulating factors, such as glucose, insulin, and other hormones or cytokines. All these factors collectively decide the fate of cancer cells *in vivo*. It will be important in future experiments to adjust all the confounders that are also altered in obesity and evaluate the independent role of leptin in cancer.

ADIPONECTIN AND CANCER

Adiponectin is a 244-amino acid hormone expressed mainly in adipoctyes. Adiponectin contains four differentiable domains: an amino-terminal signal sequence, a variable region, a collagenous domain, and a carboxy-terminal globular domain. The monomeric (30 kDa) form of adiponectin is thought to appear only in the adipocyte, because it has not yet been detected in the circulation. The most abundant configuration of adiponectin is the trimer, formed by a strong association between three monomers at their globular domains.

To date, three putative adiponectin receptors have been identified: adiponectin receptor R1 (AdipoR1), adiponectin receptor R2 (AdipoR2), and more recently, T-cadherin. AdipoR1 and AdipoR2 are ubiquitously expressed. The wide distribution of adiponectin in peripheral organs suggests that adiponectin exerts pleiotropic effects in the human other than regulating the energy metabolism. Indeed, *in vitro* assays show that a number of different cancer cell lines express one or both of the adiponectin receptors and undergo reduced growth and/or increased apoptosis in response to the treatment of adiponectin.

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CLINICAL EVIDENCE OF ADIPONECTIN RELATED TO CANCER

Adiponectin is abundant in human plasma, with the concentration ranging from 2 to 10 μg/ml and accounting for up to 0.05% of total plasma protein. In contrast to the other adipokines, plasma adiponectin concentrations are lower in obese than in lean subjects. A negative correlation between circulating adiponectin and obesity, insulin resistance, and type 2 diabetes has been well established. Accordingly, adiponectin levels are inversely associated with the risk of malignancies associated with obesity and insulin resistance in most cases. Low plasma adiponectin concentrations may be a novel risk factor for cancer.

It has been postulated that the different adiponectin conformations exert diverse effects. Thus, it is important to characterize the forms of adiponectin, that is, trimers, hexamers, or high-molecular-weight oligomers, when detecting the concentration of adiponectin in the circulation. However, the conventional analytic techniques such as ELISA or radioimmunoassay are not able to characterize the forms of adiponectin. These technical handicaps may partially explain the contradictory results concerning the relationship between adiponectin and cancer.

Adiponectin and Breast Cancer[4]

Since the female sex and hormone levels are well-recognized risk factors for breast cancer, the relationship between adiponectin levels and breast cancer risk has been investigated extensively. Although some studies found a robust inverse association between adiponectin and breast cancer risk among both premenopausal and postmenopausal women, other studies, including one prospective case-control study involving 1477 breast cancer cases and 2196 controls,[30] reported that low adiponectin levels are associated with breast cancer only in postmenopausal women. The effect of female hormones on adiponectin might help resolve this divergence because serum adiponectin concentration has a significant negative correlation with serum estradiol concentration in postmenopausal but not in premenopausal and perimenopausal women.

The relationships of adiponectin levels with breast cancer stage and prognosis have also been explored. Low serum adiponectin concentrations usually associate with larger tumor size, higher histological grade, and higher frequency of lymph node metastasis. However, some studies disproved the negative correlation of circulation and tissue adiponectin levels with tumor stage, size, and lymph node metastasis. The association between low levels of adiponectin and increased breast cancer survivor was evaluated in a multiethnic, prospective cohort study containing 527 women diagnosed with stage I–IIIA breast cancer.[9] After adjustment for possible confounders, the hazard risk of higher levels of adiponectin to longer breast cancer survival was 0.39.

Adiponectin and Colorectal Cancer

A meta-analysis has been performed to evaluate the associations between circulating adiponectin levels and colorectal cancer or adenoma.[34] After gathering data from 13 case-control studies including 3015 cases of colorectal cancer and adenoma, the authors conclude that patients with colorectal cancer and adenoma have markedly lower adiponectin values than controls, although there is obvious heterogeneity across the studies. In men, there is a 2% decreased risk of colorectal neoplasm for a 1-mg/mL increment in adiponectin levels, whereas among women, there is no evidence of such a trend.

Adiponectin and Prostate Cancer

Based on a 25-year prospective cohort, adiponectin concentrations are reversely associated with the risk of developing high grade or lethal cancer. In addition, higher adiponectin concentrations predict lower mortality independent of BMI, leptin, clinical stage, and tumor grade.[16] These observations are in line with previous clinical results showing prostate cancer patients with a Gleason score ≥8 or extraprostatic cancer had lower adiponectin concentrations.

Adiponectin and Pancreatic Cancer

In a prospective cohort study with 452 pancreatic cancer cases and 452 matched controls, higher circulating concentrations of adiponectin are associated with a reduction in pancreatic cancer risk among never smokers (OR = 0.44).[11] Adiponectin is also more specific than standard tumor markers in differentiating pancreatic cancer from chronic pancreatitis.

POTENTIAL MECHANISMS OF ADIPONECTIN AS A PROTECTIVE FACTOR OF CANCER

The role of adiponectin in cancer might include several mechanisms, including antiproliferation, apoptosis, antiangiogenesis and vascularization, regulation of inflammatory cytokines, and improvement in insulin resistance.

Adiponectin is a potent regulator of cell proliferation and apoptosis. Adiponectin significantly suppresses the proliferation of many types of cancer cell lines. The antiproliferative effect is mediated partly via AdipoRs. The binding of adiponectin to its receptor triggers various signaling pathways by which adiponectin accomplishes its antiproliferative and apoptotic activities.[13] Treatment of the cancer cells with adiponectin can cause: (1) downregulation of MAPK 1

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and 3 expression; (2) stimulation of AMPK activity by phosphorylation at Thr172; (3) a decrease in Akt phosphorylation at Thr308 leading to a reduction of mTOR activity; (4) suppression of the intracellular accumulation of β-catenin and its nuclear activities, which consequently reduces cyclin D1 and c-myc expression. Activation of AMPK has been considered as one of the critical signaling molecules in adiponectin-mediated metabolic signaling. The downstream events include the enhanced expression of Bax and p53, reduced expression of cyclin D1, blockage of c-Jun N-terminal kinase (JNK) signaling, and increased poly(ADP-ribose) polymerase (PARP) cleavage.

Some studies indicate that adiponectin is a negative regulator of angiogenesis.[4] Adiponectin potently inhibits endothelial cell proliferation and migration and remarkably prevents new blood vessel growth in chick chorioallantoic membrane and mouse corneal angiogenesis models. In a hypervascularized murine T241 fibrosarcoma mouse tumor model, adiponectin significantly inhibits primary tumor growth, which is associated with decreased neovascularization. Intraperitoneal and intravitreal injection of recombinant adiponectin dramatically inhibits the size of choroidal neovascularization in a laser-induced neovascularization model. By contrast, in some studies, adiponectin has shown pro-angiogenic effects. Adiponectin-null transgenic mice have reduced vascularization, decreased rate of tumor growth, and increased frequency of pulmonary metastases.[8]

IN VIVO RESULTS OF ADIPONECTIN AND CANCER

Adiponectin deficiency enhances colorectal carcinogenesis and liver tumor formation *in vivo*. Adiponectin knockout (KO) mice develop larger and histologically more progressive colorectal tumors with greater frequency after azoxymethane-induced treatment. In addition, KO mice show a higher incidence and frequency of liver tumors.[19] In B16F10 melanoma and Lewis Lung Carcinoma mice model, adiponectin deficiency markedly promotes the growth of both tumors. Interestingly, the anticancer effect is not related to cell mitosis, apoptosis, or tumor-associated angiogenesis but results from the reduction of macrophage recruitment to the tumor. The restoration of macrophage infiltration at an early stage of tumorigenesis reverses the increased tumor growth in adiponectin KO mice.[27] In MDA-MB-231 breast cancer xenograft nude female mice, both supplementation of recombinant adiponectin and adenovirus-mediated overexpression of adiponectin substantially reduce tumor growth and tumor weight gain. These treatments substantially decrease metastasis of tumor cells into lung tissue and decrease the development of secondary tumors in the mammary fat pads.[33] These *in vivo* results suggest adiponectin as a promising molecular target for cancer therapy.

CANCER-RELATED POLYMORPHISMS OF ADIPONECTIN AND ADIPOR1 GENES

Polymorphisms of adiponectin (ADIPOQ) and AdipoR1(ADIPOR1) genes might be related to the risk of cancer. This association has been reported in colorectal, breast, lung and prostate cancers. In a case-control study with a total of 1497 individuals, the SNP rs266729 (GG/CG genotype), which tags the 5′ flanking region of ADIPOQ, is associated with decreased colorectal cancer risk.[15] Later, this correlation was confirmed in prostate cancer. Another important SNP involved in the cancer risk is rs1501299 located in intron 2 of ADIPOQ. The low expressing rs1501299 G allele (G/G or G/T) is associated with increased risk for breast, prostate, and non-small-cell lung cancers. Interestingly, rs1501299 GG also correlates with decreased levels of serum adiponectin. To date, a total of eight SNPs, namely, rs266729, rs822395, rs822396, rs1501299, and rs2241766 of the ADIPOQ gene and rs12733285, rs1342387, and rs7539542 of the ADIPOR1 gene, have been suggested as cancer-related SNPs. In contrast to the above findings, one large case-control study from the UK failed to replicate the association between variants of ADIPOQ/ADIPOR1 and colorectal cancer.[3] After screening 82 tagging SNPs spanning 250kb around the ADIPOQ locus in 921 familial cases of colorectal tumor and 929 cancer-free controls, Cazier's group failed to find any SNP associated with colorectal tumor risk. The inconsistent results could be because of the different ethnic subjects involved in the study. More epidemiological studies are needed to elucidate the link between genetics of ADIPOQ/ADIPOR1 and cancer, and to address the potential mechanisms of genetic variations in the prognosis of cancer.

INTERPLAY BETWEEN LEPTIN AND ADIPONECTIN

Leptin and adiponectin may downregulate each other's pathway. *In vivo* treatment of adiponectin decreases leptin and Ob-R mRNA expression, and leptin inhibits the mRNA level of AdipoR1. This suggests that obesity-associated hypoadiponectinemia should increase the leptin sensitivity of cancer cells. In the HCC cell lines HepG2 and Huh7, adiponectin treatment effectively blocks leptin-induced cell proliferation and invasion and reverses the inhibitory effect of leptin on apoptosis. More importantly, adiponectin reduces leptin-induced tumor burden in nude mice.[25] The effect of the adiponectin and leptin cotreatment on breast cancer cells is however more complex. Incubation of both adiponectin and leptin increases the number of apoptotic MCF-7 cells but decreases the number of apoptotic T47D cells.[20] These results suggest a very subtle regulation in the molecular mechanisms by which the different adipokines regulate tumor cell proliferation and apoptosis.

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SUMMARY

As two major factors secreted by adipose tissue, leptin and adiponectin play important roles not only in metabolism but also in the development and progression of various types of cancers. Circulating levels of leptin and adiponectin may be novel tumor markers that can serve as useful screening tools for predicting risk for obesity-related cancers. Moreover, the leptin and adiponectin signaling pathways emerge as attractive therapeutic targets for cancer patients. A peptide-based leptin receptor antagonist has been developed to treat human breast cancer.[21] Regulating the ratio of leptin to adiponectin in circulation might be another effective approach toward cancer prevention and therapy.

ACKNOWLEDGMENTS

The authors received financial support, while compiling this chapter, by grants from the Chinese State Key Laboratory of Oncogenes and Related Genes 91-10-03.

REFERENCES

1. Aparicio T, Guilmeau S, Goiot H, Tsocas A, Laigneau JP, Bado A, et al. Leptin reduces the development of the initial precancerous lesions induced by azoxymethane in the rat colonic mucosa. *Gastroenterology* 2004;**126**:499–510.
2. Aparicio T, Kotelevets L, Tsocas A, Laigneau JP, Sobhani I, Chastre E, et al. Leptin stimulates the proliferation of human colon cancer cells in vitro but does not promote the growth of colon cancer xenografts in nude mice or intestinal tumorigenesis in Apc(Min/+) mice. *Gut* 2005;**54**:1136–45.
3. Carvajal-Carmona LG, Spain S, Kerr D, Houlston R, Cazier JB, Tomlinson I. Common variation at the adiponectin locus is not associated with colorectal cancer risk in the UK. *Hum Mol Genet* 2009;**18**:1889–92.
4. Chen X, Wang Y. Adiponectin and breast cancer. *Med Oncol* 2010.
5. Chia VM, Newcomb PA, Lampe JW, White E, Mandelson MT, McTiernan A, et al. Leptin concentrations, leptin receptor polymorphisms, and colorectal adenoma risk. *Cancer Epidemiol Biomarkers Prev* 2007;**16**:2697–703.
6. Cirillo D, Rachiglio AM, la Montagna R, Giordano A, Normanno N. Leptin signaling in breast cancer: an overview. *J Cell Biochem* 2008; **105**:956–64.
7. Cleary MP, Juneja SC, Phillips FC, Hu X, Grande JP, Maihle NJ. Leptin receptor-deficient MMTV-TGF-alpha/Lepr(db)Lepr(db) female mice do not develop oncogene-induced mammary tumors. *Exp Biol Med (Maywood)* 2004;**229**:182–93.
8. Denzel MS, Hebbard LW, Shostak G, Shapiro L, Cardiff RD, Ranscht B. Adiponectin deficiency limits tumor vascularization in the MMTV-PyV-mT mouse model of mammary cancer. *Clin Cancer Res* 2009;**15**:3256–64.
9. Duggan C, Irwin ML, Xiao L, Henderson KD, Smith AW, Baumgartner RN, et al. Associations of insulin resistance and adiponectin with mortality in women with breast cancer. *J Clin Oncol* 2011;**29**:32–9.
10. Garofalo C, Surmacz E. Leptin and cancer. *J Cell Physiol* 2006;**207**: 12–22.
11. Grote VA, Rohrmann S, Dossus L, Nieters A, Halkjaer J, Tjonneland A, et al. The association of circulating adiponectin levels with pancreatic cancer risk: a study within the prospective EPIC cohort. *Int J Cancer*.
12. Ishikawa M, Kitayama J, Nagawa H. Enhanced expression of leptin and leptin receptor (OB-R) in human breast cancer. *Clin Cancer Res* 2004;**10**:4325–31.
13. Jarde T, Perrier S, Vasson MP, Caldefie-Chezet F. Molecular mechanisms of leptin and adiponectin in breast cancer. *Eur J Cancer* 2011;**47**:33–43.
14. Jiang H, Yu J, Guo H, Song H, Chen S. Upregulation of survivin by leptin/STAT3 signaling in MCF-7 cells. *Biochem Biophys Res Commun* 2008;**368**:1–5.
15. Kaklamani VG, Wisinski KB, Sadim M, Gulden C, Do A, Offit K, et al. Variants of the adiponectin (ADIPOQ) and adiponectin receptor 1 (ADIPOR1) genes and colorectal cancer risk. *J Am Med Assoc* 2008;**300**:1523–31.
16. Li H, Stampfer MJ, Mucci L, Rifai N, Qiu W, Kurth T, et al. A 25-year prospective study of plasma adiponectin and leptin concentrations and prostate cancer risk and survival. *Clin Chem*;**56**:34–43.
17. Mauro L, Catalano S, Bossi G, Pellegrino M, Barone I, Morales S, et al. Evidences that leptin up-regulates E-cadherin expression in breast cancer: effects on tumor growth and progression. *Cancer Res* 2007;**67**:3412–21.
18. Mori A, Sakurai H, Choo MK, Obi R, Koizumi K, Yoshida C, et al. Severe pulmonary metastasis in obese and diabetic mice. *Int J Cancer* 2006;**119**:2760–7.
19. Nishihara T, Baba M, Matsuda M, Inoue M, Nishizawa Y, Fukuhara A, et al. Adiponectin deficiency enhances colorectal carcinogenesis and liver tumor formation induced by azoxymethane in mice. *World J Gastroenterol* 2008;**14**:6473–80.
20. Nkhata KJ, Ray A, Schuster TF, Grossmann ME, Cleary MP. Effects of adiponectin and leptin co-treatment on human breast cancer cell growth. *Oncol Rep* 2009;**21**:1611–9.
21. Otvos Jr L, Haspinger E, Larussa F, Maspero F, Graziano P, Kovalszky I, et al. Design and development of a peptide-based adiponectin receptor agonist for cancer treatment. *BMC Biotechnol* 2011;**11**: 90–5.
22. Park J, Scherer PE. Leptin and cancer: from cancer stem cells to metastasis. *Endocr Relat Cancer* 2011;**18**:C25–9.
23. Renehan AG, Soerjomataram I, Leitzmann MF. Interpreting the epidemiological evidence linking obesity and cancer: a framework for population-attributable risk estimations in Europe. *Eur J Cancer* 2010;**46**:2581–92.
24. Ribeiro AM, Andrade S, Pinho F, Monteiro JD, Costa M, Lopes C, et al. Prostate cancer cell proliferation and angiogenesis in different obese mice models. *Int J Exp Pathol* 2010;**91**:374–86.
25. Sharma D, Wang J, Fu PP, Sharma S, Nagalingam A, Mells J, et al. Adiponectin antagonizes the oncogenic actions of leptin in hepatocellular carcinogenesis. *Hepatology*;**52**:1713–22.
26. Somasundar P, McFadden DW, Hileman SM, Vona-Davis L. Leptin is a growth factor in cancer. *J Surg Res* 2004;**116**:337–49.
27. Sun Y, Lodish HF. Adiponectin deficiency promotes tumor growth in mice by reducing macrophage infiltration. *PLoS One* 2010;**5**: e11987–94.
28. Surmacz E. Obesity hormone leptin: a new target in breast cancer? *Breast Cancer Res* 2007;**9**:301.

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29. Tutino V, Notarnicola M, Guerra V, Lorusso D, Caruso MG. Increased soluble leptin receptor levels are associated with advanced tumor stage in colorectal cancer patients. *Anticancer Res* 2011;**31**:3381–3.
30. Tworoger SS, Eliassen AH, Kelesidis T, Colditz GA, Willett WC, Mantzoros CS, et al. Plasma adiponectin concentrations and risk of incident breast cancer. *J Clin Endocrinol Metab* 2007;**92**:1510–6.
31. Wang SN, Chuang SC, Yeh YT, Yang SF, Chai CY, Chen WT, et al. Potential prognostic value of leptin receptor in hepatocellular carcinoma. *J Clin Pathol* 2006;**59**:1267–71.
32. Wang SN, Lee KT, Ker CG. Leptin in hepatocellular carcinoma. *World J Gastroenterol* 2010;**16**:5801–9.
33. Wang Y, Lam JB, Lam KS, Liu J, Lam MC, Hoo RL, et al. Adiponectin modulates the glycogen synthase kinase-3beta/beta-catenin signaling pathway and attenuates mammary tumorigenesis of MDA-MB-231 cells in nude mice. *Cancer Res* 2006;**66**:11462–70.
34. Xu XT, Xu Q, Tong JL, Zhu MM, Huang ML, Ran ZH, et al. Meta-analysis: circulating adiponectin levels and risk of colorectal cancer and adenoma. *J Dig Dis* 2011;**12**:234–44.
35. Zheng Q, Dunlap SM, Zhu J, Downs-Kelly E, Rich J, Hursting SD, et al. Leptin deficiency suppresses MMTV-Wnt-1 mammary tumor growth in obese mice and abrogates tumor initiating cell survival. *Endocr Relat Cancer* 2011;**18**:491–503.

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Chapter 73

LHRH Analogs

Andrew V. Schally, Jorg B. Engel, Jacek Pinski and Norman L. Block

ABSTRACT

This chapter reviews the hypothalamic neuropeptide luteinizing hormore–releasing hormone (LHRH) including its discovery, elucidation of structure, and synthesis. Many analogs, both agonistic and antagonistic, have been synthesized. LHRH receptors from man through the animal kingdom are discussed, and their role is detailed. The presence of LHRH receptors on numerous human cell lines and cells of surgically explanted tumors is reviewed. These are detected by immunohistochemistry, Western blots, radioligand binding assays, and RT-PCR for mRNA of the LHRH receptor. The therapeutic implications and applications thereof are discussed. The synthesis, mechanisms, and use of targeted cytotoxic LHRH compounds are described. Phase II clinical trials of cytotoxic LHRH analogs for ovarian and endometrial cancers have been completed and Phase III trials are being formulated. Phase I/II clinical trials for castration-resistant prostate cancer and urothelial cancer are underway with promising early results. Multiple other clinical applications of LHRH analogs are briefly listed and discussed.

INTRODUCTION

Forty years have passed since the isolation, structural elucidation, and synthesis of porcine hypothalamic luteinizing hormone-releasing hormone-I (LHRH-I) was first accomplished in the laboratory of one of us (AVS).[21,22,28,29] Figure 1 shows the molecular structure of LHRH-1. The structure of hypothalamic LHRH-I in all mammalian species, including human, is identical. LHRH-I subsequently was also identified in primitive bony fish, lungfish, and amphibians.[23,28] At least 23 molecular isoforms of LHRH, each differing structurally, have been identified in birds, reptiles, amphibians, fishes, and other vertebrates, protochordata, mollusks, and cnidaria, such as coral.[23,28] Besides the hypothalamic site, LHRH-I is also found in extrahypothalamic regions of the central nervous system, and in nonneuronal tissues, such as placenta, ovary, mammary gland, and lymphoid cells, and in many cancers.

Another isoform of LHRH decapeptide, LHRH-II, differing by three amino acid residues (His^5, Trp^6, Tyr^8), called chicken LHRH, as it was first isolated from chicken, has also been reported in various animals, mammalian brain, peripheral organs, and some tumors such as breast carcinomas. LHRH-II seems to influence reproductive behavior and may have other functions.[10,23,28]

Most vertebrate species possess genes for both LHRH-I and LHRH-II. In humans, the gene for LHRH-I is located on chromosome 8p11.2-p21. LHRH-I is a central regulator in the hypothalamic–pituitary–gonadal axis and plays a vital role in vertebrate reproduction.[10,28] LHRH is also known as gonadotropin-releasing hormone (GnRH), because it controls, by pulsatile secretion, the release of luteinizing hormone (LH) and of follicle-stimulating hormone (FSH) from the anterior pituitary gland. In turn, FSH and LH are responsible for gonadal steroidogenesis and gametogenesis.[10,28] The abbreviation GnRH is too similar to GH-RH (growth hormone-releasing hormone) for which many agonistic and antagonistic analogs already exist and the use of the abbreviation LHRH is therefore recommended by us.[10,28] LHRH-I may also act as a chemokine, a growth factor, and differentiation-inducing factor, influencing cancer cells in reproductive tissues.[35]

RECEPTORS FOR LHRH-I AND LHRH-II

The actions of LHRH are mediated by high-affinity receptors for LHRH found on the plasma membrane of pituitary gonadotrophs.[10,23,28,35] The LHRH-I receptor is a member of the G protein-coupled receptor (GPCR) superfamily. The receptor consists of seven α-helical transmembrane domains and an extracellular amino-terminal domain.[23,35] The intracellular carboxy terminal domain typical of the GPCR family is absent. The LHRH type I receptor is predominantly coupled to the $G\alpha_{q/11}$-protein, through which it regulates the secretion of FSH and LH. In humans the LHRH-I receptor has 328 amino acids and its gene is located on the chromosome 4q21.2.[23,35] In rats and mice, LHRH-I receptors have 327 amino acids, and their genes are located on chromosomes 14p21 and 5e1, respectively.

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00073-7
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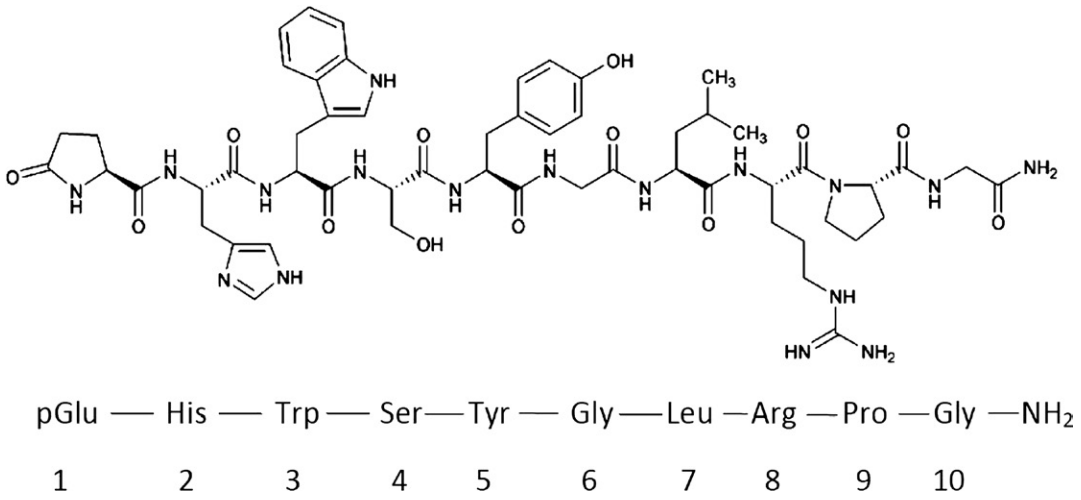

FIGURE 1 The molecular structure of LHRH (GnRH).

Specific LHRH-I receptors are also found on breast, prostatic, ovarian, and endometrial cancers as well as in malignant tumors of urogenital origin such as bladder cancer and tumors of nonreproductive tissues such as colon, pancreas, and brain. These LHRH-I receptors on tumor cells can mediate direct effects of LHRH agonists and antagonists.[10,28]

In man and other mammals, the receptor for LHRH-II (the type II LHRH receptor) is nonfunctional because it is disrupted by frame-shift mutations or premature stop codons and the signaling of LHRH-II thus occurs through type 1 receptor. Thus, in man, there is one functional receptor.[23,35]

SIGNALING MECHANISMS FROM LHRH RECEPTORS

The binding of LHRH-I to its pituitary receptors causes a microaggregation of receptors and complex formation. The complex formed is then internalized and degraded. The LHRH receptors are coupled to guanosine triphosphate GTP-binding proteins ("G proteins"). In the pituitary, these $G\alpha_{q/11}$ proteins activate phospholipase C, which leads to the production of inositol phosphates and diacylglycerol. This process induces Ca^{++} mobilization and activation of protein kinase C, resulting in the release of LH and FSH.[3,14,20,23,35]

With respect to tumoral LHRH receptors, different pathways of signal transduction have been described. In some tumors, after binding to its ligand, the LHRH receptor couples to G protein αi and activates a phosphotyrosine phosphatase, which then dephosphorylates epidermal growth factor (EGF) receptors. As a result, mitogenic signaling induced by binding of EGF to its receptor is abolished.[3,23,25,35]

Recent experimental evidence, however, suggests that LHRH-receptor levels and cell context affect tumor cell responses to LHRH agonists.[20,23,25,35] Thus, after transfection of various human tumor cell lines with LHRH receptors, marked differences in receptor levels, magnitude of inositol phosphate generation, dynamics of inositol phosphate turnover, and activation of ERK1/2 is evident in the different cells. Activation of receptors by LHRH agonists induces apoptosis only if receptor expression is high or moderate. Thus, differences in levels of LHRH receptor and signaling may differentially affect the apoptotic machinery within cell lines and contribute to the cell type-specific effects of LHRH on growth.[23,35]

Furthermore, in HEK 293 cells stably transfected with the LHRH-I-receptor, modification of the LHRH ligand peptide by amino acid substitution modulated its growth inhibiting effect.[20] Thus, different ligands on the LHRH receptor stabilize different receptor conformations mediating differential intracellular signaling and effects on cell growth; this could be the basis for the design of LHRH analogs specifically targeting tumoral LHRH receptors.

DEVELOPMENT OF ANALOGS OF LHRH

During the past 40 years, >3000 analogs of LHRH-I have been synthesized with a view to their potential medical applications.[10,28–31] The substitution of one or more amino acids in the sequence of LHRH-I can result in analogs with increased LH-releasing activity or with antagonistic action. The studies on the relationship between the structure and biologic activity show that histidine in position 2 and tryptophan in position 3 play a functional role in the biologic activity of LHRH. Positions 2 and 3 are also the preferred ones for substitutions generating inhibitory activity.[28] Amino acids in positions 1 and 4–10 exert conformational effects and are essential for binding to the receptors. Substitution of a D-amino acid for Gly[6] enhances the β-II′ type turn conformation by which the peptide is bound to the LHRH-I receptor and increases the activity. We have

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produced three categories of LHRH analogs: 1. agonistic analogs, 2. antagonistic analogs, 3. cytotoxic analogs.[28–31]

AGONISTIC ANALOGS OF LHRH-I

D-Amino acid substitution in positions 6 can lead to superactive peptides. Several LHRH analogs substituted in positions 6, 10, or both, are 50–100 times more active than LHRH and also possess prolonged activity. Of these, the most important analogs are [D-Leu^6,Pro^9-NHET]LHRH (Leuprolide, Lupron) (Takeda-Abbot, TAP), [D-Trp^6] LHRH (Decapeptyl, Triptorelin) (Ipsen, Ferring, Aventis), [D-Ser(Bu^t)^6,Pro^9-NHET]LHRH SUPREFACT (Buserelin) (formerly Hoechst Marion-Roussel, now Sanofi-Aventis), [D-Ser(Bu^t)^6,Aza-Gly^{10}]LHRH (Zoladex, goserelin) (formerly ICI, now Astra-Zeneca).[10,28,29]

The greater biologic activity of these analogs is due to both increased resistance to enzymatic degradation and an enhancement in receptor affinity. The substitution of Gly^6 by D-amino acids renders the analog more resistant to degradation by endopeptidases, which cleave LHRH at this position, and which are widely distributed in mammalian tissues.[28]

For tumor therapy, long-acting depot formulations of LHRH analogs dispersed in microcapsules of biodegradable polymers or in implants are used. Leuprolide depot and Decapeptyl LP are available in the form of slow-release formulations containing the analogs in microcapsules of poly(D,L-lactide-co-glycolide) and are administered intramuscularly at monthly intervals. Depot formulations of Leuprolide acetate and Triptorelin (Trelstar LA) are also available in forms that release the drugs over several months. Implants containing goserelin are designed for subcutaneous injection and produce continuous release over a 4- to 12-week period.[28,29]

MECHANISM OF BIOLOGICAL ACTION OF LHRH AND ITS AGONISTS

An acute injection of LHRH or a superactive agonist induces a marked and sustained release of LH and FSH, whereas a chronic administration produces inhibitory effects. Thus, continuous stimulation of the pituitary by repeated injections of superactive agonists of LHRH or monthly administration of depot preparations induces an inhibition of the hypophyseal–gonadal axis through the process of "downregulation" of pituitary receptors for LHRH, desensitization of the pituitary gonadotrophs, and creation of a state of reversible medical castration with suppression of circulating levels of LH, FSH, and the sex steroids. For the therapy of sex hormone-dependent malignant neoplasms, such as prostate and breast cancer, the inhibitory process produced by depot preparations of LHRH agonists depends on the creation of a state of sex steroid deprivation. LHRH agonists and antagonists also exert direct inhibitory effects on breast, prostate, ovarian, and endometrial cancers mediated through LHRH receptors on the tumor cells.[10,28,29]

APPLICATIONS OF AGONISTS OF LHRH-I

Downregulation of LHRH receptors, produced by repeated administration or by depot preparations of LHRH agonists, provides the basis for clinical applications in gynecology, urology, and oncology of this class of compounds. Management of central precocious puberty, polycystic ovarian syndrome, and *in vitro* fertilization and embryo transfer (IVF-ET) programs (controlled ovarian stimulation-assisted reproductive technology) (COS-ART) are based on the suppression of gonadotropin secretion (selective medical hypophysectomy). Treatment of sex hormone-dependent malignant neoplasms, represented by prostate, breast and endometrial carcinoma, and other diseases or conditions, such as benign prostatic hyperplasia, uterine leiomyomata, and endometriosis, is based on the reversible medical castration created by sex steroid deprivation.[10,28,29]

ANTAGONISTS OF LHRH

Hundreds of LHRH antagonists have been synthesized since 1971. To eliminate the undesirable anaphylactic effects caused by insertion of D-arginine in position 6, analogs with neutral D-uridoalkyl amino acids, such as D-Cit at position 6, have been synthesized in our laboratory. Among these antagonists [Ac-D-Nal(2)^1, D-Phe(4Cl)^2,D-Pal(3)^3,D-Cit^6,D-Ala^{10}]LHRH (Cetrorelix, Aeterna-Zentaris) had the highest inhibitory activity and receptor binding affinity.[10,28,29] Other groups have also synthesized LHRH antagonists with diminished anaphylactoid activity. Among antagonists that were developed are antide [N-Ac-D-Nal(2)^1, D-Phe(4Cl)^2,D-Pal(3)^3,Lys(Nic)^5,D-Lys(Nic)6,Lys(iPr)^8,D-Ala^{10}]LHRH (Ares-Serono) and Nal-Glu antagonist [Ac-D-Nal(2)^1,D-Phe(4Cl)^2,D-Pal(3)^3,Arg^5,D-Glu^6(AA),D-Ala^{10}] LHRH (NIH), Azaline-B [Ac-D-Na]^1,D-Phe(4cl)^2,D-Pal^3,Aph^5(Atz),Aph^5(Atz),Ilys^8,D-Ala^{10}0]LHRH, Ganirelix [N-Ac-D-Nal(2)^1, D-*p*-Cl-Phe^2,D-Pal(3)^3, D-hArg (Et2)^6,L-hArg(Et2)^8,D-Ala^{10}]LHRH (Organon), Abarelix (PPI-149) [N-Ac-D-Nal (^{2})-D-(p-Cl)-Phe-D-Pal(3)-Ser-Nm-Tyr-Asn-Leu-ILys-Pro-Gly-NH2] (Precis),[10,28,29] and Degarelix [N-acetyl-3-(naphtalen-2-yl)-D-alanyl-4-chloro-D-phenylalanyl-3-(pyridin-3-yl)-D-alanyl-L-seryl-4-((((4S)-2,6-dioxohexahydropyrimidin-4-yl)carbonyl) amino)-L-phenylalanyl-4-(carbamoylamino)-D-phenylalanyl-L-leucyl-N6-(1-methylethyl)-L-lysyl-L-prolyl-D-alaninamide], (Ferring Pharmaceuticals).[34]

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Antagonists of LHRH exhibit no intrinsic biologic stimulatory activity but compete with LHRH for the same receptor sites. LHRH antagonists thus produce a competitive blockade of LHRH receptors, preventing stimulation by endogenous LHRH, and causing an immediate inhibition of the release of gonadotropins and sex steroids. LHRH antagonists can greatly reduce the onset time of therapeutic effects as compared with agonists. LHRH antagonists such as Cetrorelix already have major uses in gynecology, especially in COS-ART and seem to have various advantages over the agonists.[10,28,29] The use of antagonists can prevent the undesirable clinical "flare" of disease caused by transient initial LH and sex steroid release that can occur during agonist administration in cancer patients. Degarelix is being developed by Ferring Pharmaceuticals for prostate cancer therapy.[34] Although the principal mechanism of action of LHRH antagonists is based on a competitive occupancy of LHRH receptors, administration of LHRH antagonist Cetrorelix to rats can also produce downregulation of pituitary LHRH receptors and a decrease in the levels of mRNA for LHRH receptors. An orally active nonpeptide antagonist of LHRH receptor, Elagolix, has been reported.

TARGETED CYTOTOXIC ANALOGS OF LHRH

An additional new class of antitumor compounds consisting of LHRH agonists conjugated to chemotherapeutic agents has been recently developed for targeted chemotherapy.[28–31] The hypothesis of "magic bullet"-like compounds, which could specifically eradicate cancers, was conceived by Paul Ehrlich, but remained unexplored for nearly a century. Based on the presence of LHRH receptors in breast, endometrial, ovarian, and prostatic cancers, our laboratory, >20 years ago, started the synthesis and evaluation of targeted antitumor compounds made by linking LHRH analogs to cytotoxic compounds.[30,31]

After 15 years of exploring various antineoplastic radicals and chemical linkages, we decided to conjugate the agonist [D-Lys^6]LHRH to doxorubicin (DOX), one of the most widely used anticancer agent. The conjugation was made through the ε-amino group of the D-Lys moiety and a glutaric acid spacer to the 14-OH group of doxorubicin, to form the cytotoxic LHRH analog, AN-152 (AEZS-108). Subsequently, we synthesized another cytotoxic analog (AN-207) by linking 2-pyrrolino-DOX (AN-201), a daunosamine-modified derivative of DOX, to the same [D-Lys^6] LHRH carrier. We have demonstrated that these cytotoxic LHRH analogs, AN-152 (AEZS-108) and AN-207, powerfully inhibit the growth of multiple experimental tumors. The same cytotoxic radicals were then also conjugated to analogs of somatostatin and bombesin/gastrin-releasing peptide (GRP) to form cytotoxic peptides that could be targeted to their corresponding receptors on tumors.[7,30,31]

MECHANISM OF ACTION OF CYTOTOXIC LHRH ANALOGS

After binding to receptors for LHRH, cytotoxic LHRH analogs are internalized by rat pituitary cells and by human ovarian, endometrial, or breast cancer cells. The entry of AN-152 (AEZS-108) into the cells is followed by its appearance in the cytoplasm. The labeled carrier peptide is localized mainly in the cytosols of tumor cells and not in the nucleus. However, the fluorescent label linked to AN-152 (AEZS-108) and coupled to the daunosamine nitrogen of DOX was found only in the nuclei.[30,31] The presence of LHRH receptors on cells is essential for this internalization process, because no binding and internalization of AN-152 (AEZS-108) occurs in cells, such as UCI-107 ovarian cancer, which do not express LHRH receptors.

RECEPTORS FOR LHRH ON HUMAN TUMORS

Receptors for LHRH are expressed on the cells of many human cancer specimens as well as human cancer cell lines.[3,5,28,31] One method of their detection is immunohistochemistry (IHC), which has been used mainly for the demonstration of LHRH receptors in specimens of lymphoma, renal cell carcinoma, melanoma, bladder cancer, prostate cancer, breast and ovarian and endometrial cancers. Other methods of detection include western blots, radioligand binding assays, and RT-PCR for mRNA of LHRH receptor. Inhibition *in vivo* of the growth of human cancers xenografted into nude mice in response to targeted cytotoxic LHRH analogs AN-152 (AEZS-108) and AN-207 may also be considered proof of receptor presence and suggested therapeutic applications. The blockage of the LHRH receptors with agonist D-Trp^6-LHRH abolishes these inhibitory responses. LHRH receptors are not expressed in UCI-107 and SKOV-3 ovarian cancer cell lines or in MFE 296 endometrial cancer cell line, and so these are not affected by cytotoxic or other analogs of LHRH.

The following human tumor specimens and human cancer lines are positive for LHRH receptors by IHC:

1. Non-Hodgkin's Lymphomas (NHL)—Human specimens and NHL cell lines HT and RL;[16]
2. Renal Cell Carcinoma (RCC)—Human specimens and RCC cell lines A-498, ACHN, 7860;[17]
3. Malignant Cutaneous Melanomas (MM)—Human specimens and MM cell lines MRI-H255, MRI-H187;[18]
4. Pancreatic Cancers—Human specimens and PANC-1 cell line;[28–31]
5. Urinary Bladder Cancer—Human specimens and HT-1376 cell line [Ref. 1, Keller & Schally unpublished];

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6. Prostate Cancer—Human specimens and cell lines: LNCaP, MDA-PCa-2b, C4-2, PC-82, DU-145, and LUCaP-35;[15,29,31]
7. Breast Cancer—Human specimens and cell lines: HCC-1806, HCC-1937, MDA-MB-231, MX-1, MDA-MB-435, MCF-7, and T47D;[11,15,31]
8. Ovarian Cancer—Human specimens and cell lines: OV-1063, ES-2, EFO-21, EFO-27, OVCAR-3, and HTOA;[5,14,31]
9. Endometrial Cancer—Human specimens and cell lines: HEC-1A, HEC-1B, RL-95-2, and Ishikawa line;[9,14]
10. Colorectal Cancer—Human specimens and cell lines: HT29, HCT116, HCT-15, LoVo, and COLO-320DM;[32]
11. Benign Prostatic Hyperplasia (BPH)—Human BPH Specimens and Cell line BPH-1.[27]

ANTITUMOR EFFECTS OF AGONISTS AND ANTAGONISTS OF LHRH—EXPERIMENTAL AND CLINICAL FINDINGS

Prostate Cancer

The greatest therapeutic impact of LHRH analogs came about in the field of prostate cancer.[28,29] Carcinoma of the prostate is the most common noncutaneous malignancy in men. About 70% of human prostate cancers are testosterone dependent, and the endocrine therapy of advanced prostate cancer with agonists of LHRH is based on the resultant desired androgen deprivation.[28,29]

The finding that prolonged treatment with agonistic analogs of LHRH causes testicular inhibition and chemical castration prompted us to try this method to induce the regression of prostate tumors in rat models.[26] Treatment of male rats bearing the Dunning R3327H prostate adenocarcinoma with D-Trp^6-LHRH reduces tumor volume and decreases actual tumor weight as compared with untreated controls.[26] Serum LH, FSH, and testosterone levels are significantly reduced. This demonstration led to clinical applications. The first successful palliative treatment of advanced prostatic carcinoma by agonistic analogs of LHRH was shown in a collaborative trial performed with G. Tolis in the late 1970s at the Royal Victoria Hospital in Montreal.[33] This trial showed for the first time that superactive agonistic LHRH analogs are effective therapeutic agents in patients with androgen-sensitive prostatic adenocarcinoma.[33] These findings were confirmed and extended by other clinical trials of LHRH agonists in patients with prostate cancer in Europe and North America. In addition to Decapeptyl, the agonists buserelin, leuprolide, and goserelin were tested.[2]

At present, the use of sustained-release formulations of agonists, which can be administered at intervals of 1–6 months, makes the treatment of prostate cancer even more convenient, practical and effective.[10,28,29] These regimens also ensure better patient compliance. Medical castration produced by chronic administration of LHRH analogs accounts for most of the clinical benefits of this treatment, but LHRH agonists and antagonists can also exert direct effects on prostate tumor cells. Occasional "flare" in the disease with an increase in bone pain during the first weeks of administration of LHRH agonists was reported in approximately 10% of patients, but such "flares" can be prevented by administration of an antiandrogen before and for 2–4 weeks after the first injection with the agonist.[10,28,29] The clinical acceptance of LHRH analogs is excellent. Therapy with agonists of LHRH is presently the preferred method of primary treatment for men with advanced prostate cancer, and recent surveys indicate that LHRH agonists are selected for primary treatment in approximately 70% of cases.[10,28,29] More recently, LHRH agonists with or without antiandrogens have been used before, during, or after various local treatments in patients with localized prostate cancer. Treatment with LHRH agonists is now recommended in patients who have a rising prostate specific antigen (PSA) levels after surgery and/or radiotherapy. Androgen deprivation produced by LHRH agonists however leads to a decrease in libido, impotence, hot flashes, weight gain, muscle loss, osteopenia, and other side effects concomitant with decreased sex steroids.

The use of LHRH antagonists would avoid the temporary "flare" of the disease that can occur when LHRH agonists are given as single agents. We first showed inhibitory effects of the LHRH antagonist Cetrorelix on experimental androgen-dependent Dunning R-3327-H in rats and human PC-82 prostate cancers in nude mice.[28] Subsequent clinical trials showed that a decrease in testosterone, PSA levels, and prostate size, measured by ultrasonography, can be achieved in patients with advanced prostate cancer treated with Cetrorelix. Patients who were paraplegic due to metastatic invasion of the spine show neurologic improvement during therapy with Cetrorelix. Cetrorelix or other antagonists could be indicated for patients with prostate cancer having metastases to the spine, bone marrow, bladder base and other sites in which the LHRH agonists cannot be used as single drugs because of the possibility of "flare".[10,28] Studies performed with a depot formulation of the LHRH antagonist Abarelix show that its administration leads to a faster decline of the testosterone levels than that of leuprolide with or without antiandrogens. A new LHRH antagonist, Degarelix, is presently in clinical trials for prostate cancer.

Cetrorelix may be also useful for the therapy of BPH. Cetrorelix can produce long-term improvement in patients with BPH.[10,28]

All hormonal therapies, including orchidectomy and LHRH agonists or antagonists, however, provide a cancer remission with a limited duration, and most patients relapse

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within two to three years. The relapse may be manifested clinically, radiolographically, and/or biochemically by an increase in serum PSA levels. These patients eventually die of androgen-independent prostatic cancer (CRPC) which is apparently stimulated by various growth factors. The prognosis for patients with hormone-refractory prostate cancer is very poor, and no effective treatment exists at present. Chemotherapy produces poor response rates, although Docetaxel-based therapy confers some improvement in survival.[10,28]

APPLICATIONS OF CYTOTOXIC LHRH ANALOGS

Cytotoxic analogs of LHRH could be useful in the management of relapsed prostate cancer. First, we showed inhibitory effects of the cytotoxic LHRH analog AN-207 in rats with Dunning prostate cancers and in nude mice with human PC-82 tumors.[30,31] We then showed that AN-207 inhibits growth of MDA-PCa-2B prostate cancers xenografted into nude mice and lowers PSA levels. AN-207 also inhibits growth of DU-145 and LuCaP-25 human prostate cancers and is less toxic than its cytotoxic radical used independently.[30,31] Although AN-207 could not be easily synthesized on a large scale, this work supports the concept that targeted chemotherapy based on cytotoxic LHRH analogs should be more efficacious and less toxic than the current systemic chemotherapy. We then started tests with AN-152 (AEZS-108) containing doxorubicin (DOX). In castrated nude mice bearing LNCaP androgen-sensitive prostate cancers treatment with AN-152 (AEZS-108) produces a nearly 70% reduction of tumor volume.[30,31] The cytotoxic analog, AN-152 (AEZS-108), also strongly suppresses growth of androgen-sensitive MDA-PCa-2b prostate cancers and is more effective than equimolar doses of DOX. In nude mice bearing androgen-independent intraosseous C4-2 prostate cancers, treatment with AN-152 (AEZS-108) decreases serum PSA levels, whereas DOX has no effect. These three lines are LHRH receptor positive. AN-152 (AEZS-108) also inhibits the growth of human androgen-independent DU-145 prostate cancers.[30,31] The results suggest that the cytotoxic analog, AN-152 (AEZS-108), should be effective in those patients with prostate cancer who relapse after androgen deprivation therapy. Thus, targeted cytotoxic analogs of LHRH may be useful for the treatment of advanced prostate cancer after it relapses.

CLINICAL TRIALS WITH AN-152 (AEZS-108) IN MEN WITH PROSTATE CANCER

AN-152 (AEZS-108) is the only cytotoxic hybrid that has entered early clinical study. A Phase I and two Phase II studies in women with endometrial and ovarian cancers have been completed.[4,6–8] In prostate cancer, accrual recently began in an accelerated Phase I lead-in to a Phase II trial of AN-152 (AEZS-108) [Pinski et al., unpublished]. This NIH funded trial is enrolling men with CRPC previously treated with a taxane but with no other limit on previous therapy. Eligibility criteria include adequate hematologic and organ function and an Eastern Cooperative Oncology Group performance status of 0 or 1. As LHRH agonists will compete for binding sites with AN-152 (AEZS-108), a previous washout period from LHRH agonists is required. The primary endpoint of this trial is clinical benefit rate and the trial features several unique correlative studies. Tissue from previous biopsies will be assessed for strength of LHRH receptor expression, which will then be correlated to response. Circulating tumor cells (CTCs) will be captured at several prespecified time points by a novel microfiltration assay and the FDA approved CellSearch assay for comparison. In addition to quantification of CTC number, this novel microfiltration assay will permit direct evaluation of LHRH receptor expression on the captured cells. This also will be correlated with response and changes in expression over time will be monitored during therapy. Furthermore, the unique autofluorescence properties of the doxorubicin moiety will be exploited to directly visualize internalization of AN-152 (AEZS-108) in captured CTCs for a unique pharmacokinetic study. Preliminary results from the phase I portion of this clinical trial indicate that AN-152 (AEZS-108) is well tolerated by most of the patients and has shown activity in the form of diminished PSA, other objective responses, or stabilization of the disease [Pinski et al., unpublished].

BREAST CANCER

Breast cancer is the most common malignancy in women and a leading cause of cancer related deaths among women in the Western world.[10,29] Various groups, including ours, showed an inhibition of rat and mouse mammary tumors after administration of agonists or antagonists of LHRH.[10,28] Later we showed that LHRH antagonists such as Cetrorelix can also inhibit growth of human breast cancer lines *in vitro* by direct action, mediated by LHRH receptors, on tumor cells. Thus, considerable evidence supports the view that the proliferation of human breast cancer cells *in vitro* and *in vivo* can be suppressed by LHRH agonists and antagonists.[28] In early studies *in vivo*, targeted cytotoxic LHRH analogs, AN-152 (AEZS-108) and AN-207, both inhibit growth of estrogen-independent MXT mouse mammary cancers.[31] In another investigation, AN-207 causes a regression of MX-1 hormone-independent doxorubicin-resistant human breast cancers that express significant levels of LHRH receptors in nude mice. Similar inhibition of tumor growth with AN-207 is obtained in MDA-MB-435 human estrogen-independent mammary carcinomas implanted s.c. or orthotopically in the mammary fat pad of nude mice. This effect of AN-207

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could be blocked by pretreatment of nude mice with the agonist [D-Trp^6]LHRH, which competes for LHRH receptors before the administration of AN-207.[30,31]

Because it was decided to choose AN-152 (AEZS-108) (*vide supra*) for clinical trials, we evaluated its effects on MX-1 human mammary carcinoma line xenografted into nude mice. Treatment with AN-152 (AEZS-108) significantly decreases the tumor volume and extends tumor doubling time. The levels of HER-2 and HER-3 proteins are also significantly reduced by AN-152 (AEZS-108).[30,31] AN-152 (AEZS-108) also can suppress growth of other triple-negative human breast cancer lines including MDA-MB-231 and HCC-1806.[12]

Cytotoxic LHRH analogs such as AN-152 (AEZS-108) have a selective transient inhibitory effect on pituitary LH cells. However, the dose-limiting toxicity of AN-152 (AEZS-108) will probably be myelotoxicity caused by doxorubicin. Our results show that targeted cytotoxic LHRH analogs such as AN-152 (AEZS-108) could be considered for treatment of LHRH receptor-positive advanced or metastatic breast cancers.[30,31]

Clinical Effects of Analogs of LHRH in Women with Breast Cancer

Klijn and De Jong[19] were the first to report that two of four premenopausal women with metastatic breast cancer improve after seven weeks of treatment with the LHRH agonist, buserelin (Suprafact). Clinical trials with microcapsules of triptorelin or implants of goserelin (Zoladex) in patients with advanced breast carcinoma show that the responses occurred primarily in women with ER-positive tumors.[10,28,29] Kaufman et al. used depot implants of goserelin (Zoladex) in 134 premenopausal women with breast cancer and found a 54% objective tumor response (reviewed in Refs 10, 28, 29). These studies indicated that LHRH agonists are efficacious for the treatment of premenopausal women with estrogen-dependent, ER-positive breast cancer. Subsequent trials showed beneficial effects of LHRH analogs in combination with tamoxifen in early breast cancer. Combined therapy with an LHRH agonist and tamoxifen is superior to either treatment modality alone.[10] Results from a large clinical trial indicate that goserelin alone or in combination with tamoxifen is at least as effective as chemotherapy with cytoxan, methotrexate, 5-FU (CMF) in patients with estrogen-dependent early disease.[10] In postmenopausal patients with metastatic breast cancer, the overall response rate of only about 8% can be explained by direct inhibitory effects of LHRH agonists on tumors.[10,28,29]

Epithelial Ovarian Cancer

The treatment of epithelial ovarian cancer, based on surgery and/or chemotherapy, is not very effective, and new approaches are needed.[28,29] Specific receptors for LHRH have been found in 78% of surgically removed human ovarian carcinomas as well as in EFO-21, EFO-27, and OV-1063 and ES-2 human ovarian cancer cell lines.[5,14,30,31] These receptors mediate direct inhibitory effects of LHRH analogs on the growth of ovarian cell lines *in vitro*.[5] The agonist, [D-Trp^6]LHRH, and antagonist, Cetrorelix, inhibit *in vivo* growth of human OV-1063 and ES-2 epithelial ovarian cancers xenografted into nude mice. However, in patients with advanced ovarian carcinoma, no beneficial effects of therapy with [D-Trp^6]LHRH were found in a multicenter trial.[30,31]

Targeted cytotoxic LHRH analogs, AN-152 (AEZS-108) and AN-207, in nude mice, inhibit the growth of LHRH receptor-positive OV-1063 ovarian tumors, but not of LHRH receptor-negative UCI-107 human ovarian carcinomas, showing that the presence of LHRH receptors is essential.[30,31] For clinical trials, see the next section on endometrial cancer.

Endometrial Cancer

New methods are needed for the treatment of advanced or relapsed endometrial cancer.[29] High-affinity receptors for LHRH are present on nearly 80% of human endometrial cancers and endometrial cancer cell lines.[9,14,28–30] The agonist [D-Trp^6]LHRH and the antagonist Cetrorelix inhibit the proliferation of endometrial cancer cell lines *in vitro*. Only limited clinical studies have been carried out so far, but administration of LHRH agonists produces a partial or complete remission in 35% of patients with recurrent endometrial cancer.[13]

Because LHRH receptors are found on 80% of endometrial cancers, the use of targeted cytotoxic analogs has been investigated. Thus, AN-152 (AEZS-108) inhibits the growth of HEC-1A and HEC-1B human endometrial cancers in nude mice. AN-207 also significantly suppresses the proliferation *in vivo* of HEC-1A and RL-95-2 endometrial cancers.[9]

Clinical Studies with AN-152 (AEZS-108) Women with Endometrial and Ovarian Cancers

There is one phase I[4,7] and two phase II studies on AN-152 (AEZS-108).[6,8] The phase I study includes 17 women with histologically confirmed epithelial cancer of the ovary, endometrium, or breast that is metastatic or unresectable and for which standard curative or palliative measures could not be used or are no longer effective or tolerated.[4,7] In each patient, immunohistochemistry of primary tumor or metastatic lesion confirmed that the tumors express LHRH receptors.[4,7] The study is designed as a dose-escalation trial to determine the maximum tolerated dose of AN-152 (AEZS-108). One patient each received intravenous doses of 10, 20, 40, or 80mg/m^2 of AN-152 (AEZS-108), six received

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160mg/m^2 and seven 267mg/m^2 at 3 week intervals. Dose-limiting leukopenia and neutropenia were observed at the highest dose. Although efficacy was not a primary endpoint, tumor response was assessed whenever possible. In the low dose group, none of the patients showed a response; however, a total of 6 patients of 13 in both upper dose groups (3 patients at 160mg/m^2, 3 at 267mg/m^2) showed responses to AN-152 (AEZS-108) (2 complete remissions, 1 partial remission, 3 disease stabilization). The maximum tolerated dose of AN-152 (AEZS-108) in the absence of supportive medication was 267mg/m^2, and this dose was used as starting dose for therapeutic phase II studies.[4,7]

The first phase II study was performed on behalf of Arbeitsgemeinschaft Gynäkologische Onkologie (AGO) in 43 patients with platinum-resistant advanced ovarian cancer and was presented at the ASCO symposium in 2010.[8] Patients with histologically confirmed taxane-pretreated platinum-resistant/refractory, LHRH-receptor positive, advanced (FIGO III or IV) or recurrent ovarian cancer received up to 6 cycles of AN-152 (AEZS-108) at doses of 267mg/m^2. At least 1 measurable lesion or CA-125 ≥ 200% of the upper normal range limit was required at baseline. An independent radiologic review was performed. Response rate (RECIST and/or GCIG criteria) was defined as primary endpoint. Secondary endpoints were safety, time-to-progression (TTP) and overall survival (OS). Efficacy included partial remissions in 5 patients (11.6%) and disease stabilization for > 12 weeks in 14 patients (32.6%). AN-152 (AEZS-108) was active and well tolerated in patients with heavily pretreated platinum-/taxane-resistant ovarian cancer. The safety profile confirmed the dose of 267mg/m^2.

The second phase II study was a multicenter trial also performed by AGO and includes 43 patients with histologically confirmed, LHRH-R positive, advanced (FIGO III or IV) or recurrent endometrial cancer.[6] Patients receive AN-152 (AEZS-108) for a planned duration of 6 treatment courses at doses of 267mg/m^2. At least 1 measurable lesion is required at baseline with an independent radiologic review. The response rate per RECIST is defined as the primary endpoint. Secondary endpoints are safety, time-to-progression (TTP) and overall survival (OS). Tumors of all the patients were confirmed as LHRH-R positive; percentages of tumor cells staining for LHRH-R ranged 30%–90% (median 70%); 27 patients had a primary diagnosis of EC FIGO III/IV and 31 patients had tumors with endometrioid and 8 with serous histology. Previous treatment included surgery (42 patients), radiotherapy (29 patients), chemotherapy (9 patients) and hormonal therapy (11patients). Twenty-eight patients (62.8%) received AN-152 (AEZS-108) for 6 courses (2 patients with 8 courses), only one patient was retreated at a reduced dose. Independent review, which is currently ongoing, has confirmed the target minimum of 5 responses. Definite results for further reported responses (preliminary judgment) were 4 complete remissions, 10 partial remissions and—at end of course—6–15 disease stabilizations.[6]

Renal Cell Carcinoma

The prognosis of patients with metastatic renal cell carcinoma (RCC) is poor, and the present methods of treatment of RCC should be improved.[17,28,30] Therapy with the LHRH antagonist, Cetrorelix, inhibits the growth of the CAKI-I RCC line xenografted into nude mice. The expression of LHRH receptors is found by immunohistochemistry in 28 of 28 surgically removed specimens of human renal cell carcinoma and in three RCC cell lines, A-498, ACHN, and 786-0, by Western immunoblotting and RT-PCR.[17] The cytotoxic LHRH analog, AN-207, significantly inhibits the growth of these three human RCC lines xenografted into nude mice. Our findings indicate that LHRH receptors expressed in human RCC specimens can be used for targeted chemotherapy with cytotoxic LHRH analogs.[17,30]

Non-Hodgkin's Lymphoma

Non-Hodgkin's lymphoma (NHL) is the most frequently diagnosed hematological malignancy in the U.S.[16,30] Patients with advanced disease show a five-year survival rate of only 26%, emphasizing the need for new therapies. LHRH receptors were detected in 92% of surgically removed human NHL specimens and in RL and HT NHL cell lines. AN-207 significantly inhibits the growth of RL and HT tumors.[16,30] Thus, LHRH receptors found in human NHL could also be therapeutic targets for the cytotoxic LHRH analogs.

Melanoma

The prognosis for metastatic melanoma is very poor and new effective therapies must be developed.[18,30] The first demonstration of LHRH receptors on rat melanomas was made by Moretti et al.[24] We found LHRH receptor expression by immunohistochemistry in 19 of 19 human melanoma specimens. The mRNA for LHRH receptors and LHRH receptor protein were similarly detected in transplantable MRI-H255 and MRI-H187 human melanoma tumor lines.[18] We then showed that AN-207 significantly inhibits the growth of MRI-H255 and MRI-H187 tumors xenografted into nude mice.[18] Thus, LHRH receptors expressed in human malignant melanoma could be used for targeted chemotherapy with LHRH analogs.[30]

Colorectal Cancer

Colon cancer lines including HT-29, HCT-116, HCT-15, LoVo, and Colo-320DM, show high-affinity binding sites for LHRH.[30,32] Cytotoxic LHRH analogs AN-207 and AN-152 (AEZS-108) strongly inhibit growth of these colon

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cancer cell lines.[32] Thus, targeted therapy with cytotoxic LHRH analogs could be considered for the treatment of metastatic colorectal cancer.

Pancreatic Cancers

The prognosis for patients with ductal pancreatic cancers is dismal, and it is essential to develop new therapeutic approaches.[29] Human and hamster pancreatic cancers express LHRH receptors that are localized mostly in the cell nuclei;[28,30] their function is not known. Various clinical trials with LHRH agonists in patients with pancreatic carcinoma were unsuccessful.[28–30] Treatment with cytotoxic LHRH analog, AN-152 (AEZS-108), inhibits growth of SW-1990, PANC-1 and CFPAC-1 human pancreatic cancer lines xenografted into nude mice [Szepeshazi and Schally, in press]. Phase I/II clinical trials with the cytotoxic LHRH analog, AN-152 (AEZS-108), in patients with advanced pancreatic cancers are pending.

Urinary Bladder Cancers

New therapeutic methods are needed for the treatment of metastatic bladder cancer.[30] Immunohistochemical studies indicate that human bladder cancers express receptors for LHRH [1, Keller and Schally, unpublished]. We (Szepeshazi and Schally) have shown that the growth of HT-1197, RT-4, and J-82 human bladder cancer lines xenografted into nude mice is strongly inhibited by the cytotoxic LHRH analog AN-152 (AEZS-108). A phase I/II clinical trial (Fernandez G. et al.) with AN-152 (AEZS-108) in patients with metastatic urothelial carcinoma, resistant to platinum chemotherapy, is in progress, and early patient responses have been encouraging.

SUMMARY

In the 40 years that have elapsed since the elucidation of the structure of LHRH-I, more than its 23 molecular isoforms have been identified in other species. Interest in the medical applications of LHRH derivatives led to the synthesis of >3000 analogs. Various uses in gynecology, reproductive medicine, and oncology have been established for LHRH-I agonists and antagonists. Clinical applications of LHRH agonists are based on gradual downregulation of pituitary receptors for LHRH and inhibition of the pituitary and gonads. LHRH antagonists produce an immediate suppression of the secretion of gonadotropins and sex steroids and thus achieve rapid therapeutic effects. LHRH agonists and antagonists can be also used for the treatment of endometriosis, uterine leiomyomas, and in assisted reproduction programs. The preferred primary treatment of advanced androgen-dependent prostate cancer is based on the periodic administration of depot preparations of LHRH agonists. LHRH agonists can likewise be applied for treatment of breast cancer. Besides their action on the pituitary, LHRH agonists and antagonists can also exert direct effects on tumor cells. The presence of specific receptors for LHRH on various cancers provided a rationale for the design and synthesis of cytotoxic hybrids, consisting of chemotherapeutic agents conjugated to the analogs of these peptides, which can be targeted to these tumoral receptors. Cytotoxic LHRH analogs, targeted to tumors, were tested on many human cancer lines xenografted into nude mice. Thus, cytotoxic analogs of LHRH, AN-152 (AEZS-108) and AN-207, containing DOX or 2-pyrrolino-DOX (AN-201), respectively, targeting LHRH receptors, inhibited growth of prostatic, bladder, breast, ovarian, endometrial, colorectal, and pancreatic cancers as well as lymphomas, melanomas, and RCCs. The clinical applications of the cytotoxic LHRH analog, AN-152 (AEZS-108), was successful in a phase I/II trial in women with gynecologic cancers, and this analog is now in phase III trials. Other clinical trials are pending. Continued development of targeted cytotoxic LHRH analogs should lead to a more efficacious therapy for many human cancers.

ACKNOWLEDGMENTS

Some of this research was supported in part by the Medical Research Service of the Veterans Affairs Department to AV Schally.

REFERENCES

1. Bahk JY, Kim MO, Park MS, Lee HY, Lee J, Chung BC, et al. Gonadotropin-releasing hormone (GnRH) and GnRH receptor in bladder cancer epithelia and GnRH effect on bladder cancer cell proliferation. *Urol Int* 2008;**80**:431–8.
2. Block NL, Garnick MB, Glode LM, Guinan PD, Sharifi R, Smith JA. Preliminary evaluation of efficacy and safety of gonadotropin-releasing hormone analog (Leuprolide) in management of advanced adenocarcinoma of prostate—poster summary. *Urology* 1985;**25**:58–9.
3. Emons G, Grundker C, Gunthert AR, Westphalen S, Kavanagh J, Verschraegen C. GnRH antagonists in the treatment of gynecological and breast cancers. *Endocr Relat Cancer* 2003;**10**:291–9.
4. Emons G, Kaufmann M, Gorchev G, Tsekova V, Gruendker C, Guenthert AR, et al. Dose escalation and pharmacokinetic study of AEZS-108 (AN-152), an LHRH agonist linked to doxorubicin, in women with LHRH receptor-positive tumors. *Gynecol Oncol* 2010;**119**:457–61.
5. Emons G, Ortmann O, Becker M, Irmer G, Springer B, Laun R, et al. High-affinity binding and direct antiproliferative effects of LHRH analogs in human ovarian-cancer cell-lines. *Cancer Res* 1993;**53**:5439–46.
6. Emons G, Sehouli J, Gorchev G, Hristamian A, Staehle A, Hanker LC, et al. Phase II study of AEZS-108 (AN-152), a targeted cytotoxic LHRH analog, in patients with LHRH receptor positive endometrial cancer. Protocol AGO-GYN 5, AGO Study Group, Germany. *EJC Suppl* 2010;**8**:120.

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7. Emons G, Sindermann H, Engel J, Schally AV, Gruendker C. Luteinizing hormone-releasing hormone receptor-targeted chemotherapy using AN-152. *Neuroendocrinology* 2009;**90**:15–8.
8. Emons G, Tomov S, Harter P, Sehouli J, Wimberger P, Staehle A, et al. Phase II study of AEZS-108 (AN-152), a targeted cytotoxic LHRH analog, in patients with LHRH receptor-positive platinum resistant ovarian cancer. ASCO Annual Meeting 2010. *J Clin Oncol* 2010;**28**(15s):[suppl; abstr 5035].
9. Engel JB, Keller G, Schally AV, Nagy A, Chism DD, Halmos G. Effective treatment of experimental human endometrial cancers with targeted cytotoxic luteinizing hormone-releasing hormone analogues AN-152 and AN-207. *Fertil Steril* 2005;**83**:1125–33.
10. Engel JB, Schally AV. Drug insight: clinical use of agonists and antagonists of luteinizing-hormone-releasing hormone. *Nat Clin Pract Endocrinol Metab* 2007;**3**:157–67.
11. Fekete M, Wittliff JL, Schally AV. Characteristics and Distribution of receptors for [D-Trp6]-luteinizing hormone-releasing hormone, somatostatin, epidermal growth-factor, and sex steroids in 500 biopsy samples of human breast cancer. *J Clin Lab Anal* 1989;**3**:137–47.
12. Foest C, Duwe F, Hellriegel M, Schweyer S, Emons G, Gruendker C. Targeted chemotherapy for triple-negative breast cancers via LHRH receptor. *Oncol Rep* 2011;**25**:1481–7.
13. Gallagher CJ, Oliver RTD, Oram DH, Fowler CG, Blake PR, Mantell BS, et al. A new treatment for endometrial cancer with gonadotropin-releasing-hormone analog. *Br J Obstet Gynaecol* 1991;**98**:1037–41.
14. Grundker C, Volker P, Griesinger F, Ramaswamy A, Nagy A, Schally AV, et al. Antitumor effects of the cytotoxic luteinizing hormone-releasing hormone analog AN-152 on human endometrial and ovarian cancers xenografted into nude mice. *Obstet Gynecol* 2002;**187**:528–37.
15. Halmos G, Arencibia JM, Schally AV, Davis R, Bostwick DG. High incidence of receptors for Luteinizing hormone-releasing hormone (LHRH) and LHRH receptor gene expression in human prostate cancers. *J Urol* 2000;**163**:623–9.
16. Keller G, Schally AV, Gaiser T, Nagy A, Baker B, Halmos G, et al. Receptors for Luteinizing hormone releasing hormone (LHRH) expressed in human non-Hodgkin's lymphomas can be targeted for therapy with the cytotoxic LHRH analogue AN-207. *Eur J Cancer* 2005;**41**:2196–202.
17. Keller G, Schally AV, Gaiser T, Nagy A, Baker B, Halmos G, et al. Receptors for luteinizing hormone releasing hormone expressed on human renal cell carcinomas can be used for targeted chemotherapy with cytotoxic luteinizing hormone releasing hormone analogues. *Clin Cancer Res* 2005;**11**:5549–57.
18. Keller G, Schally AV, Gaiser T, Nagy A, Baker B, Westphal G, et al. Human malignant melanomas express receptors for luteinizing hormone releasing hormone allowing targeted therapy with cytotoxic luteinizing hormone releasing hormone analogue. *Cancer Res* 2005;**65**:5857–63.
19. Klijn JGM, De Jong FH. Treatment with a luteinising-hormone–releasing-hormone analog (Buserelin) in premenopausal patients with metastatic breast-cancer. *Lancet* 1982;**1**:1213–6.
20. Lopez de Maturana R, Pawson AJ, Lu Z, Davidson L, Maudsley S, Morgan K, et al. Gonadotropin-releasing hormone analog structural determinants of selectivity for inhibition of cell growth: support for the concept of ligand-induced selective signaling. *Mol Endocrinol* 2008;**22**:1711–22.
21. Matsuo H, Arimura A, Nair RMG, Schally AV. Synthesis of porcine LH- and FSH-Releasing hormone by solid-phase method. *Biochem Biophys Res Commun* 1971;**45**:822.
22. Matsuo H, Baba Y, Nair RMG, Arimura A, Schally AV. Structure of porcine LH- and FSH-Releasing hormone 1. Proposed amino acid sequence. *Biochem Biophys Res Commun* 1971;**43**:1334.
23. Millar RP, Lu ZL, Pawson AJ, Flanagan CA, Morgan K, Maudsley SR. Gonadotropin-releasing hormone receptors. *Endocr Rev* 2004;**25**:235–75.
24. Moretti RM, Marelli MM, Van Groeninghen JC, Limonta P. Locally expressed LHRH receptors mediate the oncostatic and antimetastatic activity of LHRH agonists on melanoma cells. *J Clin Endocrinol Metab* 2002;**87**:3791–7.
25. Morgan K, Stewart AJ, Miller N, Mullen P, Muir M, Dodds M, et al. Gonadotropin-releasing hormone receptor levels and cell context affect tumor cell responses to agonist in vitro and in vivo. *Cancer Res* 2008;**68**:6331–40.
26. Redding TW, Schally AV. Inhibition of prostate tumor-growth in 2 rat models by chronic administration of D-Trp6 analog of luteinizing-hormone-releasing hormone. *Proc Natl Acad Sci USA Biol Sci* 1981;**78**:6509–12.
27. Rozsa B, Nadji M, Schally AV, Dezso B, Flasko T, Toth G, et al. Receptors for luteinizing hormone-releasing hormone (LHRH) in benign prostatic hyperplasia (BPH) as potential molecular targets for therapy with LHRH antagonist Cetrorelix. *Prostate* 2011;**71**:445–52.
28. Schally AV. Luteinizing hormone-releasing hormone analogs: their impact on the control of tumorigenesis. *Peptides* 1999;**20**:1247–62.
29. Schally AV, Comaru-Schally AM. Hypothalamic and other peptide hormones. In: Kufe DW, Pollock RE, Weichselbaum RR, Bast JRC, Gansler TS, Holland J, editors. *Cancer medicine*. 7th ed. Hamilton, Ontario: B.C. Dekker Publishers; 2006. p. 802–16.
30. Schally AV, Halmos G. Targeting to peptide receptors. In: Kratz F, Senter P, Steinhagen H, editors. *Drug delivery in oncology. From basic research to cancer therapy*. Weinheim, Germany: Wiley-VCH: 2012;**3**:1219–26.
31. Schally AV, Nagy A. Chemotherapy targeted to cancers through tumoral hormone receptors. *Trends Endocrinol Metab* 2004;**15**:300–10.
32. Szepeshazi K, Schally AV, Halmos G. LH-RH receptors in human colorectal cancers: unexpected molecular targets for experimental therapy. *Int J Oncol* 2007;**30**:1485–92.
33. Tolis G, Ackman D, Stellos A, Mehta A, Labrie F, Fazekas ATA, et al. Tumor-growth inhibition in patients with prostatic-carcinoma treated with luteinizing-hormone-releasing hormone agonists. *Proc Natl Acad Sci USA Biol Sci* 1982;**79**:1658–62.
34. Van Poppel H, Tombal B, de la Rosette JJ, Persson B, Jensen J, Olesen TK. Degarelix: a novel gonadotropin-releasing hormone (GnRH) receptor Blocker-results from a 1-yr, multicentre, randomised, phase 2 dosage-finding study in the treatment of prostate cancer. *Eur Urol* 2008;**54**:805–15.
35. White CD, Stewart AJ, Lu Z, Millar RP, Morgan K. Antiproliferative effects of GnRH agonists: prospects and problems for cancer therapy. *Neuroendocrinology* 2008;**88**:67–79.

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Chapter 74

Orexins (Hypocretins)

Marc Laburthe and Thierry Voisin

ABSTRACT

Orexin-A and orexin-B are closely related peptides expressed by hypothalamic neurons that regulate sleep, wakefulness, and other central functions. There is factual evidence for their role in the control of gastrointestinal motility, gastric acid secretion, duodenal bicarbonate secretion, jejunal glucose absorption, and pancreatic hormone (insulin, glucagon) secretion. The orexin receptor OX1R is aberrantly expressed at all steps of colorectal tumor progression and after metastasis. Treatment of colon cancer cells in culture with orexins promotes robust apoptosis even in cells that are resistant to classical chemotherapy. When human colon cancer cells are xenografted in nude mice, injections of orexins dramatically slow down tumor growth and even reverse the development of established tumors. In this respect, OX1R may be considered as an Achilles heel of colon cancer. OX1R drives mitochondrial apoptosis through a new paradigm of G protein-coupled signaling involving phosphorylation of the immunoreceptor tyrosine-based inhibitory motifs (ITIMs) and recruitment/activation of the tyrosine phosphatase SHP-2.

INTRODUCTION

Orexin-A and orexin-B, also named hypocretin-1 and hypocretin-2, are two neuropeptides encoded in a single gene and expressed in lateral hypothalamus neurons that project throughout the brain[8,28] and regulate sleep, wakefulness, feeding, energy homeostasis, breathing, reward system, or drug addiction.[20] Loss of orexin neurons or deficiency in orexin signaling results in the sleep disorder narcolepsy–cataplexy in humans, dogs, and rodents[20] and possibly other neurological disorders.[11] Functions and expression of orexins in peripheral tissues, including the digestive system, have been also documented.[4,13,35] This chapter presents and discusses the current understanding of orexin and orexin receptors with special attention on a new paradigm of G protein-coupled receptor signaling by orexin receptors through phosphorylation of the tyrosine-based motifs ITIMs. It also focuses on the possible physiological role of orexins in the digestive system, which is still under debate. Finally, it reviews recent findings showing that orexin receptors are aberrantly expressed in colon cancer in which they drive apoptosis and represent thereby a novel and promising target in colon cancer therapy.

DISCOVERY

Hypocretins[8]/orexins[28] were discovered at the same time by two independent groups using different strategies and technologies. Analysis of expression pattern of cDNA selectively expressed in the hypothalamus revealed an original 569 nucleotide clone encoding a 131-residue putative secretory protein (in humans), which can generate two homologous peptides sharing very weak sequence homology with secretin. They were named hypocretin-1 and hypocretin-2 thereby.[8] At the same time, rat brain extracts were screened for their ability to induce intracellular calcium transients in a battery of orphan G protein-coupled receptor-expressing cells.[28] Two peptides were purified and named orexin-A and orexin-B because of their orexigenic effects in rats.[28] This latter strategy allowed the description of two G protein-coupled receptors for orexins referred to as OX1R and OX2R.[28] The 28 amino acid orexin-B and 33 amino acid orexin-A were identical to hypocretin-2 and hypocretin-1, respectively. Both names are problematic because regulation of feeding and appetite is probably not the major action of the neuropeptides, and they have no clear sequence homology with secretin. In the absence of recommendations by the International Union of Pharmacology, and for the sake of clarity, we have used the names orexin and orexin receptor OXR in this chapter.

STRUCTURE OF THE mRNA/GENE

Orexin-A and orexin-B are derived from a single prepro-orexin precursor. The human mRNA was predicted to encode a 131-residue precursor peptide.[8,28] The human preproorexin gene is located at human chromosome 17q21 and consists of two exons and one intron distributed over 1432 bp.[28] The 143 bp first exon includes the 5′-untranslated region and a small part of the coding region that encodes the first seven residues of the

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00074-9
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secretory signal sequence. The second 473 bp exon contains the remaining portion of the open reading frame and a 102 bp 3′-untranslated region. The two exons are separated by an 816 bp intron.

PROCESSING OF THE PRECURSOR

The first 33 amino acids of the human 131-residue preproorexin exhibits the characteristics of a secretory signal sequence with a putative cleavage site at Ala^{33}-Gln^{34}.[35] Orexin-A sequence starts with Gln^{34}. which is cyclized into an N-terminal pyroglutamyl residue presumably by enzymatic transamidation. The last Leu^{66} residue of mature orexin-A is followed by a Gly^{67} in the precursor allowing C-terminal amidation by peptidylglycine monooxygenase and peptidylaminoglycolase. Gly^{67} is followed by a pair of basic amino acids, Lys^{68}-Arg^{69}, which constitutes a recognition site for prohormone convertase. The next segment Arg^{70} to Met^{97} constitutes orexin-B. The Met^{97} residue is again followed by Gly and a pair of basic residues which constitute a C-terminal amidation/cleavage signal. The mature 33 amino acid peptide orexin-A contains two intrachain disulfide bonds, whereas the mature 28 amino acid peptide orexin-B is devoid of a Cys and thereby of any disulfide bonds. Nevertheless, the structures of orexin-A and orexin-B exhibit high similarity with two alpha helices connected by a short linker (Fig. 1).[35]

DISTRIBUTION OF THE mRNA/PEPTIDE

The main site of orexin synthesis in the body is the lateral hypothalamus, which contains orexin neuron cell bodies that project in many areas of the brain.[8,23,28] Several lines of evidence also support the expression of orexins in the periphery, including the digestive tract, though reported results are somewhat inconsistent.[4,13,35] Preproorexin mRNA was identified by RT-qPCR in the testis and at very low levels in the heart but not in other tissues of rats including stomach, duodenum, jejunum, pancreas, and liver.[15] Similarly,

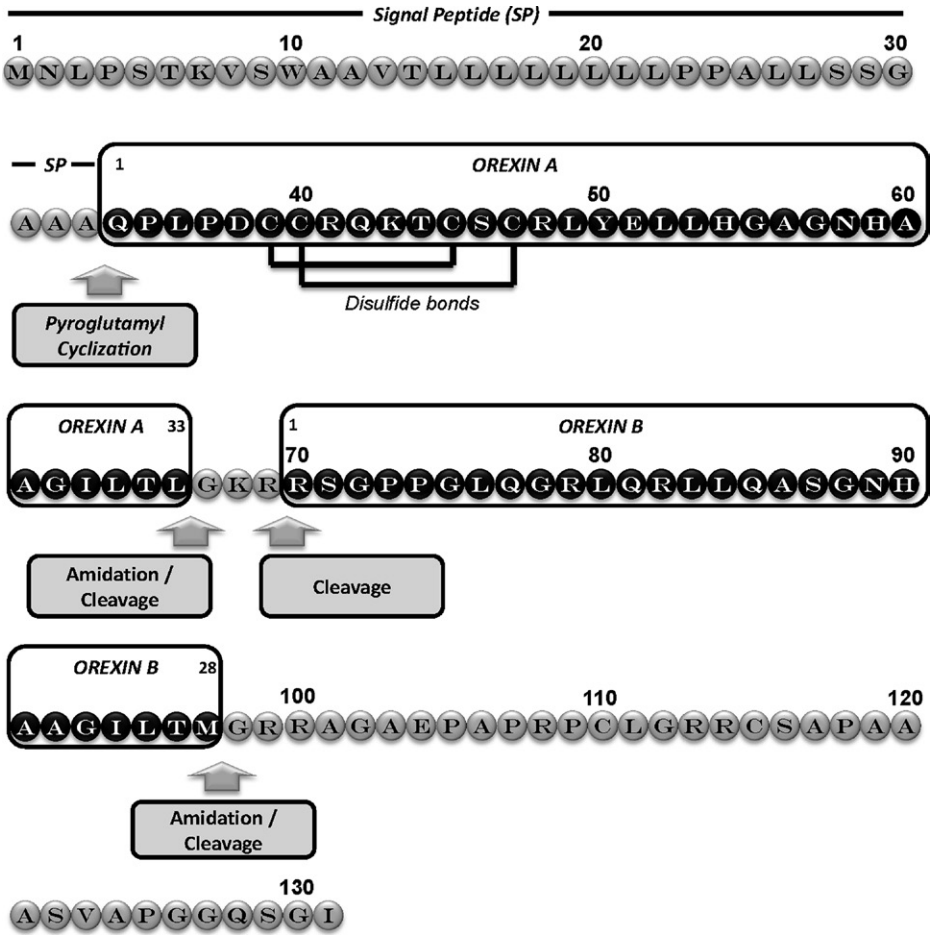

FIGURE 1 Human preproorexin processing. The 131-residue preproorexin precursor encompasses the signal peptide, orexin-A and orexin-B. Arrows indicate predictive enzymatic cleavage sites and chemical modifications. Note that orexin-A has two disulfide bonds.

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no preproorexin mRNA could be identified by RT-qPCR in the human colon and the liver.[33] These results contrast markedly with immunostaining of neurons or endocrine cells with anti-orexin antibodies in various parts of the gut mucosa and smooth muscles in several species.[16] Problems with the specificity of antibodies and/or species variations in the expression of peripheral orexins may be responsible for discrepancies in the results. Alternatively, the existence of neuronal populations that project from hypothalamus to the periphery[30] may explain the presence of orexins in the gut, at least in neurons, but the absence of preproorexin mRNA which would be present in orexin neuron cell bodies located outside the gut. Orexin-A was also identified in both β- and α-cells of the rat endocrine pancreas.[24] Orexin-B was identified in β-cells and nerves in the wall of blood vessels of rat pancreas.[2] Again, these observations contrast with the absence of detectable preproorexin mRNA in the pancreas of rats.[15] A few studies reported the radioimmunological detection of very low concentrations of orexin-A in human plasma but results probably need to be interpreted with caution.[13]

RECEPTORS AND SIGNALING CASCADES

Two orexin receptors, OX1R and OX2R, also named HCTR1 and HCTR2, have been characterized.[8,28] They have seven transmembrane domains and belong to the family A of G protein-coupled receptors.[35] In humans, the two OXRs show 64% identity in amino acid sequences. OXRs are highly selective for orexins with no significant binding of other natural peptide hormones or neuropeptides.[35] Contrary to other peptide receptors, only few reports described binding studies of orexins to OXRs.[35] In some studies, OX1R seems to have more affinity for orexin-A than for orexin-B, a selectivity that is also observed when measuring calcium transients.[29,35] However, in other studies orexin-A and orexin-B have identical potencies in promoting OX1R-mediated cell apoptosis or inhibition of cell growth with EC_{50} in the nanomolar range.[18] The OX2 receptor seems to have similar affinity for orexin-A and orexin-B in binding studies[35] and biological response such as stimulation of apoptosis.[18,34]

Although subtype-selective orexin receptor peptide agonists have been described,[3,19] the selectivity of some of them has been reported to be somewhat weak and variable[26] depending on the cellular context for native receptors or expression system for recombinant receptors. No orexin receptor small-molecule agonist has been described yet.[26] By contrast, numerous small-molecule orexin receptor antagonists have been described because of their potential interest as a novel therapy for the treatment of insomnia.[6,29] Both OX1R-selective and OX2R-selective antagonists have been synthesized and dual OX1R/OX2R antagonists are also described.[6,29]

Classically, the activation of both orexin receptors induces cellular calcium transients in receptor-transfected cells and native receptor-expressing cells.[35] This occurs through the increase of intracellular inositol triphosphate and subsequent release of calcium from intracellular stores.[32] OX1R connects to multiple phospholipase activities including phospholipases C and D.[14] OX1R has also been shown to be linked to calcium influx through the canonical transient receptor potential channel TRPC3.[25] A few reports also suggest that the activation of OXRs can result in stimulation of cAMP production.[35] These observations are consistent with the coupling of OXRs to Gq, Gi/Go and/or Gs proteins.

Although the classical calcium pathway is considered to play an important role in OXR signaling, a new paradigm of OX1R signaling has recently emerged.[10,17,32] Activation of OX1R drives apoptosis in colon cancer cells or OX1R-transfected cells through Gq protein but independently of classical Gαq activation of phospholipase C and calcium pathway. On OX1R activation, βγ dimers of Gq are freed resulting in the stimulation of Src-tyrosine kinase. Src then phosphorylates two immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in OX1R allowing the recruitment of the phosphotyrosine phosphatase SHP-2, which is activated thereby.[10,32] Activated SHP-2 triggers a cascade of downstream events resulting in mitochondrial apoptosis.[17,27] The role of ITIMs in OX1R-driven apoptosis represents a new paradigm of G protein-coupled receptor signaling.[17] Figure 2 schematizes classical and ITIM-driven OX1R signaling.

BIOLOGICAL ACTIONS WITHIN THE GASTROINTESTINAL TRACT AND THE PANCREAS

Many reports indicate that orexins are able to affect motility and secretions in the digestive tract via both central and peripheral actions. They also show that peripheral actions can be neurally mediated and/or triggered by direct interaction of orexins with smooth muscle, exocrine or endocrine cells.[4,13,35] However, although OXRs have been detected by immunohistochemistry in the gut and pancreas in different species including rats,[35] RT-qPCR experiments performed on rat tissues failed to identify any significant amounts of OX1R and OX2R mRNA in the stomach, duodenum, and pancreas, while OX1R mRNA could be identified in jejunum.[15] Again, problems associated with the specificity of antibodies and/or species variations in the expression of peripheral OXRs may be responsible for the discrepancies in the results. In this context, the possibility of a direct control of digestive functions by orexins still remains to be firmly established in many cases. The actions of orexins

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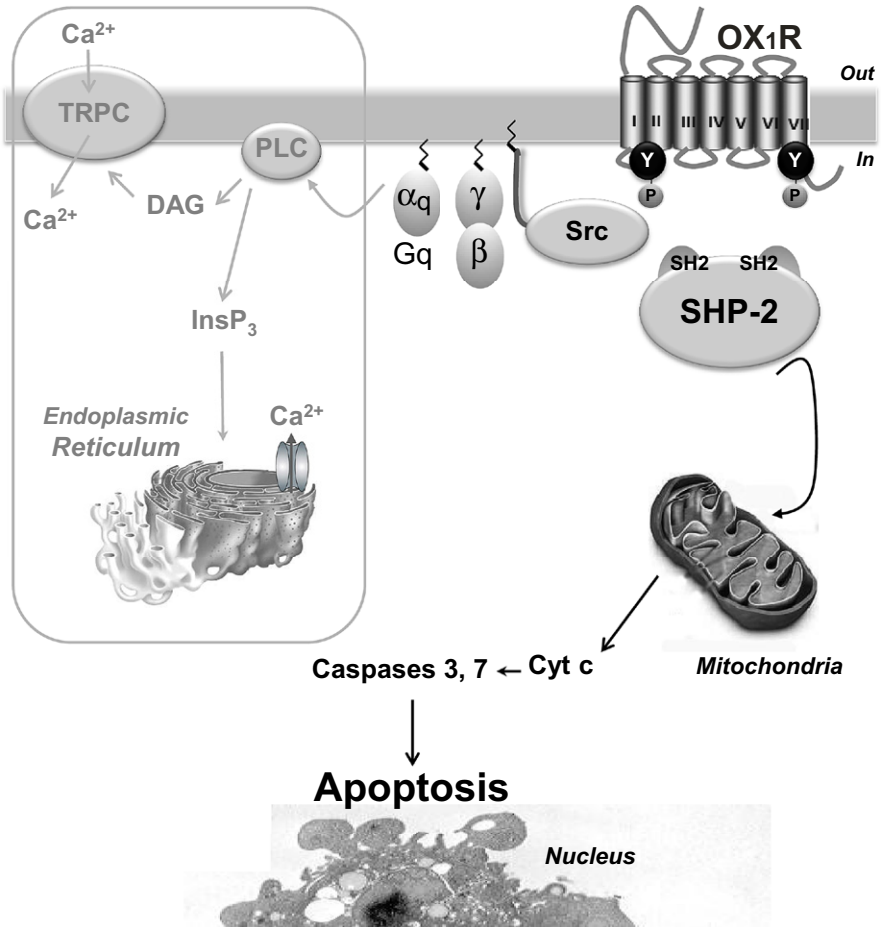

FIGURE 2 Mechanism of OX1R-driven apoptosis. Activation of the heptahelical orexin receptor OX1R by orexins promotes the dissociation of G_q protein into α_q and $\beta\gamma$ dimers. The classical pathway resulting in phospholipase C activation by αq is not involved in OX1R-mediated apoptosis and is shown in a box. Freed $\beta\gamma$ dimers stimulate the Src-tyrosine kinase resulting in the tyrosine phosphorylation of the two ITIMs of OX1R. The phosphotyrosine phosphatase SHP-2 is then recruited by OX1R and activated thereby. Activated SHP-2 is mandatory for subsequent cytochrome c-mediated mitochondrial apoptosis. Cyt c, cytochrome c; DAG, diacylglycerol; $InsP_3$, inositol triphosphate; SHP-2, SH2 domain-containing phosphotyrosine phosphate-2; TRPC, canonical transient receptor potential channel.

on gastric, intestinal, and pancreatic functions are briefly reviewed below:

- Gastrointestinal motility

Central and peripheral administrations of orexins have been shown to modify motility in stomach and small intestine and to lower gastric emptying rate.[4,13] However, because of the variability in the results and methodology used, the effects of orexins on gastrointestinal motility are still under debate.[13]

- Gastrointestinal secretion

Central, but not peripheral, administration of orexin-A causes an increase in gastric acid secretion in rats most probably by modulating the vagal system.[31] Peripheral administration of orexins stimulates duodenal bicarbonate secretion in fed but not in fasted rats and is independent of vagal control.[5]

- Small intestinal absorption

Orexin-A and orexin-B acting at OX1R or OX2R inhibit rat jejunal glucose absorption through a complex mechanism that involves distinct neuronal, endocrine, and direct epithelial pathways.[9] It is suggested that orexins are involved in a regulatory loop between enteric glucose entry and central evaluation of peripheral available energy levels[9] in agreement with results indicating that orexin neurons in the hypothalamus are specialized cells for sensing blood glucose.[7]

- Pancreatic secretions

Central administration of orexin-A stimulates pancreatic exocrine secretion of fluid and protein via the vagus.[21] Several reports also suggest that orexins can modulate the function of endocrine pancreas though, again, the variability in the results and methodology used makes it difficult to

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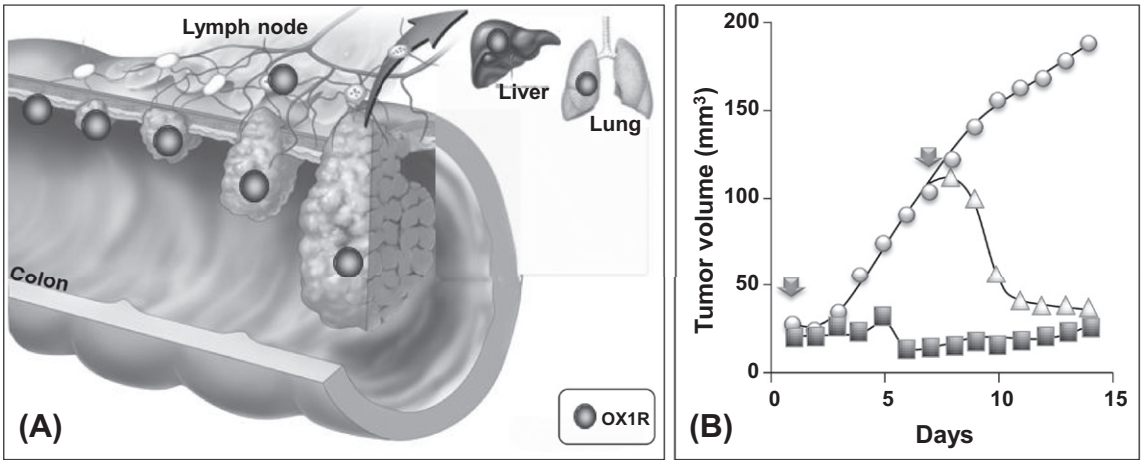

FIGURE 3 Expression of the orexin receptor OX1R during colon cancer progression and metastasis and treatment of colon tumors with orexins *in vivo*. Left, OX1R (circles) is aberrantly expressed in primary colon tumors and metastases but not in normal colon. It is expressed very early during carcinogenesis and whatever Dukes' stage of the primary tumors. After metastasis in the colon (lymph node) and in distant organs (liver, lung) OX1R is still expressed (see Ref. 33 for details). Right, effect of inoculation of orexin-A on the growth of tumors developed by xenografting human colon cancer cells LoVo in nude mice. Mice were daily injected intraperitoneally without (circles) or with 1.12 μmol of orexin-A/kg body weight starting on day 0 (squares) or day 7 (triangles). See Ref. 33 for details.

clearly understand their physiological role, if any. Subcutaneous injection of orexin-A in rats results in an increase in blood insulin and orexin-A stimulates insulin secretion in an *in vitro* perfusion system of rat pancreas.[22] Orexin-B evokes insulin and glucagon release from pancreatic tissue fragments.[2] This is in line with the ability of orexin-A to reduce glucagon secretion from perfused rat pancreas and isolated rat pancreatic islets.[12] However, all these results contrast markedly with a report suggesting that orexin-A increases glucagon secretion and decreases glucose-stimulated insulin release from isolated rat islets and that orexin-A infusion increases plasma glucagon and decreases plasma insulin in fasted rats.[24]

PATHOPHYSIOLOGICAL IMPLICATIONS

Considering that brain orexins can control gastrointestinal functions through the vagal system, the lack of orexin signaling in the brain observed in narcolepsy and perhaps other neurological diseases (see above) might trigger the development of functional gastrointestinal disorders. However, a direct relationship between orexin/orexin receptors and gastrointestinal diseases has only been firmly shown for colon cancer.[17,33]

After earlier work showing that orexins are able to promote apoptosis in human colon cancer cell lines in culture through interaction with the OX1R,[27] the role of OX1R in colorectal cancers has been clearly shown.[17,33] The OX1R is aberrantly expressed at all steps of primary colorectal tumor progression as shown in a large collection of resected tumors.[33] The same occurs after local (lymph node) and distant (liver, lung) metastasis (Fig. 3A). In sharp contrast, no OX1R is detected in normal human colonic epithelial cells from which cancer cells derive. Treatment of human colon cancer cells in culture with orexins promotes robust apoptosis and subsequent reduction of cell growth even in cells that are resistant to 5-fluorouracil, the most commonly used drug in chemotherapy.[33] When human colon cancer cells are xenografted in nude mice, daily ip injections of orexins dramatically slow down tumor growth and even reverse the development of established tumors by promoting a selective apoptosis in tumor cells (Fig. 3B).[33] Because neither colon tumors nor normal colon expresses preproorexin mRNA in humans,[33] the OX1R aberrantly expressed in colon tumors is most probably not activated by endogenous orexins in patients. In this context, it has been suggested that OX1R in colorectal cancers constitutes a gate to apoptosis that probably remains unopened *in vivo* but could be openable by therapeutic administration of exogenous orexins or OX1R agonists.[17] In this respect, OX1R may be considered as an Achilles heel of colon cancer, because targeting OX1R with agonists leads to cancer cell death by apoptosis. Therefore, forthcoming OX1R agonists might be novel candidates for colorectal cancer therapy, which could be used alone or in combination with classical chemotherapeutic drugs such as 5-fluorouracil.[33]

Ox1r in colon cancer might be considered as a new type of gene involved in cancer, because it is aberrantly expressed as a functional protein whose function, when activated by an agonist, is to promote apoptosis of the cancer cell. The colon cancer cells unexpectedly provide a new gate to promote their death, which was not present in the normal colonocyte from which they derive.[33] Whether or not orexins may be useful in the treatment of other

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digestive cancers remains an open question, but preliminary data suggest that orexins can also promote apoptosis in pancreatic tumor cells through interaction with the OX2 receptor.[34]

After initial characterization of orexins as potent inducers of cell apoptosis,[18,27] it has been shown that streptozotocin-induced diabetes mellitus in rats, which constitutes a model of type 1 diabetes, requires the orexigenic system and OX1R to cause severe apoptosis in pancreatic islets.[1] Whether or not OX1R participates in the onset and/or outcome of type 1 diabetes in humans remains an open question.

CONCLUSIONS AND PERSPECTIVES

Orexins are certainly very important peptides in the central nervous system in health and diseases. Their role in the periphery remains much less understood in physiology. However, the discovery that orexin receptors OX1R are aberrantly expressed in human colorectal cancers opens a gate to the therapeutic induction of apoptosis in cancer cells and shows that orexins may have considerable interest for cancer research. The development of long-lived peptide agonists or nonpeptide agonists of orexin receptors will represent an important advance not only in neuroscience but also in cancer research. Moreover, the identification of th e role of ITIMs in OX1R-driven apoptosis paves the way to the development of a new paradigm of G protein-coupled receptor signaling. In this respect, the presence of ITIM canonical sequences in most GPCRs[17] should encourage studies to understand the role of ITIM-driven biological responses in all classes of GPCRs.

REFERENCES

1. Adeghate E, Fernandez-Cabezudo M, Hameed R, El-Hasasna H, El WM, Abbas T, et al. Orexin-1 receptor co-localizes with pancreatic hormones in islet cells and modulates the outcome of streptozotocin-induced diabetes mellitus. *PLoS One* 2010;**5**(1):e8587.
2. Adeghate E, Hameed R. Mechanism of orexin B-stimulated insulin and glucagon release from the pancreas of normal and diabetic rats. *Pancreas* 2011;**40**(1):131–6.
3. Asahi S, Egashira S, Matsuda M, Iwaasa H, Kanatani A, Ohkubo M, et al. Development of an orexin-2 receptor selective agonist, [Ala(11), D-Leu(15)]orexin-B. *Bioorg Med Chem Lett* 2003;**13**(1):111–3.
4. Baccari MC. Orexins and Gastrointestinal Functions. *Curr Protein Pept Sci* 2010;**11**(2):148–55.
5. Bengtsson MW, Makela K, Sjoblom M, Uotila S, Akerman KE, Herzig KH, et al. Food-induced expression of orexin receptors in rat duodenal mucosa regulates the bicarbonate secretory response to orexin-A. *Am J Physiol Gastrointest Liver Physiol* 2007;**293**(2):G501–9.
6. Boss C, Brisbare-Roch C, Jenck F. Biomedical application of orexin/hypocretin receptor ligands in neuroscience. *J Med Chem* 2009;**52**(4):891–903.
7. Burdakov D, Jensen LT, Alexopoulos H, Williams RH, Fearon IM, O'Kelly I, et al. Tandem-pore K+ channels mediate inhibition of orexin neurons by glucose. *Neuron* 2006;**50**(5):711–22.
8. De Lecea L, Kilduff TS, Peyron C, Gao X, Foye PE, Danielson PE, et al. The hypocretins: hypothalamus-specific peptides with neuroexcitatory activity. *Proc Natl Acad Sci U S A* 1998;**95**(1):322–7.
9. Ducroc R, Voisin T, El Firar A, Laburthe M. Orexins control intestinal glucose transport by distinct neuronal, endocrine, and direct epithelial pathways. *Diabetes* 2007;**56**(10):2494–500.
10. El Firar A, Voisin T, Rouyer-Fessard C, Ostuni MA, Couvineau A, Laburthe M. Discovery of a functional immunoreceptor tyrosine-based switch motif in a 7-transmembrane-spanning receptor: role in the orexin receptor OX1R-driven apoptosis. *FASEB J* 2009;**23**(12):4069–80.
11. Fronczek R, Baumann CR, Lammers GJ, Bassetti CL, Overeem S. Hypocretin/orexin disturbances in neurological disorders. *Sleep Med Rev* 2009;**13**(1):9–22.
12. Goncz E, Strowski MZ, Grotzinger C, Nowak KW, Kaczmarek P, Sassek M, et al. Orexin-A inhibits glucagon secretion and gene expression through a Foxo1-dependent pathway. *Endocrinology* 2008;**149**(4):1618–26.
13. Heinonen MV, Purhonen AK, Makela KA, Herzig KH. Functions of orexins in peripheral tissues. *Acta Physiol (Oxf)* 2008;**192**(4):471–85.
14. Johansson L, Ekholm ME, Kukkonen JP. Multiple phospholipase activation by OX(1) orexin/hypocretin receptors. *Cell Mol Life Sci* 2008;**65**(12):1948–56.
15. Johren O, Neidert SJ, Kummer M, Dendorfer A, Dominiak P. Prepro-orexin and orexin receptor mRNAs are differentially expressed in peripheral tissues of male and female rats. *Endocrinology* 2001;**142**(8):3324–31.
16. Kirchgessner AL, Liu M. Orexin synthesis and response in the gut. *Neuron* 1999;**24**(4):941–51.
17. Laburthe M, Voisin T. The orexin receptor OX(1) R in colon cancer: a promising therapeutic target and a new paradigm in G protein-coupled receptor signalling through ITIMs. *Br J Pharmacol* 2011.
18. Laburthe M, Voisin T, El FA. Orexins/hypocretins and orexin receptors in apoptosis: a mini-review. *Acta Physiol (Oxf)* 2010;**198**(3):393–402.
19. Lang M, Soll RM, Durrenberger F, Dautzenberg FM, Beck-Sickinger AG. Structure-activity studies of orexin a and orexin B at the human orexin 1 and orexin 2 receptors led to orexin 2 receptor selective and orexin 1 receptor preferring ligands. *J Med Chem* 2004;**47**(5):1153–60.
20. Mieda M, Sakurai T. Integrative physiology of orexins and orexin receptors. *CNS Neurol Disord Drug Targets* 2009;**8**(4):281–95.
21. Miyasaka K, Masuda M, Kanai S, Sato N, Kurosawa M, Funakoshi A. Central Orexin-A stimulates pancreatic exocrine secretion via the vagus. *Pancreas* 2002;**25**(4):400–4.
22. Nowak KW, Mackowiak P, Switonska MM, Fabis M, Malendowicz LK. Acute orexin effects on insulin secretion in the rat: in vivo and in vitro studies. *Life Sci* 2000;**66**(5):449–54.
23. Ohno K, Sakurai T. Orexin neuronal circuitry: role in the regulation of sleep and wakefulness. *Front Neuroendocrinol* 2008;**29**(1):70–87.
24. Ouedraogo R, Naslund E, Kirchgessner AL. Glucose regulates the release of orexin-a from the endocrine pancreas. *Diabetes* 2003;**52**(1):111–7.
25. Peltonen HM, Magga JM, Bart G, Turunen PM, Antikainen MS, Kukkonen JP, et al. Involvement of TRPC3 channels in calcium oscillations mediated by OX(1) orexin receptors. *Biochem Biophys Res Commun* 2009;**385**(3):408–12.

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26. Putula J, Turunen PM, Jantti MH, Ekholm ME, Kukkonen JP. Agonist ligand discrimination by the two orexin receptors depends on the expression system. *Neurosci Lett* 2011;**494**(1):57–60.
27. Rouet-Benzineb P, Rouyer-Fessard C, Jarry A, Avondo V, Pouzet C, Yanagisawa M, et al. Orexins acting at native OX(1) receptor in colon cancer and neuroblastoma cells or at recombinant OX(1) receptor suppress cell growth by inducing apoptosis. *J Biol Chem* 2004;**279**(44):45875–86.
28. Sakurai T, Amemiya A, Ishii M, Matsuzaki I, Chemelli RM, Tanaka H, et al. Orexins and orexin receptors: a family of hypothalamic neuropeptides and G protein-coupled receptors that regulate feeding behavior. *Cell* 1998;**92**(4):573–85.
29. Scammell TE, Winrow CJ. Orexin receptors: pharmacology and therapeutic opportunities. *Annu Rev Pharmacol Toxicol* 2011;**51**:243–66.
30. Stanley S, Pinto S, Segal J, Perez CA, Viale A, DeFalco J, et al. Identification of neuronal subpopulations that project from hypothalamus to both liver and adipose tissue polysynaptically. *Proc Natl Acad Sci U S A* 2010;**107**(15):7024–9.
31. Takahashi N, Okumura T, Yamada H, Kohgo Y. Stimulation of gastric acid secretion by centrally administered orexin-A in conscious rats. *Biochem Biophys Res Commun* 1999;**254**(3):623–7.
32. Voisin T, El Firar A, Rouyer-Fessard C, Gratio V, Laburthe M. A hallmark of immunoreceptor, the tyrosine-based inhibitory motif ITIM, is present in the G protein-coupled receptor OX1R for orexins and drives apoptosis: a novel mechanism. *FASEB J* 2008.
33. Voisin T, El FA, Fasseu M, Rouyer-Fessard C, Descatoire V, Walker F, et al. Aberrant expression of OX1 receptors for orexins in colon cancers and liver metastases: an openable gate to apoptosis. *Cancer Res* 2011;**71**(9):3341–51.
34. Voisin T, Firar AE, Avondo V, Laburthe M. Orexin-induced apoptosis: the key role of the seven-transmembrane domain orexin type 2 receptor. *Endocrinology* 2006;**147**(10):4977–84.
35. Voisin T, Rouet-Benzineb P, Reuter N, Laburthe M. Orexins and their receptors: structural aspects and role in peripheral tissues. *Cell Mol Life Sci* 2003;**60**(1):72–87.

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Chapter 75

Somatostatin/NPY

Meike Körner and Jean Claude Reubi

ABSTRACT

Somatostatin and NPY are two different peptide hormones with multiple functions in neoplasia. Somatostatin exerts inhibitory effects on tumor hormonal secretion, growth, and angiogenesis, which represent the basis for the clinical use of long-acting somatostatin analogs in tumor therapy. Conversely, emerging results show variable effects of NPY on tumor cell proliferation, migration, hormonal release, and angiogenesis of unknown significance *in vivo*. Moreover, many tumors overexpress enough somatostatin receptors to be targeted with radioactive and cytotoxic somatostatin analogs for diagnostic and therapeutic purposes. Similarly, an increasing number of tumors highly overexpress NPY receptors, which may also be targeted *in vivo* with adequate NPY analogs.

INTRODUCTION

The two peptides discussed in this chapter do not have much in common at first glance. Physiologically, they control and regulate different biological systems in the body. Historically, somatostatin was discovered in 1973,[2] whereas NPY was first reported in 1982.[33] This one decade implies that much more information is available on the somatostatin than on the NPY systems, not only in general biology but also regarding clinical applications. In particular, the relation between somatostatin and cancer has been known for a relatively long time, whereas NPY has only recently been linked to neoplasia. Nevertheless, there are numerous converging features appearing that suggest that the very young NPY cancer field may learn and strongly be influenced by the decades' experience of the somatostatin cancer field (Table 1).

SOMATOSTATIN

Somatostatin and Cancer

Somatostatin is a peptide hormone that is widely distributed in nervous, endocrine, gastrointestinal, and other systems of the human body where it has mainly inhibitory regulative functions (see the Somatostatin chapters in the Brain and Gastrointestinal Peptides sections of the book). These inhibitory actions of somatostatin are not only restricted to physiologic processes but they also play a role in neoplasia, for which mainly three mechanisms have to be considered: the inhibition of tumoral hormone secretion, the direct and indirect inhibition of tumor cell proliferation, and the inhibition of tumoral angiogenesis. These aspects are of current interest, as they represent one molecular basis for the clinical use of somatostatin in tumor therapy.

Inhibition of Hormone Release from Tumors by Somatostatin

Somatostatin strongly inhibits hormonal secretion from normal endocrine cells and their neoplastic counterparts. Thus, somatostatin and somatostatin analogs are highly effective in reducing hormonal symptoms caused by hypersecretory endocrine tumors. Therefore, they form an integral part of the routine therapy of several such tumors. For somatostatin therapy, metabolically stable and/or long-acting somatostatin analogs have been developed, such as octreotide acetate (Sandostatin®), lanreotide, and their respective long-lasting release (LAR) formulations, as native somatostatin has a very short half-life in the circulation. These somatostatin analogs are generally well tolerated and cause only mild side effects. They potently inhibit tumoral growth hormone (GH) secretion and reduce GH-related symptoms in patients suffering from GH-producing pituitary adenomas. They are also the drugs of first choice to control hormone-related symptoms in several functionally active gastroenteropancreatic neuroendocrine tumors. For instance, in patients with the carcinoid syndrome, somatostatin analogs achieve symptomatic control in 73% and a significant improvement of the quality of life.[9]

Direct and Indirect Antiproliferative Effects of Somatostatin in Tumors

More than 20 years ago, Schally et al. performed seminal studies in various animal tumor models showing that somatostatin and somatostatin analogs inhibit tumor cell proliferation.[29] This effect is probably achieved via

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00075-0
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TABLE 1 Somatostatin, NPY, their Respective Receptors and Cancer

	Somatostatin	NPY
Peptide structure	14 and 28 Amino acids	36 Amino acids
Peptide distribution	Central nervous system, gastrointestinal tract, endocrine systems	Central and peripheral nervous system, adrenal gland
Receptors	sst1, sst2A, sst2B, sst3, sst4, sst5	Y1, Y2, Y4, Y5, y6
Cancers with peptide production	Somatostatinoma, neuroblastoma, pheochromocytoma	Paraganglioma, pheochromocytoma, neuroblastoma
Cancers with receptor expression	Gastroenteropancreatic, lung, and thyroid neuroendocrine tumors, pituitary adenomas, pheochromocytomas, paragangliomas, neuroblastomas, medulloblastomas, meningiomas, breast carcinomas, prostatic carcinomas, renal cell carcinomas, gastric adenocarcinomas, lymphomas	Breast carcinomas, renal cell carcinomas, Ewing sarcomas, synovial sarcomas, GIST, gliomas, meningiomas, schwannomas, adrenal cortical tumors, pheochromocytomas, paragangliomas, ovarian sex cord-stromal tumors, pituitary adenomas, medullary thyroid carcinomas, lung and gastroenteropancreatic neuroendocrine tumors, neuroblastomas, nephroblastomas, medulloblastomas, CNS-PNET
Peptide actions on tumors	↓ Hormone release ↓ Proliferation ↓ Angioneogenesis	↓/↑ Proliferation ↓/↑ Migration ↓ Hormone release ↑ Angiogenesis
Clinical applications in oncology	- Symptomatic therapy of hormone secreting neuroendocrine tumors (S) - Diagnostic tumor visualization (D) - Radiotherapeutic tumor targeting (R) - Cytotoxic tumor therapy (C)	- Diagnostic tumor visualization (D)
Tools	- Octreotide (Sandostatin®), lanreotide (S) - ^{111}In-octreotide (OctreoScan®) (D) - ^{68}Ga-DOTA-TATE, ^{68}Ga-DOTA-NOC (D) - ^{111}In-DOTA-BASS (D) - ^{90}Y-DOTATOC, ^{177}Lu-DOTA-TATE (R) - AN-238 (C)*	- (^{99m}Tc-NαHis-ac)-NPY (D) - Daunorubicin-coupled Y1 analog*

S, symptomatic; D, diagnostic; R, radiotherapeutic; C, cytotoxic.
Preclinical use only.

several independent mechanisms. Somatostatin may indirectly reduce tumor cell proliferation via the inhibition of various physiologically occurring growth stimulating pathways such as pituitary GH and prolactin release or the GH/insulin-like growth factor (IGF)-I axis. Moreover, somatostatin may directly act on tumor cells by binding to tumoral somatostatin receptors and interfering with intracellular signaling cascades of cell cycle and apoptosis.[20,29]

The tumor growth inhibitory effect of somatostatin has been documented in many experimental models. In animals bearing pancreatic, breast, or prostate carcinomas, or chondrosarcoma or osteosarcoma, somatostatin analogs reduce tumor size and prolong survival.[29] In human tumor cell lines, including pancreatic, breast, and small-cell lung cancer (SCLC) cell lines, the antiproliferative effect of somatostatin analogs could also be reproduced. Worth mentioning are the elegant studies by Susini and collaborators, showing that the introduction of the somatostatin receptor subtype 2 (sst2) in pancreatic tumor cell lines originally devoid of sst2 lead to a marked inhibition of tumor growth, indicating the pivotal role of sst2.[4] In patients, reproducible inhibitory effects of somatostatin analogs on tumor growth are observed primarily in GH-producing pituitary adenomas; overall, 42 % of patients have a mean tumor size reduction of 50%. Unfortunately, however, results of clinical trials on other tumors are disappointing. Although a reduction of tumor size under somatostatin therapy is only very rarely observed in a few patients with advanced gastroenteropancreatic neuroendocrine tumors,[9] the more recent ProMid study by Rinke et al.[28] reports that octreotide LAR can have a substantial antitumor effect, with a longer progression-free survival compared with placebo in patients with well-differentiated neuroendocrine tumors.

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Antiangiogenic Effects of Somatostatin

Induction of new blood vessels is an important feature of malignant tumors necessary for growth and metastasis, and inhibition of tumoral angiogenesis is a promising therapeutic approach. Somatostatin acts antiangiogenically by inhibiting endothelial cell proliferation. It reduces the new formation of both normal and tumor-associated blood vessels in several experimental animal and human models. In particular, octreotide can reduce tumor vascularization *in vivo* in human breast cancer.[35]

Vascular somatostatin receptors are locally upregulated in the vicinity of tumors.[6] This is important for a somatostatin-based antiangiogenic tumor therapy, as it may allow specific targeting of tumor-associated vessels while sparing normal vessels. Although the present *in vitro* and animal tumor model results look promising, no clinical experience exists on an antiangiogenic effect of somatostatin analogs in tumor patients so far.

Molecular Signaling of Somatostatin Receptors

The multiple somatostatin effects are mediated by six different somatostatin receptor (sst) subtypes, named sst1, sst2A, sst2B, sst3, sst4, and sst5 (see the Somatostatin chapter in Brain Peptides section). The molecular signaling of sst is complex, as sst couple to various different pertussis-toxin-sensitive and pertussis-toxin-insensitive G proteins, which in turn modulate multiple interdependent intracellular signal transduction pathways.[20] Correspondingly, the effect of somatostatin on normal and tumor cell proliferation can be mediated by multiple mechanisms. Cell cycle arrest is mediated by various sst subtypes, leading for instance to activation of tyrosine phosphatases, interference with the MAP kinase ERK pathway, induction of cyclin-dependent kinase inhibitors, and accumulation of hypophosphorylated Rb protein. Moreover, the antiproliferative effect of somatostatin may also be mediated indirectly by the induction of apoptosis via several sst. Endothelial cell proliferation is regulated by sst3-mediated inhibition of endothelial nitric oxide synthase activity.[20]

Only more recently was it recognized that ssts are not only activated by somatostatin agonist binding to a single sst entity but they may also undergo complex and yet poorly understood regulating mechanisms of homooligomerization and heterooligomerization.[20] Sst oligomerization not only affects intracellular signal transduction but it also alters sst binding affinity and internalization. For instance, sst3 is completely inactivated by heterodimerization with sst2A, and sst1 internalization depends on heterodimerization with sst5. These effects have been observed only in transfected cells and have not been established in native tissues so far, in particular not in sst expressing tumors. However, as tumors often coexpress multiple sst subtypes,[23] sst heterodimerization may significantly affect binding and internalization properties of the various tumoral sst.

Somatostatin Receptors in Cancer

The discovery that sst are expressed in many malignant human tumors has led to further important clinical applications that are based on sst properties independent of the regulation of hormone release, proliferation, and angiogenesis.[23] Tumoral sst overexpression allows a highly effective *in vivo* tumor targeting for diagnostic and therapeutic purposes based on the binding and internalization of radiolabeled and cytotoxic somatostatin analogs to sst.[19,21,31]

Somatostatin Receptor Expression in Human Tumors

Sst are expressed on the tumor cell surface in a broad spectrum of human cancers. As expected from the sst distribution in nonneoplastic tissues, the sst expression is very high in (neuro-)endocrine and brain tumors, particularly in gastroenteropancreatic and bronchial neuroendocrine tumors, pheochromocytomas, paragangliomas, GH-producing pituitary adenomas, neuroblastomas, medulloblastomas, meningiomas, medullary thyroid carcinomas, and SCLC. By comparison, ssts are expressed in lower incidence and density in many common epithelial tumors (breast carcinomas, prostatic carcinomas, renal cell carcinomas, gastrointestinal adenocarcinomas, hepatocellular carcinomas, ovarian carcinomas), lymphomas, and mesenchymal tumors.[23]

Ssts are also overexpressed in tumor-associated blood vessels, in particular in peritumoral veins and, more rarely, in intratumoral blood vessels, as compared with normal blood vessels in corresponding nonneoplastic tissues.[6] The degree of vascular sst expression varies according to the tumor type. Tumor-associated vascular sst expression is important because it may also render tumors that do not express sst on tumor cells[35] responsive to *in vivo* sst targeting (see below).

The six sst subtypes are differentially and often concomitantly expressed in tumors. However, sst2A is by far the most common and most predominant subtype both on tumor cells and in tumor-associated blood vessels.[23,35] This is of great clinical advantage because sst2A, compared with other sst subtypes, has a high affinity for the commercially available somatostatin analogs.

Moreover, because sst2A is the most abundant somatostatin receptor subtype, specific antibodies against sst2A have been developed for immunohistochemical sst2A detection in tumors. Antibodies such as R2–88 or UMB-1 have been able to localize sst2A at the cell membrane of neuroendocrine tumor cells.[17,25] An example is shown in Figure 1. This histopathological measurement is of great importance

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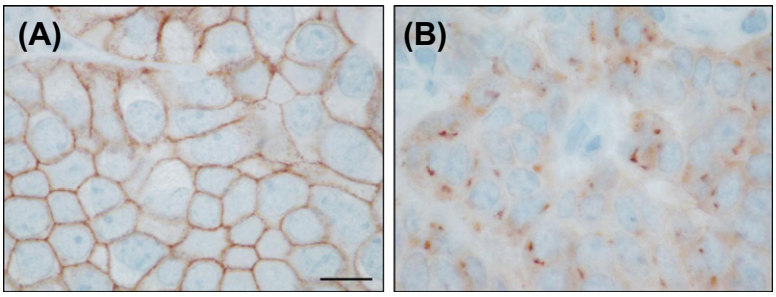

FIGURE 1 Immunohistochemical detection of somatostatin receptors in human tumors using the sst2A-specific antibody UMB-1. A shows the membrane-bound receptors in an neuroendocrine tumor from an octreotide-naive patient, B shows internalized sst2A in a tumor from an octreotide-treated patient. Bar = 0.01 mm. See color plate 21.

for the clinical management of the neuroendocrine tumor patients. Further, in patients under octreotide therapy, the sst2A receptors are trafficking from the membrane to the inside of the tumor cells, a mechanism that appears to be extremely potent and of great biological relevance.[27] Such an sst2 internalization is triggered by somatostatin agonists but not by antagonists. An example of internalized sst2 in a neuroendocrine tumor of an octreotide-treated patient is seen in Figure 1.

Somatostatin Receptor-Targeted Tumor Imaging

Sst overexpressing tumors can be specifically visualized with scintigraphy using radioactively labeled somatostatin analogs such as ^{111}In-DTPA-octreotide (OctreoScan®). This method is very effective in detecting tumors with high and homogeneous sst expression and therefore became the gold standard method for localizing and staging gastroenteropancreatic neuroendocrine tumors. Compared with computed tomography and ultrasound, OctreoScan® often shows more lesions and thus modifies tumor staging and changes surgical strategy in a substantial number of cases. For the detection of gastrinomas, OctreoScan® is superior to all other imaging techniques.

Although ^{111}In-DTPA-octreotide has been used for >15 years in the diagnosis and staging of sst-positive tumors, its performance has been compared with new SPECT and PET tracers based on ^{99m}Tc and ^{68}Ga. For instance, ^{68}Ga-based PET tracers are widely used in Europe, in particular ^{68}Ga-DOTA-TOC, ^{68}Ga-DOTA-TATE, and ^{68}Ga-DOTA-NOC; the latter has affinity for sst2, sst3, and sst5 resulting in a somewhat higher diagnostic sensitivity than ^{68}Ga-DOTA-TATE, which has the highest sst2 affinity of all these tracers.[26] Several studies comparing the ^{68}Ga-based peptides with ^{111}In-DTPA-octreotide show the distinctly higher sensitivity of the PET tracers.[7]

Although the past decade has led to the development of a large number of excellent somatostatin agonist radioligands, more recently, many observations have challenged this strategy. Potent somatostatin receptor antagonists, known to poorly internalize into tumor cells, can visualize tumors *in vivo* as well, or even better than agonists.[8] This unexpected phenomenon was found for sst2- and sst3-selective somatostatin analogs. It may result from the binding of the antagonist to a larger number of receptor sites and from its lower dissociation rate from the receptors. Figure 2 shows that the ^{177}Lu-DOTA-BASS antagonist binds to many more sst2 sites in a neuroendocrine tumor than the ^{177}Lu-DOTA-TATE agonist does. A pilot clinical trial with an ^{111}In-DOTA-linked sst2 antagonist confirms these results.[34] More potent sst2 antagonists aimed at that same purpose are in development.[3]

Somatostatin Receptor-Targeted Tumor Therapy

Somatostatin analogs labeled with cytotoxic radionuclides such as ^{111}In, ^{90}Y or ^{177}Lu, or alternatively with cytotoxic chemotherapeutic agents, may be used to specifically target tumoral sst for therapeutic purposes. They will be internalized into the cells and develop their radiotoxic and cytotoxic effects in tumor cells and tumor vessels. Such an sst targeting represents a very promising approach in cancer therapy, with the advantage of a more favorable benefit-toxicity profile compared with conventional radio- and cytotoxic therapy.[19,31] In Europe, numerous centers provide somatostatin targeted radionuclide therapy to patients suffering from metastasized or unresectable neuroendocrine tumors. The most frequently used radioactively labeled peptides are ^{90}Y-DOTA-TOC and ^{177}Lu-DOTA-TATE, and their combinations. Several Phase I and II clinical studies have been published.[19] Tumor shrinkage in almost 45%, clinical improvement in over half of the patients, as well as a median time to progression from 29 to 40 months and a median overall survival from 37 to 46 months have been reported for patients with gastroenteropancreatic neuroendocrine tumors. It is very probable that sst-targeted radiotherapy will be clinically effective also in other human tumors with high sst expression. A relative limitation of sst-targeted radiotherapy is the renal toxicity of the radionuclide compounds, a problem that has been

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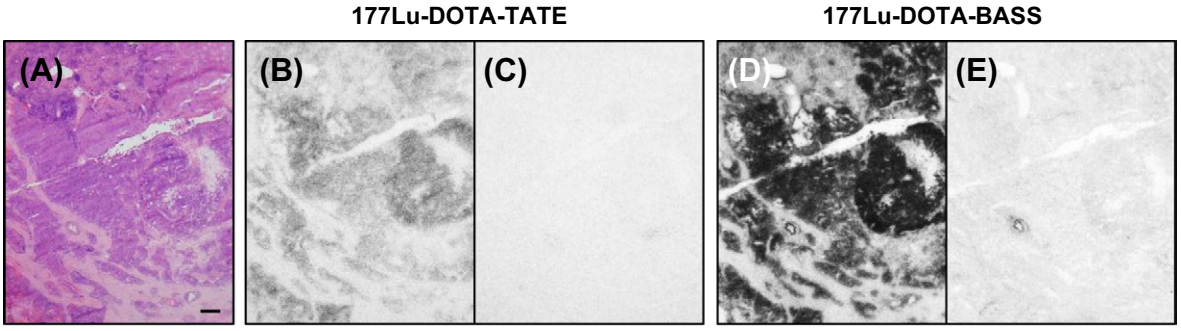

FIGURE 2 Receptor autoradiography of a somatostatin-receptor-expressing neuroendocrine tumor (A = Hematoxylin–eosin section. Bar = 1 mm.) The agonist ^{177}Lu-DOTA-TATE binds to less sst2A receptors (B) than the antagonist ^{177}Lu-DOTA-BASS (D). (C, E) Respective nonspecific binding.

given full consideration for renal protection by infusion of amino acid solutions together with the radioactively labeled tracer.

Sst-targeted cytotoxic therapy is an alternative approach unimpaired by this renal side effect. The most active compounds developed are doxorubicin derivatives coupled to somatostatin analog. They have been extensively tested in animal models, in particular in human cancer cell lines xenografted in nude mice,[30] where they achieve significant growth inhibition. More recently, first clinical trials with sst-targeted cytotoxic tumor therapy in humans with doxorubicin-coupled somatostatin analogs have been performed with encouraging results, and follow-up studies are foreseen in the near future.[30]

NPY

Neuropeptide Y (NPY) forms together with the structurally related peptide YY (PYY) and pancreatic polypeptide (PP) the NPY family of peptides (see the chapters on these peptides in the Brain, Ingestive, and Gastrointestinal Peptides sections of the book). NPY is primarily a neurotransmitter that is widely distributed in the central, peripheral, and vegetative nervous system where it regulates many diverse functions, such as feeding and anxiety behavior, reproduction, and blood pressure. Although these NPY effects have been extensively investigated and are discussed elsewhere in this book, evidence of a role of NPY in cancer has only recently been emerging.

NPY Receptors

The actions of NPY are mediated by at least five different NPY receptor subtypes that form the NPY receptor family.[22] In humans, the subtypes Y1, Y2, Y4, and Y5 are expressed, whereas a physiological correlate of y6 has not been described and the existence of the putative Y3 receptor has not been proven. All NPY receptor subtypes are members of the G-protein-coupled receptor superfamily. The NPY receptors almost universally act *via* pertussis-toxin-sensitive G_i- and G_o-proteins and inhibit adenylyl cyclase; in a restricted number of cell types they modulate calcium and potassium channels. The intracellular signaling pathways downstream to G-protein coupling are incompletely characterized and may vary depending on NPY receptor subtypes, cell lines, tissues, and species.

NPY Receptor Expression in Human Tumors

A decade ago, it was recognized for the first time that NPY receptors are expressed not only in normal tissues and in selected tumor cell lines but also at high levels in original human tumors.[24] This represents the molecular basis for potential clinical applications of NPY analogs in cancer, analogous to somatostatin receptor targeting of tumors.

NPY Receptor Expression in Tumor Cells

During the last 10 years, the NPY receptor expression has extensively been studied in a large spectrum of human tumors using *in vitro* NPY receptor autoradiography, a method that quantitatively assesses the binding site of the NPY receptor protein in tissues. NPY receptors were thus found to be variably expressed in the tumor cells of a large number of different cancer types, including epithelial, mesenchymal, brain, endocrine, and embryonal tumors (Table 1). Particularly noteworthy are breast adenocarcinomas, Ewing sarcomas, and glioblastomas because of their extremely high NPY receptor expression with respect to both incidence and density levels.[12,16,24] This is illustrated with typical examples in Figure 3. Also moderate to high NPY receptor levels are found in adrenal cortical tumors, pheochromocytomas, and paragangliomas,[13] ovarian sex cord-stromal tumors,[14] and renal cell carcinomas.[15] NPY receptors are also found in high incidence in embryonal tumors, including neuroblastomas, nephroblastomas, and medulloblastomas/CNS-PNET,[12,13,15] in accordance with NPY receptor expression during human fetal development. Conversely, in other common epithelial, endocrine, and

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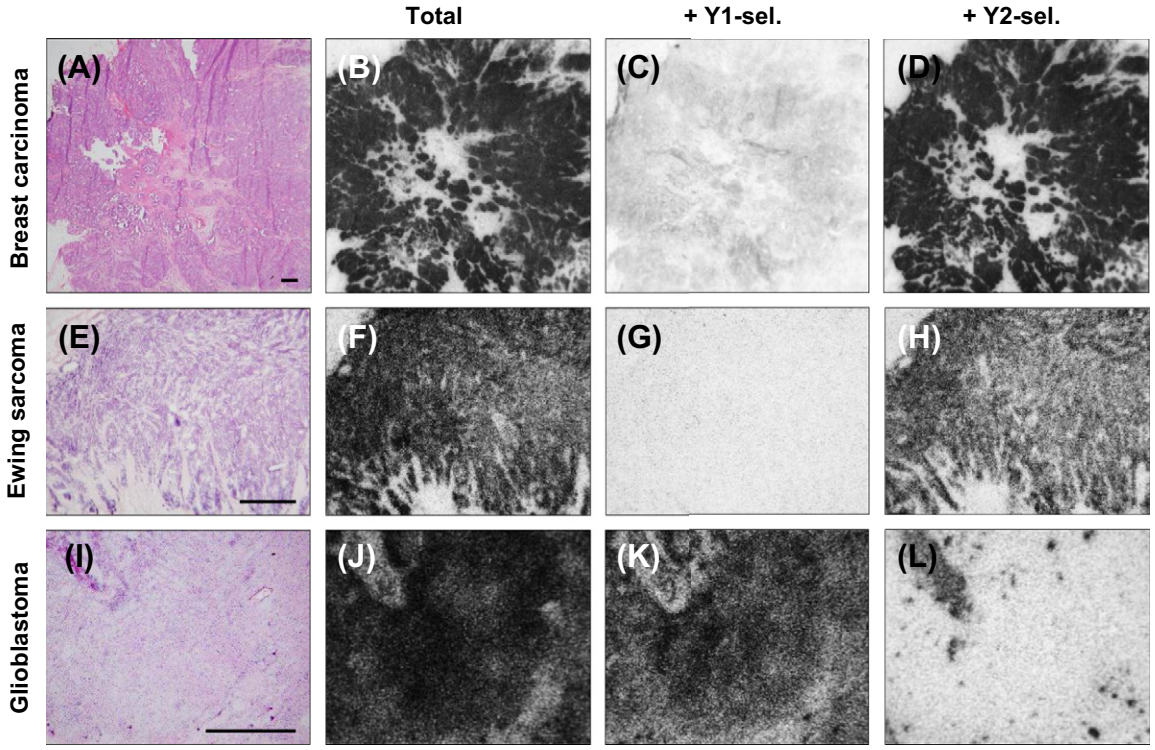

FIGURE 3 *In vitro* NPY receptor autoradiography on serial tissue sections of a breast carcinoma (A–D), an Ewing sarcoma (E–H), and a glioblastoma (I–L). A, E, I: H&E-stained tissue section showing the tumor tissues (bars = 1 mm). B, F, J: Autoradiograms showing strong total binding of ^{125}I-PYY to the tumors, corresponding to very high NPY receptor levels. C, G, K: Autoradiograms showing ^{125}I-PYY binding in the presence of 25 nM of the Y1-selective ligand [Leu^{31}, Pro^{34}]-PYY. Complete displacement of ^{125}I-PYY in the breast carcinoma (C) and the Ewing sarcoma (G) provides proof of predominant expression of the Y1 subtype. No displacement in the glioblastoma (K). D, H, L: Autoradiograms showing ^{125}I-PYY binding in the presence of 25 nM of the Y2-selective ligand PYY(3–36). No displacement in the Y1-expressing breast carcinoma (D) and Ewing sarcoma (H). Complete displacement in the glioblastoma is consistent with predominant Y2 expression (L).

mesenchymal tumors, NPY receptors have been identified in a relatively small number of cases and in low density[14,16] (Körner and Reubi, unpublished results).

NPY Receptor Expression in Intratumoral Blood Vessels

NPY receptors occur not only on tumor cells but also in intratumoral blood vessels. These vessels correspond to small arteries where the receptors are located in the muscle cells. This vascular NPY receptor expression seems to depend on the tumor type. It is particularly frequent in seminomas and liposarcomas, that is, tumors with low NPY receptor expression in tumor cells,[16,18] and also commonly observed in renal cell carcinomas and adrenal cortical adenomas, whereas it is rare or absent in breast cancers and glioblastomas.

NPY Receptor Subtypes in Tumors

Regarding NPY receptor subtypes, tumor cells express predominantly Y1 and/or Y2, and tumoral blood vessels express exclusively Y1. Although Y4 and Y5 have been detected with RT-PCR in selected tumor cell lines,[11] expression of significant amounts of these subtypes at the protein level in original tumors could be ruled out with pharmacological displacement experiments.[13,16,24] Some tumors express the same NPY receptor subtype as their nonneoplastic counterpart tissues, as is the case for adrenal cortical tumors and renal cell carcinomas. Conversely, other tumors express a different NPY receptor subtype. In particular, ductal and lobular breast carcinomas express predominantly Y1, whereas normal breast ducts and acini express Y2,[24] suggesting a switch in NPY receptor subtype expression during neoplastic transformation.

NPY Receptor Functions in Tumors

A functional role of tumoral NPY receptors is suggested by the presence of endogenous NPY in close proximity to tumoral NPY receptors in various tumor types. Indeed, NPY-containing nerve fibers are observed next to tumor cells and tumoral blood vessels particularly in renal cell carcinomas, adrenal cortical adenomas, and gliomas, suggestive of an interaction of the peripheral and central nervous system with these tumors via NPY.[12,13,15] Further,

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concomitant NPY peptide production and NPY receptor expression in tumor cells are present in pheochromocytomas, paragangliomas, and neuroblastomas, indicative of an autocrine effect of NPY on tumor cells and a paracrine action on tumor blood vessels.

Moreover, the tumoral NPY receptors could be shown to be functional using agonist-stimulated [^{35}S]-GTPγS-binding autoradiography. This method is effective in original tumor tissues if agonist binding to a G-protein-coupled receptor induces GTP binding to the receptor–ligand complex, which represents the first step in the intracellular signal transduction cascade initiated by receptor binding. It could thus be visualized that the NPY receptors in glioblastomas, neuroblastomas, and breast cancers are indeed activated by agonist binding[12] (Cescato and Reubi, unpublished results).

The functional effects of NPY on tumors, particularly on growth, invasion, hormone release, and angiogenesis, have been investigated in different tumor types *in vitro* and *in vivo* in animal models. Regarding tumor cell proliferation, NPY may exert either stimulating or inhibitory effects, possibly depending on the tumor type, the NPY receptor subtype, or the NPY concentration. An antiproliferative effect of NPY is repeatedly observed in the Y1-expressing human Ewing sarcoma cell line SK-N-MC,[11,24] in Y1-expressing human breast cancer cell lines[1] as well as in human cholangiocarcinoma cell lines via Y2.[5] Tumor growth reduction was found to be related to Y1-mediated activation of proapoptotic caspases-3/7 in Ewing sarcoma cells.[11] Conversely, stimulation of tumor growth by NPY was reported in the Y2-expressing human neuroblastoma cell line SK-N-BE(2) *via* the MAP kinase pathway.[11] Moreover, opposite effects of NPY on tumor cell migration were described. NPY inhibited migration of a Y2-expressing cholangiocarcinoma cell line,[5] whereas it stimulated it in a Y5-expressing breast cancer cell line.[32] Further, NPY may possibly affect hormone production or release from tumor cells, based on the high NPY receptor expression in steroid hormone producing tissues.

NPY may modulate not only tumor cell functions but also tumoral blood supply via several pathways. First, NPY may stimulate angiogenesis. This has extensively been investigated in normal and ischemic nonneoplastic tissues, where it may require complex activation of several NPY receptors, including 1 and Y2. Similarly, it was repeatedly shown that NPY may also induce tumoral angiogenesis: Human Ewing sarcoma and neuroblastoma cell lines xenografted in mice show increased tumor vascularity after treatment with NPY.[11] Second, it may also be speculated that NPY may have a negative effect on tumoral blood perfusion. Indeed, NPY physiologically exerts potent vasoconstrictive actions on arterial blood vessels via Y1. Similar effects may occur in intratumoral small arteries that often exhibit a prominent Y1 expression and, moreover, are accompanied by NPY-containing nerve fibers.

Of importance, the data on possible NPY-receptor-mediated actions of NPY in tumors are derived from cell line models. The *in vivo* significance of tumoral NPY receptors in humans is presently unknown.

In vivo NPY-Receptor Targeting of Tumors

By analogy to somatostatin receptor targeting of tumors, the use of NPY analogs to target NPY receptors for tumor diagnostics and therapy has been proposed. As the effects of NPY itself on tumor biology such as growth or hormone release are not yet fully elucidated, a tumor targeting with NPY analogs coupled with a radioactively labeled nuclide or a chemotherapeutic agent is more timely and promising. First choice candidate tumors for such applications are breast carcinomas, glioblastomas, and Ewing sarcomas based on their very high NPY receptor levels in the tumor cells. These tumors seem to be suitable for an NPY-receptor-targeted imaging due to the favorable tumor-to-background ratio of NPY receptor levels at the sites of tumor origin and at common sites of metastasis such as lymph nodes, liver, and lung[12,24] (Körner and Reubi, unpublished results). NPY analogs suitable for this purpose are developed in increasing numbers. An NPY analog labeled with ^{99m}Tc has been tested in the first clinical pilot study of NPY receptor targeting in breast carcinoma patients.[10] The preliminary results are favorable: primary tumors and metastases could be visualized, and no side effects occurred. Thus, the proof of principle of NPY receptor targeting of tumors is provided. Moreover, this also represents the basis for an NPY receptor-targeted therapy using NPY analogs coupled with either a cytotoxic radionuclide or a chemotherapeutic agent. Another attractive strategy for therapy may represent the targeting of tumor blood vessels, alone or together with NPY-receptor-expressing tumor cells. Finally, as many of the NPY receptor-expressing tumors can express multiple peptide receptors concomitantly, NPY receptors may be suitable for a multireceptor targeting with a cocktail containing NPY and other therapeutic peptide analogs directed against various peptide hormone receptors. For such a multireceptor approach, good candidate tumors seem to be breast tumors targeted with NPY and bombesin analogs.

REFERENCES

1. Amlal H, Faroqui S, Balasubramaniam A, Sheriff S. Estrogen up-regulates neuropeptide Y Y1 receptor expression in a human breast cancer cell line. *Cancer Res* 2006;**66**:3706–14.
2. Burgus R, Ling N, Butcher M, Guillemin R. Primary structure of somatostatin, a hypothalamic peptide that inhibits the secretion of pituitary growth hormone. *Proc Natl Acad Sci USA* 1973;**70**:684–8.
3. Cescato R, Erchegyi J, Waser B, Piccand V, Maecke HR, Rivier JE, et al. Design and in vitro characterization of highly sst2-selective somatostatin antagonists suitable for radiotargeting. *J Med Chem* 2008;**51**:4030–7.

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4. Delesque N, Buscail L, Esteve JP, Saint-Laurent N, Muller C, Weckbecker G, et al. sst2 somatostatin receptor expression reverses tumorigenicity of human pancreatic cancer cells. *Cancer Res* 1997; **57**:956–62.
5. DeMorrow S, Onori P, Venter J, Invernizzi P, Frampton G, White M, et al. Neuropeptide Y inhibits cholangiocarcinoma cell growth and invasion. *Am J Physiol Cell Physiol* 2011;**300**:C1078–89.
6. Denzler B, Reubi JC. Expression of somatostatin receptors in peritumoral veins of human tumors. *Cancer* 1999;**85**:188–98.
7. Gabriel M, Decristoforo C, Kendler D, Dobrozemsky G, Heute D, Uprimny C, et al. 68Ga-DOTA-Tyr3-octreotide PET in neuroendocrine tumors: comparison with somatostatin receptor scintigraphy and CT. *J Nucl Med* 2007;**48**:508–18.
8. Ginj M, Zhang H, Waser B, Cescato R, Wild D, Wang X, et al. Radiolabeled somatostatin receptor antagonists are preferable to agonists for in vivo peptide receptor targeting of tumors. *Proc Natl Acad Sci USA* 2006;**103**:16436–41.
9. Hejna M, Schmidinger M, Raderer M. The clinical role of somatostatin analogues as antineoplastic agents: much ado about nothing? *Ann Oncol* 2002;**13**:653–68.
10. Khan IU, Zwanziger D, Böhme I, Javed M, Naseer H, Hyder SW, et al. Breast-cancer diagnosis by neuropeptide Y analogues: from synthesis to clinical application. *Angew Chem Int Ed Engl* 2010;**49**: 1155–8.
11. Kitlinska J, Abe K, Kuo L, Pons J, Yu M, Li L, et al. Differential effects of neuropeptide Y on the growth and vascularization of neural crest-derived tumors. *Cancer Res* 2005;**65**:1719–28.
12. Körner M, Reubi JC. NPY receptors in primary human brain tumors: over-expression in high grade tumors. *J Neuropath Exp Neurol* 2008;**67**:741–9.
13. Körner M, Waser B, Reubi JC. High expression of NPY receptors in tumors of the human adrenal gland and extraadrenal paraganglia. *Clin Cancer Res* 2004;**10**:8426–33.
14. Körner M, Waser B, Reubi JC. Neuropeptide Y receptor expression in human primary ovarian neoplasms. *Lab Invest* 2004;**84**:71–80.
15. Körner M, Waser B, Reubi JC. Neuropeptide Y receptors in renal cell carcinomas and nephroblastomas. *Int J Cancer* 2005;**115**:734–41.
16. Körner M, Waser B, Reubi JC. NPY receptors in human sarcomas: high expression levels of Y1 in Ewing sarcoma family of tumors. *Clin Cancer Res* 2008;**14**:5043–9.
17. Körner M, Waser B, Schonbrun A, Perren A, Reubi JC. Somatostatin receptor subtype 2a immunohistochemistry using a new monoclonal antibody selects tumors suitable for in vivo somatostatin receptor targeting. *Am J Surg Pathol* 2012;**36**:242–52.
18. Körner M, Waser B, Thalmann GN, Reubi JC. High expression of NPY receptors in the human testis. *Mol Cell Endocrinol* 2011;**337**: 62–70.
19. Kwekkeboom DJ, de Herder WW, Kam BL, van Eijck CH, van Essen M, Kooij PP, et al. Treatment with the radiolabeled somatostatin analog [177 Lu-DOTA 0, Tyr3]octreotate: toxicity, efficacy, and survival. *J Clin Oncol* 2008;**26**:2124–30.
20. Lahlou H, Guillermet J, Hortala M, Vernejoul F, Pyronnet S, Bousquet C, et al. Molecular signaling of somatostatin receptors. *Ann N Y Acad Sci* 2004;**1014**:121–31.
21. Maecke HR, Reubi JC. Somatostatin receptors as targets for nuclear medicine imaging and radionuclide treatment. *J Nucl Med* 2011;**52**:841–4.
22. Michel MC, Beck-Sickinger A, Cox H, Doods HN, Herzog H, Larhammar D, et al. XVI. International union of pharmacology recommendations for the nomenclature of neuropeptide Y, peptide YY, and pancreatic polypeptide receptors. *Pharmacol Rev* 1998;**50**:143–50.
23. Reubi JC. Peptide receptors as molecular targets for cancer diagnosis and therapy. *Endocr Rev* 2003;**24**:389–427.
24. Reubi JC, Gugger M, Waser B, Schaer JC. Y1-mediated effect of neuropeptide Y in cancer: breast carcinomas as targets. *Cancer Res* 2001;**61**:4636–41.
25. Reubi JC, Kappeler A, Waser B, Laissue J, Hipkin RW, Schonbrunn A. Immunohistochemical localization of somatostatin receptors sst2A in human tumors. *Am J Pathol* 1998;**153**:233–45.
26. Reubi JC, Schar JC, Waser B, Wenger S, Heppeler A, Schmitt JS, et al. Affinity profiles for human somatostatin receptor subtypes SST1–SST5 of somatostatin radiotracers selected for scintigraphic and radiotherapeutic use. *Eur J Nucl Med* 2000;**27**:273–82.
27. Reubi JC, Waser B, Cescato R, Gloor B, Stettler C, Christ E. Internalized somatostatin receptor subtype 2 in neuroendocrine tumors of octreotide-treated patients. *J Clin Endocrinol Metab* 2010;**95**:2343–50.
28. Rinke A, Muller HH, Schade-Brittinger C, Klose KJ, Barth P, Wied M, et al. Placebo-controlled, double-blind, prospective, randomized study on the effect of octreotide LAR in the control of tumor growth in patients with metastatic neuroendocrine midgut tumors: a report from the PROMID Study Group. *J Clin Oncol* 2009;**27**:4656–63.
29. Schally AV. Oncological applications of somatostatin analogues. *Cancer Res* 1988;**48**:6977–85.
30. Schally AV, Engel JB, Emons G, Block NL, Pinski J. Use of analogs of peptide hormones conjugated to cytotoxic radicals for chemotherapy targeted to receptors on tumors. *Curr Drug Deliv* 2011;**8**:11–25.
31. Schally AV, Nagy A. Chemotherapy targeted to cancers through tumoral hormone receptors. *Trends Endocrinol Metab* 2004;**15**: 300–10.
32. Sheriff S, Ali M, Yahya A, Haider KH, Balasubramaniam A, Amlal H. Neuropeptide Y Y5 receptor promotes cell growth through extracellular signal-regulated kinase signaling and cyclic AMP inhibition in a human breast cancer cell line. *Mol Cancer Res* 2010;**8**:604–14.
33. Tatemoto K. Neuropeptide Y: complete amino acid sequence of the brain peptide. *Proc Natl Acad Sci USA* 1982;**79**:5485–9.
34. Wild D, Fani M, Behe M, Brink I, Rivier JE, Reubi JC, et al. First clinical evidence that imaging with somatostatin receptor antagonists is feasible. *J Nucl Med* 2011;**52**:1412–7.
35. Woltering EA. Development of targeted somatostatin-based antiangiogenic therapy: a review and future perspectives. *Cancer Biother Radiopharm* 2003;**18**:601–9.

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Chapter 76

VIP/PACAP Receptors

Terry W. Moody and Robert T. Jensen

ABSTRACT

VIP and PACAP function in a paracrine manner in the normal brain, pituitary, as well as the digestive, cardiovascular and respiratory systems. VIP and PACAP function in an autocrine manner, however, in cancer to stimulate cellular proliferation. VIP and PACAP bind with high affinity to human breast cancer and lung cancer cell lines. $VPAC_1$ and PAC_1 receptor (R) mRNA is present in lung and breast cancer cells. VIP and PACAP increase cAMP in breast and lung cancer cells, whereas PACAP causes phosphatidyl inositol (PI) turnover leading to elevated cytosolic Ca^{2+}. VIP and PACAP cause the tyrosine phosphorylation of numerous proteins, for example, the epidermal growth factor receptor, mitogen-activated protein kinases, and focal adhesion kinase. The growth of lung cancer cells is stimulated by VIP or PACAP addition, whereas $VPAC_1$-R antagonists such as VIPhybrid or PAC_1-R antagonists such as PACAP(6–38) inhibit proliferation. These results suggest that $VPAC_1$-R and/or PAC_1-R are molecular targets for breast and lung cancer.

INTRODUCTION

Pituitary adenylate cyclase–activating polypeptide (PACAP) and vasoactive intestinal peptide (VIP) are 28 and 27 amino acid peptides, respectively, which are biologically active in the normal brain, pituitary, digestive system, cardiovascular system, and respiratory system (see the chapters in these sections of the book). VIP and PACAP function as autocrine growth factors in cancer.[21] Breast cancer patients (40,000 women in the United States die annually) and lung cancer patients (160,000 patients die annually in the United States) are traditionally treated with chemotherapeutic agents and radiation therapy.[5] Because the 5-year survival rate for lung cancer patients is only 16%, new therapeutic approaches are needed. In preclinical studies, PACAP receptor antagonists such as PACAP(6–38) or VIP receptor antagonists such as VIPhybrid (VIPhyb) inhibit the growth of breast and lung cancer cells.[23,35] These results suggest that VIP/PACAP receptors may be used to impair breast and/or lung cancer progression.

The actions of VIP and PACAP are mediated by three G protein–coupled receptors (GPCR). The human $VPAC_1$ receptor (R), which binds PACAP and VIP with high affinity, is a 457-amino acid glycoprotein.[11] It has 49% homology with the human $VPAC_2$-R, which contains 438 amino acids and binds PACAP and VIP with high affinity.[9] The PAC1-R contains 467 amino acids and binds PACAP but not VIP with high affinity.[26,30] All breast cancer cells examined contain $VPAC_1$-R.[7,34] $VPAC_1$-R and PAC_1-R immunoreactivity is present in all breast tumors examined, whereas 50% of the tumors have $VPAC_2$-R immunoreactivity.[29] $VPAC_1$-R and PAC_1-R immunoreactivity are more abundant in lung cancer biopsy specimens than $VPAC_2$-R.[2] By *in vitro* autoradiographic techniques, the $VPAC_1$-R is abundant in bladder, breast, colon, liver, lung, prostate, stomach, thyroid, and uterine cancer tumors, whereas the $VPAC_2$-R is present in stomach leiomyomas.[28] PAC_1-R predominate in glioblastoma, neuroblastoma, adrenal, and pituitary cancer.[28] Seven of 8 lung cancer cell lines examined have $VPAC_1$-R and PAC_1-R mRNA including cell line NCI-H1299, whereas only 1 cell line (NCI-H345) has $VPAC_2$-R mRNA.[18] This review will focus on $VPAC_1$-R and PAC_1-R as molecular targets in breast and lung cancer.

$VPAC_1$-R

A large protein groove may be present in the $VPAC_1$-R whereby acidic amino acids (Glu^{36} and Asp^{68}) interact electrostatically with basic VIP amino acids (Arg^{14}, Lys^{15} and Lys^{21}).[24] Substitution of Ala for VIP amino acids at positions 1, 6, 12, 14, or 23 reduce binding affinity to $VPAC_1$-R by more than 2 orders of magnitude. Substitution of Ala for VIP amino acids at positions 3, 5, 7, 10, 15, 20, or 21 reduce binding affinity to $VPAC_1$-R by more than 1 order of magnitude. The analog ($Ala^{2,8,9,11,19,24,25,27,28}$) VIP is a potent $VPAC_1$-R agonist, which is resistant to degradation.[10] A VIP analog ($Ala^{2,8,9,11,19,24,25,27}$, Norleucine^{17}, Lysine^{28})VIP, (ANK) VIP,[17] inhibits specific ^{125}I-VIP binding to lung cancer cells with an IC_{50} value of 2nM (Table 1). The topoisomerase inhibitor camptothecin (CPT) coupled to the C-terminal of (ANK)VIP with a linker (L2) inhibits specific ^{125}I-VIP binding to lung cancer cells with an IC_{50} value of 150nM.[22] (ANK)VIP-L2-CPT functions as a $VPAC_1$-R agonist that is cytotoxic for lung cancer cells. The N-terminal nonapeptide of VIP and N-terminal pentapeptide of PACAP-38 can be

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00076-2
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TABLE 1 Binding of VIP/PACAP-Like Peptides to Lung Cancer Cells

	IC_{50}, nM	
Addition	^{125}I-VIP binding	^{125}I-PACAP-27 binding
PACAP-27	5 ± 1	3 ± 0.4
PACAP(6–38)	>1000	30 ± 4
VIP	11 ± 2	>1000
VIPhyb	300 ± 40	700 ± 100
VIP-LALA-E	500 ± 50	>1000
(ANK)VIP	2 ± 0.2	>1000
(ANK)VIP-L2-CPT	150 ± 20	>1000

The IC_{50} (nM) to inhibit specific ^{125}I-VIP or ^{125}I-PACAP-27 binding to NCI-H1299 lung cancer cells is indicated. The mean value ± S.D. of 3 experiments each repeated in quadruplicate is indicated. Abbreviations: Leu, L; Ala, A; nor-Leu, N; Lys, K; ellipticine, E; camptothecin, CPT; linker 2; L2. The structures of VIP and PACAP are.
VIP: His-Ser-Asp-Ala-Val-Phe-Thr-Asp-Asn-Tyr-Thr-Arg-Leu-Arg-Lys-Gln-Met-Ala-Val-Lys-Lys-Tyr-Leu-Glu-Ser-Ile-Leu-Asn-NH2.
PACAP-27: His-Ser-Asp-Gly-Ile-Phe-Thr-Asp-Ser-Tyr-Ser-Arg-Tyr-Arg-Lys-Gln-Met-Ala-Val-Lys-Lys-Tyr-Ala-Ala-Val-Leu-NH2.
Sequence homologies relative to VIP are underlined.

deleted resulting in VIP(10–28) and PACAP(6–38), which function as antagonists for $VPAC_1$-R and PAC_1-R, respectively.[14] Chimeric peptides such as neurotensin$^{6–11}$ VIP$^{7–28}$ (VIPhyb) were developed.[23] Table 1 shows that VIPhyb is a broad spectrum antagonist that binds to $VPAC_1$-R and PAC_1-R. PG 97-269, a chimeric peptide of VIP and GHRH, has 300-fold selectivity for $VPAC_1$-R relative to $VPAC_2$-R.[25] PG 97-269 inhibits the proliferation of lung cancer cells and antagonizes the increase in cAMP caused by VIP. Subsequently, the C-terminal of the antagonist was PEGylated resulting in a long-lasting $VPAC_1$-R antagonist.[25]

When VIP agonists bind to lung cancer cells, they are rapidly internalized at 37°C. Previously, we found that the $VPAC_1$-R could deliver VIP-chemotherapeutic conjugates into breast and lung cancer cells.[17] The C-terminal of VIP was conjugated with a linker containing elipticine (VIP-LALA-E) and on addition to lung cancer cells VIP-LALA-E functions as an agonist in that it causes the elevation of cAMP and increased c-fos mRNA. VIP-LALA-E is metabolized by breast cancer enzymes with E resulting in the inhibition of topoisomerase, leading to cancer cell apoptosis. The $VPAC_1$-R is localized to the plasma membrane and nucleus of breast cancer cells.[32] Traditionally, topoisomerase inhibitors prevent unwinding of DNA in the nucleus before replication of DNA. Unfortunately, toxic side effects occur because the topoisomerase inhibitors kill both normal and cancer cells. Our strategy is to use VPAC1-R, which is enriched in cancer cells, to deliver E. Alternatively, VIP was conjugated to a linker containing camptothecin (CPT). The resulting VIP-L2-CPT binds with high affinity to lung cancer cells and functions as an agonist. VIP-L2-CPT is internalized by lung cancer cells, and the CPT is released into the cells after metabolism by lysozomes. Thus, $VPAC_1$-R can be used to deliver chemotherapeutic drugs into cancer cells. Another chapter in this section of the book by Schally's group indicates that the LHRH-R can be used to deliver doxorubicin to cancer cells.

PAC_1-R

The PAC1-R regular (Reg) form contains 467 amino acids; however, splice variants (SVs) occur in intracellular loop 3 (IC3).[30] SV-1 or the hip receptor has a 28 amino acid insert in IC3. SV-2 or the hop receptor (HOP1) has a different 28-amino acid insert in IC3. SV-3 or the hip-hop receptor has both 28 amino acid inserts in IC3. PACAP addition to cells transfected with PAC_1-R SV-2 strongly increases PI turnover, whereas cells transfected with Reg, SV-1 or SV-3 had a weaker Ca^{2+} response.[27] Also, extracellular loop 1 (EC1) deletions or SV insertions exist.[15] Ushiyama et al.[31] expressed variants of the EC1 domain (N or S form) and variants of IC3 (Reg or HOP1). The resulting 4 PAC_1-R isoforms (N/Reg, N/HOP1, S/Reg or S/HOP1) binds PACAP-27 with high affinity. N/Reg, S/Reg, or S/HOP1 but not N/HOP1 strongly increases cAMP. These results indicate that alterations in EC1 or IC3 do not affect PAC_1-R binding but do affect second messenger production. Table 1 shows that PACAP and PACAP(6–38), but not VIP, inhibit ^{125}I-PACAP-27 binding to NCI-H1299 cells with high affinity (IC_{50} = 3 and 30nM, respectively). The results indicate that biologically active $VPAC_1$-R and PAC_1-R are present on lung cancer cells.

SECOND MESSENGERS

Upon binding VIP, the $VPAC_1$ receptor interacts with a stimulatory guanine nucleotide binding protein (Gs), which activates adenylyl cyclase.[18] The addition of 10nM VIP increases the cAMP 10–30-fold within minutes after addition to human breast or lung cancer cells. The elevated cAMP activates protein kinase (PK) A, resulting in increased phosphorylation of protein substrates such as Creb.[8] Figure 1 shows that when Creb is phosphorylated, it alters gene expression. Nanomolar concentrations of VIP cause increased expression of c-fos, c-jun, and c-myc oncogenes 1h after the addition to lung or breast cancer cells.[35] After translation, the c-fos and c-jun proteins may form heterodimers and activate AP-1 sites on the 5′ regulatory region of growth factor genes such as VEGF. In this regard, 10nM VIP increased VEGF expression in lung cancer cells after 4h.[3] The VEGF may be secreted from cancer cells and bind to receptors on endothelial cells, facilitating tumor

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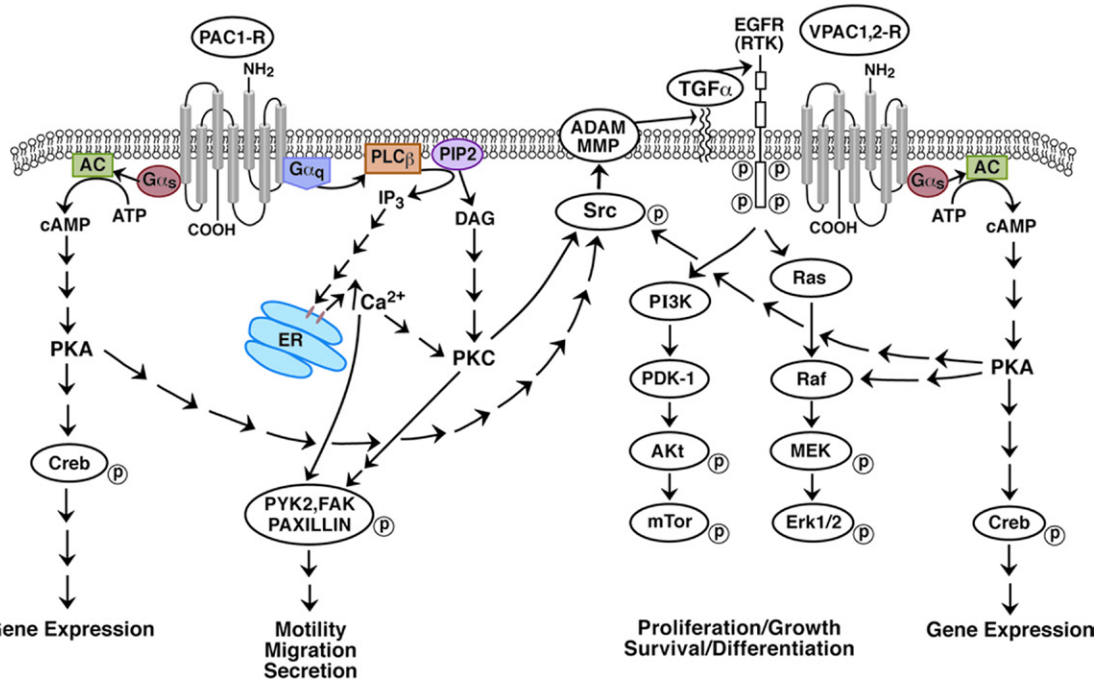

FIGURE 1 Signal transduction mechanisms for $VPAC_1$-R, $VPAC_2$-R and PAC_1-R. Activated $VPAC_1$-R and $VPAC_2$-R interacts with Gαs stimulating adenylyl cyclase (AC) leading to PKA activation, Creb phosphorylation and altered gene expression. PKA activates MMP and/or ADAM releasing TGFα from its precursor protein in the plasma membrane. VIP addition to lung or breast cancer cells causes EGFR and Her2/Neu phosphorylation. The phosphorylated EGFR causes Ras and Raf activation resulting in phosphorylation of MEK and Erk1/2 leading to proliferation. The phosphorylated EGFR causes tyrosine phosphorylation of PI3K resulting in activation of PDK-1, Akt and mTor phosphorylation leading to cancer cellular survival. Activated PAC_1-R interacts with Gαs and Gαq leading to phospholipase (PL) C stimulation and PI turnover. The IP_3 and DAG released cause the elevation of cytosolic Ca^{2+} and PKC activation, respectively. The PKC or Ca^{2+} increased tyrosine phosphorylation of FAK, PYK2, and paxillin leading to altered cellular secretion and migration.

angiogenesis. VIPhyb blocks the increase in cAMP and c-fos mRNA caused by VIP addition to lung cancer cells.[35]

When PACAP-27 binds to the PAC_1-R in NCI-H1299 cells, it causes an increase in the cAMP. Also, PACAP-27 addition to NCI-H1299 or H838 cells causes increased PI turnover (Fig. 1). The diacylglycerol (DAG) released activates PKC, whereas the inositol-1,4,5-trisphosphate (IP_3) released causes Ca^{2+} release from the endoplasmic reticulum (ER) resulting in a transient increase in cytosolic Ca^{2+}. Addition of PACAP-27 to NCI-H1299 cells increases cytosolic Ca^{2+} from 150 to 200nM within 15s.[18] The actions of PACAP-27 on cAMP and PI turnover are antagonized by addition PACAP(6–38) to lung or breast cancer cells.[14]

PROTEIN PHOSPHORYLATION

VIP and PACAP increase the tyrosine phosphorylation of a wide variety of proteins. In GH4C1 pituitary cells, VIP and PACAP increases extracellular signal-regulated kinase (Erk1/2) phosphorylation that is blocked by H89, a PKA inhibitor but not GF109203X, a PKC inhibitor.[13] Addition of PACAP-27 to NCI-H1299 lung cancer cells increases phosphorylation of Erk1/2 3-fold after 5min.[20] The increase in P-Erk1/2 caused by PACAP-27 addition to NCI-H1299 cells is inhibited by PACAP(6–38) or PD98059 (MEK inhibitor). VIP stimulates HT29 cell proliferation and induced Erk1/2 activation and activation of the small GTPase Rap1 and the 95kDa isoform of the serine/threonine kinase Raf.[1]

Figure 1 shows that VIP causes tyrosine phosphorylation of receptor tyrosine kinsases (RTK). These include the epidermal growth factor (EGF)R and HER2/Neu in MDA-MB-468 breast cancer cells.[33] The effects of VIP were reversed by JV-1-53, a nonselective antagonist of $VPAC_1$-R and $VPAC_2$-R. The transactivation of the EGFR caused by VIP is reversed by PKA inhibitors (H89) or disintegrin and metalloprotease (ADAM) inhibitor (TAPI-1).[33] Src inhibitors, e.g. PP2, prevent VIP from increasing tyrosine phosphorylation of the EGFR. Figure 1 shows that Src may be activated by PKA or PKC. The results suggest that VIP addition to breast cancer cells may cause release of an endogenous EGFR ligand, for example, transforming growth factor (TGF)α resulting in the tyrosine phosphorylation of the EGFR. When TGFα binds to the EGFR, PI 3K is phosphorylated leading to activation of PDK-1. Figure 1 shows that Akt and mTor are then phosphorylated increasing cancer cell survival.

The ability of PACAP to cause transactivation of the EGFR in lung cancer cells is reversed by gefitinib, an EGFR tyrosine kinase inhibitor (T. Moody, unpublished). Further,

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the EGFR (gefitinib) or HER2/Neu (trastuzumab) tyrosine kinase inhibitors reduces the growth of SK-BR-3 and BT-474 breast cancer cells.[4] Because the EGFR and HER2/Neu can form heterodimers, VIP or PACAP can result in the tyrosine phosphorylation of the EGFR and HER2/Neu. These results suggest that some of the growth effects of VIP and PACAP may result from RTK transactivation.

VIP and PACAP may function as promoters of lung carcinogenesis. Subsequently, lung cancer cells undergo metastasis to other organs such as the liver, lymph nodes, bone, and/or brain. Metastasis is increased by activation of protein tyrosine kinase (PYK) 2B and focal adhesion kinase (FAK). PACAP-27 causes paxillin and PYK2 tyrosine phosphorylation in human monocytes.[6] PACAP addition to monocytes significantly increases hydrogen peroxide and superoxide production, which is reduced by the phospholipase C inhibitor U73122.[6] U73122 addition to monocytes blocks PI turnover, reducing the ability of PACAP to increase PYK2 or paxillin tyrosine phosphorylation [T. Moody, unpublished]. PYK2 is a cytosolic tyrosine kinase that interacts with the SH2 domain of Src, whereas paxillin coordinates Rho family GTPases, which regulate the actin skeleton (Fig. 1). PACAP modulates NMDA receptors in CA1 hippocampal neurons as a result of Src tyrosine phosphorylation.[16] The results suggest that PACAP causes PI turnover and that both PKC activation and Ca^{2+} can stimulate Src. Activation of Src results in phosphorylation of FAK on multiple residues, causing an increase in FAK activity and its recruitment to the actin cytoskeleton. G-proteins can also activate the GTPase, Rho, which participates in cytoskeletal reorganization and tyrosine phosphorylation of FAK. When these cascades are activated, actin stress fiber formation occurs and resulting in phosphorylation of cytoskeletal proteins paxillin, p130Cas and Crk. Proteins recruited to the multimolecular signaling complex include GSK, SRK, paxillin, Grb2/Sos, p130Cas/Crk, and PI 3K. PACAP addition to PC12 pheochromocytoma cells causes phosphorylation of the tyrosine kinase TrkA, which was reversed by K252a, a tyrosine kinase inhibitor, and PP1, an inhibitor of Src.[12] The results indicate that VIP and PACAP cause the tyrosine phosphorylation of a wide variety of proteins.

PROLIFERATION

VIP or PACAP stimulate the clonal growth of breast or lung cancer cells.[18] The increase in growth is reversed by VIPhyb.[19] VIPhyb, however, inhibits the basal growth of breast or lung cancer cells. These results suggest that VIP and/or PACAP are autocrine growth factors for breast and lung cancer cells. Taxol, which interacts with microtubules, inhibited the proliferation of MDA-MB-231 breast cancer cells and was synergistic with VIPhyb at inhibiting breast cancer cellular proliferation. Table 2 shows that 1μM VIPhyb reduced the clonal growth of MCF7 cells to 74%, whereas 3ng/ml taxol reduced the clonal growth to 34%. The combination of VIPhyb and taxol reduced MCF7 growth to 27%. In nude mice bearing MDA-MB-231 xenografts *in vivo*, 0.4mg/kg of VIPhyb injected daily reduces tumor volume by 25% and 6mg/kg taxol reduces tumor volume by 30%. The combination of VIPhyb + taxol reduces tumor volume by 65%. These results indicate that VIPhyb increased the potency of taxol to cause apoptosis of breast cancer cells *in vitro* and *in vivo*.

TABLE 2 Effects of VIPhyb and Taxol on Breast Cancer Proliferation

Addition	% Proliferation	
None	100 ± 9	100 ± 12
VIPhyb	74 ± 6	75 ± 9
Taxol	34 ± 4	70 ± 8
VIPhyb + Taxol	27 ± 3	35 ± 5

The % proliferation of MDA-MB-231 was determined *in vitro* using the clonogenic assay and 0.1μM VIPhyb and/or 3ng/ml taxol. The mean value ± S.D. of 4 determinations each repeated in triplicate is indicated. The % proliferation was calculated based on *in vivo* data using nude mice with MDA-MB-231 tumors. VIPhyb (0.4mg/kg) was injected daily s.c, whereas taxol (6mg/kg) was injected daily i.p. The mean value ± S.D. of 6 determinations is indicated 5 weeks after drug treatment.

SUMMARY

VIP and PACAP are peptide growth factors synthesized in breast and lung cancer cells. After secretion, they bind to cell surface $VPAC_1$-R and/or PAC_1-R leading to second messenger production. The $VPAC_1$-R interacts with Gs leading to increased cAMP, which activates PKA. The PKA may activate Src and/or ADAM leading to EGFR and HER2/Neu tyrosine phosphorylation. Thus, GPCR can transactivate RTK leading to increased breast cancer and lung cancer proliferation. These results suggest that some of the effects of VIP on proliferation are mediated by RTK. It remains to be determined if $VPAC_1$-R antagonists and RTK inhibitors are synergistic at inhibiting breast cancer proliferation.

The PAC_1-R signaling pathway involves PI turnover in addition to adenylyl cyclase stimulation. After metabolism by PLC, IP_3 plus DAG result leading to increased lung cancer cytosolic Ca^{2+} and PKC activity, respectively. Src is activated resulting in tyrosine phosphorylation of numerous proteins such as FAK and paxillin, which play a role in the formation of focal adhesions. Also, SFK activation results in Raf, MEK, and Erk1/2 phosphorylation leading to lung cancer proliferation. It remains to be determined if PAC_1-R antagonists and Src inhibitors are synergistic at inhibiting tumor growth.

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$VPAC_1$-R antagonists are synergistic with chemotherapeutic agents at inhibiting breast and lung cancer proliferation. Alternatively, VIP-chemotherapeutic conjugates were synthesized, which were internalized into breast and lung cancer cells containing $VPAC_1$-R by receptor-mediated endocytosis. New VIP-chemotherapeutic conjugates need to be developed, which bind with high affinity to VPAC1-R and are resistant to degradation by blood proteases.

The $VPAC_1$-R is present in high densities (100,000 per cell) in almost all breast and lung cancer cell lines examined. ^{123}I and ^{99m}Tc-labeled VIP analogs have been used for imaging tumors in colon and breast cancer patients. It remains to be determined whether a radioactively labeled VIP analog can be used for early detection of breast and/or lung cancer. Alternatively, the $VPAC_1$-R can be used to deliver VIP-chemotherapeutic conjugates into breast and lung cancer cells. In preclinical studies, VIP-L2-CPT inhibited the growth of lung cancer tumors, and it remains to be determined whether VIP-L2-CPT inhibits tumor growth in clinical studies.

ACKNOWLEDGMENTS

The authors thank Drs. David Coy, Julius Leyton, and Samuel Mantey for numerous contributions. This research is supported in part by intramural funds of the NCI and NIDDK of NIH.

REFERENCES

1. Alleaume C, Eychene A, Caigneaux E, Muller JM, Philippe M. Vasoactive intestinal peptide stimulated proliferation in HT29 human colonic adenocarcinoma cells: concomitant activation of Ras/Rap1-B-Raf-ERK signaling pathway. *Neuropeptides* 2003;**37**:98–104.
2. Busto R, Prieto JC, Bodega G, Zapatero J, Fogue L, Carrero I. VIP and PACAP receptors coupled to adenylyl cyclase in human lung cancer: a study in biopsy specimens. *Peptides* 2003;**24**:429–36.
3. Casibang MC, Purdom S, Zia R, Jakowlew S, Neckers L, Ben-Av P, et al. Prostaglandin E_2 and VIP increase VEGF mRNAs in lung cancer cells. *Lung Cancer* 2001;**31**:203–12.
4. D'Alessio A, De Luca A, Maiello MR, Lamura L, Rachiglio AM, Napolitano M, et al. Effects of the combined blockade of EGFR and ErbB-2 on signal transduction and regulation of cell cycle regulatory proteins in breast cancer cells. *Breast Cancer Res Treat* 2010;**123**:387–96.
5. DeVita VT, Hellman S, Rosenberg SA. Cancer. *Principles and practice of oncology*. 6th ed. Philadelphia: Lippincott, Williams and Wilkins; 2001.
6. El Zein N, Corazza F, Sariban E. The neuropeptide pituitary adenylate cyclase activating protein is a physiological activator of human monocytes. *Cell Signal* 2006;**18**:162–73.
7. Gespach C, Bawab W, Decremoux P, Calvo F. Pharmacology, molecular identification and functional characteristics of vasoactive intestinal peptide receptors in human breast cancer cells. *Cancer Res* 1988;**48**:5079–83.
8. Guttierrez-Canas I, Juarranz MG, Collado B, Rodriguez-Hence N, Chiloeches A, Prieto JC, et al. Vasoactive intestinal peptide induces neuroendocrine differentiation in the LNCaP prostate cancer cell line through PKA, ERK and PI3K. *Prostate* 2005;**63**:44–55.
9. Harmar T, Lutz E. Multiple receptors for PACAP and VIP. *Trends Pharmacol Sci* 1993;**15**:97–9.
10. Igarashi H, Ito T, How W, Mantey SA, Pradhan TK, Ulrich II C, et al. Elucidation of vasoactive intestinal peptide pharmacophore for VPAC1 receptors in human, rat and guinea pig. *J Pharmcol Exp Ther* 2002;**301**:37–50.
11. Ishihara T, Shigemoto R, Mori K, Takahashi K, Nagata S. Functional expression and tissue distribution of a novel receptor for vasoactive intestinal polypeptide. *Neuron* 1992;**8**:811–9.
12. Lee FS, Rajagopal R, Kim AH, Chang PC, Shao MV. Activation of Trk neurotropin receptor signaling by pituitary adenylate cyclase-activating polypeptide. *J Biol Chem* 2002;**277**:9096–102.
13. Le Pechon-Vallee C, Maglalon K, Rasolonjanahary R, Enjalbert A, Gerard C. Vasoactive intestinal polypeptide and pituitary adenylate cyclase-activating polypeptide stimulate mitogen-activated protein kinase in the pituitary cell line GH4C1 by a 3′,5′-cyclic adenosine monophospate pathway. *Neuroendocrinology* 2000;**72**:46–56.
14. Leyton J, Gozes Y, Pisegna J, Coy D, Purdom S, Casibang M, et al. PACAP(6-38) is a PACAP receptor antagonist for breast cancer cells. *Breast Cancer Res Treat* 1999;**56**:177–86.
15. Lutz EM, Ronaldson E, Shaw P, Johnson MS, Holland PJ, Mitchell R. Characterization of novel splice variants of the PAC1 receptor in human neuroblastoma cells: consequences for signaling by VIP and PACAP. *Mol Cell Neurosci* 2006;**31**:193–209.
16. Macdonald DS, Weerapura M, Beazely MA, Martin L, Czerwinski W, Roder JC, et al. Modulation of NMDA receptor by pituitary adenylate cyclase activating peptide in CA1 neurons requires Gαq, protein kinase C and activation of Src. *J Neurosci* 2005;**25**:11374–84.
17. Moody TW, Czerwinski G, Tarasova NI, Michejda CJ. VIP ellipticine derivatives inhibit the growth of breast cancer cells. *Life Sci* 2001;**19**:1005–14.
18. Moody TW, Hill JM, Jensen RT. VIP as a trophic factor in the CNS and cancer cells. *Peptides* 2003;**24**:163–77.
19. Moody TW, Leyton J, Chan D, Brenneman DC, Fridkin M, Gelber E, et al. VIP receptor antagonists and chemotherapeutic agents inhibit the growth of breast cancer cells. *Breast Cancer Res Treat* 2001;**68**:55–64.
20. Moody TW, Leyton J, Casibang M, Pisegna J, Jensen RT. PACAP-27 tyrosine phosphorylates mitogen activated protein kinase and increases VEGF mRNAs in human lung cancer cells. *Reg Peptides* 2002;**109**:135–40.
21. Moody TW, Jensen RT. VIP and PACAP as autocrine growth factors in breast and lung cancer. In: Kastin A, editor. *Handbook of biologically active peptides*. : Elsevier Press; 2006. p. 493–8.
22. Moody TW, Mantey SA, Fuselier JA, Coy DH, Jensen RT. Vasoactive intestinal peptide-camptothecin conjugates inhibit the proliferation of breast cancer cells. *Peptides* 2007;**28**:1883–90.
23. Moody TW, Zia F, Brenneman D, Fridkin M, Davidson A, Gozes I. A VIP antagonist inhibits the growth of non-small cell lung cancer. *Proc Natl Acad Sci USA* 1993;**90**:4345–9.
24. Nicole P, Lins L, Rouyer-Fessard C, Drouot C, Fulcrand P, Thomas A, et al. Identification of key residues for interaction of vasoactive intestinal peptide with human $VPAC_1$ and $VPAC_2$ receptors and development of a highly selective $VPAC_1$ receptor agonist. Alanine scanning and molecular modeling of the peptide. *J Biol Chem* 2000;**275**:24003–12.
25. Pan CQ, Hamren S, Roczniak S, Tom I, DeRome M. Generation of PEGylated VPAC1-selective antagonists that inhibit proliferation of a lung cancer cell line. *Peptides* 2008;**29**:479–86.

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26. Pisegna J, Wank SA. Molecular cloning and functional expression of the pituitary adenylate cyclase activating polypeptide type I receptor. *Proc Natl Acad Sci USA* 1993;**90**:6345–9.
27. Pisegna JR, Wank SA. Cloning and characterization of the signal transduction of four splice variants of the human pituitary adenylate cyclase activating polypeptide receptor. Evidence for dual coupling to adenylate cyclase and phospholipase C. *J Biol Chem* 1996;**271**:17267–74.
28. Reubi JC, Laderach U, Waser B, Gebbers JO, Robberecht P, Laissue JA. Vasoactive intestinal peptide/pituitary adenylate cyclase-activating peptide receptor subtypes in human tumors and their tissues of origin. *Cancer Res* 2000;**60**:3105–12.
29. Schulz S, Rocken C, Mawrin C, Weise W, Hollt V, Schulz S. Immunocytochemical identification of $VPAC_1$, $VPAC_2$ and PAC_1 receptors in normal and neoplastic human tissues with subtype-specific antibodies. *Clin Cancer Res* 2004;**10**:8235–42.
30. Spengler D, Waeber C, Pantaloni C, Holsboer F, Bockaert J, Seeburg PH, et al. Differential signal transduction by five splice variants of the PACAP receptor. *Nature* 1993;**365**:170–5.
31. Ushiyama M, Ikeda R, Sugawara H, Yoshida M, Mori K, Kangawa K, et al. Differential intracellular signaling through PAC1 isoforms as a result of alternative splicing in the first extracellular domain and the third intracellular loop. *Mol Pharm* 2007;**79**:103–11.
32. Valdehita N, Bajo AM, Fernandez-Martinez AB, Arenas MI, Vacas E, Valenzyela P, et al. Nuclear localization of vasoactive intestinal peptide (VIP) receptors in human breast cancer. *Peptides* 2010;**31**:2035–45.
33. Valdehita A, Bajo AM, Schally AV, Varga JL, Carmena MJ, Prieto JC. Vasoactive intestinal peptide (VIP) induces transactivation of EGFR and HER2 in human breast cancer cells. *Mol Cell Endo* 2009;**302**: 41–8.
34. Waschek JA, Richards MI, Bravo DT. Differential expression of VIP/PACAP receptor genes in breast, intestinal and pancreatic cell lines. *Cancer Lett* 1995;**92**:143–9.
35. Zia H, Hida T, Jakowlew S, Birrer M, Gozes Y, Reubi JC, et al. Breast cancer growth is inhibited by VIP hybrid, a synthetic VIP receptor antagonist. *Cancer Res* 1996;**56**:3486–9.

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Chapter 77

Antiadhesin Peptide-Based Vaccines for *Pseudomonas aeruginosa*

Wendy J. Hartsock, Clifton Hackbarth and Robert S. Hodges

ABSTRACT

Pseudomonas aeruginosa (*Pa*), a ubiquitous Gram-negative bacterial pathogen, is responsible for a growing number of hospital-acquired infections and is highly resistant to current antibiotics. Synthetic peptide-based vaccines that target functionally relevant motifs such as the receptor binding domain (RBD) of the pilin protein of *Pa*, which is responsible for bacterial adhesion to host receptors, are a novel alternative to traditional vaccines and antibiotics to combat *Pa* infection. Structural features in the RBD are conserved across multiple strains of *Pa* making the pilin RBD an excellent candidate to design a consensus sequence immunogen. Direct comparison of a synthetic RBD peptide and its parent pilin protein shows that the peptide is able to elicit higher titer antisera against the native RBD. The consensus sequence vaccine generates highly protective antibodies in active immunization studies using a mouse model of *Pa* infection. The results show that synthetic peptides have clear advantages over traditional subunit, whole-killed, or live attenuated vaccines against *Pa*.

SIGNIFICANCE AND BACKGROUND

Pseudomonas aeruginosa (*Pa*) is an opportunistic Gram-negative pathogen that has achieved superbug status and is a member of the so-called 'ESKAPE' (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) pathogens[2] that pose an increasing threat to human health as multidrug resistant microorganisms.

Pa is a ubiquitous organism that is isolated from terrestrial and aquatic environments as well as plants and animals; it can colonize medical equipment, including ventilators, endoscopes, and implants; it can bind to and grow on stainless steel and plastics and can even grow in distilled water. *Pa* expresses multiple virulence factors that are classified as exoproducts or constitutive factors. Exoproducts, which are involved in bacterial invasion, immune suppression, and antibiotic degradation, consist of proteases, exotoxins, β-lactamases, and several other extracellular products. Constitutive factors are involved in adhesion, molecular transport, and metabolism and include the type-IV pili, flagellum, outer membrane proteins, and lipopolysaccharide. The first step in *Pa* infection is the adhesion of the bacterium to target cells by the type-IV pili. The receptor-binding domain (RBD) located in the tip of the pilus of *Pa* specifically interacts with the receptors on tracheal, buccal, and corneal epithelial cells and epidermal cells.[33] Infections are common in the respiratory tract, urinary tract, eye, and extremities of immunocompromised patients but do not occur in healthy individuals.[30] *Pa* is a major agent of nosocomial infections in the United States and is of particular burden to cystic fibrosis (CF) patients, burn victims, and soldiers injured in combat.

Common antipseudomonals include β-lactams, aminoglycosides, and fluoroquinolones; *Pa* has mechanisms to render each of these drug classes ineffective.[3,34] *Pa* possesses inherently low susceptibility to many antibiotics as a result of the low permeability of its outer membrane. It is highly adaptable and readily develops resistance to antibiotics by nearly all known mechanisms of bacterial resistance including expression of antibiotic destroying enzymes, mutation of drug targets, modification of outer membrane proteins, changes to membrane composition, and overexpression of efflux pumps. *Pa* has several intrinsic mechanisms of resistance and is also capable of acquiring resistance through transfer of genetic information.

New regimens consisting of various combinations of "old" drugs are currently used to treat multidrug resistant (MDR) strains, but the inevitable failure of our current arsenal of antibiotics to treat MDR *Pa* necessitates the discovery of new therapeutic options.[21,30] Vaccination may be the more appropriate choice to diminish *Pa* infections given the ability of *Pa* to readily develop resistance to multiple antibiotics.

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00077-4
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PSEUDOMONAS AERUGINOSA PILI AS A VACCINE TARGET

The *Pa* pilus is a key virulence factor[12] as binding to the oligosaccharide (GalNAcβ(1-4) Gal) of the target receptors (asialo-GM1 and asialo-GM2) is the first step in establishing infection. Therefore, a vaccine that elicits antibodies that target the RBD of the type-IV pili of *Pa* would prevent bacterial colonization and effectively prevent infection.

Traditional vaccines that comprise whole-killed *Pa*, live attenuated *Pa*, or whole pilin protein have shown efficacy in clinical trials.[11,31,32] However, each suffers complications arising from safety concerns and complications of production as well as the likelihood of antigenic competition and low crossreactivity. Antigenic competition results from the inhibition of the desired immune response against certain antigens or determinants when multivalent vaccines or peptide cocktails are used to immunize an individual.[5] An example of such antigenic competition was shown when multivalent vaccines targeting the type-IV pilus of *Dichelobacter nodosus* were administered to sheep to combat footrot.[16–18] Of the nine serogroups targeted by a multivalent vaccine comprised of recombinant pili, only a limited number actually elicit a robust immune response. Monovalent vaccines show superior protection against certain *D. nodosus* serogroups compared with the multivalent vaccine; the levels of antibody elicited against specific serogroups by multivalent vaccines consisting of six or more *D. nodosus* pili are reduced compared with antibody levels generated by their monovalent counterparts. Because the pili of *D. nodosus* (type-IV) are structurally similar to those of *Pa* (also type-IV), it is reasonable to assume that vaccines that target the type-IV pili of *Pa* probably also suffer from antigenic competition. This would probably occur for both traditional multivalent vaccines and synthetic peptide-based cocktail vaccines.

Thus, although synthetic peptide-based vaccines have several inherent advantages over traditional vaccines,[28] it is still necessary to design an appropriate peptide immunogen that elicits crossreactive antibodies but avoids antigenic competition. One of the main advantages of peptide vaccines is the ability to target regions of proteins that have evolved to maintain a critical function. The immunogenicities of such regions are often masked in native structures either as a result of antigenic domination by other more immunogenic regions or more accessible regions. The functional importance of these regions dramatically reduces the organism's ability to create mutations to evade antipeptide antibodies, and such mutations most probably produce dysfunctional proteins with the complete loss of function and virulence. One concern regarding peptide vaccines is that to elicit antibodies that react with the native epitope, the peptides should structurally mimic the native proteins from which they are derived and therefore may require conformational constraints to maintain the structure in the peptide immunogen. A second issue that can be potentially detrimental to the efficacy of peptide-based vaccines is the inherently low immunogenicty of small peptides. To circumvent poor immunogenicity, peptides are commonly conjugated to highly immunogenic carrier proteins such as tetanus toxiod (TT) or keyhole limpet hemocyanin (KLH).[29] The disulfide-bridged loop of the *Pa* pilin protein RBD is an intrinsically optimized epitope for the design of a synthetic peptide-based vaccine because it consists of a continuous peptide that has secondary structural elements incorporated into the sequence in the form of β-turns and a β-strand (Fig. 1). Still, an effective synthetic peptide-based vaccine

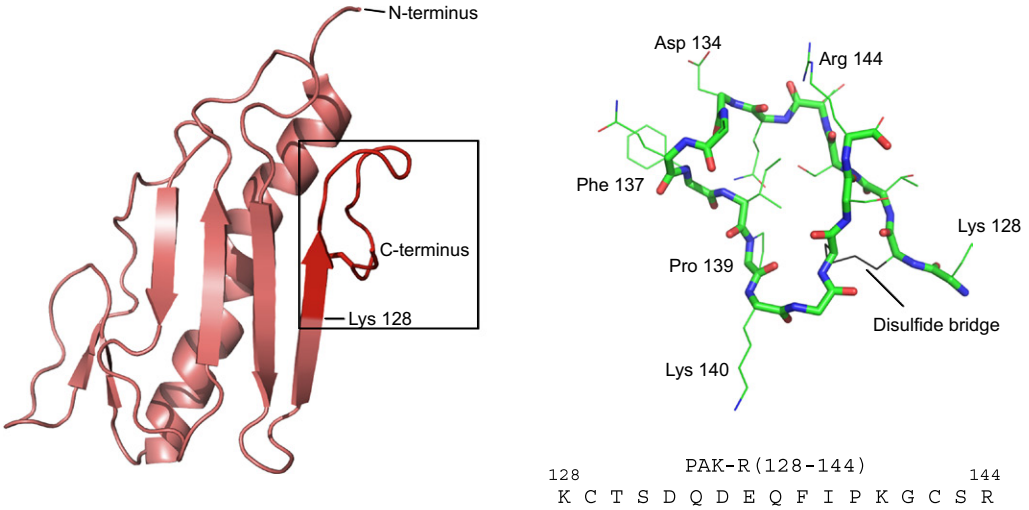

FIGURE 1 Ribbon diagram of the truncated PAK pilin protein monomer (PAK-R, residues 29–144) and the RBD is boxed (left), stick diagram of the PAK pilin RBD (PAK-R, residues 128–144) (top right) and sequence (bottom right). Residues Asp134 and Phe137 that form the type-I β-turn as well as residues Pro139, Lys140, and Cys142, which comprise the type-II β-turn and residues Lys128 and Asp132 of the β-strand are indicated along the loop (top right).

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based on the RBD must elicit antibodies that crossreact with pilin from different strains of *Pa*. Because multivalent vaccines composed of synthetic peptides targeting the pili of various *Pa* strains have the potential for antigenic competition,[5] we have focused much of our research efforts on the consensus sequence approach, which avoids antigenic competition.

The type-IV pili are filamentous structures that comprise thousands of monomeric pilin proteins arranged in a helical array whereby one turn comprises 5 protein monomers (Fig. 2).[10] Pili are polar tubes that are typically 5.2nm in outer diameter with a 1.2nm central channel and an average length of 2.5μm.[9,26] Structural elements of the type-IV pilin proteins include an N-terminal extended α-helix, a globular head region, a major β-sheet, a minor β-sheet, and a C-terminal disulfide-bridged loop (Fig. 1). The loop structure is either 14 residues or 19 residues long (including the Cys residues) depending on the *Pa* strain. The 14-residue loops, which comprise residues 129–142, consist of two Cys residues at positions 129 and 142, conserved or semiconserved residues at positions 131, 134, 137, and 139–141 and variable residues at positions 130, 132, 133, 135, 136, and 138 (Fig. 3). The N-terminus of the pilin protein monomer (residues 1–28) is responsible for oligomerization. Truncation of the pilin protein at the N-terminus allows for an X-ray structure of the *Pa* strain K (PAK) pilin (29–144),[15] which shows prominent secondary structures in the RBD loop region including a type-I β-turn that comprises residues Asp134 through Phe137, a type-II β-turn that comprises residues Pro139-Cys142 and a β-strand that comprises residues 128–132. The loop, which is exposed only at the tip of the pilus, is involved in pilus assembly and importantly in receptor recognition and surface binding.[13,22,24,33] The structural elements in the RBD are conserved in pilin proteins of various strains, specifically, the β-turns of the C-terminal disulfide loop.[1] NMR solution structures of 17 residue peptides of PAK,[25] PAO and KB7[7] pilin (128–144) and a 23-residue peptide from P1 pilin (126–148)[8] showed that all these peptides possessed consecutive type-I and type-II β-turns. The diversity of sequence of the RBDs was addressed by obtaining sequence information from 100 clinical *Pa* isolates with 14-residue disulfide loop regions. The sequence of the five native strains shown in Fig. 3, represents essentially 100% of the residues found in the RBDs at each position from 128 to 144. There is limited diversity in the six variable positions shown in Fig. 3 (positions 130, 132, 133, 135, 136, and 138). Only 3 different residues are observed at positions 130, 132, 133, and 136 and only 4 different residues at positions 135 and 138. These results further support the idea that the development of a consensus sequence vaccine is feasible.

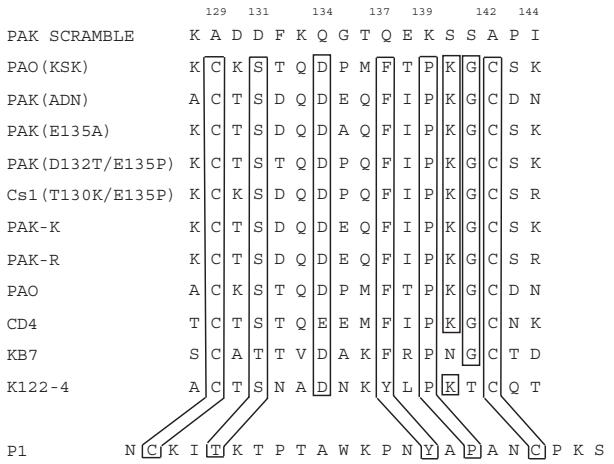

FIGURE 3 Amino acid sequences of the PAK scramble peptide, PAO(KSK) and PAK(ADN) mutants, Ala substituted PAK analog (E135A) and disubstituted PAK analog (D132T/E135P), the consensus peptide (Cs1: T130K/E135P) and seven *Pa* strains (PAK-K, PAK-R, PAO, CD4, KB7, K122-4, and P1). The sequences of PAK-K and PAK-R are identical to the exception of residue 144, which is Lys in PAK-K and Arg in PAK-R. The scramble peptide, Ala substituted analog and PAO(KSK) mutant were designed based on the PAK-K sequence, whereas the Cs1 peptide was designed from the PAK-R sequence. The boxed residues correspond to identical positions in the sequences and conservative amino acid substitutions.

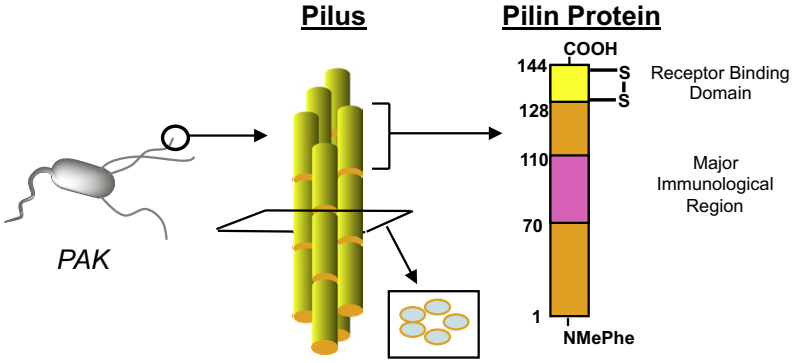

FIGURE 2 Cartoon diagram of PAK showing pili, which are located on the surface of the bacterium (left) and a magnified view of the pilus (middle), which consists of pilin protein monomers arranged in a helical array with five-fold-symmetry (cross section). The C-terminus of the pilin protein monomer (right) houses the RBD between residues 128 and 144. The major immunological region is located between the residues 70 and 110.

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SYNTHETIC CONSENSUS SEQUENCE PEPTIDE-BASED *PSEUDOMONAS AERUGINOSA* VACCINES

The antibodies to the RBD of the pilin protein are able to block and prevent the infection process.[23] Alanine substitution of the peptides representing the RBD of *Pa* strains PAK and KB7 were used to map the residues responsible for adhesion to the target receptor (known as adhesintopes).[35] NMR solution structures of these regions show that although there was sequence variation between the two strains, the structural elements are similar, probably as a result of compensatory mutations that allow changes to the RBD sequence without hindering interactions between the pilus and its target. As there is sequence and structural homology in the RBD and all *Pa* strains examined bind to the same cell surface receptor, we reasoned out that it should be possible to produce a synthetic peptide consensus sequence vaccine that would provide crossreactive antibodies from a single peptide immunogen. These antibodies would inhibit the adherence of all known strains of *Pa* and prevent *Pa* infection. Early work on *Pa* in the Hodges laboratory showed that antisera raised against a native PAK C-terminal pilin peptide (residues 128–144) also bound a PAO peptide (residues 128–144) and the PAO pilin. It was possible to enhance antibody crossreactivity to various strains of *Pa* by manipulating the amino acid sequence of the peptide immunogen.[4–6] We have developed novel technology to measure antibody affinity by surface plasmon resonance.[6] A single substitution analog of PAK peptide RBD (E135A) and a double substitution analog where two PAO residues were inserted into the PAK sequence (T130K/E135P) generated antibodies in rabbits with enhanced crossreactivity to the PAK, PAO, KB7, and P1 RBD peptides.[4–6] Active immunization in mice with RBD peptides conjugated to TT (PAK, PAO, and PAK (D132T/E135P)) showed that the PAK (D132T/E135P) peptide gives 100% protection against active challenge with PAOwt (wild-type) bacteria and that this protection is enhanced more than that from active immunization with native PAK and PAO peptide conjugates.

Kao and Hodges[20] directly compared the immunogenicity of the truncated PAK pilin protein (29–144) with a synthetic 17-residue disulfide-bridged PAK RBD peptide (Fig. 3) comprising residues 128–144 conjugated to KLH. This peptide conjugate was then used to immunize mice along with the truncated PAK pilin protein monomer. Antisera collected from mice that were immunized with the peptide immunogen show higher titers compared with the antisera from mice immunized with the pilin protein monomer. In addition, although the pilin protein generates an IgG response, a large portion of the antibodies is directed against regions other than the desired region (i.e. the RBD, residues 128–144). This was also documented by using a pilin protein construct with a scrambled RBD sequence (Fig. 3) that is not recognized by RBD specific antibodies. Furthermore, the antibodies raised against the peptide immunogen bound with greater affinity to native PAK pilin compared with antibodies raised against the pilin protein monomer. Because animals immunized with peptide conjugate only generate antibodies against the RBD and these antibodies are able to recognize the RBD on the native pilin protein, there is a clear advantage in using the synthetic peptide conjugate over the monomeric pilin protein as an immunogen.[20] This result also emphasizes the advantages of synthetic peptide vaccines not only over subunit vaccines but also over killed vaccines or attenuated vaccines all of which may not generate the desired antibodies in terms of titer and affinity to the desired target.

One of the main obstacles in the development of a vaccine against *Pa* is the requirement that it is protective against a wide range of virulent strains. Thus, we investigated our synthetic RBD peptide immunogen PAK T130K/E135P (Cs1) conjugated to TT in active vaccination of mice for protection against lethal challenge with 4 different strains of *Pa* (PAK, PAO, KB7, and P1). Mice were also immunized with a scrambled PAK sequence, native PAK and native PAO peptide conjugates.[19] The A.BY/Sn J mouse strain has a competent humoral immune response, but genetic defects make it naturally susceptible to infection by *Pa*. Thus, it is a convenient mouse model that does not require additional immunosuppressive procedures.[27] Mice immunized with PAO peptide TT-conjugate, then challenged with PAK strain bacteria or immunized with PAK peptide TT-conjugate, and then challenged with PAO strain bacteria are not as well protected as mice immunized with Cs1 peptide conjugate. The Cs1 peptide TT-conjugate provides increased protection against challenge by KB7 and P1 bacterial strains compared with immunization with adjuvant alone.[19] The amino acid sequences of KB7 and P1 RBD regions are quite different from the Cs1 sequence, which is based only on the PAK and PAO sequences (Fig. 3). The scrambled RBD sequence allows for a rigorous examination of the efficacy of the Cs1 peptide sequence in protecting mice from *Pa* challenge. As seen in Fig. 4, the PAK-scrambled RBD provides no protection from PAO or PAK challenge. However, immunization with Cs1 peptide TT-conjugate provides excellent protection from challenge with PAO or PAK bacteria (Fig. 4).

The Cs1 peptide was also engineered into the PAK pilin monomer to examine the RBD by X-ray analysis in the structural context of the monomeric pilin protein. Each of the secondary structural elements of the RBD loop, the type- I β-turn, type-II β-turn, and β-strand, when individually assessed, is similar in the PAK and Cs1 pilin structures. However, a notable difference is observed when the RDB of PAK and Cs1 are compared with K122-4. The differences exist as a result of changes in the hydrogen bond network that anchor the RBD to the pilin in Cs1 and PAK that is replaced by hydrophobic clustering in K122-4 and possibly due to the

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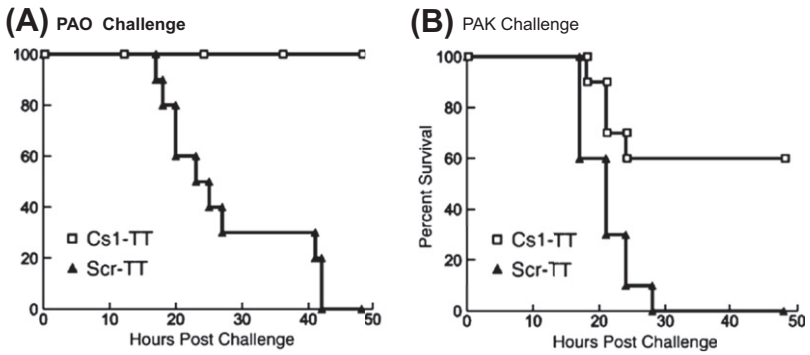

FIGURE 4 Active immunization of A.BY/SnJ mice. Protection of mice from challenge with (A) wild-type PAO and (B) wild-type PAK by the consensus peptide (Cs1)-TT-conjugate and the PAK scramble peptide TT-conjugate. Cs1 peptide affords 100% protection from PAO challenge compared with no protection from the PAK-scrambled peptide. Cs1 peptide also shows protection in mice challenged with PAKwt (60% survival), whereas the PAK scramble provides no protection from PAKwt challenge. Taken from Ref. 19.

presence of Ile, a β-branched amino acid at position 133 in K122-4 versus Leu in PAK and Cs1 (Fig. 3). These differences manifest as changes to the orientation of the RBD and of the individual β-turns in K122-4 relative to those in the PAK and Cs1 pilin proteins.[19] It is possible and even probable that the Cs1 peptide is flexible enough to adopt various conformations mimicking different pilin proteins.

To further examine the ability to produce crossreactive immunogens, a series of 17-residue peptides were made by substitution of two to five amino acid residues from the PAK sequence into the native PAO sequence as shown in Fig. 5.[14] The objective of this project was to generate a synthetic peptide immunogen that displays two distinct epitopes such that one epitope would generate antibodies that were PAO specific and the other epitope would generate antibodies that were PAK specific. Immunogen A is the native PAO (128–144) sequence, whereas immunogens B–E systematically increase the number of PAK residues substituted into the PAO template from 2 to 5 residues within the disulfide loop region. These five synthetic peptides were conjugated to KLH and used to immunize rabbits. IgG was purified from the sera and antibody crossreactivity determined using competitive enzyme-linked immunosorbent assays (ELISA) (Fig. 6). As expected, immunogen A (native PAO sequence) generates strain-specific antibodies that bind only native PAO pilin but not PAK pilin. All other immunogens B, C, D, and E generate crossreactive antibodies that bind both PAK and PAO pilins. Immunogen B has only two PAK amino acid residues E135 and Q136 transplanted into the PAO sequence (Fig. 5). Competitive ELISA clearly shows that polyclonal antibodies raised against peptide immunogen B bind to both PAO and PAK pilins (Fig. 6). Furthermore, the antibodies that bind PAO pilin are competed off with PAO pilin and not PAK pilin; antibodies that bind PAK pilin are competed off with PAK pilin and not PAO pilin. Thus, there are at least two different antibodies contained in the polyclonal sera raised against a single peptide immunogen containing two distinct epitopes, one that is specific for PAK pilin and one that is specific for PAO pilin. The affinity of the antibodies elicited by different peptide immunogens for both PAO and PAK pilins are similar, and antibodies obtained from different rabbits immunized with the same peptide immunogens bind with similar affinity to the *Pa* pilin antigens. To delineate the epitope sequence responsible for PAO and PAK pilin antibody interactions, a PAO mutant was engineered with three PAK residues, namely, K128, S143, and K144, PAO(KSK), which are located outside the disulfide loop (Fig. 3). Similarly, a PAK mutant was engineered with three PAO residues A128, D143, and N144, PAK(ADN).

PAO-specific antibodies were then analyzed for reactivity to the two mutants. PAO-specific antibodies (Immunogen A)

Peptide immunogen	Receptor binding domain (RBD) sequence 128	129	130	131	132	133	134	135	136	137	138	139	140	141	142	143	144		# of PAK specific residues	# of PAO specific residues
A	A	C	K	S	T	Q	D	P	M	F	T	P	K	G	C	D	N	- OH	0	8
B	A	C	K	S	T	Q	D	E	Q	F	T	P	K	G	C	D	N	- OH	2	6
C	A	C	K	S	T	Q	D	E	Q	F	I	P	K	G	C	D	N	- OH	3	5
D	A	C	K	S	D	Q	D	E	Q	F	I	P	K	G	C	D	N	- OH	4	4
E	A	C	T	S	D	Q	D	E	Q	F	I	P	K	G	C	D	N	- OH	5	3

FIGURE 5 Amino acid sequences of the synthetic peptide analogs of the PAO RBD (residues 128–144), which were conjugated to KLH and used to immunize rabbits. The native strain PAK residues substituted into the PAO sequence, which are also PAK specific, are boxed. Residues that are PAO specific are circled. Peptide A (PAO native), peptide B (P135E/M136Q), peptide C (P135E/M136Q/T138I), peptide D (T132D/P135E/M136Q/T138I), and peptide E (K130T/T132D/P135E/M136Q/T138I). Taken from Ref. 14.

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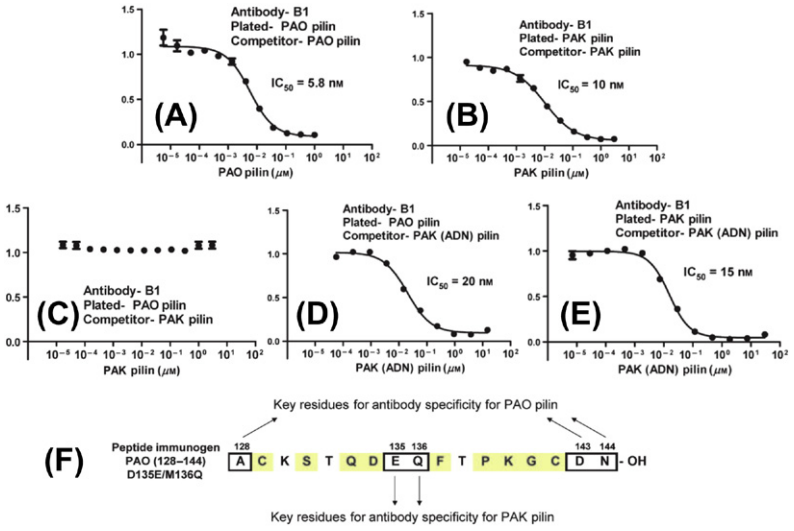

FIGURE 6 Competitive ELISA results for antibody B1 binding to PAK and PAO pilin proteins (panels A–E) and the substituted PAO peptide sequence that corresponds to peptide B (PAO 128-144 D135E/M136Q, panel F). The B1 antibody crossreacts with PAO (panel A) and PAK (panel B) pilins. The PAK pilin cannot compete for B1 antibody binding to PAO (panel C). However, the PAK(ADN) mutant is able to inhibit B1 binding to either PAO pilin (panel D) or PAK pilin (panel E). The concentration of pilin protein required for 50% inhibition of antibody binding (IC_{50}) is reported in each panel. The key residues to elicit PAK-specific antibodies (E135 and Q136) and PAO-specific antibodies (A128, D143 and N144) are boxed in the amino acid sequence of peptide B (panel F). Taken from Ref. 14.

are capable of interacting with both the PAO(KSK) mutant and the PAK(ADN) mutant with IC_{50} values in the low nanomolar range. These results show that PAO-specific antibodies recognize epitopes displayed inside the disulfide loop and also interact with an epitope located outside the disulfide-bridged loop. Antibody B1 was generated by immunizing rabbits with peptide B-KLH conjugate, a peptide that contains only 2 PAK residue substitutions into the PAO peptide and produces crossreactive antibodies to both PAK and PAO. The binding of this antibody to PAO could be competed off by native PAO but not native PAK. However, the PAK(ADN) mutant is able to inhibit B1 antibody binding to PAO pilin with an IC_{50} value of 20nM (Fig. 6D). The B1 antibodies could not bind to the PAO(KSK) mutant pilin. These results clearly indicate that residues A128, D143, and N144, which are located outside the disulfide-bridged loop, comprise part of the epitope responsible for generating PAO-specific antibodies to the peptide immunogen B1. On the other hand, the epitope responsible for generating PAK-specific antibodies is located within the disulfide loop and includes residues E135 and Q136. Therefore, this peptide generates crossreactive polyclonal sera with two specificities (Fig. 6).

The ability to rationally design effective peptide vaccines relies partly on our ability to analyze structural elements of the native protein immunogens from which they are derived. The filamentous nature of *Pa* pili make the pilin protein notably difficult to crystallize. Therefore, we used the native strain PAK, which was previously crystallized[15] as a template to insert the RBD of the PAO pilin protein using site-directed mutagenesis. The three-dimensional structures of the PAK PAO RBD chimera and native PAK are similar (Hackbarth and Hodges, unpublished results). The β-turns located in the RBD of the PAK PAO RBD chimera are nearly identical to the PAK RBD with an r.m.s.d. of 0.368Å. This result strongly suggests that the use of PAK pilin protein to incorporate RBDs from other strains is an excellent approach to generate X-ray structures. The surface-exposed residues of the PAK and PAO PAK chimera RBD are shown in Fig. 7. As expected, residues at positions 128, 143, and 144, which were deemed critical for epitope recognition by PAO-specific antibodies generated against the peptide immunogens and pilin mutants form a cluster and residues at positions 135 and 136, which make up at least part of the PAK epitope, are also clustered into a particular region on the surface of the pilin protein.

CONCLUSIONS

The RBD of the *Pa* pilin protein is an intrinsically optimized epitope for the design of a synthetic peptide-based vaccine because it consists of a continuous peptide that has secondary structural elements inherent to the sequence and is integral to *Pa* virulence. Because peptides have the potential to mimic functionally relevant domains of different pilin proteins, peptide-based vaccines may show more favorable characteristics such as enhanced crossreactivity compared with subunit, whole-killed, or live attenuated vaccines. The consensus sequence approach avoids antigenic competition by presenting a single immunogen to the immune system.

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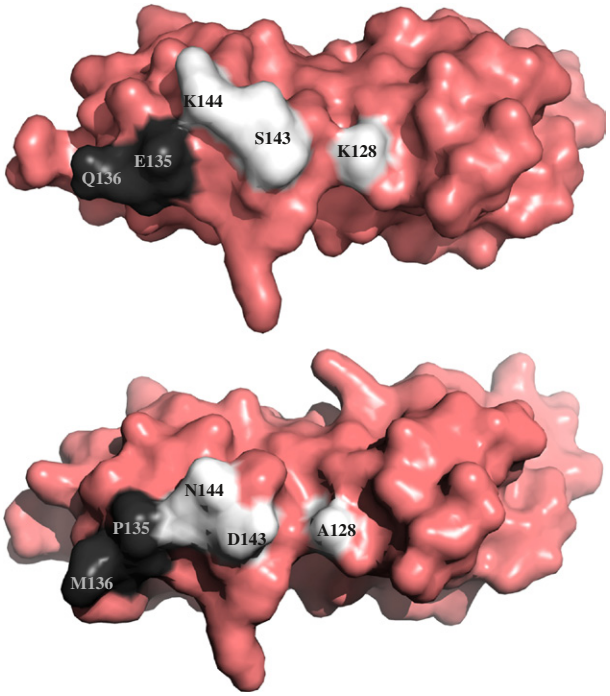

FIGURE 7 Molecular surface diagrams of PAK (top) and PAK PAO chimera (bottom) RBDs. The key residues responsible for PAK-specific antibodies (positions 135 and 136) and those responsible for PAO-specific antibodies (positions 128, 143, and 144), form separate clusters on the surface of the RBD (Hackbarth and Hodges, unpublished results).

We have clearly shown that to design a consensus sequence vaccine we can manipulate the sequence within and outside the disulfide loop region of the RBD. It is interesting that only three residues outside the disulfide loop can create high-affinity antibodies to a specific pilin strain. This suggests that we can use the epitope outside the loop (residues 128, 143, and 144) to enhance antibody crossreactivity to immunogens that lack crossreactivity to a particular strain.

To date, four X-ray crystallographic structures have been obtained for monomeric pilin proteins (29–144), native PAK[15] and K122-4[1] pilins, and two chimeric pilin proteins, PAK with a PAO RBD 128–144 and Cs1 pilin, which is PAK with two substitutions, T130K and E135P, in the RBD.[19] Based on the X-ray structures of these pilin proteins and NMR structures of the RBDs 128–144, it is clear that synthetic peptide vaccines must maintain the conserved conformation of the RBD consisting of two β-turns, one β-strand, and the disulfide bond to constrain the structure. Thus, our current hypothesis is that a consensus sequence immunogen must maintain this conserved conformation of the RBD while displaying a particular combination of variable residues on its surface that will induce antibodies that are crossreactive. Of the 6 variable positions in the RBD loop, only four are prominent on the exposed surface of the RBD (Lys130, Asp132, Pro135, and Gln136). We have shown that Pro135 in Cs1 may bolster the β-turn propensity of the RBD.[19] Thus, we propose to maintain Pro in this position in further design of a structure-based consensus sequence immunogen. Because the three variable residues Lys 130, Asp132, and Gln136 do not seem to play other structural roles in the RBD, we will focus our attention on these positions. The limited diversity at variable positions in native strains of *Pa* has led us to hypothesize that non-native substitutions will perturb the conformation of the RBD. On the other hand, substitutions derived from native strains at variable positions will maintain the conformation. Therefore, future consensus sequence RBD peptides should be derived from the residues that occur in native strains at each position.

REFERENCES

1. Audette GF, Irvin RT, Hazes B. Crystallographic analysis of the *Pseudomonas aeruginosa* strain K122-4 monomeric pilin reveals a conserved receptor-binding architecture. *Biochemistry* 2004;**43**:11427–35.
2. Boucher HW, Talbot GH, Bradley JS, Edwards JE, Gilbert D, Rice LB, et al. Bad bugs, no drugs: no ESKAPE! An update from the Infectious Diseases Society of America. *Clin Infect Dis* 2009;**48**:1–2.
3. Breidenstein EB, de la Fuente-Nunez C, Hancock RE. *Pseudomonas aeruginosa*: all roads lead to resistance. *Trends Microbiol* 2011;**19**:419–26.
4. Cachia PJ, Glasier LM, Hodgins RR, Wong WY, Irvin RT, Hodges RS. The use of synthetic peptides in the design of a consensus sequence vaccine for *Pseudomonas aeruginosa*. *J Pept Res* 1998;**52**:289–99.
5. Cachia PJ, Hodges RS. Synthetic peptide vaccine and antibody therapeutic development: prevention and treatment of *Pseudomonas aeruginosa*. *Biopolymers* 2003;**71**:141–68.
6. Cachia PJ, Kao DJ, Hodges RS. Synthetic peptide vaccine development: measurement of polyclonal antibody affinity and cross-reactivity using a new peptide capture and release system for surface plasmon resonance spectroscopy. *J Mol Recognit* 2004;**17**:540–57.
7. Campbell AP, McInnes C, Hodges RS, Sykes BD. Comparison of NMR solution structures of the receptor binding domains of *Pseudomonas aeruginosa* pili strains PAO, KB7, and PAK: implications for receptor binding and synthetic vaccine design. *Biochemistry* 1995;**34**:16255–68.
8. Campbell AP, Sheth H, Hodges RS, Sykes BD. NMR solution structure of the receptor binding domain of *Pseudomonas aeruginosa* pilin strain P1. Identification of a beta-turn. *Int J Pept Protein Res* 1996;**48**:539–52.
9. Craig L, Pique ME, Tainer JA. Type IV pilus structure and bacterial pathogenicity. *Nat Rev Microbiol* 2004;**2**:363–78.
10. Craig L, Taylor RK, Pique ME, Adair BD, Arvai AS, Singh M, et al. Type IV pilin structure and assembly: X-ray and EM analyses of *Vibrio cholerae* toxin-coregulated pilus and *Pseudomonas aeruginosa* PAK pilin. *Mol Cell* 2003;**11**:1139–50.
11. Doring G, Pier GB. Vaccines and immunotherapy against *Pseudomonas aeruginosa*. *Vaccine* 2008;**26**:1011–24.
12. Farinha MA, Conway BD, Glasier LM, Ellert NW, Irvin RT, Sherburne R, et al. Alteration of the pilin adhesin of *Pseudomonas aeruginosa* PAO results in normal pilus biogenesis but a loss of adherence to human pneumocyte cells and decreased virulence in mice. *Infect Immun* 1994;**62**:4118–23.

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13. Giltner CL, van Schaik EJ, Audette GF, Kao D, Hodges RS, Hassett DJ, et al. The *Pseudomonas aeruginosa* type IV pilin receptor binding domain functions as an adhesin for both biotic and abiotic surfaces. *Mol Microbiol* 2006;**59**:1083–96.
14. Hackbarth C, Hodges RS. Synthetic peptide vaccine development: designing dual epitopes into a single pilin peptide immunogen generates antibody cross-reactivity between two strains of *Pseudomonas aeruginosa*. *Chem Biol Drug Des* 2010;**76**:293–304.
15. Hazes B, Sastry PA, Hayakawa K, Read RJ, Irvin RT. Crystal structure of *Pseudomonas aeruginosa* PAK pilin suggests a main-chain-dominated mode of receptor binding. *J Mol Biol* 2000;**299**:1005–17.
16. Hunt JD, Brown LE, Wood PR, Stewart DJ, Jackson DC. Manipulation of the helper T cell response to influence antigenic competition occurring with a multivalent vaccine. *Immunol Cell Biol* 1996;**74**:81–9.
17. Hunt JD, Jackson DC, Brown LE, Wood PR, Stewart DJ. Antigenic competition in a multivalent foot rot vaccine. *Vaccine* 1994;**12**:457–64.
18. Hunt JD, Jackson DC, Wood PR, Stewart DJ, Brown LE. Immunological parameters associated with antigenic competition in a multivalent footrot vaccine. *Vaccine* 1995;**13**:1649–57.
19. Kao DJ, Churchill ME, Irvin RT, Hodges RS. Animal protection and structural studies of a consensus sequence vaccine targeting the receptor binding domain of the type IV pilus of *Pseudomonas aeruginosa*. *J Mol Biol* 2007;**374**:426–42.
20. Kao DJ, Hodges RS. Advantages of a synthetic peptide immunogen over a protein immunogen in the development of an anti-pilus vaccine for *Pseudomonas aeruginosa*. *Chem Biol Drug Des* 2009;**74**:33–42.
21. Kunz AN, Brook I. Emerging resistant Gram-negative aerobic bacilli in hospital-acquired infections. *Chemotherapy* 2010;**56**:492–500.
22. Lee KK, Sheth HB, Wong WY, Sherburne R, Paranchych W, Hodges RS, et al. The binding of *Pseudomonas aeruginosa* pili to glycosphingolipids is a tip-associated event involving the C-terminal region of the structural pilin subunit. *Mol Microbiol* 1994;**11**:705–13.
23. Lee KK, Yu L, Macdonald DL, Paranchych W, Hodges RS, Irvin RT. Anti-adhesin antibodies that recognize a receptor-binding motif (adhesintope) inhibit pilus/fimbrial-mediated adherence of *Pseudomonas aeruginosa* and *Candida albicans* to asialo-GM1 receptors and human buccal epithelial cell surface receptors. *Can J Microbiol* 1996;**42**:479–86.
24. Mattick JS. Type IV pili and twitching motility. *Annu Rev Microbiol* 2002;**56**:289–314.
25. McInnes C, Sonnichsen FD, Kay CM, Hodges RS, Sykes BD. NMR solution structure and flexibility of a peptide antigen representing the receptor binding domain of *Pseudomonas aeruginosa*. *Biochemistry* 1993;**32**:13432–40.
26. Paranchych W, Sastry PA, Volpel K, Loh BA, Speert DP. Fimbriae (pili): molecular basis of *Pseudomonas aeruginosa* adherence. *Clin Invest Med* 1986;**9**:113–8.
27. Pennington JE, Williams RM. Influence of genetic factors on natural resistance of mice to *Pseudomonas aeruginosa*. *J Infect Dis* 1979;**139**:396–400.
28. Purcell AW, McCluskey J, Rossjohn J. More than one reason to rethink the use of peptides in vaccine design. *Nat Rev Drug Discov* 2007;**6**:404–14.
29. Purcell AW, Zeng W, Mifsud NA, Ely LK, Macdonald WA, Jackson DC. Dissecting the role of peptides in the immune response: theory, practice and the application to vaccine design. *J Pept Sci* 2003;**9**:255–81.
30. Sanchez A, Gattarello S, Rello J. New treatment options for infections caused by multiresistant strains of *Pseudomonas aeruginosa* and other nonfermenting Gram-negative bacilli. *Semin Respir Crit Care Med* 2011;**32**:151–8.
31. Sedlak-Weinstein E, Cripps AW, Kyd JM, Foxwell AR. *Pseudomonas aeruginosa*: the potential to immunise against infection. *Expert Opin Biol Ther* 2005;**5**:967–82.
32. Sharma A, Worgall S. Recent developments for *Pseudomonas* vaccines. *Hum Vaccin* 2011;**7**.
33. Sheth HB, Lee KK, Wong WY, Srivastava G, Hindsgaul O, Hodges RS, et al. The pili of *Pseudomonas aeruginosa* strains PAK and PAO bind specifically to the carbohydrate sequence beta GalNAc(1-4)beta Gal found in glycosphingolipids asialo-GM1 and asialo-GM2. *Mol Microbiol* 1994;**11**:715–23.
34. Strateva T, Yordanov D. *Pseudomonas aeruginosa*—a phenomenon of bacterial resistance. *J Med Microbiol* 2009;**58**:1133–48.
35. Wong WY, Campbell AP, McInnes C, Sykes BD, Paranchych W, Irvin RT, et al. Structure-function analysis of the adherence-binding domain on the pilin of *Pseudomonas aeruginosa* strains PAK and KB7. *Biochemistry* 1995;**34**:12963–72.

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Chapter 78

Lipo-Peptides/Saccharides for Peptide Vaccine Delivery

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ABSTRACT

A subunit vaccine is a vaccine that contains only the minimal microbial elements. Despite the fact that elimination of redundant components brings several advantages, it also removes the "danger signal" necessary to trigger the desired immune response. To overcome this problem, adjuvants (immunostimulants) have been widely used in vaccine formulations. Many currently available adjuvants suffer from side toxicity or poor efficacy; therefore, numerous vaccine delivery systems have been developed to improve the immunogenicity of peptide subunit vaccines. Among them, self-adjuvanting lipopeptides/saccharides-based delivery systems have shown high efficiency in inducing strong immune responses to incorporated peptide-based immunogens and are considered a promising platform for development of human vaccines.

INTRODUCTION

Vaccination is considered to be one of the most effective public health interventions since the eradication of smallpox through a worldwide vaccination campaign during the last century. Considering that until the 18th century smallpox caused about 10% of global mortalities in Europe, the success of this vaccine can be only compared with the introduction of penicillin. The first "controlled" infections were made in India, China, and North America hundreds of years ago, but the beginning of vaccination has been attributed to the time Jenner's cow-derived smallpox vaccine (the word vaccination is derived from the Latin word for cow = *vacca*) was introduced to the public. Vaccinology has changed greatly since that time, but the classical vaccine strategy based on attenuated or inactivated pathogens is still used. Problems associated with conventional vaccines include risks of infection especially in the case of immune compromised humans, difficulties with the production of pathogens *in vitro*, and instability of the biological material. The use of whole microorganisms is associated with the exposure of the patient to unnecessary and possibly harmful oncogenic microbial components, and the possible elicitation of autoimmunity or strong allergic responses. Concerns about vaccine safety are especially high because vaccines are usually administered to healthy humans, including children, for prophylaxis. Therefore, more recently, the use of whole pathogens has fallen out of favor, and most of the newly licensed vaccines are recombinant products.

SUBUNIT VACCINES

A subunit vaccine is a vaccine that contains only the minimal microbial elements necessary to stimulate long-lasting protective/therapeutic immune responses. In such vaccines, the disadvantages of classical vaccines are diminished. The absence of whole microorganisms eliminates the risk of its reversion to a virulent state while improving storage stability. Potentially oncogenic, allergenic, or toxic materials are removed. In addition, some pathogens are problematic to culture (e.g. sporozoites for malaria vaccines), and a subunit-based vaccine might be the only solution in such cases. However, such vaccines usually need several injections (primary and boosts) for long-lasting protection. Moreover, subunit-based vaccines generally require the use of an immunostimulant (adjuvant) as elimination of redundant components often removes the "danger signal" necessary to trigger the desired immune response. Currently available experimental adjuvants suffer from side toxicity, while commercially available (safe for human) adjuvants are mostly limited to aluminum derivatives that have limited potency. Aluminum-type adjuvants are also not suitable for eliciting cell-mediated immunity, and therefore cannot be used in therapeutic vaccines. Only recently, Cervarix®, a vaccine containing a new adjuvant, aluminum hydroxide and 3-*O*-desacyl-4′-monophosphoryl lipid A, was approved in the USA. This event did not change global demand for new and safe adjuvants, which are able to improve not only vaccine humoral responses but also cell-mediated immune responses. Despite the fact that modern vaccines consist mainly of proteins, such vaccines have major weaknesses such as cost of production, purity, and stability problems and in some cases the use of whole protein is prohibited because of the risk of triggering of autoimmune responses. For example, development of a vaccine against Group A

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00078-6
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Streptococcus (*Streptococcus pyogenes*; GAS) bacteria, based on the GAS virulence factor, M Protein, was suppressed because of the production of antibodies that cross-reacted with human heart muscle.[3] Considering that the minimal microbial elements necessary to stimulate the required immune response can be as small as a short synthetic peptide, peptide subunit-based vaccines have seemed to be ideal candidates to solve all of the above-mentioned problems.[22,30] Peptide-based vaccines can be accurately designed to induce only the desired immune responses, as the sequences possessing similarity with human proteins can be easily excluded, eliminating the risk of autoimmunity. Such vaccines can be more precisely designed to recognize cancer specific targets. Nowadays, peptides can be easily and relatively inexpensively synthesized using solid phase peptide synthesis (SPPS) in a highly reproducible manner and pure state, in linear or dendritic form, incorporating only natural amino acids or synthetic analogs. Such compounds are usually exceptionally well defined and characterized and more stable during postproduction (including freeze drying), and under storage and transportation conditions than whole proteins. Peptide vaccines can include several different epitopes to target diverse subtypes of a pathogen during any stage of its life cycle. Peptide-based vaccines can incorporate B-cell epitopes to induce humoral response (antibody-mediated immunity), and/or T-cell epitopes for cellular adaptive immune response (cytotoxic T-lymphocytes/CTL, CD8+ T-cells/ mediated immunity), as well as helper T-lympocytes (TH cells, CD4+ cells) for eliciting long-lasting immune responses in outbred populations. Functional dissection and proteomic analysis of the peptide epitopes from pathogens have broadened the choice of peptide epitopes. Genomic information has allowed identification of proteins and antigens from pathogens, enabling the prediction of CD4+ T-cell and CD8+ T-cell epitopes using bioinformatic methods. These epitopes have been analyzed for the relevance of their immunogenicity via synthesis of overlapping peptide sequences. While the prediction of B-cell epitopes is a more challenging task, major progress has been made in this field. Despite many of well-defined peptide antigens being available for vaccine applications, those antigens are not immunogenic themselves and require appropriate delivery systems and even stronger adjuvants than those required to stimulate immune responses against recombinant vaccines. Indeed, the development of potent but safe adjuvants has become the holy grail of modern vaccinology. Lipoproteins and bacterial lipids such as lipopolysaccharide (LPS, a cell-wall component of Gram-negative bacteria), predominantly lipid A, have been identified as natural adjuvants.[8] However, because of the significant toxicity associated with natural bacterial lipids, synthetic analogs have been investigated for some time. This had resulted in the lipidation of peptides emerging as a promising strategy for delivery of peptide subunit vaccines.[16,30]

Dendritic Cell Toll-Like Receptor – The Keyhole for Lipopeptide Vaccines

Dendritic cells (DCs) are bone marrow-derived leukocytes that can be found throughout lymphoid and nonlymphoid tissues, afferent lymph vessels, and in peripheral blood. DCs are a group of heterogeneous multifunctional antigen presenting cells (APCs). Functionally, DCs are potent stimulators of B- and T-lymphocytes, the key mediators of immunity. T-lymphocytes require the antigen to be processed and presented to them by an APC such as a DC. After recognition of pathogen associated molecular patterns, DCs undergo a process called maturation, where they express high levels of several cell surface molecules including major histocompatibility complex (MHC) I and II, CD40, CD80, etc. The T-cell antigen receptors (TCRs) recognize antigen-specific peptide fragments bound to MHC molecules on the surface of DCs. MHC class I and MHC class II molecules stimulate cytotoxic T-cells (CTLs) and helper T-cells, respectively. Thus, intracellular antigens (such as tumor specific protein), when processed and bound to MHC class I molecules and recognized by CTLs, directly kill a target cell after activation. Extracellular antigens (e.g. from bacteria) are presented by the MHC class II molecules on DCs to T-helper cells. Activated T-helper cells then interact with antigen-specific B-cells causing the B-cells to proliferate and to differentiate into antibody-secreting plasma cells. Activated T-helper cells also play various roles in the quality of immunity including switching to certain immunoglobulin isotypes. Given the important role of DCs in mediating immunity, significant scientific focus has been on targeting DCs in vaccine development. Several lipid-based vaccine candidates have been examined and their efficacy in different systems was initially attributed to the ability of the lipid tail to enhance intracellular epitope delivery into APCs. A more recent understanding of how the lipid component enables the potent adjuvanting properties by targeting DCs has provided a rational basis for lipid-based vaccine development.

The endocytic route of entry is usually triggered by receptor-mediated events and lipid moiety mediated endocytosis of antigens toward Toll-like receptors (TLRs) as an obvious candidate for the mechanism of action.[4] There are 10 TLRs in humans that recognize different microbial molecular patterns during infection and trigger signaling pathways that lead to activation of transcription factors such as nuclear factor-κB (NF-κB). This consequently leads to the induction of immune and inflammatory genes. TLR2 is responsible for the recognition of bacterial lipoprotein and lipopeptides. The interaction between TLR2 present on DCs and its ligand initiates a signaling cascade that results in the translocation of NF-κB to the nucleus and activation of NF-κB-dependent genes responsible for the phenotypic and functional changes associated with DC maturation. Lipopeptide/saccharide vaccine candidates mimic bacterial

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lipoproteins, which results in their self-adjuvanting activity.[32] The discovery of TLRs and their significance in the recognition of pathogens and the consequent immune response has expanded the spectrum of vaccine adjuvants. This is exemplified by current investigations incorporating a range of lipid moieties to form lipopeptide/saccharide-based vaccine candidates.

LIPIDATION

Lipidation has been extensively used for peptide delivery. Lipidation involves increasing peptide hydrophobicity, which improves their permeation of biological membranes and therefore increases their bioavailability. Modification with lipids often increases the chemical stability of peptides and protects them against enzymatic degradation.[24] Importantly, lipidic moieties can target peptides to particular cells, such as dentritic[8] or cancer cells,[26] thus increasing peptide immunogenicity or antitumor efficacy, respectively. There are several different strategies to incorporate lipid moieties into peptide epitopes, such as introduction of lipid via an *S*-glyceryl cysteine moiety, and use of a carbohydrate scaffold, or lipopeptide units.[16,34]

Lipid Core Peptide

The lipid core peptide (LCP) system for drug and vaccine delivery was first reported in the early 1990s. Since then, many modifications and improvements have been implemented but the LCP vaccine delivery system still consists of three major persistent components: (a) a nonmicrobial lipopeptide moiety (composed of synthetic lipidic amino acids (LAAs)), which might be arranged into the peptide sequence with a glycine spacer; (b) a branching moiety (usually based on polylysine); and (c) appropriate peptide epitopes.[28] The typical LCP system is presented in Fig. 1. The lipidic self-adjuvanting moiety can be easily modified in terms of the presence of a spacer, the number of LAAs and the length of their alkyne chains.[6] The lysine branching, allows the advantageous use of a multiple antigenic peptide (MAP) system. The level of the branching can be adjusted to suit the requirements. The lysine carrier permits the conjugation of multiple copies of the same peptide epitope and conjugation of many different peptide antigens.[34] LCP vaccine candidates can be freeze dried, are generally stable against peptidase degradation, and can be stored at room temperature.[16]

The first LCP system was tested *in vivo*[35] and was designed by Toth et al. as a vaccine against *Chlamydia trachomatis*, bacteria associated with infectious eye disease and the commonest infectious cause of blindness. The LCP system is composed of three C16-LAAs (2-amino-*R*,*S*-hexadecanoic acid), no glycine spacers, and branched lysines to which 8 copies of peptide epitope (A ETIFD VTTLN PTIAG or SAETI FDVTT LNPTI AGSDV AGLQN DPTTN), derived from the variable domains of *Chlamydia trachomatis* outer membrane protein, are attached. Compounds with a single epitope attached to two lipoamino acids were also synthesized. All antigens were administered with external adjuvant. The multiepitopic LCP constructs show significantly enhanced immunogenicity compared with monomeric counterparts or peptides alone.[35] Despite high antibody titers elicited by LCP constructs, these antibodies have only limited neutralizing ability, most probably because of conformational differences between the synthetic peptides and the corresponding native antigens.

Most research involving LCP delivery systems has been focused on development of a vaccine against GAS. This Gram-positive bacterial pathogen causes a variety of diseases that range from common bacterial pharyngitis to life-threatening streptococcal toxic shock syndrome and autoimmune postinfectious diseases such as rheumatic fever (RF) and rheumatic heart disease (RHD). In 2010, it was estimated that around 70 million individuals worldwide currently had RHD, which would result in 1.4 million deaths per year from RHD and its complications. Classical intervention with antibiotics is not very effective, especially in developing countries; therefore, there is high interest in the development of a vaccine to prevent streptococcal infections. As protection against GAS infection was correlated with opsonic antibody against the GAS major virulence factor, M protein, most designs of the vaccine candidates worldwide are based on M protein or its fragments. The use of whole protein in vaccine development was abandoned because of the high risk of autoimmune side reactions caused by antibodies and T-cells against whole protein because of the molecular mimicry between cardiac myosin and streptococcal M protein.

M protein, a coiled-coil protein consists of three major domains: an N-terminal domain, which is highly variable and able to induce production of protective antibodies; a B-repeat domain, which induces production of nonopsonic antibodies; and a highly conserved C-repeat domain. In 2002, Hyman et al. reported LCP constructs incorporating a p145 epitope (LRRDL DASRE AKKQV EKALE) from the C-terminal region of M protein.[7] Using a different number of LAAs and LAAs with different lengths of side chains, more lipophilic compounds induce stronger immune responses against p145. The level of antibody titers after subcutaneous immunization with LCP constructs, without use of any adjuvant, is similar to that when p145 was administered with toxic Complete Freund's Adjuvant (CFA). Increasing, the number of antigen copies from 4 to 8 does not improve humoral immune response. After further structure–activity relationship (SAR) study, the LCP core with C12-LAAs (Fig. 1, $n=8$), and glycine spacer was suggested as the most promising construct for vaccine delivery.[12] More hydrophobic structures ($n>8$) were difficult to synthesize and purify

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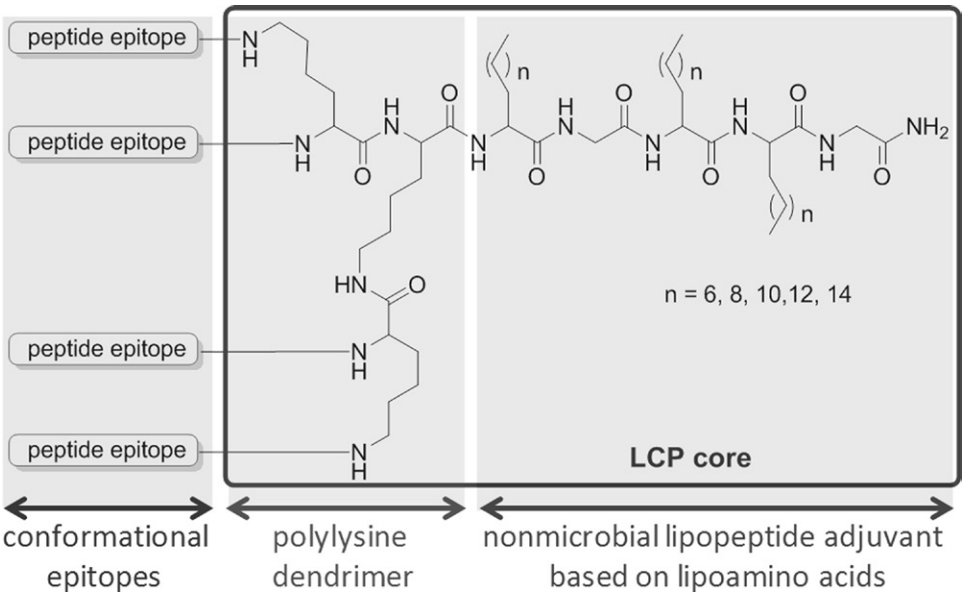

FIGURE 1 The typical structure of peptide subunit vaccine based on a lipid core peptide (LCP) system.

and were poorly water soluble making them less practical as components of vaccine candidates. Further studies were discontinued as T-cells specific to the p145 epitope were able to crossreact with human heart. To overcome problems associated with potential autoimmune responses to the p145 peptide epitope, the minimal B-cell epitopes have been identified in the p145 peptide. Because M protein has an α-helical coiled-coil structure and minimal epitopes, in contrast to p145, and does not demonstrate helical potential, the coil-promoting sequences from the yeast GCN4 protein were attached to the N- and C-termini of the minimal epitopes. Two of them were chosen as the most promising/immunogenic and non-crossreactive with human heart epitopes for vaccine design: J8 (QAE DKVKQ *SREAK KQVEK AL*KQL EDKVQ, minimal epitope is in italics) and J14 with a similar sequence (KQAE DKVK*A SREAK KQVEK ALE*QL EDKVK). However, like other short peptides, these epitopes do not stimulate an immune response without the help of adjuvant. With the incorporation of multiple copies of J8 epitope into LCP system,[28] the conjugates induce strong immune responses in mice without the need for coadministration with additional adjuvant.[10,12,17] Similar diepitopic constructs possessing both J8, and 8830 (DNGKA IYERA RERAL QELGP), epitope from the N-terminal domain elicit high antibody titers against both epitopes.[10] The system possessing these two epitopes protect, to almost 100%, mice that were challenged intraperitoneally with the 8830 GAS strain after intranasal immunization.[20] The additional epitope (in this case 8830) was incorporated to further increase the strain coverage of these vaccine candidates, as the conserved-region determinant (J8) is identical in at least 70% of GAS isolates but not all of them.

To examine the potential of the LCP system to incorporate more than two peptide epitopes and to analyze when each component (epitope) induced specific and strong immune responses, three and four epitopic constructs (**1**, **2**) were designed and synthesized (Fig. 2). These conjugates incorporate the conserved J8 epitope, and two or three epitopes from the variable N-terminal region of M protein, namely, 8830, NS1 (RVTTR SQAQD AAGLK EKAD), and PL1 (EVLTR RQSQD PKYVT QRIS) serotype-specific peptide epitopes.[18] All compounds were synthesized using a simple branching procedure during SPPS. After coupling of Boc-Lys(Boc)-OH to the lipopeptide, both Boc protective groups were removed. The LCP constructs were branched using α-Fmoc-Lys(ε-Boc)-OH which was at first coupled to the Lys-lipopeptide. After selective deprotection of the ε-amine group of α-Fmoc-Lys(ε-Boc)-Lys-lipopetide, two copies of the first peptide epitope (J8) were coupled using standard SPPS and protected at the N-termini by an acetyl group. After Fmoc deprotection, α-Fmoc-Lys(ε-Boc)-OH was coupled to the construct on the resin, and the whole process was repeated until the desired number of epitopes was attached to the LCP core. All compounds induce significant immune responses. After administration of the compounds (presented in Fig. 2), mice are protected against the challenge of GAS strain 8830 but the level of protection is not significantly different when four epitopic compounds are compared with a mixture of two diepitopic compounds. These results suggest that antibody response levels are not unfavorably affected by mixing different LCP constructs. The physical mixture of simpler LCP compounds might be advantageous in terms of simplicity of synthesis and purification when compared with sophisticated tetravalent

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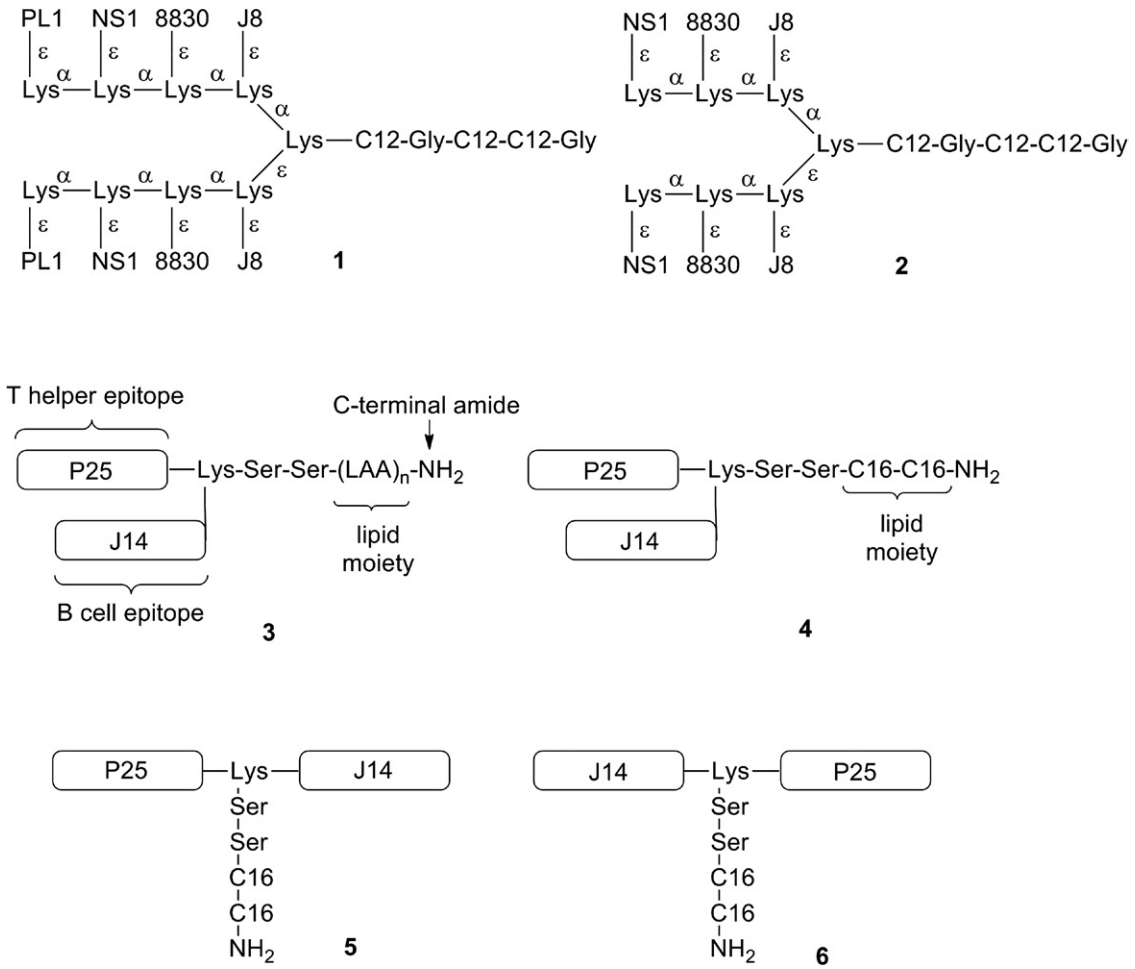

FIGURE 2 Examples of multiepitopic LCP vaccine candidates against GAS infection (1, 2) and the lipopeptide GAS vaccine candidates 3-6 from a "one lysine branching" series (where C12, C16 = LAAs possessing 12 and 16 carbon atoms, respectively).

or even more branched constructs. A tetraepitopic vaccine candidate was also examined for its ability to induce long-lasting immune responses (290 days) and showed high antibody titers, especially when administered with CFA.[18]

Another GAS virulent factor SfbI, a fibronectin-binding protein, plays a key role in bacterial attachment to host cells and colonization of pharyngeal mucosa, as well as in cellular invasion. The highly conserved fibronectin-binding repeats region (FNBR) is the minimal protein domain required to elicit protective immunity. The constructs possessing, J8 or FNBR epitopes (positions 373–402 and 393–422), from M protein and SfbI, respectively, were synthesized.[19] Mice were immunized intranasally with a physical mixture of monoepitopic J8-LCP and FNBR-LCP or with each construct alone. In all cases, LCPs were coadministered with the TLR2/6 agonist MALP-2 as the mucosal adjuvant. Immune responses stimulated at systemic and mucosal levels are strongest when mice are immunized with the mixture of both antigens. In the challenge experiment, the mice are fully protected against *S. pyogenes* only when the mixture of both LCPs is used for immunization. The mixture without external mucosal adjuvant (MALP-2) is not able to protect mice, even partially, against the bacteria.

The Toth group synthesized and examined a library of lipopeptide vaccine candidates consisting of B-cell epitope J14, a CD4+ T-helper epitope P25 (to improve immune responses in outbred populations), and a lipid (LAAs) variously attached to a central lysine core via its carboxylic acid or alpha- or episilon-amino groups to create different molecular geometries (see compounds **3**–**6**, Fig. 2). The position of epitopes was optimized to elicit a higher immune response. At the same time, the influence of the length of the alkyl chain on the immune response was investigated. Molecular geometry has a significant effect on the immune response after subcutaneous immunization.[1] Vaccine candidates featuring C-terminus lipid moiety (**3**) are optimal for systemic antibody production. The length of the alkyl chain and number of lipoamino acids in the sequence have more variable influence on the level of IgG elicited.

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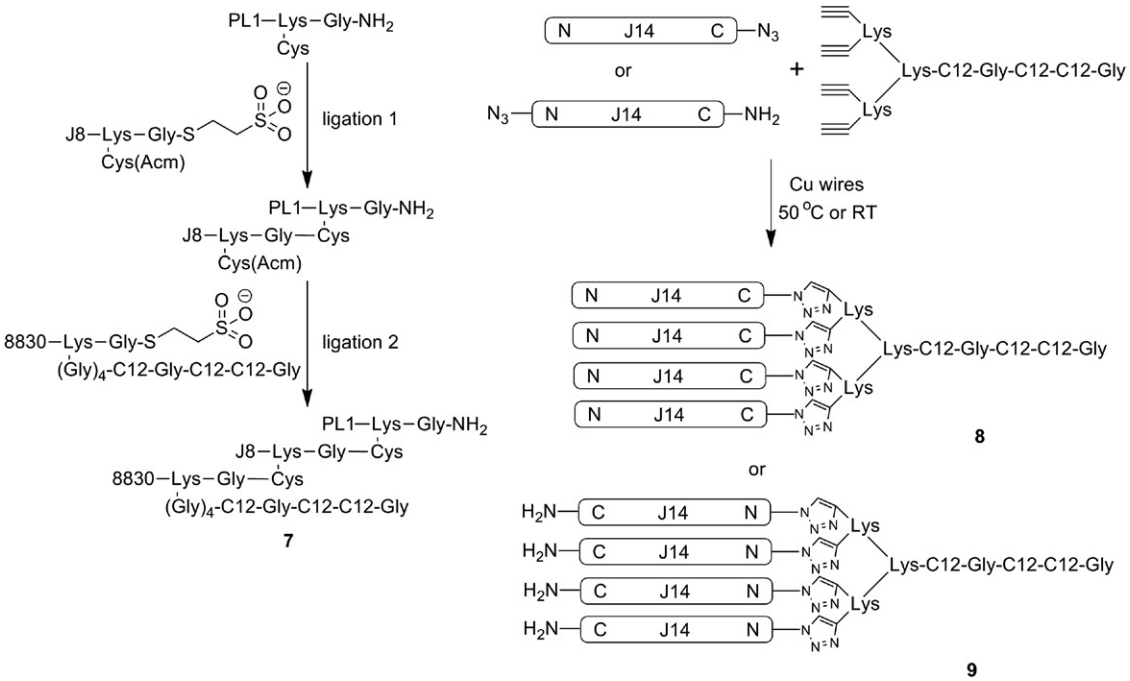
After intranasal administration of the same library of lipopeptides, the best systemic antibody response is seen with a lipopeptide featuring a lipid moiety consisting of two 16 carbon chains incorporated at the *C*-terminus of the lipopeptide and the J14 epitope attached to the epsilon group of lysine (see **4**, Fig. 2).[2] However, this candidate does not protect mice against bacterial challenge. The best protection is by a lipopeptide featuring a *C*-terminal J14, conjugated through a lysine residue to P25 at the *N*-terminus, and the C16 lipoamino acids attached on the lysine side chain (see **4**, Fig. 2). Lipopeptide **5**, unlike **4**, maintains the native conformation of the J14 epitope required to produce protective J14-specific antibodies. These studies provide important information for the design of effective lipopeptide-based vaccines and highlight the influence of molecular geometry on induction of a protective immune response.

To overcome the problems related to synthesis and purification of LCP vaccine candidates using stepwise SPPS, especially in the case of the systems possessing various epitopes, conjugation techniques were applied. These methods involve synthesis of individual unprotected peptide blocks that are then purified before conjugation. At first, native chemical ligation for the selective conjugation of epitopes and LCP core was employed. Aniko Horvath et al. synthesized three epitope constructs (J8+PL1+8830) without lipid moiety. As expected, upon immunization of mice, this polypeptide elicited immune responses only with the help of an external adjuvant.[9] Incorporation of the LCP core into the constructs via ligation was difficult because of the highly lipophilic nature of LCP. Poor water solubility of the lipid fragment in the aqueous reaction environment blocked the conjugation. To overcome this obstacle, sodium dodecyl sulfate (SDS) as a solubilizing agent was used during the conjugation of the LCP core with peptide epitopes.[13] This technique was applied successfully to the ligation of multiple single epitopes to the lipidic core and to polyvalent epitopes[13,14] as shown in Scheme 1. Subcutaneous immunization of mice with compound **7** without the addition of adjuvant elicited high antibody titers against the incorporated epitopes (J8+PL1+8830). A compound that possesses four copies of 8830 epitopes attached to classical LCP (Fig. 1) was easily synthesized using SDS-assisted ligation, but the incorporation of two copies of two different epitopes was more challenging because of steric hindrance arising during conjugation of the first two peptide epitopes. The difficulties were mostly overcome by using alternative protective groups for the cysteine moieties.

The use of a copper-catalyzed alkyne–azide 1,3-dipolar cycloaddition "click" reaction was successfully exploited to obtain a polymer-based delivery system binding J14 GAS epitope to the hydrophobic core.[29] The use of copper wires, instead of the typically employed copper/ligand catalyst system, allowed reduction of copper and ligand impurities, and therefore simplified the purification of final products. The problem with components' solubility reported in the case of native chemical ligation was eliminated by the use of organic solvent (*N*,*N*,-dimethylformamide), which was able to readily solubilize polar and hydrophobic starting



SCHEME 1 Synthesis of LCP constructs using native chemical ligation (**7**) and click reaction (**8**, **9**).

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materials. When this technique was used for the synthesis of LCP constructs almost quantitative conjugation was observed. [25,33] Upon conjugation with LCP core (Scheme 1), the J14 epitope adopts the desired (native) α-helical conformation and compounds are able to self-assemble into large nanoparticles (>100nm) under aqueous conditions. In contrast to the compounds from the series shown in Fig. 2, molecular geometry does not play a significant role in compounds **8** and **9**, and both constructs elicit similar titers of systemic IgG antibodies against the J14 epitope without the need of adjuvant. The Particle size of the peptide-based subunit vaccines usually plays a role in immunogenicity, and in most cases, 20- to 40-nm nanoparticles are favorable for the induction of strong immune responses.[22,30] The LCP constructs were examined for their ability to form small nanoparticles.[27] With the help of the click reaction, five LCP conjugates were prepared possessing J14, FNBR-B (VETED TKEPG VLMGG QSESV EFTKD TQTGM), FNBR-BT (EFTKD TQTGM SGQTT PQVET EDTKE PGVLM), J14+FNBR-B, and J14+FNBR-BT epitopes. Only diepitopic compounds formed small nanoparticles (5–15nm). Both FNBR compounds formed large particles, and the J14 construct did not aggregate. The ability to form small nanoparticles for diepitopic compounds is related to the balance between hydrophilic and hydrophobic properties of individual segments of the constructs. The immunological evaluation of these compounds has not been reported yet.

The mode of action of LCP-based vaccine candidates has been analyzed.[21,31] MHC II levels increase in splenic DCs after administration of LCP in mice. Furthermore, LCP enhanced DC-mediated proliferation of T-cells and cytokine production by DCs. Using cells expressing TLR2 and a luciferase reporter gene under the transcriptional control of NF-κB, the LCP system triggers activation of NF-κB in a TLR2-specific manner.[31] As such LAA-based lipid moieties exert their self-adjuvanting activity similarly in part to other lipopeptides via TLR2 mediated DC activation.[32]

GLYCOLIPOPEPTIDES

Carbohydrates have been used as building blocks in lipopeptide delivery systems to: serve as scaffold/branching moieties for epitopes display, or in the form of liposaccharides as self-adjuvanting moiety, to possibly protect peptides against enzymatic degradation, and as targeting moieties to specific carbohydrate-recognizing receptors.[24,34] They have been used as branching moieties because multiple copies of the peptide antigen can be easily attached to their hydroxy groups, the antigen can be attached in different spatial orientations depending on carbohydrate configuration, and the use of suitable protective groups allows attachment of different antigens to a single carbohydrate moiety. Several linkers have been designed to conjugate peptide to carbohydrate hydroxyls.[34] The rigid three-dimensional geometry of carbohydrates has been used to predetermine the conformation of attached peptides. Glucose, mannose, and galactose-based LCP vaccine candidates incorporating 8830 or J14i (ASRE AKKQV EKALE) GAS epitopes adopt an α-helical conformation[34] Such constructs (**10**, Fig. 3) are able to elicit high titers of systemic antibody after immunization in mice. The immune responses against those glycolipopeptides are similar to those against constructs based on lysine branching moiety showing that the carbohydrate scaffold is an interesting alternative to polylysine dendrimers.[23]

Glycosylated proteins and their interaction with carbohydrate-binding proteins (lectins) play a major role in the cellular recognition processes. Mannose receptors are highly expressed in cells responsible for immune responses such as macrophages and dendritic cells.[11] Mannosylation of an antigen to target receptors on the surface of dendritic cells improves vaccine efficacy.[28] A mannose receptor-targeting strategy was used for the development of a vaccine against human papillomavirus (HPV) infection, which is one of the leading causes of cancer deaths in women. The mannosylated antigen (8Q: QAEP DRAHY NIVTF CCKCD possessing CTL, Th-cell, and B-cell epitopes from the HPV-16 E7 protein) was incorporated by stepwise SPPS into a classical LCP structure (Fig. 1, where $n=8$).[15] Constructs without the help of additional adjuvant were evaluated for their capacity to reduce the size of HPV-16 associated tumors in mice. The mannosylated LCP vaccine candidates are more effective in reducing tumor sizes when compared with the nonmannosylated analog. In contrast to glycolipopeptides, the nonmannosylated compound is poorly water soluble. Thus, the improved protection of mice under tumor challenge might result from improvement in water solubility of the construct and not from the targeting properties of mannose. To examine this possibility, mannosylated, mannosylated (with acetylated mannose), nonmannosylated, and glucosylated constructs were examined in an additional experiment, but no significant difference was observed in the efficacy of the compounds.[15] Noticeably, the LCP-based constructs are able to offer superior anticancer activity and usually complete prevention of the formation of detectable tumors but improve vaccine efficacy because of the ability of mannosylated compounds to target dendritic cells yet to be clearly confirmed.

Monophosphoryl lipid A (MPL), a less toxic version of a major cell-wall component of Gram-negative bacteria lipid A, is an adjuvant which stimulates TLR4 mediated DC activation. MPL has been approved for use in vaccines for human use relatively recently (Fig. 3). Synthetic analogs of lipid A and MPL have been studied with the goal to maintain high adjuvant activity of these glycolipids but to produce fully defined, highly pure compounds and to

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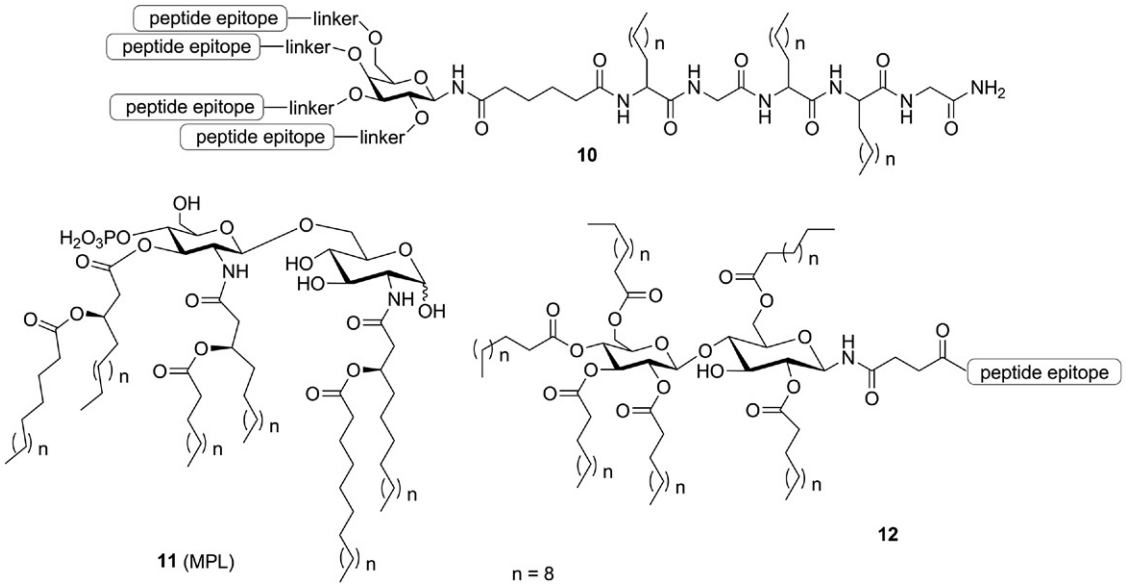

FIGURE 3 Vaccine candidates with carbohydrate as a branching scaffold for epitope display (10) and glycolipid adjuvants (11: monophosphoryl lipid A derived from cell wall lipopolysaccharide of Gram-negative *Salmonella minnesota;* 12: the most immunogenic liposaccharide from the series reported by Fujita et al.).

avoid any toxic bacterial traces. Thus, carbohydrates are not only attractive scaffolds for peptide antigens, but they can also be used to display lipid moieties in an organized fashion and with potential adjuvant activity. A synthetic simplified mimic of MPL was introduced to the LCP-like construct **12** (Fig. 3) and its efficacy to induce immune responses was compared with a physical mixture of glycolipid and peptide (J8).[5] Interestingly, although the new glycolipid does not show any adjuvant activity in the mixture, but when conjugated to the one and only peptide epitope is equally efficient as classical LCP-J8 with four copies of the peptide epitope. Disaccharide derivative **12** elicits a high level of J8-specific antibody after subcutaneous immunization of mice, similar to that elicited by LCP-J8 or J8 mixed with CFA while monosaccharide replacements are less immunogenic.

CONCLUSIONS

Numerous vaccine delivery systems have been developed to improve immunogenicity of peptide-based vaccines. Among them self-adjuvanting lipid- and glycolipid-based delivery systems have shown high efficiency in inducing strong immune responses to incorporated peptide-based immunogens. In most cases, the chemical conjugation between lipid moiety and peptide epitopes is necessary to induce these strong immune responses after the administration of subunit vaccines. Thus, LCP units serve usually as self-adjuvanting moieties but not as adjuvants in the physical mixtures. Many of these experimental vaccines have been analyzed in the context of their size, lipophilicity, spatial arrangement, and morphological properties, showing that physicochemical properties of the delivery system can have an important influence on vaccine efficacy. These ongoing studies expect to provide vaccinologists with more alternatives to develop new vaccines against a wide range of diseases. The use of lipopeptides/saccharides in peptide vaccine delivery should allow production of highly customized multicomponent, while still caged in a single chemical molecule, human vaccines with the ability to induce immune responses in an acceptably controllable fashion.

REFERENCES

1. Abdel-Aal ABM, Batzloff MR, Fujita Y, Barozzi N, Faria A, Simerska P, et al. Structure-activity relationship of a series of synthetic lipopeptide self-adjuvanting group A streptococcal vaccine candidates. *J Med Chem* 2008;**51**:167–72.
2. Abdel-Aal ABM, Zaman M, Fujita Y, Batzloff MR, Good MF, Toth I. Design of three-component vaccines against group a streptococcal infections: importance of spatial arrangement of vaccine components. *J Med Chem* 2010;**53**:8041–6.
3. Batzloff MR, Sriprakash KS, Good MF. Vaccine development for group a *Streptococcus* infections and associated diseases. *Curr Drug Targets* 2004;**5**:57–69.
4. Brown LE, Jackson DC. Lipid-based self-adjuvanting vaccines. *Curr Drug Deliv* 2005;**2**:383–93.
5. Fujita Y, Abdel-Aal ABM, Wimmer N, Batzloff MR, Good MF, Toth I. Synthesis and immunological evaluation of self-adjuvanting glycolipopeptide vaccine candidates. *Bioorg Med Chem* 2008;**16**:8907–13.
6. Gibbons WA, Hughes RA, Charalambous M, Christodoulou M, Szeto A, Aulabaugh AE, et al. Lipidic peptides.1. Synthesis, resolution and structural elucidation of lipidic amino-acids and their homo-oligomers and heterooligomers. *Liebigs Ann Chem* 1990:1175–83.

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7. Hayman WA, Toth I, Flinn N, Scanlon M, Good MF. Enhancing the immunogenicity and modulating the fine epitope recognition of antisera to a helical group A streptococcal peptide vaccine candidate from the M protein using lipid-core peptide technology. *Immunol Cell Biol* 2002;**80**:178–87.
8. Hennessy EJ, Parker AE, O'Neill LAJ. Targeting toll-like receptors: emerging therapeutics? *Nat Rev Drug Discovery* 2010;**9**:293–307.
9. Horvath A, Olive C, Karpati L, Sun HK, Good M, Toth I. Toward the development of a synthetic group A streptococcal vaccine of high purity and broad protective coverage. *J Med Chem* 2004;**47**:4100–4.
10. Horvath A, Olive C, Wong A, Clair T, Yarwood P, Good M, et al. Lipoamino acid-based adjuvant carrier system: enhanced immunogenicity of group a streptococcal peptide epitopes. *J Med Chem* 2002;**45**:1387–90.
11. Irache JM, Salman HH, Gamazo C, Espuelas S. Mannose-targeted systems for the delivery of therapeutics. *Exp Opin Drug Deliv* 2008;**5**:703–24.
12. Moyle PM, Horvath A, Olive C, Good MF, Toth I. Development of lipid-core-peptide (LCP) based vaccines for the prevention of group A streptococcal (GAS) infection. *Lett Pept Sci* 2003;**10**:605–13.
13. Moyle PM, Olive C, Ho MF, Burgess M, Karpati L, Good MF, et al. Method for the synthesis of multi-epitopic *Streptococcus pyogenes* lipopeptide vaccines using native chemical ligation. *J Org Chem* 2006;**71**:6846–50.
14. Moyle PM, Olive C, Ho MF, Good MF, Toth I. Synthesis of a highly pure lipid core peptide based self-adjuvanting triepitopic group A streptococcal vaccine, and subsequent immunological evaluation. *J Med Chem* 2006;**49**:6364–70.
15. Moyle PM, Olive C, Ho MF, Pandey M, Dyer J, Suhrbier A, et al. Toward the development of prophylactic and therapeutic human papillomavirus type-16 lipopeptide vaccines. *J Med Chem* 2007;**50**:4721–7.
16. Moyle PM. Toth I self-adjuvanting lipopeptide vaccines. *Curr Med Chem* 2008;**15**:506–16.
17. Olive C, Batzloff M, Horvath A, Clair T, Yarwood P, Toth I, et al. Potential of lipid core peptide technology as a novel self-adjuvanting vaccine delivery system for multiple different synthetic peptide immunogens. *Infect Immun* 2003;**71**:2373–83.
18. Olive C, Ho MF, Dyer J, Lincoln D, Barozzi N, Toth I, et al. Immunization with a tetraepitopic lipid core peptide vaccine construct induces broadly protective immune responses against group A *Streptococcus*. *J Infect Dis* 2006;**193**:1666–76.
19. Olive C, Schulze K, Sun HK, Ebensen T, Horvath A, Toth I, et al. Enhanced protection against *Streptococcus pyogenes* infection by intranasal vaccination with a dual antigen component M protein/SfbI lipid core peptide vaccine formulation. *Vaccine* 2007;**25**:1789–97.
20. Olive C, Sun HK, Ho MF, Dyer J, Horvath A, Toth I, et al. Intranasal administration is an effective mucosal vaccine delivery route for self-adjuvanting lipid core peptides targeting the group A streptococcal M protein. *J Infect Dis* 2006;**194**:316–24.
21. Phillipps KS, Wykes MN, Liu XQ, Brown M, Blanchfield J, Toth I. A novel synthetic adjuvant enhances dendritic cell function. *Immunology* 2009;**128**(Suppl. 1):e582–8.
22. Purcell AW, McCluskey J, Rossjohn J. More than one reason to rethink the use of peptides in vaccine design. *Nat Rev Drug Discovery* 2007;**6**:404–14.
23. Simerska P, Abdel-Aal ABM, Fujita Y, Moyle PM, McGeary RP, Batzloff MR, et al. Development of a liposaccharide-based delivery system and its application to the design of group a streptococcal vaccines. *J Med Chem* 2008;**51**:1447–52.
24. Simerska P, Moyle PM, Toth I. Modern lipid-, carbohydrate-, and peptide-based delivery systems for peptide, vaccine, and gene products. *Med Res Rev* 2011;**31**:520–47.
25. Skwarczynski M, Ahmad Fuaad AAH, Rustanti L, Ziora ZM, Aqil M, Batzloff MR, et al. Group A streptococcal vaccine candidates based on the conserved conformational epitope from M protein. *Drug Deliv Lett* 2011;**1**:2–8.
26. Skwarczynski M, Hayashi Y, Kiso Y. Paclitaxel prodrugs: toward smarter delivery of anticancer agents. *J Med Chem* 2006;**49**:7253–69.
27. Skwarczynski M, Parhiz BH, Soltani F, Srinivasan S, Kamaruzaman KA, Lin IC, et al. Lipid peptide core nanoparticles as multivalent vaccine candidates against *Streptococcus pyogenes*. *Aust J Chem* 2011:early view.
28. Skwarczynski M, Toth I. Lipid-core-peptide system for self-adjuvanting synthetic vaccine delivery. In: Mark SS, *Bioconjugation protocols: strategies and methods*. 2nd ed., vo. 751; Springer: 2011. p. 297-308
29. Skwarczynski M, Zaman M, Urbani CN, Lin IC, Jia ZF, Batzloff MR, et al. Polyacrylate dendrimer nanoparticles: a self-adjuvanting vaccine delivery system. *Angew Chem Int Ed Engl* 2010;**49**:5742–5.
30. Skwarczynski M, Toth I. Peptide-based subunit nanovaccines. *Curr Drug Deliv* 2011;**8**:282–9.
31. Zaman M, Abdel-Aal AM, Phillipps KSM, Fujita Y, Good MF, Toth I. Structure-activity relationship of lipopeptide group A *Streptococcus* (GAS) vaccine candidates on toll-like receptor 2. *Vaccine* 2010;**28**:2243–8.
32. Zaman M, Skwarczynski M. Toth I toll-like receptor 2 mediated dendritic cell activation—key target for lipopeptide vaccines design. In: Welles LC, editor. *Dendritic cells: types, life cycles and biological functions*. New York: Nova Science Publishers Inc.; 2010. p. 63–80.
33. Skwarczynski M, Malcolm JM, Urbani CN, Jia ZF, Batzloff MR, Good MF, et al. Self-adjuvanting polyacrylic nanoparticulate delivery system for group A streptococcus (GAS) vaccine. *Nanomed-Nanotechnol Biol Med* 2011;**7**:168–73.
34. Zhong W, Skwarczynski M, Toth I. Lipid core peptide system for gene, drug, and vaccine delivery. *Aust J Chem* 2009;**62**:956–67.
35. Zhong GM, Toth I, Reid R, Brunham RC. Immunogenicity evaluation of a lipidic amino acid-based synthetic peptide vaccine for chlamydia-trachomatis. *J Immunol* 1993;**151**:3728–36.

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Chapter 79

Novel Tumor Derived Peptides in Vaccines

Anthony W. Purcell, Nathan P. Croft and Nadine L. Dudek

ABSTRACT

Traditionally, cancer vaccines have consisted of recombinant antigens or tumor cell lysates. Whole tumor-derived preparations contain predominantly normal self-proteins of no therapeutic benefit or material with the potential to induce further malignancy. Thus, the identification and administration of tumor-specific antigens rather than crude tumor preparations are desirable. The most precise selection of vaccine components exists in epitope-based peptide vaccines, which represent the minimal immunogenic region of an antigen and allow exquisite control of immune responses. The difficulties associated with peptide-based vaccines; including stability, delivery, and the diversity of human immunogenetics have now mostly been overcome by high throughput sequencing of haplotype-specific peptide ligands and the ability to modify peptides to enhance both their immunogenicity and stability. Such recent progress makes it very probable that peptide-based cancer vaccines will enter the human therapeutics marketplace in the near future.

INTRODUCTION

The cellular immune response depends on recognition of small protein fragments presented on the cell surface by Major Histocompatibility Complex (MHC) molecules. These peptides are derived from the degradation of proteins from either intracellular or extracellular sources with tens to hundreds of thousands of different MHC-bound peptides (the immunopeptidome) simultaneously displayed for scrutiny by T-cells. Traditionally, T-cell epitopes have been defined using peptide libraries that span a given antigen and less frequently by biochemical definition using Edman sequencing or tandem mass spectrometry (MS/MS).[6] However, for many human diseases, including most cancers, the vast majority of peptide epitopes remain poorly defined.[23] One reason for this is the great complexity of the immunopeptidome and the relatively small proportion of tumor-specific peptides presented on the surface of cancer cells. The complexity of the immunopeptidome arises from both the large number of endogenous and exogenous peptides sampled by antigen presentation and the chemical diversity of these peptides. In particular, the immunopeptidome contains a significant proportion of peptides of atypical or heterogeneous length and peptides bearing a multitude of posttranslational modifications (PTMs). The factors that predispose to heterogeneous peptide length and the impact of PTMs on antigen processing are poorly understood. Mass spectrometry offers a powerful approach to dissect this complexity, not only to identify new targets of immunity but to also provide accurate quantitative measurement of antigen presentation. We discuss how mass spectrometry can be used to identify novel tumor-specific peptide antigens and how such information can be harnessed in vaccine design.

ANTIGEN PROCESSING AND ITS ROLE IN PEPTIDE-BASED VACCINOLOGY

The adaptive immune system is regulated by the recognition of antigenic peptides complexed to MHC molecules by clonally distributed T-cell receptors (TcRs). This interaction occurs at the immune synapse, where intimate membrane contacts are made between the T-cell and the antigen-presenting cell (APC). The outcome of this interaction may lead to the eradication of infected or malignant cells, the secretion of cytokines that recruit, and activate additional immune effector cells, or lead to the generation of antibodies. Immune surveillance relies upon accurate reporting of molecular events occurring within the cell via antigen presentation. As such, viral infection or malignant transformation alters the array of peptides presented on the cell surface bound to MHC molecules. There are two classes of MHC molecules that sample distinct peptides and present them to T-cells, namely, MHC class I molecules (MHC I) and class II molecules (MHC II).

Intracellular Immunosurveillance and the MHC Class 1 Pathway

Cytotoxic T-lymphocytes are generally activated by antigens derived from intracellular proteins, presented on the surface

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00079-8
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of cells complexed with MHC I molecules. Intracellular proteins (including, e.g. nuclear or cytoplasmic tumor antigens) are degraded to oligopeptides in the cytoplasm through the action of a multicatalytic protease structure known as the proteasome. These peptides are transported into the lumen of the endoplasmic reticulum (ER) where they assemble with MHC I and transit through the Golgi bodies to the cell surface (Fig. 1). The MHC I molecules themselves consist of a complex of a polymorphic heavy chain, a monomorphic light chain (β-2 microglobulin) and an antigenic peptide. The heavy chain forms an antigen-binding groove that accommodates peptides most commonly 8–10 amino acid residues long. The amino acid residues that line the binding groove are the focus for the majority of MHC polymorphism and determine allelic differences in peptide binding. Structural and biochemical studies of MHC-bound peptides have underpinned our knowledge of the binding characteristics of different MHC I-alleles (Table 1). In particular, these studies have highlighted the role of conserved amino acids at different positions of all allele-specific peptide ligands that are involved in binding to the cleft of MHC I molecules (anchor residues). Other more variable amino acids at different positions within the peptide project out of the antigen-binding cleft and make critical contacts with TcRs. The identification of specific MHC I binding motifs has been used to successfully predict T-cell epitopes with listings and search tools conveniently web based (e.g. http://www.immuneepitope.org/).[10] However, the success rate for de novo prediction of T-cell epitopes, even for well-studied MHC allotypes, is only about 60%, whereas for newly

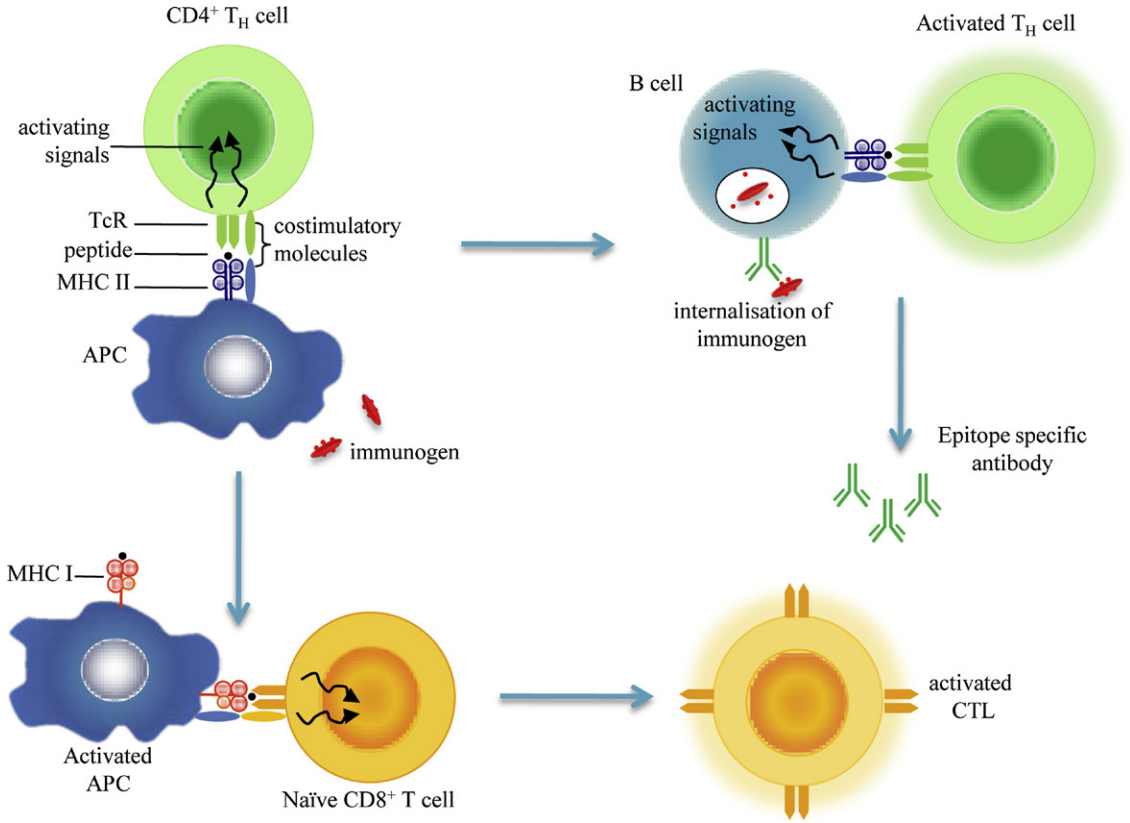

FIGURE 1 Key concepts of peptide vaccine immunology The adaptive immune system comprises two arms, responsible for the cytotoxic and the humoral immune response, respectively. Cytotoxic T-lymphocytes (CTLs, also referred to as CD8+ T-cells) eradicate tumor cells via direct cytotoxic action, and B-cells are the main players of the humoral immune response by generating antibodies toward tumor-specific molecules. Both processes are further dependent on T helper cells (T_H cells, also referred to as CD4+ T-cells). CTLs detect malignant cells by recognition of MHC class I molecules in complex with peptides derived from proteins expressed within the cell, whereas T_H cells recognize MHC class II molecules complexed to peptides derived from predominantly exogenous proteins. All nucleated cells present peptides derived from intracellular proteins on their surface bound to MHC I, whereas peptides derived from extracellular proteins are mainly presented by MHC II on specialized antigen-presenting cells (APC) such dendritic cells and macrophages. In both cases, the T-cell receptor (TcR) on the surface of the CTL or T_H cell ligates an MHC I/peptide complex or MHC II/peptide complex, respectively, aided by the CD8/CD4 coreceptors, respectively. The complex interplay of these peptide dependent recognition events results in the propagation of immune responses that ideally control cancer. The goal of vaccination is to induce immunity toward these states by selectively stimulating antigen-specific CTL or B-cells and T_H cells. A vaccine must therefore contain two antigenic epitopes—a T_H-epitope and an epitope that will either induce specific B-cell- or CTL responses. In some cases, these can overlap substantially within the sequence of an antigen and in others they may be present in discrete regions of the antigen or present in different antigens from the tumor cell.

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TABLE 1 HLA Supertypes and Their Peptide-Binding Specificities

HLA Supertype	Common alleles that are members of supertype	Peptide-binding specificity	Population coverage
A1	A*010102, A*2501, A*2601, A*2602, A*2604, A*3201, A*3601, A*4301, A*8001	XTXXXXXX. Y I W F	14.7 – 47.1%
A2	A*0201, A*0202, A*0203, A*0204, A*0205, A*0206, A*0207, A*6802, A*6901	XLXXXXXX. L I I V V M M A A T T	39.0 – 45.9%
A3	A*0301, A*1101, A*3101, A*3303, A*6801	XAXXXXXX. F L Y I W M V	37.5 – 52.7%
A24	A*2402-04, A*3001-03	XFXXXXXX. F Y I	23.9 – 58.6%
B7	B*0702-05, B*1508, B*3501-03, B*51, B*5301, B*5401, B*5501-02, B*5601-02, B*6701, B*7801	XPXXXXXX. A L I V M Y F W	43.0 – 57.1%
B27	B*1401-02, B*1503, B*1509, B*1510, B*1518, B*3801-02, B*3901-04, B*4801-02, B*7301, B*2701-08	XRXXXXXX. F H L K Y	13.3 – 35.3%
B44	B*3701, B*4402-05, B*4001, B*4006	XEXXXXXX. Y F W	21.2 – 43.0%
B58	B*1516-17, B*5701-02, B*58	XAXXXXXX. F S W T Y	1.6 – 25.1%
B62	B*1501-02, B*1513	XQXXXXXX. F L W Y	4.8 – 36.5%

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described, rare or poorly studied MHC I molecules no binding motifs are available. More advanced approaches have yielded higher success rates for some alleles.[10–12] One reason for the poor predictability resides in the occurrence of non-motif-based ligands, peptides of unusual length, PTM ligands, or of the failure of antigen processing to liberate all predicted peptides. Furthermore, many T-cell responses focus on a handful of immunodominant peptides despite numerous potential MHC ligands derived from a given antigen.[3] Such immunodominance limits the number of distinct epitopes required in a peptide-based vaccine to elicit protective immunity and also provides a challenge in the correct selection of candidate epitopes. Despite this, epitope prediction remains a popular first screening method to identify candidate epitopes for subsequent biological validation. Thus, in silico design of peptide-based vaccines must consider MHC-peptide binding, the abundance of specific MHC-peptide complexes on the surface of both APC and target tumor cells, the time of expression of the antigen during pathogenesis, correct processing and luminal transport of the epitope as well as the available T-cell repertoire in the host organism.

Extracellular Immunosurveillance and the MHC Class II Antigen Presentation Pathway

Unlike the more ubiquitous distribution of MHC I, class II molecules are only expressed on the surface of professional APC (e.g. dendritic cells, macrophages, B-cells). Extracellular antigen endocytosed via receptor-mediated processes is taken up less selectively by highly phagocytic APC like dendritic cells, and macrophages are degraded in the early and late endosomes. These compartments provide a rich source of antigenic peptides that are transported to the cell surface bound to MHC II for display and scrutiny by T helper (T_H) cells. This process is mechanistically and physically distinct to the MHC class I processing pathway.[6] Likewise, MHC II are physically distinct to MHC I molecules and are composed of two polymorphic polypeptide chains forming an αβ heterodimer, which combine to form a binding cleft that accommodates peptide antigens. B-cells that bind to a given antigen via their clonally distributed surface immunoglobulin receptor, and display a specific MHC II/peptide complex by virtue of the capture of antigen, can be triggered to differentiate into antibody producing plasma cells with the help of T_H cells (Fig. 1).

The mode of peptide binding and ligand repertoire of MHC II differs from that of MHC I molecules. The peptide-binding cleft of MHC II is not closed like that observed in MHC I molecules, which allows the termini of the bound peptides to hang out of the ends of the cleft. As a result, MHC II peptides are typically longer than class I ligands, averaging around 13–30 amino acids in length. Like MHC I, polymorphic amino acid residues line the pockets of the binding cleft, with amino acid side chains at residues 1, 4, 6, and 9 of the class II–bound peptide typically interacting with these pockets conferring allelic specificity and 'anchoring' the peptide into the cleft. It has also been suggested that the binding of ligands to MHC II is more promiscuous than the binding of peptides to MHC I, because of their free termini and ability to use alternate anchor residues or shift binding register.[18] This observation makes it more difficult to define anchor residues and to predict which peptides will be able to bind particular MHC II molecules. Amino- and C-terminal exopeptidase activities can trim the peptides bound to class II molecules during their transit to the cell surface further hampering the efforts to define MHC II binding motifs and reliably predict MHC II ligands.

New Insights into the Immunopeptidome

Although the mechanisms of peptide liberation and binding to MHC have been elucidated,[22] the relationship between the composition of the immunopeptidome and the cellular proteome remains controversial. The establishment of nanoscale preparative techniques and cutting edge mass spectrometry to study the immunopeptidome has provided critical insights into this complexity. We have focussed on naturally processed and presented MHC-bound peptides that contain PTMs. Our initial results indicate that up to 10% of MHC class I–bound peptides are greater than the expected length (Fig. 2), the majority of MHC class II–bound peptides show N- or C-terminal heterogeneity, and 5–10% (and in extreme cases up to 30%) of the immunopeptidome contains PTM peptides. These modifications are commonplace and play a crucial role in regulating cellular processes. PTMs can influence protein structure, protein–protein interactions, protein location, half life, and enzymatic function.[20] Their representation in the immunopeptidome has significant implications for traditional methods of epitope discovery, which do not normally take such peptides into account, as it implies that on an average 5–10% of immunogenic epitopes could be missed.

CTL Epitopes of Unusual Length

Despite antigen processing in the class I compartment predisposing toward peptides of 8–10 amino acids in length, peptides considerably longer than this bind to and are naturally presented by MHC I. These include naturally presented self-peptides of which at least 5% are over ten amino acids in length. The structures of several peptides considerably longer than ten residues in complex with MHC I molecules have been studied and, in each case, the peptide adopts a bulging conformation while maintaining a conserved hydrogen bonding network at the peptide amino and carboxy termini.[27] These bulged epitopes can be rigid and display a defined conformation or exhibit a considerable degree of flexibility

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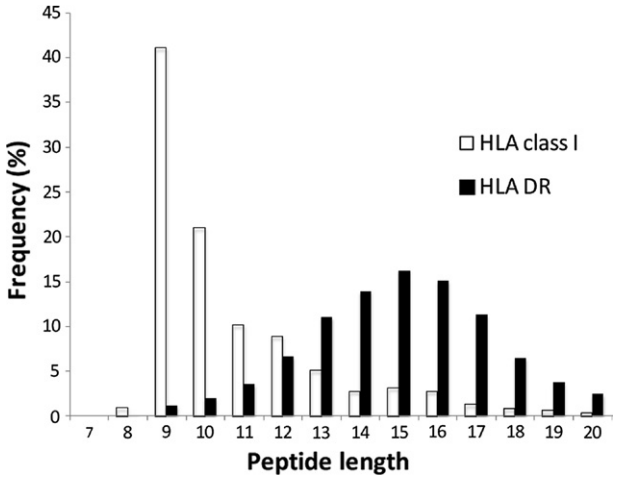

FIGURE 2 Length heterogeneity in MHC class I and class II peptides. The length of MHC-bound peptides was analyzed from approximately 5000 nonredundant peptide sequences isolated from HLA class I and HLA DR molecules. MHC–peptide complexes were purified from 5×10^8 9022 B-lymphoblastoid cells using solid phase bound monoclonal antibodies specific for HLA-DR and HLA-class I and the bound complexes eluted with 10% acetic acid. The eluted material was fractionated by reversed-phase HPLC and peptide-containing fractions analyzed by LC–MS/MS (Eksigent NanoUltra cHiPLC and an AB SCIEX 5600 TripleTOF mass spectrometer). Data were analyzed with ProteinPilot™ (AB SCIEX) software and peptide identities determined subject to strict bioinformatic criteria that included the use of a decoy database to calculate the false discovery rate (FDR). An FDR cut-off of 5% was applied, and the dataset was further filtered to exclude redundant peptides and known contaminants.

when bound to the antigen-binding cleft. For example, we have studied a highly immunogenic region from the BZLF1 antigen of Epstein–Barr Virus (EBV) that is presented by HLA-B35 family members in a length and conformation-specific manner.[29] Some of these longer epitopes contain within their sequences shorter core peptides of more conventional length that can bind to MHC I and are frequently naturally presented yet fail to induce a CTL response.[27,28] Why these longer peptides are preferentially selected as targets of CTL is most probably the result of biases in antigen processing and T cell repertoire toward the longer peptide.[29,30] The existence of such epitopes presents new challenges to vaccine design, both in terms of controlling the processing of these long peptides and ensuring the correct conformation is assumed by the epitope bound to MHC I. There are already several examples of unusually long CTL epitopes from tumor antigens including NY-ESO, M-CSF, tyrosinase, MART-2, CAMEL, and p53.[7] Thus, inclusion of such epitopes may be essential for optimal peptide-based cancer vaccines

T-cell Recognition of Posttranslationally Modified Peptides

The recognition of modified antigens by antibodies and T-cells can have important implications in disease.[20] Several studies have linked immune recognition of PTM peptides to disease states,[14,15,21] and the biology surrounding the formation and accumulation of PTM proteins and the peptides liberated from them has emerged as a new paradigm in autoimmunity. A role for immune recognition of PTMs has also been established in tumor immunology.[17,21,35] For example, HLA A2-bound peptides resulting from the differential phosphorylation of β-catenin, associated with nuclear accumulation of this molecule and the malignant phenotype, have been isolated from several tumor cell lines.[35] This highlights the link between deregulated signaling and the ability of antigen processing to alert T-cells to such molecular events. Moreover, T-cell epitopes have been characterized that include other PTMs including glycopeptides, deamidated peptides (both through the action of N-glycanases and enzymes such as transglutaminase), peptides exhibiting asparagine bond isomerism, acetylation, methylation, cysteinylation, nitration, and peptides containing disulphide bonds.[20] Such PTMs can be reliably and accurately incorporated into peptides at the time of their synthesis, allowing the end product of complex antigen presentation to be mimicked by a simple compound and the desired immune response to be elicited. Perhaps the most extreme form of PTM can be evidenced from recent reports of epitopes generated from discontinuous peptide sequences via a protein splicing mechanism.[5] Such processing may occur in a tissue-specific manner, and therefore, such epitopes are not only impossible to predict but also peptide-based vaccines may be the only reliable way of delivering such epitopes to the immune system.

T-cell Recognition of Tumor-Associated Peptides

There are at least five classes of tumor-associated antigens of relevance to peptide-based immunotherapy that include differentiation antigens, reactivated embryonic antigens, oncogene or suppressor gene products, mutated proteins, and some cancer-associated viral proteins. For instance, mutated tumor suppressor gene products may not only play a key role in tumorigenesis but may also create new T-cell epitopes. A prime example of such a protein is the p53 oncoprotein. Mutated p53 is found in >50% of all human cancers and a number of T-cell epitopes have been reported representing such mutated peptides.[9,19] Likewise, there has been much interest in defining epitopes derived from differentiation antigens such as the NY-ESO[16,24] and from cancer-associated viruses such as EBV found in lymphomas.[31] The identification of such peptides using peptide libraries or mass spectrometry is only complicated by the necessity to use both human and viral protein databases in library design or data search workflows and by the sometimes relatively low levels of presentation of

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such species. Once identified, such peptides are frequently engineered to produce more potent immunogens through the enhancement of MHC binding. As discussed later, the generation of these peptides often substituted at their anchor residue positions induce heteroclitic immunity often breaking tolerance to the often poorly immunogenic native tumor peptides.

MASS SPECTROMETRY AND THE IDENTIFICATION OF T-CELL EPITOPES

There are a number of methods that have been used for epitope identification, and recent advances in tandem mass spectrometry (MS/MS) have allowed cell-free methods to study the antigen processing and presentation. These approaches typically involve the purification of cell-surface MHC/peptide complexes using immunoaffinity chromatography followed by the extraction of the peptides and their analysis by MS/MS.[6] Recent improvements in this approach allow up to ten thousand peptides to be identified from a single MHC-peptide isolation experiment. These new methods incorporate several key changes including (i) serial purification of MHC allotypes using specific monoclonal antibodies to delineate allele-specific peptidomes; (ii) multiple dimensions of liquid chromatography (LC) of the peptides extracted from the individually purified MHC allotype-peptide complexes to enhance peptide separation and aid in MS/MS analysis; and (iii) fast, high resolution mass spectrometers to deal with the sample complexity (Scull et al. *Mol. Immunol*., in press; Dudek et al., in preparation). This depth of coverage of the immunopeptidome not only allows a significant proportion of the constitutive peptide repertoire to be identified, but this analysis also allows changes in the immunopeptidome to be examined during infection, drug treatment, tumorigenesis and metastasis. Such studies will open up personalized medicine and allow specific treatment of cancer and prevention of metastatic disease.

Despite considerable improvements in the number of peptides that can be confidently identified in the global LC–MS/MS analysis of MHC-bound peptides, the analysis of antigen- or disease-specific peptides can often still be challenging. This results from the relatively low abundance of many disease-specific epitopes compared with constitutively presented self-peptides. For the same reasons, modified peptides are also difficult to detect, and the interpretation of their spectra is complicated by the computational penalties associated with searching raw data while considering multiple potential PTMs. One way to circumvent this problem is to use affinity techniques to enrich for subpopulations of peptides. For example, Zarling et al. have used immobilized metal affinity chromatography to selectively capture phosphopeptides from HLA A2-peptide complexes isolated from the surface of tumor cell lines.[35] This study identified 36 HLA A2-bound phosphopeptides, including 5 that were common to several different cancers. Importantly, some of these phosphopeptides could be recognized by antitumor CTL.[35] Although affinity capture is a powerful method, only one modification can be targeted at a time, and a specific affinity technique is required for each modification. Alternatively, the mass spectrometer can be used to selectively screen for modified peptides using experiments such as neutral loss or precursor ion scanning.[34] For example, monitoring for the presence of phosphopeptides can be accomplished by detecting the m/z -79 ion derived from phosphate. Likewise, monitoring at m/z 216 can selectively identify phosphotyrosine-containing peptides in a complex mixture. Thus, by the use of appropriate diagnostic fragment ions, peptides with different PTMs can be detected. We have recently extended this approach to enable antigen-specific peptides to be identified using characteristic low mass reporter ions for precursor scanning experiments. This approach coined NIIPe (^{15}N immonium ion precursor scanning) takes advantage of unique MS/MS signatures of isotopically labeled antigen to effectively fish out antigen-specific peptides from cells pulsed with full length ^{15}N- labeled antigen by targeting heavy immonium ions for precursor scanning experiment. This approach has recently been used to successfully identify MHC-bound antigen-specific peptides (Reilly et al., submitted).

In addition to discovery-based workflows, we have used multiple reaction monitoring (MRM) to detect and quantitate peptide epitopes. The incorporation of MRM into LC–MS analyses provides the benchmark in sensitivity of detection of peptides isolated from biological samples. It combines a knowledge of the mass of the targeted peptide with fragmentation information to provide highly sensitive and specific peptide detection – in essence, it searches for the mass spectral fingerprint of peptide(s) being monitored and when combined with an isotopically labeled peptide standard allows absolute quantitation in the same experiment (Fig. 3). Thus, MRM can be used in experiments in which the presence of a particular peptide epitope needs to be confirmed or where the abundance of a peptide epitope is under consideration.[26] Moreover, MRMs may be designed in silico to probe antigen presentation by combining epitope prediction with experimental analysis of the resultant immunopeptidome postinfection or on introduction of a tumor-specific antigen. Finally, the sensitivity of this technique promises to allow rapid translation from *in vitro* studies using cultured cells to directly examine antigen presentation in biopsies or from tissues. Such information will provide a critical link between laboratory-based epitope discovery and clinical validation and will guide the composition of personalized peptide-based vaccines in the future.

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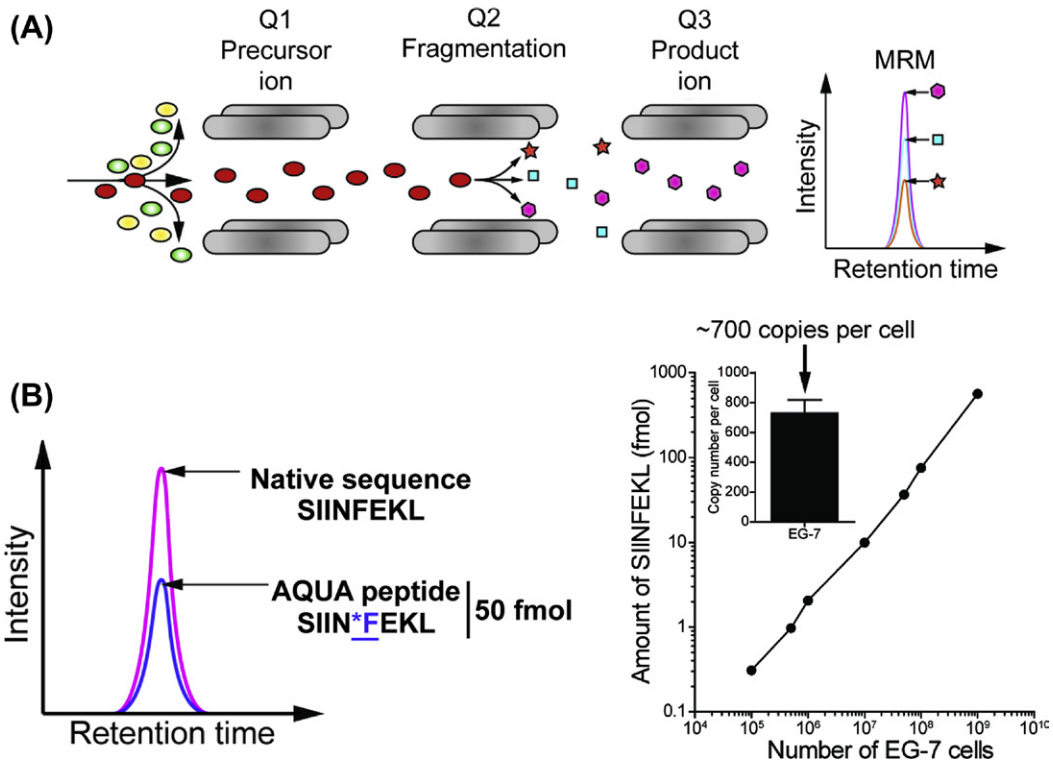

FIGURE 3 Multiple reaction monitoring and epitope quantitation (A). The concept of multiple reaction monitoring (MRM) Using a triple quadrupole mass spectrometer each mass analyzer can be used to spatially manipulate ions for the specific detection of a given analyte. The first quadrupole (Q1) is used to isolate a precursor ion of known m/z, in Q2 these ions are fragmented and in Q3 only fragment ions characteristic of the desired target analyte are detected. In a typical experiment, 3–4 fragment ions characteristic of the target peptide are monitored. The intensity of each Q1/Q3 mass pair (transition) is then plotted against retention time, and the area under each curve provides quantitative information. When used in combination with a heavy isotope labeled–peptide standard, the absolute quantity of the peptide can be determined (B). Shown in panel (b) is the estimation of the concentration of the SIINFEKL epitope derived from the surrogate tumor antigen chicken ovalbumin expressed by EG/7 thymoma cells.[1] For MRM analysis, peptide fractions were analyzed using a Eksigent TempoNanoLC cHiPLC coupled to a 5500 QTRAP mass spectrometer (AB SCIEX). The acquisition method was set to perform an MRM scanning experiment and to trigger a full mass MS/MS experiment for transitions that exceeded 500 counts. To quantify the amount of peptide present in each HPLC fraction, the area under each MRM transition peak was compared with an internal standard AQUA peptide. The copy number of SIINFEKL epitope per cell was calculated using Multiquant software by taking the molar quantity of peptides, multiplying by Avogadro's number and dividing by the cell number.

SYNTHETIC PEPTIDES AS VACCINES

Synthetic peptides offer several advantages over other forms of vaccines, particularly concerning safety and ease of production.[23] However, there are several disadvantages, the main issue being the poor immunogenicity of simple peptides and the need to more potently stimulate T-cells and elicit immunological memory. Adjuvant science, lipopeptide conjugation, and direct delivery to dendritic cells are some of the approaches currently being used to overcome this problem. Epitope-based vaccines are also restricted to use in patients of a given tissue type (HLA haplotype) and as such need to be personalized to accommodate natural variation in HLA genes. Although initially thought to be a major impediment, new technologies have made this personalized medicine approach quite feasible.[23] Moreover, the existence of HLA supertypes can simplify the use of epitope-based vaccines that target MHC I–restricted cytotoxic T-cells to essentially 9 super families, with shared peptide-binding specificity and shared epitope presentation (Table 1). Supertype motifs generally allow a significant reduction in the number of epitopes required to give good population coverage. However, supertypes are not always predictive of stable peptide binding, and significant variation even between closely related alleles can occur.[13] The binding of class II MHC ligands is much more promiscuous and as such motifs have been difficult to delineate with any great reliability. An alternative approach to the delivery of peptides with broad relevance to many different HLA allotypes is to use longer peptides and rely on their accurate processing to shorter allele-specific peptides. However, the use of longer peptide precursors is not always desirable

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because tissue-specific processing and the frequent non-concordance of processing of longer exogenous peptides compared with endogenous antigens can result in unwanted immune responses.

Finally, the path that peptide vaccines follow is less well understood than that for endogenous peptides liberated from intact antigens either within the cell or captured from the extracellular environment. On vaccination, peptides are immediately exposed to relatively harsh conditions rich in proteases and peptides are thus rapidly degraded and cleared from the body. Peptide-based vaccination strategies therefore must take into account the poor stability of native peptides and their requirements for incorporation into their relevant MHC/peptide complex.

Incorporation of Tumor-Derived Peptides into Vaccines

Because most known tumor-specific antigens recognized by T-cells represent overexpressed or aberrantly expressed proteins, which are not mutated and to which immunological tolerance has been developed, many vaccine efforts have focussed on substitution of specific amino acid residues to produce more immunogenic analogs. Typically, this has been done using the 19 remaining naturally occurring amino acids (for a review of this area see Ref. 25) with the goal being to improve MHC-binding affinity. The rationale here is that the majority of immunogenic tumor epitopes fail to elicit full immunological tolerance. This may result from either weak MHC binding or that the available T-cell repertoire comprises low-affinity TcRs after thymic selection. Although such substituted peptides can readily elicit T-cells, they do not in themselves possess improved bioavailability. The authors and others have chosen additional chemical augmentation of peptides to not only enhance MHC binding but to also improve their bioavailability. Such modification of tumor epitope sequences can include the incorporation of nonnatural amino acids and peptide bond modifications. The synthesis of these peptidomimetic vaccines still has all the advantages of synthetic peptide vaccines but with the added benefit of improved bioavailability predominantly through protease resistance.[4] Peptidomimetic vaccine research began in earnest in the early 1990s through the analysis of the effect of introducing stereoisomers of amino acids into peptide sequences, that is, replacing L-amino acids with their D-enantiomers. This represented an empirical strategy to increase peptide bioavailability based on the observation that cellular proteases are unable to cleave peptide bonds incorporating D-amino acids. More rational approaches then undertook the analysis of retroinversion of a peptide whereby all amino acids are substituted with their D-enantiomer but in reverse sequence. This inverts the side-chain topochemistry into a conformation that closely resembles that of the L-amino acid side chains. It should be noted that for studies utilizing retroinverso peptides, N- and C-terminal modifications are required particularly for CTL epitopes. In addition to retroinversion, other forms of peptide bond alteration are also possible. These so-called pseudopeptides often have reduced peptide bonds whereby the carbonyl moiety in the natural peptide bond is replaced by a methylene group. A separate class of peptide mimetics that undergo backbone modification are *N*-hydroxylated peptides—modification of the amide nitrogen to contain a hydroxyl group, a process that renders the resultant peptide resistant to proteolytic degradation.

In addition to direct alteration of the peptide backbone, there is considerable interest within the peptide vaccine design in the replacement of individual or multiple amino acids with nonnatural amino acids. These may be functional homologs of natural amino acids or entirely novel chemical entities. For example, we have used a steroisomer of cysteine, L-amino butyric acid (Abu), as a functional homolog in several epitopes[2,32] resulting from the natural amino acids propensity to oxidize and mask immunogenicity. For example, the C-terminal cysteine residue in the HLA A2 tumor epitope NY-ESO$_{157\text{-}165}$[32] was replaced using this substitution. The Abu modification enabled the mimetic to maintain HLA-A2 binding and up to 1000-fold improvements in T-cell recognition by wild-type epitope-specific CD8$^+$ T-cells. This primarily results from a small increase in binding affinity and the prevention of undesirable consequences associated with synthetic cysteine-containing peptides; namely epitope-dimerization, oxidative damage and cysteinylation.[32]

Ideally, for agonistic peptides, the closer a peptidomimetic is to the native unaltered epitope, the more likely it is to remain immunogenic. We have explored the use of homologous β-amino acid substitution as a generic platform for generating peptidomimetic based vaccines. A β-amino acid contains an extra methyl group within the amino acid backbone, with the R group positioned on either the resulting second or third carbon atom. As a consequence, the side chain remains unaltered, yet the substitution results in a broad resistance to degradation by a wide range of proteases even with just a single substitution in a CTL epitope.[33] The authors and others have used sequential substitution of each individual residue with β-amino acids followed by monitoring for protease resistance, MHC binding, MHC thermal stability, and recognition by an epitope-specific CTL line as a screening strategy for vaccine components.[33] Results from these approaches have shown that changes introduced by the β-amino acid residue substitutions had independent effects on MHC binding and T-cell recognition but consistently engendered protease resistance. Likewise, Guichard et al. have applied a positional β-amino acid scan on the MART-1$_{27\text{-}35}$ melanoma epitope.[8] The optimal position for the β-amino acid was determined to be at the postulated

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TCR contact residue Ile_{30}; interestingly, as a consequence, HLA-A2 binding was also enhanced.[8] Despite the success of these studies, none of the β-analogs described above have been tested *in vivo*. The only study so far to analyze this type of modification for *in vivo* immunogenicity has used the SIINFEKL epitope as a surrogate tumor antigen. By using a β-amino acid scan of this epitope, the MHC anchor residues were found to be the most efficacious site for β-amino acid replacement, with single substitutions at positions 5 and 8 conferring strong MHC binding and maintaining T cell recognition. Importantly, immunization of mice with the βPhe_5 analog generated crossreactive T-cells capable of lysing tumor cells expressing the native epitope.[33] Collectively, these studies suggest that β-amino acid-containing epitopes show promise as highly stable mimetics which, through careful manipulation, retain MHC binding and immunogenicity.

CONCLUSIONS

In this chapter, we have discussed the relevance of peptides to immune recognition and contrasted peptide vaccination with the processing and presentation of endogenous antigens. The advantages of peptides as vaccines have often been counterbalanced by their disadvantages, namely, poor bioavailability and the requirement to personalize vaccines because of the tremendous immunogenetic diversity in human populations. Relatively simple chemical modification of these peptides can overcome problems with bioavailability and generate potent immunogens. Moreover, the use of mass spectrometry to identify antigen-specific peptides both *in vitro* and *in vivo* will fast track the identification of tumor-specific epitopes from selected HLA haplotypes. In the near future, one can foresee where tumor biopsies may be probed by mass spectrometry using targeted approaches such as MRM to examine the actual presentation of tumor-specific epitopes on the surface of the patient's tumor and a cocktail of tumor peptides selected for immunotherapy. This will then allow chemically optimized forms of the peptide epitopes to be administered to patients. Thus, whether as a standalone treatment or used in combination with other antitumor strategies, there is a bright future for peptide-based vaccine in the treatment of cancer.

ACKNOWLEDGMENTS

A.W. Purcell is supported by a National Health and Medical Research Council (Australia) Senior Research Fellowship and project grants 1009326 and 1022509. N.L. Dudek is supported by grants from Diabetes Australia Research Trust and the National Health and Medical Research Council (Australia) project 637349. N.P. Croft is supported by National Health and Medical Research Council (Australia) project 1023141.

REFERENCES

1. Alleva DG, Crowe PD, Jin L, Kwok WW, Ling N, Gottschalk M, et al. A disease-associated cellular immune response in type 1 diabetics to an immunodominant epitope of insulin. *J Clin Invest* 2001;**107**:173–80.
2. Butler NS, Theodossis A, Webb AI, Dunstone MA, Nastovska R, Ramarathinam SH, et al. Structural and biological basis of CTL escape in coronavirus-infected mice. *J Immunol* 2008;**180**:3926–37.
3. Chen W, McCluskey J. Immunodominance and immunodomination: critical factors in developing effective CD8+ T-cell-based cancer vaccines. *Adv Cancer Res* 2006;**95**:203–47.
4. Croft NP, Purcell AW. Peptidomimetics: modifying peptides in the pursuit of better vaccines. *Expert Rev Vaccines* 2011;**10**:211–26.
5. Dalet A, Stroobant V, Vigneron N, Van den Eynde BJ. Differences in the production of spliced antigenic peptides by the standard proteasome and the immunoproteasome. *Eur J Immunol* 2011;**41**:39–46.
6. Dudek NL, Perlmutter P, Aguilar MI, Croft NP, Purcell AW. Epitope discovery and their use in peptide based vaccines. *Curr Pharm Des* 2010;**16**:3149–57.
7. Ebert LM, Liu YC, Clements CS, Robson NC, Jackson HM, Markby JL, et al. A long, naturally presented immunodominant epitope from NY-ESO-1 tumor antigen: implications for cancer vaccine design. *Cancer Res* 2009;**69**:1046–54.
8. Guichard G, Zerbib A, Le Gal FA, Hoebeke J, Connan F, Choppin J, et al. Melanoma peptide MART-1(27-35) analogues with enhanced binding capacity to the human class I histocompatibility molecule HLA-A2 by introduction of a beta-amino acid residue: implications for recognition by tumor-infiltrating lymphocytes. *J Med Chem* 2000;**43**:3803–8.
9. Kawakami Y, Rosenberg SA. T-cell recognition of self peptides as tumor rejection antigens. *Immunol Res* 1996;**15**:179–90.
10. Kim Y, Sette A, Peters B. Applications for T-cell epitope queries and tools in the immune epitope database and analysis resource. *J Immunol Methods* 2011;**374**:62–9.
11. Lafuente EM, Reche PA. Prediction of MHC-peptide binding: a systematic and comprehensive overview. *Curr Pharm Des* 2009;**15**:3209–20.
12. Lundegaard C, Lund O, Buus S, Nielsen M. Major histocompatibility complex class I binding predictions as a tool in epitope discovery. *Immunology* 2010;**130**:309–18.
13. Macdonald WA, Purcell AW, Mifsud NA, Ely LK, Williams DS, Chang L, et al. A naturally selected dimorphism within the HLA-B44 supertype alters class I structure, peptide repertoire, and T cell recognition. *J Exp Med* 2003;**198**:679–91.
14. Mannering SI, Harrison LC, Williamson NA, Morris JS, Thearle DJ, Jensen KP, et al. The insulin A-chain epitope recognized by human T cells is posttranslationally modified. *J Exp Med* 2005;**202**:1191–7.
15. Mannering SI, Pang SH, Williamson NA, Naselli G, Reynolds EC, O'Brien-Simpson NM, et al. The A-chain of insulin is a hot-spot for CD4+ T cell epitopes in human type 1 diabetes. *Clin Exp Immunol* 2009;**156**:226–31.
16. Matsuzaki J, Qian F, Luescher I, Lele S, Ritter G, Shrikant PA, et al. Recognition of naturally processed and ovarian cancer reactive CD8+ T cell epitopes within a promiscuous HLA class II T-helper region of NY-ESO-1. *Cancer Immunol Immunother* 2008;**57**:1185–95.
17. Mohammed F, Cobbold M, Zarling AL, Salim M, Barrett-Wilt GA, Shabanowitz J, et al. Phosphorylation-dependent interaction between antigenic peptides and MHC class I: a molecular basis for the presentation of transformed self. *Nat Immunol* 2008;**9**:1236–43.

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18. Mohan JF, Petzold SJ, Unanue ER. Register shifting of an insulin peptide-MHC complex allows diabetogenic T cells to escape thymic deletion. *J Exp Med* 2011;**208**:2375–83.
19. Pedersen AE, Stryhn A, Justesen S, Harndahl M, Rasmussen S, Donskov F, et al. Wildtype p53-specific antibody and T-cell responses in cancer patients. *J Immunother* 2011;**34**:629–40.
20. Petersen J, Purcell AW, Rossjohn J. Post-translationally modified T cell epitopes: immune recognition and immunotherapy. *J Mol Med (Berl)* 2009;**87**:1045–51.
21. Petersen J, Wurzbacher SJ, Williamson NA, Ramarathinam SH, Reid HH, Nair AK, et al. Phosphorylated self-peptides alter human leukocyte antigen class I-restricted antigen presentation and generate tumor-specific epitopes. *Proc Natl Acad Sci U S A* 2009;**106**:2776–81.
22. Purcell AW, Elliott T. Molecular machinations of the MHC-I peptide loading complex. *Curr Opin Immunol* 2008;**20**:75–81.
23. Purcell AW, McCluskey J, Rossjohn J. More than one reason to rethink the use of peptides in vaccine design. *Nat Rev Drug Discov* 2007;**6**:404–14.
24. Robson NC, McAlpine T, Knights AJ, Schnurr M, Shin A, Chen W, et al. Processing and cross-presentation of individual HLA-A, -B, or -C epitopes from NY-ESO-1 or an HLA-A epitope for Melan-A differ according to the mode of antigen delivery. *Blood* 2010;**116**:218–25.
25. Sette A, Fikes J. Epitope-based vaccines: an update on epitope identification, vaccine design and delivery. *Curr Opin Immunol* 2003;**15**:461–70.
26. Tan CT, Croft NP, Dudek NL, Williamson NA, Purcell AW. Direct quantitation of MHC-bound peptide epitopes by selected reaction monitoring. *Proteomics* 2011;**11**:2336–40.
27. Tynan FE, Borg NA, Miles JJ, Beddoe T, El-Hassen D, Silins SL, et al. High resolution structures of highly bulged viral epitopes bound to major histocompatibility complex class I. Implications for T-cell receptor engagement and T-cell immunodominance. *J Biol Chem* 2005;**280**:23900–9.
28. Tynan FE, Burrows SR, Buckle AM, Clements CS, Borg NA, Miles JJ, et al. T cell receptor recognition of a 'super-bulged' major histocompatibility complex class I-bound peptide. *Nat Immunol* 2005;**6**:1114–22.
29. Tynan FE, Elhassen D, Purcell AW, Burrows JM, Borg NA, Miles JJ, et al. The immunogenicity of a viral cytotoxic T cell epitope is controlled by its MHC-bound conformation. *J Exp Med* 2005;**202**:1249–60.
30. Tynan FE, Reid HH, Kjer-Nielsen L, Miles JJ, Wilce MCJ, Kostenko L, et al. T-cell receptor deformation of a bulged antigenic peptide enables MHC restricted recognition. 2007:377.
31. Villegas E, Santiago O, Sorlozano A, Gutierrez J. New strategies and patent therapeutics in EBV-associated diseases. *Mini Rev Med Chem* 2010;**10**:914–27.
32. Webb AI, Dunstone MA, Chen W, Aguilar MI, Chen Q, Jackson H, et al. Functional and structural characteristics of NY-ESO-1-related HLA A2-restricted epitopes and the design of a novel immunogenic analogue. *J Biol Chem* 2004;**279**:23438–46.
33. Webb AI, Dunstone MA, Williamson NA, Price JD, de Kauwe A, Chen W, et al. T cell determinants incorporating beta-amino acid residues are protease resistant and remain immunogenic in vivo. *J Immunol* 2005;**175**:3810–8.
34. Williamson NA, Reilly C, Tan CT, Ramarathinam SH, Jones A, Hunter CL, et al. A novel strategy for the targeted analysis of protein and peptide metabolites. *Proteomics* 2011;**11**:183–92.
35. Zarling AL, Polefrone JM, Evans AM, Mikesh LM, Shabanowitz J, Lewis ST, et al. Identification of class I MHC-associated phosphopeptides as targets for cancer immunotherapy. *Proc Natl Acad Sci U S A* 2006;**103**:14889–94.

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Chapter 80

Peptide Vaccine for Alzheimer's Disease

Beka Solomon

ABSTRACT

The pathology of Alzheimer's disease (AD) shows a significant correlation between β-amyloid peptide (AβP) deposition and severity of dementia. We have shown that antibodies toward the N-terminal region of β-amyloid peptide suppress *in vitro* and *in vivo* formation of toxic β-amyloid (Aβ). The epitope of these antiaggregating antibodies displayed on phage proved to be a potent antigen which induces antibodies against the whole AβP. In Alzheimer's diseased transgenic mice, these antibodies are delivered from the periphery to the CNS, preventing β-amyloid formation and/or dissolving such aggregates. Performance of such antigens offers possibilities for development of an efficient, long-lasting immunization for AD treatment.

INTRODUCTION

The pathology of AD is characterized primarily by extracellular senile plaques and intracellular neurofibrillary tangles.[26,27] The presence of amyloid in senile plaques and in cerebral blood vessels (amyloid angiopathy) has long been pathologically recognized. In 1984, Glenner and Wong were the first to sequence the deposits, showing them to be β-pleated sheets of amyloid (Aβ).[9] Soluble amyloid beta-peptide is a normal metabolite of approximately 4-kDa that is produced by processing a large transmembrane glycoprotein called amyloid beta-protein precursor (APP),[11,28] The pathological conditions and mechanisms that transform soluble beta-amyloid peptide into the fibrillary, toxic, beta-sheet form found in the plaques and vessels of patients with AD is not yet completely understood, but it is clear that the same amino acid sequence of beta-amyloid peptide can have both a fibrillar and soluble conformation.

Various theories have suggested that besides amyloid plaques, circulating amyloid, vascular amyloid and, more recently, soluble oligomeric species of amyloid may be responsible for the cognitive impairment and neural degeneration in AD.[12,14,26,27]

The most studied hypothesis, amyloid cascade,[14] states that overproduction of AβP, or failure to clear this peptide, leads to AD primarily through amyloid deposition, which is presumed to be involved in neurofibrillary tangles formation,[2,13] These lesions are then associated with cell death, which is reflected in memory impairment, the hallmark of this dementia.[12,13]

IN VITRO MODULATION OF β-AMYLOID FORMATION

Many investigators have studied the propensity of beta-amyloid peptide or its fragments to assemble into insoluble aggregates.[16,20] Beta-amyloid peptide can exist in two alternative conformations, depending on the secondary structure adopted by the N-terminal domain,[15,32] under various environmental conditions.[1] The N-terminal domain contains sequences that permit the existence of a dynamic equilibrium between the alpha-helix and the beta-strand conformations[32] (Fig. 1). The perturbations of the equilibrium of various conformational states of the beta-amyloid peptide can be caused by local pH changes, alterations of environmental hydrophobicity, or binding of other proteins.[17,32]

The involvement of the N-terminal region in conformational transformations of beta-amyloid peptide was confirmed by studies using synthetic peptides bearing the beta-amyloid peptide sequence in various solvents and in studies with synthetic beta-amyloid peptides containing single amino acid substitutions.[16] The polar domain of beta-amyloid peptide (1–28) is capable of stable self-association to form fibrils at acid pH, even in the absence of the stabilizing influence of the hydrophobic carboxyl terminus.[17] The importance of the N-terminal region in maintaining the solubility of the beta-peptide has been confirmed by studies showing that amino-terminal deletions within the (1–12) and (1–17) peptides enhance the aggregation of the beta-amyloid peptide *in vitro* in parallel with the neurotoxicity effect.[23]

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00080-4
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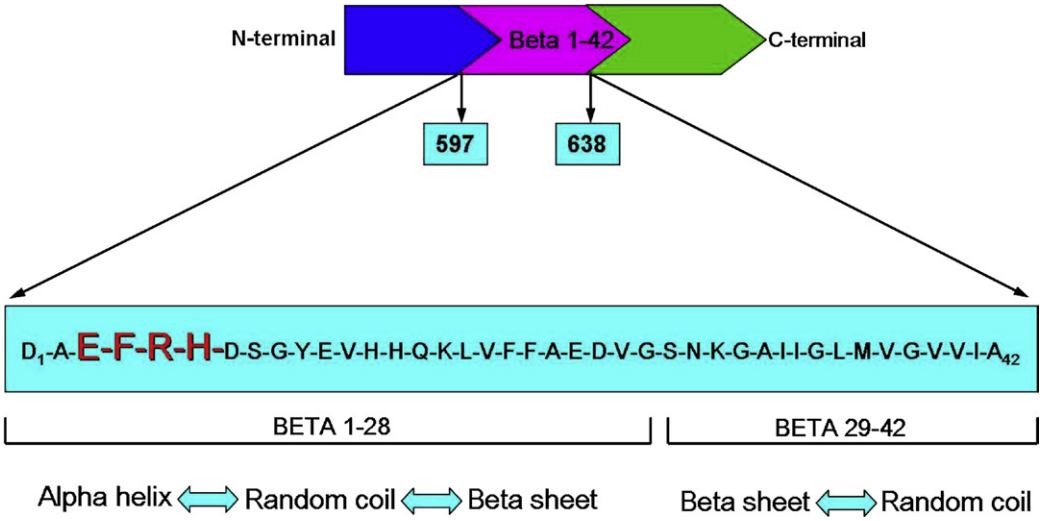

FIGURE 1

As beta-amyloid peptide might be critical for inducing the pathology seen in AD, its accumulation may result in a cascade effect, thereby allowing for intervention at multiple different points to slow disease progression. The treatment may be directed toward decreasing beta-amyloid peptide production, increasing its removal, and decreasing beta-amyloid aggregation.[3]

We showed for the first time that antibodies raised against the N-terminal region of the AβP peptide prevent amyloid formation and bind to preformed Aβ fibrils, leading to their disaggregation and inhibition of their neurotoxic activity.[29,30]

Antibodies interact at strategic sites where protein aggregation is initiated and are able to stabilize the antigen by preventing aggregation and resolubilizing already formed protein aggregates. For such an active role, the mAbs require a high binding constant to the "strategic" positions on the antigen molecule, and to be noninhibitors of the biological activity of the respective antigen.[31]

The ability of these antibodies to modulate βA formation *in vitro* and *in vivo* was found to be epitope dependent. We localized the epitope of antiaggregating antibodies in the N-terminal region of beta-amyloid peptide, which modulates amyloid formation,[4,5] using the phage libraries. The construction and application of several epitope libraries in which different peptides are expressed on the surface of filamentous phage (fd) has been described.[21,25] The minimal epitope for these mAbs was determined by using combinatorial phage-displayed six and/or fifteen peptide libraries. The immunopositive peptide-presenting phages were selected by AβP antiaggregating antibody according to the higher affinity binding, of about 10–9 M (half maximal binding). A consensus sequence of four residues, namely, EFRH (Glu–Phe–Arg–His), was found and this motif is sequentially located at positions 3–6 of the beta-amyloid molecule. The fact that the EFRH epitope is available for mAbs both when beta-amyloid peptide is either in solution or is an aggregate suggests that the N-terminal is exposed for antibody binding in both forms.[4,5]

We found that the epitope EFRH, which is located at the soluble tail of the N-terminal region, is involved in the aggregation process and acts as a regulatory site controlling both the solubilization and the disaggregation process of the beta-amyloid molecule. Locking of this epitope by antibodies modulates the dynamics of aggregation and resolubilization of already formed aggregates.

If the so-called pathological chaperones, such as ApoE and heparan sulfate, increase the extent of β-amyloid formation, we propose the use of site-directed monoclonal antibodies against β-amyloid peptides, which decrease β-amyloid formation as therapeutic chaperones.

Finding the "aggregating epitopes" as sequences that are related to the physiological protein regions where conformational changes are initiated, and preparation of antibodies against them, *are the bases of the immunological approach in treatment of conformational diseases, such as Alzheimer's disease*.

EFRH PHAGE ELICITS ANTIBODIES AGAINST β-AMYLOID PEPTIDE

We developed an immunization procedure for the production of effective antiaggregating AβP antibodies based on filamentous phages displaying on their surface the EFRH (Glu–Phe–Arg–His) peptide as the antigen,[6,7] which was found to be the main regulatory site for amyloid modulation.[5] Small synthetic peptides, consisting of antibody epitopes, are generally poor antigens and need to be coupled

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to a large carrier, but even then they may induce only a low affinity immune response. The generation of antisera to define peptide sequences usually involves the chemical synthesis of a peptide and its conjugation to a carrier molecule.

Much effort has been made to circumvent the low affinity response with limited success. A novel turn has been accomplished by using filamentous bacteriophage as peptide carrier. Filamentous bacteriophages offer an obvious advantage over other immunocarriers.[35]

Filamentous bacteriophages have been used extensively in recent years for the "display" on their surface of large repertoires of peptides generated by cloning random oligonucleotides at the 5′-end of the genes coding for the phage coat proteins,[21,25] Filamentous phages are long thread-like single-stranded DNA phages, which infect bacteria via sex pili. The best known, the Ff phages, are a group of three phages (M13, fd and fl). The phage protein that is responsible for binding to the pilus tip, pIII, is present in four copies, encoded by gene 3. If foreign DNA that encodes a peptide or protein is inserted downstream of the leader sequence of gene 3, it will be translated and exposed at the N terminus of the mature pIII without compromising the ability of pIII to mediate infection via the F pilus. Although pIII is the protein most used for phage display, the major coat protein, pVIII, has also been used. The main difference between pIII and pVIII is the copy number of the displayed protein; although pIII is present in four copies, pVIII is found in 2700 copies.

Parenteral administration of filamentous phages in animals induced a strong immunological response to the phage proteins in all animals tested.[35] Mimotopes displayed on filamentous phages could be used as immunogens and form the basis for developing a wide range of vaccines.

Immunization with the EFRH phage of BALB/c mice may, in a short period of time (a few weeks), increase the high concentration of high affinity (IgG) antiaggregation antibodies. Seven days after each injection of 1011 phages displaying the epitope without the adjuvant, mice were bled, and their sera were tested by ELISA for antibody IgG reactivity against wildtype (WT) phage (not bearing the peptide EFRH on its surface) and against whole beta-amyloid peptide. Highly specific antibodies (IgG) against the beta-amyloid peptide were achieved after the first injection and the titers increased with the second and the third injection till 1:50,000.[7] Moreover, that specific IgA antibodies against the beta-amyloid peptide antiaggregation epitope EFRH were obtained seven days after only one boost through intranasal administration.[6] The level of antibody in the sera is related to the number of copies per phage. The high immunogenicity of filamentous phages enables the raising of antibodies against self-peptides or antigens. Immunization of guinea pigs with EFRH phage as antigen, in which the beta-amyloid peptide sequence is similar to that in humans, results in the production of antibodies against self-peptides. Although in mice there is a switch of one amino acid (glycine instead of arginine in position 5-EFGH), in guinea pigs, the amino acid sequences are identical to those in humans.[7]

Highly specific antibodies (IgG) against the AβP are achieved after the fourth injection effective in concentrations as dilute as 1:5000. Similar results are obtained after immunization with 1011 phage per intranasal injection without the adjuvant with an additional administration of antigen.

The antibodies resulting from EFRH phage immunization are similar regarding their immunological properties to monoclonal antibodies previously studied, and to the antibodies raised by direct injection with fibrillar toxic beta-amyloid.[6]

PEPTIDES AS VACCINES FOR THE PREVENTION AND/OR REDUCTION OF AMYLOID PLAQUES IN AD TRANSGENIC MICE

The abundant evidence that AβP aggregation is an essential early event in AD pathogenesis has prompted an intensive search for therapeutics that target amyloid β-peptide, especially after the development of animal models of the disease. Several laboratories have bred AD diseased models of transgenic mice that produce human AβP, which develop plaques and neuron damage in their brains, as reviewed.[34] Although they do not develop the widespread neuronal death and severe dementia seen in the human disease, they are used as models for the study of AD and putative treatments. With the development of transgenic mice (Tg), the immunological concept in the treatment of conformational diseases became immunization approaches being pursued to stimulate clearance of brain Aβ plaques.[30] They include both active and passive immunization techniques. Active immunization approaches use AβP epitopes and/or immunogenic AβP conjugates, as well as various routes of administration and types of adjuvants. Passive immunization approaches include monoclonal antibodies or specific antibody fractions (Fabs) directed against specific AβP epitopes.

The idea of eliciting an immune response to exogenously administered Aβ-peptide in humans was originally described in a 1990 U.S. patent by a physician and an experimental immunologist.[18] They proposed to parenterally administer low amounts (up to 10 μg/day) of Aβ-peptide to slow the development of amyloid plaques.

Later, Schenk et al.[24] showed that immunization with human synthetic fibrillar Aβ 42 in transgenic mice (PDAPP) harboring a mutant version of human APP770 (V717F) produces high serum antibody titers against Aβ 42 (1:10,000) and inhibits the formation of amyloid plaques and associated histopathologic lesions.

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ACTIVE IMMUNIZATION OF HAPP TRANSGENIC MICE WITH EFRH PHAGE AS PEPTIDE VACCINE

We developed a series of antigens displaying the EFRH epitope on filamentous phages, which enabled modulating the immune response against AβP, according to the number of EFRH copies.[19]

APP[V717I] transgenic mice (16 months old) were immunized with the EFRH phage and analyzed at age 21 months.[8] Amyloid burden in the brain was significantly reduced in the immunized APP[V717I] transgenic mice that developed anti-AβP titers of at least 1:100, indicating that a relatively low antibody titer may be enough to reduce brain amyloid load.[8]

Another set of experiments was performed on transgenic mice which express human APP751 regulated by the neuronal murine Thy1 promoter. The hAPP gene carries both the London (717) and the Swedish (670/671) mutations, resulting in age-dependent increase in Aβ. Overexpression of the mutated human APP results in the development of typical AβP depositions as amyloid plaques in the neocortex and hippocampus. The mice were provided by JSW Research Co., Austria.[19]

hAPP transgenic mice were immunized with phages displaying EFRH sequences by intraperitoneal administration and analyzed at age 15 months. At this age, the amyloid plaque pathology is maximally and stably established.

Titer levels, although moderate, are positively correlated to the copy number of the EFRH epitopes displayed by the phage antigens. Titers obtained from sera of non transgenic mice or mice that were injected only with PBS are at background levels.

The amyloid burden in the brain is reduced in the immunized hAPP transgenic mice that developed anti-AβP titers of at least 1:100, similar to that in previous experiments,[6] showing a dose–response relationship between antibody titer and reduced amyloid load.

A considerable difference in plaque load content exists between treated and untreated mice. The reduction in total amyloid burden is related to the level of antibodies. We showed that a low but continuous titer of anti-EFRH is sufficient to reduce the amyloid burden.[19]

The development of AβP antiaggregating antibodies via immunization with phage-EFRH of AD models of transgenic mice show not only a considerable reduction of amyloid in the brains of the affected animals but also a considerable improvement in cognitive functions of the treated mice.[19]

The advantages of phage-EFRH antigen in raising antiaggregating β-amyloid antibodies versus whole β-amyloid can be summarized as follows:

1. The high immunogenicity of the phage enables production of high titers of IgG antibodies in a short period of weeks without need for adjuvant administration. Self-expression of the antigen led to long-lasting immunization.
2. The key role of the EFRH epitope in β-amyloid formation and its high immunogenicity leads to antiaggregating antibodies which recognize whole β-amyloid peptide and can substitute for whole toxic β-amyloid fibrils.

FUTURE OUTLOOK

In spite of uncertainty regarding the efficacy of AβP immunization in humans, the very promising results with APP Tg mice generated by several laboratories led ELAN/AHP to start human clinical trials after two and a half years of preclinical animal studies by injection of fibrillar Aβ 42 into participants in the clinical trials.

Patients with mild to moderate AD were immunized with AN1792 (fibrillar Aβ 1–42 plus adjuvant) in single- and multiple-dose Phase 1 safety studies, which were well tolerated and showed no significant safety concerns. Elan suspended inoculations in mid-January 2002 after some volunteers fell ill with what is called "clinical signs consistent with inflammation in the central nervous system."

Clinical trials showed stabilized cognition and slowed progression of dementia; however, there was neuroinflammation in some patients. Meningoencephalitis is not a feature of AD pathology and is probably a consequence of the immunotherapy. Vaccination seemed to have both beneficial and harmful effects, suggesting that the vaccine can have potent clinical utility if an appropriate immunological response can be generated.

An inflammatory reaction was probably triggered by T-lymphocyte activation after immune system stimulation with fibrillar Aβ 1–42. The adverse events observed in patients with AN1792 could reflect an autoimmune inflammatory response[22] because of T-cell epitopes to AβP 1–42, which reside in the mid-to-carboxy-terminal region of the beta-peptide.[10]

Improved immunotherapeutic strategies may be used to obtain a beneficial effect without untoward side effects. It is possible to induce AβP antibodies with no AβP T-cell response, as the N-terminal of AβP (1–15) contains the dominant B-cell epitopes that bind to Aβ plaques with no T-cell epitopes,[22] or with only small sequences from this region,[19] as described below.

In contrast, humans may develop self-antibodies when immunized with whole or fragments of AβP. These antibodies are capable of binding to a variety of Aβ species in the brain. Thus, immunization could have contradictory effects: the desired inhibition of amyloid fibril formation versus Fc microglial overactivation leading to neuroinflammation.[33]

How many antibodies are required for an effective immunization? No data are available on the quantitative decrease of AβP concentration necessary to reduce

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Aβ deposition. An increase of approximately 30% of Aβ concentration shifts the disease onset earlier by several decades.

Taking these arguments into consideration, the proposed EFRH sequence devoid of T-cell epitopes displayed on phage as antigens and able to provide a relatively low titer of AβP antiaggregating antibodies may become a real alternative for AD vaccination.

Despite the setbacks in the halted phase II Elan/Wyeth vaccination trial, amyloid immunotherapy strategies remain a novel and promising approach for the treatment and/or prevention of AD. To move forward, it will require considerable effort to determine whether immunotherapy will work in humans.

Amino acid sequence of β-amyloid peptide–C-terminal domain of AβP between 29 and 42 amino acids develops an β-strand structure in aqueous solutions independent of pH or temperature conditions, while the N-terminal region, 1–28 amino acids, can exhibit different conformations and solubility properties depending on environmental conditions. **EFRH** epitope of antiaggregating antibodies located at positions 3–6 is in bold.

ACKNOWLEDGMENTS

The author wishes to thank Rachel Cohen-Kupiec for phage-EFRH construction, Vered Lavie and Maria Becker for neuropathology staining, Rela Koppel for technical assistance, and Faybia Margolin for editing.

REFERENCES

1. Barrow CJ, Zagorski MG. Solution structures of β-peptide and its constituent fragments: relation to amyloid deposition. *Science* 1991;**253**:179–82.
2. Busciglio J, Lorenzo A, Yeh J, Yankner BA. β-amyloid fibrils induce tau phosphorylation and loss of microtubule binding. *Neuron* 1995;**14**:879–88.
3. Drachman DA. Preventing and treating Alzheimer's disease: strategies and prospects. *Expert Rev. Neurotherapeutics* 2003;**3**:565–9.
4. Frenkel D, Balass M, Solomon B. N-terminal EFRH sequence of Alzheimer's β-amyloid peptide represents the epitope of its anti-aggregating antibodies. *J Neuroimmunol* 1998;**88**:85–90.
5. Frenkel D, Balass M, Kachalsky-Katzi RE, Solomon B. High affinity binding of monoclonal antibodies to the sequential epitope EFRH of β-amyloid peptide is essential for modulation of fibrillar aggregation. *J Neuroimmunol* 1999;**95**:136–42.
6. Frenkel D, Katz O, Solomon B. Immunization against Alzheimer's β-amyloid plaques via EFRH phage administration. *Proc Natl Acad Sci USA* 2000;**97**:11,455–9.
7. Frenkel D, Kariv N, Solomon B. Generation of auto-antibodies towards Alzheimer's disease vaccination. *Vaccine* 2001;**19**:2615–9.
8. Frenkel D, Dewachter I, Van Leuven F, Solomon B. Reduction of beta-amyloid plaques in brain of transgenic mouse model of Alzheimer's disease by EFRH-phage immunization. *Vaccine* 2003;**21**:1060–5.
9. Glenner GG, Wong CW. Alzheimer's disease: initial report of the purification and characterization of a novel cerebrovascular amyloid protein. *Biochem Biophys Res Commun* 1984;**120**(3): 885–90.
10. Grubeck-Loebenstein B, Blasko I, Marx FK, Trieb I. Immunization with beta-amyloid: could T-cell activation have a harmful effect? *Trends Neurosci* 2000;**23**:114.
11. Haass C, Schlossmacher MG, Hung AY, et al. Amyloid β-peptide is produced by cultured cells during normal metabolism. *Nature* 1992;**359**:322–4.
12. Hardy J, Allsop D. Amyloid deposition as the central event in the aetiology of Alzheimer's disease. *Trends Pharmacol Sci* 1991;**12**:383–8.
13. Hardy J, Duff K, Hardy KG, Perez-Tur J, Hutton M. Genetic dissection of Alzheimer's disease and related dementias: amyloid and its relationship to tau. *Nat Neurosci* 1998;**1**:355–8.
14. Hardy J, Selkoe DJ. The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. *Science* 2002;**297**:353–6.
15. Hollosi M, Otvos L, Kaijtar J, Percell A, Lee VMY. Is amyloid deposition in Alzheimer's disease preceded by an environment induced double conformational transition? *Pept Res* 1989;**2**: 109–13.
16. Kirschner DA, Inouye H, Duffy LK, Sinclair A, Lind M, Selkoe DJ. Synthetic peptide homologous to β-protein from Alzheimer disease forms amyloid-like fibres in vitro. *Proc Natl Acad Sci USA* 1987;**84**:6953–7.
17. Kirshenbaum K, Daggett V. PH-Dependent conformations of the amyloid β (1–28) peptide fragment explored using molecular dynamics. *Biochemistry* 1995;**34**:7629–39.
18. Kline EL, McMichael J. Method and composition for treatment of central nervous systems disease states associated with abnormal amyloid β protein. WO 91/16819 (1991).
19. Lavie V, Becker M, Cohen-Kupiec R, Yacoby I, Koppel R, Wedenig M, et al. EFRH—phage immunization of Alzheimer's disease animal model improves behavioral performance in Morris Water Maze trials. *J Molec Neurosci* 2004;**24**(1):105–13.
20. Maggio JE, Mantyh PW. Brain amyloid—a physicochemical perspective. *Brain Pathol* 1996;**6**:147–62.
21. Medynski D. Phage display: all dressed up and ready to role. *Biol Technol* 1994;**12**:1134–6.
22. Monsonego A. Immunogenic aspects of amyloid beta peptide: implications for pathogenesis and treatment of Alzheimer's disease. *Neurobiol Aging* 2002;**23**:S112.
23. Pike CJ, Overman MJ, Cotman CW. Amino-terminal deletions enhance aggregation of β-amyloid peptides in vitro. *J Biol Chem* 1995;**270**:23,895–8.
24. Schenk D, Barbour R, Dunn W, et al. Immunization with amyloid-β attenuates Alzheimer's disease-like pathology in the PDAPP mouse. *Nature* 1999;**400**(1):73–177.
25. Scott JK, Smith GP. Searching for peptide ligands with an epitope library. *Science* 1990;**249**:386–90.
26. Selkoe DJ. Amyloid β protein and the genetics of Alzheimer's disease. *J Biol Chem* 1996;**271**:18,295–8.
27. Selkoe DJ. Toward a comprehensive theory for Alzheimer's disease. Hypothesis: Alzheimer's disease is caused by the cerebral accumulation and cytotoxicity of amyloid beta-protein. *Ann NY Acad Sci* 2000;**924**:17–24.

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28. Seubert P, Vigo-Pelfrey C, Esch F, et al. Isolation and quantification of soluble Alzheimer's β-peptide from biological fluids. *Nature* 1992;**359**:325–7.
29. Solomon B, Koppel R, Hanan E, Katzav T. Monoclonal antibodies inhibit in vitro fibrillar aggregation of the Alzheimer's β-amyloid peptide. *Proc Natl Acad Sci USA* 1996;**93**(1):452–5.
30. Solomon B, Koppel R, Frankel D, Hanan-Aharon E. Disaggregation of Alzheimer β-amyloid by site-directed mAb. *Proc Natl Acad Sci USA* 1997;**94**:4109–12.
31. Solomon B. Alzheimer's disease and immunotherapy. *Curr Alz Res* 2004;**1**:149–63.
32. Soto C, Castano EM, Frangione B, Inestrosa NC. The α-helical to β-strand transition in the amino-terminal fragment of the amyloid β-peptide modulates amyloid formation. *J Biol Chem* 1995;**270**:3063–7.
33. Ulvestad E, Williams K, Matre R, et al. Fc receptors for IgG on cultured human microglia mediate cytotoxicity and phagocytosis of antibody-coated targets. *J Neuropathol Exp Neurol* 1994;**53**:27–36.
34. Van Leuven F. Single and multiple transgenic mice as models for Alzheimer's disease. *Prog Neurobiol* 2000;**61**(3):305–12.
35. Willis EA, Perham NR, Wraaith D. Immunological properties of foreign peptides in multiple display on a filamentous bacteriophage. *Gene* 1993;**128**:79–83.

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Chapter 81

Peptide Vaccines for Myeloid Leukemias

Pratap Neelakantan and Katayoun Rezvani

ABSTRACT

Despite recent advances in the treatment of hematological malignancies, allogeneic stem cell transplant remains the only curative option for many patients with acute or chronic leukemias. The successful identification of many leukemia-associated antigen (LAA) and advances in understanding the fundamentals of tumor immunology have fostered the progress of immunotherapy. Preliminary results from many clinical studies suggest that vaccination using peptides derived from a number of LAAs is safe and induces immune responses, which may translate into clinical responses. In this chapter, we focus on the results of clinical trials of peptide vaccination in leukemia and discuss strategies to optimize the efficacy of immunotherapy in the future.

INTRODUCTION

Allogeneic stem cell transplant remains the only curative option for many patients with acute or chronic leukemia. Although the advent of tyrosine kinase inhibitors (TKIs) has significantly improved the outlook for patients with chronic myeloid leukemia (CML), the majority of patients continue to harbor residual disease. The potent graft versus leukemia effect (GVL), mediated by donor derived lymphocytes after allogeneic stem cell transplantation (SCT), supports a role for immunotherapy in leukemia. Evidence that the GVL effect plays a significant role in determining the risk of relapse after SCT came from studies reporting that relapse rates after allogeneic transplantation are markedly less in patients who developed graft-versus-host disease (GvHD) compared with those who did not. Further, the potential of allogeneic donor lymphocyte infusions to induce remissions in patients relapsing after SCT is strong evidence that T cells could destroy leukemia.[15] This realization led to efforts by many groups to generate leukemia-specific T cells for adoptive transfer in the treatment of leukemia relapse. Despite a successful demonstration of proof of principle, however, the approach has not been applied widely because of its cost and impracticability.[8] Meanwhile, other groups have explored the use of peptide vaccines derived from defined leukemia protein antigens, because of their potential to establish universal and practical vaccines for myeloid leukemias. In the last decade, a variety of proteins giving rise to leukemia-specific antigenic peptides have been characterized and several clinical peptide-based vaccine trials have been initiated in acute myeloid leukemia (AML), CML, and myelodysplastic syndrome (MDS) (Table 1).

LEUKEMIA-ASSOCIATED ANTIGENS (LAAs)

The successful identification of a range of LAAs that drive the GVL response in recent years has stimulated research in the development of vaccines to treat hematological malignancies.

LAA can be broadly categorized as: (1) unique leukemia-specific antigens or neoantigens, arising as a consequence of mutations or chromosomal translocations (e.g. BCR-ABL);[4] (2) aberrantly expressed or overexpressed self-antigens e.g. Wilms tumor (WT1)[9] proteinase-3 (PR3),[18,26] the receptor for hyaluronic-acid-mediated motility (RHAMM)[32] and preferentially expressed antigen of melanoma (PRAME);[24,30] (3) Viral antigens e.g. Ebstein-Barr virus (EBV); and 4) Allo antigens such as the minor histocompatibility antigens (mHAgs), HA-1 and HA-2.[10] An ideal target antigen for immunotherapy should be preferentially expressed on the leukemic progenitor cells, be intrinsic to leukemic survival so that viable tumor escape by down regulation of the antigen cannot occur and induce a strong cytotoxic T-cell (CTL) response.

Expression of LAA: Targeting neoantigens such as fusion proteins arising from specific chromosomal recombinations or mutated proteins for vaccination has the advantage that it is probably safe because of being restricted entirely to leukemia cells. However, the majority of LAAs identified to date are self-antigens that are also expressed by other normal tissues, raising concerns regarding the risk of autoimmunity. Interestingly, the requirement for exclusive expression by the leukemia is not absolute: the overexpressed self-antigen WT1, expressed on stem cells and at low levels on renal podocytes

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00081-6
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TABLE 1 Summary of Peptide Vaccine Studies

Study, year	Vaccine	No. of patients	Diagnosis	Status before vaccination	Comments
BCR-ABL vaccines, phase I/II					
Bocchia et al.,[3] 2005	b3a2 breakpoint-derived peptides + QS21 + GM-CSF	16	CML CP	MCyR on imatinib or INF-α	Patients continued on imatinib or INF-α; 5/10 achieved CCyR (Imatinib group)
Bocchia et al.,[2] 2009	b3a2 breakpoint-derived peptides + GM-CSF	57	CML CP	CcyR on imatinib	Patients continued on imatinib; 67% had CD4+ T-cell proliferation. 0.5 log reduction in the BCR-ABL transcript level after 9 months
Rojas et al.,[31] 2007	PADRE-linked b3a2 breakpoint-derived peptides + GM-CSF	19	CML CP	MCyR on imatinib	Patients continued on imatinib. One log reduction in 13/14 patients in MCyR
Maslak et al.,[16] 2008	b3a2 or b2a2 breakpoint-derived heteroclitic peptides + Montanide + GM-CSF	13	CML CP	CcyR or MCyR on imatinib	CD8+ T cell responses. No objective responses
WT1 vaccines, phase I/II					
Oka et al,[20] 2004	WT1-235, heteroclitic + Montanide	20	MDS, AML	Measurable disease	12/20 had clinical responses + WT1 specific CTL response
Keilholz et al.,[14] 2009	WT1-126 peptide + KLH +GM-CSF	19	Breast, lung Ca AML (17), MDS (2)	PR, SD, PD	8/18 WT1-specific CD8+ T-cell responses; 6/10 objective responses
Maslak et al.,[17] 2010	Polyvalent WT1 peptide vaccine + GM-CSF	10	AML	CR	7/8 WT1-specific CD8+ T-cell responses. Median disease-free survival not reached at median follow-up of 30 months from diagnosis
PR1 vaccine, phase I/II					
Qazilbash et al.,[22] 2004	PR1 peptide + Montanide + GM-CSF	66	AML, CML, MDS	Measurable disease (53), CR (13)	Patients with immune response had better EFS: 8.7 vs 2.3 months ($P = 0.03$)
Combination of PR1 – Combined PR1 and WT1 vaccines					
Rezvani et al.,[27,28] 2008, 2011	PR1 + WT1-126 peptide + Montanide + GM-CSF	8	AML, MDS, CML	CR	PR1 or WT1-specific CD8+ T cells were associated with transient reduction in MRD
RHAMM vaccine – Phase I/II					
Schmitt et al.,[32] 2008	RHAMM/CD1 68-R3 peptide + Montanide + GM-CSF	10	AML, MDS, MM	PR, PD	5/10 immunological response, 3/10 objective response.

Abbreviations: CML CP: chronic myeloid leukemia in chronic phase, DTH: delayed type hypersensitivity, MCyR: major cytogenetic remission, IFN-α: interferon-alpha, CCyR: complete cytogenetic remission, AML: acute myeloid leukemia, MDS: myelodysplastic syndrome, Ca: cancer, PR: partial remission, PD: progressive disease, CR: complete remission, SD: stable disease, EFS: event-free survival, DC: dendritic cell, MRD: minimal residual disease.

and gonadal cells, behaves in a leukemia-restricted manner because only the malignant cells which overexpress WT1 are susceptible targets of T cell attack.[9] Similar concerns that immune reactions against proteins such as PR3 and neutrophil elastase (NE) may target not only the leukemia cells, which overexpress the protein but also normal tissues that express lesser amounts of the protein such as normal early myeloid precursors have so far been unjustified.[23,27,28]

There is an assumption that effective immunotherapy requires the target antigen to be expressed by the earliest

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leukemic progenitor. Although this seems to be a reasonable criterion for antigen selection, it may not be a prerequisite in every case: individual leukemias have diverse hierarchical patterns of progenitor and progeny. CML progression, for example, seems to derive from the committed granulocytic progenitor.[12] We have delineated the spectrum of expression of mHAgs and candidate LAA including PR1, WT1, and PRAME, within the CD34+ primitive stem cell and the committed progenitor pool of CML cells.[35] We found heterogeneity in the expression of leukemia antigens, whereas mHAs are expressed in all CD34+ stem cell populations, the expression of LAA is varied depending on CML disease phase and CD34 progenitor maturity. WT1 and PRAME are preferentially expressed in the advanced stages of CML, whereas PR3 and NE are expressed in chronic phase CML.

Functional importance for the leukemic phenotype: It is possible for malignant cells to escape immune control by down-regulating antigen expression. Selecting antigenic targets that are intrinsic to the maintenance of the leukemic phenotype (e.g. BCR-ABL, WT1, PR3) may render it impossible for the leukemia cell to escape and still retain leukemic function.[5,21,34] It should be remembered, however, that it is possible for leukemic stem cells to evade immune surveillance by down regulating the appropriate major histocompatibility (MHC) molecules, allowing them to conserve essential leukemic features.

Immunogenicity: The ability of the LAA to elicit an immune response in the form CTLS is the key to successful immunotherapies. CTLs directed against mHAg and a number of LAA including PR1, WT1, RHAMM, and PRAME have been shown to selectively eliminate leukemic cells while sparing normal CD34+ cells.[18,24–26,29,30,32] Further, the presence of memory T cells directed against LAA further strengthens the evidence for *in vivo* immunogenicity of these antigens.[25,26]

VACCINATION

The increasing array of both allo antigens (mHAg) and LAA that can elicit antileukemia T-cell responses provides the basis for the development of vaccines for leukemia. Vaccination offers a less complicated alternative to the expense and technical complexity of alternative forms of immunotherapy such as adoptive T-cell transfer; however, little is known about the best approach for a successful vaccination strategy.

WHY PEPTIDES?

Peptide vaccines are attractive to use in clinical trials because they are relatively cheap, easily manufactured to clinical grade, and easy to administer. We will focus on clinical trials of peptide vaccination in leukemia.

BCR-ABL PEPTIDE VACCINE

BCR-ABL offers an attractive approach to vaccination, as BCR-ABL is central to leukemogenesis and expressed by leukemic cells only. A number of phase I/II studies have investigated the safety and immunogenicity of BCR-ABL peptide vaccination in patients with CML.[3,6,16,31] Pinilla-Ibarz and colleagues at the Memorial Sloan-Kettering Cancer Center developed the first BCR-ABL vaccine, composed of a pool of six peptide fragments derived from the b3a2 junctional region. The vaccine and its adjuvant, QS-21, were well tolerated and elicited T-cell immune responses to BCR-ABL with no major cytotoxicity.[6] In a subsequent phase 2 study involving 14 CML patients, delayed type hypersensitivity or CD4+ proliferative responses to the peptide vaccines were noted in all patients.[6] Reduction in marrow Philadelphia (Ph) positive chromosomes occurred in four patients, but three of these continued other treatments such as IFN-α or imatinib. Three patients with molecular relapse after SCT had transient molecular remissions, but two had also been treated with DLIs. These results, while promising, could not convincingly demonstrate the efficacy of the vaccine.

In a subsequent Italian study, 16 CML patients who had achieved stable residual disease after a minimum of 12 months' treatment with imatinib or 24 months with IFN-α received six vaccinations with a peptide vaccine derived from the b3a2 junctional region plus recombinant granulocyte macrophage colony-stimulating factor (GM-CSF) and QS-21 as adjuvant (CMLVAX100).[3] All patients had improvements in their cytogenetic response, with five patients achieving complete cytogenetic remission (CCyR). Three of these five patients also had negative polymerase chain reaction (PCR) for BCR-ABL. The interim analysis for a follow up phase 2 study in 57 CP CML patients who had achieved a CCyR on at least 18 months of imatinib treatment was presented at the Annual Meeting of the American Society of Hematology (ASH) in 2009. The investigators reported that 67% of 43 evaluable patients had a significant *in vitro* b3a2-peptide-specific CD4+ T-cell proliferation.[2] Fourteen of 29 evaluable patients who reached the ninth month evaluation had at least a 0.5 log reduction in the BCR-ABL transcript levels. It must be emphasized that patients continued with their imatinib therapy throughout the vaccination period.

Similarly, in a phase I/II study from the UK, Rojas et al. reported that the administration of peptides spanning the b3a2 junctional region, linked to the pan DR epitope (PADRE) to enhance CD4 T-cell responses, consistently induced T-cell response in vaccinated patients, as measured by IFN-γ ELISPOT and tetramer staining.[31] Of the 14 patients in major cytogenetic response (MCyR) at baseline, 13 developed at least 1 log fall in BCR-ABL transcripts, though this occurred several months after completing vaccination and no molecular

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benefit was seen in five patients who were not in MCyR at baseline.

By contrast, in a pilot study of vaccination with heteroclitic class I and II peptides, modified to enhance their binding to HLA class I and class II molecules, despite the successful induction of CD8+ T-cell responses that cross-reacted with native junctional peptides, Maslak and colleagues failed to show a clinically relevant activity, even in the setting of minimal residual disease (MRD).[16]

Taken together, these studies support the immunogenicity and safety of BCR-ABL peptide vaccination in patients with CML, although in the setting of current treatments with TKIs, data on possible clinical responses to BCR-ABL peptide vaccination are difficult to interpret.

WT1 PEPTIDE VACCINE

WT1 is a zinc finger transcription factor that is normally expressed in the tissues of mesodermal origin during embryogenesis. In normal adult tissues, WT1 expression is limited to low levels in normal CD34+ve hematopoietic stem cells, myoepithelial progenitor cells, renal podocytes and some cells in testis and ovary. Although originally described as a tumor suppressor gene, the WT1 protein also seems to be involved in tumorigenesis, as WT1 is overexpressed in most cases of AML, CML, MDS and acute lymphoblastic leukemia (ALL), and in several solid tumors.[33] Further, expression increases as disease progresses, making WT1 an attractive vaccine candidate in otherwise untreatable advanced leukemias.[35] Several investigators reported the presence of both humoral and cellular immune responses to WT1 in patients with hematopoietic malignancies, indicating that WT1 is a highly immunogenic antigen *in vivo*.[7,25,26,29] These observations suggest that WT1 is an interesting vaccine candidate for hematological malignancies.

Oka et al. first reported the outcome of a phase I clinical study of WT1 peptide-based immunotherapy in patients with breast or lung cancer, MDS, or AML.[20] Patients received a heteroclitic HLA-A24 restricted 9-mer WT1 peptide (WT-235), modified to enhance its affinity binding to HLA class I. The vaccine was administered in Montanide adjuvant at 2 weekly intervals in a dose escalation study. The vaccine was well tolerated and 12 of 20 evaluable patients had clinical responses, including reductions in blood or marrow leukemic blasts, tumor size, or tumor markers, associated with increases in WT1-specific CTL frequencies.

In a similar study, Keilholz and colleagues in Germany investigated the safety and immunogenicity of vaccination with an HLA-A0201-restricted WT1 peptide (WT-126) with keyhole limpet hemocyanin (KLH) and GM-CSF as adjuvants. A total of 19 patients (17 AML and 2 with MDS) received a median of 11 vaccinations. Treatment was well tolerated. Clinical responses were seen in 6 of 10 patients, including 4 stable disease (SDs) with >50% blast reduction and 2 with hematological improvement. An additional four patients had clinical benefit after initial progression, including one complete remission (CR) and three SDs. WT1 transcripts as molecular disease marker decreased in 35% of patients.[14]

Maslak and colleagues at the memorial Sloan-Kettering investigated the immunogenicity of a polyvalent WT1 vaccine containing a cocktail of WT1 peptides to stimulate both CD8+ and CD4+ T-cell responses.[17] Ten AML patients in CR but with molecular evidence of WT1 transcript received six vaccinations plus immune adjuvants over 12 weeks. Of the nine evaluable patients, seven completed six vaccinations and WT1-specific T-cell responses were noted in seven of eight patients. Three patients who were HLA-A0201-positive showed significant increases in the frequency of WT1-tetramer-positive CD8+ T cells. The median disease-free survival for the group had not been reached at the time of publication.

Although the lack of a control group limits the analysis of these trials, the promising results indicate that WT1 vaccination can induce functional CTL responses, which may be associated with clinical improvement in some patients, and support further studies using WT1 as a target cancer antigen.

PR1 PEPTIDE VACCINE

PR1 is a nine amino acid, HLA-A0201-restricted peptide, shared by two myeloid LAAs, PR3, and NE. PR3 and NE are found in the primary (azurophil) granule proteins of normal granulocytes and are overexpressed in myeloid leukemia cells. PR1 induces powerful HLA-A0201-restricted CD8+ T-cell responses that selectively kill myeloid leukemia cells *in vitro*.[18] The detection of low frequencies of PR1-specific CD8+ T cells in patients with CML and at higher frequencies in patients entering molecular remission after allogeneic SCT supports the concept that there is natural immunity to PR1, which can be boosted further by vaccination.[19,26]

Highly encouraging preliminary data from a pilot study of PR1 peptide vaccination in patients with myeloid malignancies were first presented by Molldrem and colleagues at ASH in 2004[22] and updated at ASH in 2007.[23] A total of 66 HLA-A0201-positive patients with AML (42), CML (13) or MDS (11) received one of three dose levels of PR1 peptide vaccine: 0.25, 0.5, or 1.0 mg per vaccination every 3 weeks, together with Montanide adjuvant and GM-CSF. The vaccine was well tolerated. Of the 53 patients with active disease, 25 (47%) had an immune response. Clinical responses ranging from improvements in blood counts to CCyR were observed in nine of 25 responders (36%), compared with three of the 28 nonresponders (10%).

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Analysis of the effectiveness of this approach in a subgroup of 20 patients with myeloid leukemia vaccinated after SCT showed a PR1 response to the vaccine in 11/20 (55%) patients. Nine of 11 (82%) patients with a PR1 response compared with one of nine (11%) patients who failed to mount an immunological response to the vaccine had clinical responses ($P = 0.005$). Importantly, a significant PR1 response to the vaccine was associated with significantly better clinical response and longer event-free survival (EFS). These encouraging results have culminated in a Phase 3 multicenter study of PR1 vaccination in patients with myeloid malignancies.

COMBINED PR1 AND WT1 PEPTIDE VACCINES

Our group conducted a phase I study to assess the safety and immunogenicity of vaccination with a combination of PR1 and WT1-126 peptides in eight HLA-A0201-positive patients with low risk myeloid malignancies.[28] Five patients with AML in CR (one following a second unrelated donor SCT), two patients with MDS (refractory anemia and refractory anemia with ring sideroblasts) and one patient with CML in chronic phase (BCR-ABL negative on imatinib) were recruited. Patients received a single subcutaneous injection of PR1 (0.5 mg) and WT1-126 (0.2 mg) peptide vaccines in Montanide adjuvant, administered concomitantly with GM-CSF. Patients were reviewed weekly to monitor for toxicity and immunological responses. Toxicity was limited to grade 1–2. CD8+ T-cell responses against PR1 were detected in 7/8 and against WT1-126 in 5/8 vaccinated patients. Vaccine-induced T-cell responses were detected as early as 1–2 weeks postvaccination in most patients, but these responses were short-lived. Postvaccination, the emergence of PR1 or WT1-specific CD8+ T cells was associated with a significant but transient reduction in MRD as assessed by WT1 gene expression, suggesting a vaccine-induced antileukemia response.

These encouraging results led to the initiation of a phase II study of biweekly injections with PR1 and WT1 peptide vaccines in patients with myeloid malignancies. Again, the combination of PR1 and WT1 peptide vaccines was found to be immunogenic in all patients. However, repeated vaccination led to selective deletion of high avidity vaccine-induced T cells and additional boosting did not increase the frequency of PR1 or WT1-specific CD8+ T cells further. In four of six patients, the vaccine-induced T-cell response was lost after the fourth dose and in all patients after the sixth dose of vaccine.[27] Therefore, although these results support the immunogenicity of PR1 and WT1 peptide vaccination, novel approaches will be needed to induce long-term immune responses against these leukemia antigens.

RHAMM PEPTIDE VACCINE

RHAMM, also known as CD168, was first described as a soluble binding protein released by subconfluent migrating cells that interact with hyaluronic acid (HA) to promote cell motility and invasion.[32] Because RHAMM lacks a signal peptide, it interacts with CD44 to bind its ligand HA. RHAMM has been related to motility, transformation, adhesion, wound healing, metastasis, and growth. RHAMM is overexpressed in AML and CML and is immunogenic, making it an ideal LAA.

A phase I/II study investigated the safety and immunogenicity of vaccination using the HLA-A0201-restricted RHAMM/CD168-R3-peptide in ten patients with AML, MDS, or multiple myeloma.[32] Vaccination with 300 mg of R3 peptide induced immunological responses in five of ten patients. Clinical responses were observed in three patients; one patient with AML and one with MDS had a significant reduction in blasts in the bone marrow and one patient with MDS no longer required erythrocyte transfusions after four doses of vaccine. The study was therefore extended and further patients were vaccinated with an increased dose of 1000 mg of R3 peptide.[11] Nine patients were vaccinated, of whom four had positive immunological responses. However, higher doses of peptide did not improve the frequency and intensity of immune responses in this trial.

IMPLICATIONS AND FUTURE PERSPECTIVES

Taken together, these studies support the safety and immunogenicity of peptide vaccination in patients with leukemia. However, although encouraging antileukemia responses (mainly in the setting of MRD) have been reported, convincing results on sustained clinical efficacy are lacking. The current trials have so far failed to identify the best target antigen and the optimal schedule for vaccination.[20,22]

The transient immunological and clinical responses seen in many trials of peptide vaccination with CD8 epitopes may be partly explained by the lack of CD4+ T cell help in these studies. CD4+ T cells are essential for secondary CTL expansion and the induction of immunological memory.[13] It is therefore possible that inclusion of MHC class II binding peptides in a vaccine to elicit CD4+ T cells may improve the success of vaccination. The choice of adjuvant also seems to be very important in determining the peptide-specific immune response. Bijker et al. demonstrated that vaccination with CD8 epitopes derived from self-antigens mixed with oil-in-water adjuvants such as Montanide results in transient effector CD8 T-cell responses and fails to induce CD8 T cell memory in mice.[1] They proposed that the sustained systemic presentation of the peptides gradually

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leaking out of the oil-in-water depot without systemic danger signals leads to tolerance induction. This hypothesis was further supported by the demonstration that vaccination with peptides admixed with PBS, resulting in relatively short duration of antigen presentation, induced memory CD8+ T cells that were able to expand on secondary antigen stimulation *in vitro*.

Although both the clinical setting in which vaccines are used as well as their design and mode of application (e.g. CD4 epitopes, adjuvants) clearly are important parameters, it is likely that this is an oversimplification. The fact that the majority of LAA under investigation are nonmutated self-antigens and as such are likely to induce immune tolerance further adds to the challenges of peptide vaccination. Well-designed multicenter clinical studies are imperative to improve the results of peptide vaccination by addressing basic questions such as the ideal target antigen, the optimal vaccine dose and vaccination schedule, the inclusion of CD4+ T epitopes and the role of novel immune modulatory agents to enhance the vaccine-induced T-cell response.

REFERENCES

1. Bijker MS, van den Eeden SJ, Franken KL, Melief CJ, Offringa R, van der Burg SH. CD8+ CTL priming by exact peptide epitopes in incomplete Freund's adjuvant induces a vanishing CTL response, whereas long peptides induce sustained CTL reactivity. *J Immunol* 2007;**179**:5033–40.
2. Bocchia M, Defina M, Ippoliti M, Amabile M, Breccia M, Iuliano F, et al. BCR-ABL derived peptide vaccine in chronic myeloid leukemia patients with molecular minimal residual disease during imatinib: interim analysis of a phase 2 multicenter GIMEMA CML working party trial [abstract]. *ASH Annual Meet Abst* 2009:114–648.
3. Bocchia M, Gentili S, Abruzzese E, Fanelli A, Iuliano F, Tabilio A, et al. Effect of a p210 multipeptide vaccine associated with imatinib or interferon in patients with chronic myeloid leukaemia and persistent residual disease: a multicentre observational trial. *Lancet* 2005;**365**:657–62.
4. Bocchia M, Korontsvit T, Xu Q, Mackinnon S, Yang SY, Sette A, et al. Specific human cellular immunity to bcr-abl oncogene-derived peptides. *Blood* 1996;**87**:3587–92.
5. Bories D, Raynal MC, Solomon DH, Darzynkiewicz Z, Cayre YE. Down-regulation of a serine protease, myeloblastin, causes growth arrest and differentiation of promyelocytic leukemia cells. *Cell* 1989;**59**:959–68.
6. Cathcart K, Pinilla-Ibarz J, Korontsvit T, Schwartz J, Zakhaleva V, Papadopoulos EB, et al. A multivalent bcr-abl fusion peptide vaccination trial in patients with chronic myeloid leukemia. *Blood* 2004;**103**:1037–42.
7. Elisseeva OA, Oka Y, Tsuboi A, Ogata K, Wu F, Kim EH, et al. Humoral immune responses against Wilms tumor gene WT1 product in patients with hematopoietic malignancies. *Blood* 2002;**99**:3272–9.
8. Falkenburg JH, Wafelman AR, Joosten P, Smit WM, van Bergen CA, Bongaerts R, et al. Complete remission of accelerated phase chronic myeloid leukemia by treatment with leukemia-reactive cytotoxic T lymphocytes. *Blood* 1999;**94**:1201–8.
9. Gao L, Bellantuono I, Elsasser A, Marley SB, Gordon MY, Goldman JM, et al. Selective elimination of leukemic CD34(+) progenitor cells by cytotoxic T lymphocytes specific for WT1 leukemia-derived immature dendritic cells differentiate into functionally competent mature dendritic cells that efficiently stimulate T cell responses. *Blood* 2000;**95**:2198–203.
10. Goulmy E. Human minor histocompatibility antigens. *Curr Opin Immunol* 1996;**8**:75–81.
11. Greiner J, Schmitt A, Giannopoulos K, Rojewski MT, Gotz M, Funk I, et al. High-dose RHAMM-R3 peptide vaccination for patients with acute myeloid leukemia, myelodysplastic syndrome and multiple myeloma. *Haematologica* 2010;**95**:1191–7.
12. Jamieson CH, Ailles LE, Dylla SJ, Muijtjens M, Jones C, Zehnder JL, et al. Granulocyte-macrophage progenitors as candidate leukemic stem cells in blast-crisis CML. *N Engl J Med* 2004;**351**: 657–67.
13. Janssen EM, Lemmens EE, Wolfe T, Christen U, von Herrath MG, Schoenberger SP. CD4+ T cells are required for secondary expansion and memory in CD8+ T lymphocytes. *Nature* 2003;**421**:852–6.
14. Keilholz U, Letsch A, Busse A, Asemissen AM, Bauer S, Blau IW, et al. A clinical and immunologic phase 2 trial of Wilms tumor gene product 1 (WT1) peptide vaccination in patients with AML and MDS. *Blood* 2009;**113**:6541–8.
15. Kolb HJ, Mittermuller J, Clemm C, Holler E, Ledderose G, Brehm G, et al. Donor leukocyte transfusions for treatment of recurrent chronic myelogenous leukemia in marrow transplant patients. *Blood* 1990;**76**:2462–5.
16. Maslak PG, Dao T, Gomez M, Chanel S, Packin J, Korontsvit T, et al. A pilot vaccination trial of synthetic analog peptides derived from the BCR-ABL breakpoints in CML patients with minimal disease. *Leukemia* 2008;**22**:1613–6.
17. Maslak PG, Dao T, Krug LM, Chanel S, Korontsvit T, Zakhaleva V, et al. Vaccination with synthetic analog peptides derived from WT1 oncoprotein induces T-cell responses in patients with complete remission from acute myeloid leukemia. *Blood* 2010;**116**:171–9.
18. Molldrem J, Dermime S, Parker K, Jiang YZ, Mavroudis D, Hensel N, et al. Targeted T-cell therapy for human leukemia: cytotoxic T lymphocytes specific for a peptide derived from proteinase 3 preferentially lyse human myeloid leukemia cells. *Blood* 1996;**88**:2450–7.
19. Molldrem JJ, Lee PP, Wang C, Felio K, Kantarjian HM, Champlin RE, et al. Evidence that specific T lymphocytes may participate in the elimination of chronic myelogenous leukemia. *Nat Med* 2000;**6**:1018–23.
20. Oka Y, Tsuboi A, Taguchi T, Osaki T, Kyo T, Nakajima H, et al. Induction of WT1 (Wilms' tumor gene)-specific cytotoxic T lymphocytes by WT1 peptide vaccine and the resultant cancer regression. *Proc Natl Acad Sci USA* 2004;**101**:13885–90.
21. Puil L, Liu J, Gish G, Mbamalu G, Bowtell D, Pelicci PG, et al. Bcr-Abl oncoproteins bind directly to activators of the Ras signalling pathway. *EMBO J* 1994;**13**:764–73.
22. Qazilbash MH, Weider E, Rios R, Lu S, Kant S, Giralt S, et al. *Vaccination with the PR1 leukemia-associated antigen can induce complete remission in patients with myeloid leukemia. ASH abstract*; 2004. American Society of Hematology: Blood Conference, San Diego, CA, USA.
23. Qazilbash MH, Weider ED, Thall PF, Wang X, Rios RL, Lu S, et al. *PR1 peptide vaccine-induced immune response is associated with better event-free survival in patients with myeloid leukemia. ASH abstract*; 2007. American Society of Hematology: Blood Conference, Atlanta, GA, USA.

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24. Quintarelli C, Dotti G, De Angelis B, Hoyos V, Mims M, Luciano L, et al. Cytotoxic T lymphocytes directed to the preferentially expressed antigen of melanoma (PRAME) target chronic myeloid leukemia. *Blood* 2008;**112**:1876–85.
25. Rezvani K, Brenchley JM, Price DA, Kilical Y, Gostick E, Sewell AK, et al. T-cell responses directed against multiple HLA-A*0201-restricted epitopes derived from Wilms' tumor 1 protein in patients with leukemia and healthy donors: identification, quantification, and characterization. *Clin Cancer Res* 2005;**11**:8799–807.
26. Rezvani K, Grube M, Brenchley JM, Sconocchia G, Fujiwara H, Price DA, et al. Functional leukemia-associated antigen-specific memory CD8+ T cells exist in healthy individuals and in patients with chronic myelogenous leukemia before and after stem cell transplantation. *Blood* 2003;**102**:2892–900.
27. Rezvani K, Yong AS, Mielke S, Jafarpour B, Savani BN, Le RQ, et al. Repeated PR1 and WT1 peptide vaccination in Montanide-adjuvant fails to induce sustained high-avidity, epitope-specific CD8+ T cells in myeloid malignancies. *Haematologica* 2011;**96**:432–40.
28. Rezvani K, Yong AS, Mielke S, Savani BN, Musse L, Superata J, et al. Leukemia-associated antigen-specific T-cell responses following combined PR1 and WT1 peptide vaccination in patients with myeloid malignancies. *Blood* 2008;**111**:236–42.
29. Rezvani K, Yong AS, Savani BN, Mielke S, Keyvanfar K, Gostick E, et al. Graft-versus-leukemia effects associated with detectable Wilms tumor-1 specific T lymphocytes after allogeneic stem-cell transplantation for acute lymphoblastic leukemia. *Blood* 2007;**110**:1924–32.
30. Rezvani K, Yong AS, Tawab A, Jafarpour B, Eniafe R, Mielke S, et al. Ex vivo characterization of polyclonal memory CD8+ T-cell responses to PRAME-specific peptides in patients with acute lymphoblastic leukemia and acute and chronic myeloid leukemia. *Blood* 2009;**113**:2245–55.
31. Rojas JM, Knight K, Wang L, Clark RE. Clinical evaluation of BCR-ABL peptide immunisation in chronic myeloid leukaemia: results of the EPIC study. *Leukemia* 2007;**21**:2287–95.
32. Schmitt M, Schmitt A, Rojewski MT, Chen J, Giannopoulos K, Fei F, et al. RHAMM-R3 peptide vaccination in patients with acute myeloid leukemia, myelodysplastic syndrome, and multiple myeloma elicits immunologic and clinical responses. *Blood* 2008;**111**:1357–65.
33. Sugiyama H. WT1 (Wilms' tumor gene 1): biology and cancer immunotherapy. *Jpn J Clin Oncol* 2010;**40**:377–87.
34. Yamagami T, Sugiyama H, Inoue K, Ogawa H, Tatekawa T, Hirata M, et al. Growth inhibition of human leukemic cells by WT1 (Wilms tumor gene) antisense oligodeoxynucleotides: implications for the involvement of WT1 in leukemogenesis. *Blood* 1996;**87**:2878–84.
35. Yong AS, Keyvanfar K, Eniafe R, Savani BN, Rezvani K, Sloand EM, et al. Hematopoietic stem cells and progenitors of chronic myeloid leukemia express leukemia-associated antigens: implications for the graft-versus-leukemia effect and peptide vaccine-based immunotherapy. *Leukemia* 2008;**22**:1721–7.

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Chapter 82

Peptide Vaccines for Otitis Media

Laura A. Novotny and Lauren O. Bakaletz

ABSTRACT

Nontypeable *Haemophilus influenzae* (NTHI) is a predominant causative agent of bacterial otitis media (OM). Of interest for vaccine development are several conserved or semiconserved NTHI surface proteins. We have focused our efforts on two of the multiple adhesins expressed by NTHI, outer membrane protein P5 (OMP P5) and the Type IV pilus (Tfp). NTHI OMP P5 is a fimbrial structure shown to be expressed by 100% of clinical NTHI isolates recovered from children with chronic OM. This adhesin mediates bacterial binding to epithelial cells, mucin, CEACAM1, and ICAM-1. NTHI Tfp is a filamentous polymer that mediates adherence to epithelial cells, competence, twitching motility, and biofilm formation. Because of amino acid conservation within specific domains, surface location, and important biological functions, we targeted each adhesin for vaccine development, generating highly efficacious, synthetic peptide, and recombinant chimeric peptide immunogens.

DISEASE TARGET

Otitis media, both acute and chronic, are highly prevalent pediatric diseases worldwide. In 1990, 24.5 million physician office visits were made for OM in the U.S. which represented a >200% increase over those reported in the 1980s.[26] OM is the most frequently diagnosed illness in children less than 15 years of age and is the primary cause for emergency room visits.[11] It is estimated that 83% of all children will experience at least one episode of acute OM (AOM) by age 3 and that >40% of children will experience 3 or more episodes of AOM by this age.[32] Worldwide, it is reported that between 65 and 330 million children suffer from chronic secretory OM, a disease characterized by chronically draining ears and long-term hearing loss.[19] Shockingly, in 1990, approximately 28,000 childhood deaths were attributed to OM, primarily in developing countries.[26] Although in developed countries OM is rarely associated with mortality, the associated morbidity is significant. Hearing loss is the most common complication of OM with behavioral, educational, and language development delays being additional consequences of early onset OM with effusion, or OME.[32] The socioeconomic impact of OM is also significant, with direct and indirect costs of diagnosing and managing OM exceeding $5 billion annually in the U.S. alone.[3,15]

BACKGROUND OF AVAILABLE THERAPIES

To date, antibiotic use, both therapeutically and prophylactically has been largely relied upon for medical management of the spectrum of clinical entities known collectively as OM. With 20 million antibiotic prescriptions annually in the U.S., OM is the primary reason for a child to receive an antimicrobial.[1] Due to the emergence of multiple antibiotic resistant bacterial strains, clinical practice guidelines were revised in 2004 to implement a period of 'watchful waiting' for otherwise healthy children diagnosed with OM with effusion who were likely to resolve disease without treatment, versus immediate prescription of antibiotics for children with more severe acute OM.[2]

Surgical management of OM involves the insertion of tympanostomy tubes through the tympanic membrane (or eardrum) while a child is anesthetized. Although this procedure is commonplace and is highly effective in terms of relieving painful symptoms by draining the middle ear of accumulated fluids, it has been met with criticism because of the invasive nature of the procedure and the incumbent risks of putting a child under general anesthesia.

Clearly, there is a tremendous need to develop more effective and novel approaches to the management as well as prevention of OM. Vaccine development holds the greatest promise and would be the most cost-effective method to accomplish this goal. However, progress in terms of vaccine development for NTHI, the Gram-negative pathogen that predominates both in *chronic* OM with effusion, as well as being a significant etiologic agent of *acute* OM, is hampered by our incomplete understanding of the pathogenesis and immunobiology of OM, a polymicrobial disease caused by one or more of three predominant bacterial pathogens, whose ability to invade the tympanum is facilitated by virtually any upper respiratory tract virus and some enteroviruses. Thus, pathogenesis is dependent on interactions

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00082-8
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FIGURE 1 Synthetic chimeric peptide immunogens, LB1 and LB2.

between microbes, within distinct microenvironments of the host, and the evolving innate and acquired immune responses to these microbes. Adding to the complexity is the ability of the bacteria that cause OM to form biofilms, communities of bacteria that are recalcitrant to standard therapies, and resistant to host defenses. As NTHI are an extremely heterogeneous group of microorganisms, identification of appropriate and conserved targets for vaccine development has proved to be quite challenging. Because adherence and colonization are of primary importance in the disease course of OM, we have focused our vaccine development efforts to date on two candidates that target two of several known NTHI adhesins.

WHY PEPTIDES?

In 1988, we made the observation that 100% of a panel of clinical NTHI isolates recovered from children with chronic OM expressed a filamentous OMP P5-homologous adhesin.[9] To begin to characterize the biological functions of this structure, we cloned and sequenced the gene that encodes the adhesin in NTHI strain 1128. The nucleotide sequence of OMP P5 encodes a mature protein of 338 amino acids with a calculated molecular mass of 36.4 kDa.[31] Expression of OMP P5 facilitates NTHI adherence to human oropharyngeal cells and nasopharyngeal mucin, as well as to CEACAM1- and ICAM-1-expressing cells.[4,10,28] Upon disruption of *ompP5*, the mutant shows reduced adherence to human oropharyngeal cells *in vitro*. In animal models of experimental OM, the OMP P5 mutant is cleared more rapidly from the chinchilla middle ears after direct challenge and is significantly less able to induce OM after intranasal inoculation.[31]

Although OMP P5 is an adhesin and a virulence factor for OM in chinchilla models, immunization with the whole native protein was only effective against challenge with the homologous isolate,[7,8,31] with no (or limited) protection conferred against heterologous challenge, as had been shown with many other NTHI OMPs when used in their entirety as an immunogen. To overcome this obstacle and identify a minimal region of the protein that could elicit a protective response, we subjected the translated amino acid sequence of OMP P5 to multiple algorithmic analyses and determined that there were two predominant putative B-cell epitopes of interest that resided within two of four predicted surface-exposed loops within this protein (loops 3 and 4).[35] With the rationale that inclusion of both B- and T-cell epitopes within the design of a synthetic peptide immunogen can induce antibodies of higher affinity to the incorporated B-cell epitope, combined with the knowledge that particular T-cell epitopes are broadly permissive in binding to human MHC class II molecules,[16] we synthesized two 40-mer peptides called 'LB1' and 'LB2' that comprised each B-cell epitope colinearly synthesized with a 'promiscuous' T-cell epitope from the fusion protein of measles virus[8] (Fig. 1). Sera obtained from immunized rabbits and chinchillas demonstrated significant reciprocal titers to LB1, and this antiserum also immunolabels native OMP P5 expressed by whole unfixed NTHI.[8] LB1 (but not LB2) induces significant protection against homologous and heterologous NTHI challenge in chinchilla and rat models of direct middle ear or lung challenge[7,8,18] as well as the prevention of ascending OM in a chinchilla passive transfer-superinfection model[17] compared to sham treated controls. Additionally, transcutaneous immunization of chinchillas with LB1 results in significantly earlier eradication of NTHI from the nasopharynx and middle ears of chinchillas when utilized in preventative and therapeutic immunization strategies.[23]

Although these were encouraging results, we wanted to determine the relative amino acid conservation within the B-cell epitope incorporated within LB1 as an indicator for potential breadth of protective coverage this immunogen might provide. To do so, a panel of 99 strains from the U.S. and Europe were subjected to PCR amplification and nucleotide sequence analysis of the 19-mer B-cell epitope incorporated into the peptide immunogen, LB1. Upon alignment of sequences (each 13–22 amino acids long), strains segregated into three groups based on at least 75% identity to the consensus sequence of that group. Approximately 76% of the NTHI strains clustered into majority Group 1, 16% to Group 2 (limited, but consistent sequence differences further divide this group into subgroups 2a and 2b) and 8% to Group 3 (Table 1).[8] Therefore, an immunogen that incorporated the B-cell epitope derived from majority Group 1 could be predicted to induce antibodies that would potentially crossreact with approximately 76% of circulating NTHI isolates.

Although LB1 is highly efficacious against OM resulting from NTHI in animal models, the incorporation of a T-cell promiscuous epitope was considered potentially problematic for a pediatric vaccine against OM, although its inclusion could be useful for older patient populations and/or other diseases of the respiratory tract due to NTHI. We

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TABLE 1 Aligned NTHI Group 1, 2, and 3 Peptide Sequences

NTHI strain	Group	Consensus sequence	No. of amino acids	No. of U.S. strains (% of total)[a]	No. of European strains (% of total)[a]
1128	1	RSDYKFYEDANGTRDHKKG---	19	53 (76)	22 (76)
1715	2a	RSDYKLYNKNSSSNSTLKNLGE	22	7 (10)	4 (14)
183	2b	RSDYKLYNKNSS---TLKDLGE	19	3 (4)	2 (7)
1729	3	RSDYKFYDN------KRID---	13	7 (10)	1 (3)

[a]*Of 99 U.S. and European strains tested*

therefore sought to replace the measles virus fusion protein moiety without reducing the overall efficacy observed for LB1. In 2005, ongoing efforts within the laboratory to identify novel NTHI virulence determinants revealed that NTHI strain 86-028NP possesses a four-gene cluster, *pilABCD*, which is highly homologous to gene loci in other bacterial species that express Type IV pili (Tfp).[6] Upon further interrogation, the *comABCDEF* competence gene cluster was also identified, one gene of which encodes the ComE secretin, a protein required for passage of an assembled pilus through the cell membrane. Thus, NTHI 86-028NP possesses the genetic material required to assemble and express functional Tfp. However, as Tfp had not yet been described for NTHI, we first confirmed that the bacterium produced this structure. When cultured under defined nutrient conditions and at an alkaline pH, >80% of NTHI express several Tfp-like structures of an approximately 6- to 7-nm diameter, localized to a slightly off polar location.[6] Further, no Tfp are observed upon disruption of the gene that encodes the majority subunit of the pilus, PilA. Functionally, Tfp are involved in competence, adherence, biofilm formation, twitching motility, and colonization. Upon disruption or deletion of *pilA*, NTHI is unable to take up exogenous DNA, shows reduced adherence to normal human bronchial epithelial cells, forms a biofilm of diminished height and density and is attenuated in its ability to twitch on solid medium, compared to the parent strain.[6,14,33] Moreover, the *pilA* mutant is compromised in its ability to maintain long-term colonization (>10 days) within the chinchilla nasopharynx and forms a biofilm within the middle ear with reduced structural integrity, compared to the parent strain.[14]

We therefore focused on developing a novel chimeric immunogen wherein NTHI PilA could serve as both a carrier peptide for the OMP P5 B-cell epitope and a powerful co-immunogen. Toward that end, we confirmed that within a panel of 24 clinical NTHI isolates recovered from patients in the U.S. and Europe with a variety of acute or chronic respiratory tract diseases that a single *pilA* gene was present within the genome of each strain.[6] Moreover, alignment of the deduced amino acid sequences of the PilA proteins revealed >81% homology among the strains with only two regions of limited diversity (Fig. 2) (unpublished observations).[6] Antibodies against an approximately 14 kDa N-terminally truncated and recombinant soluble form of PilA, 'rsPilA', recognize native Tfp expressed by viable NTHI *in vitro*.[21] Moreover, rsPilA affords significant protection against NTHI-induced OM in the chinchilla passive transfer–superinfection model[21] and as observed with LB1, transcutaneous immunization of chinchillas with rsPilA was significantly efficacious in both preventative and therapeutic immunization regimes.[23] As a result of these findings, we designed and expressed a novel

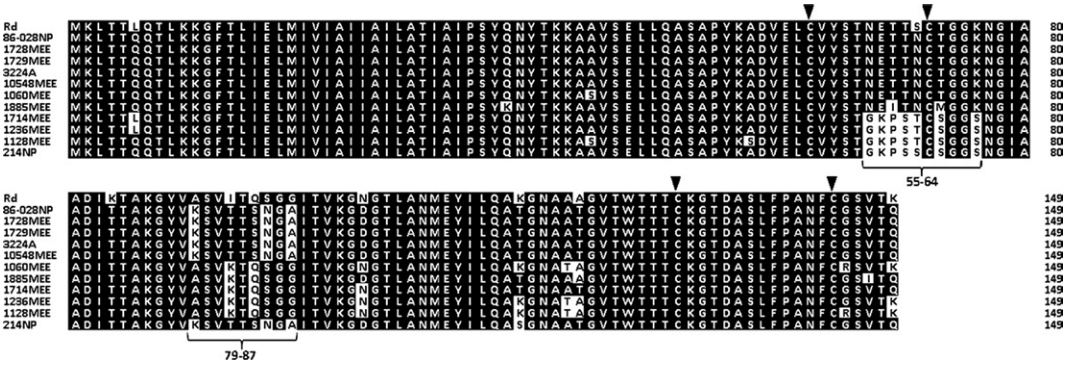

FIGURE 2 Aligned deduced amino acid sequences for PilA of 11 NTHI clinical isolates and *H. influenzae* strain Rd. *Copyright © American Society for Microbiology,* Infection and Immunity*, vol 73, 2005, pg 1635–1643, 10.1128/IAI.73.3.1635–1643.2005.*

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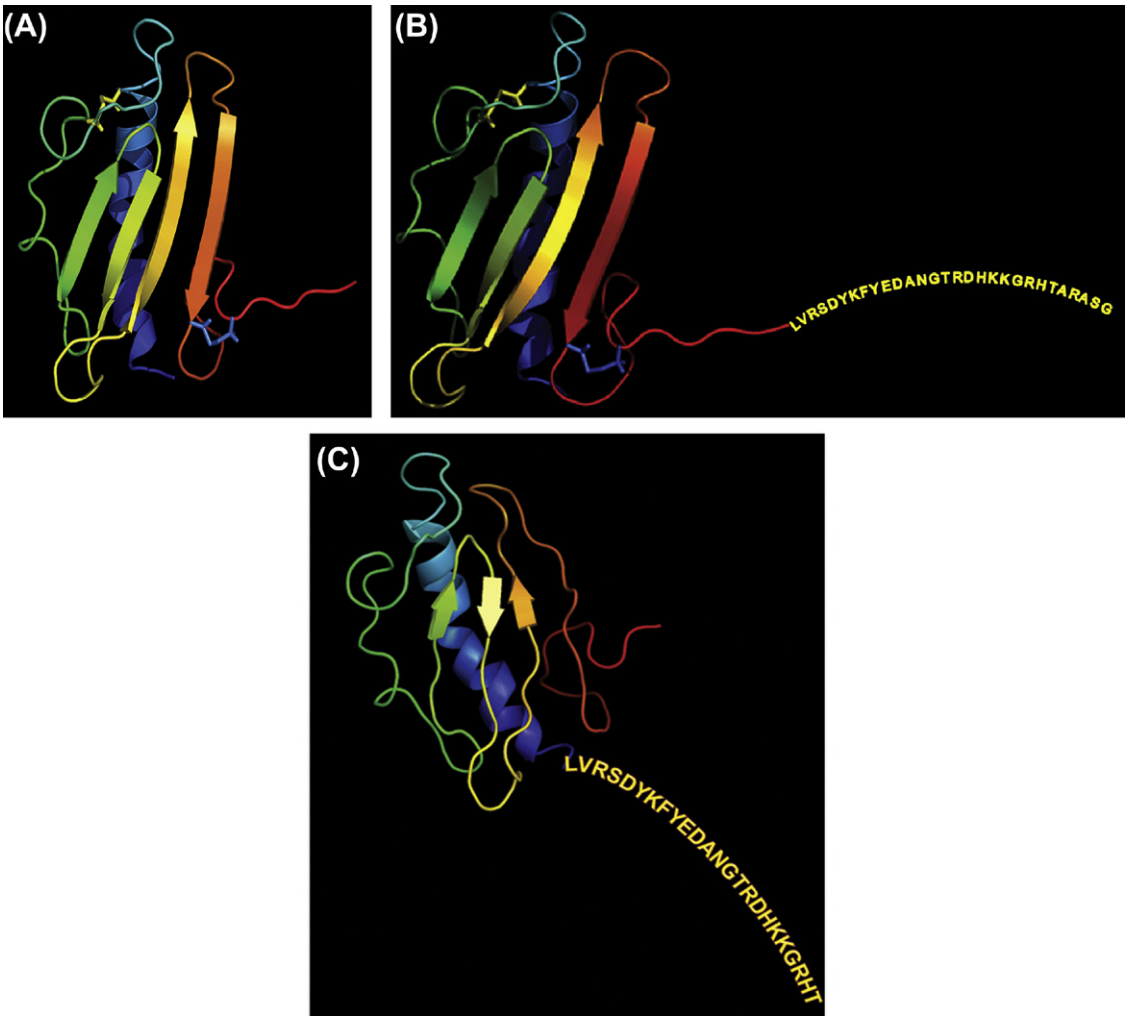

FIGURE 3 Computer model of N-terminally truncated structure of (A) PilA from NTHI strain 86-028NP and the chimeric recombinant immunogens, (B) chimV3 and (C) chimV4. *Reprinted from* Vaccine*, vol 28, Novotny et. al., Epitope mapping immunodominant regions of the PilA protein of nontypeable* Haemophilus influenzae *(NTHI) to facilitate the design of two novel chimeric vaccine candidates, pg 279–289, 2009, with permission from Elsevier.*

chimeric immunogen in which rsPilA served as a unique immunogenic protein that carries the slightly larger (24-mer) OMP P5 B-cell epitope at its N-terminus (Fig. 3C). The resultant chimeric recombinant immunogen, called 'chimV4,' thus has the potential to simultaneously induce the formation of protective antibodies directed against two key NTHI virulence determinants within a single, approximately 18-kDa protein.

In support of this premise, we showed that serum from animals immunized with chimV4 contains antibodies that recognized both rsPilA and the OMP P5 B-cell epitope individually.[21] Passive transfer of serum raised against chimV4 to adenovirus-compromised chinchillas by cardiac puncture, followed by NTHI challenge, confers significant protection against ascending NTHI-induced OM.[21] No statistically significant differences were observed between cohorts that received anti-rsPilA or anti-chimV4 serum pools; however, this chinchilla model of experimental OM may not be sensitive enough to detect an additive effect. Interestingly, immunization with chimV4 by transcutaneous immunization (TCI) results in significantly fewer NTHI detected within directly challenged middle ears and complete eradication of bacteria within 10 days after challenge.[23] Moreover, after a therapeutic immunization regime, receipt of chimV4 by TCI induces a significant reduction or eradication of NTHI biofilms already resident within the middle ear. Although no statistically significant differences in resolution of OM is observed after transcutaneous immunization after preventative or therapeutic strategies between cohorts that received either rsPilA or chimV4, receipt of chimV4 is associated with a greater reduction in the number of NTHI within the middle ear and enhanced resolution of signs of active disease compared to immunization with rsPilA.

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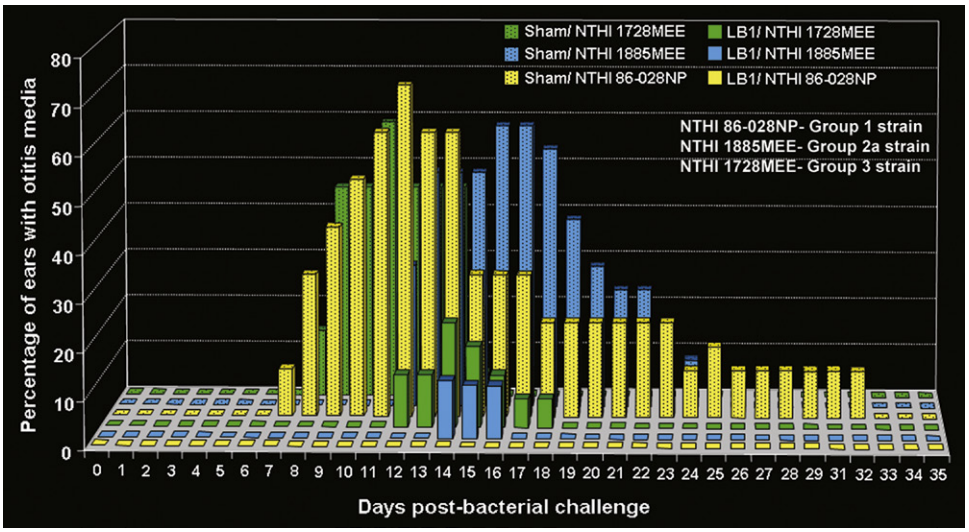

FIGURE 4 Crossstrain protection against ascending OM afforded by passive transfer of antiserum directed against the peptide immunogen LB1 that was derived from the majority Group 1 isolate NTHI strain 1128. *Reprinted from* The Journal of Immunology[22] *with permission of the publisher. Copyright 2003. The American Association of Immunologists, Inc.*

OUR CONTRIBUTIONS TO THE AREA

Active immunization with either isolated native OMP P5 or with the P5-derived synthetic peptide immunogen LB1 induces significant protection in chinchilla models against homologous challenge. However, to assay for potentially greater breadth of protective efficacy, we conducted a passive protection study in which antiserum directed against LB1 was now assayed for ability to confer protection against heterologous challenge using NTHI strains representing majority Group 1 but also a minority Group 2 and 3 isolate as well.[17] We found that passive immunization of chinchillas with serum specific for LB1 before challenge with heterologous NTHI isolates significantly inhibits the signs and incidence of OM ($p \leq 0.01$) induced by all three challenge isolates (Fig. 4). These results suggest that the approximately nine-residue N-terminal portion of the 19-mer sequence that is shared among all three NTHI groups (Table 1) and is incorporated into LB1 [RSDYFK(L)YE(N,D)D(K,N) is potentially an immunodominant region of this epitope or that it perhaps comprises a key protective epitope.[17]

In addition to noting the ability of anti-LB1 antiserum to provide significant crossprotection relevant to induction of culture-positive OM, in this study, we also observed that the ability of this antiserum pool to induce total eradication of NTHI from the nasopharynx was not equivalent among challenged cohorts despite the noted protection against development of OM. Importantly, these data thus suggested that while early, complete eradication of NTHI from the nasopharynx (as seen in the homologously challenged cohort) was highly protective against OM, reduction of the bacterial load to below a critical threshold level (as seen in the heterologously challenged cohorts) seemed to be equally protective. This observation has very important clinical implications, in that induction of sterilizing immunity did not seem to be necessary for protection against ascending OM, and instead, by immunizing in a manner that simply reduced the load of bacteria present in the NP, one could have a similar protective effect against disease. Given the serotype replacement issues that are currently confounding the efficacy of multivalent pneumococcal capsular-conjugate based vaccines posteradication of vaccine types of pneumococci from the NP after immunization,[29,34] this was a valuable piece of information to have and keep in mind as we moved forward with our vaccine development efforts.

Having now shown that LB1 could induce the formation of antibodies that were protective against both homologous and heterologous challenge in chinchilla models after parenteral immunization, variants of this vaccine candidate were then blindly evaluated in a rat model of mucosal immunization.[18] For this study, the 18-mer B-cell epitope contained within LB1 was conjugated to either keyhole limpet hemocyanin or bovine serum albumin and delivered to rats via direct immunization of the Peyer's patches followed 14 days later by an intratracheal boost. All immunogens tested induced a high-titered and specific response in the rat. Clearance of NTHI, measured 4 hours after challenge, occurred significantly earlier from the lungs of rats immunized with a combination of three LB1 peptides (representing each of the groups, see Table 1) than from sham-immunized controls. Clearance of NTHI from the middle ears of rats challenged intrabullarly also occurred

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significantly earlier for those rats administered the combination of three LB1-derived peptides. The efficacy shown for LB1 in a second, independent rodent model lent strong support to the merits of its continued development and evaluation as a component of a vaccine directed against NTHI-induced OM.

Given the significant protective efficacy of LB1, delivered parenterally in chinchilla models and mucosally in rat models of OM, we now wanted to examine the response induced when LB1 was delivered intranasally to chinchillas. We also wanted to determine the optimal prime-boost regimen for this immunogen when admixed with the adjuvant monophosphoryl lipid A (MPL). Thereby, we immunized cohorts of chinchillas with either 30 μg of LB1 plus MPL, 10 μg LB1 plus MPL, or MPL alone. Formulations were administered as either three intranasal (IN) doses via microaerosolized spray, or as one subcutaneous (SQ) dose followed by two IN doses, all at biweekly intervals. Chinchillas were then challenged IN with first adenovirus, then NTHI to better mimic the polymicrobial nature of natural OM where children typically develop OM approximately 1 week after having a viral URT infection.[5]

Clearance of NTHI from the NP occurred most rapidly in the cohort immunized mucosally with 10 μg LB1/dose. In the directly challenged middle ear, significantly earlier clearance of NTHI ($p<0.01$) occurred in cohorts immunized with either 10 μg LB1 delivered IN/IN/IN or with 30 μg LB1 delivered SQ/IN/IN. Immunization via either regimen induced LB1-specific serum antibody in all cohorts except those that received MPL only. We also detected immunogen-specific immunoglobulins in effusions recovered from the middle ears of chinchillas after NTHI challenge, with greatest levels occurring in those that cleared bacteria most rapidly. Although total serum antibody was greatest in cohorts receiving 30 μg LB1/dose, regardless of the immunization regime, based on induced augmented clearance observed among cohorts, LB1-specific immunoglobulins available locally seem to also play a key role in mediating bacterial clearance from both the NP and middle ears.

Antibody levels induced by IN immunization with LB1 were modest compared with parenteral immunization strategy, yet significant efficacy against NTHI-induced OM was achieved. Both characteristics of the immunogen itself and antigen processing by dendritic cells (DCs) can influence vaccine efficacy, as upon migration to lymphoid tissues, DCs present antigen in the context of MHC molecules to naive T-cells, thus serving to initiate an immune response or induce T-cell tolerance. The gene that encodes NTHI OMP P5 shares homology with *E. coli ompA*.[31] It is reported that OmpA proteins induce dendritic cell maturation and activation, and this has led to the designation of this family of proteins as a new pathogen-associated molecular pattern (PAMP).[13] As NTHI OMP P5 protein is an OmpA protein homolog, we wanted to determine whether OMP P5-derived LB1 showed similar properties of those of a PAMP (i.e., the ability to interact with and induce the maturation of DCs), thereby providing a mechanism for the observed efficacy afforded by IN administration. Briefly, we identified that chinchilla bone marrow-derived DCs exhibit phenotypic and functional activation on incubation with LB1, including preferential binding and uptake of LB1, greater expression of costimulatory markers and ability to effectively present antigen to T-cells, compared with a control peptide of comparable size.[25] Collectively, these results suggest that the 19-mer motif derived from OMP P5 and incorporated within LB1 may serve as a PAMP and that preferential processing and presentation of LB1 by DCs probably underlies the efficacy achieved by immunization with this peptide.

At the time we made the above observations, research within the laboratory had identified that NTHI expressed Tfp. As the majority subunit of Tfp is highly conserved among diverse NTHI clinical isolates and Tfp are located on the bacterial surface where they are involved in multiple key biologic functions, we targeted PilA in the design of a second novel vaccine candidate. Moreover, as described earlier, we sought to replace the potentially problematic T-cell promiscuous epitope within LB1 with PilA to serve as both a carrier for the OMP P5 B-cell epitope and immunogenic partner. To do so, several iterations of a chimeric immunogen were designed to determine the optimal location within PilA to incorporate the P5 epitope. Two chimeric recombinant immunogens were successfully expressed, 'chimV3' in which rsPilA carries the OMP P5 B-cell epitope at its C-terminus, and 'chimV4,' in which the epitope is positioned at the truncated N-terminus (Fig. 3). Disappointingly, antiserum against chimV3 does not confer protection against NTHI-induced OM in a chinchilla model and is less protective than either rsPilA or the B-cell epitope individually. Ongoing epitope mapping studies reveal that the C-terminus of PilA and the N-terminus of the P5 B-cell epitope are immunodominant and thereby within the context of chimV3, these moieties are blocked and potentially unavailable for presentation by antigen-presenting cells.[21,24] Within chimV4, however, the critical regions of both immunogens were exposed. The improvement of this design strategy is evident as significant protective efficacy against NTHI-induced OM is achieved in the chinchilla model upon immunization with chimV4 ($p<0.001$).

Finally, our most recent work examines transcutaneous immunization (TCI) as a noninvasive but potentially equally efficacious strategy to immunize against OM. This route targets the numerous dendritic cells within the skin and incorporation of a novel adjuvant to induce both mucosal and

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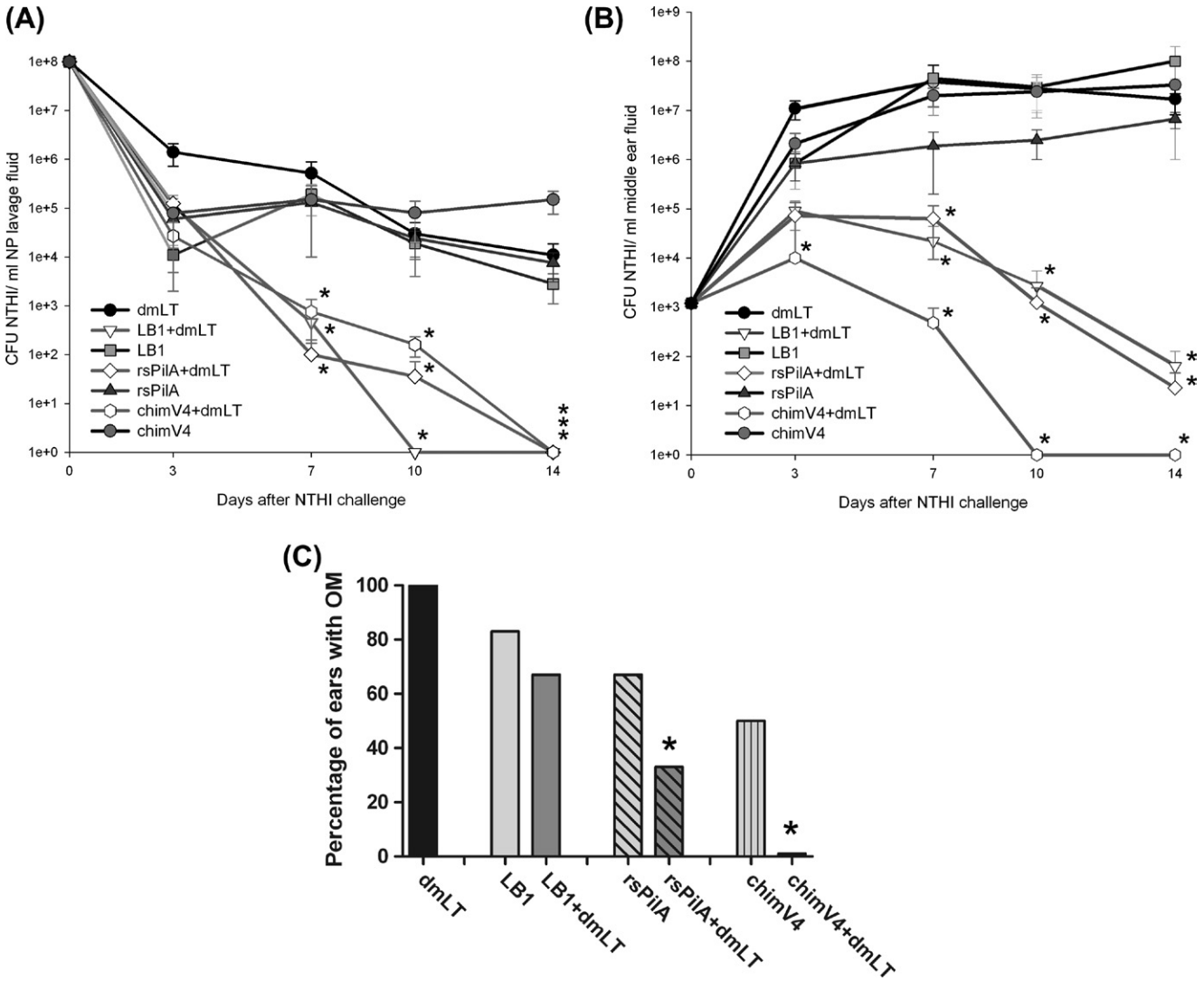

FIGURE 5 Transcutaneous immunization via a preventative regimen induced clearance if NTHI from (A) the nasopharynx and (B) the middle ear of chinchillas. (C) Transcutaneous immunization via a therapeutic regimen induces clearance of fluid from the middle ear and resolution of signs of experimental OM. *Reprinted from* Mucosal Immunology.[23]

systemic immune responses. The immunization regimen is performed as follows: both pinnae of alert chinchillas were hydrated with saline to facilitate adsorption of formulations through the barrier of the *stratum corneum*. Vaccine formulations are then applied, the pinnae folded in half and opposing surfaces gently rubbed together. Formulations consist of our three most promising immunogens LB1, rsPilA, and chimV4 delivered either alone or admixed with a potent adjuvant, a double mutant of *E. coli* heat-labile enterotoxin, called dmLT.[20] Animals are immunized either before NTHI challenge to examine the ability of TCI to *prevent* OM, or after direct inoculation of the middle ear to determine the resolution of active OM induced by a *therapeutic* regime. TCI with LB1, rsPlA or chimV4 delivered with dmLT before bacterial challenge induces a rapid reduction in the bacterial load within the NP of immune animals, with the cohort that received LB1+dmLT eradicating NTHI from this site within 10 days of challenge (Fig. 5A).[23] Within middle ears although an increase in bacterial concentration is typically observed after direct challenge because of initial bacterial replication, only a modest increase in CFU NTHI is observed in cohort that receive any of the three antigens plus dmLT and is followed by rapid elimination of NTHI from the middle ear (Fig. 5B). Strikingly, beginning 3 days after direct challenge of the middle ear, significantly fewer NTHI are detected in animals that received chimV4+dmLT, with complete eradication within 10 days, compared with receipt of adjuvant alone.

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The chronicity and recurrence of OM is a consequence of biofilms established by NTHI within the middle ear. Based on the efficacy afforded by TCI with LB1, rsPilA and chimV4 before bacterial challenge when used in a standard preventative regime, we then wondered whether TCI after challenge might also effectively resolve OM by eradicating an already established biofilm from the middle ear. Notably, immunization via a therapeutic regimen results in a reduction in the signs of OM based on a clinically relevant scoring system, in addition to the overall resolution of an established NTHI biomass in the middle ear (Fig. 5C). Moreover, only cohorts that received rsPilA or chimV4 admixed with adjuvant show a significant reduction in signs of disease as determined by video otoscopy and tympanometry. The strategy to resolve active disease by therapeutic vaccination is highly promising.

Collectively, our results show that a protective immune response could be elicited after delivery of LB1 or the combined Tfp- and OMP P5-targeted immunogen, chimV4 via multiple immunization strategies. Whether or not the efficacy shown for LB1 and chimV4 in animal models will translate into similar protection in children awaits clinical evaluation.

FUTURE OUTLOOK

Despite their limitations, peptide-based vaccines have the potential to direct the immune response against prespecified immunodominant or protective epitopes of native proteins.[12] Reports from several laboratories, including ours, show that the inclusion of both B- and T-cell epitopes in synthetic peptide immunogens can induce antibodies of higher affinity for the incorporated B-cell epitopes.[27,30] The orientation, number, and secondary character of peptide sequences included in these immunogens influence antigen processing and presentation to T-cells, thus affecting the specificity and affinity of the antibodies produced for native proteins.[12,27,30] Using this epitope-targeted, peptide-based strategy to develop the immunogens LB1 and chimV4, we have shown in multiple preclinical trials that this approach to the rational design of vaccine candidates is highly successful.

REFERENCES

1. Diagnosis and management of acute otitis media. *Pediatrics* 2004;**113**:1451–65.
2. Otitis media with effusion. *Pediatrics* 2004;**113**:1412–29.
3. Alsarraf R, Jung CJ, Perkins J, Crowley C, Alsarraf NW, Gates GA. Measuring the indirect and direct costs of acute otitis media. *Arch Otolaryngol Head Neck Surg* 1999;**125**:12–8.
4. Avadhanula V, Rodriguez CA, Ulett GC, Bakaletz LO, Adderson EE. Nontypeable *Haemophilus influenzae* adheres to intercellular adhesion molecule 1 (ICAM-1) on respiratory epithelial cells and upregulates ICAM-1 expression. *Infect Immun* 2006;**74**:830–8.
5. Bakaletz LO. Viral potentiation of bacterial superinfection of the respiratory tract. *Trends Microbiol* 1995;**3**:110–4.
6. Bakaletz LO, Baker BD, Jurcisek JA, Harrison A, Novotny LA, Bookwalter JE, et al. Demonstration of Type IV pilus expression and a twitching phenotype by *Haemophilus influenzae*. *Infect Immun* 2005;**73**:1635–43.
7. Bakaletz LO, Kennedy BJ, Novotny LA, Duquesne G, Cohen J, Lobet Y. Protection against development of otitis media induced by nontypeable *Haemophilus influenzae* by both active and passive immunization in a chinchilla model of virus-bacterium superinfection. *Infect Immun* 1999;**67**:2746–62.
8. Bakaletz LO, Leake ER, Billy JM, Kaumaya PT. Relative immunogenicity and efficacy of two synthetic chimeric peptides of fimbrin as vaccinogens against nasopharyngeal colonization by nontypeable *Haemophilus influenzae* in the chinchilla. *Vaccine* 1997;**15**:955–61.
9. Bakaletz LO, Tallan BM, Hoepf T, DeMaria TF, Birck HG, Lim DJ. Frequency of fimbriation of nontypable *Haemophilus influenzae* and its ability to adhere to chinchilla and human respiratory epithelium. *Infect Immun* 1988;**56**:331–5.
10. Bookwalter JE, Jurcisek JA, Gray-Owen SD, Fernandez S, McGillivary G, Bakaletz LO. A carcinoembryonic antigen-related cell adhesion molecule 1 homologue plays a pivotal role in nontypeable *Haemophilus influenzae* colonization of the chinchilla nasopharynx via the outer membrane protein P5-homologous adhesin. *Infect Immun* 2008;**76**:48–55.
11. Cassell GH, Archer GL, Beam TR, Gilchrist MJ, Goldmann D, Hooper DC, et al. Report of the ASM Task Force on Antibiotic Resistance. Washington D.C., 1994.
12. Craig L, Sanschagrin PC, Rozek A, Lackie S, Kuhn LA, Scott JK. The role of structure in antibody cross-reactivity between peptides and folded proteins. *J Mol Biol* 1998;**281**:183–201.
13. Jeannin P, Magistrelli G, Goetsch L, Haeuw JF, Thieblemont N, Bonnefoy JY, et al. Outer membrane protein A (OmpA): a new pathogen-associated molecular pattern that interacts with antigen presenting cells-impact on vaccine strategies. *Vaccine* 2002;**20**(Suppl. 4): A23–7.
14. Jurcisek JA, Bookwalter JE, Baker BD, Fernandez S, Novotny LA, Munson Jr RS, et al. The PilA protein of non-typeable *Haemophilus influenzae* plays a role in biofilm formation, adherence to epithelial cells and colonization of the mammalian upper respiratory tract. *Mol Microbiol* 2007;**65**:1288–99.
15. Kaplan B, Wandstrat TL, Cunningham JR. Overall cost in the treatment of otitis media. *Pediatr Infect Dis J* 1997;**16**:S9–11.
16. Kaumaya PT, Kobs-Conrad S, Seo YH, Lee H, VanBuskirk AM, Feng N, et al. Peptide vaccines incorporating a 'promiscuous' T-cell epitope bypass certain haplotype restricted immune responses and provide broad spectrum immunogenicity. *J Mol Recognit* 1993;**6**:81–94.
17. Kennedy BJ, Novotny LA, Jurcisek JA, Lobet Y, Bakaletz LO. Passive transfer of antiserum specific for immunogens derived from a nontypeable *Haemophilus influenzae* adhesin and lipoprotein D prevents otitis media after heterologous challenge. *Infect Immun* 2000;**68**:2756–65.
18. Kyd JM, Cripps AW, Novotny LA, Bakaletz LO. Efficacy of the 26-kilodalton outer membrane protein and two P5 fimbrin-derived immunogens to induce clearance of nontypeable *Haemophilus influenzae* from the rat middle ear and lungs as well as from the chinchilla middle ear and nasopharynx. *Infect Immun* 2003;**71**:4691–9.

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19. Leach AJ, Morris PS, Mathews JD. Compared to placebo, long-term antibiotics resolve otitis media with effusion (OME) and prevent acute otitis media with perforation (AOMwiP) in a high-risk population: a randomized controlled trial. *BMC Pediatr* 2008;**8**:23.
20. Norton EB, Lawson LB, Freytag LC, Clements JD. Characterization of a mutant *Escherichia coli* heat-labile toxin, LT(R192G/L211A), as a safe and effective oral adjuvant. *Clin Vaccine Immunol* 2011;**18**:546–51.
21. Novotny LA, Adams LD, Kang DR, Wiet GJ, Cai X, Sethi S, et al. Epitope mapping immunodominant regions of the PilA protein of nontypeable *Haemophilus influenzae* (NTHI) to facilitate the design of two novel chimeric vaccine candidates. *Vaccine* 2009;**28**:279–89.
22. Novotny LA, Bakaletz LO. The fourth surface-exposed region of the outer membrane protein P5-homologous adhesin of nontypeable *Haemophilus influenzae* is an immunodominant but nonprotective decoying epitope. *J Immunol* 2003;**171**:1978–83.
23. Novotny LA, Clements JD, Bakaletz LO. Transcutaneous immunization as preventative and therapeutic regimens to protect against experimental otitis media due to nontypeable *Haemophilus influenzae*. *Mucosal Immunol* 2011;**4**:456–67.
24. Novotny LA, Jurcisek JA, Pichichero ME, Bakaletz LO. Epitope mapping of the outer membrane protein P5-homologous fimbrin adhesin of nontypeable *Haemophilus influenzae*. *Infect Immun* 2000;**68**:2119–28.
25. Novotny LA, Partida-Sanchez S, Munson Jr RS, Bakaletz LO. Differential uptake and processing of a *Haemophilus influenzae* P5-derived immunogen by chinchilla dendritic cells. *Infect Immun* 2008;**76**:967–77.
26. Organization WH. *Chronic suppurative otitis media: burden of illness and management options*. : Publications of the World Health Organization: World Health Organization; 2004.
27. Partidos C, Stanley C, Steward M. The influence of orientation and number of copies of T and B cell epitopes on the specificity and affinity of antibodies induced by chimeric peptides. *Eur J Immunol* 1992;**22**:2675–80.
28. Reddy MS, Bernstein JM, Murphy TF, Faden HS. Binding between outer membrane proteins of nontypeable *Haemophilus influenzae* and human nasopharyngeal mucin. *Infect Immun* 1996;**64**:1477–9.
29. Reinert RR. Pneumococcal conjugate vaccines—a European perspective. *Int J Med Microbiol* 2004;**294**:277–94.
30. Shaw DM, Stanley CM, Partidos CD, Steward MW. Influence of the T-helper epitope on the titre and affinity of antibodies to B-cell epitopes after co-immunization. *Mol Immunol* 1993;**30**:961–8.
31. Sirakova T, Kolattukudy PE, Murwin D, Billy J, Leake E, Lim D, et al. Role of fimbriae expressed by nontypeable *Haemophilus influenzae* in pathogenesis of and protection against otitis media and relatedness of the fimbrin subunit to outer membrane protein A. *Infect Immun* 1994;**62**:2002–20.
32. Teele DW, Klein JO, Chase C, Menyuk P, Rosner BA. Otitis media in infancy and intellectual ability, school achievement, speech, and language at age 7 years. Greater Boston Otitis Media Study Group. *J Infect Dis* 1990;**162**:685–94.
33. Tracy E, Ye F, Baker BD, Munson Jr RS. Construction of non-polar mutants in *Haemophilus influenzae* using FLP recombinase technology. *BMC Mol Biol* 2008;**9**:101.
34. Watson M, Roche P, Bayley K, Bell JM, Collignon P, Gilbert GL, et al. Laboratory surveillance of invasive pneumococcal disease in Australia, 2003 predicting the future impact of the universal childhood conjugate vaccine program. *Commun Dis Intell* 2004;**28**:455–64.
35. Webb DC, Cripps AW. Secondary structure and molecular analysis of interstrain variability in the P5 outer-membrane protein of non-typable *Haemophilus influenzae* isolated from diverse anatomical sites. *J Med Microbiol* 1998;**47**:1059–67.

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Chapter 83

Therapeutic Peptides Targeting HER-2/*neu* and VEGF Signaling Pathways in Breast Cancer

Kevin Chu Foy, Daniele Vicari and Pravin T.P. Kaumaya

ABSTRACT

HER-2/*neu* is a member of the epidermal growth factor receptor family and is overexpressed in 30% of breast cancers. Trastuzumab and Pertuzumab are humanized monoclonal antibodies that target the extracellular domain of the HER-2, but limitations still exist with these treatments due to its high cost and short half-life of the drugs. The overexpression of HER-2 leads to overexpression of VEGF, which turns on angiogenesis, causing increased blood flow to the tumors. This chapter is mainly centered on the use of peptides that are cost effective, specific and nontoxic to target both HER-2 and VEGF signaling pathways. We have designed conformational peptides of the pertuzumab epitope and also peptides that mimic the VEGF binding sites. With the use of *in vitro* assays and two *in vivo* tumor models, we have shown that combination treatment aimed at targeting the HER-2/*neu* and VEGF signaling pathways produces additive effects with no cardiotoxicity.

PEPTIDES AS THERAPEUTIC CANDIDATES

Peptides are small protein-like chains of amino acids, and a peptide mimic is a peptide or a peptide-like molecule that intends to mimic one portion of the entire protein. In most cases, the region of interest is biologically active such as the binding sites or active site of enzymes.[20] Protein–protein interactions play a key role in the activation of several mechanisms such as viral replication, cellular growth, and differentiation. Thus, it is the great interest in the development of peptides that would retain the protein binding ability, acting as an antagonist or agonist.[28] Smaller molecules such as peptides can be chemically synthesized and easily modified to such molecules. Synthetic peptides that disrupt protein–protein interaction inhibit HIV-1 proteases, HIV-1 reverse transcriptase, herpes simplex virus ribonucleotide reductase, and thymine synthase. Peptides that can block receptor–ligand interaction can be obtained by the screening of combinatorial libraries of compounds but the most preferred approach is to use a structure-based design. The spatial configuration of side-chain groups is the key for recognition and biological activity. The aim is to maintain the conformational space and orientation of the bioactive surface while retaining sufficient flexibility to bind cooperatively with a given receptor.[16] Peptide mimics offer the benefits of being water soluble, nonimmunogenic, and the ability to easily cross tissue barriers.[30] Natural peptides such as insulin, vancomycin, oxytocin, and cyclosporine and synthetically produced ones such as Fuzeon® (enfuvirtide) and Integrilin® (eptifibatide) are among the FDA-approved peptide-based drugs. Fuzeon® is a peptide derived from the HIV-1 envelope glycoprotein transmembrane subunit gp41 C-terminal heptad repeat (CHR) region and blocks the entry of HIV into cells,[32] whereas Integrilin® is an antiplatelet drug that selectively blocks the platelet glycoprotein IIb/IIIa receptor. Peptide-based therapy has the inconveniency that frequent doses need to be administrated because of proteosomal degradation *in vivo* of the peptides.[9] However, this problem can be overcome by the use of retro-inverso D-amino acid peptides.

HER-2 SIGNALING PATHWAYS, CANCER, AND STRATEGY FOR THE DESIGN OF HER-2 PEPTIDE MIMIC

HER-2/*neu* is a self-antigen that is overexpressed in different types of epithelial tumors including breast and is associated with a worse prognosis in several malignancies. Overexpression of HER-2 has been reported in about 30% of all breast cancers[29] and seems to predict a decreased disease free and overall rates of survival. Many therapeutic strategies targeting the HER-2 receptor have been devised, and most outstanding among them are a group of humanized monoclonal antibodies which include Trastuzumab and Pertuzumab. Trastuzumab is a growth inhibitory monoclonal Ab that binds the extracellular domain (ECD) of the HER-2 receptor and blocks

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00083-X
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intracellular signaling. Pertuzumab also binds the ECD of HER-2 thereby preventing dimerization of the receptor, and it is currently being evaluated in human clinical trials. HER-2 is an attractive therapeutic target for several reasons. The amount of HER-2 expressed on cancer cells is much higher than in normal adult tissues potentially reducing the toxicity of HER-2 targeting drugs.[5] Tumors with a high expression of HER-2 often show homogenous intense IHC staining,[4] signifying that HER-2 targeted therapy will target most cancer cells in a given patient. HER-2 is always observed in metastatic disease suggesting its implication in most aggressive tumors though it is not observed in benign tumors. However, evidence suggesting the continuous use of this humanized Abs is lacking because of their instability, repeated administration, and effects of cardiotoxicity, poor penetration across tissue barriers and their high cost of treatment with potential immunogenicities associated with them.[21] Prediction of novel B-cell epitopes from the ECD of HER-2 has been the focus of our laboratory for the past decade. Through such an approach, we have identified two novel HER-2 vaccine candidates that were translated into a Phase I clinical trial thereby validating our overall strategy. A subset of the patients (6 out of 24) showed clinical responses, and some of these patients were given more doses of the vaccine because of their clinical responses.[17] After the publication of the crystal structure of HER-2 in complex trastuzumab[3] and HER-2 in complex with pertuzumab,[11] we synthesized a second generation of peptide vaccines based on the interaction of these abs with the ECD of the HER-2 receptor. We have extensively showed the efficacy of these new vaccine candidates in preclinical studies.[1,12] Two of the vaccine candidates are currently being evaluated in a Phase Ib NCI funded clinical trial at the James Cancer Hospital. Pertuzumab bind to different epitopes in the extracellular domain near the junction of domains I, II, and III of the HER-2 extracellular domain amino acids **266–333**. This interaction of pertuzumab with HER-2 was shown to sterically hinder the association of HER-2 with other receptors such as HER3[11] sterically blocking a binding pocket necessary for receptor dimerization and signaling. Based on these studies, we have selected one HER-2 peptide mimic pertuzumab-like sequence 266–296, which has been shown to be a good candidate for a neutralizing, blocking peptide.[1] The B-cell peptide epitope alone can therefore be used to inhibit HER-2 dimerization just like pertuzumab. We showed that the HER-2-266–296 peptide mimic is able to prevent tumor growth and development in a transplantable mouse model of breast cancer. Peptide treatments caused a significant delay in the onset of tumor growth and also significantly increased the tumor-free survival rates when compared with mice in the untreated groups or groups treated with the irrelevant peptide. Also the peptide interrupts many HER-2 signaling pathways *in vitro* like inhibition of proliferation, dimerization, and cell viability in a dose-dependent manner.[10]

VEGF, CANCER, AND THE DESIGN OF VEGF PEPTIDE INHIBITOR

VEGF is the most proangiogenic factor studied because of its specificity and important role in the activation of all steps of angiogenesis in the endothelium vasculature,[7] and it is upregulated in the tumor microenvironment by hypoxia or activation of altered oncogenes like HER-2.[24] Examples of FDA-approved cancer treatments with anti-angiogenic agents targeting VEGF pathway include monoclonal antibody bevacizumab (Avastin®), small tyrosine kinase inhibitors SU11248 (Sunitinib®), and BAY 43-9006 (Sorafenib®).[6] Although the clinical applications of these drugs in cancer therapy are promising, drug resistance development and long-term side effects such as hypertension and endothelium dysfunction remain a concern. VEGF binds with higher affinity to VEGFR-1, in the endothelial cells, but the majority of angiogenesis signaling (proliferation, migration, and survival) occurs via the interaction between VEGF and VEGFR-2.[18] The VEGF binding site to its receptors and neutralizing antibodies has been characterized by crystal structure analysis as well as alanine scanning, revealing overlapping regions located at the poles in each homodimer. VEGF: VEGFR-2 interaction has been extensively targeted by antibodies that bind VEGF and the extracellular domain of VEGFR-2. These studies have identified VEGF sequences in the binding region that inhibit VEGF activated pathways. The interaction between VEGF and VEGFR-2 comprises residues within the loop region formed by the antiparallel β-sheets β5–β6 in the VEGF protein and blockade of receptor–ligand interaction offers a validated and proven approach in drug development because receptor:ligand interaction is usually confined to a defined portion of the ligand and the receptor, and recent technologies have allowed the accurate identification of these binding regions. In an attempt to block the VEGF and VEGFR-2 interaction, we designed several peptides that mimic the VEGF loop region containing the binding sites residues. Our hypothesis is that inhibiting binding of the VEGFR-2 and in activating the tyrosine kinase activity can be achieved by blocking the binding site of the receptor using peptide mimics containing the structural binding region of VEGF. To confirm the effect of the VEGF peptide mimics as angiogenesis inhibitors, we used several *in vitro* angiogenic assays such as the scratch wound (migration), Matrigel (tube formation) and HUVEC proliferation assay (proliferation). All three VEGF peptide mimics are able to inhibit cell migration in the presence of exogenous rhVEGF at the wound assay, showing that they can block migration induced by rhVEGF, throughout blocking VEGF: VEGFR-2 interaction. We tested whether the VEGF peptide mimics could inhibit the tube formation in Matrigel. All three peptide mimics inhibit tube formation with

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VEGF-P3(CYC) displaying the largest inhibition. Proliferation of endothelial cells is essential in the formation of new blood vessels, and inhibition of HUVEC proliferation occurs in a dose-dependent manner with VEGF-P3(CYC) being the most potent inhibitor. The conformational peptide VEGF-P3(CYC) shows the best inhibitory effects and the highest binding affinity, suggesting that the loop stabilization by the disulfide bond between the extra cysteines, may be responsible for this effect. Our biochemical and *in vitro* experiment results are in agreement and confirm that VEGF-P3(CYC) has the best potential of inhibiting angiogenesis. To investigate whether these results would be reproduced *in vivo*, we tested the VEGF peptide mimic as a potential treatment in VEGF overexpressing mice. The *in vivo* studies were performed in the double transgenic mice VEGF$^{-/+}$/Neu2–5$^{-/+}$, which has acceleration (resulting from VEGF overexpression) of spontaneous mammary gland tumor development (resulting from the *neu* mutation).[23] The delay in tumor burden seen in the VEGF-P3(CYC)-treated mice indicates that the conformational loop designed structure may be necessary for blocking VEGF and its receptor interaction and consequently, delaying the tumor growth. Combining *in vitro* and *in vivo* results VEGF-P3(CYC) is the best candidate for the development of angiogenesis peptide inhibitors. The design of the peptide, VEGF-P3 (CYC) that would mimic a structural binding site of VEGF to its receptor is important in obtaining a better inhibitory molecule in several *in vitro* assays. The conformational peptide mimic VEGF-P3 (CYC) also shows better affinity to the receptor and is efficient in delaying tumor burden in a VEGF overexpressing animal model.[31] These findings motivate the development and potential of using VEGF-P3 (CYC) as a peptide therapeutic drug to inhibit angiogenesis. Still, future analysis involving other animal models more specific to angiogenesis will give an insight into the efficacy of these peptides in inhibiting angiogenesis given the complexity of the tumor microenvironment.

ADDITIVE/SYNERGISTIC EFFECTS OF TARGETING BOTH HER-2 AND VEGF PATHWAYS IN CANCER

The upregulation of HER-2 is associated with increased expression of vascular endothelial growth factor, or VEGF at both the RNA and protein level in human breast cancer cells and exposure of HER-2 positive cells to Trastuzumab significantly decreases VEGF expression.[26] Shc, a downstream adaptor protein of the HER-2 signaling pathway has been identified as a critical switch for VEGF production[15] showing that VEGF is a downstream target of the HER-2 signaling pathway. This shows that, the effects of HER-2 on tumor cell behavior may be mediated in part through the stimulation of angiogenesis. Angiogenesis is the growth of new blood vessels from preexisting ones and contributes to the development of numerous types of tumors and their metastasis. VEGF, a well-known proangiogenic factor is secreted by most tumor cells.[25] VEGF expression is increased in many different types of cancer and most tumor cells secrete VEGF.[7] It is also believed that VEGF is a key promoter of metastasis.[27] VEGF is a 34- to 42-kDa, homodimeric, heparin-binding, disulfide-bonded glycoprotein that has several isoforms arising from splice variants.[8] Therefore, VEGF and its receptors VEGFR-1 and VEGFR-2 are prime targets for antiangiogenic intervention, which is believed to be one of the most promising approaches in cancer therapy. Blocking angiogenesis is an attractive strategy to inhibit tumor growth, invasion, and metastasis. Bevacizumab has been tested in several cancer clinical trials and showed some promising results in a phase II clinical trial for breast cancer.[26] Other VEGF inhibitors in clinical trials in progress today include several receptor tyrosine kinase inhibitors, VEGF-Trap, and anti-VEGF-R2. VEGF stimulates angiogenesis by binding to its receptor VEGFR-2, which is expressed by both endothelial and tumor cells.[2] A two-pronged approach to target cancer by coimmunizing with defined tumor-associated antigens and angiogenesis associated antigens have been shown to have synergistic effects. All these show that combination treatment targeting both HER-2 and VEGF is a promising strategy because angiogenic therapy alone will only delay tumor growth[19] and targeting HER-2 and VEGF will destroy two different tumor-dependent subpathways. In a study, we evaluated the effects of combination treatment with both HER-2 and VEGF peptide mimics, and the results obtained showed that targeting these two proteins seems to be a promising strategy for treating HER-2-positive cancers. Combination treatments with both peptides causes superior antitumor and antiangiogenic effects *in vitro* and *in vivo*. This dual therapeutic benefit is clearly shown by the increased proliferation and phosphorylation inhibition as well as by a decrease in cell viability. Combination treatment also causes a greater delay in tumor growth and development in a transplantable tumor model. These results are indicative that the selected inhibitory peptides have great therapeutic effects in targeting both HER-2 and VEGF by eliciting potent antitumor and antiangiogenic effects.

CONCLUSIONS

Drug therapies, which target specific tumor cell types of different signaling pathways, have been the mainstay of pharmaceutical companies. Development of monoclonal antibodies and small tyrosine kinase inhibitors has been the spotlight of cancer therapeutics, bringing huge revenue to biotech companies. R&D investments to develop such novel drugs are really enormous because it involves a long way between discovery and implementation of the drug, with the entire

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process being covered under patents.[14] This reflects the cost of treatment using target therapeutic drugs and difficulties in broad utilization. Also, these drugs did not eliminate high-risk long-term toxicity and development of drug resistance. These obstacles combined with economical stagnation have put pressure on biotech companies to decrease drug development costs and increase productivity. Also, many companies have turned their attention to the use of peptides as alternatives to the development of therapeutic drugs. Advances in the peptide synthesis technology have allowed the application of peptides as antimicrobial agents or antagonists in protein–protein interaction.[13] We decided to evaluate rationally designed peptides as cancer and angiogenesis inhibitors. VEGF peptide mimics designed in our laboratory show potential in inhibiting angiogenesis. Many studies have shown the beneficial effects of using a combination strategy to target cancer cells and *in vitro* studies have shown that a combination of pertuzumab and trastuzumab synergistically inhibit the survival of breast cancer cells.[22] The combination of these peptide mimics could be translated into the clinic to investigate the efficacy of targeting both HER-2 and VEGF in HER-2 positive cancers. Additionally, future studies could be performed to investigate possible combination of our peptide mimics with low doses of chemotherapy.

ACKNOWLEDGMENTS

Research in the authors' laboratory was funded in part by National Institutes of Health (NIH), OSU Comprehensive Cancer Center Pelotonia funds and Ob/Gyn Peptide funds.

REFERENCES

1. Allen SD, Garrett JT, Rawale SV, Jones AL, Phillips G, Forni G, et al. Peptide vaccines of the HER-2/neu dimerization loop are effective in inhibiting mammary tumor growth in vivo. *J Immunol* 2007;**179**:472–82.
2. Casella I, Feccia T, Chelucci C, Samoggia P, Castelli G, Guerriero R, et al. Autocrine–paracrine VEGF loops potentiate the maturation of megakaryocytic precursors through Flt1 receptor. *Blood* 2003;**101**: 1316–23.
3. Cho HS, Mason K, Ramyar KX, Stanley AM, Gabelli SB, Denney Jr DW, et al. Structure of the extracellular region of HER2 alone and in complex with the Herceptin Fab. *Nature* 2003;**421**:756–60.
4. Dakappagari NK, Lute KD, Rawale S, Steele JT, Allen SD, Phillips G, et al. Conformational HER-2/neu B-cell epitope peptide vaccine designed to incorporate two native disulfide bonds enhances tumor cell binding and antitumor activities. *J Biol Chem* 2005;**280**:54–63.
5. Dakappagari NK, Sundaram R, Rawale S, Liner A, Galloway DR, Kaumaya PT. Intracellular delivery of a novel multiepitope peptide vaccine by an amphipathic peptide carrier enhances cytotoxic T-cell responses in HLA-A*201 mice. *J Pept Res* 2005;**65**:189–99.
6. Dancey JE, Chen HX. Strategies for optimizing combinations of molecularly targeted anticancer agents. *Nat Rev Drug Discov* 2006;**5**: 649–59.
7. Ferrara N. Vascular endothelial growth factor as a target for anticancer therapy. *Oncologist* 2004;**9**(Suppl. 1):2–10.
8. Ferrara N. Vascular endothelial growth factor: basic science and clinical progress. *Endocr Rev* 2004;**25**:581–611.
9. Fischer PM. The design, synthesis and application of stereochemical and directional peptide isomers: a critical review. *Curr Protein Pept Sci* 2003;**4**:339–56.
10. Foy KC, Liu Z, Phillips G, Miller M, Kaumaya PT. Combination treatment with HER-2 and VEGF peptide mimics induces potent anti-tumor and anti-angiogenic responses in vitro and in vivo. *J Biol Chem* 2011;**286**:13,626–37.
11. Franklin MC, Carey KD, Vajdos FF, Leahy DJ, de Vos AM, Sliwkowski MX. Insights into ErbB signaling from the structure of the ErbB2-pertuzumab complex. *Cancer Cell* 2004;**5**:317–28.
12. Garrett JT, Rawale S, Allen SD, Phillips G, Forni G, Morris JC, et al. Novel engineered trastuzumab conformational epitopes demonstrate in vitro and in vivo antitumor properties against HER-2/neu. *J Immunol* 2007;**178**:7120–31.
13. Hans D, Young PR, Fairlie DP. Current status of short synthetic peptides as vaccines. *Med Chem* 2006;**2**:627–46.
14. Hennig IM, Twelves C. Loading doses for costly cancer biologicals: a cause for concern or tilting at windmills? *Eur J Cancer* 2008;**44**: 1493–6.
15. Holash J, Davis S, Papadopoulos N, Croll SD, Ho L, Russell M, et al. VEGF-Trap: a VEGF blocker with potent antitumor effects. *Proc Natl Acad Sci U S A* 2002;**99**:11.393–8.
16. Hruby VJ. Conformational and topographical considerations in the design of biologically active peptides. *Biopolymers* 1993;**33**:1073–82.
17. Kaumaya PT, Foy KC, Garrett J, Rawale SV, Vicari D, Thurmond JM, et al. Phase I active immunotherapy with combination of two chimeric, human epidermal growth factor receptor 2, B-cell epitopes fused to a promiscuous T-cell epitope in patients with metastatic and/or recurrent solid tumors. *J Clin Oncol* 2009;**27**:5270–7.
18. Keyt BA, Nguyen HV, Berleau LT, Duarte CM, Park J, Chen H, et al. Identification of vascular endothelial growth factor determinants for binding KDR and FLT-1 receptors. Generation of receptor-selective VEGF variants by site-directed mutagenesis. *J Biol Chem* 1996;**271**:5638–46.
19. Kim DW, Huamani J, Fu A, Hallahan DE. Molecular strategies targeting the host component of cancer to enhance tumor response to radiation therapy. *Int J Radiat Oncol Biol Phys* 2006;**64**:38–46.
20. Mizejewski GJ. Peptides as receptor ligand drugs and their relationship to G-coupled signal transduction. *Expert Opin Investig Drugs* 2001;**10**:1063–73.
21. Nahta R, Esteva FJ. *In vitro* effects of trastuzumab and vinorelbine in trastuzumab-resistant breast cancer cells. *Cancer Chemother Pharmacol* 2004;**53**:186–90.
22. Nahta R, Hung MC, Esteva FJ. The HER-2-targeting antibodies trastuzumab and pertuzumab synergistically inhibit the survival of breast cancer cells. *Cancer Res* 2004;**64**:2343–6.
23. Oshima RG, Lesperance J, Munoz V, Hebbard L, Ranscht B, Sharan N, et al. Angiogenic acceleration of Neu induced mammary tumor progression and metastasis. *Cancer Res* 2004;**64**:169–79.
24. Petit AM, Rak J, Hung MC, Rockwell P, Goldstein N, Fendly B, et al. Neutralizing antibodies against epidermal growth factor and ErbB-2/neu receptor tyrosine kinases down-regulate vascular endothelial growth factor production by tumor cells *in vitro* and *in vivo*: angiogenic implications for signal transduction therapy of solid tumors. *Am J Pathol* 1997;**151**:1523–30.

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25. Riemer AB, Klinger M, Wagner S, Bernhaus A, Mazzucchelli L, Pehamberger H, et al. Generation of peptide mimics of the epitope recognized by trastuzumab on the oncogenic protein Her-2/neu. *J Immunol* 2004;**173**:394–401.
26. Rugo HS. Bevacizumab in the treatment of breast cancer: rationale and current data. *Oncologist* 2004;**9**(Suppl. 1):43–9.
27. Saito H, Tsujitani S, Ikeguchi M, Maeta M, Kaibara N. Relationship between the expression of vascular endothelial growth factor and the density of dendritic cells in gastric adenocarcinoma tissue. *Br J Cancer* 1998;**78**:1573–7.
28. Sillerud LO, Larson RS. Design and structure of peptide and peptidomimetic antagonists of protein–protein interaction. *Curr Protein Pept Sci* 2005;**6**:151–69.
29. Slamon DJ, Godolphin W, Jones LA, Holt JA, Wong SG, Keith DE, et al. Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. *Science* 1989;**244**:707–12.
30. Taylor EM, Otero DA, Banks WA, O'Brien JS. Retro-inverso prosaptide peptides retain bioactivity, are stable In vivo, and are blood–brain barrier permeable. *J Pharmacol Exp Ther* 2000;**295**:190–4.
31. Vicari D, Foy KC, Liotta EM, Kaumaya PT. Engineered conformation-dependent VEGF peptide mimics are effective in inhibiting VEGF signaling pathways. *J Biol Chem* 2011;**286**:13,612–25.
32. Williams IG. Enfuvirtide (Fuzeon): the first fusion inhibitor. *Int J Clin Pract* 2003;**57**:890–7.

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Chapter 84

Use of Positional Scanning Libraries to Identify Immunologically Relevant Peptides

Mireia Sospedra, Valeria Judkowski, Jon R. Appel and Clemencia Pinilla

ABSTRACT

Many receptor–ligand interactions in biology involve the binding of peptidic molecules to complex surface receptors. Among these, the immune system is a particularly interesting example, because peptides are the main targets for T lymphocytes and can also be recognized by antibodies. The systematic identification of ligands for biologically relevant receptors has been greatly facilitated by the introduction of combinatorial peptide chemistry. Here, we introduce the underlying principles of the use of positional scanning libraries and report their application to elucidate the specificity of antibody, T cell, and MHC molecules. The first reports as well as representative work from different research groups and recent contributions are presented.

DEFINITION OF MIXTURE-BASED LIBRARIES

Mixture-based libraries are synthesized in a combinatorial manner and are made up of mixtures having one or more positions defined with a given amino acid, while other positions have amino acids incorporated as mixtures. These libraries represent a very large number of compounds—in this case synthetic peptides. They are generated by the use of the simultaneous multiple peptide synthesis method known as the "tea bag approach."[7] Then, they are cleaved from the solid support and assayed in solution; this allows each peptide within each mixture to freely interact with a given receptor and thus these libraries can be used in virtually all existing assay systems. Two different deconvolution methods can be used to identify individual active compounds from these mixture-based libraries. The first described method, the iterative approach,[9] uses a selection and enhancement process to identify individual compounds. After the identification of active mixtures having defined positions, the remaining positions are defined, one diversity position at a time, through a synthesis and selection process, until individual compounds are identified. The second deconvolution approach, the positional scanning method,[22] simultaneously addresses each position of the library by testing at the same time individual sublibraries in which each diversity position is defined by a single amino acid, while the remaining positions are composed of mixtures of amino acids. The assay data derived from each position provide information about the most important amino acid for every diversity position of the positional scanning library.

An illustration of the positional scanning library using a tripeptide combinatorial library is shown in Fig. 1. Four different amino acids are incorporated at each of the three diversity positions resulting in a diversity of 64 individual peptides. When the same diversity is arranged as a positional scanning library, only 12 peptide mixtures (4 amino acids × 3 positions) need to be synthesized. Each of the three positional sublibraries, namely, OXX, XOX, and XXO and the same diversity of peptides, is synthesized, and they differ only in the location of the defined amino acid position. The O positions represent one of the four amino acids, whereas the remaining two diversity positions are mixtures (X) of the same four amino acids. Shown below each mixture contains the 16 peptides (4^2) that make up that mixture. Assuming that ART is the only active tripeptide in this library that is recognized, the ART tripeptide (outlined below each sublibrary in Fig. 1) is present in all three positional sublibraries. Thus, the only mixtures with activity are AXX, XRX, and XXT because the ART tripeptide is present only in those mixtures. The combination of these amino acids in their respective positions yields the tripeptide ART, which would then be synthesized and tested for its activity against the receptor. The activity observed for each of the three mixtures (AXX, XRX, and XXT) results from the presence of

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00084-1
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Tripeptide Library

Four amino acids in each position

$4^3 = 64$

A = Alanine
R = Arginine
T = Threonine
W = Tryptophan

Individual Peptides

1	A	A	A	**17**	R	A	A	**33**	T	A	A	**49**	W	A	A
2	A	A	R	**18**	R	A	R	**34**	T	A	R	**50**	W	A	R
3	A	A	T	**19**	R	A	T	**35**	T	A	T	**51**	W	A	T
4	A	A	W	**20**	R	A	W	**36**	T	A	W	**52**	W	A	W
5	A	R	A	**21**	R	R	A	**37**	T	R	A	**53**	W	R	A
6	A	R	R	**22**	R	R	R	**38**	T	R	R	**54**	W	R	R
7	A	R	T	**23**	R	R	T	**39**	T	R	T	**55**	W	R	T
8	A	R	W	**24**	R	R	W	**40**	T	R	W	**56**	W	R	W
9	A	T	A	**25**	R	T	A	**41**	T	T	A	**57**	W	T	A
10	A	T	R	**26**	R	T	R	**42**	T	T	R	**58**	W	T	R
11	A	T	T	**27**	R	T	T	**43**	T	T	T	**59**	W	T	T
12	A	T	W	**28**	R	T	W	**44**	T	T	W	**60**	W	T	W
13	A	W	A	**29**	R	W	A	**45**	T	W	A	**61**	W	W	A
14	A	W	R	**30**	R	W	R	**46**	T	W	R	**62**	W	W	R
15	A	W	T	**31**	R	W	T	**47**	T	W	T	**63**	W	W	T
16	A	W	W	**32**	R	W	W	**48**	T	W	W	**64**	W	W	W

Tripeptide Positional Scanning Library

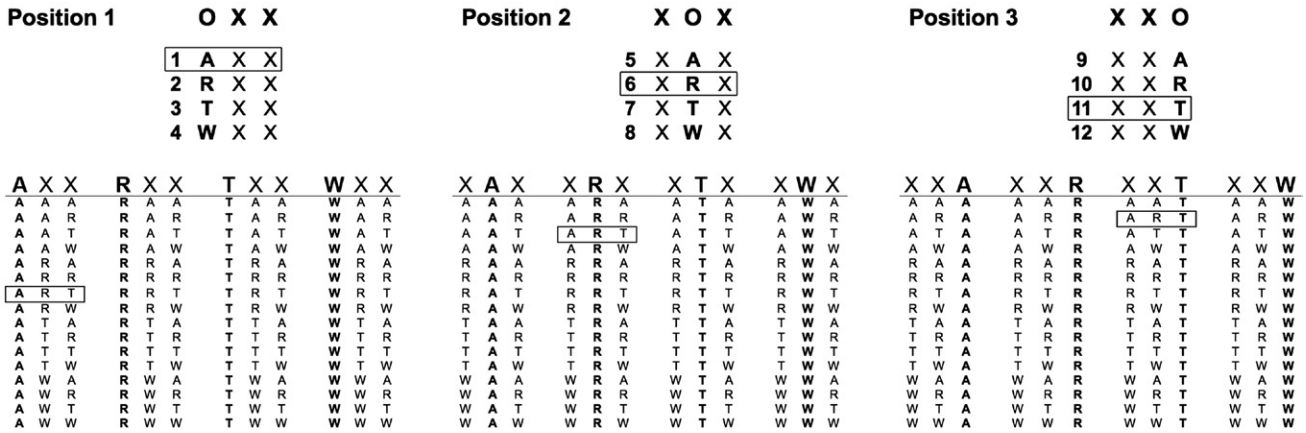

FIGURE 1 Conceptual illustration of a tripeptide positional scanning library.

the tripeptide ART within each mixture and not from the individual amino acids (A, R, and T) that occupy the defined positions. In more complex libraries, more than one mixture is often found to have activity at each position. Selection of the amino acids for the synthesis of individual peptides is based first on activity and then on differences in the chemical character. Although the above example is a simple representation of the arrangement and use of positional scanning libraries, the same concept applies to all types of mixture-based libraries having defined and mixture positions. To give an estimate of the complexity of these libraries, a hexapeptide library using 20 amino acids represents a total diversity of approximately 50 million (19^6) (cysteine is not included in the mixture positions) individual peptides, while a decapeptide library using the same number of amino acids represents a total diversity of approximately 6 trillion (19^{10}) individual peptides, a number of peptides that could clearly not be synthesized or tested individually. A hexapeptide positional scanning library can be formatted into 120 mixtures (20 amino acids × 6 positions) and a decapeptide library into 200 mixtures (20 amino acids × 10 positions).

Positional scanning libraries provide an invaluable resource for characterizing the molecular interactions that underlie many biological processes and for identifying novel sequences that have a desired biological activity. In this chapter, we describe the use of positional scanning libraries in the elucidation of antibody, T cell, and MHC specificity. The first report of their use for each of these molecular interactions, contributions by different research groups and some of the most recent reports are presented.

USE OF POSITIONAL SCANNING LIBRARIES TO DETERMINE ANTIBODY SPECIFICITY

Antibodies are glycoproteins synthesized and secreted by B cells that mediate humoral immunity. Monoclonal antibodies are identical antibodies produced by one type of B cell that recognizes antigens with high affinity and

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specificity. The remarkable specificity of antibodies makes them very valuable for protection against disease organisms and also attractive candidates to target other types of molecules found in the body and promising agents for human therapy. Furthermore, antibodies have an important medical application in the diagnosis of infectious diseases and autoimmune disorders. In this context, the identification of antibody ligands is of great interest. Despite the high specificity that characterizes antibodies, it is important to notice that crossrecognition of peptides by single antibodies also exists. The identification of synthetic mimics recognized by an antibody is also very interesting in the development of more effective immunodiagnostics and synthetic vaccines.

The epitopes recognized by antibodies can be classified as either continuous or discontinuous. It is believed that antibodies mainly recognize native discontinuous or conformational epitopes, and therefore, linear peptides might represent part of the complete epitope. In addition, it has been described that antibodies that recognize discontinuous epitopes can crossreact with linear peptide fragments, indicating that peptides can be used effectively to determine antibody specificity. The classical approach used to identify antibody ligands involves the synthesis and testing of overlapping peptides. Although this methodology allows the identification of ligands, it requires substantial time and resources. Several different peptide combinatorial library approaches, including synthetic and phage display, have emerged as more straightforward and useful approaches for mapping antigen–antibody interactions. Among these approaches positional scanning libraries offer an important advantage because no prior information regarding the specificity of the antibody is needed.

The first use of positional scanning libraries in immunology was for the identification of high-affinity peptides recognized by monoclonal antibodies of known specificity. In 1992, Pinilla et al.[22] showed that the antigenic determinants recognized with high affinity by two antibodies could be correctly identified upon a single screening using hexapeptide positional scanning library. In 1994, Pinilla et al.[23] extended the practical range to decapeptide libraries. With a decapeptide library, the known six-residue antigenic determinant sequence recognized by an antibody was found, with the most specific residues appearing to "walk through" the 10 positions of the peptide library. Interestingly, the antibody recognition in this system was stronger when the antigenic determinant was located at the C-terminus of the decapeptide library. Individual decapeptides corresponding to sequences derived from the most active peptide mixtures at each position were synthesized to confirm the results of the screening. Four peptides were 5–10 times more active than the known control peptides. Additional studies were performed using hexa- and decapeptides by us and others to identify the antigen determinants of antibodies (reviewed in Refs. 8,24).

One of the main advantages of the use of positional scanning libraries for the study of antibody specificity is that no previous specificity information is required. In 1996, two studies reported the use of positional scanning libraries to determine the epitope determinant for antibodies for which the target protein was known, but not the epitope. The first study, Appel et al. reported the identification of high-affinity, crossreactive peptides recognized by a monoclonal antibody (mAb 12) known to recognize the surface antigens of hepatitis B virus (HBsAg). The binding specificity of mAb 12 was studied with a hexapeptide library that was screened by competitive enzyme-linked immunosorbent assay (ELISA). This led to the identification of the peptides STTSMM (IC50 = 170nM) and SVGPPH (IC50 = 165nM), which specifically inhibited the interaction between HBsAg and mAb 12. Because motifs from these two hexapeptides, –STTS– and –GP–, are located in the primary sequence of the protein (residues 114–120), overlapping hexapeptides of this region were used to identify the most active hexapeptide, TTSTGP (IC50 = 2.3μM), which is 10-fold less active than either hexapeptide found from the library (included in review[24]). In the second study, Gao et al. reported on the identification of an epitope for a monoclonal antibody 201/9, raised against betafactor XIIa, which was determined with octapeptide mixture–based libraries including some in positional scanning format (reviewed in Ref. 24).

One important observation was that highly active peptide sequences having little or no resemblance to the known immunogen were identified for some antibodies. Park et al. in 1997 using a hexapeptide library identified peptide sequences that inhibit the binding of IgG antibodies specific of thyroid-stimulating-hormone receptor (TSHR) obtained from patients with Graves' disease. Interestingly, these peptides do not resemble the linear sequence of TSHR and thus may mimic a spatial arrangement of the key antigenic residues (reviewed in Ref. 24). The structural basis for the molecular mimicry shown by monoclonal antibodies remains unclear. It has been suggested that the flexible hypervariable loops of antibodies and the fact that they recognize functional conformation rather than identical structures can facilitate crossrecognition.

It is often assumed that crossreactive antigens share some structural similarity that is specifically recognized by a monoclonal antibody. Antibody crossreactivity was further explored by Pinilla et al. in 1998 for a monoclonal antibody (mAb HGAC 39.G3), which binds to *N*-acetyl-D-glucosamine (GlcNAc) on a polyrhamnose backbone. Hexapeptide positional scanning libraries consisting of all-L or all-D amino acids were screened and the inhibitory activity by mixtures from the all-D hexapeptide library is much greater than the activity from the all-L libraries. The sequence Ac-yryygl-NH_2 is specifically recognized by mAb HGAC 39.G3 with a relative affinity of 300nM when

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measured in a competitive binding assay. This peptide shares little homology with the carbohydrate antigen, suggesting that the peptide might interact differently with the antibody (reviewed in Ref. 24).

USE OF POSITIONAL SCANNING LIBRARIES FOR T CELL EPITOPE MAPPING

T lymphocytes play a central role in controlling the acquired immune response orchestrating both the "cellular" and "humoral" (helping B cells) arms of acquired/specific immunity. Like B lymphocytes, T cells express a clonal antigen-specific receptor that recognizes fragmented, linear antigenic peptides when these are bound to proteins encoded by major histocompatibility complex (MHC) genes. There are two major subsets of T lymphocytes that differ in effector function and MHC restriction. $CD8^+$ T cells recognize peptides bound to MHC class I molecules and act as effector cells during viral infections through antigen-specific cytotoxic activity. $CD4^+$ T cells recognize peptides bound to MHC class II molecules and act as inducers of T and B cell functions producing cytokines. Although T cell recognition was previously considered exquisitely specific for single peptides or close homologs with similar sequence, it is now clear that T cell recognition is very flexible and crossreactivity is a common phenomenon.

T lymphocytes play important roles in infectious diseases, autoimmunity, and also immune responses against tumors. For many of these disorders, the relevant target antigens are not known. Designing effective methods that allow the search for T cell epitopes is therefore an important goal, and several approaches have been applied with variable success. Epitope mapping with series of overlapping peptides can be applied only when the antigenic protein is known. Biochemical analysis of pools and peptides eluted from MHC molecules is another approach but requires sophisticated instrumentation. Molecular biology approaches using expression libraries or the search of epitopes using MHC-binding motifs are biased because they require the previous selection of an organism or tissue of interest.

Several studies have shown the efficacy of using positional scanning libraries for identifying target antigens and highly active peptide mimics and naturally occurring peptides for both $CD4^+$ and $CD8^+$ T cells of known and unknown specificity. Initially $CD8^+$ T cells received more attention not only because they often show higher antigen affinities but also because the class I-binding groove accommodates shorter peptides (8–10 amino acids) and less complex peptide libraries can be used to determine their specificity. In 1996, Gundlach et al.[3] presented the use of a nonapeptide positional scanning library to elucidate the specificity of cytotoxic T cells (CTL). Sequences for potential T cell epitopes were deduced from scan profiles by the use of combinations of the active amino acids. An octapeptide positional scanning library was successfully used by this group to identify self-MHC-restricted antigenic peptides crossrecognized by an allo-MHC-specific CTL clone and also by Udaka et al. in 1996 to study the specificity and degeneracy of H-3-specific, H-2K(b)-restricted CTL clones (reviewed in Refs. 1, 24).

The identification of defined human tumor antigens recognized by autologous CTL opened new opportunities for the development of antigen-specific cancer vaccines. In 2000, Linnemann et al. (reviewed in Ref. 1) used a nonapeptide library in a standard chromium release assay to identify a synthetic epitope for the My-La CTL specific for cutaneous T cell lymphoma. The response toward this epitope was comparable with the response toward their natural target My-La. The identification of synthetic epitopes for tumor-specific CTL clones that are recognized in an MHC-restricted manner can be used for the development of vaccines for immune therapies of cancer.

Positional scanning libraries can also be used to improve vaccines, for example, enhancing the activity of peptide ligands to reduce the amount of peptide vaccine needed to obtain a clinical CTL response in an immunization strategy. In this context, the study by La Rosa et al. 2001, using a nonapeptide library identified a series of peptide analogs for the cytomegalovirus (CMV)-specific T Cell Clone 3-3F4 that were recognized better than the native peptide sequence reviewed in Ref. 1. Moreover, the study by Lustgarten et al. in 2006 reported the use of a decapeptide positional scanning library to identify optimal amino acids substitutions in the native peptide. This strategy led to the identification of superagonist peptides that expand antitumor T cells with higher killing efficiency than the native peptide.[19]

The identification of the specificity of $CD4^+$ class II-restricted T cell clones seemed more difficult because the affinity of these cells is lower than described for $CD8^+$ T cells and peptides bound to class-II molecules are longer (typically 12–15 amino acids). $CD4^+$ class II-restricted T cells specific for self-antigens are thought to be involved in the pathogenesis of most human autoimmune diseases, and molecular mimicry between foreign and self-ligands has been implicated as a possible mechanism for their activation. The identification of peptide mimics for these autoreactive $CD4^+$ T cells is crucial to understand the autoimmune process and to develop future therapies. In 1997, Hemmer et al.[4] showed that a 11-mer library could be used to identify crossreactive artificial peptides for a $CD4^+$ T cell clone (TCC) TL5G7, specific for the myelin basic protein peptide (MBP) (86–96) and derived from the peripheral blood of a patient with multiple sclerosis. Interestingly, these artificial peptides induced proliferative responses at much lower concentrations than the original peptide MBP (86–96) used to generate the TCC. In addition stimulatory

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ligands derived from protein sequences of self and microbial proteins were identified, some of them more potent agonists than MBP (86–96). A more extensive study was performed with a decapeptide library to study the specificity of two additional MBP (86–96)-specific TCC, also established from peripheral blood lymphocytes of MS patients: TL5F6 that is DR2b restricted, and TL3A6 that is DR2a restricted.[6] The use of positional scanning libraries allowed the identification of TCR motifs and stimulatory antigenic peptides. In this study, the authors suggested for the first time that each amino acid residue contributes to the overall potency of the antigenic peptide ligand. The decapeptide library was also successfully used to identify peptide mimics for CD4+ T cell clones of known specificity in two additional studies. The first study published in 1999 by Wilson and colleagues showed that the use of a 10-mer library to identify peptide epitopes is more effective than the native peptide in stimulating proliferative responses in a CD4+ T cell clone derived from transgenic mice. This TCC expresses a TCR specific for the 88–104 peptide fragment of pigeon cytochrome c and the Ek class II molecule, reviewed in Ref. 1. A second study showing the power of positional scanning libraries in identifying epitopes for CD4+ T cell clones was published in 2001.[12] Nonobese diabetic (NOD) mice spontaneously develop destruction of the insulin-producing pancreative islet beta cells similar to type-1 diabetes mellitus in humans. The diabetogenic NOD CD4+ T cell clone BDC2.5 adoptively transfers disease after being activated *in vitro* with syngeneic islets cells. The identity of the islet cell antigen responsible for pathogenicity is not known. Positional scanning libraries were used to identify decapeptides able to stimulate BDC2.5 cells and transfer disease to NOD-scid mice. Surprisingly, some of these peptides include sequences similar to residues 528–539 of glutamic acid decarboxylase 65, a candidate autoantigen in diabetes.

An enormous step forward was achieved in the use of positional scanning libraries to identify T cell epitopes when Hemmer et al.[5] incorporated biometrical analysis to this approach. The positional scanning libraries based biometrical analysis developed as a strategy that systematically compares the data derived from the screening with the library with all peptide fragments present in protein databases. The response of T cell clones to positional scanning synthetic combinatorial libraries is analyzed with a mathematical approach that is based on a model of independent contribution of individual amino acids to peptide antigen recognition. This biometric analysis compares the information derived from these libraries consisting of trillions of decapeptides with all the millions of decapeptides contained in a protein database to rank and predict the most stimulatory peptides for a given T cell clone. In chronic infectious diseases such as Lyme disease, immune-mediated damage may add to the effects of direct infection by means of molecular mimicry to tissue autoantigen. Using this strategy, the authors effectively identified both microbial epitopes, from *Borrelia burgdorferi*, and candidate autoantigens, from the host, recognized by TCC CSF-3 of unknown specificity that was established from the cerebrospinal fluid (CSF) of a patient with chronic neuroborreliosis using *Borrelia burgdorferi* lysate. The positional scanning libraries based biometrical analysis was analyzed in detail by Zhao et al.,[35] and its predictive power was shown with CD4+ TCC GP5F11 that was established from the peripheral blood of a patient with multiple sclerosis using influenza virus hemagglutinin (HA) peptide 309–318 as antigen. The experimental data from the screening of the library was used to generate a matrix, which was used to score all the decapetides in all the viral proteins in the Genpept database. The information obtained was a list of peptides ranked by their score, and the native peptide ranked sixth. The selected peptides were synthesized and tested, and their stimulatory capacity from TCC GP5F11 recognition was confirmed. More recently, the crossreactivity of this TCC against 14 Flu-HA variants, 11 viral, 15 human, and 3 myelin-derived naturally occurring peptides was reported.[20] Six of these peptides have higher stimulatory potency than the native ligand, showing that high crossreactivity can be a feature even of virus-specific memory TCC. Furthermore, positional scanning libraries in combination with protein database analyses were used to identify the specificity of TCC of completely unknown specificity that were generated from a relevant compartment and expanded using a universal T cell stimulus such as phytohemagglutinin. Using this strategy the specificity of CSF infiltrating and *in vivo* expanded TCC from an untreated MS patient[29] and of CSF infiltrating TCC from a Lyme disease's patient[18] was successfully identified. In addition, the use of positional scanning libraries in combination with B cell lines expressing only a single class II restriction element as APC was used to study the promiscuous restriction of T cell recognition.[27] The ability of T cells to recognize peptides in the context of more than one HLA class II molecule is a feature of some T cells, and this crossrecognition seems to be behind the low predictive capacity of this approach for some T cell clones.[28] The use of B cell lines expressing single HLA class II molecules as antigen-presenting cells instead of autologous peripheral blood mononuclear cells to test the mixtures of the positional scanning libraries markedly improves the capacity to identify target peptides for this type of T cells. In addition, Judkowski et al. reported in 2004 the use of the screening data obtained with the BDC2.5 T cell clone together with the biometrical analysis for the identification of viral and bacterial peptides that are capable of activating BDC2.5 CD4+ T cells and transfer diabetes in NOD-scid mice.[13]

Recent studies have extended the use of positional scanning libraries together with the biometrical analysis

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to determine the specificity of the CD4+ T cell response upon smallpox vaccination in humans. Using a single *in vitro* stimulation of PBMC with vaccinia virus and PHA stimulation, CD4+ T cell clones from vaccinated donors were generated. A comprehensive analysis of the cytokine expression profile by vaccinia specific CD4+ T cells showed that GM-CSF is produced at high levels in the supernatant of pathogen stimulated cultures. Based on this observation, GM-CSF was used as readout of CD4+ activation and was shown, for the first time, to be an excellent cytokine to measure the response to mixtures of positional scanning libraries. The biometrical analysis was performed with a custom Western Reserve vaccinia protein database and all clones were found to respond to the first or second vaccinia ranked predicted peptide. Interestingly, analysis of the performance efficacy of the T cell driven strategy presented in this study showed that this approach is very efficient in the identification of immunogenic and predominantly recognized proteins. Because the analysis of the whole pathogen proteome to identify relevant antigens might not be feasible for larger pathogens (>1,000 proteins), there is a clear advantage in using positional scanning libraries to elucidate the antigen specificity upon infection or vaccination in humans.[11] Finally, it is clear that further efforts on the development of miniature T cell activation assays will be extremely beneficial to reduce the number of clonal CD4+ T cells required to test combinatorial peptide libraries and to be able to define the spectrum of specificities with higher throughput.

The effectiveness of positional scanning library-based biometrical analysis has also been shown with tumor antigen-specific $CD8^+$ clones of known (Melan-A, Pinilla et al., 2001 and Tyrosinase, Rubio-Godoy et al., 2002) and unknown specificity (SSX-2, Rubio-Godoy et al., 2002). These studies as well as an overview of the positional scanning library-based biometrical analysis of the study of T cell specificity and crossreactivity are reviewed in Refs. 1, 21. In addition, positional scanning libraries in combination with motif analysis have also been used to study the specificity of CD8+ T cells. Anderson et al. published in 1999 a study in which nonapeptide mixtures were designed to have the $H\text{-}2K^d$ or $H\text{-}2D^b$ anchor residues fixed at positions 2 and 9 or 5 and 9, respectively. Screening of the $H\text{-}2K^d$ library derived in the identification of a mimotope targeted by diabetogenic 8.3-CD8+ T cells reviewed in Ref. 1. Screening of the $H\text{-}2D^b$ restricted positional scanning library led to the identification of a set of active peptides, including a natural occurring sequence within the widely expressed dystrophia miotonica kinase for the A14-pathogenic CD8+ T cell clone. In addition, the analysis of the screening results using both libraries with A14 T cells was used to confirm that the A14 TCR is highly promiscuous having the capacity to interact with both the $H\text{-}2K^d$ and $H\text{-}2D^b$ Class I MHC molecules.[17]

USE OF POSITIONAL SCANNING LIBRARIES TO IDENTIFY MHC-BINDING MOTIFS

$CD8^+$ and $CD4^+$ T lymphocytes recognize peptides bound to class I or class II MHC molecules, respectively. These complexes are assembled intracellularly during the biosynthesis and trafficking of MHC molecules. MHC class I and class II molecules have different peptide binding sites. MHC class I molecules have a closed groove that only allows short peptides of 8–10 amino acids in length. In contrast MHC class II molecules bind peptides that are 10–25 amino acids in length. Considerable interest has been focused on understanding how MHC specificity is generated with the ultimate goal of predicting peptide binding.

Positional scanning libraries have emerged as a highly efficient, universal and unbiased approach to address MHC specificity. In the first report in 1995, Udaka et al.[32] identified patterns of amino acid preferences from testing an octapeptide library against the mouse MHC-I molecule H2-Kb using stabilization experiments. A year later, Stryhn et al.[30] used an octapeptide library and competitive binding assays to describe peptide binding motifs for class I molecules, including primary and secondary anchor residues. The quantification of the effect of any amino acid in each position also allowed the identification of disfavored residues very important in shaping MHC class I specificity. In 1996, Pridzun et al. reported on the determination of the impact of the amino acids in every sequence position of octapeptides using stabilization experiments for the mouse MHC class I molecule H2-Ld (reviewed in Ref. 24). Later, Udaka et al.[31] also used this approach to determine the specificities of three mouse major MHC class I molecules, Kb, Db, and Ld. The results of this study was used to create a scoring program to predict MHC-binding peptides in proteins. However, the authors frequently found MHC-binding peptides with incomplete or no anchor amino acid(s) suggesting a substantial bias that is introduced by natural antigen processing in peptide selection by MHC class I molecules. More recently, the efficacy of the positional scanning libraries was shown for 15 human and 4 class I alleles for the definition of the binding specificity and the identification of high-affinity-binding peptides.[25] In addition, a novel matrix similarity derived from these studies was used to develop a new MHC Class I prediction method.[16] Also, the characterization of the peptide binding to MHC alleles in rhesus macaques was reported for frequently expressed allele, namely, Mam-A*02201.[26]

Positional scanning libraries have also been used to study the binding characteristics of MHC class II molecules. In 1996, Fleckenstein et al.[2] used an undecapeptide library to estimate the influence of every amino acid on the binding to human MHC-DRB1*0101 molecules (HLA-DRI) and to identify new peptide ligands. The amino acids favorable or

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unfavorable for DR1-binding at every sequence position were defined and individual peptides were identified. The same research group using an undecapeptide library elucidated the specificity of HLA-DRB1*0301.[14] More recently, the use of nonapeptide libraries for the identification of ligands for two human MHC alleles, namely, HLA-DQ4[33] and HLA-DQ2[15] has been reported. Also, the predictive performance of a 13 amino acid long positional scanning peptide library, having alanine residues in positions 1, 2, 12, and 13 and the central nine residues in positional scanning format, was compared with several other predicting algorithms. The authors concluded that positional scanning libraries are an efficient approach to derive MHC class II binding profiles.[34]

CONCLUSIONS

This chapter presents a summary of the use of positional scanning libraries to elucidate the specificity of a number of immunologically relevant receptor–peptide interactions. These receptors include antibody, T cell receptors, and MHC molecules. Peptide libraries have also been successfully used for the identification of the substrates of several proteases relevant in immunology such as human caspases and granzyme B (reviewed in Refs. 8,10). Furthermore, nonpeptidic and small molecule positional scanning libraries represent a source of diversity for the identification of protein–protein or protein–receptor interactions in which inhibitors or modulators of nonpeptidic composition would be favorable. The authors and others have successfully used these libraries for the identification of highly active agonists and antagonists of G protein-coupled receptors, antimicrobials, enzyme inhibitors, and other biological targets.[8,10]

REFERENCES

1. Borras E, Martin R, Judkowski V, Shukaliak J, Zhao Y, Rubio-Godoy V, et al. Findings on T cell specificity revealed by synthetic combinatorial libraries. *J Immunol Methods* 2002;**267**:79–97.
2. Fleckenstein B, Kalbacher H, Muller CP, Stoll D, Halder T, Jung G, et al. New ligands binding to the human leucocyte antigen class II molecule DRB*0101 based on the activity pattern of an undecapeptide library. *Eur J Biochem* 1996;**240**(1):71–7.
3. Gundlach BR, Wiesmüller KH, Junt T, Kienle S, Jung G, Walden P. Determination of T cell epitopes with random peptide libraries. *J Immunol Meth* 1996;**192**(1–2):149–55.
4. Hemmer B, Fleckenstein B, Vergelli M, Jung G, McFarland H, Martin R, et al. Identification of high potency microbial and self ligands for a human autoreactive class II-restricted T cell clone. *J Exp Med* 1997;**185**(9):1651–9.
5. Hemmer B, Gran B, Zhao Y, Marques A, Pascal J, Tzou A, et al. Identification of candidate T cell epitopes and molecular mimics in chronic Lyme disease. *Nature Med* 1999 Dec;**5**(12):1375–82.
6. Hemmer B, Pinilla C, Gran B, Vergelli M, Ling N, Conlon P, et al. Contribution of individual amino acids within MHC molecule or antigenic peptide to TCR ligand potency. *J Immunol* 2000 Jan;**164**:861–71.
7. Houghten RA. General method for the rapid solid-phase synthesis of large numbers of peptides: specificity of antigen–antibody interaction at the level of individual amino acids. *Proc Natl Acad Sci USA* 1985;**82**:5131–5.
8. Houghten RA, Pinilla C, Appel JR, Blondelle SE, Dooley CT, Eichler J, et al. Mixture-based synthetic combinatorial libraries. *J Med Chem* 1999 Sep;**42**(19):3743–78.
9. Houghten RA, Pinilla C, Blondelle SE, Appel JR, Dooley CT, Cuervo JH. Generation and use of synthetic peptide combinatorial libraries for basic research and drug discovery. *Nature* 1991;**354**:84–6.
10. Houghten RA, Pinilla C, Giulianotti MA, Appel JR, Dooley CT, Nefzi A, et al. Strategies for the use of mixture-based synthetic combinatorial libraries: scaffold ranking, direct testing in vivo, and enhanced deconvolution by computational methods. *J Comb Chem* 2008 Jan;**10**(1):3–19.
11. Judkowski V, Bunying A, Ge F, Appel JR, Law K, Sharma A, et al. GM-CSF production allows the identification of immunoprevalent antigens recognized by human CD4+ T cells following smallpox vaccination. *PLoS One* 2011;**6**(9):e24091.
12. Judkowski V, Pinilla C, Schroder K, Tucker L, Sarvetnick N, Wilson DB. Identification of MHC class II-restricted peptide ligands, including a glutamic acid decarboxylase 65 sequence, that stimulate diabetogenic T cells from transgenic BDC2.5 nonobese diabetic mice. *J Immunol* 2001;**166**:908–17.
13. Judkowski VA, Allicotti GM, Sarvetnick N, Pinilla C. Peptides from common viral and bacterial pathogens can efficiently activate diabetogenic T-cells. *Diabetes* 2004 Sep;**53**(9):2301–9.
14. Jung C, Kalbus M, Fleckenstein B, Melms A, Jung G, Wiesmüller KH. New ligands for HLA DRB1*0301 by random selection of favourable amino acids ranked by competition studies with undecapeptide amide sublibraries. *J Immunol Methods* 1998;**219**:139–49.
15. Juse U, van de WY, Koning F, Sollid LM, Fleckenstein B. Design of new high-affinity peptide ligands for human leukocyte antigen-DQ2 using a positional scanning peptide library. *Hum Immunol* 2010 May;**71**(5):475–81.
16. Kim Y, Sidney J, Pinilla C, Sette A, Peters B. Derivation of an amino acid similarity matrix for peptide: MHC binding and its application as a Bayesian prior. *BMC Bioinformatics* 2009;**10**:394.
17. Lieberman SM, Takaki T, Han B, Santamaria P, Serreze DV, DiLorenzo TP. Individual nonobese diabetic mice exhibit unique patterns of CD8+ T cell reactivity to three islet antigens, including the newly identified widely expressed dystrophia myotonica kinase. *J Immunol* 2004 Dec;**173**(11):6727–34.
18. Lunemann JD, Gelderblom H, Sospedra M, Quandt JA, Pinilla C, Marques A, et al. Cerebrospinal fluid-infiltrating CD4+ T cells recognize *Borrelia burgdorferi* lysine-enriched protein domains and central nervous system autoantigens in early lyme encephalitis. *Infect Immun* 2007 Jan;**75**(1):243–51.
19. Lustgarten J, Dominguez AL, Pinilla C. Identification of cross-reactive peptides using combinatorial libraries circumvents tolerance against Her-2/neu-immunodominant epitope. *J Immunol* 2006;**176**:1796–805.
20. Markovic-Plese S, Hemmer B, Zhao Y, Simon R, Pinilla C, Martin R. High level of cross-reactivity in influenza virus hemagglutinin-specific CD4+ T-cell response: implications for the initiation of autoimmune response in multiple sclerosis. *J Neuroimmunol* 2005 Dec;**169**(1–2):31–8.
21. Nino-Vasquez JJ, Allicotti G, Borras E, Wilson DB, Valmori D, Simon R, et al. A powerful combination: the use of positional scanning libraries and biometrical analysis to identify cross-reactive T cell epitopes. *Mol Immunol* 2004 Feb;**40**(14–15):1063–74.

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22. Pinilla C, Appel JR, Blanc P, Houghten RA. Rapid identification of high affinity peptide ligands using positional scanning synthetic peptide combinatorial libraries. *Biotechniques* 1992;**13**:901–5.
23. Pinilla C, Appel JR, Houghten RA. Investigation of antigen-antibody interactions using a soluble nonsupport-bound synthetic decapeptide library composed of four trillion sequences. *Biochem J* 1994;**301**:847–53.
24. Pinilla C, Martin R, Gran B, Appel JR, Boggiano C, Wilson DB, et al. Exploring immunological specificity using synthetic peptide combinatorial libraries. *Curr Opin Immunol* 1999;**11**:193–202.
25. Sidney J, Assarsson E, Moore C, Ngo S, Pinilla C, Sette A, et al. Quantitative peptide binding motifs for 19 human and mouse MHC class I molecules derived using positional scanning combinatorial peptide libraries. *Immunome Res* 2008;**4**:2.
26. Solomon C, Southwood S, Hoof I, Rudersdorf R, Peters B, Sidney J, et al. The most common Chinese rhesus macaque MHC class I molecule shares peptide binding repertoire with the HLA-B7 supertype. *Immunogenetics* 2010;**62**:451–64.
27. Sospedra M, Muraro PA, Stefanova I, Zhao Y, Chung K, Li Y, et al. Redundancy in antigen-presenting function of the HLA-DR and -DQ molecules in the multiple sclerosis-associated HLA-DR2 haplotype. *J Immunol* 2006 Feb;**176**(3):1951–61.
28. Sospedra M, Zhao Y, Giulianotti M, Simon R, Pinilla C, Martin R. Combining positional scanning peptide libraries, HLA-DR transfectants and bioinformatics to dissect the epitope spectrum of HLA class II cross-restricted CD4+ T cell clones. *J Immunol Methods* 2010 Feb;**353**(1–2):93–101.
29. Sospedra M, Zhao Y, zur Hausen H, Muraro PA, Hamashin C, de Villiers EM, et al. Recognition of conserved amino acid motifs of common viruses and its role in autoimmunity. *PLoS Pathog* 2005 Dec;**1**(4):e41.
30. Stryhn A, Pedersen LO, Romme T, Holm CH, Holm A, Buus S. Peptide binding specificity of major histocompatibility complex class I resolved into an array of apparently independent subspecificities: quantitation by peptide libraries and improved prediction of binding. *Eur J Immunol* 1996;**26**(8):1911–8.
31. Udaka K, Wiesmüller KH, Kienle S, Jung G, Tanamura H, Yamagishi H, et al. An automated prediction of MHC class I-binding peptides based on positional scanning with peptide libraries. *Immunogenetics* 2000;**51**:816–28.
32. Udaka K, Wiesmüller KH, Kienle S, Jung G, Walden P. Tolerance to amino acid variations in peptides binding to the major histocompatibility complex class I protein H-2K^{b}. *J Biol Chem* 1995;**270**:24130–4.
33. Volz T, Schwarz G, Fleckenstein B, Schepp CP, Haug M, Roth J, et al. Determination of the peptide binding motif and high-affinity ligands for HLA-DQ4 using synthetic peptide libraries. *Hum Immunol* 2004 Jun;**65**(6):594–601.
34. Wang P, Sidney J, Kim Y, Sette A, Lund O, Nielsen M, et al. Peptide binding predictions for HLA DR, DP and DQ molecules. *BMC Bioinformatics* 2010;**11**:568.
35. Zhao Y, Gran B, Pinilla C, Markovic-Plese S, Hemmer B, Tzou A, et al. Combinatorial peptide libraries and biometric score matrices permit the quantitative analysis of specific and degenerate interactions between clonotypic TCR and MHC peptide ligands. *J Immunol* 2001;**167**:2130–41.

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Chapter 85

Anaphylatoxins

De Yang

ABSTRACT

Anaphylatoxins, namely, C3a and C5a, are complement-derived, cationic, inflammatory peptides that can cause anaphylactic reaction. C3a and C5a are generated on the enzymatic cleavage of complement C3 and C5 during the course of complement activation through classic, lectin, or alternative pathways. C3a and C5a mediate diverse biological actions by interacting with their receptors that are G protein coupled receptors differentially expressed by many subsets of leukocytes. Anaphylatoxins play important roles in inflammation and immunity and are implicated in the pathogenesis of numerous diseases.

HISTORICAL PERSPECTIVE AND SCOPE

Anaphylatoxin is a substance derived from complement activation that causes smooth muscle contraction, capillary leakage, and even anaphylactic shock. Historically, the development of our knowledge of anaphylatoxin is closely linked with the advancement in the understanding of complement system, which parallels the history of immunology. In the late ninteenth century, two theories on the mechanisms involved in protecting the host from microbial attack emerged: the "cellular theory," based on the existence of blood cells capable of ingesting bacteria, and the "humoral theory," based on the capacity of cell-free serum to cause bacteriolysis. Toward the end of the ninteenth century, Bordet, working at the Pasteur Institute, found that the serum from guinea pigs immunized with cholera organisms lost its bacteriolytic activity after heating but that the activity could be fully restored by the addition of nonimmune serum.[4] Bordet hypothesized that two humoral factors were involved, one of which was heat liable and the other a thermostable substance present in immune serum. Later, the thermostable factor present only in immune serum was identified as antibody and the heat-labile factor present in both nonimmune and immune sera was termed complement because it 'complemented' the activity of antibody in bacteriolysis. At the beginning of the twentieth century, anaphylatoxin was first described by Friedberger, who showed that the treatment of fresh serum with immune precipitates resulted in the formation of a substance that elicits anaphylactic shock in animals and speculated that the substance was derived from complement.[13] In the first half of the twentieth century, it was established that the complement contains more than one proteinaceous component. The 1960s and 1970s witnessed the purification and characterization of the individual components of the complement system including the identification of anaphylatoxic peptides, the establishment of the classic and alternative pathways of the complement activation cascade, as well as the biochemical characterization of the receptors used by complement components including the anaphylatoxins.[21,25] The genes for the complement components, anaphylatoxin, and their receptors were cloned in the 1980s and 1990s.[30]

Anaphylatoxins include C3a and C5a. Although C4a is also considered an anaphylatoxin,[21,25] C4a has only been shown to bind to the C3a receptor (C3aR) with low affinity and no C4a-specific receptor has been identified. In addition, human C4a has no agonistic effect on human C3aR despite showing anaphylactic activity in guinea pigs.[23]

PRECURSOR mRNA/GENE STRUCTURE, EXPRESSION, AND REGULATION OF ANAPHYLATOXINS

The genome organization, transcription, translation, and processing of human C3 and C5 are schematically illustrated in Fig. 1. Human C3 and C5 genes are located on chromosome 19p13.3–p13.2 and 9q33, respectively.[32,33] Although C3 (41 kb) and C5 (79 kb) genes are quite different in size, their intron–exon organization is very similar, with each consisting of 41 exons.[7,11] Transcription of the genes leads to the generation of C3 and C5 mRNAs of approximately 5.0–5.4 kb, which contain C3- and C5-coding sequence of 4992 and 5031 bases (including three bases of the stop codon), respectively.[10,17]

The major site for both C3 and C5 synthesis is hepatocytes; however, many other cell types, such as monocytes/macrophages, dendritic cells, and alveolar type II epithelial cells, can also produce C3 and C5.[2,12,25,30] Translation of

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00085-3
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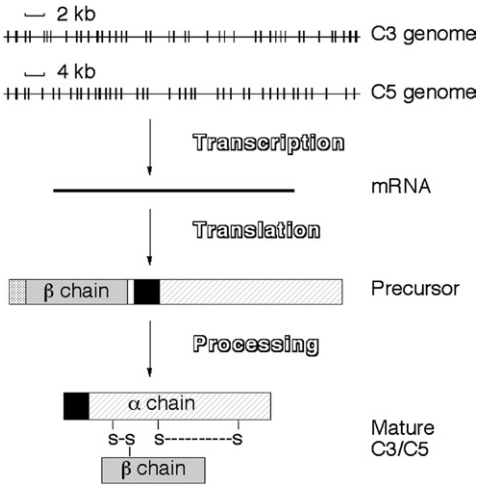

FIGURE 1 Schematic of the genome, synthesis, and processing of the precursors of human anaphylatoxins. The vertical bars in the genome represent exons.

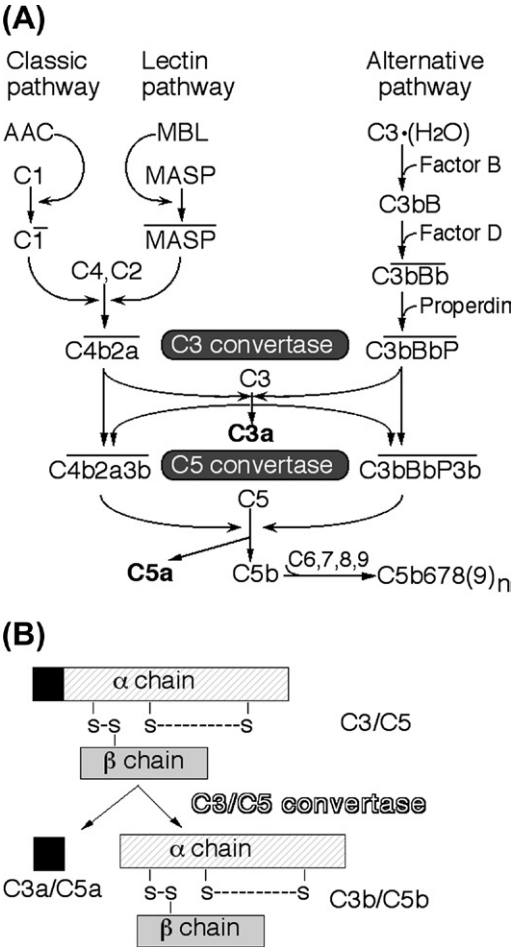

FIGURE 2 Generation of anaphylatoxins. (A) Outline of the complement activation cascade. Overlined components represent activated enzymes. AAC, antigen–antibody complex; MBL, mannose-binding lectin; MASP, MBL-associated serine protease. (B) cleavage of the α chain of C3 or C5 by respective convertases leads to the release of C3a or C5a anaphylatoxin.

C3 mRNA gives rise to a single-chain C3 precursor of 1663 amino acid (aa) residues consisting of a 22 aa leader peptide, a 645 aa β chain peptide, a 4 aa (Arg–Arg–Arg–Arg) linker sequence, and a 992 aa α chain peptide.[10] Similarly, C5 mRNA is translated into a single-chain procursor of 1676 aa including an 18 aa leader peptide, a 655 aa β chain peptide, a 4 aa (Arg–Pro–Arg–Arg) linker sequence, and a 999 aa α chain peptide.[17] Both the leader peptide and the linker sequence are removed during intracellular processing, leading to the generation of mature C3 or C5, which possesses a similar two-chain structure held together by one interchain disulfide bond and noncovalent interactions.[10,17,25,30]

Mature C3 and C5 are secreted into the extracellular fluids. In the serum, C3 is the most abundant complement component with a concentration ranging from 1 to 1.5 mg/ml, whereas C5 is present at approximately 70–80 μg/ml.[12,25,30]

The expression of C3 by many types of cells can be upregulated by inflammatory stimuli such as proinflammatory cytokines (e.g., TNFα, IL-1, IL-6, and IFNγ) and microbial products (e.g., LPS).[30] Therefore, C3 is considered to be one of the prominent acute response proteins. The expression of C3 is regulated at both transcriptional and posttranscriptional levels. By contrast, proinflammatory cytokines and LPS do not modulate the hepatic expression of C5.

GENERATION OF C3A AND C5A ANAPHYLATOXINS

C3a and C5a are generated in the course of complement activation.[12,21,25,30] The complement system is activated through three different pathways: classic, lectin, and alternative, as illustrated in Fig. 2A [Reviewed in detail in Ref. 30]. Although initiated uniquely and differing in the generation of C3 and C5 convertases, the three activation pathways converge on the activation of C5. The classic pathway is initiated by the formation of antigen–antibody complex (AAC), which activates C1 by binding C1q through the exposed CH2 region of certain antibodies (IgG1–3 and IgM). By contrast, the lectin pathway is initiated by the activation of mannose-binding lectin associated serine proteases, which is triggered by the binding of mannose-binding lectin to mannose-containing carbohydrate moieties on the microbial cell surfaces. Activated C1 and MASP sequentially cleave C4 and C2, leading to the generation of C4b2a C3 convertase. The alternative pathway is initiated by many microbial cell wall components, such as zymosan and LPS. The exact mechanism of initiation of this pathway is not completely understood; however, the most probable explanation is the "tickover" hypothesis.

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Based on this hypothesis, C3 is believed to be constantly hydrolyzed [C3?(H_2O)] at a low-rate ("tickover") to form C3b, which is extremely unstable unless it binds to targets such as bacteria. Subsequently, factor B is recruited to the bound C3b and followed by factor D that cleaves factor B to form C3bBb, which is further stabilized by properdin to form C3bBbP, the C3 convertase of the alternative complement activation pathway. Once C3 is cleaved by C3 convertases, C3b binds with C3 convertase to form a complex termed C5 convertase [either C4b2a3b or C3bBbP3b], which, in turn, cleaves C5. C5b continues the complement activation cascade by binding to C6–8 and multiple C9 to ultimately form a cylindrical transmembrane channel, C5b678$(9)_n$, which is termed the membrane attack complex, for the destruction of target cell membrane.

In the complement activation cascade, cleavage of either C3 or C5 by C3 or C5 convertases occurs at a site close to the N-termini of their α chains,[12,21,25,30] leading to the generation of a big fragment, C3b or C5b, and a small peptide fragment, C3a or C5a (Fig. 2A and B). C3b and C5b continue the complement activation cascade on the surface of microbial or cell surfaces, whereas C3a and C5a are released into the fluid phase to act as anaphylatoxins. The life span of C3a and C5a *in vivo* is limited by carboxypeptidase N, an enzyme in the plasma that cleaves off the C-terminal Arg residue from both C3a and C5a, leading to the formation of desArg derivatives, C3adesArg and C5adesArg. Although C3adesArg is inactive, C5adesArg retains about 1% of the anaphylactic activity of C5a.

STRUCTURE OF ANAPHYLATOXINS

Anaphylatoxins purified from many vertebrate species are 74–77 aa residue peptides, highly cationic (with pIs of 8.6–9.6), and characteristically contain six cysteine residues.[21,25,30] The primary sequences of human C3a (77 aa) and C5a (74 aa) are shown in Fig. 3A. Elucidation of the tertiary structures of C3a and C5a by X-ray crystallography and nuclear magnetic resonance spectroscopy has revealed that both C3a and C5a adopt a compact globular folding, which consists of four antiparallel α-helices connected by three peptide loops,[27,35] as illustrated by the structure of human C5a (Fig. 3B). The globular core of four antiparallel α-helices is stabilized by three intrachain disulfide bonds, whereas the C-terminal tail is relatively disordered and flexible (Fig. 3B).

For both C3a and C5a, the C-terminal tail, particularly the C-terminal pentapeptide (LGLAR for C3a and MQLGR for C5a), plays an important role in mediating both binding and activation of their receptors.[16,20,21,24,25,35] However, other regions of surface of the globular core, such as the first and second loops, also participate in receptor binding.[8,16,20,24,27,35]

(A)

C3a SVQLTEKRMDKVGKYPKELRKCCEDGM
RENPMRFSCQRRTRFISLGEACKKVFL
DCCNYITELRRQHARASHLGLAR

C5a TLQKKIEEIAAKYKHSVVKKCCYDGAC
VNNDETCEQRAARISLGPRCIKAFTEC
CVVASQLRANISHKDMQLGR

(B)

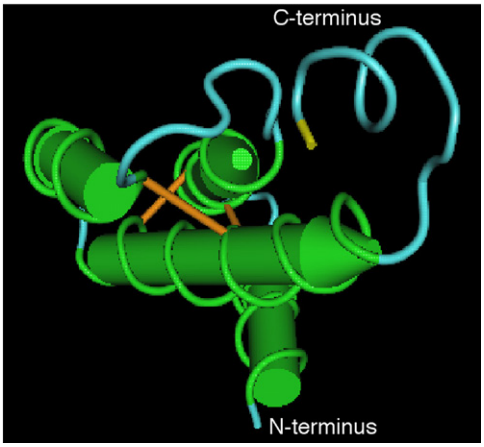

FIGURE 3 Structure of anaphylatoxins. (A) Primary sequences of human C3a (NCBI accession no. = P01024) and C5a (NCBI accession no. = P01032). (B) The tertiary structure of human C5a. Green, α-helices; blue, N-terminus, the three loops connecting the α-helices, and the C-terminal tail; brown, disulfide bonds; yellow, C-terminal arginine residue. See color plate 22.

RECEPTORS FOR ANAPHYLATOXINS

C3a and C5a exert their biological actions by binding to their receptors. To date, one C3a receptor (C3aR) and two C5a receptor (C5aR, also known as CD88, and C5L2) have been cloned and characterized.[3,5,6,9,15] As illustrated by Fig. 4, all three receptors belong to the family of G-protein-coupled receptors (GPCRs), which share a common structural motif of seven transmembrane domains joined together by three extracellular and three intracellular hydrophilic loops. These receptors also have an extracellular N-terminal portion that contains an N-glycosylation site, and an intracellular C-terminal tail that harbors multiple Ser/Thr phosphorylation sites. C3aR contains a large second extracellular loop.

C3aR is expressed by many tissues including those of the central nervous system and by myeloid leukocytes such as neutrophils, eosinophils, basophils, mast cells, and monocytes/macrophages.[3,9,21,25,30] C5aR is expressed by many leukocytes such as neutrophils, eosinophils, basophils, mast cells, monocytes/macrophages, dendritic cells, and certain subset of lymphocytes, as well as nonleukocytes such as endothelial, epithelial, glial, and blood vessel smooth muscle cells.[5,15,20,21,25,30,34]

The N-terminal part, the second extracellular loops, and some residues of the transmembrane domains of the receptors

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```
  1 ---------- ------MASF SAETNSTDLL SQPWNEPPVI LSMVILSLTF LLGLPGNGLV LWVAGLKMQR TVNTIWFLHL TLADLLCCLS  hC3aR
  1 --MNSFNYTT PDYGHYDDKD TLDLNTPVDK TSNTLRVPDI LALVIFAVVF LVGVLGNALV VWVTAFEAKR TINAIWFLNL AVADFLSCLA  hC5aR
  1 MGNDSVSYEY GDYSDLSDRP VDCLDGAC-L AIDPLRVA-- -PLPLYAAIF LVGVPGNAMV AWVAGKVARR RVGATWLLHL AVADLLCCLS  hC5L2
                                                I                                   II

 75 LPFSLAHLAL QGQWPYGRFL CKLIPSIIVL NMFASVFLLT AISLDRCLVV FKPIW-CQNH RNVGMACSIC GCIWVVAFVM CIPVFVYREI  hC3aR
 89 LPILFTSIVQ HHHWPFGGAA CSILPSLILL NMYASILLLA TISADRFLLV FKPIW-CQNF RGAGLAWIAC AVAWGLALLL TIPSFLYRVV  hC5aR
 87 LPILAVPIAR GGHWPYGAVG CRALPSIILL TMYASVLLLA ALSADLCFLA LGPAWWSTVQ RACGVQ-VAC GAAWTLALLL TVPSAIYRRL  hC5L2
       II                                III                                                IV

       EMNDRLDPSSFQTNDHPWDYTVPTVFQPQTFQRPSADSLPRGSARLTSQNLYSNVFKPADVVSPKIPSGFPIED
       GPPQVINELSRNELPDGYFVLPTPVQDDDTFQGLNYYDQFGQPLSEYFSNSSASPFLKLHTSLFADSNDLPSTEH
164 FTTDNHNRCG YKFGLSSSLD YPDAITITRL VVGFLLPSVI MIACYSFIVF RMQRGRFAKS QSKTFRVAVV VVAVFLVCWT PYHIFGVLSL  hC3aR
178 REEYFPPKVL CGVDYSHDKR RERAVAIVRL VLGFLWPLLT LTICYTFILL RTWSRRATRS -TKTLKVVVA VVASFFIFWL PYQVTGIMMS  hC5aR
176 HQEHFPARLQ CVVDYGGSSS TENAVTAIRF LFGFLGPLVA VASCHSALL- -CWAARRCRP ------LGTA IVVGFFVCWA PYHLLGLVLT  hC5L2
                                  V                                                   VI

402 LTDPETPLGK TLMSWDHVCI ALASANSCFN PFLYALLGKD FRKKARQSIQ GI----LEAA FSEELTRSTH CPSNNVISER NSTTV      hC3aR
267 FLEPSSPTFL LLNKLDSLCV SFAYINCCIN PIIYVVAGQG FQGRLRKSLP SL----LRNV LTEESVVRES KSFTRSTVDT MAQKTQAV   hC5aR
258 VAAPNSALLA RALRAEPLIV GLALAHSCLN PMLFLYFGRA ---QLRRSLP AACHWALRES QGQDESVDSK KSTSHDLVSE MEV        hC5L2
    VI                        VII
```

FIGURE 4 Sequence alignment of human C3aR (NCBI accession no. = Q16581), C5aR (NCBI accession no. = P21730), and C5L2 (NCBI accession no. = NP060955). The seven transmembrane domains (I–VII) are underlined. Residues involved in ligand-induced receptor activation in C5aR and conserved in C3aR and C5L2 are boxed. The "DRY" motif residues are bold-faced. The intracellular loop 3 Ser/Thr-containing motif in C3aR and C5aR is both boxed and bold-faced. The extra residues of large extracellular loop of C3aR are shown as a budding out loop for appropriate alignment.

contribute to the binding of their respective ligands.[6,8,20,30] Binding of ligands to C3aR and C5aR, in particular through interacting with the boxed residues (Fig. 4), triggers the conformational change of the receptor, which leads to the activation of heterotrimeric G proteins. Subsequently, multiple intracellular kinases (e.g., PLCs, PI-3Ks, PKC, PKB, MAPKs) and small G proteins (e.g., Ras, Rac, Rho, Cdc42) are activated, which ultimately lead to diverse biological actions. Although it can bind with both C5a and C5adesArg with high affinity, C5L2 does not elicit the biological actions that C5aR does,[6,14] raising the possibility that C5L2 may act as a decoy receptor for C5a.

BIOLOGICAL ACTIONS OF ANAPHYLATOXINS

Binding and activation of C3aR and C5aR by their respective ligands have diverse effects on various target cells, which play important roles in inflammation and immunity.

Anaphylatoxic Effect

C3a and C5a can cause smooth muscle contraction, increase in capillary permeability, vasodilation, and anaphylactic shock if systemically generated or applied. This anaphylatoxic effect is predominantly due to the activation of anaphylatoxin receptors on mast cells and basophils, which leads to their degranulation and release of histamine and other vasoactive or inflammatory mediators such as arachidonic acid metabolites (prostaglandins, leukotrienes, lipoxins), lipid mediators (e.g., platelet-activating factor), and the nucleoside adenosine.[12,21,25,30] Together with the direct action of anaphylatoxins on smooth muscle and vessel endothelial cells, these mediators elicit the constriction of bronchial, intestinal, and uterine smooth muscles, promote local vasodilatation, augment capillary permeability, and induce mucous secretion from bronchial, gastric, and intestinal epithelial cells.

Chemotactic Effect

Anaphylatoxins are potent chemoattractants and can induce the migration of leukocytes expressing their receptors.[8,12,15,20,21,25,30,34] C5a is more potent than C3a, and is actually one of the most potent peptide chemoattractants that are active at concentrations as low as 0.1 nM. Both C3a and C5a are chemotactic for granulocytes (neutrophils, eosinophils, and basophils), monocytes/macrophages, and mast cells. In addition, C5a is also chemotactic for dendritic cells (DCs), germinal center B cells, and T cells.[22,26,34] Thus, anaphalatoxins participate in the recruitment of various leukocytes into sites of inflammation during infection and tissue injury.

Phagocyte-activating Effect

Anaphylatoxins act as strong activators for neutrophils and monocytes/macrophages.[12,21,30] Activation of neutrophils by anaphylatoxins increases their adhesiveness, causes neutrophil aggregation, triggers the release of antimicrobial substances such as lysozyme, defensins, and cathelicidns, and remarkably stimulates neutrophil oxidative metabolism and the production of toxic reactive oxygen species. Activation of monocytes or macrophages by anaphylatoxins augments their cytotoxic activity and stimulates their production of inflammatory mediators such as cytokines and chemokines.

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Immunoregulatory Effect

Interaction of C3a and C5a with their receptors on antigen-presenting cells (APCs) such as macrophage or DC promotes APC recruitment, surface upregulation of MHC and costimulatory molecules, and production of inflammatory cytokines.[28,29,34] C3a and C5a modulate the activation of APCs induced by various Toll-like receptor ligands, and hence altering the subsequent polarization of T cells.[28] C3aR and C5aR expressed by T cells on interacting with C3a and C5a can provide a survival signal. Therefore, anaphylatoxins modulate both innate and adaptive immune responses.

PATHOPHYSIOLOGICAL IMPLICATIONS OF ANAPHYLATOXINS

Based on their multiple biological actions, it is clear that the physiological function of anaphylatoxins is participation in immune defense and inflammation of the host.[21,25,28,30] During infection, anaphylatoxins contribute to the establishment of inflammatory responses with the ultimate goal of elimination of invading pathogens by facilitating bacterial killing or the induction of adaptive immunity. Therefore, it is not unexpected that individuals genetically deficient in either C3 or C5 display severe recurrent bacterial infection, likely due to the lack of both terminal membrane attack complex and anaphylatoxins. The critical role of C5a in host antimicrobial immune defense and inflammation has been clearly demonstrated by the use of C5aR knockout mice that, in comparison with wild-type mice, exhibit elevated susceptibility to mucosal bacterial challenge and an impaired inflammatory response in experimental reverse Arthus reactions.[18,19]

Anaphylatoxins are associated with nearly all types of inflammation.[14,21,25,30,31] Their inflammatory effects, particularly when excessive, are harmful and play a role in the pathogenesis of many diseases such as anaphylaxis, adult respiratory distress syndrome, acute transplant ejection, ischemia reperfusion, sepsis, brain inflammation, and many autoimmune diseases such as psoriasis, rheumatoid arthritis, and systemic lupus erythematosus.[30,31] Blockade of anaphylatoxins by neutralizing antibody or antagonists ameliorates the pathogenesis and symptoms of these diseases in experimental animal models. Therefore, great effort has been made to develop effective antagonists for anaphylatoxins.[1,31] Thus, anapylatoxic peptides have moved from gross observation to the understanding of their genomic complexity, regulation at the molecular level of their expression and action, as well as roles in inflammation and immunity in the twentieth century, and finally culminating in the twenty-first century with the development of molecularly targeted antagonists for the potential treatment of various inflammatory diseases.

ACKNOWLEDGMENTS

This publication has been funded in whole or in part with federal funds from the National Cancer Institute, National Institute of Health under HHSN261200800001E.

REFERENCES

1. Allegretti M, Moriconi A, Beccari AR, Di Bitondo R, Bizzarri C, Bertini R, et al. Targeting C5a: recent advances in drug discovery. *Curr Med Chem* 2005;**12**:217–36.
2. Alper CA, Johnson AM, Birtch AG, Moore FD. Human C′3: evidence for the liver as the primary site of synthesis. *Science* 1969;**163**:286–8.
3. Ames RS, Li Y, Sarau HM, Nuthulaganti P, Foley JJ, Ellis C, et al. Molecular cloning and characterization of the human anaphylatoxin C3a receptor. *J Biol Chem* 1996;**271**:20,231–4.
4. Bordet J, Gengou O. Sur l'existence de substances sensibilisatrices dans la plupart des serum antimicrobiens. *Ann Inst Pasteur (Paris)* 1901;**15**:289–302.
5. Boulay F, Mery L, Tardif M, Brouchon L, Vignais P. Expression cloning of a receptor for C5a anaphylatoxin on differentiated HL-60 cells. *Biochemistry* 1991;**30**:2993–9.
6. Cain SA, Monk PN. The orphan receptor C5L2 has high affinity binding sites for complement fragments C5a and C5a des-Arg(74). *J Biol Chem* 2002;**277**:7165–9.
7. Carney DF, Haviland DL, Noack D, Wetsel RA, Vik DP, Tack BF. Structural aspects of the human C5 gene: intron/exon organization, 5′-flanking region features, and characterization of two truncated cDNA clones. *J Biol Chem* 1991;**266**:18,786–91.
8. Chao TH, Ember JA, Wang M, Bayon Y, Hugli TE, Ye RD. Role of the second extracellular loop of human C3a receptor in agonist binding and receptor function. *J Biol Chem* 1999;**274**:9721–8.
9. Crass T, Raffetseder U, Martin U, Grove M, Klos A, Kohl J, et al. Expression cloning of the human C3a anaphylatoxin receptor (C3aR) from differentiated U-937 cells. *Eur J Immunol* 1996;**26**:1944–50.
10. de Bruijn MH, Fey GH. Human complement component C3: cDNA coding sequence and derived primary structure. *Proc Natl Acad Sci USA* 1985;**82**:708–12.
11. Fong KY, Botto M, Walport MJ, So AK. Genomic organization of human complement component C3. *Genomics* 1990;**7**:579–86.
12. Frank MM. Complememt and kinin. In: Stites DP, Terr AI, Parslow TG, editors. *Medical immunology*. 9th ed. Stamford: Appleton & Lange; 1997. p. 169–81.
13. Friedberger E. Weitere Untersuchungen uber Eiweissanaphylaxie. *Z Immunforsch Exp Ther* 1910;**4**:636–89.
14. Gao H, Neff TA, Guo RF, Speyer CL, Sarma JV, Tomlins S, et al. Evidence for a functional role of the second C5a receptor C5L2. *Faseb J* 2005;**19**:1003–5.
15. Gerard NP, Gerard C. The chemotactic receptor for human C5a anaphylatoxin. *Nature* 1991;**349**:614–7.
16. Gerardy-Schahn R, Ambrosius D, Saunders D, Casaretto M, Mittler C, Karwarth G, et al. Characterization of C3a receptor-proteins on guinea pig platelets and human polymorphonuclear leukocytes. *Eur J Immunol* 1989;**19**:1095–102.
17. Haviland DL, Haviland JC, Fleischer DT, Hunt A, Wetsel RA. Complete cDNA sequence of human complement pro-C5: evidence of truncated transcripts derived from a single copy gene. *J Immunol* 1991;**146**:362–8.

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18. Hopken UE, Lu B, Gerard NP, Gerard C. The C5a chemoattractant receptor mediates mucosal defence to infection. *Nature* 1996;**383**:86–9.
19. Hopken UE, Lu B, Gerard NP, Gerard C. Impaired inflammatory responses in the reverse Arthus reaction through genetic deletion of the C5a receptor. *J Exp Med* 1997;**186**:749–56.
20. Huber-Lang MS, Sarma JV, McGuire SR, Lu KT, Padgaonkar VA, Younkin EM, et al. Structure-function relationships of human C5a and C5aR. *J Immunol* 2003;**170**:6115–24.
21. Hugli TE. Biochemistry and biology of anaphylatoxins. *Complement* 1986;**3**:111–27.
22. Kupp LI, Kosco MH, Schenkein HA, Tew JG. Chemotaxis of germinal center B cells in response to C5a. *Eur J Immunol* 1991;**21**:2697–701.
23. Lienenklaus S, Ames RS, Tornetta MA, Sarau HM, Foley JJ, Crass T, et al. Human anaphylatoxin C4a is a potent agonist of the guinea pig but not the human C3a receptor. *J Immunol* 1998;**161**:2089–93.
24. Lu ZX, Fok KF, Erickson BW, Hugli TE. Conformational analysis of COOH-terminal segments of human C3a: evidence of ordered conformation in an active 21-residue peptide. *J Biol Chem* 1984; **259**:7367–70.
25. Muller-Eberhard HJ. Molecular organization and function of the complement system. *Annu Rev Biochem* 1988;**57**:321–47.
26. Nataf S, Davoust N, Ames RS, Barnum SR. Human T cells express the C5a receptor and are chemoattracted to C5a. *J Immunol* 1999; **162**:4018–23.
27. Nettesheim DG, Edalji RP, Mollison KW, Greer J, Zuiderweg ER. Secondary structure of complement component C3a anaphylatoxin in solution as determined by NMR spectroscopy: differences between crystal and solution conformations. *Proc Natl Acad Sci USA* 1988; **85**:5036–40.
28. Ricklin D, Hajishengallis G, Yang K, Lambris JD. Complement: a key system for immune surveillance and homeostasis. *Nat Immunol* 2010;**11**:785–97.
29. Strainic MG, Liu J, Huang D, An F, Lalli PN, Muqim N, et al. Locally produced complement fragments C5a and C3a provide both costimulatory and survival signals to naive CD4+ T cells. *Immunity* 2008;**28**:425–35.
30. Volanakis JE, Frank MM, editors. *Human complement system in health and disease*. New York: Marcel Dekker; 1998.
31. Ward PA. The dark side of C5a in sepsis. *Nat Rev Immunol* 2004; **4**:133–42.
32. Wetsel RA, Lemons RS, Le Beau MM, Barnum SR, Noack D, Tack BF. Molecular analysis of human complement component C5: localization of the structural gene to chromosome 9. *Biochemistry* 1988;**27**:1474–82.
33. Whitehead AS, Solomon E, Chambers S, Bodmer WF, Povey S, Fey G. Assignment of the structural gene for the third component of human complement to chromosome 19. *Proc Natl Acad Sci USA* 1982;**79**:5021–5.
34. Yang D, Chen Q, Stoll S, Chen X, Howard OM, Oppenheim JJ. Differential regulation of responsiveness to fMLP and C5a upon dendritic cell maturation: correlation with receptor expression. *J Immunol* 2000;**165**:2694–702.
35. Zuiderweg ER, Nettesheim DG, Mollison KW, Carter GW. Tertiary structure of human complement component C5a in solution from nuclear magnetic resonance data. *Biochemistry* 1989;**28**:172–85.

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Chapter 86

Annexin-A1 Peptide Ac2–26

M Perretti, F D'Acquisto and R.J. Flower

ABSTRACT

Annexin A1 *N*-Acetyl 2–26 (m.w. 3089.5 kDa; empirical formula $C_{141}H_{210}N_{32}O_{44}S$, hereafter referred to as Acetyl 2–26), is a peptide sequence derived from the N-terminus of the glucocorticoid-regulated protein Annexin (Anx) A1. Although it does not occur naturally as such, longer peptides containing this sequence are found in inflammatory exudates and other biological fluids. The significance of the peptide is that it seems to contain the major Anx-A1 pharmacophore and can reproduce many of the biological properties of the full-length protein. The peptide is available commercially and is commonly used in many experimental systems in its capacity as an Anx-A1 mimetic, albeit with less potency.

DISCOVERY

The discovery of the 37-kDa protein Annexin A1 (Anx-A1; formerly macrocortin, Lipocortin 1) arose from investigations into the mechanism by which anti-inflammatory glucocorticoids suppressed eicosanoid synthesis in intact but not disrupted cells. In the authors' own laboratory, it was determined that glucocorticoids, acting on cells through a receptor-dependent mechanism, promoted the synthesis and release of a proteinaceous factor that, when partially purified, was found to duplicate the glucocorticoid-like activity in other untreated cells. Although originally observed in guinea-pig lung tissue, further sources were soon discovered. The unknown protein was found to possess anti-inflammatory properties in rodent models of acute inflammation. Similar factors (renomedullin, lipomodulin) were reported by other laboratories and were, in due course, found to be structurally or immunologically related.[13]

Anx-A1 itself was subsequently[32] purified to homogeneity from peritoneal lavage fluid obtained from glucocorticoid-treated rats,[23] cloned and sequenced. The mammalian native protein is acetylated after removal of the N-terminal Met residue.

A comparison of the Anx-A1 sequence with several other partially purified proteins led to the recognition that all these species formed part of a much larger family of proteins, subsequently dubbed the "Annexin super-family."[21] This embraces some thousand proteins distributed throughout most eukaryotic (but not prokaryotic) phyla although it seems to be largely absent from yeasts.

Family members are characterized by the presence of four (eight in the case of Anx-A6) homologous domains of approximately 70 residues (the "core") that usually harbor "type 2" calcium-binding sites but distinguished by a unique N-terminal region believed to confer individual properties upon each member of the family. These N-termini are of variable length with the shortest being that of Anx-A5 (six amino acids) and the longest, Anx-A7 (100 amino acids).

The early history of the discovery of Anx-A1 has been documented,[13] and there are several reviews relating to its action as an anti-inflammatory agent,[10,26] as a mediator of glucocorticoid feedback in the HPA axis[3] and as a regulator of other cell functions.[15] The detailed information in these reviews will not be reiterated here, but Table 1 shows the main actions of Anx-A1 as established by experiments using transgenic mice, neutralizing anti-Anx-A1 antibodies and antisense agents.

SCOPE OF THIS CHAPTER

This review restricts itself to a discussion of the usage of Acetyl 2–26 as a pharmacologically active agent. Other peptides based on the Anx-A1 structure have been used and some have been found to have anti-inflammatory or other biologically active properties. These include Anx-A1 1–188 (which includes the Acetyl 2–26 sequence) and peptides from some other regions of the protein including the antiflammins such as AF2, which is derived (His^{246}-Glu^{254}) from the core domain of Anx-A1.[2] These will not be discussed further in this chapter.

DISCOVERY OF IMPORTANCE OF THE N-TERMINAL PEPTIDE

The notion that the N-terminal domain was of particular significance to the biological activity of Anx-A1 originally arose from the work of Cirino et al.[6] who observed that an N-terminal truncated form of native Anx-A1 (~33 kDa),

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00086-5
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TABLE 1 A Summary of Some Biological Actions of Native Anx-A1

System	Inhibits	Promotes
In vitro	• $cPLA_2$ activation • Eicosanoid production • Superoxide production • Phagocytosis • Th2 cell differentiation • Anterior pituitary hormone release • Cell (e.g. A549) growth and differentiation	• L-selectin shedding • PMN apoptosis • Efferocytosis • T-cell proliferation • TCR downstream signaling • Th1 cell differentiation
In vivo	• PMN trafficking • PMN adherence • Histamine release • Cytokine generation • Acute inflammation (some models) • Chronic inflammation (some models) • Hyperalgesia/ nociception • Fever (some pyrogens) • NMDA-induced neuronal damage	• T-cell dependent inflammatory responses

These were established by experiments using recombinant Anx-A1, Anx-A1 null mice, specific neutralizing antibodies, or antisense agents. The results are collected from a variety of sources as reviewed in Refs 3,10,13,26.

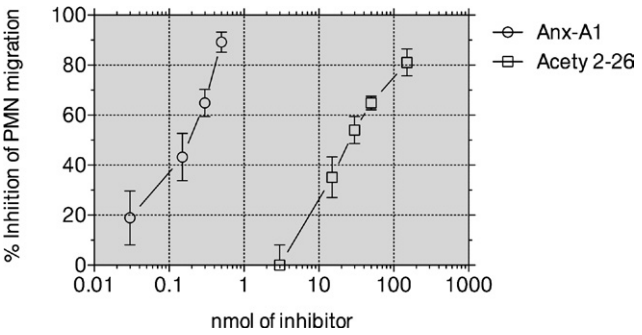

FIGURE 1 Anti-inflammatory action of acetyl 2–26 in comparison with the full-length Anx-A1 protein. The model used is a murine air pouch model in which neutrophil emigration is stimulated with IL-1β. Anx-A1, or its acetyl 2–26 N-terminal peptide are administered 15 min before the cytokine and the neutrophil emigration in the pouch counted 4 h later. The ED_{50} doses are approximately 5.2 and 88 μg per mouse, respectively. In this experiment, a longer N-terminal peptide (1–188) seemed to have an intermediate (ED_{50} 38 μg per mouse) potency although a full dose-response curve could not be established. Acetyl 2–26 has a potency of some 200-fold less than the native protein although it evidently has the same efficacy. From Perretti et al.[24]

prepared by plasmin cleavage of the native protein, was biologically inactive while a synthetic peptide corresponding to the first 26 amino acids retained activity in three different models of acute rodent inflammation, although it was at least an order of magnitude less active. In a related article,[24] the same group showed using a murine air pouch technique that Acetyl 2–26 shows a similar efficacy to the full-length recombinant protein in murine peritonitis, neutrophil migration, and skin inflammation models. The authors observed that the efficacy of the peptide was similar that of the native protein but that it was some 200-fold less potent (Fig. 1).

The conclusive demonstration that this fragment was responsible for much of the observed biological actions of Anx-A1 came from a study using a chimeric protein. Anx-A5, another member of the annexin family with a very short N-terminus, lacks the biological activity associated with Anx-A1, but a fusion protein comprising the Anx-A5 "core" domain and the Anx-A1 N-terminus exhibited very similar biological activity to the intact Anx-A1 protein itself.[18]

THE STRUCTURE OF ANX-A1 AND ACETYL 2–26

Anx-A1 itself is a monomeric amphipathic protein comprising 346 amino acids. The crystal structure has been determined at the 1.8 Å level[29] and reveals the protein to exist in a lenticular configuration with a central pore. The N-terminus (44 amino acids) is folded into the core domain on the concave surface, but can "flip" out in the presence of calcium. On the convex aspect of the protein, six calcium-binding sites may mediate the binding to negatively charged phospholipids in a pH-dependent fashion.

The peptide itself has a complex predicted structure (Table 2) in aqueous solution with a strong tendency to form a helical structure in residues Ala^2-Ile^{14} a potential "turn" in the structure at residues Glu^{15}-Glu^{18} and a propensity to form a sheet structure from residues Gln^{19}-Thr^{24}. The majority of the peptide consists of hydrophobic sequences interspersed with a strongly hydrophilic region at residues Asn^{16}-Tyr^{21}. It is likely that the actual structure adopted by the peptide in biological solutions will depend on the presence of lipids such as phosphatidyl serine.

Biochemistry of the Anx-A1 N-Terminus Domain

The full-length N-terminal domain contains several Ser, Thr, His, and Tyr phosphorylation sites. Several protein kinases including PKC and the EGF and other receptor tyrosine kinases have been implicated in the widespread occurrence of phosphorylated species in biological fluids

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TABLE 2 Physicochemical Properties of Acetyl 2–26

	2	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*	*17*	*18*	*19*	*20*	*21*	*22*	*23*	*24*	*25*	*26*
Acetyl 2-26	**A**	**M**	**V**	**S**	**E**	**F**	**L**	**K**	**Q**	**A**	**W**	**F**	**I**	**E**	**N**	**E**	**E**	**Q**	**E**	**Y**	**V**	**Q**	**T**	**V**	**K**
Helix	H	H	H	H	H	H	H	H	H	H	H	H	H	h	h	h	h	h	h	h	h	h	H		
Sheet	s	s	s	s	s	s	s	s	s	s	s	S						s	S	S	S	S	S	s	
Turn									t					T	T	T	T								
Coil																									C
Hydrophilic						1	4	2							8	12	20	11	9	4					2
Hydrophobic			2	4	6				8	8	8	8	2								1	4	4		

The propensity of the Acetyl 2–26 to form helices, sheets, turns, or coils in aqueous solution is shown. These parameters were calculated using conventional algorithms. The upper case and lower case letters indicate the approximate likelihood of these structures occurring. The relative hydrophilicity/-phobicity of the sequence is represented numerically on a scale of 1–20 where 1 represents the least, and 20 the most hydrophobic/-philic residues. From Croxtall et al.[8]

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and cell extracts. Acetyl 2–26 itself has many residues that are putative phosphorylation targets (Ser^3, Thr^{24}, and Tyr^{21}).

Consensus sequences for glycosylation (Asn^{43}-Ser^{45}) and transglutamination (Glu^{19}, Gln^{23}) are also found within the N-terminal domain. Both glycosylated and transglutaminase-linked species have been reported in the placenta and elsewhere, although these species are not apparently widespread.

The N-terminus also contains several proteolytic motifs. An N-terminal truncated moiety is commonly encountered in inflammatory fluids probably as a result of cleavage at Lys^{26}-Lys^{29}, or Val^{36} by serine proteases such as neutrophil elastase or PR3.[10] Proteolysis in the N-terminal domain profoundly modifies the physical properties of the protein and is probably associated with the inactivation given the dramatic reduction in potency of Acetyl 2–26 when compared with that of the native protein.

The N-terminal domain of Anx-A1 is important for another reason. Certain annexins, including Anx-A1, can form heterotetramers with members of the S100 family of EF-hand calcium-binding proteins. It is believed that the Anx-A1 1–12 sequence is the region involved in binding to these proteins.[21]

The significance of these observations is that Acetyl 2–26 could have secondary actions by blocking one or more of these interactions in addition to its chief, receptor mediated, effect (see below).

RECEPTORS FOR ANX-A1 AND N-ACETYL 2–26

Native Anx-A1 and its bioactive N-terminal peptides exert extracellular actions on cells to mimic some of the effects of glucocorticoids both *in vivo* and *in vitro*. It was therefore a reasonable assumption that there was a cell surface receptor for the proteins.

The first experimental evidence for such a receptor arose from the observation that there were discrete, saturable binding sites for human recombinant Anx-A1 on the surface of human peripheral blood monocytes and neutrophils,[10] which disappeared during the inflammatory response and which coprecipitated with Anx-A1 from membrane extracts. Receptor-like proteins were later described in the endocrine pituitary cells.[5]

A key article from Walther et al.[33] led the field in a different direction by implicating the formylated peptide (f-Met-Leu-Phe; FMLP) receptor (FPR) in the transduction of the Anx-A1 signal in leukocytes. FPR is a member of a family of G-protein-coupled receptors expressed in migratory cells and many other tissues. In humans, there are three known species: FPR itself (now known as FPR1), FPRL-1 (FPR-like-1, also termed FPR2 or ALXR) and FPRL-2 (now known as FPR3). Although FPR1 probably mediates the proinflammatory effects of the bacterial tri-peptide FMLP, FPR2/ALX is more promiscuous binding lipoxin A_4, an anti-inflammatory mediator generated from arachidonic acid, as well as serum amyloid A, a liver-derived acute phase protein.[4]

Anx-A1 and the peptide Acetyl 2–26 induce calcium transients in human neutrophils suggesting that their effects are mediated through FPR1 itself.[33] However, unlike FMLP, Anx-A1-related peptides do not stimulate superoxide generation at concentrations that provoke calcium fluxes and L-selectin shedding, and FMLP does not compete with Anx-A1 binding in cells transfected with the human FPR1.

"Boc" antagonists (Boc-Met-Leu-Phe and Boc-Phe-D-Leu-Phe-D-Leu-Phe) of the FPR family of receptors inhibit Anx-A1-induced calcium fluxes and L-selectin shedding by PMN and also prevent the Acetyl 2–26 induced inhibition of leucocyte adhesion and emigration in murine inflammatory models and elsewhere.[17] However, these antagonists lack specificity between members of the FPR family making it difficult to determine which subtype is being used. Acetyl 2–26 also retains anti-inflammatory activity in FPR1 null mice[14] suggesting that although the FPR family mediates some Anx-A1 effects the receptor responsible is unlikely to be FPR1 itself.

Of the three products of the FPR gene cluster in humans, only FPR2/ALXR binds the anti-inflammatory lipoxin A_4. This binding can be displaced by SAA, and high concentrations of FMLP or the synthetic peptide MMK1.[4] Both Anx-A1 and Acetyl 2–26 also bind FPR2/ALX in human PMN and compete specifically with lipoxin A_4. Endogenous Anx-A1 and FPR2 co-immunoprecipitate from human and murine PMN.[25] Like lipoxin A_4, Acetyl 2–26 retains the ability to promote the detachment of adherent leucocytes in FPR1 null mice, again suggesting that the receptor for Anx-A1 is not FPR1 but a further subtype.[14]

In the mouse, the fpr gene cluster has undergone differential expansion and six genes have been identified.[11] Two are not expressed, and another is found only in the skeletal muscle. The remaining three genes (now termed Fpr1, Fpr2, and Fpr3) are expressed in leucocytes, spleen and lung and are therefore potential candidate murine receptors for Anx-A1. In mouse, however, the functions of human FPR2/ALX seem to be split between Fpr2 and Fpr3. It is probable that the mRNA for Fpr2 and Fpr3 is formed by differential transcription of the same gene.

Dufton et al.[11] generated an Fpr2/3 null mouse strain and used this to assess the role of these receptors in the action of Anx-A1 and Acetyl 2–26. These transgenic mice fail to respond to the N-terminal peptide Acetyl 2–26 and other agonists at these receptors and exhibit an enhanced inflammatory response when compared with that in WT control animals. These results are reminiscent of the behavior of the Anx-A1 null mouse and taken, together with other results,[2]

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point to the existence of an anti-inflammatory loop centered upon this receptor.

BIOLOGICAL PROPERTIES OF THE ACETYL-26 PEPTIDE

After the discovery that the biological activity of Anx-A1 could be reproduced by *N*-acetyl 2–26, and by some shorter peptides, it has become a common practice to use these instead of the full-length recombinant molecule which, despite its greater potency, exhibits more instability in use and can prove difficult to prepare in a correctly folded and endotoxin-free manner. Acetyl 2–26 does exhibit a slightly different affinity profile for the FPR family of receptors but nevertheless possesses very similar anti-inflammatory properties to the full-length molecule.[2] Tables 3 and 4 give an idea of the different systems in which this peptide has been tested and found to be effective.

TABLE 3 Some *In Vitro* Systems in Which Acetyl 2–26 Has Biological Properties

Year	Species	System	Concentration	Comments	Refs
1995	Rat	Release of prolactin from the anterior pituitary gland.	3 pM–3 nM	Inhibition of prolactin release in a stimulus dependent fashion.	Taylor et al.[31]
2004	Human	Monocyte activation and chemotaxis.	50 μM	Activation of chemotaxis through FPR receptors.	Ernst et al.[12]
2005	Human	Phagocytosis of neutrophils by monocyte-derived macrophages.	32 μM	Stimulated phagocytosis through an FPR-dependent mechanism.	Maderna et al.[20]
2005	Rat	Ischemic damage to cardiac myocytes.	0.3 μM	Cardio-protective effect through an FPR-mediated mechanism.	Ritchie et al.[28]
2006	Human	Neutrophil–endothelium interaction under flow conditions.	3–30 μM	Attenuated capture and rolling but not adhesion to endothelium.	Hayhoe et al.[16]
2007	Human	Growth of Hep-2 larynx cancer cells.	2 μM	Inhibition of cell growth.	Silistino-Souza et al.[30]
2010	Equine	Blood neutrophil superoxide production.	10–50 μM	Stimulation at higher concentration: inhibition at lower concentration. Through an FPR-dependent mechanism.	Pickles et al.[27]

TABLE 4 Some *In Vivo* Systems in Which Acetyl 2–26 Has Biological Activity

Year	Species	System	Dose	Comments	Refs
1993	Mouse	Models of inflammation: air pouch, skin edema and zymosan peritonitis	88–200 μg per mouse	Anti-inflammatory actions in all models.	Perretti et al.[24]
1997	Rat	Ischemia reperfusion injury of intestine	0.125–0.5 mg/kg	Reduced GI damage and cardiovascular disturbances.	Cuzzocrea et al.[9]
2001	Rat	Myocardial ischemia-perfusion injury	0.5–1.0 mg/kg	Cardioprotective in an FPR dependent fashion.	La et al.[17]
2005	Rat	Allergic response	50–200 μg topically	Inhibition of mast cell histamine release and cell accumulation.	Bandeira-Melo et al.[1]
2007	Rat	Spinal cord injury	5–20 μg topically	Neuroprotective	Liu et al.[19]
2011	Rat	Asthma	1.0 mg/kg	Inhibited eosinophil migration and the release of cytokines and PGD in lungs. Effect reversed by PGD administration.	Wang et al.[34]

Some *in vivo* systems in which Acetyl 2–26 has biological activity.

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In Vitro Systems

Table 3 shows a representative (though necessarily incomplete) selection of *in vitro* systems, drawn from the literature, in which Acetyl 2–26 has been found to possess biological activity. The examples illustrate the wide range of biological activities achieved by this peptide, which includes inhibition of hormone release from neuroendocrine tissues, cardioprotection, inhibition of cell growth, and PMN and monocyte activation. The peptide is active across a wide range of species including human, mouse, rat, and horse.

In general, the peptide displays FPR-mediated biological activity in the 1 μM range, although there are exceptions. Of importance is the finding by several groups of the concentration-dependency of the actions of the peptide. Several groups[27] have observed, for example, that high concentrations of the peptide can activate neutrophil superoxide production, whereas low concentrations can inhibit the response. This is most probably because Acetyl 2–26 (unlike full-length Anx-A1) is not specific for the FPR2/ALXR subtype and can also activate other FPR family receptors at concentrations of 10–100 μM.[12,16]

The question of which portion of the Acetyl 2–26 structure interacts with the FPR1 receptor to activate PMN NADPH oxidase has been addressed by Movitz et al.[22] with the suggestion that the sequence Acetyl Gln^{10}–Phe^{13} is the minimum sequence required to activate the receptor and to block the action of FMLP.

In Vivo Systems

Table 4 shows a selection of systems drawn from the literature, in which the peptide has been used *in vivo*, mainly as an anti-inflammatory agent. Of necessity, most work has been performed in rodents but the range of models used is wide, including allergic disorders, cardioprotection, and neuroprotection. Routes of administration, vary but the peptide is effective systemically in doses in the general range of 0.1–5.0 mg/kg and topically in the range 5–200 μg. The frequency of dosing varies, but most investigators have deployed a single administration given before the inflammatory stimulus.

STRUCTURE–ACTIVITY RELATIONSHIPS

The structure–activity relationships between different fragments of Acetyl 2–26 have been investigated by several authors, but there are some anomalies and gaps in the results that make definitive conclusions regarding the minimum active sequence, difficult at this time.

One problem has been that some of the peptides tested have been N-acetylated, while others have not. Additionally, it has not been established in every case whether or not the FPR receptor system is involved in the action of the peptide in all test systems. As explained above, it is conceivable that Acetyl 2–26 may have a secondary pharmacology through its ability to interfere with heterodimerization, phosphorylation, or proteolysis of the native Anx-A1 and such factors may account for some of the discrepancies.

Generally speaking, the results from *in vivo* models seem fairly straightforward. As Table 5 shows, not only Acetyl 2–26 but also the fragment Acetyl 2–12 is biologically active in models of reperfusion injury *in vivo* through an FPR mechanism, whereas the fragments Acetyl 2–6 and 9–25 are not. This would imply that the key sequence for achieving bioactivity *in vivo* is located somewhere in the region of Glu^{6}–Trp^{12}. This sequence is similar to the sequence proposed by Movitz et al.[22] to be the highly hydrophobic core sequence required to bind to the transmembrane binding pocket of FPR1, Gln^{10}–Phe^{13}. Although the FPR receptors are not identical, there is a high degree of homology between them, so this could be a significant region for interaction with all the FPR family.

In vitro however, the situation is more complex. Croxtall et al.[7,8] examined the activity of a range of peptides drawn from the N-terminal domain for their effect on A549 cell growth, proliferation, arachidonic acid, and eicosanoid release under either resting or EGF-stimulated conditions. Native Anx-A1 is highly active in this assay (EC_{50} ~50 nM) and (unacetylated) Met^{1}–Trp^{12} is inactive, whereas Phe^{13}–Val^{25} is fully active (although ~1000 less potent than the native protein) through an effect that depends on the inhibition of $cPLA_2$ activation. The role of Tyr^{21} seems crucial for the inhibition of cell growth as replacement with a Phe residue, or methoxylation, abolishes activity of the peptide. However, these peptides retain inhibitory activity when EGF proliferation is measured instead as does Met^{1}–Trp^{12}, which is inactive on cell growth per se.

When a range of 25 (nonacetylated) peptides derived from the sequence Phe^{13}–Val^{25} is systematically tested, the sequence common to all active peptides is Glu^{18}–Val^{22}, and the minimum sequence that is required for biological action is Glu^{18}–Val^{25}. This sequence contains important phosphorylation sites and is at least partially homologous to several other peptides such as middle T antigen, MT peptide and the amphibian anti-inflammatory peptide sauvagine all of which that have been shown to exert similar biological effects in some systems.

The most probable possibility is that the activity of these peptides in this particular system is not dependent on FPR receptor occupation but rather on some secondary interference with signal transduction systems by, for example, acting as decoy substrates for kinases. Tyr^{21} is a substrate for EGF receptor kinase and these sequences contain other

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TABLE 5 Structure–Activity Relationships

Peptide	N-	Sequence	Comments
1–44	Ac	A M V S E L F L K Q A W F I E N E E Q E Y V Q T V K S S K G G P G S A V S A V S P Y T F N P	Complete N-terminal sequence.
2–26	Ac	A M V S E L F L K Q A W F I E N E E Q E Y V Q T V K	Acetyl 2–26 peptide (Tables 3 and 4).
2–12	Ac	A M V S E L F L K Q A W	Bioactive *in vivo*[17]
2–6	Ac	A M V S E	Inactive *in vivo*[17]
13–25	-	F I E N E E Q E Y V Q T V	Active *in vitro* in some assays[7,8]
18–25	-	E Y V Q T V	Active *in vitro* in some assays[7,8]
21–33	-	Y V Q T V K S S K G G P G	Active *in vitro* in some assays[7,8]

TABLE 6 Homology of the Acetyl 2–26 Peptide among Common Laboratory Species

Species	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
Human	A	M	V	S	E	F	L	K	Q	A	W	F	I	E	N	E	E	Q	E	Y	V	Q	T	V	K
Mouse	A	M	V	S	E	F	L	K	Q	A	R	F	L	E	N	Q	E	Q	E	Y	V	Q	A	V	K
Rat	A	M	V	S	E	F	L	K	Q	A	C	Y	I	E	K	Q	E	Q	E	Y	V	Q	A	V	K
Dog	A	M	V	S	E	F	L	K	Q	A	W	F	I	E	N	E	E	Q	E	Y	I	E	T	V	K
Guinea pig	S	M	V	S	E	F	L	K	Q	A	Y	F	I	D	N	Q	E	Q	D	Y	V	K	T	V	K
Horse	S	M	V	S	A	F	L	K	Q	A	W	F	I	E	N	E	E	Q	E	Y	I	K	A	V	K

Key.

- Favored substitution
- Favored substitution in membrane proteins
- Unfavorable substitution

Sequence data were obtained from public databases. Substitutions were ascertained on the basis of the known occurrence in different types of proteins (http://www.russelllab.org/aas/).

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phosphorylation sites also (e.g. Thr^{24}). Because many of the peptide fragments used in these experiments are generally hydrophilic and have a tendency to form helical structures in solution, it may be the conformation of the peptide rather than its sequence that is crucial to the effects seen in this type of assay. Some kinases such as $PP60^{v\text{-}src}$ have a strong preference for substrates in this conformation.In summary, it is possible that the effects of short peptides in this system reflect a capacity to interfere with cell signaling downstream from G protein activation rather than the FPR-mediated effects that probably account for most of their biological actions.

The structure of the Acetyl 2–26 is highly conserved between humans and common laboratory species (Table 6) particularly in the region Ala^2–Ala^{11}. Only in the horse was there an "unfavorable" substitution for Glu at Ala^6. This does not affect the activity of Acetyl 2–26 in equine cells (Table 4). Elsewhere, the overall homology is very high with the overwhelming number of substitutions being "favorable" replacements.

SUMMARY

Acetyl 2–26 is a convenient and readily obtained peptide with which to explore the functionality of the Anx-A1—FPR2/ALX anti-inflammatory system *in vivo* and *in vitro* and there is a substantial literature on the subject. The main mode of action of its anti-inflammatory action is through the activation of FPR2/ALX receptors and in this respect the key sequence seems to be Ala^2–Trp^{12}. The peptide is not, however, specific for this receptor especially at higher concentrations. Activation of other FPR receptors may complicate interpretation of results if it is not used with care. Further, the peptide may have secondary pharmacology through its ability to interfere with signal transduction systems, at least under defined conditions *in vitro*.

REFERENCES

1. Bandeira-Melo C, Bonavita AG, Diaz BL, E Silva PM, Carvalho VF, Jose PJ, et al. A novel effect for annexin 1-derived peptide ac2–26: reduction of allergic inflammation in the rat. *J Pharmacol Exp Ther* 2005;**313**:1416–22.
2. Brancaleone V, Dalli J, Bena S, Flower RJ, Cirino G, Perretti M. Evidence for an anti-inflammatory loop centered on polymorphonuclear leukocyte formyl peptide receptor 2/Lipoxin A4 receptor and operative in the inflamed microvasculature. *J Immunol* 2011.
3. Buckingham JC, Solito E, John C, Tierney T, Taylor A, Flower R, et al. Annexin 1: a paracrine/juxtacrine mediator of glucorticoid action in the neuroendocrine system. *Cell Biochem Funct* 2003;**21**:217–21.
4. Chiang N, Fierro IM, Gronert K, Serhan CN. Activation of lipoxin A(4) receptors by aspirin-triggered lipoxins and select peptides evokes ligand-specific responses in inflammation. *J Exp Med* 2000;**191**:1197–208.
5. Christian HC, Taylor AD, Flower RJ, Morris JF, Buckingham JC. Characterization and localization of lipocortin 1-binding sites on rat anterior pituitary cells by fluorescence-activated cell analysis/sorting and electron microscopy. *Endocrinology* 1997;**138**:5341–51.
6. Cirino G, Cicala C, Sorrentino L, Ciliberto G, Arpaia G, Perretti M, et al. Anti-inflammatory actions of an N-terminal peptide from human lipocortin 1. *Br J Pharmacol* 1993;**108**:573–4.
7. Croxtall JD, Choudhury Q, Flower RJ. Inhibitory effect of peptides derived from the N-terminus of lipocortin 1 on arachidonic acid release and proliferation in the A549 cell line: identification of E-Q-E-Y-V as a crucial component. *Br J Pharmacol* 1998;**123**:975–83.
8. Croxtall JD, Waheed S, Choudhury Q, Anand R, Flower RJ. N-terminal peptide fragments of lipocortin-1 inhibit A549 cell growth and block EGF-induced stimulation of proliferation. *Int J Cancer* 1993;**54**:153–8.
9. Cuzzocrea S, Tailor A, Zingarelli B, Salzman AL, Flower RJ, Szabo C, et al. Lipocortin 1 protects against splanchnic artery occlusion and reperfusion injury by affecting neutrophil migration. *J Immunol* 1997;**159**:5089–97.
10. D'Acquisto F, Perretti M, Flower RJ. Annexin-A1: a pivotal regulator of the innate and adaptive immune systems. *Br J Pharmacol* 2008;**155**:152–69.
11. Dufton N, Hannon R, Brancaleone V, Dalli J, Patel HB, Gray M, et al. Anti-inflammatory role of the murine formyl-peptide receptor 2: ligand-specific effects on leukocyte responses and experimental inflammation. *J Immunol* 2010;**184**:2611–9.
12. Ernst S, Lange C, Wilbers A, Goebeler V, Gerke V, Rescher U. An annexin 1 N-terminal peptide activates leukocytes by triggering different members of the formyl peptide receptor family. *J Immunol* 2004;**172**:7669–76.
13. Flower RJ. Eleventh Gaddum memorial lecture. Lipocortin and the mechanism of action of the glucocorticoids. *Br J Pharmacol* 1988;**94**:987–1015.
14. Gavins FN, Yona S, Kamal AM, Flower RJ, Perretti M. Leukocyte antiadhesive actions of annexin 1: ALXR- and FPR-related anti-inflammatory mechanisms. *Blood* 2003;**101**:4140–7.
15. Gerke V, Moss SE. Annexins: from structure to function. *Physiol Rev* 2002;**82**:331–71.
16. Hayhoe RP, Kamal AM, Solito E, Flower RJ, Cooper D, Perretti M. Annexin 1 and its bioactive peptide inhibit neutrophil endothelium interactions under flow: indication of distinct receptor involvement. *Blood* 2006;**107**:2123–30.
17. La M, D'Amico M, Bandiera S, Di Filippo C, Oliani SM, Gavins FN, et al. Annexin 1 peptides protect against experimental myocardial ischemia-reperfusion: analysis of their mechanism of action. *FASEB J* 2001;**15**:2247–56.
18. Lim LH, Solito E, Russo-Marie F, Flower RJ, Perretti M. Promoting detachment of neutrophils adherent to murine postcapillary venules to control inflammation: effect of lipocortin 1. *Proc Natl Acad Sci U S A* 1998;**95**:14535–9.
19. Liu NK, Zhang YP, Han S, Pei J, Xu LY, Lu PH, et al. Annexin A1 reduces inflammatory reaction and tissue damage through inhibition of phospholipase A2 activation in adult rats following spinal cord injury. *J Neuropathol Exp Neurol* 2007;**66**:932–43.
20. Maderna P, Yona S, Perretti M, Godson C. Modulation of phagocytosis of apoptotic neutrophils by supernatant from dexamethasone-treated macrophages and annexin-derived peptide Ac(2–26). *J Immunol* 2005;**174**:3727–33.

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21. Moss SE, Morgan RO. The annexins. *Genome Biol* 2004;**5**:219.
22. Movitz C, Brive L, Hellstrand K, Rabiet MJ, Dahlgren C. The annexin I sequence gln(9)-ala(10)-trp(11)-phe(12) is a core structure for interaction with the formyl peptide receptor 1. *J Biol Chem* 2010;**285**:14338–45.
23. Pepinsky RB, Sinclair LK, Browning JL, Mattaliano RJ, Smart JE, Chow EP, et al. Purification and partial sequence analysis of a 37-kDa protein that inhibits phospholipase A2 activity from rat peritoneal exudates. *J Biol Chem* 1986;**261**:4239–46.
24. Perretti M, Ahluwalia A, Harris JG, Goulding NJ, Flower RJ. Lipocortin-1 fragments inhibit neutrophil accumulation and neutrophil-dependent edema in the mouse. A qualitative comparison with an anti-CD11b monoclonal antibody. *J Immunol* 1993;**151**:4306–14.
25. Perretti M, Chiang N, La M, Fierro IM, Marullo S, Getting SJ, et al. Endogenous lipid- and peptide-derived anti-inflammatory pathways generated with glucocorticoid and aspirin treatment activate the lipoxin A4 receptor. *Nat Med* 2002;**8**:1296–302.
26. Perretti M, D'Acquisto F. Annexin A1 and glucocorticoids as effectors of the resolution of inflammation. *Nat Rev Immunol* 2009;**9**:62–70.
27. Pickles KJ, Brooks AC, Rickards KJ, Cunningham FM. Expression of annexin-1 in equine leucocytes and the effects of the N-terminal annexin-1 peptide, Ac2–26, on equine neutrophil superoxide production. *Vet Immunol Immunopathol* 2010;**135**:226–33.
28. Ritchie RH, Sun X, Bilszta JL, Gulluyan LM, Dusting GJ. Cardioprotective actions of an N-terminal fragment of annexin-1 in rat myocardium in vitro. *Eur J Pharmacol* 2003;**461**:171–9.
29. Rosengarth A, Gerke V, Luecke H. X-ray structure of full-length annexin 1 and implications for membrane aggregation. *J Mol Biol* 2001;**306**:489–98.
30. Silistino-Souza R, Rodrigues-Lisoni FC, Cury PM, Maniglia JV, Raposo LS, Tajara EH, et al. Annexin 1: differential expression in tumor and mast cells in human larynx cancer. *Int J Cancer* 2007;**120**:2582–9.
31. Taylor AD, Cowell AM, Flower RJ, Buckingham JC. Dexamethasone suppresses the release of prolactin from the rat anterior pituitary gland by lipocortin 1 dependent and independent mechanisms. *Neuroendocrinology* 1995;**62**:530–42.
32. Wallner BP, Mattaliano RJ, Hession C, Cate RL, Tizard R, Sinclair LK, et al. Cloning and expression of human lipocortin, a phospholipase A2 inhibitor with potential anti-inflammatory activity. *Nature* 1986; **320**:77–81.
33. Walther A, Riehemann K, Gerke V. A novel ligand of the formyl peptide receptor: annexin I regulates neutrophil extravasation by interacting with the FPR. *Mol Cell* 2000;**5**:831–40.
34. Wang LM, Li WH, Xu YC, Wei Q, Zhao H, Jiang XF. Annexin 1-derived peptide Ac2–26 inhibits eosinophil recruitment in vivo via decreasing prostaglandin D. *Int Arch Allergy Immunol* 2011;**154**:137–48.

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Chapter 87

Immunoregulatory Neuropeptides

Mario Delgado

ABSTRACT

The immune system is faced with the daunting job of defending the organism against invading pathogens, while at the same time maintaining tolerance to the body's own tissues, thereby preserving its integrity. Multiple mechanisms act in concert to ensure self-tolerance. Loss of immune tolerance compromises immune homeostasis and results in the onset of autoimmune disorders. Identification of the endogenous factors that control antigen-specific tolerance and inflammation is a key goal in the field of immunology. In the last decade, various neuropeptides that are produced by immune cells with potent anti-inflammatory actions have been found to participate in the maintenance of immune tolerance at multiple levels, especially in immunological disorders. Here, I review the most recent advances in this field and highlight the therapeutic potential of using neuropeptides in a wide range of inflammatory/autoimmune situations.

INTRODUCTION

The immune system is a protective device perfectly designed to recognize and respond to a wide variety of foreign pathogens and injuries. However, because foreign and self-proteins are chemically similar, strict discrimination between self and non-self is neither possible nor desirable. The inflammatory process is vital to the survival of all complex organisms and although it is a localized protective response that serves to destroy the injurious agents, once the pathogen is eliminated, cells participating in innate and adaptive immunity have to be deactivated or eliminated, to reestablish tissue homeostasis.[20] The accumulation and subsequent activation of leukocytes are central events in the pathogenesis of virtually all forms of inflammation. Uncontrolled activation of the immune system and the sustained production of inflammatory mediators lead to serious consequences for the host, such as tissue destruction, organ failure, even death if it is systemically disseminated (i.e., septic shock) (Fig. 1A). A further damage arises from potential autoimmune responses occurring during chronic inflammatory responses.[20] Most of the immune-mediated destruction of self-tissues occurs via specific recognition of self-antigens, followed by a typical effector immune response, and/or occasionally as a byproduct of unchecked nonspecific inflammation or overly vigorous responses to chronic infections (Fig. 1A). In addition, genetic predisposition and environmental factors are critical triggers of self-reactive responses in a chronic inflammatory scenario. Generally, autoimmunity is initiated by differential processing of self-antigens/peptides, which occurs under inflammatory conditions, in which cytokines activate proteases that increase the processing and presentation of previously cryptic self-antigens. Alternatively, the activation of crossreactive lymphocytes that recognize the original pathogen antigen and self-antigen leads to release of cytokines and chemokines, which enhance local inflammation, activate antigen-presenting cells, and indirectly or directly cause tissue destruction and spreading of the autoimmune process (Fig. 1A). The initial stages of the autoimmune disease take place in peripheral lymphoid organs, are associated with initiation and establishment of autoimmunity to self-components, and involve the development of self-reactive T helper 1 (Th1) and Th17 cells by dendritic cells (DCs) presenting self-antigens. Moreover, Th1 cells promote the secretion of IgG2 autoantibodies by self-reactive B-cells and the deposition of immune complexes in the target tissue where they activate complement, neutrophils, and mast cells (Fig. 1A). Progression of the autoimmune response involves the entry of self-reactive Th1/Th17 cells into the affected organ or organs (if systemic), release of proinflammatory cytokines and chemokines, and subsequent recruitment and activation of inflammatory cells (macrophages and neutrophils). Later events are associated with the evolving immune and destructive inflammatory responses, where inflammatory mediators (cytokines, oxygen and nitrogen reactive species, and free radicals) produced by infiltrating cells and resident cells participate in tissue destruction (Fig. 1A).

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00087-7
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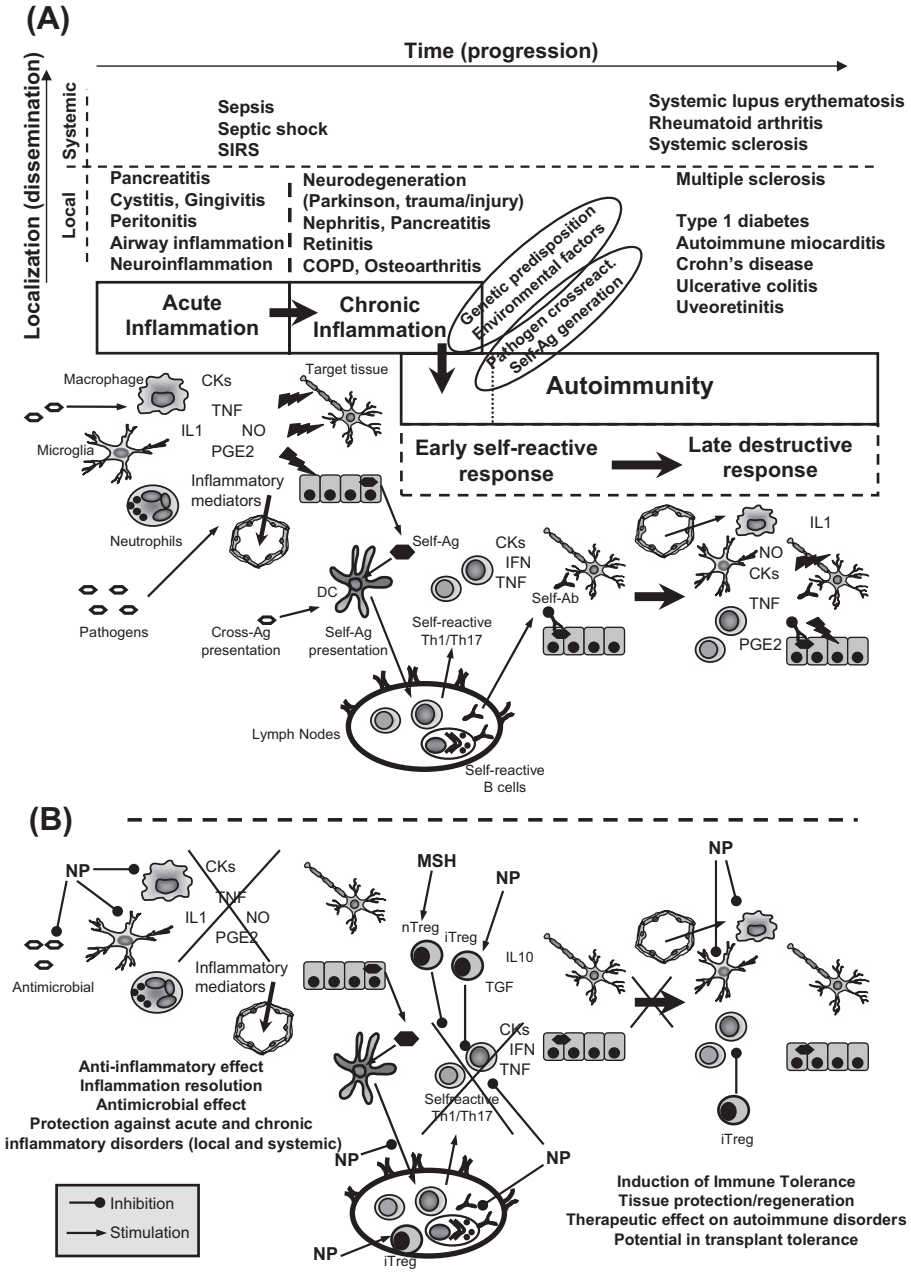

FIGURE 1 Mechanisms involved in the protective effect of neuropeptides on autoimmunity. (A) Inflammation is a necessary process for the elimination of pathogens, but it can lead to serious deleterious effects in the host if left unchecked. A sustained inflammatory response causes tissue damage, which is especially serious in some tissues/organs such as the central nervous system with limited regenerative capacities (i.e., neurodegenerative disorders). Systemically disseminated inflammatory mediators could cause sepsis, septic shock, systemic inflammatory response syndrome (SIRS), and eventually death. Besides, autoimmunity is the result of the progression from acute to chronic inflammation, is initiated by aberrant immune responses against self-antigens (self-Ag), and genetic predisposition and environmental factors play a role in its triggering. (B) To reinstate tolerance on inflammatory and autoimmune disorders, certain neuropeptides (NP), including VIP, PACAP, ghrelin, cortistatin, αMSH, urocortin, and adrenomedullin, reinforce various mechanisms. (**1**) Neuropeptides inhibit the production of a plethora of inflammatory cytokines, chemokines (CK), and free radicals (NO: nitric oxide) by macrophages and resident cells (i.e., microglia and synovial cells), avoiding the perpetuation/dissemination of the inflammatory response and its cytotoxic effect against self-tissue components. (**2**) Through a direct cell-independent antimicrobial action, neuropeptides eliminate pathogens and subsequently avoid chronic inflammation and self-antigen crosspresentation. (**3**) If autoimmune responses occur, neuropeptides can decrease Th1/Th17 cell functions through direct actions on differentiating T-cells, or indirectly by regulating DC functions in the peripheral lymphoid organs and locally in the affected tissue. Consequently, the inflammatory and autoimmune responses are inhibited because the infiltration/activation of neutrophils and macrophages and deposition of immune complexes (self-Ab) are avoided. (**4**) Neuropeptides induce the new generation of peripheral Treg (iTreg) that suppress the activation of self-reactive T-cells, expand naturally occurring Treg (nTreg), and indirectly generates iTreg through the differentiation of tolerogenic DCs. COPD: chronic obstructive pulmonary disease.

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To protect against autoimmunity without inhibiting its ability to mount effective responses to pathogens, the immune system has evolved several mechanisms to maintain tolerance to self-tissues that normally operate at multiple and successive layers of regulation.[20] Central clonal deletion, effector T-cell balance, regulatory T-cells (Treg), and cytokine deviation are some of the multiple mechanisms that act in concert to ensure self-tolerance (Table 1). Despite the plethora of safeguards that exists to ensure that tolerance is enforced in the immune system, they can occasionally be overcome, and autoimmune diseases develop.

Because the maintenance of this tolerance is critical for the homeostasis of the immune system and at the end the survival of the organism, it is logical to assume that the immune system must produce endogenous factors involved in such a role to accomplish this task. For example, inflammatory responses are self-controlled by anti-inflammatory mediators secreted during the ongoing process. Although traditional anti-inflammatory and/or immunosuppressive cytokines and interleukins (i.e., IL-10, TGFβ, and IFNα) are the immediate candidates,[20] numerous researchers have lately focused their effort in the identification of these endogenous inductors of tolerance on mediators nontraditionally classified as immune factors. In this sense, considerable evidence indicates that the immune system is not totally autonomous in generating an immune response and that the neuroendocrine system communicates with the immune system mounting a variety of coordinated responses to the "danger" of pathogen infection, injury, or inflammation.[29] This communication is bidirectional and is based in a mutual biochemical language. Thus, cytokines/interleukins produced by immune cells are recognized by receptors expressed on neuroendocrine cells, and vice versa, cells of the immune system recognize neurotransmitters, neuropeptides, and hormones produced by the nervous system. In this scenario, the immune system acts as sixth sense signaling the brain to respond to danger, orchestrating the febrile response and its subsequent effects on behavior. The nervous system conversely regulates the immune system in response to physical or psychological stress. Glucocorticoids and adrenergic neurotransmitters secreted by the hypothalamic-pituitary-adrenergic axis and sympathetic nervous system are the classical example of endogenous immunosuppressive agents produced in response to stress or systemic inflammation.[13,29] Besides, peripheral endings of sensory and efferent nerves release various neuropeptides capable of acting on nearby immune cells in response to various danger signals.[14,29]

From the growing list of about 40 neuropeptides currently thought to posses immunomodulatory actions, some of them (i.e., opioids and galanin) show a clear anti-inflammatory profile, acting on macrophages, microglia, and neutrophils, in an attempt to keep under control the innate inflammatory response; other neuropeptides (i.e., substance P and neurokinins) contribute to the inflammatory response, especially associated with neurogenic inflammation and hyperalgesia; and more limited is the number of neuropeptides that modulates the acquired immune response.[14,25] Most of these neuropeptides have been addressed in detail by Dr. Zhang in another chapter in this section of the book.

TABLE 1 Mechanisms of Induction of Immune Tolerance

Mechanism	Definition
Central deletion	Developing self-reactive lymphocytes generated by the random genetic recombination, with a high affinity to ubiquitously expressed self-antigens, are eliminated in the central lymphoid organs (thymus for T-cells, bone marrow for B-cells) by apoptosis. Before being clonally deleted, the self-reactive B-cell might be rescued by further gene rearrangements that replace the light chains of the self-reactive receptor with a new receptor that is not autoreactive, a mechanism termed receptor editing.
Anergy and ignorance	T-cells that recognize a self-antigen in the periphery in the absence of sufficient costimulatory signals or in the presence of coinhibitory (e.g., CTLA4) signals are functionally inactivated. Persistent and repetitive stimulation with a self-antigen also causes inadequate T-cell responses.
Regulatory T-cells (Treg)	Various T-cell populations that can functionally suppress an immune response by influencing the activity of effector autoreactive cells. Several phenotypically distinct regulatory T-cell populations exist: thymus-generated natural $CD4^+CD25^+FOXP3^+$ Treg, peripherally induced $CD4^+CD25^+FOXP3^+$ Treg, IL-10-producing type 1 regulatory cells induced by tolerogenic dendritic cells and transforming growth factor-β1-producing Th3 cells.
Cytokine deviation	Inflammatory responses are counteracted by anti-inflammatory mediators secreted during the ongoing process. The ability to control an inflammatory state depends on the local balance between proinflammatory and anti-inflammatory factors. An insufficient response can be life threatening, but an excessive response may lead to pathological conditions. Differentiation of T-cells that express noninflammatory Th2 cytokines and inhibit the generation of self-reactive Th1 cells that produce inflammatory cytokines contributes to this immune modulation.

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This chapter only covers the neuropeptides that have been shown to be clearly involved in the maintenance of immune tolerance and that regulate the autoimmune response. Autoreactivity, by definition, designates a specific immune response to self-antigens. Therefore, antigen-non-specific responses such as inflammation and innate immune processes should not be considered autoimmune in the strict sense, although they may accompany, enhance, or even trigger adaptive autoimmune responses. Thus, I will focus on the neuropeptides showing a prominent role in the regulation of the self-antigen-specific T- or B-cell immunity, which underlie the genuine autoimmune disorders.

ANTI-INFLAMMATORY NEUROPEPTIDES THAT REGULATE THE AUTOIMMUNE RESPONSE

With the exception of VIP and pituitary adenylate cyclase-activating polypeptide (PACAP), the sequence and structure of the neuropeptides, melanocyte-stimulating hormone (MSH), urocortin, adrenomedullin, cortistatin, ghrelin, and neuropeptide Y (NPY) are not related to each other, and their source and main actions in the body generally differ (see other chapters of this book). However, from an immunological point of view, they have some characteristics in common that make them attractive as potential regulators of immune tolerance: they are produced by immune cells, generally in response to inflammation, antigen stimulation, or injury; their receptors are expressed on various immune cells; besides their potent anti-inflammatory effects, they show diverse regulatory effects on T-cell–mediated responses (Table 2).

Recent studies examining these neuropeptides have shown their relevance to health, proving a crucial clinical significance in autoimmune diseases. Treatment with VIP, MSH, NPY, urocortin, adrenomedullin, cortistatin, or ghrelin decreases disease frequency/severity in various experimental models of rheumatoid arthritis, inflammatory bowel disease, type I diabetes mellitus, multiple sclerosis, Sjogren's syndrome, and uveoretinitis (Table 2).[4,8,15] The therapeutic effects of these neuropeptides are associated with the reduction of the two main phases of these diseases. They downregulate early events that are associated with the initiation and establishment of autoimmunity to self-tissue components and later phases that are associated with the evolving immune and destructive inflammatory responses (Fig. 1B). These neuropeptides impair the development of self-reactive Th1 and Th17 cells, their entry into the target organ, the release of inflammatory cytokines and chemokines, and the subsequent recruitment and activation of macrophages and neutrophils. This results in a decreased production of destructive inflammatory mediators (cytokines, nitric/oxygen radicals, and matrix metalloproteinases) by infiltrating and resident (i.e., microglia or synoviocytes) inflammatory cells.[8,15]

Various *in vitro* and *in vivo* studies indicate that some of these neuropeptides regulate the inflammatory response by directly acting on inflammatory cells. Thus, VIP, PACAP, MSH, urocortin, adrenomedullin, cortistatin, and ghrelin downregulate the production of a wide spectrum of inflammatory mediators, including inflammatory cytokines (i.e., TNFα, IL-12, IL-6, and IL-1β), various chemokines, PGE2 (by decreasing cycloxygenase 2), nitric oxide (by inhibiting the induced form of nitric oxide synthase), and the late inflammatory mediator high mobility group box-1 (Table 2, Fig. 1B) by macrophages, microglia, synovial cells, and DCs.[5,8,9,15–17,21,23] At the same time, they stimulate the production of anti-inflammatory cytokines such as IL-10 by macrophages, microglia, and DCs.

Although the mechanisms involved in the suppressive effects on Th1-cell responses of most of these neuropeptides are not fully elucidated, most results coming from VIP studies show that neuropeptides may regulate Th1/Th2 balance through various nonexcluding mechanisms with direct actions on differentiating T-cells and indirect regulation of functions of antigen-presenting cells (Table 2). Thus, they directly reduce the proliferation of activated Th1 cells, inhibit the production of the Th1-associated cytokine IL-12, inhibit the transcription factor T-bet, which is required for Th1 differentiation, and impair the recruitment of Th1 cells by inhibiting the secretion of CXC-chemokine ligand 10 by DCs and macrophages.[8,9] VIP also induces a sustained anergic state in human T-cells at multiple levels by inducing cell cycle arrest, inhibiting IL-2 secretion, and increasing the immunosuppressive molecule CTLA4.[2] Some evidence indicates that the induction of Th2 responses by VIP, PACAP, cortistatin, and ghrelin could contribute to the impairment of Th1-mediated responses. For example, VIP and PACAP induce CD86 expression in resting DCs, which is important for Th2 differentiation, promote specific Th2 recruitment by increasing CC-chemokine ligand 22, inhibit FasL- and granzyme B-mediated apoptosis of Th2 but not of Th1, and finally, induce the Th2 master transcription factors c-MAF, GATA-3, and JUNB in differentiating CD4 T-cells.[8,9]

Although the inhibitory effect of VIP, PACAP, urocortin, cortistatin, adrenomedullin, and ghrelin on Th17-cell response is evident *in vivo*, no results exist *in vitro* about the mechanisms involved in such effect and whether it is simply a consequence of an impaired recruitment of these cells to the target tissue and lymphoid organs resulting from the inhibitory effect exerted by these neuropeptides in the production of inflammatory chemokines by macrophages and inflammatory resident cells.

Another important issue that contributes to the amelioration of the destructive responses on the target tissue after the treatment of autoimmunity with these neuropeptides is the decrease in the levels of autoantibodies (especially IgG2a antibodies),[8,15–17] because this isotype of antibodies

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TABLE 2 Anti-Inflammatory Neuropeptides that Regulate Immune Tolerance: Source, Receptors, Functions, and Therapeutic Effects in Immune System[a]

Neuropeptide	Immune source[b]	Receptor type: immune cell[c]	Immune function[d]	Therapy[e]
VIP/PACAP	CD4 Th2, CD8 T2, PMNC, Mast cells	VPAC1: T,Mϕ,Mo,DC,PMNC VPAC2: T,Mϕ (inducible) PAC1: Mϕ,Mo	▼ inflammatory cytokines ▼ chemokines ▼ costimulation macrophages and DCs ▲ IL-10, TGFβ ▼ T-cell proliferation ▼ T_H1 response: IL-2 and IFNγ production and differentiation ▼ T_H1 recruitment ▼ T_H2 apoptosis ▼ T_H2 response and recruitment ▲ iTreg cells ▲ generation tolDCs Antimicrobial activity	RA EAE EAU IBD T1DM SD
αMSH	T,Mo,DC	MC1R: T,Mϕ,Mo,DC,PMNC,B MC3R: Mϕ,Mo MC5R: T,B	▼ antigen presentation and costimulation by DCs ▲ IL-10 production ▼ T-cell proliferation ▲ expansion Treg cells ▲ generation tolDCs Antimicrobial activity	EAE RA EAU IBD
Neuropeptide Y	T,Mϕ,DC	Y1: T,Mϕ, DC,B	▲ antigen presentation and costimulation by DCs ▼ T-cell proliferation ▼ T_H1 response: activation and differentiation	EAE
Urocortin	T,B, Mϕ,Mo, Mast cells	CRFR2: T,Mϕ,Mo,DC,PMNC	▼ inflammatory factors ▼ chemokines ▲ IL-10/TGFβ1 production ▼ T-cell proliferation ▼ T_H1 response: IL-2 and IFNγ ▲ Treg cells Antimicrobial activity	IBD RA EAE
Adrenomedullin	Mϕ,Mo	CRLR-RAMP2/3: T,Mϕ,Mo,DC	▼ inflammatory factors ▼ chemokines ▲ IL-10/TGFβ production ▼ T-cell proliferation ▼ T_H1 response: IL-2 and IFNγ ▲ Treg cells Antimicrobial activity	IBD RA
Cortistatin	T,Mo,Mϕ	Sst1-5: T,Mϕ,Mo,DC GHSR: T,Mϕ,Mo	▼ inflammatory factors ▼ chemokines ▲ IL-10/TGFβ1 production ▼ T-cell proliferation ▼ T_H1 response/differentiation ▲ Treg cells	RA IBD
Ghrelin	Mo,Mϕ	GHSR: T,Mϕ,Mo,DC	▼ inflammatory cytokines ▼ chemokines ▲ IL-10/TGFβ1 production ▼ T-cell proliferation ▼ HMGB1 ▼ T_H1 response: IL-2 and IFNγ production ▲ Treg cells Antimicrobial activity	EAE IBD RA

Data of the table are reviewed in references 5,8,9,15–18,21,23,32,33.

[a]*Abbreviations: MCR, melanocortin receptors; sst1–5, somatostatin receptors 1–5; CRLR, calcitonin-related ligand receptor; αMSH, melanocyte-stimulating hormone; CRFR, CRH receptor; VPAC, VIP/PACAP receptor; VIP, vasoactive intestinal peptide; PACAP; pituitary adenylate cyclase–activating polypeptide; RAMP, receptor-activity–modifying proteins; GHSR, growth hormone-secretagogue receptor; Y1, type 1 Neuropeptide Y-receptor; T, T-cell; Mϕ, macrophage; Mo, Monocyte; DC, dendritic cell; PMNC, polymorphonuclear cell; B, B-cell; EAE, experimental autoimmune encephalomyelitis; RA, rheumatoid arthritis; EAU, experimental autoimmune uveitis; IBD, inflammatory bowel disease; T1DM, type 1 diabetes mellitus; SD, Sjögrend's disease; T_H, T helper cell.*

[b]*Immune cells producing neuropeptides.*

[c]*Immune cells expressing neuropeptide receptor subtypes.*

[d]*Major roles of the neuropeptides related with the autoimmune response. ▲, stimulation; ▼, inhibition.*

[e]*Beneficial effects of neuropeptides in various experimental models of autoimmunity.*

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is involved in the activation of complement and neutrophils. It is still unknown whether neuropeptides could affect B-cell responses directly, but some results with VIP and PACAP indicate that this action could be indirectly exerted through the inhibition of self-reactive Th1-cell responses.[8]

On the other hand, another interesting effect of some neuropeptides that could limit the perpetuation and/or dissemination of chronic inflammatory responses and the triggering of an autoimmune response is the recently discovered antimicrobial properties exerted by VIP, αMSH, ghrelin, and adrenomedullin against a wide variety of gram-positive and gram-negative bacteria, essentially present in the gastrointestinal tract.[5,12] This is especially important in autoimmune disorders of the bowel, such as Crohn's disease, where an imbalance between commensal and pathogenic bacteria seems to be related to the initiation of the autoimmune response. Based on their amphipathic design, amino acid composition and cationic charge, these neuropeptides form pores on the plasma membrane of the bacteria compromising its viability. VIP, PACAP, urocortin, and adrenomedullin have also shown significant antiparasitic actions against tropical protozoa[7] that are frequently related to the initiation of myocardial autoimmune disorders.

INDUCTION OF IMMUNE TOLERANCE BY GENERATING REGULATORY T-CELLS

Considerable evidence indicates that the generation of Treg plays a major role in the beneficial effect of αMSH, VIP, ghrelin, PACAP, urocortin, adrenomedullin, and cortistatin in several models of autoimmune diseases, including experimental autoimmune encephalomyelitis (EAE), experimental colitis, experimental uveoretinitis, and experimental arthritis.[15–19] These neuropeptides induce the peripheral expansion of new antigen-specific $CD4^+CD25^+FoxP3^+$ Treg that suppresses the activity of self-reactive T- and B-cells and tissue-localized inflammatory responses.[2] With the exception of αMSH, all of them promote the differentiation of $CD4^+CD25^+$ Treg from the $CD4^+CD25^-$ T-cell repertoire.[15–19] αMSH seems to expand the thymus-occurring natural Treg.[32] The suppression of self-reactive effector T-cells by neuropeptide-induced Treg involves direct cellular contacts dependent on CTLA-4 and/or the production of the immunosuppressive cytokines IL-10 and/or TGFβ. In addition, αMSH, VIP, and PACAP induce the differentiation of tolerogenic DCs (tolDCs) from bone marrow precursors or monocytes.[6,18,24] The tolDCs induced by these neuropeptides have the ability to generate antigen-specific Treg with suppressive actions on effector T-cells and to transfer antigen-specific tolerance *in vivo*. As far as I know, the effect of NPY on Treg has not been reported yet. Although results from human studies are scarce, recent results show that it is possible to induce alloantigen-specific human Treg with VIP-differentiated tolDCs[18] and to convert conventional human $CD4^+CD25^-$ lymphocytes into antigen-specific $CD4^+CD25^+FoxP3^+$ Treg in the presence of VIP.[2] Importantly, the VIP-converted human Tregs show a potent suppressive capacity on effector antigen-specific T-cells. More definitive are the results obtained in a recent phase I/II clinical trial performed in patients with sarcoidosis, a chronic inflammatory/Th1-driven disease, showing that inhalation of VIP for four weeks increased the number and activity of Treg in the lung of these patients, and at the same time, decreased the levels of inflammatory and Th1 cytokines and chemokines in the bronchoalveolar fluid.[27]

On the other hand, various reports have described in experimental models of autoimmunity the beneficial effects of Treg and tolDCs generated *ex vivo* with neuropeptides.[6,18,19] The therapeutic application of this strategy has been also extended to other immune disorders, such as allogeneic transplantation, in which the induction of tolerance against alloantigens is required,[18] and to neurodegenerative diseases, such as Parkinson's disease,[28] in which the neuroprotective effect of VIP-induced Treg is antigen specific, as occurs in autoimmune disorders.

REDUNDANT AND PLEIOTROPIC EFFECTS ON IMMUNE HOMEOSTASIS: A MATTER OF RECEPTOR SPECIFICITY AND SIGNALING

Now, the obvious question is, how do VIP, α-MSH, urocortin, NPY, adrenomedullin, ghrelin, and cortistatin regulate this plethora of inflammatory and immunomodulatory mediators? The answer could lie with the cAMP/PKA pathway that is the major intracellular signaling pathway involved in most of the effects exerted by all these neuropeptides, including the downregulation of inflammatory mediators.[17] cAMP-inducing agents have been described as potent anti-inflammatory factors, inhibitors of the Th1 response, and inducers of Treg function.[17] These neuropeptides, through the elevation of intracellular cAMP, downregulate the activation of several signal transduction pathways and transcription factors essential for the transcriptional activation of most of the inflammatory cytokines, chemokines, and costimulatory factors, including nuclear factor-κB (NFκB), mitogen-activated protein kinases (MAPK), interferon regulatory factor 1 (IRF1), and the activator protein 1 (AP1).[9,17,23] Moreover, they can also affect common upstream elements located very early in the signaling of these pathways, such as inhibition of the expression of Toll-like receptors (TLRs) and associated proteins (i.e., endotoxin-binding factor CD14), or of proximal intracellular signaling molecules of TLR4 such as IRAK-1 in macrophages.[17] A cAMP/PKA-dependent inhibition of NFκB, AP-1, NF-AT, and Akt pathways seems to be critically involved in the induction of T-cell anergy, the generation of Treg cells, and the induction of tolDCs by VIP.[2,15,18] Most

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of these findings have been reported for VIP, PACAP, and αMSH. However, studies in other cell types show that urocortin, NPY, adrenomedullin, and ghrelin regulate a number of the described pathways, suggesting that they can also be involved in their immunosuppressive effects.[17]

That most of the modes of action of these neuropeptides in inflammation primarily depend on the activation of the cAMP/PKA pathway translates a relevant importance to the differential expression of the specific receptors for these neuropeptides in different immune cells, as well as their regulation by inflammatory and antigenic stimuli. The differential expression and signaling of these receptors in immune cells, the dimerization with other receptors, or the association with certain receptor-activity-modifying proteins (RAMPs) have also shed light on the existing controversy about the emergence of new antinflammatory neuropeptides, such as urocortin, adrenomedullin, and cortistatin, which show more potent, in some cases contrary actions on immune tolerance compared with their respective structurally related peptides, corticotropin-releasing hormone (CRH), calcitonin gene-related peptide (CGRP), and somatostatin.

For example, although the anti-inflammatory action of urocortin is well documented,[8,15–17] CRH regulates the immune and inflammatory responses in two antithetical ways, depending on the site of secretion (central vs. peripheral).[21] The different relevance of CRH and urocortin in the CNS and periphery might result from the differential expression of specific receptors for both neuropeptides in immune cells. CRH and urocortin bind to two receptors: CRH receptor type 1 (CRHR1) and CRHR2. In general, CRHR1 is expressed abundantly in the CNS with limited expression in peripheral tissues, whereas CRHR2 is more widespread in the periphery, including in most immune cells (Table 2). Urocortin binds to CRHR2 with much higher affinity than CRH. CRHR1 and CRHR2 signal through cAMP/PKA, but only CRHR1 stimulates the proinflammatory p38MAPK pathway, whereas only CRHR2 inhibits ERK signaling.[17] Indeed, specific CRHR2 agonists have shown a clear antinflammatory action *in vivo*.[17] Therefore, the fact that urocortin is produced by immune cells, and that CRHR2 is the predominant receptor in immune cells, indicates that urocortin can act peripherally in the immune system as an anti-inflammatory factor.

Another example of differential effects between two structurally related neuropeptides is the case of CGRP and adrenomedullin. CGRP has been traditionally considered a neuropeptide with a general anti-inflammatory effect, although others have described the contrary.[14] By contrast, adrenomedullin clearly acts as an anti-inflammatory and immunosuppressive factor.[15,17] Indeed, although CGRP fails to prevent sepsis, inflammatory bowel disease and arthritis and to downregulate the inflammatory and T-cell-mediated responses in these disorders, adrenomedullin shows striking therapeutic effects.[15,17] Interestingly, although CGRP induces inflammatory response by stimulating NFκB, adrenomedullin deactivates the NFκB and p38MAPK pathways in target cells.[17] The difference in responses induced by adrenomedullin and CGRP might reside in the differential binding of both neuropeptides to different binding complexes, which are formed by the calcitonin-related-like receptor (CRLR) and various RAMPs (RAMP1, RAMP2, and RAMP3). The RAMP subtype associated with CRLR determines the binding specificity for the ligand and the activity of the receptor: CGRP specifically binds to the CRLR–RAMP1 complex, whereas adrenomedullin preferentially binds to CRLR associated with RAMP2 and RAMP3 and coupled to the cAMP/PKA pathway. Macrophages specifically express the adrenomedullin-specific CRLR-RAMP2/3 complexes (personal communication). This partially explains the higher potency of adrenomedullin versus CGRP in the inflammatory response.

Finally, cortistatin shows a high homology with somatostatin and binds to all five somatostatin receptor subtypes (SST1–5), sharing many of the functional properties of somatostatin, including the suppression of neuronal activity and cell proliferation. However, cortistatin has many properties distinct from somatostatin in the nervous system (induction of slow-wave sleep and reduction of locomotor activity) and in the immune system. Although contradictory results exist about the production of somatostatin by immune cells, cortistatin expression by immune cells is well established,[15] suggesting that cortistatin might be a major endogenous immunoregulatory factor. Somatostatin and different SST agonists have emerged as attractive anti-inflammatory factors, in some cases with clinical validation. However, cortistatin downregulates inflammation more efficiently than somatostatin, and cortistatin, but not somatostatin, induces antigen-specific Treg.[8,15] This might reside in its capacity to activate different receptors and signal transduction pathways. Although somatostatin only binds to SST, cortistatin can also activate other receptors, including the ghrelin receptor (GHSR).[17] Ghrelin binding to GHRS and activation of the cAMP/PKA pathway are associated with antinflammatory and immunosuppressive actions.[15,17] Therefore, cortistatin might exert its immunoregulatory effect through both SST- and GHSR-mediated signaling pathways, one GHSR-mediated cAMP-dependent and one SST-mediated cAMP-independent.

A HEALTHY IMMUNE SYSTEM REQUIRES A PHYSIOLOGICALLY FUNCTIONAL NEUROPEPTIDE SYSTEM

In view of this plethora of actions mediated by these neuropeptides, an important issue is related to the physiological role played by endogenous neuropeptides in the maintenance of immune homeostasis. Previous evidence, obtained

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primarily in animals deficient in one of these neuropeptides or their receptors, indicates that the absence of adrenomedullin, VIP, urocortin, or some of their receptors results in higher susceptibility to endotoxemia and airway inflammation.[8,15] Adaptive immune response is also affected, and mice deficient in the VIP receptor VPAC2 show exacerbated Th1-mediated responses *in vivo*, and conversely, animals overexpressing this receptor show eosinophilia, elevated IgE and IgG1 levels, and increased cutaneous anaphylaxis.[9] PACAP-deficient mice support these findings in an autoimmune scenario, because they show a higher incidence and severity with EAE, increased inflammatory/Th1-driven encephalitogenic responses, and decreased numbers of antigen-specific Treg than wild-type mice.[31] Interestingly, mice deficient for NPY-receptor Y1 reveal a bimodal effect of NPY on the immune system, in which signaling through Y1 in T-cells results in T-cell deactivation and through Y1 in DCs results in antigen-presenting cell activation.[34] These results could explain why NPY treatment shows a beneficial effect on EAE,[4] whereas paradoxically, Y1-KO mice are resistant to Th1-mediated inflammatory responses and colitis.[34] Similarly, VIP-KO mice show resistance to EAE, although they show an increased number of encephalitogenic T-cells[1] and improved survival after viral infections associated with a marked increase in MHCII and CD80, increased Th1/Tc1 cells, and fewer IL-10 producing T-cells.[22] Finally, ghrelin seems to play a major role on the function and evolution of the thymus, because mice deficient in ghrelin or its receptor have compromised the thymic stromal cell microenvironment, which plays a critical role in the education/selection of T-cells in the thymus (central tolerance and generation of natural Treg).[35]

Even more interesting is the biological relevance of the neuropeptides produced by one's own immune cells. Various studies reported the capacity of the VIP and ghrelin secreted by T-cells to decrease, in an autocrine and paracrine way, the production of inflammatory Th1 and Th17 cytokines by murine and human T-cells.[9,11,22] Similarly, NPY secreted by lymphocytes and antigen-presenting cells actively regulates T-cell activation.[34] Various studies with VIP suggest that similar conclusions can be made in human conditions. Monocytes, synovial cells, and T-cells isolated from patients with rheumatoid arthritis, osteoarthritis, and multiple sclerosis express lower VPAC1 levels and respond poorly to VIP.[10,26,30] The altered expression of VIP receptors is probably related to an aberrant autoreactive Th1-driven inflammatory response in these patients. Moreover, there is a genetic association between multiple-marker haplotypes of the VIP receptor VPAC1 and susceptibility to suffer rheumatoid arthritis and ankylosing spondylitis[10,26] and also a correlation between reduced VIP levels and high amounts of autoantibodies with VIPase activity in patients with autoimmune thyroiditis and systemic lupus erythematosus.[3]

CONCLUSIONS

Because there are no particular molecular signatures of self, autoimmunity is the inevitable evolutionary price of being able to make effective responses against a wide variety of pathogens by the immune system. Without the various phenomena referred to as immune tolerance, the organism would surely self-destruct. The findings discussed in this chapter indicate that various endogenous neuropeptides play a major role in the education of our immune system to be self-tolerant. That neuropeptides regulate, with apparent redundancy as cytokine-like factors, various layers involved in maintenance of tolerance, balancing proinflammatory and antinflammatory responses and decreasing self-reactive Th1/Th17 cells versus Treg/Th2 cells, makes them attractive candidates for the development of new therapies for the treatment of inflammatory and autoimmune disorders. Once the problems of instability inherent in their peptidic nature are solved (i.e., aminoacid substitutions, cycling structure, gene therapy, combination with endopeptidase inhibitors, biocompatible and degradable depots), interest by the pharmaceutical industry for neuropeptide-based therapies in immune disorders will be high because of their potency and minimal side effects. In addition, the possibility of manipulating Treg generation and/or expansion raises the possibility of a cell-based strategy to treat autoimmunity by providing long-term tolerance to self-antigens. This is tremendously attractive from a therapeutic point of view, because the translation of important biological findings about Treg to a clinic setting has been limited by the scarcity of circulating Treg. Beside their therapeutic potential, recent evidence suggests that neuropeptides and their receptors are intrinsic regulators of immune tolerance and that a defect in their signaling system affects the maintenance of immune homeostasis. Even if neuropeptides are not commonly used under normal circumstances, they could come into play when the immune system is trying to adapt to various types of challenges (e.g., autoimmunity), representing, together with other cytokines, the language of the stressed immune system.

ACKNOWLEDGMENTS

The authors thank the many researchers and laboratories that have contributed to our current understanding of the fundamental roles that neuropeptides play in immunology. This work was supported by grants from the Spanish Ministry of Health and Junta de Andalucia.

REFERENCES

1. Abad C, Tan YV, Lopez R, Nobuta H, Dong H, Phan P, et al. Vasoactive intestinal peptide loss leads to impaired CNS parenchymal T-cell infiltration and resistance to experimental autoimmune encephalomyelitis. *Proc Natl Acad Sci USA* 2010;**107**:19555–60.

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2. Anderson P, Gonzalez-Rey E. Vasoactive intestinal peptide induces cell cycle arrest and regulatory functions in human T cells at multiple levels. *Mol Cell Biol* 2010;**30**:2537–51.
3. Bangale Y, Karle S, Planque S, Zhou YX, Taguchi H, Nishiyama Y, et al. VIPase autoantibodies in Fas-defective mice and patients with autoimmune disease. *FASEB J* 2003;**17**:628–35.
4. Bedoui S, Miyake S, Lin Y, Miyamoto K, Oki S, Kawamura N, et al. Neuropeptide Y (NPY) suppresses experimental autoimmune encephalomyelitis: NPY1 receptor-specific inhibition of autoreactive Th1 responses in vivo. *J Immunol* 2003;**171**:3451–8.
5. Chorny A, Anderson P, Gonzalez-Rey E, Delgado M. Ghrelin protects against experimental sepsis by inhibiting high-mobility group box 1 release and by killing bacteria. *J Immunol* 2008;**180**:8369–77.
6. Chorny A, Gonzalez-Rey E, Fernandez-Martin A, Pozo D, Ganea D, Delgado M. Vasoactive intestinal peptide induces regulatory dendritic cells with therapeutic effects on autoimmune disorders. *Proc Natl Acad Sci USA* 2005;**102**:13562–7.
7. Delgado M, Anderson P, Garcia-Salcedo JA, Caro M, Gonzalez-Rey E. Neuropeptides kill African trypanosomes by targeting intracellular compartments and inducing autophagic-like cell death. *Cell Death Differ* 2009;**16**:406–16.
8. Delgado M, Ganea D. Anti-inflammatory neuropeptides: a new class of endogenous immunoregulatory agents. *Brain Behav Immun* 2008;**22**:1146–51.
9. Delgado M, Pozo D, Ganea D. The significance of vasoactive intestinal peptide in immunomodulation. *Pharmacol Rev* 2004;**56**:249–90.
10. Delgado M, Robledo G, Rueda B, Varela N, O'Valle F, Hernandez-Cortes P, et al. Genetic association of vasoactive intestinal peptide with rheumatoid arthritis. Altered expression and signal in immune cells. *Arthritis Rheum* 2008;**58**:1010–9.
11. Dixit VD, Yang H, Cooper-Jenkins A, Giri BB, Patel K, Taub DD. Reduction of T cell-derived ghrelin enhances proinflammatory cytokine expression: implications for age-associated increases in inflammation. *Blood* 2009;**113**:5202–5.
12. El Karim IA, Linden GJ, Orr DF, Lundy FT. Antimicrobial activity of neuropeptides against a range of micro-organisms from skin, oral, respiratory and gastrointestinal tract sites. *J Neuroimmunol* 2008;**200**:11–6.
13. Elenkov IJ, Wilder RL, Chrousos GP, Vizi S. The sympathetic nerve-An integrative interface between two supersystems: the brain and the immune system. *Pharmacol Rev* 2000;**52**:595–638.
14. Fernandes ES, Schmidhuber SM, Brain SD. Sensory-nerve-derived neuropeptides: possible therapeutic targets. *Handb Exp Pharmacol* 2009;**194**:393–416.
15. Gonzalez-Rey E, Chorny A, Delgado M. Regulation of immune tolerance by anti-inflammatory neuropeptides. *Nat Rev Immunol* 2007;**7**:52–63.
16. Gonzalez-Rey E, Chorny A, Varela N, O'Valle F, Delgado M. Therapeutic effect of urocortin on collagen-induced arthritis by downregulating inflammatory and Th1 response and inducing regulatory T cells. *Arthritis Rheum* 2007;**56**:531–43.
17. Gonzalez-Rey E, Delgado M. Anti-inflammatory neuropeptide receptors: new therapeutic targets for immune disorders? *Trends Pharmacol Sci* 2007;**28**:482–91.
18. Gonzalez-Rey E, Delgado M. Vasoactive intestinal peptide and regulatory T-cell induction: a new mechanism and therapeutic potential for immune homeostasis. *Trends Mol Med* 2007;**13**:241–51.
19. Gonzalez-Rey E, Fernandez-Martin A, Chorny A, Delgado M. Vasoactive intestinal peptide induces CD4+CD25+ regulatory T cells with therapeutic effect on collagen-induced arthritis. *Arthritis Rheum* 2006;**54**:864–76.
20. Goodnow CC. Multistep pathogenesis of autoimmune diseases. *Cell* 2007;**130**:25–35.
21. Gravanis A, Margioris AN. The corticotropin-releasing factor (CRF) family of neuropeptides in inflammation: potential therapeutic applications. *Curr Med Chem* 2005;**12**:1503–12.
22. Li JM, Southerland L, Hossain MS, Giver CR, Wang Y, Darlak K, et al. Absence of vasoactive intestinal peptide expression in hematopoietic cells enhances Th1 polarization and antiviral immunity in mice. *J Immunol* 2011;**187**:1057–65.
23. Lipton JM, Catania A. Anti-inflammatory actions of the neuroimmunomodulator alpha-MSH. *Immunol Today* 1997;**18**:140–5.
24. Luger TA, Scholzen TE, Brzoska T, Bohm M. New insights into the functions of α-MSH and related peptides in the immune system. *Ann NY Acad Sci* 2003;**994**:133–40.
25. Lühder F, Lee DH, Gold R, Stegbauer J, Linker RA. Small but powerful: short peptide hormones and their role in autoimmune inflammation. *J Neuroimmunol* 2009;**217**:1–7.
26. Paladini F, Cocco E, Cauli A, Cascino I, Vacca A, Belfiore F, et al. A functional polymorphism of the vasoactive intestinal peptide receptor 1 gene correlates with the presence of HLA-B*2705 in Sardinia. *Genes Immun* 2008;**9**:659–67.
27. Prasse A, Zissel G, Lutzen N, Schupp J, Schmiedlin R, Gonzalez-Rey E, et al. Inhaled vasoactive intestinal peptide exerts immunoregulatory effects in sarcoidosis. *Am J Respir Crit Care Med* 2010;**182**:540–8.
28. Reynolds AD, Stone DK, Hutter JA, Benner EJ, Mosley RL, Gendelman HE. Regulatory T cells attenuate Th17 cell-mediated nigrostriatal dopaminergic neurodegeneration in a model of Parkinson's disease. *J Immunol* 2010;**184**:2261–71.
29. Sternberg EM. Neural regulation of innate immunity: a coordinated nonspecific host response to pathogens. *Nat Rev Immunol* 2006;**6**:318–28.
30. Sun W, Hong J, Zang YC, Liu X, Zhang JZ. Altered expression of vasoactive intestinal peptide receptors in T lymphocytes and aberrant Th1 immunity in multiple sclerosis. *Int Immunol* 2006;**18**:1691–700.
31. Tan YV, Abad C, Lopez R, Dong H, Liu S, Lee A, et al. Pituitary adenylyl cyclase-activating polypeptide is an intrinsic regulator of Treg abundance and protects against experimental autoimmune encephalomyelitis. *Proc Natl Acad Sci USA* 2009;**106**:2012–7.
32. Taylor A, Namba K. In vitro induction of CD25 + CD4+ regulatory T cells by the neuropeptide alpha-melanocyte stimulating hormone (alpha-MSH). *Immunol Cell Biol* 2001;**79**:358–67.
33. Wheway J, Herzog H, Mackay F. The Y1 receptor for NPY: a key modulator of the adaptive immune system. *Peptides* 2007;**28**:453–8.
34. Wheway J, Mackay CR, Newton RA, Sainsbury A, Boey D, Herzog H, et al. A fundamental bimodal role for neuropeptide Y1 receptor in the immune system. *J Exp Med* 2005;**202**:1527–38.
35. Youm YH, Yang H, Sun Y, Smith RG, Manley NR, Vandanmagsar B, et al. Deficient ghrelin receptor-mediated signaling compromises thymic stromal cell microenvironment by accelerating thymic adiposity. *J Biol Chem* 2009;**284**:7068–77.Chapter | 87 Immunoregulatory Neuropeptides

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Chapter 88

Chemerin

Marc Parmentier

ABSTRACT

Chemerin is a 16 kDa polypeptide containing a cystatin-like fold and a disordered C-terminus required for bioactivity. It is secreted as an inactive precursor (prochemerin) requiring processing by various proteases to become a high affinity ligand for its main receptor ChemR23. Chemerin binds two other receptors, GPR1 and CCLR2, with high affinity, promoting only weak signaling through GPR1, and no signaling through CCRL2. Chemerin promotes chemotaxis of leukocyte populations expressing ChemR23, including monocytes, macrophages, myeloid and plasmacytoid dendritic cells (pDC), and NK cells. Bioactive chemerin is generated in inflammatory conditions, and ChemR23-positive cells are recruited to affected tissues in a number of inflammatory diseases in humans. In animal models, chemerin displays both pro- and anti-inflammatory properties. Chemerin is also an adipokine secreted by adipocytes, and its serum concentratons are correlated with body mass index (BMI) and other parameters of the metabolic syndrome. It regulates lipid and glucose metabolism in different tissues.

DISCOVERY

Tazarotene-induced gene 2 (Tig2) was first described as a gene induced by retinoic acid and synthetic analogs in keratinocytes.[23] The protein encoded by this gene was not recognized initially as a secreted factor and its function remained unknown. The Tig2 product was subsequently purified as a natural ligand stimulating the orphan G protein-coupled receptor (GPCR) ChemR23, starting from ascitic fluid secondary to ovary cancer.[30] The requirement for proteolytic processing of the protein was shown, and the processed ligand displayed chemotactic properties for leukocyte populations expressing ChemR23. The bioactive processed product of the Tig2 gene was therefore named chemerin. Chemerin displays weak similarity with a set of extracellular proteins sharing the so-called "cystatin" fold. These include the cysteine protease inhibitors cystatins type 2, the bactericidal peptide precursors cathelicidins, and kininogens. All these proteins share four conserved cysteines per cystatin domain, which form two disulfide bonds in the crystal structures that have been determined (e.g. chicken cystatin, 1CEW; human cystatin D, 1ROA; porcine protegrin 3, 1PFP). Chemerin contains two additional cysteines, suggesting the existence of an additional disulfide bond, and some variation from the classical cystatin fold. An NMR assignment of human chemerin was published, although the tridimensional structure of the protein was not reported.[2]

STRUCTURE OF THE mRNA/GENE

The gene encoding preprochemerin (RARRES2) is located on 7q36.1 in human and 6B2.3 in mouse. The human gene spans 3357 base pairs (2973 in mouse) from the transcriptional start site to the poly-adenylation site. It contains six exons and five introns, and the length of the mature transcript is 741 nucleotides (excluding the poly-A tail). Three of the introns split the coding region, while one exon is located in the 5′-untranslated region and another in the 3′-untranslated region. The location of the exons is similar to that found in other genes encoding proteins sharing with chemerin the cystatin fold. The position of the three introns splitting the open reading frame is conserved relative to codons (between codons for all three), and relative to the conserved cysteines involved in the disulfide bonds that stabilize the cystatin fold. The promoter of the mouse chemerin gene was shown to contain a DR-1-type PPAR response element between −61 and −49.[22] A binding site for the sterol regulatory element-binding protein 2 (SREBP2) transcription factor was also reported in the chemerin gene, potentially explaining the induction of chemerin expression by free fatty acids.[5]

PROCESSING OF THE PRECURSOR

The Tig2 gene product contains a signal peptide of 20 amino acids, which is cleaved during biosynthesis (Fig. 1). Chemerin is generated as a precursor, prochemerin (21-163), displaying very poor activity toward its receptors. To become an agonist of its main receptor, ChemR23, prochemerin requires precise proteolytic processing at its C-terminus. Several proteases activate or inactivate (pro)chemerin by cleaving at specific sites of the protein C-terminus (Fig. 2).

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00088-9
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                        1.                  21.                                                                  78.
Human                    mrrlliplalwlgavg--vgvaELTEAQRRGLQVALEEFHKHPPVQWAFQETSVESAVDTPFPAGI-FVRLEFKLQQTSCR
Mouse                    mkcllislalwlgtvgtrgTEPELSETQRRSLQVALEEFHKHPPVQLAFQEIGVDRAEEVLFSAGT-FVRLEFKLQQTNCP
Rat                      mkcllislalwlgtadihgTELELSETQRRGLQVALEEFHRHPPVQWAFQEIGVDSADDLFFSAGT-FVRLEFKLQQTSCL
Bos                      mwqlllplalglgtmg--lgraELTTAQHRGLQVALEEFHKHPPVLWAFQVTSVDNAADTLFPAGQ-FVRLEFKLQQTSCR
Sus                      mwqlllplalwlgtmg--lgraELTAAQLRGLQVALEEFHKHPPVQWAFRETGVNSAMDTPFPAGT-FVRLEFKLQQTSCR
Rabbit                   mrrlmipvalwlgvvg--vssTELTRAQQRGLQVALEEFHKHPPVQWAFQETGVDSAVDTPFPAGT-FVRLEFKLQQTNCR
Chicken                     mklllgiavavla--ladaGQSPLQRRVVKDVLDYFHSRSNVQFLFREQSVEGAVERVDSSGT-FVQLHLNLAQTACR
Xenopus            meitgawsllgmvgllvtvrg-QVPTEELSDIQTKALTLVTEDFHNVDNIYSRFQVTSVLNAAEEKFPGGI-FVLLHLRKKQTNCA
Fugu     maapppplplpmmmmmmmisvgalLPSETQTNPYSQLSPSLKGGVDLALEKLHSHAAIQQHLVFLRSLSTSDVQAGFGVTFIYHHFYLKPTNCP

          79.                                                                  141.   148.     157.  163.
Human       KRDWKKP--ECKVRPNGRKRKCLACIKLGSED--KVLGRLVHC-PIETQVLREAEEHQETQCLRVQRAGEDPHSFYFPGQFAFSKALPRS
Mouse       KKDWKKP--ECTIKPNGRRRKCLACIKMDPKG--KILGRIVHC-PILKQGP---QDPQELQCIKIAQAGEDPHGYFLPGQFAFSRALRTK
Rat         KKDWKKP--ECTIKPNGRKRKCLACIKLDPKG--KVLGRMVHC-PILKQGP--QQEPQESQCSKIAQAGEDSRIYFFPGQFAFSRALQSK
Bos         KKDWRKE--DCKVKPNGRKRKCLACIKLDSKD--QVLGRMVHC-PIQTQVQRELDDAQDAQCSRVERAGEDPHSYYLPGQFAFIKALSP
Sus         KRDWKKA--ECKVKPNGRKRKCLACIKLNSED--KVLGRMVHC-PIETQVQREPEERQEAQCSRVERAGEDPHSYYFPGQFAFFKALPPS
Oryctolagus KKDWKKP--ECKVKPNGRKRKCLACIKLDPED--KVLGRMVHC-PIQTQIRRELEEHQGTQCSRVERAGEDPHSYYFPGQFAFFKALPPS
Chicken     KQAQRKQ--NCRIMENRRKPVCLACYKFDSSDVPKVLDKYYNCGPSHHLAMKDIKHRDEAECRAVEEAGKMSDVLYLPGMFAFSKGLPA
Xenopus     KTEWKSK--VCKALRGGRVFNCFACLKFEYGS-HKVLAKAIDCVPDRQVSP-EVEQKRRQRCTTMESVNETG--LRLPGALSFVKSQ
Fugu        KRTADVS--GCRHRNDRPLIDCAVCYKTLRGEIEPQPKPYVHCIHKTALTQ-EMKDTRVEHCNQLSYSSGDTTLLASTGNEEA
Human chemerin-9 peptide                                                                      YFPGQFAFS
Mouse C15 peptide                                                                   AGEDPHGYFLPGQFA
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FIGURE 1 Alignment of preprochemerin amino acid sequences from a set of mammalian and non-mammalian species. A set of preprochemerin sequences was aligned. The signal peptides are displayed as italic lowercase letters. Cleavage sites were predicted on the SignalP 4.0 server (http://www.cbs.dtu.dk/services/SignalP/). For human preprochemerin, the cleavage site of the signal peptidase was confirmed by mass spectrometry analysis of bioactive chemerin purified from natural sources.[30] The cysteines stabilizing the core structure of chemerin, presumably adopting a cystatin-like fold, are indicated in bold. The nonapeptides characterized in human and mouse as displaying nanomolar potencies toward ChemR23 and GPR1 are indicated in bold and underlined. The sequence of human chemerin-9 and mouse C15, two peptides described in the text, are also indicated.

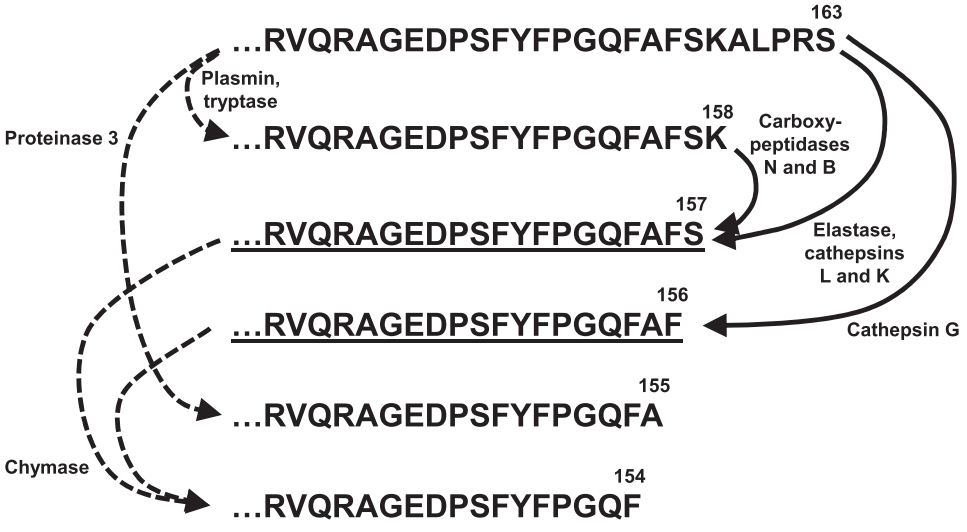

FIGURE 2 Multiple proteases regulate human (pro)chemerin bioactivity. The C-terminal sequence of known human chemerin variants is displayed, with numbering of the last amino acid relative to the full preprochemerin sequence (see Fig. 1). The two active forms are underlined. On the right, proteases generating bioactive forms of chemerin are indicated, while on the left, proteases resulting in inactive (or poorly active) forms of chemerin are displayed.

Two neutrophil serine proteases, elastase and cathepsin G, remove respectively the last 6 and 7 amino acids from prochemerin.[31] The two resulting forms of chemerin (21-157 and 21-156) are bioactive, although the longer form is slightly more potent, according to the pharmacological characterization of peptides derived from chemerin C-terminus. Serine proteases of the coagulation and fibrinolytic cascades, other cathepsins, and mast cell tryptase were also described as activators of prochemerin.[34] Plasmin and tryptase remove the last 5 amino acids (generating an inactive form), while carboxypeptidases N and B efficiently remove the resulting carboxy-terminal lysine, generating the active chemerin 20-157 form.[13] Cathepsins L and K generate the 20-157 form directly from prochemerin.[20] Urokinase and tissue plasminogen activator (TPA) as well as factor XIIa at high concentrations activate prochemerin, but the precise cleavage sites were not reported.[34] Staphopain B, a cysteine protease from *Staphylococcus aureus*, also activates plasma

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prochemerin. Other proteases inactivate bioactive chemerin or compete for prochemerin with the activating proteases. Mast cell chymase targets chemerin 21-157 and chemerin 21-156 (but not prochemerin) and generates the inactive variant chemerin 21-154. Neutrophil proteinase three cleaves prochemerin (but not the bioactive chemerin forms), generating the inactive 21-155 form.[17]

Short peptides derived from chemerin C-terminus retain significant bioactivity *in vitro* (Fig. 1). A chemerin nonapeptide (Y^{149}FPGQFAFS^{157}, chemerin-9) displays an EC_{50} of 5 nM for ChemR23. This is at least 10-fold higher than that of full-size chemerin, which has an EC_{50} of 0.1 to 0.2 nM in the same assays. Y^{149}, F^{150}, G^{152}, F^{154}, F^{156} and the terminal carboxyl group were shown as essential moieties for the bioactivity of these peptides. These C-terminal peptides were used for generating binding tracers, such as ^{125}I-YHSFFPGQFAFS, but the stability of these peptides in plasma, cell cultures and *in vivo* is very short (half-life < 10 min[26]), as a result of proteolytic degradation. The requirement for C-terminal proteolytic processing is identical in human and mouse, and the C-terminal nonapeptide of mouse chemerin, although slightly different (L^{148}FPGQFAFS^{156}), displays properties similar to that of human chemerin-9.[21,26] A peptide analog was developed (D-Tyr^{147}-[D-Ser^{151}, D-Ala^{154}, Tic^{155}] mouse chemerin 148-156), displaying good potency (EC_{50}: 22 nM) and a considerable improvement of stability in biological media (half-life > 240 min in plasma).[26] Another peptide derived from the mouse chemerin C-terminus, C15 (A^{140}GEDPHGYFLPGQFA^{154}), was described to display anti-inflammatory properties *in vitro* and *in vivo* through ChemR23.[9,10] However, this peptide was later shown to be devoid of any biological activity toward ChemR23 (see below), using either binding or functional assays.[21,26]

DISTRIBUTION OF THE mRNA/PEPTIDE

Prochemerin is expressed by most tissues. The transcript is detected at high levels in liver, white adipose tissue and placenta, but is also present in skin, adrenal gland, all parts of the gut, pancreas, the airways, and the kidney. Epithelial cells, endothelial cells and fibroblasts were shown to express prochemerin. The precursor is present at relatively high concentrations in plasma. Immunoassays, measuring all (active and inactive) forms of chemerin in serum, have provided averages of 100–200 ng/ml in control subjects according to studies, corresponding to 6–12 nM. Concentrations of bioactive chemerin forms are more troublesome to measure, and rarely assayed. Liver and adipose tissue probably constitute the main contributors to serum (pro)chemerin, and it was suggested that modifications of these levels might arise mostly from production by adipocytes.[15,22] Prochemerin transcripts were also detected in platelets, and chemerin is stored in platelet granules and released after activation by thrombin, collagen or ADP, partially in an activated form.[13]

RECEPTORS AND SIGNALING CASCADES

Three GPCR have been described so far to bind chemerin with high affinity (K_D around 1 nM). The first of these receptors, ChemR23 (also known as CMKLR1), is expressed by a number of leukocyte populations, including monocytes, macrophages, immature myeloid (mDC) and plasmacytoid dendritic cells (pDC) and NK cells. ChemR23 expression is significantly higher in pDCs than in mDCs, and ChemR23 is one of the few functional receptors in pDCs.[28] Chemerin may therefore play an important and nonredundant role in the recruitment of pDCs, which are professional type I interferon producers involved in antiviral responses. Epidermal Langerhans cells do not express ChemR23. ChemR23 is down regulated during maturation of dendritic cells and after stimulation of macrophages by proinflammatory cytokines and TLR ligands, while TGF-β upregulates the receptor in macrophages.[30,28] ChemR23 expression was also described in adipocytes, endothelial cells, osteoclasts, and vascular smooth muscle cells [reviewed in Ref. 33].

ChemR23 was reported to bind another ligand, resolvin E1 (RvE1). RvE1 is an anti-inflammatory lipid mediator, derived from eicosapentaenoic acid, that promotes the resolution of inflammation. RvE1 was described as an agonist of ChemR23, using an assay based on the inhibition of TNF-induced NF-κB activation in HEK293 cells. Subsequent studies have failed to confirm the activity of RvE1 on ChemR23, using binding and diverse functional assays.[21] Besides, ChemR23 is not expressed by neutrophils, the main target of RvE1, while another receptor (BLT1, a leukotriene B4 receptor), was later shown to respond to RvE1[3] and is present in neutrophils. Furthermore, blockade of neutrophil recruitment by RvE1 in a model of zymosan-induced peritonitis was abrogated in BLT1 knockout mice,[3] suggesting that BLT1 is indeed the sole relevant receptor of RvE1.

ChemR23 is coupled to the G_i family of G proteins. In cells expressing this receptor, chemerin inhibits accumulation of cAMP, promotes phospholipase C activation, IP_3 release and calcium mobilization, and activates the PI3K and MAPK pathways, with EC_{50} values in the low or subnanomolar range.[30] All these activities are blocked by Pertussis toxin.

GPR1 was described as a second receptor for chemerin, using a β-arrestin recruitment screening assay (Tango).[4] GPR1, which is structurally related to ChemR23, binds chemerin with high affinity, but signaling is inefficient. Chemerin promotes relatively modest mobilization of Ca^{2+} and MAPK activation in cell lines expressing the receptor, and no signaling mediated by GPR1 has so far been

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described in primary cells. GPR1 is not present in leukocytes, but expressed in the central nervous system, adipose tissue, skeletal muscle and skin. As a receptor poorly signaling through classical intracellular pathways, but able to bind chemerin with high affinity and internalize as a result of this binding, GPR1 might be considered as a decoy receptor or chemerin scavenger. However, the demonstration of such role has not yet been demonstrated.

A third receptor, chemokine (C-C motif) receptor-like 2 (CCRL2, also called CKRX, HCR or CRAM in human, and L-CCR in mice), was described to bind chemerin with a K_D of about 1 nM.[35] CCRL2 is an atypical receptor belonging to the chemokine receptor family, with an unusually high variability across species (51% identity between human and mouse). Chemerin does not seem to promote any signaling pathway through CCRL2, nor internalization of the ligand-receptor complex. It was therefore suggested that CCRL2 might increase local concentrations of chemerin and present the protein to other nearby cells displaying ChemR23 on their surface.[35] CCRL2 is expressed by human monocytes, macrophages, DCs, neutrophils, T cells, NK cells, mast cells and $CD34^+$ bone marrow precursors.[33] The receptor is generally upregulated upon activation of these cells, by LPS or TNF-α.

BIOLOGICAL ACTIONS

Chemerin was first described as a chemoattractant agent, promoting chemotaxis of leukocyte populations that express ChemR23. Macrophages, immature mDCs and pDCs, and NK cells from human and mouse respond to chemerin in chemotaxis assays ex vivo, including in transmigration assays across endothelial monolayers.[30,28,25,21] These cells are also responsive to chemerin in other functional assays, such as calcium mobilization or MAP kinase activation. Maturation of mDCs and pDCs abrogates their functional response to chemerin, which is partially explained by the down regulation of ChemR23 in these cells.[28] Chemerin stimulates adhesion of macrophages to VCAM-1 and fibronectin, through a pathway involving G_i proteins, PI3K, Akt, and p38, and the clustering of the VLA-4 and VLA-5 integrins at the cell surface.[18] Despite its cystatin-like structure, chemerin does not seem to inhibit cysteine proteases.[20] *In vivo*, chemerin was reported to display both pro- and anti-inflammatory actions (see below), depending on the animal model studied. ChemR23 expression in endothelial cells was reported to be upregulated by inflammatory cytokines (TNF-α and IL-6), and chemerin was described to promote angiogenesis.[19]

Chemerin was more recently described as an adipokine regulating adipogenesis and adipocyte metabolism.[15] As stated above, both the protein and its main receptor, ChemR23, are expressed by adipocytes, and upregulated during differentiation from bone marrow mesenchymal stem cells (BMSC) or 3T3-L1 cells. The chemerin/ChemR23 autocrine/paracrine stimulatory loop plays an important role during the early clonal expansion phase of BMSC differentiation into adipocyte precursors, as knock down of chemerin or ChemR23, as well as blocking antibodies, inhibit differentiation of adipocytes in culture. Chemerin upregulation during adipocyte differentiation from BMSCs is correlated to that of the PPARγ transcription factor, the master regulator of adipogenesis.[22] Chemerin synthesis and secretion is increased by TNF-α in 3T3-L1 cells, primary human adipocytes, and mouse fat tissue *in vivo*.[24] Chemerin expression is also promoted by IL-1β in 3T3-L1 cells and brown fat adipocytes, through a pathway involving NFκB and ERK1/2, and free fatty acids were reported to upregulate chemerin expression in adipocytes, through the transcription factor SREBP2.[5]

In mature adipocytes, chemerin regulates key effectors of the glucose and lipid metabolism, including the glucose transporter GLUT4, diacylglycerol acyltransferase (DGAT2), an enzyme involved in triglyceride synthesis, and the adipokines leptin and adiponectin.[15] Chemerin was reported to both promote and inhibit basal or isoproterenol-evoked lipolysis in 3T3-L1 adipocytes or primary cultures of mouse white adipocytes.[15,26] Similarly, the effect of chemerin on glucose metabolism is a matter of debate. On 3T3-L1 adipocytes, a moderate increase of insulin-promoted glucose uptake and IRS-1 phosphorylation was reported after a short stimulation by low concentrations (nM) of chemerin, while longer stimulation by higher chemerin concentrations (μM) decreased insulin-promoted glucose uptake in the same cells.[15] Inhibition of glucose uptake by chemerin was also reported in primary muscle cells, and a moderate increase in glucose intolerance was observed after chemerin treatment in *ob/ob* and *db/db* mice, as a result of decreased glucose uptake in adipose tissue, liver and skeletal muscle.[14,15] Further studies will therefore be required to clarify the discrepancies regarding the effects of chemerin on lipid and glucose metabolism.

ChemR23 is also expressed in endothelial cells and upregulated following stimulation by proinflammatory cytokines, including TNF-α, IL-1β and Il-6.[19] Chemerin promotes proliferation of endothelial cells and the release of metalloproteases (MMP-2 and MMP-9), suggesting a contribution of the chemerin/ChemR23 system in angiogenesis. In fibroblast/endothelial cell cocultures, chemerin promotes the formation of endothelial tubules in a MAPK-dependent manner.[8]

PATHOPHYSIOLOGICAL IMPLICATIONS

As stated above, chemerin is generated as an inactive precursor requiring activation by proteolysis. Prochemerin-processing enzymes are activated during inflammation

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(neutrophil proteases), coagulation, fibrinolysis, and cancer. High levels of bioactive chemerin were therefore found in inflammatory conditions, and ChemR23-expressing cells are recruited in a number of chronic inflammatory diseases.

The presence of chemerin was reported by immunohistochemistry in the endothelium of dermal blood vessels in lupus erythematous skin lesions, and in high endothelial venules of secondary lymphoid organs, suggesting that it is involved in the recruitment of leukocyte populations, particularly pDCs in this disease.[28] Infiltration by ChemR23-expressing pDCs was further reported in the kidney of patients with severe lupus nephritis.[12] Chemerin was found in epithelial cells from proximal tubules and lymphatic endothelium from lupus-affected kidneys, but not in normal kidneys.

The presence of chemerin was also shown in fibroblasts, mast cells and endothelial cells in prepsoriatic skin adjacent to active lesions, and early psoriatic lesions, which are strongly infiltrated by neutrophils and pDCs.[1] Low chemerin expression and low pDC infiltration occurs in chronic plaques. Cultured fibroblasts from psoriatic skin lesions release bioactive chemerin, and elevated serum chemerin concentrations occur in psoriatic patients. These observations support the involvement of the chemerin/ChemR23 system in the early phases of psoriasis development, which are characterized by pDC recruitment and the production of type I interferons. In oral lichen planus, expression of chemerin in dermal endothelium is associated with the co-recruitment of pDCs and NK cells, suggesting that chemerin might promote the important cross-talk between these two cell populations in inflammatory diseases.[25]

Chemerin was described to inhibit the production of inflammatory cytokines by mouse peritoneal macrophages after stimulation by LPS and IFN-γ. Similar inhibition of macrophage activation (but inefficient chemotaxis) was observed with the mouse C15 peptide.[9] C15 was also described to display anti-inflammatory activities at surprisingly low doses in a zymosan-induced peritonitis model in mice, preventing the recruitment of neutrophils and monocytes and the expression of proinflammatory cytokines.[9] The absence of effects of C15 in ChemR23$^{-/-}$ mice suggested a specific activity through this receptor. The same group reported that C15, but not full-size chemerin, could enhance phagocytosis of microbial particles and apoptotic cells by ChemR23$^{+/+}$ (but not ChemR23$^{-/-}$) macrophages.[10] C15 was however shown to be devoid of biological activities toward recombinant ChemR23-expressing cells (using functional and binding assays) and native leukocyte populations expressing the receptor in the hands of other groups, up to μM concentrations,[21,26] casting doubt on the biological activities attributed to this peptide. Although the anti-inflammatory role assigned to chemerin on the basis of these experiments seem to fit with some later studies *in vivo* (see below), the observations made with C15 need to be clarified.

In a model of acute lung injury promoted by lipopolysaccharide instillation into the airways, chemerin displays anti-inflammatory properties in a ChemR23-dependent manner. Co-administration of chemerin with LPS reduces the number of neutrophils recruited to the lung, as well as the production of chemokines (CXCL1) and cytokines (TNF-α, IL-1β, IL-6). Chemerin has no effect in ChemR23 KO mice, and the number of recruited neutrophils is higher than in wildtype mice, suggesting that endogenous production of chemerin dampens the inflammatory response in ChemR23$^{+/+}$ mice.[21] ChemR23$^{-/-}$ mice were also tested in a mouse model of viral pneumonia.[6] Pneumonia virus of mice (PVM) is considered as the mouse counterpart of the human respiratory syncytial virus (RSV), which is a major cause of acute bronchiolitis in infants and young children. ChemR23$^{-/-}$ mice displayed a higher mortality, a severe impairment of lung function, and increased neutrophilic infiltration in the lung. Lower recruitment of pDCs, reduction in type I interferon production and delayed viral clearance were observed in these mice, showing that the chemerin/ChemR23 system is involved in the recruitment of pDCs *in vivo*. Adoptive transfer of ChemR23$^{+/+}$ pDCs restored the type I interferon response, but increased further the morbidity and mortality. Similar observations were made in chimeric mice expressing ChemR23 in bone marrow-derived cells. These observations suggest therefore an anti-inflammatory role of the chemerin/ChemR23 system, which is independent from marrow-derived leukocyte populations. Although the cells and precise mechanisms involved in this process are still unknown, chemerin and its receptor appear to play important roles in the physiopathology of viral pneumonia, and might be considered as a therapeutic target for anti-inflammatory strategies in this frame.

By contrast, chemerin and ChemR23 contribute to the pathogenesis of experimental autoimmune encephalomyelitis (EAE), a model for multiple sclerosis induced by immunization with the myelin oligodendrocyte glycoprotein (MOG). ChemR23 expression is found in microglial cells and infiltrating myeloid DCs, and chemerin upregulation is observed in mice with EAE. ChemR23$^{-/-}$ mice are partially protected, displaying lower leukocytic infiltration of the brain and less clinical signs.[16] Adoptive transfer of MOG-reactive lymphocytes between ChemR23$^{+/+}$ and ChemR23$^{-/-}$ mice show that ChemR23 is involved both in the induction and effector phases of the disease.

The chemerin/ChemR23 system was also studied in a model of chronic obstructive pulmonary disease (COPD) secondary to cigarette smoke exposure.[11] Acute and chronic exposure of mouse airways to cigarette smoke increase the levels of chemerin in the bronchoalveolar lavage (BAL) fluid, while chemerin and ChemR23 transcripts are down regulated in the lung of these mice. In ChemR23$^{-/-}$ mice, proinflammatory chemokine levels in BAL, and recruitment of neutrophils,

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DCs and CD4+ T cells to the lung are decreased after exposure to cigarette smoke. These observations suggest that the chemerin/ChemR23 system contributes significantly to the recruitment of innate and adaptive immune cells to cigarette smoke-exposed lungs and plays a proinflammatory role in this context.

Obesity is frequently associated with a chronic systemic inflammatory state. As adipocytes enlarge, they start to secrete a number of cytokines (TNF-α, IL-6) that are found elevated in the bloodstream, while the adipose tissue becomes infiltrated by various leukocyte populations, particularly macrophages, but also T cells, NK cells and immature mDCs and pDCs. Macrophages also contribute to the production of proinflammatory cytokines that in turn interfere with insulin signaling and promote insulin resistance. Chemerin, as an adipokine able to promote chemotaxis of most leukocyte populations found in adipose tissue from obese individuals, might therefore constitute a link between obesity and inflammation, although this role is not yet formally established. Proteases known to activate prochemerin have been described in adipose tissue,[15] but it is not known presently which forms of chemerin predominate in fat tissue, and whether these forms evolve with the development of obesity.

Chemerin concentrations in human serum are elevated in obese and type 2 diabetic patients, and in a number of studies correlate positively with body mass index (BMI), fasting glucose, fasting insulin, triglycerides, and total cholesterol, and negatively with HDL cholesterol.[15] Serum chemerin concentrations also correlate with levels of TNF-α, IL-6, and C-reactive protein (CRP), it was suggested that they may reflect more the inflammatory status associated with obesity than obesity itself.[29] Serum chemerin does not correlate with the extent of atherosclerosis lesions, but local production of chemerin was suggested to play a role by promoting the recruitment of macrophages to atherosclerotic plaques.[27] Circulating chemerin levels are also increased in nonalcoholic fatty liver disease.[32] In animal models, obesity and diabetes are associated with increased chemerin concentrations in serum.[14] High chemerin levels are found in genetically obese *ob/ob* and *db/db* mice, which are characterized by high TNFα production in adipose tissue,[15,24] and chemerin is also overexpressed in adipose tissue of obese *Psammomys obesus*.[7]

ACKNOWLEDGMENTS

This work performed in the laboratory of the author was supported by the Interuniversity Attraction Poles Programme (P6/14)—Belgian State—Belgian Science Policy, the Actions de Recherche Concertées of the Communauté Française de Belgique, the European Union (LSHB-CT-2005-518167/INNOCHEM), the Fonds de la Recherche Scientifique Médicale of Belgium, and the Walloon Region (Programme d'excellence "CIBLES").

REFERENCES

1. Albanesi C, Scarponi C, Pallotta S, Daniele R, Bosisio D, Madonna S, et al. Chemerin expression marks early psoriatic skin lesions and correlates with plasmacytoid dendritic cell recruitment. *J Exp Med* 2009;**206**:249–58.
2. Allen SJ, Zabel BA, Kirkpatrick J, Butcher EC, Nietlispach D, Handel TM. NMR assignment of human chemerin, a novel chemoattractant. *Biomol NMR Assign* 2007;**1**:171–3.
3. Arita M, Ohira T, Sun YP, Elangovan S, Chiang N, Serhan CN. Resolvin E1 selectively interacts with leukotriene B4 receptor BLT1 and ChemR23 to regulate inflammation. *J Immunol* 2007;**178**:3912–7.
4. Barnea G, Strapps W, Herrada G, Berman Y, Ong J, Kloss B, et al. The genetic design of signaling cascades to record receptor activation. *Proc Natl Acad Sci USA* 2008;**105**:64–9.
5. Bauer S, Wanninger J, Schmidhofer S, Weigert J, Neumeier M, Dorn C, et al. Sterol regulatory element-binding protein 2 (SREBP2) activation after excess triglyceride storage induces chemerin in hypertrophic adipocytes. *Endocrinology* 2011;**152**:26–35.
6. Bondue B, Vosters O, De Nadai P, Glineur S, De Henau O, Luangsay S, et al. ChemR23 dampens lung inflammation and enhances antiviral immunity in a mouse model of acute viral pneumonia1. *PLoS Pathog* 2012;**7**:e40043.
7. Bozaoglu K, Bolton K, McMillan J, Zimmet P, Jowett J, Collier G, et al. Chemerin is a novel adipokine associated with obesity and metabolic syndrome. *Endocrinology* 2007;**148**:4687–94.
8. Bozaoglu K, Curran JE, Stocker CJ, Zaibi MS, Segal D, Konstantopoulos N, et al. Chemerin, a novel adipokine in the regulation of angiogenesis. *J Clin Endocrinol Metab* 2010;**95**:2476–85.
9. Cash JL, Hart R, Russ A, Dixon JP, Colledge WH, Doran J, et al. Synthetic chemerin-derived peptides suppress inflammation through ChemR23. *J Exp Med* 2008;**205**:767–75.
10. Cash JL, Christian AR, Greaves DR. Chemerin peptides promote phagocytosis in a ChemR23- and Syk-dependent manner. *J Immunol* 2010;**184**:5315–24.
11. Demoor T, Bracke KR, Dupont LL, Plantinga M, Bondue B, Roy MO, et al. The role of ChemR23 in the induction and resolution of cigarette smoke-induced inflammation. *J Immunol* 2011;**186**:5457–67.
12. De Palma G, Castellano G, Del Prete A, Sozzani S, Fiore N, Loverre A, et al. The possible role of ChemR23/Chemerin axis in the recruitment of dendritic cells in lupus nephritis. *Kidney Int* 2011;**79**:1228–35.
13. Du XY, Zabel BA, Myles T, Allen SJ, Handel TM, Lee PP, et al. Regulation of chemerin bioactivity by plasma carboxypeptidase N, carboxypeptidase B (activated thrombin-activable fibrinolysis inhibitor), and platelets. *J Biol Chem* 2009;**284**:751–8.
14. Ernst MC, Issa M, Goralski KB, Sinal CJ. Chemerin exacerbates glucose intolerance in mouse models of obesity and diabetes. *Endocrinology* 2010;**151**:1998–2007.
15. Ernst MC, Sinal CJ. Chemerin: at the crossroads of inflammation and obesity. *Trends Endocrinol Metab* 2010;**21**:660–7.
16. Graham KL, Zabel BA, Loghavi S, Zuniga LA, Ho PP, Sobel RA, et al. Chemokine-like receptor-1 expression by central nervous system-infiltrating leukocytes and involvement in a model of autoimmune demyelinating disease. *J Immunol* 2009;**183**:6717–23.
17. Guillabert A, Wittamer V, Bondue B, Godot V, Imbault V, Parmentier M, et al. Role of neutrophil proteinase 3 and mast cell chymase in chemerin proteolytic regulation. *J Leukoc Biol* 2008;**84**:1530–8.

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18. Hart R, Greaves DR. Chemerin contributes to inflammation by promoting macrophage adhesion to VCAM-1 and fibronectin through clustering of VLA-4 and VLA-5. *J Immunol* 2010;**185**:3728–39.
19. Kaur J, Adya R, Tan BK, Chen J, Randeva HS. Identification of chemerin receptor (ChemR23) in human endothelial cells: chemerin-induced endothelial angiogenesis. *Biochem Biophys Res Commun* 2010;**391**:1762–8.
20. Kulig P, Kantyka T, Zabel BA, Banas M, Chyra A, Stefanska A, et al. Regulation of chemerin chemoattractant and antibacterial activity by human cysteine cathepsins. *J Immunol* 2011;**187**:1403–10.
21. Luangsay S, Wittamer V, Bondue B, De Henau O, Rouger L, Brait M, et al. Mouse ChemR23 is expressed in dendritic cell subsets and macrophages, and mediates an anti-inflammatory activity of chemerin in a lung disease model. *J Immunol* 2009;**183**:6489–99.
22. Muruganandan S, Parlee SD, Rourke JL, Ernst MC, Goralski KB, Sinal CJ. Chemerin, a novel peroxisome proliferator-activated receptor γ (PPAR γ) target gene that promotes mesenchymal stem cell adipogenesis. *J Biol Chem* 2011;**286**:23982–95.
23. Nagpal S, Patel S, Jacobe H, DiSepio D, Ghosn C, Malhotra M, et al. Tazarotene-induced gene 2 (TIG2), a novel retinoid-responsive gene in skin. *J Invest Dermatol* 1997;**109**:91–5.
24. Parlee SD, Ernst MC, Muruganandan S, Sinal CJ, Goralski KB. Serum chemerin levels vary with time of day and are modified by obesity and tumor necrosis factor-{alpha}. *Endocrinology* 2010;**151**:2590–602.
25. Parolini S, Santoro A, Marcenaro E, Luini W, Massardi L, Facchetti F, et al. The role of chemerin in the colocalization of NK and dendritic cell subsets into inflamed tissues. *Blood* 2007;**109**:3625–32.
26. Shimamura K, Matsuda M, Miyamoto Y, Yoshimoto R, Seo T, Tokita S. Identification of a stable chemerin analog with potent activity toward ChemR23. *Peptides* 2009;**30**:1529–38.
27. Spiroglou SG, Kostopoulos CG, Varakis JN, Papadaki HH. Adipokines in periaortic and epicardial adipose tissue: differential expression and relation to atherosclerosis. *J Atheroscler Thromb* 2010;**17**:115–30.
28. Vermi W, Riboldi E, Wittamer V, Gentili F, Luini W, Marrelli S, et al. Role of ChemR23 in directing the migration of myeloid and plasmacytoid dendritic cells to lymphoid organs and inflamed skin. *J Exp Med* 2005;**201**:509–15.
29. Weigert J, Neumeier M, Wanninger J, Filarsky M, Bauer S, Wiest R, et al. Systemic chemerin is related to inflammation rather than obesity in type 2 diabetes. *Clin Endocrinol (Oxf)* 2010;**72**:342–8.
30. Wittamer V, Franssen JD, Vulcano M, Mirjolet JF, Le Poul E, Migeotte I, et al. Specific recruitment of antigen-presenting cells by chemerin, a novel processed ligand from human inflammatory fluids. *J Exp Med* 2003;**198**:977–85.
31. Wittamer V, Bondue B, Guillabert A, Vassart G, Parmentier M, Communi D. Neutrophil-mediated maturation of chemerin: a link between innate and adaptive immunity. *J Immunol* 2005;**175**:487–93.
32. Yilmaz Y, Yonal O, Kurt R, Alahdab YO, Eren F, Ozdogan O, et al. Serum levels of omentin, chemerin and adipsin in patients with biopsy-proven nonalcoholic fatty liver disease. *Scand J Gastroenterol* 2011;**46**:91–7.
33. Yoshimura T, Oppenheim JJ. Chemokine-like receptor 1 (CMKLR1) and chemokine (C-C motif) receptor-like 2 (CCRL2); two multifunctional receptors with unusual properties. *Exp Cell Res* 2011;**317**:674–84.
34. Zabel BA, Allen SJ, Kulig P, Allen JA, Cichy J, Handel TM, et al. Chemerin activation by serine proteases of the coagulation fibrinolytic and inflammatory cascades. *J Biol Chem* 2005;**280**:34661–6.
35. Zabel BA, Nakae S, Zuniga L, Kim JY, Ohyama T, Alt C, et al. Mast cell-expressed orphan receptor CCRL2 binds chemerin and is required for optimal induction of IgE-mediated passive cutaneous anaphylaxis. *J Exp Med* 2008;**205**:2207–20.

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Chapter 89

Chemokines

Sunny C. Yung and Joshua M. Farber

ABSTRACT

Chemokines belong to a family of phylogenetically related, small inducible proteins that recruit leukocytes to sites of inflammation and infection. Their name is derived from *chemo*tactic cyto*kines*. In addition to promoting host defense and mediating inflammation, some chemokines can affect tissue repair and organ development, and many can be shown to have antimicrobial properties.

ORGANIZATION OF CHEMOKINES

Chemokines belong to the largest subgroup of cytokines, with approximately 45 present in humans. Chemokine genes are found in vertebrates from teleost fish to humans. Chemokines are classified according to the number and arrangement of conserved cysteine residues. All but two chemokines contain four conserved cysteine residues, and disulfide bonds link C1–C3 and C2–C4[20] (Figure 1). In the four-cysteine group, the first two are adjacent (CC motif, $n=24$), or separated by one amino acid (CXC motif, $n=16$), or by three amino acids (CX3C motif, $n=1$). The XC chemokines ($n=2$) have only two cysteines, without the paired arrangement close to the N-terminus. Sequence identity is <30% between members of different classes, but within the same class, sequence identity ranges from approximately 30% to 99% within species. Most chemokines are cationic with basic isoelectric points and approximately 7–12 kDa in molecular weight.

The CXC chemokines can be divided into ELR positive and ELR negative. ELR denotes glutamatic acid–leucine–arginine amino acids that are N-terminal to C1 in these CXC chemokines. They have >40% amino acid sequence identity, are the principal chemokines for recruiting neutrophils, are angiogenic, and share a receptor, CXCR2.

STRUCTURE OF CHEMOKINES

Tertiary structures of chemokines are similar because of the conserved disulfide bonds. Structural analysis of chemokines reveals a flexible N-terminal region, an N-terminal loop, three antiparallel beta sheets, and a C-terminal alpha helix (Figure 1). Chemokines are believed to interact with their receptors through two domains.[19] The N-region loop of chemokines interacts with the receptor N-domain residues (receptor Site-I), and the N-terminal flexible region of the chemokine associates with the extracellular loops and/or transmembrane residues of the receptor (receptor Site-II).

Although homodimers and aggregation of chemokines are often observed in structural studies, functional analysis of chemokines by *in vitro* assays (calcium flux, chemotaxis, internalization of receptor) shows that at least some chemokines can function as monomers to activate their receptors.[5] Nonetheless, it has been reported that oligomerization is required for binding glycosaminoglycans (GAGs), which is in turn required for recruiting leukocytes *in vivo*. GAGs such as heparan sulfate are highly expressed on cell surfaces and extracellular matrix, and by binding to GAGs, chemokines can form high local concentrations. Site-directed mutagenesis in CCL2, CCL3, and CCL5 showed that mutants incapable of binding GAGs or incapable of forming oligomers could not recruit leukocytes into the peritoneum of mice.[2]

CHEMOKINE RECEPTORS

Chemokines signal by activating seven-transmembrane-domain receptors, and the responses of leukocytes to particular chemokines is determined by their repertoire of chemokine receptors. The chemokine system shows bidirectional promiscuity: Some chemokines can activate more than one chemokine receptor, and many chemokine receptors can be activated by more than one chemokine. In general, however, a receptor binds chemokines within only a single class. Chemokine receptors are denoted by the motif of their ligands (e.g., CXC, CC) + R (for receptor) + a number in approximate order of discovery.[20] One consequence of receptor activation is a change in integrin conformation that leads to the arrest of leukocytes on endothelium and subsequent transendothelial migration. In addition, in the presence of a concentration gradient of chemokine in tissue, signals are produced that lead to cell polarization and directional migration up the gradient of

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00089-0
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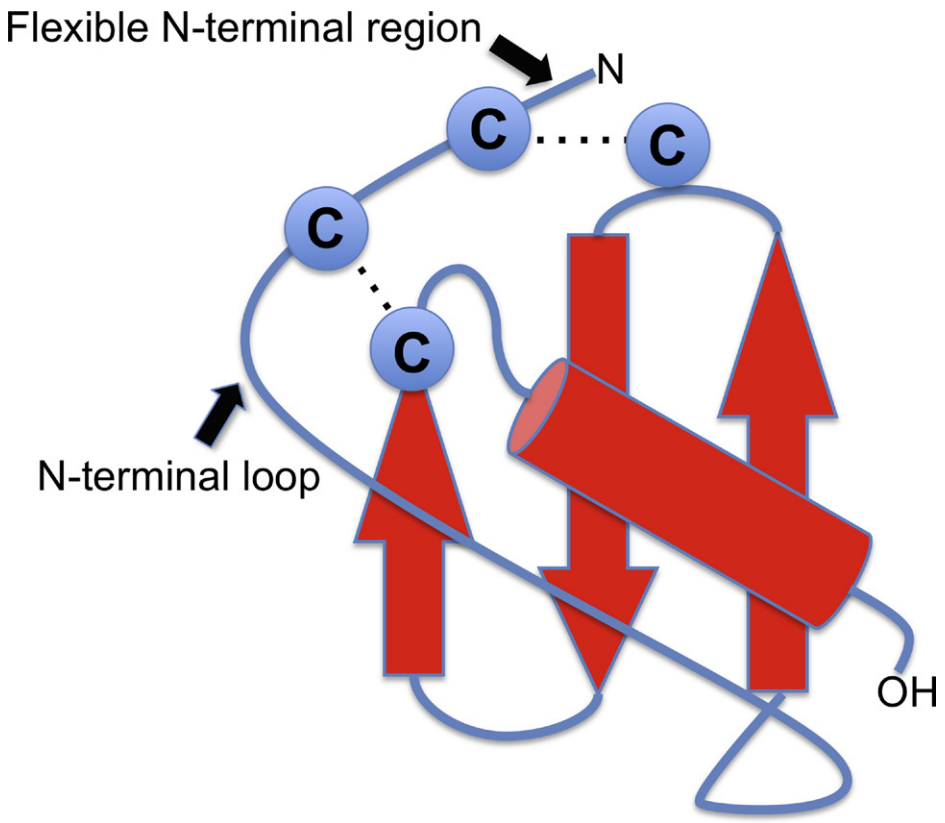

FIGURE 1 Tertiary structure of chemokines. For chemokines with four conserved cysteines, intramolecular disulfide bonds connect cysteines one and three and two and four. The first two cysteines are either adjacent (in CC chemokines) or separated by one residue (in CXC chemokines). The disulfide bonds (dotted lines), flexible N-terminal region, and the N-loop are important for receptor binding and activation. The amphipathic alpha helix (cylinder) is probably important for structural stability and antimicrobial properties. The large arrows represent antiparallel beta sheets. Adapted from Kohidai L. http://en-wikipedia.org/wiki/File:ChtxCheminStr2.png

chemoattractant. In neutrophils, chemokines can also induce the exocytosis of granules.

BIOLOGY OF CHEMOKINES

The principal biological function of the chemokine system is to coordinate the homing and trafficking of hematopoietic cells under homeostatic and inflammatory conditions. However, some chemokines can also regulate the migration of nonhematopoietc cells, affecting organ development and angiogenesis, and chemokines may have activities beyond the control of cell movement. In addition, several chemokines have antimicrobial properties that affect viruses, bacteria, and fungi.

In one commonly used scheme, chemokines are denoted as either homeostatic or inflammatory depending on whether they are constitutively expressed or induced during inflammation—although the distinctions are not absolute.[27] Homeostatic chemokines are important in hematopoiesis and immune surveillance, and, based on experiments in mice, include CXCL12, CXCL13, CCL2, CCL7, CCL19, CCL21, and CCL25. CXCL12 is made constitutively in the bone marrow by stromal cells and is important in myelopoiesis and B cell development. Mice genetically deficient in CXCL12 die close to birth with abnormalities in the cardiac ventricular septum and major vessels to the gastrointestinal tract, as well as in the architecture of the cerebellum. The CCR2 ligands, CCL2 and CCL7, which have prominent roles in recruiting cells to inflammatory sites, are also important for monocyte homeostasis by controlling the movement of monocytes from bone marrow to blood. The CXCR5 ligand, CXCL13, and the CCR7 ligands, CCL19 and CCL21, are important for immune system homeostasis by helping to establish the B and T cell compartments, respectively, within secondary lymphoid organs. CCL25, the ligand for CCR9, is unusual in serving a homeostatic role in a specific peripheral tissue, namely in recruiting cells to the small bowel.

Although CXCL12 is the only chemokine that has a role in vasculogenesis during development, several chemokines also have activity in assays of angiogenesis, such as the rat corneal micropocket assay. ELR$^+$ CXC chemokines (CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7, CXCL8) have scored as proangiogenic, whereas

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ELR⁻ CXC chemokines (CXCL4, CXCL4L1, CXCL9, CXCL10, CXCL11, CXCL14) have angiostatic activities. Blockage of CXCR2, a receptor for all the ELR⁺ CXC chemokines, results in the inhibition of angiogenesis and impaired wound healing. CC chemokines such as CCL2, CCL11, and CCL16 may also participate in angiogenesis.[14]

Effects on angiogenesis is one of the mechanisms proposed for the activities of the chemokine system in cancer, although there are many other ways in which chemokines could influence malignancies. The most extensive result for chemokine receptors in cancer is, again, for CXCR4, which is expressed in >23 human cancers and has been implicated in enhancing tumor cell survival and/or growth and/or metastasis. In general, the expression levels of CXCR4 on cancer cells are correlated with resistance to therapy and poor prognosis. Other chemokine receptors expressed on cancers of hematopoietic and nonhematopoietic origin, such as CCR6 and CCR7, are also believed to influence patterns of metastasis. Given the roles for leukocytes in tumor stroma as both: (1) promoters of cancer in the context of chronic inflammation, and (2) agents of immunologically mediated antitumor activity, the chemokine system is certain to play a broad role in cancer biology.[22]

The majority of chemokines are inducible under inflammatory conditions, often in response to pleiotropic cytokines. These chemokines are involved in host defense (both innate and adaptive immunity), and in acute and chronic inflammation.

CHEMOKINES IN HOST DEFENSE

Host defense against pathogens can be divided into innate and adaptive immune responses. Chemokines are involved in the development and recruitment of immune cells in both systems.

Innate Immunity

Neutrophil Activation and Trafficking

The ELR⁺ CXC chemokines attract and activate neutrophils. There are seven members in this family (CXCL1, 2, 3, 5, 6, 7, and 8). Separate chemokine domains were found to be critical for different aspects of neutrophil activation. Such observations probably stem from roles for these domains in the activation of different chemokine receptors. For example, CXCL8, in contrast to other ELR⁺ CXC chemokines, can bind and activate both CXCR1 and CXCR2 (although affinities for the two receptors differ). Mutational analysis of CXCL8 shows that the ELR domain is critical for neutrophil degranulation (an effect mediated by CXCR1) but not neutrophil chemotaxis (an effect mediated by CXCR2). The C-terminal domain probably also participates in receptor binding, because C-terminal truncation of CXCL8 decreases receptor affinity. Interestingly, a sequence that includes the GP dipeptide and is found just N-terminal to the third conserved cysteine in most ELR+ positive chemokines is active as a chemoattractant and resembles a proteolytic fragment of collagen, PGP, that is able to recruit neutrophils via both CXCR1 and CXCR2.[28]

Monocyte and Macrophage Activation

Historically, CC chemokines were first identified as having chemotactic activity for monocytes and macrophages. Monocyte chemoattractantants (CCL2, CCL7, CCL8, and CCL13) recruit monocytes to sites of trauma, infection, toxin exposure, and ischemia.

Within the blood, there exist subpopulations of monocytes expressing different chemokine receptor profiles. Classical or "inflammatory" monocytes ($CD14^{high}CD16^{-}$ cells) preferentially express CCR2, whereas "resident'' monocytes ($CD14^{low}CD16^{+}$ cells) preferentially express CX3CR1.[11] CCR2 is activated by CCL2, CCL7, and CCL13, and these chemokines recruit inflammatory monocytes to the sites of inflammation. By contrast, the resident-monocyte subset is thought to replace tissue-resident macrophage and dendritic cell populations. The only ligand for CX3CR1 is CX3CL1, an unusual chemokine that is membrane bound and contains a mucin-like stalk.

One "nonimmunologic" process that depends on cells of the innate immune system is wound healing, which involves inflammation, cell proliferation, and tissue remodeling. These steps require the coordinated movement and activities of neutrophils, monocytes, and macrophages. As such, chemokines have an important role in tissue repair. CXCL1 and CXCL8 along with their receptor, CXCR2, are expressed in the human epidermis during inflammation. These chemokines as well as CCL2 and CX3CL1 are involved in leukocyte recruitment and neovascularization during wound repair.[22]

Adaptive Immunity

T cells

Chemokine receptors CCR9, CCR7, and CXCR4 are important in the development of α/β T cells in the thymus. T cells begin as $CD4^{-}CD8^{-}$ and go through a $CD4^{+}CD8^{+}$ stage before becoming mature $CD4^{+}CD8^{-}$ or $CD4^{-}CD8^{+}$ cells. During the double-negative stage, T cells undergo rearrangement of the TCRβ gene and formation of the pre-T cell receptor (TCR), a developmental checkpoint known as β-selection. CXCR4 serves to localize T cell precursors within the thymus during this process and serves as an essential costimulator of pre-TCR signals.[26] CCR9 is a receptor expressed on late double-negative and on

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double-positive T cells. However, as double-positive T cells transition to mature single-positive T cells, CCR9 is downregulated. This regulated expression of CCR9 may be critical for T cell development, because forced transgenic expression of CCR9 resulted in fewer double-positive and single-positive cells. CCL19 and CCL21 are highly expressed in the medulla of the thymus,[30] and their receptor, CCR7, is important for the inward migration of T cells from the cortex to medulla, which occurs during maturation and before the single-positive cells leave the thymus.

Naive T cells migrate through the T cell zones of secondary lymphoid organs, and as discussed above, localization in T cell areas requires CCL19/CCL21/CCR7. After the activation of a naive helper ($CD4^+$) T cell, some progeny migrate to B cell follicles (so-called T_{FH}, or follicular helper cells), which depends on CXCL13 and its receptor CXCR5. Other activated T cells will traffic to peripheral sites. For activated $CD4^+$ T cells, some will differentiate into "lineages" of effector/memory cells defined by patterns of cytokine production—Th1, Th2, and Th17 cells. Each of these subpopulations is associated with certain chemokine responses and chemokine receptors.

Th1 cells, whose signature cytokine is IFN-γ, all express CXCR3. Not all $CXCR3^+$ $CD4^+$ T cells are Th1 cells, however, so that the Th1 population is a subset of the $CXCR3^+$ cells. Analogous relationships pertain for the other lineages. Th2 cells, whose signature cytokine is IL-4, form a subset within the $CCR4^+$ cells, and Th17 cells, whose signature cytokine is IL-17, form a subset within the $CCR6^+$ cells. Of additional interest, the chemokine ligands for these receptors are inducible by the subsets' signature cytokines. CXCL9, CXCL10, and CXCL11, the ligands for CXCR3, are inducible by IFN-γ; CCL17 and CCL22, the ligands for CCR4, are inducible by IL-4; and CCL20, the ligand for CCR6, is inducible by IL-17. These relationships suggest that the chemokine/receptor groups can serve as components of positive feedback loops to establish Th1 or Th2 or Th17 cell accumulations that yield type-specific inflammatory environments.[15,24,25]

B Cells

CXCL12 (ligand for CXCR4) is important for B cell development in the bone marrow. Mice deficient in CXCL12 are defective in B lymphopoiesis in the fetal liver and bone marrow.[21] Naive B cells express high levels of CXCR4 and are attracted to secondary lymphoid organs via CXCL12 (and CCR7 ligands). CXCL13 is crucial for defining B cell follicles, and both B cells and T_{FH} are recruited to the follicles by signaling through CXCR5. In the germinal centers, CXCL12/CXCR4 is again important for the localization of centroblasts to the dark zones, where these antigen-activated B cells are undergoing proliferation and somatic hypermutation important for generating high-affinity antibodies. Finally, CXCL12/CXCR4 is important for the localization of plasma cells to the bone marrow.[1]

CHEMOKINES IN INFLAMMATORY DISEASES

Many diseases, such as rheumatoid arthritis, atherosclerosis, diabetes, chronic obstructive pulmonary diseases, asthma, psoriasis, and multiple sclerosis have major inflammatory components, and, not surprisingly, the chemokine system is involved in each.[4,27] Some of the chemokine activity is through the recruitment of cells of innate immunity. For example, macrophage-rich plaques in atherosclerosis exhibit high expression of CCL2 and deletion of CCL2 decreased diet-induced atherosclerosis in mice. Further, higher transcription of CCL2 resulting from a mutation in the promoter is associated with an increased risk for coronary artery disease. Chemokines are also involved in the autoimmune component of these diseases, because effector/memory T cells, such as the $CD4^+$ T cell subsets discussed above, can express as many as 15 different chemokine receptors in combinatorial fashion to mediate trafficking toward inflammatory chemokines.

CHEMOKINES AS ANTIMICROBIAL PEPTIDES

Certain chemokines have intrinsic antimicrobial properties *in vitro* (Table 1). At micromolar concentrations, and at low salt concentrations, these chemokines can inhibit bacterial and fungal growth. The mechanism is believed to involve the cationic chemokine binding to the negatively charged membrane of the pathogen to disrupt membrane integrity, and electron microscopy shows membrane damage after chemokine exposure. However, as the salt concentration is increased, the antimicrobial effect of chemokines is greatly diminished or disappears. In some respects, chemokines resemble β-defensins, a group of host antimicrobial peptides that are active at mucosal surfaces. Both are cationic peptides with intrachain disulfide bonds and amphipathic regions, and both have antimicrobial and immunomodulatory activities.[29] α- and β-Defensins have been reported to attract leukocytes by acting on chemokine receptors.[23]

In addition to the reports of antimicrobial properties *in vitro*, there is also some recent evidence that these antimicrobial effects can be important in host defense *in vivo*. For example, the related, IFN-γ-inducible chemokines CXCL9, 10, and 11 protect mice against pulmonary infection with *B. anthracis* through a receptor-independent mechanism.[8]

Chemokines have also been reported to block viral infection. The most celebrated example is in the inhibition of cell entry by HIV-1. The experiments identifying the chemokines CCL3, CCL4, and CCL5 as agents able to inhibit

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TABLE 1 Antibacterial and Antifungal Properties of Chemokines from the Literature[7,9,12,16–18,31,32]

Chemokine			MRSA		S. coag neg	*S. pyogenes*		*E. faecium*	*B. subtilis*	*L. monocytogenes*		*E. coli*		*P. aeruginosa*	*C. neoformans*	*C. glabrata*	*C. albicans*
			[Na+]		[Na+ or K+]	[Na+ or K+]		[K+]	[Na+]	[Na+]		[Na+ or K+]		[Na+ or K+]	[Na+ or K+]	[Na+]	[Na+ or K+]
Family	Member	pI	10 mM	137 mM	10 mM	10 mM	150 mM	10 mM	10 mM	10 mM	100 mM	10 mM	100 mM	10 mM	10 mM	10 mM	10 mM
CXC ELR+	CXCL1	10.09	–	–								++++					
	CXCL2	10.45	+	–								+++					
	CXCL3	10.15	+	–								+++					
CXC ELR-	CXCL4	8.71	–	–					–			–			–		
CXC ELR+	CXCL5	11.4	–	–		–						–					
	CXCL6	10.44	++	–		+						+		+			
	CXCL7	8.73	–	–		–			+ (Cleaved)			+ (Cleaved)			+ (Cleaved)	– (Cleaved)	
	CXCL8	9.85	+	–	–							–, +	+				
CXC ELR-	CXCL9	11.1	++++	–		+	+			+	–	++++	–				
	CXCL10	10.77	++++	–		+	+			+	–	++++	–				
	CXCL11	11.1	++++	+		+	+			+	–	+++	–				
	CXCL12	10.48	++++	++								++++					
	CXCL13	10.92	++++	+								++++					
	CXCL14	10.47	–	–	+							++, ++++	+++				+
	CXCL16	8.75	+	–													
CX3C	CX3CL1	10.24	–	+								–					
C	XCL1	11.28	–	–								++++					

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CC	CCL1	10.48	++	–				++++				
	CCL2	9.74	–	–				–				
	CCL3	4.48	–	–				–				
	CCL3L1	4.47	–	–								
	CCL4	4.47	–	+				–				
	CCL5	8.61	–	–				–				
	CCL7	10.33	–	+				–				
	CCL8	9.88	–	–				+				
	CCL11	10.74	–	–				+++				
	CCL13	10.62	+++	++				+++				
	CCL14	8.25	+	–								
	CCL15	8.01	–	–								
	CCL16	9.86	–	–				–				
	CCL17	9.81	–	+				+++				
	CCL18	9.47	+	–				++++				
	CCL19	10.31	+++	–				++++				
	CCL20	10.26	+++	–	+	+	+	++++	++	+	+	+
	CCL21	10.71	+	+				++				
	CCL22	8.98	+	–				++++				
	CCL23	9.09	+	+								
	CCL24	10.76	–	–								
	CCL25	10.92	+++	+				+++				
	CCL26	10.85	++++	+								
	CCL27	9.07	–	–	–	– in 1 mM K		–, +		– in 1 mM K		– in 1 mM K
	CCL28	10.88	++++	–		+ in 1 mM K				+ in 1 mM K		+ in 1 mM K

Gram-positive organisms (methicillin-resistant ***S. aureus*** (MRSA), ***Staphylococcus coagulase*** negative species***, S. pyogenes, E. faecium, B. subtilis, L. monocytogenes***) are arranged on the left side, followed by Gram-negative organisms (***E. coli***, ***P. aeruginosa***) and fungi (***C. neofomans***, ***C. glabrata***, ***C. albicans***). Only for assays with MRSA and ***E. coli*** do the following symbols of inhibition apply: ++++, >80%; +++, 60–79%; ++, 40–59%; +, 20–39%; –, <20%. For all other organisms "+, –/+, and –" denote >50%, 20–50%, and <20% antimicrobial activity, respectively. The "–, +" and "++, ++++" symbols represent discrepancies in antimicrobial properties from the literature. Most antimicrobial assays were performed at low salt concentration (10 mM). The CXCL7 cleaved peptide "(cleaved)" with antimicrobial properties was purified from platelet granules and is a C-terminal processed form. The pI of each chemokine is also shown.

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some strains of HIV-1 contributed to the discovery that chemokine receptors, and in particular CCR5, are essential coreceptors for HIV-1 entry.[3,6] There have been suggestions that increased production of the HIV-blocking chemokines may be effective against the virus *in vivo*, because higher production of CCL3, CCL4, and CCL5 by HIV-antigen-stimulated peripheral blood mononuclear cells has been associated with a more favorable clinical status in HIV/AIDS.[10] There are also recent results for antiviral activity of CXCL10, again by blocking viral entry. CXCL10 binds to heparan sulfate on cell membranes and can prevent dengue virus from infecting cells. Deletion of the *Cxcl10* gene makes mice more susceptible to dengue virus infection.[13]

In summary, the chemokines are a group of phylogenetically and structurally related proteins with diverse biological properties. Both the innate and adaptive immune systems use chemokines to regulate specific leukocyte populations, and chemokines are critical for host defense and inflammation. In addition, chemokines may have more direct effects against pathogens. Given these activities, chemokines could be used to optimize host defense or for immunotherapy, or could be targeted to block inflammatory damage.

REFERENCES

1. Allen CD, Ansel KM, Low C, Lesley R, Tamamura H, Fujii N, et al. Germinal center dark and light zone organization is mediated by CXCR4 and CXCR5. *Nat Immunol* 2004;**5**:943–52.
2. Allen SJ, Crown SE, Handel TM. Chemokine: receptor structure, interactions, and antagonism. *Annu Rev Immunol* 2007;**25**:787–820.
3. Berger EA, Murphy PM, Farber JM. Chemokine receptors as HIV-1 coreceptors: roles in viral entry, tropism, and disease. *Annu Rev Immunol* 1999;**17**:657–700.
4. Charo IF, Ransohoff RM. The many roles of chemokines and chemokine receptors in inflammation. *New Engl J Med* 2006;**354**:610–21.
5. Clark-Lewis I, Kim KS, Rajarathnam K, Gong JH, Dewald B, Moser B, et al. Structure-activity relationships of chemokines. *J Leukoc Biol* 1995;**57**:703–11.
6. Cocchi F, DeVico AL, Garzino-Demo A, Arya SK, Gallo RC, Lusso P. Identification of RANTES, MIP-1 alpha, and MIP-1 beta as the major HIV-suppressive factors produced by CD8+ T cells. *Science* 1995;**270**:1811–5.
7. Cole AM, Ganz T, Liese AM, Burdick MD, Liu L, Strieter RM. Cutting edge: IFN-inducible ELR-CXC chemokines display defensin-like antimicrobial activity. *J Immunol* 2001;**167**:623–7.
8. Crawford MA, Burdick MD, Glomski IJ, Boyer AE, Barr JR, Mehrad B, et al. Interferon-inducible CXC chemokines directly contribute to host defense against inhalational anthrax in a murine model of infection. *PLoS Pathog* 2010;**6**:e1001199.
9. Egesten A, Eliasson M, Johansson HM, Olin AI, Morgelin M, Mueller A, et al. The CXC chemokine MIG/CXCL9 is important in innate immunity against Streptococcus pyogenes. *J Infect Dis* 2007;**195**:684–93.
10. Garzino-Demo A, Moss RB, Margolick JB, Cleghorn F, Sill A, Blattner WA, et al. Spontaneous and antigen-induced production of HIV-inhibitory beta-chemokines are associated with AIDS-free status. *Proc Natl Acad Sci USA* 1999;**96**:11986–91.
11. Gordon S, Taylor PR. Monocyte and macrophage heterogeneity. *Nat Rev Immunol* 2005;**5**:953–64.
12. Hieshima K, Ohtani H, Shibano M, Izawa D, Nakayama T, Kawasaki Y, et al. CCL28 has dual roles in mucosal immunity as a chemokine with broad-spectrum antimicrobial activity. *J Immunol* 2003;**170**:1452–61.
13. Ip PP, Liao F. Resistance to dengue virus infection in mice is potentiated by CXCL10 and is independent of CXCL10-mediated leukocyte recruitment. *J Immunol* 2010.
14. Keeley EC, Mehrad B, Strieter RM. Chemokines as mediators of tumor angiogenesis and neovascularization. *Exp Cell Res* 2011;**317**:685–90.
15. Kim CH, Rott L, Kunkel EJ, Genovese MC, Andrew DP, Wu L, et al. Rules of chemokine receptor association with T cell polarization *in vivo*. *J Clin Invest* 2001;**108**:1331–9.
16. Krijgsveld J, Zaat SA, Meeldijk J, van Veelen PA, Fang G, Poolman B, et al. Thrombocidins, microbicidal proteins from human blood platelets, are C-terminal deletion products of CXC chemokines. *J Biol Chem* 2000;**275**:20374–81.
17. Linge HM, Collin M, Nordenfelt P, Morgelin M, Malmsten M, Egesten A. The human CXC chemokine granulocyte chemotactic protein 2 (GCP-2)/CXCL6 possesses membrane-disrupting properties and is antibacterial. *Antimicrob Agents Chemother* 2008;**52**:2599–607.
18. Maerki C, Meuter S, Liebi M, Muhlemann K, Frederick MJ, Yawalkar N, et al. Potent and broad-spectrum antimicrobial activity of CXCL14 suggests an immediate role in skin infections. *J Immunol* 2009;**182**:507–14.
19. Murphy PM. The molecular biology of leukocyte chemoattractant receptors. *Annu Rev Immunol* 1994;**12**:593–633.
20. Murphy PM, Baggiolini M, Charo IF, Hebert CA, Horuk R, Matsushima K, et al. International union of pharmacology. XXII. Nomenclature for chemokine receptors. *Pharmacol Rev* 2000;**52**:145–76.
21. Nagasawa T, Hirota S, Tachibana K, Takakura N, Nishikawa S, Kitamura Y, et al. Defects of B-cell lymphopoiesis and bone-marrow myelopoiesis in mice lacking the CXC chemokine PBSF/SDF-1. *Nature* 1996;**382**:635–8.
22. Raman D, Sobolik-Delmaire T, Richmond A. Chemokines in health and disease. *Exp Cell Res* 2011;**317**:575–89.
23. Rohrl J, Yang D, Oppenheim JJ, Hehlgans T. Human beta-defensin 2 and 3 and their mouse orthologs induce chemotaxis through interaction with CCR2. *J Immunol* 2010;**184**:6688–94.
24. Singh SP, Zhang HH, Foley JF, Hedrick MN, Farber JM. Human T cells that are able to produce IL-17 express the chemokine receptor CCR6. *J Immunol* 2008;**180**:214–21.
25. Syrbe U, Siveke J, Hamann A. Th1/Th2 subsets: distinct differences in homing and chemokine receptor expression? *Springer Semin Immunopathol* 1999;**21**:263–85.
26. Trampont PC, Tosello-Trampont AC, Shen Y, Duley AK, Sutherland AE, Bender TP, et al. CXCR4 acts as a costimulator during thymic beta-selection. *Nat Immunol* 2010;**11**:162–70.
27. Viola A, Luster AD. Chemokines and their receptors: drug targets in immunity and inflammation. *Annu Rev Pharmacol Toxicol* 2008;**48**:171–97.

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28. Weathington NM, van Houwelingen AH, Noerager BD, Jackson PL, Kraneveld AD, Galin FS, et al. A novel peptide CXCR ligand derived from extracellular matrix degradation during airway inflammation. *Nat Med* 2006;**12**:317–23.
29. Wiesner J, Vilcinskas A. Antimicrobial peptides: the ancient arm of the human immune system. *Virulence* 2010;**1**:440–64.
30. Witt CM, Robey EA. The ins and outs of CCR7 in the thymus. *J Exp Med* 2004;**200**:405–9.
31. Yang D, Chen Q, Hoover DM, Staley P, Tucker KD, Lubkowski J, et al. Many chemokines including CCL20/MIP-3alpha display antimicrobial activity. *J Leukoc Biol* 2003;**74**:448–55.
32. Yung SC, Parenti D, Murphy PM. Host chemokines bind to Staphylococcus aureus and stimulate protein A release. *J Biol Chem* 2011; **286**:5069–77.

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Chapter 90

DP IV/CD26 Effector Peptides

Siegfried Ansorge and Dirk Reinhold

ABSTRACT

Many biologically active peptides of the immune system like cytokines and chemokines and also a number of neuropeptides are generated or inactivated by a concerted action of proteolytic enzymes. One of the most prominent peptidases is the dipeptidyl aminopeptidase IV (DP IV, CD26, EC 3.4.14.5), which is expressed as an ectoenzyme on all lymphocytes and that plays a crucial role in the regulation of the immune response. In this chapter, we describe the activities of biologically active peptides that are regulated by soluble and membrane-bound DP IV/CD26 and that themselves may specifically act on this peptidase and thereby modulate the immune response. Recent results show that the cellular role of DP IV/CD26 is regulated not only via its active site but much more by its newly discovered binding site in the antechamber of the central pore of this peptidase.

INTRODUCTION

The ectoenzyme dipeptidyl peptidase IV (DP IV, E.C. 3.4.14.5) is identical to the leukocyte surface antigen CD26 and belongs to the group of postproline dipeptidyl aminopeptidases, which consist of the gene family of DP IV, FAP (fibroblast activation protein), DP8 and DP9, and DPII (E.C.3.4.14.2). DP IV is a homodimeric Type II transmembranic glycoprotein with a total molecular mass of 220–240 kD. It seems that DP IV is associated with glycolipid- and cholesterol-rich microdomains known as "rafts."[25] FAP (Seprase) is also a dimeric peptidase expressed in the plasma membrane, whereas DP8 and DP9 are soluble, monomeric cytoplasmic proteins.[3,34]

DP IV/CD26 is a serine exopeptidase catalyzing the release of N-terminal dipeptides from oligopeptides and polypeptides preferentially with proline, hydroxyproline, and, with less efficiency, alanine or serine in the penultimate position (Fig. 1). The postproline cleaving substrate specificity makes DP IV/CD26 and the related peptidases relatively unique among other proteases. The crystal structure of DP IV homodimers has been published recently (cf. Refs 2, 34).

Each subunit consists of two domains: an α/β-hydrolase domain and an eight-bladed ß-propeller domain. The active site is accessible via two openings from which the central pore opening in the center of the propeller domain, opposite to the cell membrane, is playing a crucial role in the function of cellular DP IV.[2]

DP IV is ubiquitously distributed and highly expressed in the intestine, kidney, and liver. Lower expression is detectable in nearly every tissue.[1] Functionally relevant is also its localization on the endothelial cells of all organs examined and on intestinal epithelial cells.

Membrane-bound DP IV on the surface of cells is embedded in a number of other membrane proteins, and the activation of cells as T lymphocytes leads to a recruitment of DP IV/CD26 to detergent-insoluble glycolipid-enriched domains or lipid rafts.[25] This raft distribution results in an association of raft-associated signal-transducing molecules (cf. Ref. 2). It is highly probable that, in contrast to the soluble DP IV, the access for peptide substrates to the side pore of membrane DP IV is restricted. Consequently, the main central access in the propeller domain could be the only access to the active site of the enzyme for those substrates. By the use of docking approaches and computational techniques, surprisingly, a special binding site within an antechamber of this central access was identified in our laboratory.[2] This binding site was named "central pore-binding site," which is located approximately 20Å from the active site. It is capable of interacting with a number of peptide substrates and DP IV inhibitors. By using docking approaches, it was found that the most frequent amino acids involved in interaction with inhibitors/ligands are Glu361, Pro362, His363, and Glu 408.[2] By the use of central pore-binding site-selective ligands, it could be shown that the central pore-binding site seems to be the sterical control and binding point that regulates the access of substrates to the active site and DP IV-mediated signal pathways.[2,14]

The unique enzymatic substrate specificity of DP IV results in the peptidase playing a key role in the catabolism

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00090-7
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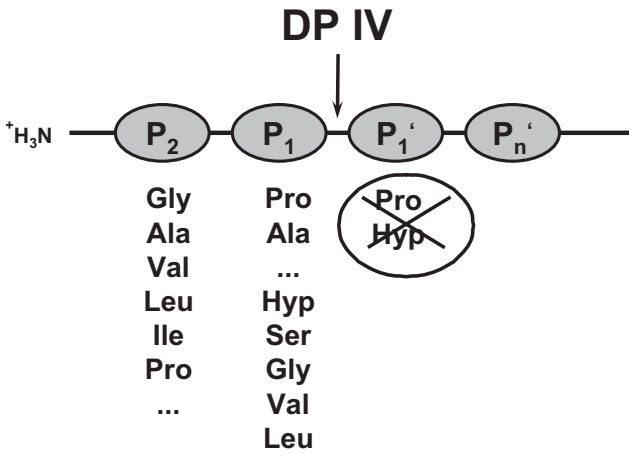

FIGURE 1 Substrate specificity of DP IV.

of a number of neuropeptides, immunopeptides, and peptide hormones, discussed elsewhere in this book, containing the X-Pro or X-Ala amino-terminal sequences, such as substance P, neuropeptide Y, peptide YY, enterostatin, glucose-dependent insulinotropic polypeptide (GIP), and glucagon-like peptide-1 (GLP-1).[9,31] Recently, the emphasis of the research related to DP IV has been concentrated on its ability to cleave a dipeptide from the N-terminus of the GLP-1 (see chapters in Ingestive Peptides and Gastrointestinal Peptides sections). This incretin seems to play an important role in the management of glucose levels. Therefore, GLP-1–based therapies are of potential use in the treatment of Type II diabetes.[9,31] As described in detail above, in addition to the active site of DP IV, which is directly involved in enzymatic hydrolysis of peptides, the central pore-binding site in the antechamber of the central access to the active site of cellular DP IV is involved in the regulation of its cellular function.[2] This holds true particularly for the immune functions of DP IV/CD26.

DP IV/CD26 IN THE IMMUNE SYSTEM

Within the immune system, DP IV/CD26 is expressed on the surface of resting and activated T-cells, activated B-cells, and activated NK cells.[2,14] DP IV/CD26 expression is upregulated after the stimulation of T-cells with mitogen, antigen, anti-CD3 antibodies, or IL–2, after B-cell stimulation with staphylococcal proteins and IL-2 stimulation of NK cells. Thus, activated antigen-specific CD4+T-cell clones express high levels of DP IV/CD26.[24] In 1985, Schön et al.[26] showed for the first time that the inhibitor N-Ala-Pro-O-(nitrobenzoyl)-hydroxylamine, which irreversibly inhibits DP IV, is capable of suppressing the proliferation of human peripheral blood mononuclear cells (PBMCs) stimulated with mitogens. This was the first evidence that DP IV/CD26 plays a critical role in the regulation of DNA synthesis of immune cells and that the enzymatic activity of DP IV is involved in this process.

In the meantime, a multitude of biochemically distinct synthetic compounds inhibiting DP IV and/or DP IV-like enzymes have been studied under different stimulation conditions with a variety of target cells. The most frequent reversible inhibitors used were Lys[Z(NO_2)]-thiazolidide, -piperidide, and -pyrrolidide, which cause a 50% inhibition of DP IV in the range of 0.2–0.5μM. The inhibitory effects of these substances on leukocyte proliferation and cytokine production were confirmed in different mitogen- or antigen-stimulated T-cells and T-cell clones from human and mouse.[24,27,28] Besides their influence on DNA synthesis, inhibitors of DP IV enzymatic activity also exhibit strong suppressive effects on the production of various cytokines. It was shown that Lys[Z(NO_2)]-thiazolidide, -piperidide, and -pyrrolidide inhibit the production of IL-2, IL-5, IL-10, IL-17, IL-12, and IFN-γ of mitogen-stimulated PBMCs and purified T-cells[1,2] as well as the production of IL-2, IL-6, and IL-10 of PHA (phytohemagglutinin)-stimulated mouse splenocytes and Con A (concanavalin A)-stimulated mouse thymocytes (cf. Ref. 14). These inhibitors also reduce in a dose-dependent manner IFN-γ, IL-4, and TNF-α production of myelin basic protein (MBP)-stimulated T-cell clones from MS patients.[24] DP IV inhibitors, on the other hand, elicit the enhanced production and secretion of latent transforming growth factor-ß1 (TGF-ß1), which partially explain the suppressive effects on DNA synthesis and cytokine production.[14,24]

In vivo, the administration of DP IV inhibitors suppresses antibody production in mice after stimulation with bovine serum albumin,[16] and hind paw swelling in an arthritis model in rats,[29] acute rejection in a rat cardiac transplantation model,[15] and experimental autoimmune encephalomyelitis in mice.[28] In all, the results convincingly demonstrate the involvement of DP IV or DP IV-like proteolytical activity in immune functions.

It should be mentioned that in addition to DP IV, lymphocytes do express the cytosolic peptidases DP8 and DP9, which have similar substrate specificity as DP IV. Both are also inhibited by a number of typical DP IV inhibitors and also by ligands that selectively interact with the central pore-binding site but not with the active site of DP IV.[1,2] This means that many DP IV inhibitors are selective for the DP IV family rather than DP IV itself. However, a functional relevance of the cytosolic DP8 and DP9 in regulating growth and cytokine production is difficult to explain.[3] Moreover, it is highly probable that the newly discovered central pore-binding site of DP IV/CD26 is the most crucial target site in the regulation of growth and cytokine production of immune and other cells. Recent results show that combined targeting of DP IV/CD26 and the alanyl-aminopeptidase N (APN/CD13) have much stronger synergistic anti-inflammatory effects compared with DP IV inhibition alone.[1]

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DP IV EFFECTOR PEPTIDES

Although the detailed molecular mechanism of DP IV action in the immune system is not known yet, the most probable explanation for its role lies in the processing of immunorelevant bioactive oligo- and polypeptides and/or the inhibitory effects of DP IV-binding peptides, that is, peptides with N-terminal X-Pro- or X-Ala- motifs, on the enzymatic activity of DP IV itself. During the past years, a number of bioactive peptides were experimentally identified as substrates of DP IV. Most of them belong to the group of chemokines and endocrine factors (Table 1). However, the group of potential DP IV effector peptides is much larger. A number of bioactive polypeptides like cytokines have the N-terminal X-Pro or X-Ala sequence required for cleavage by DP IV but are not processed by soluble DP IV (Table 2). Probably their N-terminus is not susceptible under *in vitro* conditions. The N-terminal peptides of some, if not all, of them are, however, susceptible to DP IV cleavage after endoproteolytical processing of the native peptide.

Moreover, potential DP IV substrates can be also generated from any proteins or peptides, which express the -X-Pro sequence within the peptide chain, namely, via endo- or exo-proteolytical processing. One example is the endogenous peptide hemorphin-7 belonging to the family of atypical opioid peptides released from hemoglobin B-chain sequence 35-41.[4] From this, one can conclude that the DP IV substrate scenario is characterized by a vast number of oligo- and polypeptides, which potentially can act either as substrates, as competitive inhibitors or as ligands for the central pore-binding site.

PRIMARY DP IV EFFECTOR PEPTIDES

In vitro studies on processing of immunorelevant peptides by soluble DP IV have identified a number of candidates where DP IV plays a critical role in regulating their bioactivity (Table 1). *In vivo*, bioactive peptides are acting in nanomolar or picomolar concentration ranges. The physiological relevance of *in vitro* inactivation of such peptides is defined by the physiological efficiency or selectivity constant kcat/Km. Substances with higher kcat/Km values are better substrates than those with lower values.

Chemokines play a fundamental role in the immune system, particularly in traffic and homing of lymphocytes

TABLE 1 The *in vitro* Truncation of Natural Immunorelevant DP IV Peptide Substrates (Primary DP IV Effector Peptides)

Peptide	N-terminus	Residues	k_{cat}/K_m ($10^6 M^{-1}s^{-1}$)	References
CXCL12 (SDF-1α)	KP↓VSLS	68	5	18
CCL22 (MDC)	GP↓YGAN	69	4	18
CXCL11 (I-TAC)	FP↓MFKR	73	1.2	18
CXCL10 (P-10)	VP↓LSRT	77	0.5	18
CXCL9 (Mig)	TP↓VVRK	103	>0.4	18
CCL11 (Eotaxin)	GP↓ASVP	74	0.08	18
CCL5 (RANTES)	SP↓YSSD	68	0.04	18
CCL3L1 (LD78ß)	AP↓LAAD	70	0.003	18
Peptide YY	YP↓IKPE	36	1.9	19
GRP	VP↓LPAG	27	1.8	17
Substance P	RP↓KPQE	11	0.91	20
Neuropeptide Y	YP↓SKPD	36	0.76	17,18
PACAP38	HS↓DGIF	38	0.024	17
VIP	DA↓VFTD	28	0.012	17
PACAP27	HS↓DGIF	27	0.0042	17
Hemorphin-7	YP↓WTQR	7	0.137	4
PCT	AP↓FRSA	116	n.d.	32

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TABLE 2 Secondary Immunorelevant Peptide Substrates and Ligands of DP IV

Peptide	N-terminus	Residues
IL-1ß	AP↓VRSL	153
IL-1Ra	RP↓SGRK	152
IL-2	AP↓TSSS	133
IL-3	AP↓MTQT	133
IL-5	IP↓TEIP	115
IL-8	SA↓KELR	72
IL-10	SP↓GQGT	160
IL-11	AP↓GPPG	179
IL-13	GP↓VPPS	112
G-CSF	TP↓LGPA	177
GM-CSF	AP↓ARSP	127
LIF	SP↓LPIT	180
TNF-ß	LP↓GVGL	171
Thrombopoietin	SP↓APPA	332
MCP-1 (CCL2)	QP↓DAIN	76
MCP-2	QP↓DSVS	76
MCP-3	QP↓DSVS	76
MIP-1α (CCL3)	AS↓ LAAD	70
MIP-1ß (CCL4)	AP↓MGSD	69
IP-10	VP↓LSRT	77
MIF	MP↓MFIV	115
VEGF	AP↓MAEG	189
CNTF	MA↓FTEH	200
aFGF	MA↓EGEI	155
bFGF	MA↓AGSI	155
NT-3	YA↓EHKS	119
OSM	AA↓IGSC	196
PF-4	EA↓EEDG	70
EGF	NS↓DSEC	53
IFN-ß	MS↓YNLL	166
NGF	SS↓SHPI	120

and other immune cells. They bind to specific receptors on the surface of endothelial or immune cells or to the extracellular matrix. Many chemokines exhibit a DP IV susceptible N-terminal sequence, and some of them have been found in a DP IV truncated form in blood and tissues. Therefore, it is highly probable that DP IV-mediated truncation is an important mechanism of regulating chemokinesis.

One of the most rapidly DP IV-processed chemokine **is CXCL12 (stromal-derived factor–1α and -1ß)**. The CXCL12 form found in blood is predominantly the truncated form generated by DP IV cleavage.[5] CXCL12 is also a ligand of the HIV coreceptor CXCR4 and inhibits infection of macrophages by special HIV strains.[7,11,17,18]

CCL11/Eotaxin is a significant factor for the recruitment of eosinophils to inflammatory sites, particularly in allergic reactions. DP IV-mediated processing of CCL11 results in a clear loss of its biological activity.[7] Coincubation of CCL11 with human T-cells shows a processing of full-length CCL11 to CCL11(3–74) reflecting the specific cleavage of CCL11 by DP IV. Intravenous administration of CCL11 in wild-type Fischer 344 rats leads to the mobilization of eosinophils into the blood, peaking at 30min. This mobilization is significantly increased in DP IV–deficient Fischer 344 mutants. This result clearly underscores the role of DP IV in controlling eosinophilia.[11]

Neuropeptide Y (**NPY**) and **peptide YY** (**PYY**) belong to the best natural substrate of DP IV. Several lines of evidence suggest that NPY exerts regulatory effects in local inflammatory response. Lymphocytes express NPY Y1 receptor. It was shown that NPY, PYY, and an NPY Y5 receptor agonists potentiate Con A-induced paw edema in the rat. The DP IV inhibitor Ile-thiazolidide exerted synergistic and potentiating effects *in vivo*. This clearly shows an involvement of DP IV in NPY- and PYY-mediated immune response.[8] Recent data showed that NPY has a bimodal role as a negative regulator of T-cells and as an activator of antigen-presenting cell function[23] and is capable of promoting the production of the immunosuppressive cytokine TGF-β1.[35]

Hemorphins are endogenous peptides belonging to the family of atypical opioid peptides released from sequentially hydrolyzed hemoglobin. Hemorphin-7 (heptapeptide (35–41) of human hemoglobin) has been shown to be capable of inhibiting the inflammatory response in acute but not chronic injury conditions. Inhibition kinetic studies conducted with purified DP IV demonstrated that hemorphin-7 constitutes a good substrate for this enzyme but could also act as a selective competitive inhibitor by substrate binding site competition. Therefore, this group of blood peptides could represent endogenous regulators of DP IV activity.[4]

Substance P (SP) is an undecapeptide that is secreted by nerves and inflammatory cells such as macrophages, lymphocytes, and dendritic cells. It has proinflammatory effects in immune and epithelial cells and plays a crucial role in inflammatory diseases.[21,22] Besides the neutral endopeptidase (NEP) and the angiotensin converting enzyme (ACE), DP IV is one of the most important peptidases involved in the degradation of SP.

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In many studies, it has been reported that **vasoactive intestinal peptide (VIP)** could play an important role in the modulation of the immunological response. VIP can be produced by immunological cells, and the receptors for this neuropeptide are also present in many of these cells. Recent work has demonstrated that VIP is produced by Th2 cells, and that through specific receptors, it exerts immunological functions typically ascribed to Th2 cytokines in nervous and immune systems. VIP strongly reduces the inflammatory response under different conditions. Thus, it was added to the list of endogenous anti-inflammatory agents.[22]

The **pituitary adenylate cyclase-activating polypeptide (PACAP)** is a neuropeptide belonging to the VIP/secretin glucagon family of peptides produced by immune cells, which exerts a wide spectrum of immunological functions controlling the homeostasis of immune system through different receptors expressed on various immunocompetent cells. Like VIP, PACAP has been identified as a potent anti-inflammatory factor that exerts its function by regulating the production of both anti-inflammatory and proinflammatory mediators.[6] DP IV inhibitors are presumed to protect both PACAP and VIP from inactivation and thereby to favor the anti-inflammatory status.

The tetradecapeptide **gastrin-releasing peptide (GRP)** is the mammalian homolog of bombesin and has been shown to affect cytokine production and release from various immune cells. It is present in joint fluids in rheumatoid arthritis and correlates clearly with proinflammatory cytokines.[12]

Procalcitonin (PCT), a protein of 116 amino acids, was discovered as a prohormone of calcitonin produced by C-cells of the thyroid gland. Circulating levels of PCT in healthy subjects are below the detection limit. Blood concentration of PCT is increased in systemic inflammation, especially when this is caused by bacterial infection, such as sepsis.[10] Recently, it was found that not PCT(1–116) but only the truncated form, lacking the dipeptide Ala–Pro, PCT (3–116), is present in the serum of patients with bacterial sepsis and that this DP IV-catalyzed processing of PCT can be imitated also *in vitro*.[32]

SECONDARY IMMUNORELEVANT DP IV PEPTIDE SUBSTRATES

A number of cytokines, chemokines, endocrine factors, and other bioactive peptides exhibit X-Pro or X-Ala sequences at the N-terminus (Table 2) but were not processed by soluble DP IV under *in vitro* conditions.[13,14]

A number of candidates are yet to be studied with respect to their DP IV susceptibility *in vitro*. In the context of DP IV action, it is of interest that biological activity is changed strongly by short deletions and mutations at the N-terminal part of the bioactive peptide [cf. Ref. 13]. Using IL-2 oligopeptides with different chain lengths, it was shown that the soluble DP IV-catalyzed hydrolysis rate was negatively correlated to their chain length.[13] Similar effects were observed with IL-6 and other peptides.[13] Obviously, the free and flexible N-terminus of the peptide is required. Probably, binding of the bioactive peptide to the receptor could change conformation and susceptibility.

It should be mentioned, however, that all data on hydrolysis of these peptides available as yet were generated with soluble DP IV, which represents <1% of the total DP IV and not with the cellular DP IV.

Moreover, it is also not known how strong native peptides do interact with the central pore-binding site of cellular DP IV/CD26 and whether these interactions have a direct effect on cellular functions after binding to this site. Docking results from our laboratory show the binding of shorter N-terminal hexapeptides of some of these peptides to the central pore-binding site of DP IV/CD26, which suggests a functional role of these natural peptides via this binding site. This would be a totally new mechanistic aspect on the role of DP IV/CD26 in the regulation of growth and inflammatory processes.

Nevertheless, it should be taken also into consideration that, in any case, soluble DP IV-resistant bioactive peptides can be processed by endoproteases *in vivo* to suitable substrates for DP IV, which are then capable of acting as competitive inhibitors and immune suppressors. The large number of such peptides makes them a powerful natural tool in the regulation of immune response via DP IV/CD26 *in vivo*.

PEPTIDE INHIBITORS OF DP IV

In contrast to the increasing number of nonpeptide inhibitors of DP IV,[9,31] the examples of natural peptide inhibitors already studied are limited (Table 3). One should remember, however, that the above-discussed peptide substrates of DP IV can principally act also as competitive inhibitors of this peptidase. This means that the number of potential DP IV inhibitors with proline in the second position is much larger. A special feature of DP IV inhibition is the finding that peptides with proline in the third position can also act as potent inhibitors of DP IV particularly if tryptophan is present in the second position of the N-terminus (Table 3). Examples are Tat protein and its derivatives of the human immunodeficiency virus 1.[33]

Peptides described in this chapter probably represent only a small portion of the total number of peptide candidates related to DP IV. Future work will show that the number of immunorelevant peptides that are actively (DP IV inhibitors as suppressor peptides) or passively (cytokines and chemokines as instructor peptides) involved in DP IV/Cd26-mediated immune regulation is much larger.

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TABLE 3 DP IV Peptide Inhibitors

Peptide name	Peptide sequence	Ki value [μM]	References
Diprotin A	IPI	2.2	30
Diprotin B	VPV	7.6	30
Hemorphin 7	YPWTQR	n.d.	4
HIV-Tat(1–86)	MDPVDP	n.d.	33
Tat(1–9)	MDPVDP	267	33
Trp^2-Tat(1–9)	MWPVDP	2.12	33
TXA2-R(1–9)	MWPNGS	5.06	33
Met-IL-2(1–6)	MAPTSS	100	13

REFERENCES

1. Ansorge S, Bank U, Heimburg A, Helmuth M, Koch G, Tadje J, et al. Recent insights into the role of dipeptidyl aminopeptidase IV (DPIV) and aminopeptidase N (APN) families in immune functions. *Clin Chem Lab Med* 2009;**47**:253–61.
2. Ansorge S, Nordhoff K, Bank U, Heimburg A, Julius H, Breyer D, et al. Novel aspects of cellular action of dipeptidyl peptidase IV/CD26. *Biol Chem* 2011;**392**:163–8.
3. Bank U, Heimburg A, Wolfarth A, Koch G, Nordhoff K, Julius H, et al. Outside or inside—role of the sub-cellular localization of DPIV-like enzymes for substrate conversion and inhibitor effects. *Biol Chem* 2011;**392**:169–87.
4. Cohen M, Fruitier-Arnaudin I, Piot JM. Hemorphins: substrates and/or inhibitors of dipeptidyl peptidase IV. Hemorphins N-terminus sequence influence on the interaction between hemorphins and DPPIV. *Biochimie* 2004;**86**:31–7.
5. De La Luz Sierra M, Yang F, Narazaki M, Salvucci O, Davis D, Yarchoan R, et al. Differential processing of stromal-derived factor-1alpha and stromal-derived factor-1beta explains functional diversity. *Blood* 2004;**103**:2452–9.
6. Delgado M, Abad C, Martinez C, Juarranz MG, Leceta J, Ganea D, et al. PACAP in immunity and inflammation. *Ann N Y Acad Sci* 2003;**992**:141–57.
7. De Meester I, Lambeir AM, Proost P, Scharpe S. Dipeptidyl peptidase IV substrates. An update on *in vitro* peptide hydrolysis by human DPPIV. *Adv Exp Med Biol* 2003;**524**:3–17.
8. Dimitrijevic M, Stanojevic S, Vujic V, Kovacevic-Jovanovic V, Beck-Sickinger A, Demuth H, et al. Effect of neuropeptide Y on inflammatory paw edema in the rat: involvement of peripheral NPY Y1 and Y5 receptors and interaction with dipeptidyl-peptidase IV (CD26). *J Neuroimmunol* 2002;**129**:35–42.
9. Evans DM. Dipeptidyl peptidase IV inhibitors. I. *Drugs* 2002;**5**:577–85.
10. Florianczyk B. Structure and diagnostic value of procalcitonin. *Ann Univ Mariae Curie Sklodowska* 2003;**58**:338–42.
11. Forssmann U, Stoetzer C, Stephan M, Kruschinski C, Skripuletz T, Schade J, et al. Inhibition of CD26/dipeptidyl peptidase IV enhances CCL11/eotaxin-mediated recruitment of eosinophils in vivo. *J Immunol* 2008;**181**:1120–7.
12. Grimsholm O, Rantapaa-Dahlqvist S, Forsgren S. Levels of gastrin-releasing peptide and substance P in synovial fluid and serum correlate with levels of cytokines in rheumatoid arthritis. *Arthritis Res Ther* 2005;**7**:R416–26.
13. Hoffmann T, Faust J, Neubert K, Ansorge S. Dipeptidyl peptidase IV (CD 26) and aminopeptidase N (CD 13) catalyzed hydrolysis of cytokines and peptides with N-terminal cytokine sequences. *FEBS Lett* 1993;**336**:61–4.
14. Kähne T, Lendeckel U, Wrenger S, Neubert K, Ansorge S, Reinhold D. Dipeptidyl peptidase IV: a cell surface peptidase involved in regulating T cell growth (review). *Int J Mol Med* 1999;**4**:3–15.
15. Korom S, De Meester I, Stadlbauer TH, Chandraker A, Schaub M, Sayegh MH, et al. Inhibition of CD26/dipeptidyl peptidase IV activity in vivo prolongs cardiac allograft survival in rat recipients. *Transplantation* 1997;**63**:1495–500.
16. Kubota T, Flentke GR, Bachovchin WW, Stollar BD. Involvement of dipeptidyl peptidase IV in an in vivo immune response. *Clin Exp Immunol* 1992;**89**:192–7.
17. Lambeir AM, Durinx C, Proost P, Van Damme J, Scharpe S, De Meester I. Kinetic study of the processing by dipeptidyl-peptidase IV/CD26 of neuropeptides involved in pancreatic insulin secretion. *FEBS Lett* 2001;**507**:327–30.
18. Lambeir AM, Proost P, Durinx C, Bal G, Senten K, Augustyns K, et al. Kinetic investigation of chemokine truncation by CD26/dipeptidyl peptidase IV reveals a striking selectivity within the chemokine family. *J Biol Chem* 2001;**276**:29839–45.
19. Mentlein R, Dahms P, Grandt D, Krüger R. Proteolytic processing of neuropeptide Y and peptide YY by dipeptidyl peptidase IV. *Regul Pept* 1993;**49**:133–44.
20. Nausch I, Heymann E. Substance P in human plasma is degraded by dipeptidyl peptidase IV, not by cholinesterase. *J Neurochem* 1985;**44**:1354–7.
21. O'Connor TM, O'Connell J, O'Brien DI, Goode T, Bredin CP, Shanahan F. The role of substance P in inflammatory disease. *J Cell Physiol* 2004;**201**:167–80.
22. Pozo D, Delgado M. The many faces of VIP in neuroimmunology: a cytokine rather a neuropeptide? *FASEB J* 2004;**18**:1325–34.
23. Prod'homme T, Weber MS, Steinman L, Zamvil SS. A neuropeptide in immune-mediated inflammation, Y? *Trends Immunol* 2006;**27**:164–7.
24. Reinhold D, Hemmer B, Gran B, Born I, Faust J, Neubert K, et al. Inhibitors of dipeptidyl peptidase IV/CD26 suppress activation of human MBP-specific CD4+ T cell clones. *J Neuroimmunol* 1998;**87**:203–9.
25. Salgado FJ, Lojo J, Alonso-Lebrero JL, Lluis C, Franco R, Cordero OJ, et al. A role for interleukin-12 in the regulation of T cell plasma membrane compartmentation. *J Biol Chem* 2003;**278**:24849–57.
26. Schön E, Mansfeld HW, Demuth HU, Barth A, Ansorge S. The dipeptidyl peptidase IV, a membrane enzyme involved in the proliferation of T lymphocytes. *Biomed Biochim Acta* 1985;**44**:K9–15.
27. Schön E, Born I, Demuth HU, Faust J, Neubert K, Steinmetzer T, et al. Dipeptidyl peptidase IV in the immune system. Effects of specific enzyme inhibitors on activity of dipeptidyl peptidase IV and proliferation of human lymphocytes. *Biol Chem Hoppe Seyler* 1991;**372**:305–11.
28. Steinbrecher A, Reinhold D, Quigley L, Gado A, Tresser N, Izikson L, et al. Targeting dipeptidyl peptidase IV (CD26) suppresses autoimmune encephalomyelitis and up-regulates TGF-beta 1 secretion in vivo. *J Immunol* 2001;**166**:2041–8.

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29. Tanaka S, Murakami T, Nonaka N, Ohnuki T, Yamada M, Sugita T. Anti-arthritic effects of the novel dipeptidyl peptidase IV inhibitors TMC-2A and TSL-225. *Immuno-pharmacology* 1998;**40**:21–6.
30. Umezawa H, Aoyagi T, Ogawa K, Naganawa H, Hamada M, Takeuchi T. Diprotins A and B, inhibitors of dipeptidyl aminopeptidase IV, produced by bacteria. *J Antibiot* 1984;**37**:422–5.
31. Weber AE. Dipeptidyl peptidase IV inhibitors for the treatment of diabetes. *J Med Chem* 2004;**47**:4135–41.
32. Wrenger S, Kähne T, Bohuon C, Weglöhner W, Ansorge S, Reinhold D. Amino-terminal truncation of procalcitonin, a marker for systemic bacterial infections, by dipeptidyl peptidase IV (DP IV). *FEBS Lett* 2000;**466**:155–9.
33. Wrenger S, Faust J, Mrestani-Klaus C, Fengler A, Stöckel-Maschek A, Lorey S, et al. Down-regulation of T cell activation following inhibition of dipeptidyl peptidase IV/CD26 by the N-terminal part of the thromboxane A2 receptor. *J Biol Chem* 2000;**275**:22180–6.
34. Yu DM, Yao TW, Chowdhury S, Nadvi NA, Osborne B, Church WB, et al. The dipeptidyl peptidase IV family in cancer and cell biology. *FEBS J* 2010;**277**:1126–46.
35. Zhou J-R, Xu Z, Jiang C-L. Neuropeptide Y promotes TGF-ß1 production in RAW264.7 cells by activating PI3K pathway via Y1 receptor. *Neurosci Bull* 2008;**24**:155–9.

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Chapter 91

FPR Ligands

Ying Liu, Keqiang Chen and Ji Ming Wang

ABSTRACT

Formyl peptide receptors (FPRs) belong to the classical G protein-coupled chemoattractant receptor family. They are mainly expressed in mammalian phagocytic leukocytes and play important roles in inflammatory and immune responses. *N*-formyl peptides produced by Gram-negative bacteria were among the first chemotactic factors identified for two FPRs in human beings: FPR1 (originally termed FPR) and FPR2 (originally termed FPRL1). During the past few years, a variety of novel pathogen- and host-derived agonists as well as antagonists for the FPR family have been identified, indicating a broader spectrum of the biological significance of these receptors. Activation of FPRs in leukocytes by agonists induces cell chemotaxis, phagocytosis, release of proinflammatory mediators and gene transcription. Despite these new developments, the *in vivo* functions of FPRs and their ligands in disease states are not yet fully understood. This chapter summarizes the pharmacological characterization of FPR ligands and their implications in pathophysiological conditions.

INTRODUCTION

Inflammation is essentially a protective attempt by the organisms to remove the injurious stimuli and to initiate the healing process. The migration of leukocytes is crucial in the progression and resolution of inflammation and is tightly regulated. Leukocytes accumulate at the sites of inflammation by responding to pathogen- and host-derived chemotactic factors named chemoattractants. Numerous chemoattractants have been described including chemokines, activated complement fragments, lipid mediators, and pathogen-derived molecules.[32]

Most chemoattractants use G protein-coupled receptors (GPCRs) classified as GPCRs for chemokines and for classical chemoattractants. Formyl peptide receptors (FPRs) belong to the family of classical GPCRs. These receptors are named based on their capacity to recognize the *N*-formyl methionine motif in synthetic neutrophil chemotactic peptides and in peptide fragments released into the culture supernatants by *Escherichia coli.*[32] The FPR family members are coupled to pertussis toxin (PTX)-sensitive Gi proteins and mediate neutrophil and monocyte chemotaxis and activation.[11] Although FPRs were identified and cloned in the early 1990s,[19] the biological function of these receptors remains poorly understood. However, with the identification of a number of novel nonformylated and host-derived agonists for FPRs, it is becoming evident that these receptors and their peptide ligands play important roles in innate and adaptive immune responses.

FORMYL PEPTIDE RECEPTORS (FPRs)

The prototype human FPR was cloned from differentiated HL-60 myeloid leukemia cells.[32] Two additional human FPRs were subsequently cloned and were named as FPR like-1 (FPRL1) and FPRL2. Recently, these GPCRs were renamed as FPR1, FPR2/ALX (FPRL1), and FPR3 (FPRL2). FPR1 and FPR2 share 69% identity at the amino acid level, whereas FPR3 has 56% amino acid sequence identity to human FPR1 and 83% to FPR2.[32] Both human FPR1 and FPR2 are expressed in many cell types, such as myeloid cells including monocytes/macrophages, neutrophils and immature dendritic cells (DCs), endothelial cells, hepatocytes, astrocytes, microglial cells, and fibroblasts.[32] The expression of human FPR3 is more restricted and is detected in monocytes/macrophages and DCs but not in neutrophils.[32] FPR3 is also expressed in plasmacytoid DCs and is upregulated on cell maturation.[10] The signal transduction pathways of FPRs have been more extensively studied with FPR1 in phagocytic cells and in cell lines transfected with cloned receptor. The interaction of FPR1 with its agonist peptide fMLF uncouples trimeric G proteins to elicit a signaling cascade that activates phospholipase C (PLC), protein kinase C (PKC) and PI3 kinases (PI3Ks), which are translated into biological functions of receptor-bearing cells including chemotaxis and mediator release. FPR1 and FPR2 also crosstalk with some receptor tyrosine kinases, such as nerve growth factor (NGF) receptor TrkA and the epidermal growth factor receptor (EGFR).[12,15]

In the mouse FPR gene family, there are at least eight members, *Fpr1* and *Fpr-rs1* to *Fpr-rs7*.[32] *Fpr1* encodes mFPR1, which is the ortholog of human FPR1 and binds fMLF with a relatively high affinity, whereas both *Fpr-rs1* and *Fpr-rs2* encode receptors most similar to human FPR2.[32] The gene product of mouse *Fpr-rs2*, mFPR2, is a

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00091-9
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low-affinity receptor for fMLF.[19] The biological functions of other six mouse *Fpr* gene homologs have not been characterized.[32] The high sequence divergence between species orthologs (~25–30% between human and mouse) indicates the complicated evolution of the FPR gene family.[32] *In vivo*, mFPR1-KO mice were more susceptible to infection by *Listeria monocytogenes*, indicating the role of FPR1 in antibacterial defense.[32] More recently, FPR1 (mFPR1) was shown to mediate trauma reaction in severe inflammatory response syndrome[34] and neutrophil accumulation in the necrotic center of the liver injury, presumably by responding to N-formylated mitochondrial peptides released by ruptured cells.[24] mFPR2-KO mice have also been generated recently and show diminished allergic airway inflammation and accompanying immune responses.[2] FPR2 (mFPR2) expressed in human and mouse neutrophils interacts with an acute-phase protein serum amyloid A (SAA) produced by liver and also by melanoma cells to promote the release of the anti-inflammatory cytokine IL-10, which increases the interaction of neutrophils with invariant natural killer (NK) T cells. These NKT cells limit the immunosuppressive activity of neutrophils by decreasing IL-10, but increasing IL-12 production, thereby enhancing the anti-melanoma host responses.[8] These new developments highlight the critical function of FPR1 and FPR2 in innate and adaptive host responses.

FPR AGONISTS

FPRs recognize many agonists including peptides and nonpeptides. The peptides comprise three subtypes, pathogen-derived, host-derived and synthetic. The nonpeptides are either synthetic or derived from the host[32] (Table 1).

TABLE 1 The Agonists of FPRs

Agonists	Origin	Receptors
Peptides		
Bacteria-derived *N*-formyl peptides		
N-formyl-MLF	*E. coli*	FPR1, FPR2
N-formyl-MIFL	*S. aureus*	mFPR1
N-formyl-MIVIL	*L. monocytogenes*	FPR1, FPR2
N-formyl-MIGWI	*L. monocytogenes*	FPR1, FPR2
N-formyl-MIVTLF	*L. monocytogenes*	FPR1, FPR2
N-formyl-MIGWII	*L. monocytogenes*	FPR1
N-formyl-MFEDAVAWF	*M. avium*	FPR1
Mitochondria-derived *N*-formyl peptides		
N-formyl-MMYALF	Mitochondria, ND6	FPR1, FPR2
N-formyl-MLKLIV	Mitochondria, ND4	FPR1, FPR2
N-formyl-MYFINILTL	Mitochondria, ND1	FPR2
N-formyl-MFADRW	Cytochrome *c* oxidase subunit	FPR1, FPR2
N-formyl-Nle-LF-Nle-YK	Synthetic	FPR1, FPR2
Mitocryptide-2 (MCT-2)	Mitochondria cytochrome b	FPR2
Microbe-derived peptides		
Hp (2–20)	*H. pylori*	FPR2, FPR3
T20 (DP178)	HIV-1 gp41 aa. 643–678	FPR1
T21 (DP107)	HIV-1 gp41 aa. 558–595	FPR2
V3 peptide	HIV-1 gp120, V3 loop	FPR2
N36 peptide	HIV-1 gp41 aa. 546–581	FPR2
F peptide	HIV-1 gp120 aa. 414–434	FPR2
gG-2p20	Herpes simplex virus type 2	FPR1
N-formyl HKU-1 coronavirus peptide	Respiratory syndrome coronavirus	FPR1

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TABLE 1 The Agonists of FPRs—*Cont'd*

Agonists	Origin	Receptors
Host-derived peptides		
CKβ8–1 (human CCL23)	Chemokine	FPR2, CCR1
SHAAGtide	CCL23 *N*-terminal 18 aa.	FPR2, CCR1
Humanin (HN)	Neuroprotective peptide	FPR2, FPR3
F2L	Heme-binding protein	FPR2, FPR3
SAA	Acute-phase protein	FPR2
Annexin 1 / lipocortin 1	Glucocorticoid-regulated protein	FPR1
Ac1-25	Annexin 1	FPR1, FPR2, FPR3
Ac2–26	Annexin 1	FPR1, FPR2, FPR3
Ac9–25	Annexin 1	FPR1, FPR2
Antiflammin-2 (AF2)	Annexin 1	FPR2
Aβ (1–42)	Amyloid precursor	FPR2
D2D3	uPAR (88–274)	FPR2
SRSRYp	D2D3	FPR1
LL-37	Cathelicidin	FPR2
PrP (106–126)	Prion protein	FPR2
Temporin A	*Rana temporaria*	FPR2
PACAP27	Pituitary adenylate cyclase activating polypeptide	FPR2
Agonists from peptide library		
WKYMVm	Peptide library	FPR1, FPR2, FPR3
WKYMVM	Peptide library	FPR2, FPR3
MMK-1	Peptide library	FPR2
MMWLL, formyl-MMWLL	Peptide library	FPR1
CGEN-855A	Peptide library	FPR2
Nonpeptides		
Host-derived nonpeptides		
Lipoxin A4 and aspirin-triggered lipoxins	Eicosanoids	FPR2, AhR
Agonists from nonpeptide library		
Quinazolinone derivative (Quin-C1)	Combinatorial library	FPR1, FPR2
Pyrazolone, 4-iodo-substituted, no. 43	Combinatorial library	FPR1, FPR2
AG-14	Drug-like molecule library	FPR1
Compound 1 and 2	Arylcarboxylic acid hydrazide derivatives	FPR2
Others		
PD168368	Gastrin-releasing	FPR1, FPR2,
PD176252	peptide/neuromedin B receptors (BB1/BB2)	FPR1, FPR2, FPR3
Trp-and Phe-based analogs	PD168368/ PD176252	FPR1, FPR2, FPR3
Related nonpeptide/nonpeptoid analogs	PD168368/ PD176252	FPR1, FPR2
A-71623	Cholecystokinin-1 receptor agonist	

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Peptides

Microbial Peptides

Synthetic or *E. coli*-derived fMLF is most widely used to characterize the function of FPR1 and FPR2. The chemotactic activity of fMLF for neutrophils is increased by an oligoethylene glycol substituent.[16] In addition to its chemotactic activity for myeloid cells, fMLF also induces intestinal epithelial cell migration and participates in epithelial restitution and wound closure.[32] The activity of fMLF on intestinal epithelial cells is attributed to FPR1, which is located along actin filaments in lamellipodial and filopodial extrusions associated with activated PI3K, Rac1, and Cdc42.[32] In human mesenchymal stem cells, fMLF stimulates signaling pathways coupled to FPR1 that drive phospholipase C (PLC)/phospholipase D (PLD)-Ca^{2+}-calmodulin-dependent kinase II-ERK-CREB during osteogenic differentiation. By contrast, fMLF inhibits the expression of PPARγ1 and suppresses adipocytic commitment during differentiation.[28] Thus, FPR1 and its agonist fMLF also display important functions in nonhematopoietic cells. In addition to fMLF, there are other *N*-formyl peptides derived from bacteria. For example, a pentapeptide fMIVIL from *L. monocytogenes* and a tetrapeptide fMIFL from *Staphylococcus aureus* preferentially activate mFPR1.[32] fMFEDAVAWF derived from *Mycobacterium avium* also activates FPR1.[32]

Although formylated peptides are believed to be mainly of bacterial origin, three hexapeptides fMLKLIV, fMMYALF, and fMFADRW corresponding to the N terminus of mitochondrial NADH dehydrogenase subunits 4 and 6 and cytochrome *c* oxidase subunit I[32] are also potent chemotactic agonists for FPR1 and FPR2. Other mitochondrial *N*-formyl peptides (e.g. mitochondrial transcription factor A (TFAM) released during cell necrosis) also activate monocytes to release IL-8 (CXCL8), a neutrophil specific chemokine.[4]

Mitochondria are considered to be evolutionary endosymbionts derived from bacteria and may thus contain bacterial molecular motifs. On cell injury, mitochondrial "damage"-associated molecular patterns (DAMPs) are released into the circulation and elicit neutrophil-mediated organ injury. The effect of mitochondrial DAMPs (MTDs) containing formyl peptides on neutrophils is mediated by FPR1, while mitochondrial DNA in MTDs activates Toll-like receptor (TLR) 9.[34] Some functional "cryptic" peptides hidden in protein structures are termed "cryptides," one of which, mitocryptide-2 (MCT-2) in mitochondrial cytochrome *b*, was a specific agonist for FPR2 and may play a role in inflammatory host responses.[27]

Non-formylated Peptides

Many peptides without *N*-formyl group have been identified as agonists for FPRs. A *Helicobacter pylori* peptide Hp (2–20) activates FPR2 and FPR3 and may contribute to the development of pyloritis by the recruitment of monocytes and basophils to the gastric mucosa in response to bacterial infection.[19] Hp (2–20) also induces the migration, proliferation, and the expression of vascular endothelial growth factor (VEGF) in gastric epithelial cells, suggesting that this *H. pylori* peptide may promote gastric mucosal healing.[32]

HIV-1 envelope proteins contain segments capable of interacting with either or both FPR1 and FPR2, including at least three domains in gp41, as well as two sequences from gp120. Although T20/DP178 from gp41 specifically activates human FPR1 *in vitro*[19] and the murine FPR1 homolog mFPR1 *in vivo*, T21/DP107 from gp41 uses both FPR1 and FPR2 with higher efficacy on FPR2. N36 from gp41, which partially overlaps with T21/DP107, solely signals through FPR2.[32] Two peptide domains in HIV-1 gp120 are potent chemoattractants and activators for FPR2, but not for FPR1, in human phagocytic leukocytes.[3] One peptide domain, F peptide, consists of 20-amino acid residues and is located in the C4–V4 region of gp120 of the HIV-1 LAI strain. Another peptide of 33 amino acids (V3 peptide) is derived from a linear sequence of the V3 region of the HIV-1 MN strain.[3] However, despite the existence of peptide domains in HIV-1 envelope proteins that interact with FPRs, it remains unclear whether such domains are released by enzymatic cleavage of the envelope proteins *in vivo* during HIV-1 infection.

In addition to peptides from HIV protein, there are also some other viral proteins containing sequences that act as FPR ligands. gG-2p20, derived from *Herpes simplex* virus type 2 (HSV-2), is a chemoattractant for both monocytes and neutrophils by activating FPR1.[32] It is noteworthy that an HKU coronavirus peptide, MYVKWPWYVWL, is a potent antagonist, but *N*-formyl-MYVKWPWYVWL is a potent agonist for FPR1,[32] suggesting the importance of N-terminal modification in the biological activity of a synthetic peptide.

Host-Derived Peptides

Peptides Associated with Amyloidogenic Diseases

At least 3 amyloidogenic polypeptides associated with chronic inflammation and amyloidosis have been identified as agonists for FPR2. They are serum amyloid A (SAA), β amyloid peptide $A\beta_{42}$, and $PrP_{106–126}$.

SAA, an acute-phase protein, increases in serum by as much as 1000-fold in inflammatory diseases, e.g. trauma, infection, and other environmental stress.[32] Recombinant human SAA as the first mammalian host-derived peptide ligand identified for FPR2[19] is chemotactic for myeloid cells and T lymphocytes. In synovial tissues of patients with rheumatoid arthritis (RA), highly expressed SAA promotes the proliferation of human fibroblast-like synoviocytes (FLS)

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and induces the expression of matrix metalloproteinases by FLS.[32] SAA also protects RA FLS from apoptotic death induced by serum starvation, anti-Fas IgM, and sodium nitroprusside[32] by interacting with FPR2. In addition, SAA stimulates CCL-2 production via FPR2 by human umbilical vein endothelial cells and monocytes contributing to the progression of atherosclerosis.[21,22] SAA also stimulates M-CSF and CCL-2 expression in human and mouse hepatocellular carcinoma cells.[23] SAA may interact with other receptors; the nucleotide receptor P2X7 was reported to mediate the protective role of SAA in human neutrophil apoptosis.[1]

$A\beta_{42}$ is a 42-amino acid cleavage product of the amyloid precursor protein in the brain and a pathogenic factor for Alzheimer's disease (AD).[19] FPR2 mediates the migration and activation of monocytic phagocytes, including macrophages and brain microglia, induced by $A\beta_{42}$. FPR2 also promotes the endocytosis of $A\beta_{42}$ by macrophages and microglia in the form of receptor and ligand complexes. If the exposure of macrophages to $A\beta_{42}$ is transient, the internalized $A\beta_{42}$ is degraded and FPR2 is rapidly recycled back to the cell surface. However, prolonged exposure results in accumulation of $A\beta_{42}$ and FPR2 complexes in macrophages, which culminates in progressive fibrillary aggregation of $A\beta_{42}$ and the macrophage death.[3] These observations suggest that FPR2 not only mediates the proinflammatory activity of $A\beta_{42}$ but it may also play an important role in the fibrillary deposition of $A\beta_{42}$, a typical pathologic feature of AD.[3]

$PrP_{106–126}$ is a prion protein fragment that is produced in human brains with prion disease.[19] $PrP_{106–126}$ induces the migration and activation of human myeloid cells through FPR2 and may thus contribute to the inflammatory responses seen in the prion disease.

Although the interaction of FPRs with peptide agonists in general elicits proinflammatory responses, FPR2 may exert a neuroprotective effect by recognizing a small peptide, humanin (HN), which was identified through a functional expression screening based on its ability to suppress neuronal cell death seen in familial AD.[32] HN is chemotactic for monocytes through the use of FPR2 and by competitive blockage of FPR2 abrogates the intracellular fibrillary aggregation of $A\beta_{42}$. In neuronal cells, HN protects the cells from apoptosis induced by $A\beta_{42}$. Thus, FPR2 may transduce life and death signals in neuronal cells depending on the nature of the agonists it encounters and may determine the outcome of AD. However, it has also been reported that HN-mediated protection of F11 neurohybrid cells is mediated through tyrosine kinases and STATs which are unlikely coupled to FPR2.[32]

Peptides Associated with Inflammatory and Antibacterial Responses

Urokinase-type plasminogen activator[18] is a serine protease known for its ability to regulate fibrinolysis. uPA is required for leukocyte trafficking to sites of inflammation *in vivo*, and it indirectly activates FPR2 through the liberation of a chemotactic peptide $D2D3_{88–274}$ from uPA receptor (uPAR) (CD87).[19] Consistent with this, the presence of both uPAR and FPR2 on the cell surface is required for the chemotactic activity of uPA, whereas FPR2 alone is sufficient for the effect of $D2D3_{88–274}$. Thus, uPAR may facilitate fibrinolysis and serve as a source of chemotactic proinflammatory peptides necessary for host defense. SRSRY, a peptide corresponding to residues 88–92 in uPAR is an agonist of FPR1.[32] In addition, $uPAR_{84–95}$ induces basophil migration by interacting with both FPR2 and FPR3 and mobilizes hematopoietic stem cells by activating FPR1 by desensitizing the chemokine receptor CXCR4.[32]

FPRs interact with some bactericidal peptides contained in human neutrophil granules. LL37, an enzymatic cleavage fragment of neutrophil granule protein cathelicidin and its mouse homolog CRAMP (see the Cathelicin Chapters in Bacterial/Antibiotic Peptides section of this book), activate FPR2 to promote myeloid cell chemotaxis.[32] LL-37 also induces angiogenesis via FPR2 expressed on endothelial cells. In CRAMP-deficient mice, neovascularization is decreased during cutaneous wound repair.[32] Another antibacterial neutrophil granule protein, cathepsin G, is a serine protease (see the Peptide Biosynthesis/Processing section of this book) and a specific agonist for FPR1.[32] It is therefore conceivable that the capacity of antimicrobial neutrophil granule peptides to interact with FPRs may aid in the recruitment of phagocytic leukocytes to the sites of infection and thus accelerate the killing and clearance of the invading bacteria.

FPR2 has also been reported to interact with a chemokine variant (CKβ8) that activates phagocytic leukocytes. CKβ8 (CCL23/MPIF-1) uses a typical GPCR, CCR1, for its leukocyte chemotactic activity. However, an N-terminal truncation product of the CKβ8 splice variant CKβ8-1 (22–137 aa) activates myeloid cells and FPR2-transfected cell lines at low nanomolar concentration range and is thus considered one of the most potent FPR2 agonists identified so far.[3] However, the cell sources of CKβ8 and the circumstances for its release *in vivo* remain unclear. Interestingly, an 18-amino acid peptide from CKβ8, SHAAG, activates FPR2 activity but not CCR1, revealing a structural basis for the receptor specificity of CKβ8.[25]

Annexin I (Anx A1) and its N-Terminal Peptides

It is intriguing that some FPR agonists have dual roles in inflammatory responses. Anx A1, also named lipocortin I, is a glucocorticoid-regulated, phosphorylated-binding protein possessing both proinflammatory and anti-inflammatory activity mediated in part by FPR1. Expressed in a variety of cell types, Anx A1 is particularly abundant in neutrophils where it is located on the outer cell surface and serves to inhibit neutrophil transendothelial migration. At low

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concentrations, Anx A1 and its N-terminal peptides (Ac2–26 and Ac9–25) elicit Ca^{2+} transients through FPR1 to desensitize neutrophils and inhibit its transendothelial migration induced by the chemokine IL-8 (CXCL8).[32] At high concentrations, the Anx A1 peptides activate neutrophils as potent proinflammatory stimulants. The antimigratory activity of exogenous and endogenous Anx A1 has been shown in both acute and chronic models of inflammation. More recent studies revealed that Anx A1 peptides may also use FPR2 and, to a lesser extent, FPR3, suggesting a complex role for FPRs in Anx A1–mediated regulation of host responses. In addition to neutrophils, Anx A1 also regulates T cell receptor[30] signaling. Stimulation of T cells via TCR leads to the secretion of endogenous Anx A1 and a simultaneous externalization of its receptor FPR2 on the plasma membrane, highlighting a "coordinated" signaling of "Anx A1_FPR" system.[5] In the presence of Anx A1, naive T cells differentiate toward Th1 cells, whereas Anx-A1-deficient T cells produce high levels of Th2 cytokines.[5,7] Also, in Anx A1-deficient T cells, the production of IL-17 as well as FPR2 signaling is impaired during differentiation in Th17-skewing conditions.[6] In addition, tumor growth and metastasis are markedly decreased in Anx A1-KO mice, whereas the survival and tumor necrosis are significantly increased, due to impaired angiogenesis.[33] These observations demonstrate a complex role of Anx A1 and FPR2 in multiple pathophysiological processes.

Frog Skin Peptides (see Amphibian/Skin Peptides Section of this Book)

Temporin A (TA) is a frog-derived antimicrobial peptide that was found to induce the migration of human monocytes, neutrophils, and macrophages.[32] Characterization of the signaling characteristics of TA in monocytes and the use of receptor transfected HEK293 epithelial cell line revealed that this peptide uses FPR2 as a receptor. TA is also chemotactic *in vivo*, because it elicited infiltration of neutrophils and monocytes into the injection site in mice. Another temporin peptide Rana-6 is chemotactic for human phagocytes also by using FPR2. Thus, frog-derived temporins have the capacity to chemoattract phagocytes through human FPR2 and the mouse homolog mFPR2, suggesting the participation of amphibian antimicrobial peptides in host innate immunity. Because the expression of FPR homolog(s) has not been reported in species other than mammals, it will be interesting to clarify whether such receptor exists in amphibians and other species such as insects, which normally rely on the secretion of antimicrobial peptides as a natural host defense.[3]

Other Host-Derived Peptides

It is important that highly efficacious agonist peptide for FPR3 has been isolated from spleen extracts. F2L, an acetylated amino-terminal 21-amino acid peptide, is derived from the cleavage of the human heme-binding protein, an intracellular tetrapyrolle-binding protein. F2L chemoattracts and activates monocyte-derived DCs, thus seems to be a novel and unique natural chemoattractant peptide for FPR3 in DCs and monocytes, in agreement with the selective expression of FPR3 in these cells,[32] suggesting its role in linking innate and adaptive immune responses by activating antigen-presenting $FPR3^{+}$ DCs, which express reduced levels of FPR1 and FPR2. In mice, mouse F2L activates neutrophils by using mFPR2, a homolog of human FPR2, because neutrophils from $mFPR2^{-/-}$ mice lost all responsiveness to F2L. These results suggest that mFPR2 in mice is likely a homolog of both human FPR2 and FPR3.[2]

Agonists from Peptide Library

Random small peptide libraries are rich sources of FPR agonists and have yielded a number of potent chemotactic stimulants for leukocytes. For instance, WKYMVm, a hexapeptide representing a modified sequence isolated from a random peptide library, was initially reported to be an efficacious stimulant of human B lymphocytes, monocytic cell lines, as well as peripheral blood neutrophils. It was subsequently found that WKYMVm uses both FPR1 and FPR2, and is by far the most potent peptide agonist for FPR2 to chemoattract and activate human phagocytic cells.[32] The WKYMVm analog, produced by converting the D-amino acid methionine at the C-terminus into to an L-amino acid, becomes a more selective agonist of FPR2 and also a weaker activator of FPR3.[3] Another peptide, MMK-1, which is also derived from a random peptide library, is a potent and very selective chemotactic agonist for FPR2. Although FPR agonists identified in random peptide libraries seem not to be pathophysiologically relevant to diseases, they nevertheless constitute useful pharmacological tools for the studies of FPR signaling pathways, structural basis of the ligands versus receptors, and identification of receptor antagonists that could be valuable in conditions resulted from receptor overactivation.

Nonpeptides

Host-derived Nonpeptide FPR Agonists

LXA4, biosynthesized from arachidonic acid metabolism, is the first described endogenous lipid ligand for FPR2. However, despite the original observation of LXA4 being a chemotactic agonist for FPR2, this lipid metabolite has been shown to mainly exert inhibitory activities on a variety of inflammatory responses. LXA4 inhibits the production of inflammatory cytokines by epithelial cell lines in response to TNFα and LPS, while it simulates the expression of the anti-inflammatory cytokine IL-10.[32] In DCs, LXA4/ATL activates the suppressor of cytokine signaling (SOCS)-2 and

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in T cells, LXA4 attenuates TNFα release by impairing ERK signaling.[32] LXA4 also prevents renal fibrosis by inhibiting PDGF-induced TGF-β production in mesangial cells.[32] In addition, LXA4 attenuates inflammation-induced pain and displays proresolution properties in various disease models, including dermal inflammation, ischemia/reperfusion injury, peritonitis, colitis, cystic fibrosis, asthma, parasitic infection and glomerulonephritis.[32] The multiplicity of the anti-inflammatory activity of LXA4 raises the question of how this lipid mediator shares FPR2 with other peptide agonists that trigger a proinflammatory signaling pathway. One plausible explanation was that LXA4 binds to an FPR2 domain, which is different from the domains utilized by peptide agonists. However, because LXA4 has been reported to interact with multiple receptors depending on the cell types, it remains to be clarified whether the biological function of LXA4 is the consequence of interaction with more than one receptor.

Agonists from Small Compound Library

In addition to recognizing peptide agonists and the lipid agonist LXA4, nonpeptide small compounds may also be recognized by FPRs. For instance, a quinazolinone derivative Quin-C1 was reported as a highly selective agonist for FPR2.[32] High-throughput screening of compound library has yielded compound 43 that reduces inflammation in an ear swelling model in mice through FPR2, but it was later shown as a preferential agonist for FPR1 that activates neutrophils.[14] In addition, AG-14 was found to activate neutrophils by using FPR1.[32] Compounds 1 and 2, arylcarboxylic acid hydrazide derivatives, are agonists for FPR2 that induce the production of TNFα by macrophages.[32]

Others

PD168368 and PD176252, antagonists of gastrin-releasing peptide/neuromedin B receptors (BB1/BB2), are potent agonists of both FPR1 and FPR2.[26] Their Trp-and Phe-based analogs and related nonpeptide/nonpeptoid analogs are also agonists of FPRs.[26] In addition, the cholecystokinin-1 receptor agonist A-71623 activates both FPR1 and FPR2.[26] These physiologically relevant nonpeptide agonists for FPRs may contribute to the regulation of inflammation and immune responses. The similarities and divergence of their binding domains in FPRs and signal transduction pathways as compared with that of peptide agonists will be of great pathophysiological and pharmacological interests.

FPR ANTAGONISTS

The importance of FPRs in microbial infection and host immune responses suggests that these GPCRs may be targets of pharmacological intervention to attenuate complications associated with undesirable receptor overactivation. This consideration leads to the search and development of FPR antagonists, which have yielded many molecules as listed in Table 2.

Microbial FPR Antagonists

Cyclosporin H (CsH) produced by a fungus is an inverse agonist (negative antagonist) that suppresses the constitutive activity of FPR1.[19] Similar to CsH, CsA also inhibits fMLF-induced neutrophil degranulation and cell activation, although the inhibitory effect is less potent than CsH. FPR2-inhibitor protein (FLIPr), a staphylococcal anti-inflammatory protein, directly binds to FPR1 and FPR2 and attenuates fMLF-induced cell activation.[32] At low concentrations, FLIPr selectively inhibits the binding of some FPR2 agonists including MMK-1, WKYMVM, PrP (106–126), and $A\beta_{42}$. At high concentrations, it reduces fMLF binding to FPR1.[32] Iodinated chemotaxis inhibitory protein of *S. aureus* (CHIPS) is a potent peptide antagonist of FPR1.[32] LDLLDL is an immunosuppressive hexapeptide derived from retroviral trans-membrane envelope protein p15E and is a specific FPR1 antagonist.[20] Peptides derived from the membrane proximal region of fusion proteins from HIV 1 and 2, Coronavirus 229 E, severe acute respiratory syndrome (SARS) coronavirus and Ebola virus are all potent antagonists of FPR1.[32] These observations suggest the capacity of microbia to produce molecules that inhibit FPR-mediated function of host cells that are directly involved in antimicrobial responses.

Host-derived Antagonists

Several "endogenous" FPR antagonists have been reported. Deoxycholic acid (DCA) and chenodeoxycholic acid (CDCA) identified as antagonists for FPR1 with unclear pathophysiological significance. CDCA also partially inhibits FPR2 agonist-induced myeloid cell activation. Spinorphin (LVVYPWT), which belongs to the family of hemorphins, is a peptide antagonist for FPR1.[32] In addition, Quin-C7, a synthetic nonpeptide compound, is a highly selective antagonist for FPR2. Interestingly, Quin-C7 is derived from FPR2 agonist Quin-C1 through chemical modification.[32]

Synthetic Peptide Antagonists

Boc1 (t-Boc-MLF) and Boc2 (t-Boc-FLFLF) were originally found as antagonists for FPR1.[11] However, a recent study shows that at low micromolar concentrations, both Boc1 and Boc2 are selective for FPR1; at high micromolar concentrations, Boc2 also partially inhibits the function of FPR2.[32] The WRW4 obtained from hexapeptide library is a more selective antagonist for FPR2.[29] Further, a cell permeable peptide PBP10, which is derived from a PIP2-binding

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TABLE 2 FPR Antagonists

Antagonists	Origin	Receptors
Microbial antagonists		
Chemotaxis inhibitory protein of *S. aureus* (CHIPS)	*Staphylococcus aureus*	FPR1
FPRL1-inhibitor protein (FLIPr)	*Staphylococcus aureus*	FPR1, FPR2
Cyclosporin A (CsA)	Fungi	FPR1
Cyclosporin H (CsH)	Fungi	FPR1
HKU coronavirus peptides	Coronavirus	FPR1
Coronavirus 229E peptides	Coronavirus	FPR1
SARS coronavirus peptides	Coronavirus	FPR1
HIV-1 peptides	HIV-1	FPR1
HIV-2 peptides	HIV-2	FPR1
Ebola peptide	Ebola virus	FPR1
LDLLDL	Retrovirus	FPR1
Synthetic peptide antagonists		
i-Boc-MLF	Synthetic peptide	FPR1
t-Boc-MLF (Boc1)	Synthetic peptide	FPR1
t-Boc-FLFLF (Boc2)	Synthetic peptide	FPR1, FPR2
Trp-Arg-Trp-Trp-Trp-Trp (WRW4)	Random peptide library	FPR1
PBP10	Gelsolin	FPR2
Host-derived antagonists		
Spinorphin	Bovine spinal cord	FPR1
Chenodeoxycholic acid (CDCA)	Endogenous (bile acid)	FPR1, FPR2
Deoxycholic acid (DCA)	Endogenous (bile acid)	FPR1
Others		
Piroxicam	Nonsteroid anti-inflammatory drugs (NSDAIDs)	FPR1
SDS	Amphiphile	FPR1

domain of the cytoskeleton protein gelsolin, displays FPR2 antagonist activity by its ability to penetrate cell membrane to act on the intracellular domains in FPR2.[13]

Other Antagonists

The nonsteroidal anti-inflammatory drug (NSDAID) piroxicam, a nonselective cyclooxygenase (COX)-inhibitor, can reduce neutrophil superoxide production induced by FPR1 agonists but had no significant effect on FPR2-mediated responses. However, piroxicam inhibits WKYMVm-induced neutrophil intracellular calcium mobilization and superoxide release when the drug was combined with the FPR2 specific antagonist WRW4.[32] The inhibitory effect of piroxicam is dependent on its binding to the receptor, therefore seems to be FPR specific.

PERSPECTIVES

In this chapter, we attempted to provide a comprehensive overview of FPR ligands including agonists and antagonists. It is now clear that FPRs play important roles in the regulation of inflammatory and immune responses. The process of understanding of the biological roles of FPRs has been

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considerably prompted by the identification of a variety of ligands from different sources, in particular, from microbes and human. In addition to the evidence that the FPRs are involved in antimicrobial defense and inflammation, they are also implicated in the progression of cancer. For example, FPR1 is selectively expressed by highly malignant human glioma cells and increases the motility, growth, and angiogenesis of human glioblastoma by interacting with host-derived agonist(s) released by necrotic tumor cells.[35] An effort to identify potential FPR1 agonist(s) in glioma cell supernatant revealed Anx A1 as a major component.[31] Therefore, FPR1 and Anx A1 axis may constitute targets for novel antiglioma therapy. Human FPR2 is also expressed in astrocytoma cell line,[9] hepatocellar carcinoma,[23] and breast cancer[17] with yet unclear pathophysiological implications.

It is intriguing that FPR1 and FPR2 exhibit a promiscuity to interact with a broad range of ligands that do not share significant primary or tertiary structure similarities. How a single FPR could bind such a diverse array of ligands, from small peptides to nonpeptide compounds, remains to be elucidated, possibly through classical receptor chimera approaches and site-directed mutagenesis. Further investigation should not only benefit better understanding of the role of FPRs and their ligands in disease states but also the identification of pharmacological targets for the development of therapeutic agents.

ACKNOWLEDGMENTS

The authors thank Dr. Joost J Oppenheim for reviewing the chapter and Sondra Sheriff and Cheryl Fogle-Lamb for secretarial assistance. This work was supported in part with Federal funds from the National Cancer Institute, National Institutes of Health, under Contract No. HHSN261200800001E and was supported in part by the Intramural Research Program of the NCI, NIH.

REFERENCES

1. Bjorkman L, Karlsson J, Karlsson A, Rabiet MJ, Boulay F, Fu H, et al. Serum amyloid A mediates human neutrophil production of reactive oxygen species through a receptor independent of formyl peptide receptor like-1. *J Leukoc Biol* 2008;**83**:245–53.
2. Chen K, Le Y, Liu Y, Gong W, Ying G, Huang J, et al. A critical role for the G protein-coupled receptor mFPR2 in airway inflammation and immune responses. *J Immunol* 2010;**184**:3331–5.
3. Chen K, Wang JM. Chemotactic peptide ligands for formylpeptide receptors influencing inflammation. *Handbook of biologically active peptides* 2006;**78**:547–52.
4. Crouser ED, Shao G, Julian MW, Macre JE, Shadel GS, Tridandapani S, et al. Monocyte activation by necrotic cells is promoted by mitochondrial proteins and formyl peptide receptors. *Crit Care Med* 2009;**37**:2000–9.
5. D'Acquisto F, Merghani A, Lecona E, Rosignoli G, Raza K, Buckley CD, et al. Annexin-1 modulates T-cell activation and differentiation. *Blood* 2007;**109**:1095–102.
6. D'Acquisto F, Paschalidis N, Sampaio AL, Merghani A, Flower RJ, Perretti M. Impaired T cell activation and increased Th2 lineage commitment in Annexin-1-deficient T cells. *Eur J Immunol* 2007;**37**:3131–42.
7. D'Acquisto F, Perretti M, Flower RJ. Annexin-A1: a pivotal regulator of the innate and adaptive immune systems. *Br J Pharmacol* 2008;**155**:152–69.
8. De Santo C, Arscott R, Booth S, Karydis I, Jones M, Asher R, et al. Invariant NKT cells modulate the suppressive activity of IL-10-secreting neutrophils differentiated with serum amyloid A. *Nat Immunol* 2010;**11**:1039–46.
9. Decker Y, McBean G, Godson C. Lipoxin A4 inhibits IL-1beta-induced IL-8 and ICAM-1 expression in 1321N1 human astrocytoma cells. *Am J Physiol Cell Physiol* 2009;**296**:C1420–7.
10. Devosse T, Guillabert A, D'Haene N, Berton A, De Nadai P, Noel S, et al. Formyl peptide receptor-like 2 is expressed and functional in plasmacytoid dendritic cells, tissue-specific macrophage subpopulations, and eosinophils. *J Immunol* 2009;**182**:4974–84.
11. Dufton N, Perretti M. Therapeutic anti-inflammatory potential of formyl-peptide receptor agonists. *Pharmacol Ther* 2010;**127**:175–88.
12. El Zein N, D'Hondt S, Sariban E. Crosstalks between the receptors tyrosine kinase EGFR and TrkA and the GPCR, FPR, in human monocytes are essential for receptors-mediated cell activation. *Cell Signal* 2010;**22**:1437–47.
13. Forsman H, Dahlgren C. The FPR2-induced rise in cytosolic calcium in human neutrophils relies on an emptying of intracellular calcium stores and is inhibited by a gelsolin-derived PIP2-binding peptide. *BMC Cell Biol* 2010;**11**:52.
14. Forsman H, Onnheim K, Andreasson E, Dahlgren C. What formyl peptide receptors, if any, are triggered by compound 43 and Lipoxin A(4)? *Scand J Immunol* 2011;**74**:227–34.
15. Huang J, Hu J, Bian X, Chen K, Gong W, Dunlop NM, et al. Transactivation of the epidermal growth factor receptor by formylpeptide receptor exacerbates the malignant behavior of human glioblastoma cells. *Cancer Res* 2007;**67**:5906–13.
16. Jiarpinitnun C, Kiessling LL. Unexpected enhancement in biological activity of a GPCR ligand induced by an oligoethylene glycol substituent. *J Am Chem Soc* 2010;**132**:8844–5.
17. Khau T, Langenbach SY, Schuliga M, Harris T, Johnstone CN, Anderson RL, et al. Annexin-1 signals mitogen-stimulated breast tumor cell proliferation by activation of the formyl peptide receptors (FPRs) 1 and 2. *FASEB J* 2011;**25**:483–96.
18. Koczulla R, von Degenfeld G, Kupatt C, Krotz F, Zahler S, Gloe T, et al. An angiogenic role for the human peptide antibiotic LL-37/hCAP-18. *J Clin Invest* 2003;**111**:1665–72.
19. Le Y, Murphy PM, Wang JM. Formyl-peptide receptors revisited. *Trends Immunol* 2002;**23**:541–8.
20. Le Y, Wang JM, Liu X, Kong Y, Hou X, Ruan L, et al. Biological active peptides interacting with the G protein-coupled formylpeptide receptors. *Protein Peptide Lett* 2007;**14**:846–53.
21. Lee HY, Kim SD, Shim JW, Lee SY, Lee H, Cho KH, et al. Serum amyloid A induces CCL2 production via formyl peptide receptor-like 1-mediated signaling in human monocytes. *J Immunol* 2008;**181**:4332–9.
22. Lee HY, Kim SD, Shim JW, Yun J, Kim K, Bae YS. Activation of formyl peptide receptor like-1 by serum amyloid A induces CCL2 production in human umbilical vein endothelial cells. *Biochem Biophys Res Commun* 2009;**380**:313–7.

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23. Li Y, Cai L, Wang H, Wu P, Gu W, Chen Y, et al. Pleiotropic regulation of macrophage polarization and tumorigenesis by formyl peptide receptor-2. *Oncogene* 2011.
24. McDonald B, Pittman K, Menezes GB, Hirota SA, Slaba I, Waterhouse CC, et al. Intravascular danger signals guide neutrophils to sites of sterile inflammation. *Science* 2010;**330**:362–6.
25. Miao Z, Premack BA, Wei Z, Wang Y, Gerard C, Showell H, et al. Proinflammatory proteases liberate a discrete high-affinity functional FPRL1 (CCR12) ligand from CCL23. *J Immunol* 2007;**178**:7395–404.
26. Schepetkin IA, Kirpotina LN, Khlebnikov AI, Jutila MA, Quinn MT. Gastrin-releasing peptide/neuromedin B receptor antagonists PD176252, PD168368, and related analogs are potent agonists of human formyl-peptide receptors. *Mol Pharmacol* 2011;**79**:77–90.
27. Seki T, Fukamizu A, Kiso Y, Mukai H. Mitocryptide-2, a neutrophil-activating cryptide, is a specific endogenous agonist for formyl-peptide receptor-like 1. *Biochem Biophys Res Commun* 2011;**404**:482–7.
28. Shin MK, Jang YH, Yoo HJ, Kang DW, Park MH, Kim MK, et al. *N*-Formyl-methionyl-leucyl-phenylalanine (FMLP) promotes osteoblast differentiation via the *n*-formyl peptide receptor 1-mediated signaling pathway in human mesenchymal stem cells from bone marrow. *J Biol Chem* 2011;**286**:17133–43.
29. Stenfeldt AL, Karlsson J, Wenneras C, Bylund J, Fu H, Dahlgren C, et al. Boc-MLF and Boc-FLFLF are antagonists that preferentially inhibit activity triggered through the formyl peptide receptor. *Inflammation* 2007;**30**:224–9.
30. Stone RA, Quinn GE, Francis EL, Ying GS, Flitcroft DI, Parekh P, et al. Diurnal axial length fluctuations in human eyes. *Invest Ophthalmol Vis Sci* 2004;**45**:63–70.
31. Yang Y, Liu Y, Yao X, Ping Y, Jiang T, Liu Q, et al. Annexin 1 released by necrotic human glioblastoma cells stimulates tumor cell growth through the formyl peptide receptor 1. *Am J Pathol* 2011.
32. Ye RD, Boulay F, Wang JM, Dahlgren C, Gerard C, Parmentier M, et al. International Union of Basic and Clinical Pharmacology. LXXIII. Nomenclature for the formyl peptide receptor (FPR) family. *Pharmacol Rev* 2009;**61**:119–61.
33. Yi M, Schnitzer JE. Impaired tumor growth, metastasis, angiogenesis and wound healing in annexin A1-null mice. *Proc Natl Acad Sci U S A* 2009;**106**:17886–91.
34. Zhang Q, Raoof M, Chen Y, Sumi Y, Sursal T, Junger W, et al. Circulating mitochondrial DAMPs cause inflammatory responses to injury. *Nature* 2010;**464**:104–7.
35. Zhou Y, Bian X, Le Y, Gong W, Hu J, Zhang X, et al. Formylpeptide receptor FPR and the rapid growth of malignant human gliomas. *J Natl Cancer Inst* 2005;**97**:823–35.

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Chapter 92

Neuroimmune Peptides

Ning Zhang, Hua Geng and Joost J. Oppenheim

ABSTRACT

Increasing evidence has shown that many neuropeptides modulate immune responses in addition to their well-documented function in the central and peripheral nervous systems. Surprisingly, RT-PCR, Western blotting, and functional assays reveal the production of these neuropeptides by the immune system and the expression of their receptors on leukocyte surface. This chapter will provide a brief summary of the immunological activities of neuropeptides.

INTRODUCTION

The nervous and immune systems are tightly integrated and orchestrated to serve the complicated tasks of host sensation and response. A disturbance in one system often has a profound effect on the other. For example, activation of the hypothalamic–pituitary–adrenal (HPA) axis by long-term stress often elicits suppression of both innate and adaptive immunities.[6] Conversely, inflammatory immune responses activate central nervous system sensory pain pathways.[34,35] Increasing evidence suggests that a family of immunoactive neuropeptides provides key signals between these two systems. Beyond their well-characterized effects in the nervous system, these neuropeptides also interact with their receptors on leukocytes, thus modulating the function of these pivotal mediators of host defense. Further, leukocytes are also capable of producing many of these peptides in the peripheral immune system. The structure, properties, and roles of neuropeptides in the central and peripheral nervous systems are discussed in other chapters of this book. This section will briefly describe the immunological effects of these neuropeptides as summarized in Table 1. Other actions of these peptides are discussed elsewhere in this book.

Neuropeptides influence host immune defenses based on two mechanisms. First, these peptides indirectly regulate the immune response by acting on the different axis of the central nervous system. For example, Met-enkephalin, an endogenous opioid, stimulates the HPA-axis-mediated production of corticosterone, a potent immunosuppressive hormone.[19] Opioids also activate the sympathetic nervous system, resulting in an increase in the level of circulating epinephrine from the adrenal medulla and secretion of the norepinephrine by sympathetic nerve terminals. Increased catecholamine levels have been linked to the suppression of natural killer cell and lymphocyte function. Second, all the peptides listed in Table 1 are able to directly regulate the functions of receptor expressing leukocytes, independent of their neuronal activities. Studies using reverse transcription-polymerase chain reaction (RT-PCR) or western blotting analysis reveal the expression of a wide variety of neuropeptide receptors on various types of leukocytes. For example, all three subtypes of opioid receptors, μ-, δ-, and κ-receptors, have been detected on T cell, B cells, monocytes, neutrophils, and natural killer cells.[23] Stimulation of these neuropeptide receptors can modulate leukocyte production of proinflammatory cytokines. Interestingly, most neuropeptide receptors are seven-transmembrane domain-containing receptors, which exert their function by activating heterotrimeric G proteins.

Neuropeptides are mainly expressed and active in the central and peripheral nervous systems. However, these peptides can also be unexpectedly produced and are functional in other peripheral tissues. For example, adrenocorticotropic hormone/corticotropin (ACTH) is not only produced by pituitary cells but also to a limited extent by leukocytes.[2] Endogenous opioids are expressed by leukocytes and play an important role in peripheral analgesic effects.[29] In addition to being produced in the pituitary gland, α-melanocyte-stimulating hormones (αMSH) are also highly expressed in the skin and the gastro intestinal (GI) tract cells.[21] Substance P mRNA and protein have been detected in the thymic medulla and purified CD5+ thymocytes.[27]

The neuropeptides of the HPA axis play a profound role in inflammation. Stressful stimuli activate the HPA axis through the release of a sequential series of neuropeptide hormones, beginning with the production of hypothalamic corticotropin-releasing hormone (CRH) and followed by pituitary derived ACTH, culminating in the secretion of glucocorticoids, potent anti-inflammatory hormones produced by the adrenal cortex.[6] Vasopressin (AVP) potentiates

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00092-0
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TABLE 1 Neuropeptides Regulating Receptor Expressing Leukocytes

Neuropeptides	Size	Receptors in immune system	Immune effects	Nonimmune effects
CRH	41aa	Neutrophils, eosinophils, thymus, spleen	Enhances the production of immunosuppresive glucocorticoids through the HPA axis, enhances peripheral inflammation. Urocortin (Ucn), UcnI, and UcnII share similar properties.	Endocrine-signaling peptide involved in the stress response
Urocortin (Ucn)	40aa	Monocytes, macrophages, dendritic cells, polymorphonuclear cells, T cells	Inhibits inflammatory cytokines and chemokines production, T cell proliferation, and TH1 response; enhances IL-10/transforming growth factor (TGF)-β1 production; induces regulatory T cells.	Regulates vasodilatation, bronchodilatation, cardiac output, smooth muscle relaxation, food intake, and ACTH secretion.
Somatostatin	14aa, or 28aa	T cells, monocytes	Inhibits the secretion of proinflammatory cytokines and antibodies by immune cells; inhibits lymphocyte proliferation.	Inhibits secretion of numerous growth hormones.
Cortistatin	17aa	Monocytes, macrophages, dendritic cells, T cells	Inhibits inflammatory cytokines and chemokines production, T cell proliferation, and TH1 response; induces regulatory T cells.	Inhibits locomotor activity, regulates growth hormone.
Vasopressin (AVP)	9aa	T cells, B cells, monocytes	Enhances production of immunosuppresive glucocorticoids through the HPA axis, enhances secretion of IFN-γ by T-cells.	Potentiates CRH effects, responds to osmotic and hemodynamic stress.
Gastrin-releasing peptides (GRP)	27aa	Macrophage, T cells, natural killer cells	Stimulates phagocytic activities of macrophages, inhibits Con-A induced proliferation of lymphocytes. Bombesin and neuromedin C share similar properties.	Activates the sympathetic nervous system to modulate stress, fear, and anxiety responses.
Met-enkephalin and endorphins	5aa	T cell, B cells monocytes, neutrophils, natural killer cells	Induces heterologous desensitization of chemokine receptors, inhibits natural killer cell activity, suppresses IL2 and IFNγ production.	Analgesic peptides, induces the secretion of corticosterone.
ACTH	39aa	T helper cells, B cells, macrophages	Enhances production of immunosuppresive glucocorticoids through the HPA axis.	Endocrine-signaling peptides involved in the response to stress.
αMSH (melanocortin)	13aa	Monocytes, macrophages, natural killer cells, T helper cells, B cells, and dendritic cells	Downregulates the production of proinflammatory cytokines, downregulates the expression of costimulatory molecules (CD86, CD40, ICAM-1) on antigen-presenting dendritic cells, upregulates IL-10. inhibits cutaneous inflammation, inhibits T cell proliferation, and induces regulatory T cells.	Regulation of energy homeostasis.

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Neuropeptide Y	36aa	Monocytes, T cells, B cells, natural killer cells	Elicits neurogenic inflammation by inducing the secretion of substance P, contributes to colitis, potentiates paw edema; enhances T cell adhesion to fibronectin; increases release of oxidative reagents by neutrophils, suppresses NK cell activity; enhances phagocytosis by monocyte.	Regulates energy balance, food intake, and antihyperalgesia, antimicrobial peptide, affects skin color, antipyretic effects.
VIP/PACAP	28aa	T cells, mast cells, macrophages, monocytes, dentritic cells, polymorphonuclear cells	Inhibits leukocyte recruitment, cytokine production; polarizes TH2 responses, induces T lymphocyte adhesion and chemotaxis, inhibits TH1 response and recruitment, enhances tolerogenic DC generation. PACAP possesses similar anti-inflammatory activities.	Neurotransmitter, secretagogue, neuroprotective, neurotrophic, and vasodilator.
Substance P (neurokinin1, tachykinin 1)	11aa	T cells, macrophages, dendritic cells	Contributes to neurogenic inflammation, involves in various respiratory diseases and pancreatitis. Substance K (neurokinin A) possesses similar proinflammatory activities.	Induces hyperalgesia
CGRP	37aa	T cells, B cells, dendritic cells	Contributes to neurogenic inflammation, involves in various aspiratory diseases.	Induces vasodilatation
Bradykinin	9aa	Monocytes, neutrophils, eosinophils, T cells	Induces chemotaxis of neutrophils, stimulates the secretion of superoxide radicals, exacerbates infection, contributes to ischemia reperfusion, involved in respiratory diseases.	Induces hyperalgesia; vasodilator.
Adrenomedullin (AM)	52aa	Monocytes, macrophages	Inhibits inflammatory cytokines and chemokine production, T cell proliferation, and TH1 response; induces regulatory T cells; Stimulates IL-10/TGF-β production.	Regulates vasodilatation, bronchodilatation, cardiac output, and smooth muscle relaxation.
Ghrelin	28aa	Monocytes, macrophages, dendritic cells, T cells	Inhibits inflammatory cytokines and chemokine production; suppresses T cell proliferation, and TH1 response (IL-2 and IFN-γ production); induces regulatory T cells	Regulates cardiac output, appetite, adiposity, vasodilatation; enhances GI secretion, and gastric motility.

CRH: corticotropin-releasing hormone; αMSH: alpha-melanocyte-stimulating hormone; ACTH: adrenocorticotropic hormone/corticotropin; CGRP: calcitonin-gene-related peptide; VIP: vasoactive intestinal polypeptide.

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the capacity of CRH to activate the HPA axis. The resultant elevated glucocorticoid concentrations in the circulation contribute to stress-induced immunosuppression. Surprisingly, CRH and AVP are also produced by leukocytes, and their receptors are expressed by leukocytes. Although hypothalamic CRH and AVP have indirect anti-inflammatory effects through the stimulation of glucocorticoids, these peptides seem to have direct proinflammatory effects in the peripheral tissues. AVP stimulates the secretion of interferon-γ by T-lymphocytes.[18] CRH elicits the production of interleukin (IL)-1 by human monocytes and enhances mouse *natural killer* (NK) cell activity.[5] CRH and its family members, urocortin (Ucn), UcnI, and UcnII have been shown to exacerbate symptoms of experimental autoimmune encephalomyelitis and toxin A-induced inflammatory diarrhea.[1] The expression of Ucn is upregulated during inflammation.[17] Ucn induces apoptosis of macrophage during inflammation, suggesting its important anti-inflammatory effect.[31] Regarding innate immunity, Ucn inhibits the production of several proinflammatory cytokines, such as *tumor necrosis factor* (TNF)α, IL-6, and IL-12, and further *in vivo* disease-associated (i.e. sepsis and inflammatory bowel disease) studies support the anti-inflammatory role of Ucn.[14] Thus, their antagonists have been tested as therapeutics for arthritis and preliminary results are encouraging.[33]

Inflammation induces hyperalgesia. This is in part attributable to the chemokine receptor-induced desensitization of analgesic opioid receptors and concomitant sensitization of algesic vanilloid receptors transient receptor potential vanilloid (TRPV)1 on neurons.[34,35] Conversely, opioid receptors on leukocytes have immunosuppressive effects. Met-enkephalin, an endogenous opioid, is an analgesic peptide of 5 amino acids.[3] It exerts immunosuppressive effects by heterologous desensitization of chemokine receptors and enhancing the secretion of corticosteroids.[23] TRPV1 is also expressed by leukocytes, but its immune function remains unknown.

Painful stimuli activate peripheral neurons to release several immunostimulatory peptides, including substance P and calcitonin-gene-related peptide (CGRP). These peptides have proinflammatory effects and are associated with neurogenic inflammation. Substance P, a hyperalgesic peptide, belongs to the tachykinin family. It stimulates the influx of neutrophils and eosinophils into the human dermis, induces the secretion of TNFα by macrophages, and it enhances leukocyte adhesion to endothelial cells, which may contribute to its proinflammatory effects in various respiratory diseases, pancreatitis, and inflammatory bowel disease.[24] Substance K, another peptide in the tachykinin family, possesses similar proinflammatory effects. CGRP is also involved in neurogenic respiratory diseases such as asthma, chronic obstructive pulmonary disease, or chronic cough.[28] Adrenomedullin (AM) could function as an endogenous anti-inflammatory immune modulator with a high structural homology to CGRP.[11] Unlike CGRP, AM could effectively prevent arthritis and sepsis through inhibiting the inflammatory response in these autoimmune/inflammatory diseases.[12,14] Neuropeptide Y (NPY), a key peptide regulating energy balance and food intake in *central nervous system* (CNS), also induces the secretion of substance P by peripheral neurons, contributing to colitis and paw edema in animal models of inflammation.[10] Further, NPY directly enhances T cell adhesion to fibronectin[20] and plays a major role in the regulation of Th1 responses.[26] Bradykinin, another hyperalgesic peptide, facilitates septic shock, exacerbates ischemia reperfusion, and enhances airway inflammation based on its vasodilatory effects.[4]

There several other neuropeptides that downregulate immune responses. Vasoactive intestinal polypeptide (VIP) has immunosuppressive effects based on its capacity to inhibit leukocyte recruitment to inflammatory sites. VIP also induces polarization to the T helper (TH)2 type of antibody-based allergic responses.[25] In addition, VIP induces the differentiation of tolerogenic dentritic cells that promote generation of antigen specific Treg cells.[15] αMSH downregulates the production of proinflammatory cytokines, inhibits the expression of costimulatory molecules (CD86, CD40, and intercellular adhesion molecule (ICAM)-1) on dendritic cells, and upregulates IL-10, resulting in immunosuppression.[22] An increasing body of evidence shows that VIP and αMSH could regulate TH1/TH2/Treg immune homeostasis and induce immune tolerance and inhibit autoimmune response through inhibiting the production of inflammatory cytokines and chemokines, polarizing the differentiation of T cells toward TH2 or Treg, and suppressing the activation of autoreactive T cells by IL-10 and TGFβ.[11,13] Moreover, both VIP and αMSH inhibit phagocytic activity as well as the migration and adherence of macrophages and neutrophils.[9] Ghrelin is one of the anti-inflammatory neuropeptides with an immunomodulatory role through binding to its receptor *growth hormone secretagog receptor* (GHSR), and subsequently activating the cyclic adenosine monophosphate-protein kinase A signaling pathway that is associated with anti-inflammatory actions.[13] Somatostatin inhibits the secretion of interferons (IFN)-γ and *cerebrospinal fluid*, enhances the secretion of IL-10 and IL-4, downregulates the secretion of antibodies by B lymphocytes, and impairs lymphocyte proliferation.[30] Cortistatin has a strong structural similarity to somatostatin. Cortistatin is expressed in various immune cells, including monocytes, macrophages, lymphocytes, and dentritic cells, with its expression upregulated on cell differentiation and activation, suggesting that cortistatin may act as a major immune regulator.[11] In addition, both *in vitro* and *in vivo* studies show that cortistatin functions more efficiently than somatostatin in its anti-inflammatory responses, probably because of the capacity

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of cortistatin to activate different receptors and signaling pathways.[7,16] Cortistatin cannot only bind to somatostatin receptor subtypes (SSTs), but also to GHSR, thus leading to the activation of both cAMP-independent (SST mediated) and -dependent (GHSR-mediated) signaling.[13,32] Gastrin-releasing peptide (GRP) and neuromedin C belong to the bombesin peptide family. They stimulate phagocytic activities of macrophages but inhibit concanavalin-A induced proliferation of lymphocytes.[8]

In summary, increasing evidence has confirmed the production of neuropeptides and the expression of their receptors by cells of the immune system. Further, neuropeptides directly contribute to a wide range of diseases, such as stress-induced immunosuppresion, asthma, and inflammatory bowel disease. However, many more studies are needed to connect *in vitro* observations, based on the use of isolated leukocytes, to the *in vivo* systemic effects of neuropeptides in disease. Further, it is critical to understand that the anatomical and temporal distribution of these peptides may determine their overall effects on immune responses. For example, CRH acts as an anti-inflammatory agent when it exerts its effects in the CNS to stimulate the production of ACTH and then glucocorticoids. However, in the peripheral tissues, CRH is proinflammatory, and its antagonists can be used to treat arthritis in animal models. Experiments from various animal models with knockout of VIP, AM, VIP/PACAP receptors, or Ucn result in endotoxemia and lung inflammation as well as Th1-mediated responses *in vivo*, and further highlight the physiological relevance of these neuroimmune peptides in certain inflammatory and autoimmune conditions. In summary, a broad range of neuropeptides is capable of influencing immune responses. They may serve to integrate a host's immune and nervous system response to injurious environmental stimuli. The physiological relevance of immunoregulatory activity of these neuropeptides needs further investigation.

ACKNOWLEDGMENTS

The authors thank Dr. Esther M. Sternberg for reviewing the manuscript and constructive discussion. This research was supported [in part or whole] by the Intramural Research Program of the National Institute Health (NIH), National Cancer Institute.

REFERENCES

1. Anton PM, Gay J, Mykoniatis A, Pan A, O'Brien M, Brown D, et al. Corticotropin-releasing hormone (CRH) requirement in *Clostridium difficile* toxin A-mediated intestinal inflammation. *Proc Natl Acad Sci U S A* 2004;**101**:8503–8.
2. Blalock JE. Production of peptide hormones and neurotransmitters by the immune system. *Chem Immunol* 1992;**52**:1–24.
3. Bodnar RJ, Klein GE. Endogenous opiates and behavior. *Peptides* 2003;**2004**(25):2205–56.
4. Calixto JB, Medeiros R, Fernandes ES, Ferreira J, Cabrini DA, Campos MM. Kinin B1 receptors: key G-protein-coupled receptors and their role in inflammatory and painful processes. *Br J Pharmacol* 2004;**143**:803–18.
5. Carr DJ, DeCosta BR, Jacobson AE, Rice KC, Blalock JE. Corticotropin-releasing hormone augments natural killer cell activity through a naloxone-sensitive pathway. *J Neuroimmunol* 1990;**28**:53–61.
6. Charmandari E, Tsigos C, Chrousos G. Endocrinology of the stress response. *Annu Rev Physiol* 2005;**67**:259–84.
7. Deghenghi R, Papotti M, Ghigo E, Muccioli G. Cortistatin, but not somatostatin, binds to growth hormone secretagogue (GHS) receptors of human pituitary gland. *J Endocrinol Invest* 2001;**24**:RC1–3.
8. Del Rio M, Hernanz A, de la Fuente M. Bombesin, gastrin-releasing peptide, and neuromedin C modulate murine lymphocyte proliferation through adherent accessory cells and activate protein kinase C. *Peptides* 1994;**15**:15–22.
9. Delgado M, Pozo D, Ganea D. The significance of vasoactive intestinal peptide in immunomodulation. *Pharmacol Rev* 2004;**56**:249–90.
10. Dimitrijevic M, Stanojevic S, Vujic V, Kovacevic-Jovanovic V, Beck-Sickinger A, Demuth H, et al. Effect of neuropeptide Y on inflammatory paw edema in the rat: involvement of peripheral NPY Y1 and Y5 receptors and interaction with dipeptidyl-peptidase IV (CD26). *J Neuroimmunol* 2002;**129**:35–42.
11. Gonzalez-Rey E, Chorny A, Delgado M. Regulation of immune tolerance by anti-inflammatory neuropeptides. *Nat Reviews Immunol* 2007;**7**:52–63.
12. Gonzalez-Rey E, Chorny A, O'Valle F, Delgado M. Adrenomedullin protects from experimental arthritis by down-regulating inflammation and Th1 response and inducing regulatory T cells. *Am J Pathol* 2007;**170**:263–71.
13. Gonzalez-Rey E, Delgado M. Anti-inflammatory neuropeptide receptors: new therapeutic targets for immune disorders? *Trends Pharmacol Sci* 2007;**28**:482–91.
14. Gonzalez-Rey E, Fernandez-Martin A, Chorny A, Delgado M. Therapeutic effect of urocortin and adrenomedullin in a murine model of Crohn's disease. *Gut* 2006;**55**:824–32.
15. Gonzalez-Rey E, Ganea D, Delgado M. Neuropeptides: keeping the balance between pathogen immunity and immune tolerance. *Curr Opin Pharmacol* 2010;**10**:473–81.
16. Gonzalez-Rey E, Varela N, Sheibanie AF, Chorny A, Ganea D, Delgado M. Cortistatin, an antiinflammatory peptide with therapeutic action in inflammatory bowel disease. *Proc Natl Acad Sci U S A* 2006;**103**:4228–33.
17. Gravanis A, Margioris AN. The corticotropin-releasing factor (CRF) family of neuropeptides in inflammation: potential therapeutic applications. *Curr Med Chem* 2005;**12**:1503–12.
18. Johnson HM, Torres BA. A novel arginine vasopressin-binding peptide that blocks arginine vasopressin modulation of immune function. *J Immunol* 1988;**141**:2420–3.
19. Law PY, Wong YH, Loh HH. Molecular mechanisms and regulation of opioid receptor signaling. *Annu Rev Pharmacol Toxicol* 2000;**40**:389–430.
20. Levite M, Cahalon L, Hershkoviz R, Steinman L, Lider O. Neuropeptides, via specific receptors, regulate T cell adhesion to fibronectin. *J Immunol* 1998;**160**:993–1000.

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21. Loir B, Bouchard B, Morandini R, Del Marmol V, Deraemaecker R, Garcia-Borron JC, et al. Immunoreactive alpha-melanotropin as an autocrine effector in human melanoma cells. *Eur J Biochem* 1997; **244**:923–30.
22. Luger TA, Scholzen TE, Brzoska T, Bohm M. New insights into the functions of alpha-MSH and related peptides in the immune system. *Ann N Y Acad Sci* 2003;**994**:133–40.
23. McCarthy L, Wetzel M, Sliker JK, Eisenstein TK, Rogers TJ. Opioids, opioid receptors, and the immune response. *Drug Alcohol Depend* 2001;**62**:111–23.
24. O'Connor TM, O'Connell J, O'Brien DI, Goode T, Bredin CP, Shanahan F. The role of substance P in inflammatory disease. *J Cell Physiol* 2004;**201**:167–80.
25. Pozo D, Delgado M. The many faces of VIP in neuroimmunology: a cytokine rather a neuropeptide? *FASEB J* 2004;**18**:1325–34.
26. Prod'homme T, Weber MS, Steinman L, Zamvil SS. A neuropeptide in immune-mediated inflammation, Y? *Trends Immunol* 2006;**27**:164–7.
27. Santoni G, Amantini C, Lucciarini R, Pompei P, Perfumi M, Nabissi M, et al. Expression of substance P and its neurokinin-1 receptor on thymocytes: functional relevance in the regulation of thymocyte apoptosis and proliferation. *Neuroimmunomodulation* 2002;**10**:232–46.
28. Springer J, Geppetti P, Fischer A, Groneberg DA. Calcitonin gene-related peptide as inflammatory mediator. *Pulm Pharmacol Ther* 2003;**16**:121–30.
29. Stein C, Schafer M, Machelska H. Attacking pain at its source: new perspectives on opioids. *Nat Med* 2003;**9**:1003–8.
30. ten Bokum AM, Hofland LJ, van Hagen PM. Somatostatin and somatostatin receptors in the immune system: a review. *Eur Cytokine Netw* 2000;**11**:161–76.
31. Tsatsanis C, Androulidaki A, Dermitzaki E, Charalampopoulos I, Spiess J, Gravanis A, et al. Urocortin 1 and urocortin 2 induce macrophage apoptosis via CRFR2. *FEBS Lett* 2005;**579**:4259–64.
32. Varela N, Chorny A, Gonzalez-Rey E, Delgado M. Tuning inflammation with anti-inflammatory neuropeptides. *Expert Opin Biol Ther* 2007;**7**:461–78.
33. Webster EL, Barrientos RM, Contoreggi C, Isaac MG, Ligier S, Gabry KE, et al. Corticotropin releasing hormone (CRH) antagonist attenuates adjuvant induced arthritis: role of CRH in peripheral inflammation. *J Rheumatol* 2002;**29**:1252–61.
34. Zhang N, Inan S, Cowan A, Sun R, Wang JM, Rogers TJ, et al. A proinflammatory chemokine, CCL3, sensitizes the heat- and capsaicin-gated ion channel TRPV1. *Proc Natl Acad Sci U S A* 2005;**102**: 4536–41.
35. Zhang N, Rogers TJ, Caterina M, Oppenheim JJ. Proinflammatory chemokines, such as C-C chemokine ligand 3, desensitize mu-opioid receptors on dorsal root ganglia neurons. *J Immunol* 2004;**173**: 594–9.

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Chapter 93

Peptide Loading of MHC

Melissa J. Call

ABSTRACT

The proteins of the major histocompatibility complex (MHC) are central to the ability of an organism to protect itself from foreign invasion, be it viral, bacterial, or parasitic. This "protection," unfortunately for many, also mediates transplant rejection and, when mistakenly directed, results in autoimmune pathology. The two classes of MHC molecules source peptides in different ways and use different strategies to promote peptide loading. This chapter focuses on how immunogenic peptides are generated and the molecules that mediate peptide loading onto MHC proteins.

INTRODUCTION

There are two main classes of MHC molecules that display peptide epitopes. Their roles in immunity are distinct and perhaps the simplest way to illustrate their function is to consider some of the roles each plays during the course of a viral infection. A viral infection begins when infectious viral particles penetrate protective barriers such as the skin or mucosal membranes. Once in contact with a cell that supports viral entry, invasion followed by viral replication within the cytoplasm can begin. Any given cell has many countermeasures available to respond to infection, but MHC-mediated responses are particularly important in efficient pathogen clearance and the generation of lasting immunity. In almost all cells of the body, MHC-class I molecules are constitutively expressed and loaded with peptides derived from proteins being synthesized and degraded within the cell, and thus, it is extremely challenging for a virus to replicate using host machinery without supplying viral peptides to the MHC-class I loading pathway. This provides an elegant system for cells to display a "snapshot" of the proteome at the cell surface for perusal by circulating lymphocytes. The role of $CD8^+$ T-cells is to lyse cells that show signs of infection through the display of foreign peptides on surface MHC-class I molecules. Although this drastic response is extremely effective in limiting viral replication, it also has the potential to create catastrophic tissue damage if inappropriately deployed, and therefore, $CD8^+$ T-cell responses are highly regulated. Before a $CD8^+$ T-cell can kill an infected cell it must first become activated in the lymph node by dendritic cells (DCs).

Conventional migratory DCs are found with high frequency in peripheral tissues near protective barriers to sense when these are disrupted and to direct T-cell responses accordingly.[33] These sentinels straddle the innate and adaptive immune responses. DCs in the periphery are highly phagocytic and responsive to innate "danger signals"[22] that warn of infection. Dying cells and virions are rich sources of antigen for DCs, and once activated by the danger signals, DCs undergo changes to initiate an immune response. They alter their chemokine and cytokine expression profiles, leading to the recruitment of other immune cells to the site of infection. They migrate to the draining lymph node and can pass antigen on to other specialized DC subsets that reside there or interact directly with a large population of naïve T-cells. Costimulatory molecules that are required for the productive stimulation of T-cells are upregulated. Antigen processing within the lysosomal pathway is also upregulated, as is MHC-class II, which is loaded with peptides from endocytic compartments before export to the cell surface. In a specialized mechanism of "crosspresentation" that occurs efficiently in certain DC subsets, the peptides from the phagocytic pathway are also loaded onto MHC-class I molecules. The end result of these processes yields a potent antigen-presenting cell that can present peptides to both $CD4^+$ and $CD8^+$ T-cells. Unlike $CD8^+$ T-cells, $CD4^+$ T-cells do not generally lyse the infected cells through direct cytotoxicity, but rather exert their important effects by directing the quality of the adaptive immune response. Through recognition of peptides displayed on MHC-class II molecules, $CD4^+$ T-cells provide positive feedback in the form of cytokines to the DC, enhancing its ability to provoke long-lived and sustainable activation from naïve $CD8^+$ T-cells, which then traffic to the site of infection to lyse infected cells. Activated $CD4^+$ T-cells also promote antibody producing B-cell activation[21] through recognition of MHC-class II-peptide complexes generated from processed antigen acquired through surface immunoglobulin receptors. Subsequent antibody class-switching and affinity maturation is critical to the ability of the host to generate sterilizing immunity.

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00093-2
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Although this simplistic overview of some MHC-directed processes only touches on a few of the events required for viral clearance, it highlights the features of the MHC system that share critical information among the many cell types that must cooperate to generate a successful immune response. In this context, MHC-class I is available to all cell types to display fragments of proteins currently being synthesized within the cell, while MHC-class II is reserved for the "professional" antigen-presenting cells and endows them with the special ability to display foreign epitopes without being directly infected. DCs are the most efficient antigen-presenting cells and have the ability to transfer the peptides derived from extracellular antigens into the MHC-class I pathway, representing an important link in the crosstalk between MHC-class I and MHC-class II mediated responses. In the following sections, I will go into more detail on how the peptides that are presented by MHC-class I and MHC-class II are generated and the sources from which they are derived.

THE MHC LOCUS

The MHC locus, located on chromosome 6 in humans and chromosome 17 in mice, is a clustering of around 150 genes that are largely involved in immune regulation and encode many different proteins including both MHC-class I and MHC-class II molecules. In humans, the classical MHC-class I genes are extremely polymorphic and are encoded by HLA-A, HLA-B and HLA-C, the gene products of which dimerize with β2 microglobulin to form functional MHC-class I proteins. More than a thousand polymorphisms have been reported for each gene and many more will probably be found. There are currently 5400 distinct sequences reported for MHC-class I molecules. An individual possesses two alleles at each MHC-class I gene locus, one maternal and one paternal. Thus, an individual expresses up to six different classical MHC-class I gene products at the cell surface.

MHC-class II proteins are formed by hetero-dimerization of α- and β-chains. Humans have three classical MHC-class II proteins, HLA-DR, HLA-DP, and HLA-DQ. The most polymorphic gene encodes the HLA-DR β-chain and the products of this gene combine with an invariant α-chain to make HLA-DR molecules. In HLA-DP and HLA-DQ both chains are polymorphic. There are currently around 1600 alleles reported for MHC-class II α- and β-chains, although the number of productive combinations that can form functional proteins is more difficult to estimate. Discussion of the nomenclature and diversity of MHC molecules is complex and beyond the scope of this chapter, and the reader is directed to this reference for further information.[28]

MHC-CLASS I

The MHC-Class I Peptide-Binding Site

MHC-class I gene products are type I membrane proteins. Each has a short cytoplasmic sequence, transmembrane domain, a membrane-proximal immunoglobulin domain and a membrane-distal peptide-binding groove. This groove is formed by an eight-strand antiparallel β-pleated sheet "floor" supporting two α-helices that flank the bound peptide. The folded protein is joined by β2-microglobulin which assumes a membrane-proximal position next to the class I immunoglobulin domain, underneath the floor of the peptide-binding groove[2] (Fig. 1A).

The α-helices that line the MHC-class I peptide-binding groove are closely apposed at both ends and MHC-class I molecules are therefore only able to "comfortably" bind peptides of 8–10 amino acids in length. The peptide is maintained in an extended conformation through a series of interactions. The N- and C-termini of the peptide are bound through a conserved network of hydrogen bonds, and key "anchor" residues have their side-chains buried in pockets found along the peptide-binding groove (Fig. 1B). The interactions mediated by the N- and C-termini represent the bulk of the peptide-binding energy[4] and this sequence-independent feature allows MHC-class I to bind extremely diverse peptides (each allele is estimated to have the ability to bind many thousands of different sequences). The series of pockets in the floor of the peptide-binding groove (Fig. 1C) provide distinct chemical environments that favor certain residues over others, and the polymorphisms in MHC-class I are concentrated in these regions to endow each MHC allele with a unique peptide specificity.[10] These pockets place restrictions on size, charge, and polarity of anchor residues and can stabilize the peptides through hydrogen bonds, electrostatic interactions, hydrophobic packing, and van der Waals forces. Importantly, in most human MHC-class I alleles, the pocket that houses the peptide C-terminal residue favors hydrophobic residues and allows basic residues, but is incompatible with peptides that have an acidic residue at this position.

MHC-Class I Peptide-Loading Complex

MHC-class I proteins are directed to the endoplasmic reticulum (ER) on synthesis for folding and secretion to the cell surface. MHC-class I molecules are unable to reach the cell surface until they are loaded with peptide. Immediately after synthesis, MHC-class I heavy chain associates with calnexin, a molecular chaperone that resides in the ER (Fig. 2a). Calnexin is associated with ERp57, a protein thiol oxoreductase that influences disulfide bond formation in the nascent MHC-class I heavy chain. Through interaction with these proteins, the heavy chain is folded

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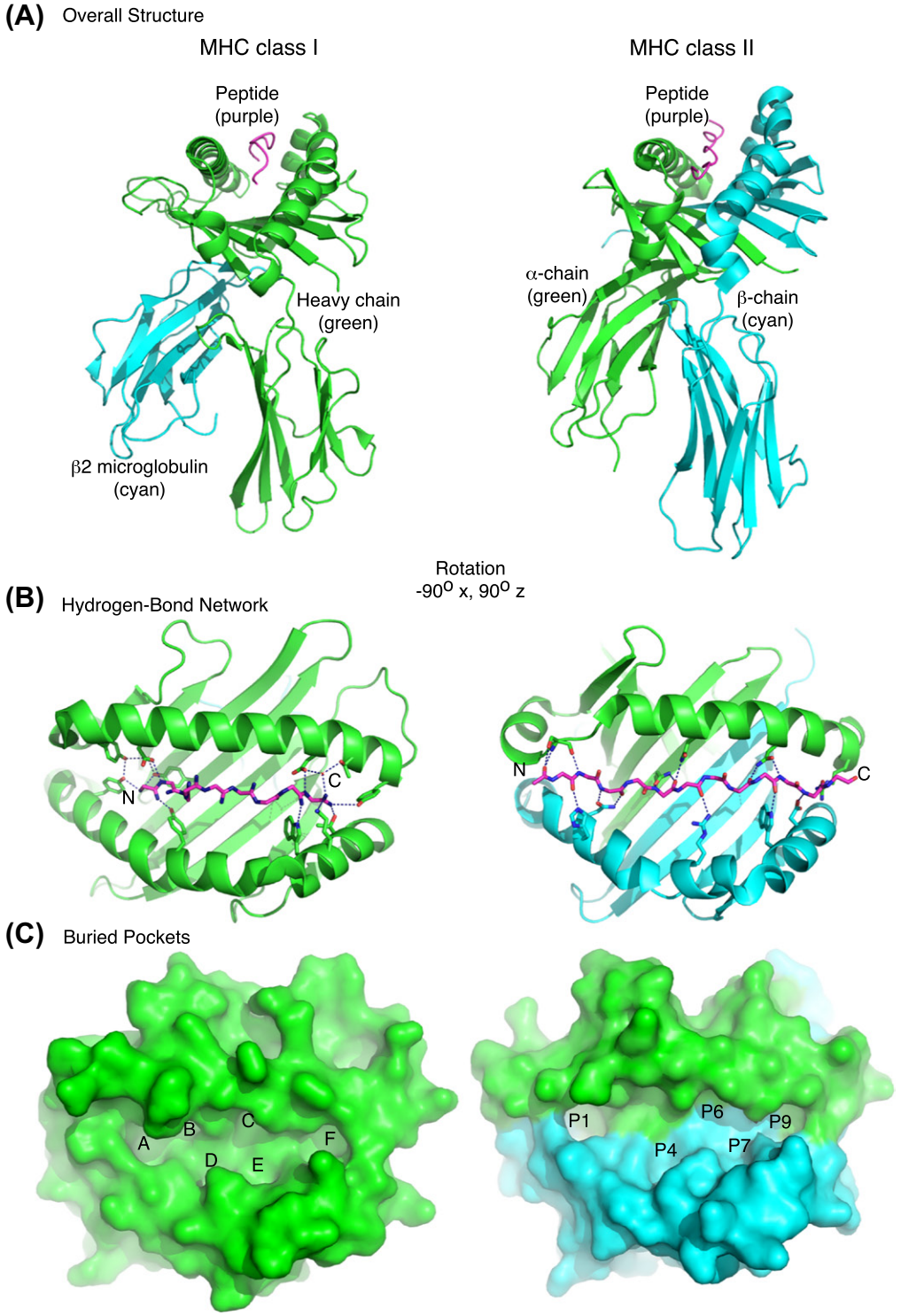

FIGURE 1 (A) Side view of MHC-class I (left) and MHC-class II (right) showing the peptide-binding site at the top and the membrane proximal immunoglobulin domains at the bottom. (B) Looking down on the peptide-binding site, which is formed by α-helices that flank the peptide and supported by an eight-strand β-sheet. Conserved hydrogen bonds that stabilize the peptide backbone are indicated as dashed lines in each structure. Note that in MHC-class II, the α- and β-chains together combine to form the groove, and peptide extends out of the peptide-binding site. The peptide N- and C-termini are indicated with N and C respectively. (C) The same view as (B), but in surface rendering with the peptide removed. The pockets that serve as anchor points for peptide side chains are labeled A–F for MHC-class I and P1–P9 for MHC-class II. PBD files 1S9Y and 1DLH were used to generate this figure. See color plate 23.

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allowing recruitment of β2-microglobulin, signaling that the MHC-class I molecule is ready for peptide loading (Fig. 2b). In humans, the folded MHC-class I – β2-microglobulin heterodimer then dissociates from calnexin and joins the peptide-loading complex containing calreticulin, transporter associated with antigen processing (TAP), tapasin, and ERp57 (Fig. 2c).[19] ERp57 is covalently linked to tapasin through a disulfide bond and this complex bridges the empty MHC heavy chain to TAP and calreticulin. In addition to its structural role in the peptide-loading complex, biochemical studies suggest that tapasin-ERp57 can modulate the quality of the peptide repertoire by favoring the loading of high-affinity peptides and promoting dissociation of prebound, low affinity peptides.[34]

TAP is an ATP-dependent transporter that delivers peptides from the cytosol to the ER lumen and comprises two

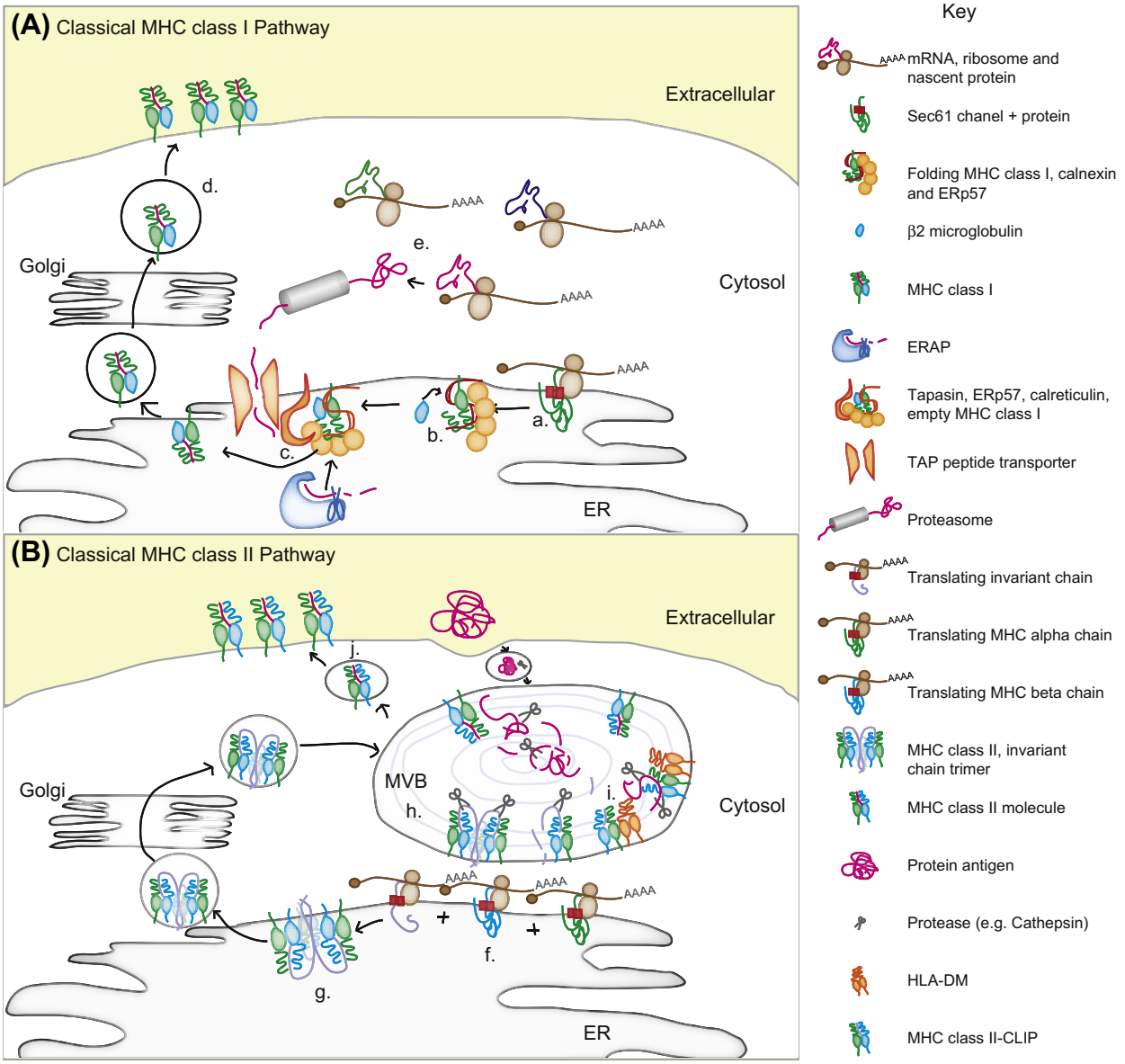

FIGURE 2 (A) MHC-class I heavy chains are synthesized in the ER (a) and then partially folded with assistance from calnexin and ERp57 (b). After β2 microglobulin joins the MHC-class I heavy chain the heterodimer is incorporated into the peptide-loading complex and loaded with peptides transported from the cytoplasm to the ER lumen by TAP and trimmed to the correct size by ERAP family proteases (c). Peptide-loaded MHC-class I is then transported via the Golgi to the cell surface (d). Proteasomes cleave proteins in to peptides, which can be selected by TAP for transport into the ER (e). (B) MHC-class II chains and invariant chain are synthesized in the ER (f) and fold together into a nonameric complex with 3 invariant chains, and 3 MHC-class II molecules (g). A region of the invariant chain (CLIP) occupies the peptide-binding groove of MHC-class II. This complex is exported via the Golgi to multivesicular bodies in the endocytic compartment (h). Proteolytic cleavage of invariant chain occurs to release MHC-class II molecules and allow HLA-DM mediated release of CLIP and replacement with peptides from endocytozed proteins (i). MHC-class II molecules are transported to the cell surface for display to CD4+ T-cells (j). See color plate 24.

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proteins, TAP1 and TAP2.[27] Like MHC-class I, TAP exhibits selectivity for peptides that have hydrophobic or basic C-terminal residues and are between 8 and 16 amino acids in length.[13] TAP has the ability to transport peptides that are longer than those preferred by MHC-class I, and proteases that trim the N-terminus are present in the ER lumen to ensure peptides of optimal length can be generated. In humans, ERAP1 and ERAP2 fulfill this role. Studies with ERAP1 have determined that this protease exhibits selectivity for peptides between 9 and 16 amino acids in length and displays little activity for peptides longer or shorter than this. A model has been proposed that suggests the peptide C-terminal residue is bound by ERAP1 in a pocket distinct from the active site and that the distance between these two sites determines the selectivity of ERAP1 for peptide length. If accurate, it would suggest that peptide trimming occurs before loading into MHC-class I.[20]

The Classical MHC-Class I Pathway

The source of peptides displayed by classical MHC-class I molecules and the pathways that generate them have been topics of intense research. Initially, it was assumed that peptides were simply "borrowed" from natural turnover processes, but because proteins often have half-lives of hours to days within a cell, the kinetics of this process did not seem to be congruous with the observation that viral peptides could be displayed by MHC-class I molecules in less than an hour after infection. Although natural turnover processes do account for some of the peptides displayed by MHC-class I, it is now thought that the bulk of peptides are acquired through the cleanup of incomplete synthesis products. Estimates indicate that up to 40% of the products generated by protein synthesis are rapidly degraded because of errors that might include early termination, misfolding, missplicing, and frameshift errors. Whether protein synthesis is simply a messy business or whether these errors are made specifically to generate peptides for the MHC-class I pathway is not known and remains a matter of debate.[9]

Proteins are targeted to the proteasome after becoming tagged with poly-ubiquitin chains, the reasons for which are diverse and include normal protein turnover, the destruction of improperly synthesized proteins described above, as well as regulatory proteins controlling cell cycle progression and intracellular signaling. The proteasome is a multisubunit complex[11] with a cylindrical catalytic core that is gated at each end by regulatory cap particles that are defined by their sedimentation values. The addition of the 19S regulatory caps to the 20S proteasomal core constitutes the 26S proteasome. Poly-ubiquitin chains of the proteins to be degraded are recognized by proteins in the 19S regulatory cap and, in an ATP-dependent process, ubiquitin is removed and the protein unwound to allow entry into the 20S proteasomal core. It is here that cleavage occurs, generating peptide fragments between 2 and 25 amino acids in length[18] (Fig. 2e). In humans the cylindrical 20S proteasomal core is made up of four heptameric rings. The alpha subunits, α1–-7, flank each end of the cylinder and form narrow openings that are modulated by the 19S regulatory cap. Sandwiched between the α rings are two antiparallel β rings made up of β1–β7 subunits.[11] Only three of these contain catalytically active sites, but between them they are able to cut after almost any amino acid: β5 is chymotrypsin-like, cleaving after bulky hydrophobic residues; β2 is trypsin-like and prefers to cleave after basic residues; and β1, which is caspase-like, cleaves after acidic and branched amino acids.

The pattern of cleavage for a particular protein is not random, and the same fragments are generated each time a protein is processed because the activity of the catalytic triad in each β subunit is sensitive to the sequence context around a cleavage site. The combination of limited time within the proteasome chamber and preferred sequences determining initial cleavage sites results in consistent, though difficult to predict, peptide products for any given protein. Peptides that are longer than seven amino acids in length are potential ligands for MHC-class I molecules, but most do not survive exposure to cytosolic proteases. A nine amino acid peptide in the cytosol has a half-life of less than 10 s, and therefore only peptides that are rapidly transported into the ER lumen have the opportunity to fold with MHC-class I heavy chains awaiting export to the cell surface.

The Immunoproteasome

Almost all cells in the body have the ability to express interferon (IFN) α/β in response to infection. This hallmark of stress, among others, is recognized by natural killer (NK) cells, which in turn secrete IFNγ. IFNγ has potent effects on antigen processing in both the MHC-class I and II pathways and is particularly important in antiviral responses.

Under normal cellular conditions, the proteasome is actually quite poor at generating peptides suitable for MHC-class I binding because most products are smaller than the minimal length required for ER import and MHC-class I loading. Additionally, all MHC-class I molecules studied to date prefer hydrophobic or basic anchors at the peptide C-terminus and acidic residues in particular seem to be excluded. This means that peptides generated by β1 cleavage, which occurs after acidic residues, are essentially lost from the MHC-class I repertoire. However, IFNγ exposure causes expression of three new β subunits, β1i, β2i, and β5i, which replace β1, β2, and β5 in the 20S proteasome catalytic core and all but eliminate the caspase-like activity. Thus most peptides processed by this alternative proteasome, termed the "immunoproteasome," contain suitable C-terminal anchor residues for MHC-class I binding. In addition to replacement of three β subunits, the immunoproteasome also uses an alternative regulatory cap known

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as PA28, REG, or 11S Activator, which is IFNγ-inducible and significantly increases the maximal velocity of peptide hydrolysis compared with the normal 26S proteasome.[6] Interestingly, although the expression of the immunoproteasome is IFNγ dependent in most cell types, the alternative subunits are constitutively expressed in some cells of hematopoietic origin, including DCs and lymphoid cells, emphasizing their specialization for antigen processing and presentation. Mice lacking all three alternative catalytic subunits exhibited alterations in the MHC-class I peptide repertoire that were significant enough to cause the rejection of splenocytes transferred from wild type mice despite being matched at all MHC loci.[17]

A Specialized Thymic Proteasome

Within the thymus, the organ where T-cells reactive to the self are deleted to prevent autoimmunity, the cells that mediate this "negative selection" express both the proteasome and the immunoproteasome. Interestingly, there is a third "thymoproteasome" that uses an alternative β5 subunit in a distinct population of thymic stromal cells responsible for "positive selection," a process that ensures T-cells receive tonic stimulation by MHC for survival in the periphery. The thymoproteasome generates peptides that display significantly reduced affinity for MHC-class I, and it has been noted that mice deficient in this alternative β5 subunit have severe defects in positive selection.[26] The mechanism behind this observation is not yet understood, but it has been proposed that unstable MHC-class I peptide complexes may be somehow required for this process or that the thymoproteasome generates unique stimulatory peptides to drive positive selection.

Crosspresentation

MHC-class I is expressed ubiquitously and, for the most part, displays peptides derived from intracellular sources, with the notable exception of certain specialized cell types such as CD8αα$^+$ DCs that have the ability to load proteins derived from extracellular sources onto MHC-class I. This specialized pathway, termed crosspresentation, allows priming of cytotoxic CD8$^+$ T-cells by professional antigen-presenting cells that are not directly infected. Endocytic receptors seem to be critical regulators of crosspresentation, with antigens targeted to DEC205, for instance, being efficiently processed for MHC-class I loading, while those targeted to DCIR2 are not. Although it was initially assumed that these markers simply distinguished cells with different inherent abilities to crosspresent, follow-up experiments determined that introduction of DEC205 into the CD8$^-$ DC subset that does not normally cross-present, is sufficient to enable cross-presentation to occur.[16] The evidence suggests that crosspresentation is proteasome dependent, and therefore a mechanism by which antigen can escape the phagolysosome to reach the cytoplasm must exist. It is considered unlikely that antigens access the cytoplasm through permeabilized phagosomal membranes and research has instead focused on channels that have the ability to translocate proteins to the cytoplasm. The process of ER associated protein degradation (ERAD), which translocates misfolded proteins from the ER to the cytoplasm for degradation by the proteasome provides an attractive solution to this problem, but requires either the recruitment of ER membrane, or more controlled delivery of ERAD components to the phagosomal pathway. Once antigen has reached the cytoplasm it may intersect with the classical MHC-class I loading pathway, but evidence suggests that peptide loading during crosspresentation may actually occur in the phagocytic compartment itself, with the recruitment of proteasomes and peptide-loading machinery directly to the vesicles.[15] Although the mechanics of cross-presentation remain controversial, it is clear that this process is extremely important for CD8$^+$ T-cell function.

Nonclassical MHC-Class I

Although the products of the classical MHC-class I alleles HLA-A, -B, and -C have a crucial role in binding peptides for display to cytotoxic T-cells, the nonclassical HLA-E, -F, and -G alleles have more poorly defined roles. These proteins do bind peptide, but they are much less polymorphic than HLA-A, -B, and -C, and this is reflected in their restricted peptide repertoire. The best studied of these alleles is HLA-E, which is recognized by the CD94/NKG2 receptor system and seems to have a major role in regulating NK cell activity.[32] HLA-E primarily binds peptides that are derived from the signal sequences of other MHC-class I molecules and is believed to convey information about normal MHC biosynthesis to NK cells. NK cells are reactive to alterations in the MHC-class I pathway that are commonly caused by viral proteins evolved to inhibit MHC-mediated immune responses. Normal operation of the pathway results in peptide-loaded HLA-E binding to inhibitory receptors on NK cells and suppression of cytotoxicity, but loss of this signal in virally infected cells can trigger killing. In a striking evolutionary "tit-for-tat," human cytomegalovirus encodes a homolog of the MHC-class I leader peptide to maintain HLA-E at the cell surface while other virulence factors downregulate classical MHC-class I expression.[31]

MHC-CLASS II

MHC-Class II Peptide-Binding Site

Nascent MHC-class II molecules form from α- and β-chains that are assembled in the ER. Like the MHC-class I heavy chain, the β-chain forms a membrane-proximal immunoglobulin domain, but unlike its class I counterpart,

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only contributes to half of the peptide-binding domain. The α-chain helps complete the peptide-binding domain and also contributes a membrane-proximal immunoglobulin domain so that, unlike MHC-class I, MHC-class II does not require β2 microglobulin (Fig. 1). Although the overall fold of MHC-class II molecules resembles that of MHC-class I, there are significant differences that endow these closely related structures with very different peptide-binding properties.[5] The peptide is accommodated in the groove in an extended conformation similar to that of a type II poly-proline helix and importantly, because the helices in MHC-class II molecules do not close together at each end of the peptide-binding groove as with MHC-class I proteins, MHC-class II molecules can bind much longer peptides. This means that polar contacts cannot always be made to free N- and C-termini of the peptide and interactions are instead dominated by a conserved network of hydrogen bonds to polar backbone atoms along the entire length of the peptide (Fig. 1B). Structural and biochemical studies indicate that the strongest binding energy is contributed by hydrogen bonds concentrated at the N-terminus of the bound peptide. These sequence-independent interactions are further complemented by anchoring pockets that, like MHC-class I, favor peptides with compatible amino acids (Fig. 1C). The register that any given peptide binds to MHC class II is defined by the residue that binds in the P1 pocket. The peptide residues are sequentially numbered in an N- to C-terminal direction. P1, P4, P6, and P9 residues interact with the major pockets in MHC, while P2, P5, and P8 are major TCR contact points. The P1 and P4 pockets of HLA-DR molecules contribute the most to peptide stability. A natural polymorphism at residue 86 of the HLA-DR β-chain influences the size of the P1 pocket. A glycine at this position provides a large pocket that can accommodate bulky hydrophobic residues while a valine at this position presents a shallower pocket that prefers smaller aliphatic residues.

Invariant Chain Processing and HLA-DM-Mediated Peptide Exchange

Each MHC-class II heterodimer assembles in the ER with the invariant chain, a trimeric chaperone that is exported through the Golgi and trans-Golgi network to intersect with the endosomal pathway in multivesicular antigen-processing compartments (Fig. 2f–h).[30] The invariant chain serves a number of purposes. A fragment of the invariant chain is embedded in the peptide-binding site to prevent opportunistic binding of proteins/peptides in the ER[29] and to stabilize the MHC-class II peptide-binding site, which is prone to collapse and aggregation if left unoccupied. The invariant chain cytoplasmic domain also contains trafficking signals that target MHC-class II to the appropriate subcellular location for peptide loading to occur.

As the invariant chain occupies the peptide-binding groove, it must be dislodged from MHC-class II to allow new peptide acquisition, and two important processes ensure that this is achieved. First, proteolytic degradation of invariant chain occurs which releases MHC-class II from all but the invariant-chain-derived fragment protecting the peptide-binding site. This fragment is termed CLIP and typically corresponds to residues approximately 80–105 depending on the fine mapping of protease cleavage sites. Degradation of invariant chain involves a number of proteases that are sensitive to the protease inhibitor leupeptin,[3] and although the complete pathway has not been elucidated, two proteases in particular play nonredundant roles in the latter steps of invariant chain processing: these are cathepsin S (in the periphery) and cathepsin L (in the thymus).[14] Attempts to identify proteases that have a nonredundant role in early steps of invariant chain processing have failed, and this no doubt reflects the susceptibility of the invariant chain to cleavage by multiple enzymes.

HLA-DM ensures that the protective CLIP peptide is then exchanged for exogenously derived peptides from the endocytic pool. HLA-DM is a heterodimeric MHC-class II protein that does not itself bind peptides.[23] HLA-DM resides in late endosomal compartments and is responsible for "editing" the peptide repertoire to promote formation of high-affinity complexes with very stably bound peptides. To achieve this, HLA-DM binds to MHC-class II molecules that contain suboptimal P1 anchor residues and stabilizes the empty groove to encourage bound peptides to leave and give new peptides access to sample the site. Peptides that supply optimal P1 anchor residues then cause HLA-DM to dissociate from MHC-class II and are protected from further editing by stable P1 interactions between peptide and MHC that block HLA-DM binding.[1] HLA-DM is negatively regulated by HLA-DO in a process that is thought to be mediated by competitive binding to HLA-DM in a way that prohibits interaction with MHC-class II complexes. HLA-DO expression depends on cell type and activation state, and is thought to be an important regulatory pathway in B-cells.[8]

Properties of CLIP

The CLIP peptide binds promiscuously to many MHC-class II alleles, albeit with widely variable affinity. For instance, the half-life of a MHC-class II allele that is associated with susceptibility to rheumatoid arthritis (DRB1*0401) in complex with CLIP is in the order of minutes, while another common allele (DRB1*0101) forms complexes that are stable for days. These differences in stability are unsurprising given that MHC-class II is highly polymorphic and those polymophisms are concentrated within the peptide-binding groove. In this context, perhaps what is more surprising is that there exists a single peptide that seems to interact

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with most of the MHC-class II molecules. Only one crystal structure of an MHC-class II molecule in complex with CLIP has been determined. In this structure, methionine 90 of the invariant chain occupies the P1 pocket of the class II molecule HLA-DR3 (DRB1*0301). Almost all of the MHC-class II alleles analyzed thus far through mutagenesis studies appear to bind CLIP in this register, with the notable exceptions of two HLA-DQ alleles associated with celiac disease in which threonine 94 occupies the P1 pocket.[35] The peptide-binding site of MHC-class II molecules is open at each end, giving peptides the ability to shift register in the binding site. This, no doubt, allows different alleles to select different segments of the CLIP sequence according to variable motif preferences.

Generation of Peptides for MHC-Class II Loading

Antigen that is taken up by phagocytosis, receptor-mediated endocytosis or pinocytosis must traverse the endocytic pathway before loading onto MHC-class II molecules can be accomplished. The endocytic pathway has distinct compartments. Early endosomes are the principal sorting organelles where decisions are made as to whether molecules are exported back to the cell surface (recycling endosomes) or delivered to late endosomes. Early endosomes are mildly acidic and while MHC peptide complexes exhibit extraordinarily long half-lives, under acidic conditions peptide release is accelerated and therefore recycling endosomes are a possible site of peptide exchange. Late endosomes, however, are the principle location of MHC-class II loading (pH ~5.5) (Fig. 2h). There is some debate as to whether there are distinct late endosomes that are specialized for MHC-class II loading (typically referred to as MIIC) or whether the components required for loading are distributed throughout all late endosomes in antigen-presenting cells.[25] The final destination in the endocytic pathway is the lysosome. Lysosomes are highly acidified and even MHC-class II molecules, which are acid resistant, are unable to survive for long in this environment. In immature DCs, MHC-class II is targeted to lysosomes for degradation until maturation signals are encountered. MHC-class II is then directed to the late endosomes for peptide loading and then to the cell surface for peptide display.

The proteases with the largest role in generating antigenic peptide fragments for MHC-class II are the cathepsins. Cathepsins are primarily cysteine proteases with pH- and redox-dependent activity and are distributed throughout the endosomal compartment.[14] Expressed as proforms, they require proteolytic cleavage themselves to become activated. Although some cathepsins can accomplish their own activation through autocatalytic proteolysis, others require a second protease, which itself might display pH dependence. In this manner, the activity of certain cathepsins can be restricted to different parts of the endocytic pathway, and this is believed to influence the rate of proteolysis to ensure large enough protein fragments reach the compartments where peptide loading takes place.

MHC-class II molecules bind peptides in extended conformations, and for this to occur, denaturation of secondary structure must take place. In the endosomal pathway proteins are denatured by a combination of events: acidification interferes with salt bridges and hydrogen bonds; IFNγ-inducible lysosomal thiol reductase (GILT) reduces cysteine-mediated disulfide bonds;[12] and endoproteases such as cathepsins B, D, F, G, H, K, S, or asparagine endopeptidase (AEP) cleave exposed flexible areas of proteins to cause catastrophic destabilization.[14] It is probable that MHC-class II molecules then bind to large polypeptide fragments, which are trimmed from the C- and N-termini by cathepsins with carboxy and amino proteolytic activity, respectively. This allows MHC-class II, which is assisted by HLA-DM, to bind securely to a peptide epitope and protect it from further proteolysis.[7]

Model antigens have been used in an attempt to determine the relative contribution of proteases to the generation of epitopes recognized by T-cells. Each cathepsin's activity destroys some epitopes while creating others, but mice that are deficient in individual cathepsins (with the exception of Cathepsin S and L, which are required for invariant chain processing) show only minor defects in antigen presentation, indicating a high degree of redundancy in the system. It has been particularly difficult to ascribe unique roles to individual proteases in the generation of defined peptide-MHC-class II complexes because effects on the MHC-class II pathway may be caused by impaired invariant chain processing, impaired epitope generation, or impaired activation of proteases involved in these processes.

Autophagy and MHC-Class II

Autophagy, which means to "eat oneself," is the mechanism by which cells break down and recycle intracellular contents and is important for the maintenance of cellular homeostasis. Normally, autophagic activity is low, but during starvation, cellular stress, or infection, autophagy is upregulated and provides a source of peptides to the MHC-class II pathway. Autophagy is initiated by the recruitment of membrane, probably from the ER, to encircle a large volume of cytosol and its organellar contents. Initiation of this process in response to starvation is controlled by mTOR, which is released from its inhibitory complex when energy levels in the cell fall below a certain threshold. The recruited membrane then fuses to generate a double-membrane vesicle that is targeted to the lysosomal pathway. Access to the lysosomal pathway not only allows the contents to be degraded into building blocks that can be used by the cell to form new proteins and lipids, but also delivers

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them to the MHC-class II loading pathway described above. Autophagy by macrophages and DCs in response to infection can be mediated by pattern recognition receptors in the cytoplasm and at the cell surface. Almost all the toll-like receptors studied to date influence the autophagic pathway, and this represents an important defense mechanism for the cell to rid itself of intracellular bacteria and parasites that might otherwise evade detection.[24]

CONCLUDING REMARKS

The MHC system and the peptides it presents to T-cells are at the heart of the adaptive immune response, and while we understand the basics of peptide processing and acquisition, there is much left to be learned. The outcome of a thorough and detailed understanding of these processes should provide the ability to manipulate the timing and location of peptide presentation. The ability to do this has implications in vaccine development, tumor immunity, transplant rejection, and autoimmunity.

REFERENCES

1. Anders AK, Call MJ, Schulze MS, Fowler KD, Schubert DA, Seth NP, et al. HLA-DM captures partially empty HLA-DR molecules for catalyzed removal of peptide. *Nat Immunol* 2011;**12**:54–61.
2. Bjorkman PJ, Saper MA, Samraoui B, Bennett WS, Strominger JL, Wiley DC. The foreign antigen binding site and T cell recognition regions of class I histocompatibility antigens. *Nature* 1987;**329**: 512–8.
3. Blum JS, Cresswell P. Role for intracellular proteases in the processing and transport of class II HLA antigens. *Proc Natl Acad Sci U S A* 1988;**85**:3975–9.
4. Bouvier M, Wiley DC. Importance of peptide amino and carboxyl termini to the stability of MHC class I molecules. *Science* 1994;**265**:398–402.
5. Brown JH, Jardetzky TS, Gorga JC, Stern LJ, Urban RG, Strominger JL, et al. Three-dimensional structure of the human class II histocompatibility antigen HLA-DR1. *Nature* 1993;**364**:33–9.
6. Cascio P, Call M, Petre BM, Walz T, Goldberg AL. Properties of the hybrid form of the 26S proteasome containing both 19S and PA28 complexes. *EMBO J* 2002;**21**:2636–45.
7. Castellino F, Zappacosta F, Coligan JE, Germain RN. Large protein fragments as substrates for endocytic antigen capture by MHC class II molecules. *J Immunol* 1998;**161**:4048–57.
8. Denzin LK, Sant'Angelo DB, Hammond C, Surman MJ, Cresswell P. Negative regulation by HLA-DO of MHC class II-restricted antigen processing. *Science* 1997;**278**:106–9.
9. Dolan BP, Bennink JR, Yewdell JW. Translating DRiPs: progress in understanding viral and cellular sources of MHC class I peptide ligands. *Cell Mol Life Sci* 2011;**68**:1481–9.
10. Garrett TP, Saper MA, Bjorkman PJ, Strominger JL, Wiley DC. Specificity pockets for the side chains of peptide antigens in HLA-Aw68. *Nature* 1989;**342**:692–6.
11. Groll M, Ditzel L, Lowe J, Stock D, Bochtler M, Bartunik HD, et al. Structure of 20S proteasome from yeast at 2.4 A resolution. *Nature* 1997;**386**:463–71.
12. Hastings KT, Cresswell P. Disulfide reduction in the endocytic pathway: immunological functions of gamma-interferon-inducible lysosomal thiol reductase. *Antioxid Redox Signal* 2011;**15**:657–68.
13. Heemels MT, Schumacher TN, Wonigeit K, Ploegh HL. Peptide translocation by variants of the transporter associated with antigen processing. *Science* 1993;**262**:2059–63.
14. Honey K, Rudensky AY. Lysosomal cysteine proteases regulate antigen presentation. *Nat Rev Immunol* 2003;**3**:472–82.
15. Houde M, Bertholet S, Gagnon E, Brunet S, Goyette G, Laplante A, et al. Phagosomes are competent organelles for antigen cross–presentation. *Nature* 2003;**425**:402–6.
16. Kamphorst AO, Guermonprez P, Dudziak D, Nussenzweig MC. Route of antigen uptake differentially impacts presentation by dendritic cells and activated monocytes. *J Immunol* 2010;**185**:3426–35.
17. Kincaid EZ, Che JW, York I, Escobar H, Reyes-Vargas E, Delgado JC, et al. Mice completely lacking immunoproteasomes show major changes in antigen presentation. *Nat Immunol* 2011;**13**:129–35.
18. Kisselev AF, Akopian TN, Woo KM, Goldberg AL. The sizes of peptides generated from protein by mammalian 26 and 20 S proteasomes. Implications for understanding the degradative mechanism and antigen presentation. *J Biol Chem* 1999;**274**:3363–71.
19. Koch J, Tampe R. The macromolecular peptide-loading complex in MHC class I-dependent antigen presentation. *Cell Mol Life Sci* 2006;**63**:653–62.
20. Kochan G, Krojer T, Harvey D, Fischer R, Chen L, Vollmar M, et al. Crystal structures of the endoplasmic reticulum aminopeptidase-1 (ERAP1) reveal the molecular basis for N-terminal peptide trimming. *Proc Natl Acad Sci U S A* 2011;**108**:7745–50.
21. McHeyzer-Williams LJ, McHeyzer-Williams MG. Antigen–specific memory B cell development. *Annu Rev Immunol* 2005;**23**:487–513.
22. Medzhitov R. Recognition of microorganisms and activation of the immune response. *Nature* 2007;**449**:819–26.
23. Morris P, Shaman J, Attaya M, Amaya M, Goodman S, Bergman C, et al. An essential role for HLA-DM in antigen presentation by class II major histocompatibility molecules. *Nature* 1994;**368**:551–4.
24. Munz C. Enhancing immunity through autophagy. *Annu Rev Immunol* 2009;**27**:423–49.
25. Neefjes J. CIIV, MIIC and other compartments for MHC class II loading. *Eur J Immunol* 1999;**29**:1421–5.
26. Nitta T, Murata S, Sasaki K, Fujii H, Ripen AM, Ishimaru N, et al. Thymoproteasome shapes immunocompetent repertoire of CD8+T cells. *Immunity* 2010;**32**:29–40.
27. Powis SJ, Townsend AR, Deverson EV, Bastin J, Butcher GW, Howard JC. Restoration of antigen presentation to the mutant cell line RMA-S by an MHC-linked transporter. *Nature* 1991;**354**:528–31.
28. Robinson J, Mistry K, McWilliam H, Lopez R, Parham P, Marsh SG. The IMGT/HLA database. *Nucleic Acids Res* 2011;**39**:D1171–6.
29. Roche PA, Cresswell P. Invariant chain association with HLA-DR molecules inhibits immunogenic peptide binding. *Nature* 1990;**345**: 615–8.
30. Stern LJ, Potolicchio I, Santambrogio L. MHC class II compartment subtypes: structure and function. *Curr Opin Immunol* 2006;**18**:64–9.
31. Tomasec P, Braud VM, Rickards C, Powell MB, McSharry BP, Gadola S, et al. Surface expression of HLA-E, an inhibitor of natural killer cells, enhanced by human cytomegalovirus gpUL40. *Science* 2000;**287**:1031.
32. van Hall T, Oliveira CC, Joosten SA, Ottenhoff TH. The other Janus face of Qa-1 and HLA-E: diverse peptide repertoires in times of stress. *Microbes Infect* 2010;**12**:910–8.

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33. Villadangos JA, Schnorrer P. Intrinsic and cooperative antigen–presenting functions of dendritic-cell subsets in vivo. *Nat Rev Immunol* 2007;**7**:543–55.
34. Wearsch PA, Cresswell P. Selective loading of high-affinity peptides onto major histocompatibility complex class I molecules by the tapasin-ERp57 heterodimer. *Nat Immunol* 2007;**8**:873–81.
35. Wiesner M, Stepniak D, de Ru AH, Moustakis AK, Drijfhout JW, Papadopoulos GK, et al. Dominance of an alternative CLIP sequence in the celiac disease associated HLA-DQ2 molecule. *Immunogenetics* 2008;**60**:551–5.

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Chapter 94

Peptide Recognition by T Cells

Malte Mohme and Roland Martin

ABSTRACT

Adaptive immune responses are mediated by either soluble immunoglobulin molecules or thymus-mediated, antigen-specific immune recognition, and orchestrate almost every aspect of physiological and pathological immune responses including the destruction of virus-infected cells, the differentiation of antibody-forming B cells, the lysis of tumor cells, and also allergic responses, autoimmune diseases, transplant rejection, and graft-versus-host disease. During the last three decades, a large proportion of research in immunology has been devoted to understanding T cell specificity. After the description that T cells recognize antigen in the context of self-major histocompatibility complex (MHC) molecules, which was coined "self-MHC restriction of T cells," the nature of the antigen was studied in detail. As is briefly described in this chapter, T cells respond with their clonotypic T cell antigen receptor to short peptides that are generated by the antigen-processing machinery of antigen-presenting cells and displayed on the surface of these in the context of self-MHC molecules.

INTRODUCTION

Different from antibodies that are largely specific for conformational determinants of macromolecules, T cells do not recognize antigen derived from, for example, a foreign pathogen itself, but only in the context of self-major histocompatibility complex (MHC; in humans also referred to as human leukocyte antigen; HLA) molecules on the surface of antigen-presenting cells (APC) such as dendritic cells (DC), B cells, or macrophages. This central observation by Zinkernagel and Doherty,[35] Bevan,[3] and others is now referred to as MHC/HLA-restriction of T cells. There are many subtypes of T cells that are characterized by specific surface molecules such as differentiation markers or by their functional phenotype such as the secretion of cytokines or chemokines. However, recent work has shown that especially with respect to secretion of soluble mediators T helper cells are more plastic than was previously believed. For the purpose of this chapter, only the two main populations will be mentioned, and these are defined by the expression of either one of the two coreceptors, the cluster of differentiation (CD) markers CD4 or CD8. CD4+ T cells are also referred to as T helper cells, whereas CD8+ T cells are often referred to as cytotoxic T cells. The subdivision into these two main categories of T cells is important not only because of their differing functions, but also because they recognize antigen in the context of different MHC/HLA (from here on these abbreviations will be used interchangeably) molecules. CD8+ T cells are restricted by MHC-class I molecules, and CD4+ by MHC-class II molecules with very few exceptions to these rules. Both CD4+ and CD8+ T cells express on their surface a clonotypic T cell receptor (TCR), a heterodimeric transmembrane receptor composed of an alpha (TCR-α) and beta-chain (TCR-β) with remarkable homologies to immunoglobulin molecules with respect to genetic organization of the genes that form the TCR-α/β chains and their molecular structure.[21] The site-specific recombination of the variable (V-), diversity (D-), and joining (J-) gene segments during thymic maturation of T cells results in the random reassortment of a limited number of germ-line encoded complementary determining region (CDR) 1 and 2 loops of their TCR together with a large number of somatically encoded CDR3 loops.[16] Generation of T cells in the thymus results in a T cell repertoire expressing a diverse set of clonally distributed TCR and in the first step of positive selection, only a few thousands of self peptides seem to be enough to generate a fully functional TCR repertoire. In both, positive and negative selection, survival of a T cell is determined by multiple variables within the trimolecular complex, like MHC surface-expression, TCR affinity, peptide abundance, -processing and -binding to MHC. Although the theoretical number of different TCR molecules has been estimated to be in the range of 10^{14}, thymic negative and positive selection processes eliminate the majority of TCR molecules with the propensity to recognize self-antigens in the context of self-MHC, and the resulting peripheral T cell repertoire is much smaller, in the order of 10^7 or even less different specificities.[1] As a consequence TCR crossreactivity

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00094-4
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has to be an intrinsic feature of the physiological T cell repertoire.

After the observation that cytotoxic, virus-specific T cells recognize short overlapping peptides of viral proteins,[32] a combination of cell biology-, cellular immunology-, biochemical-, and later structural biology-techniques led to the discovery that CD8+ cytotoxic T cells recognize with their TCR nonamer or decamer peptides embedded in the antigen binding groove of MHC-class I molecules, whereas CD4+ T cells recognize longer, usually 12–20-mer, peptides in the binding groove of MHC-class II molecules.[25] The restriction of CD8+ T cells by MHC-class I molecules and vice versa CD4+ T cells by MHC-class II results from the observation that the coreceptors CD4 and CD8 interact with MHC-class I and II, respectively, independent of the interaction of the TCR with the MHC–peptide complex. They serve a pivotal role in stabilizing TCR–peptide:MHC engagement and therefore in initializing the immunological synapse, which can be seen as the most important interface, not only triggering but also regulating effector functions of the adaptive T cell response.[18]

It is impossible to summarize here the many studies and reviews on the general subject of T cell antigen recognition, details as to how exogenous proteins or endogenously synthesized antigens are processed, loaded onto nascent MHC molecules, and later displayed at the surface, or details on the structural interactions between TCR and MHC–peptide complex. Instead this chapter highlights a few aspects that are important, particularly the MHC molecules and peptides that are involved in T cell recognition.

MAJOR HISTOCOMPATIBILITY COMPLEX CLASS I AND CLASS II MOLECULES

The human HLA complex is encoded on the short arm of chromosome 6 (6p21.1–21.3), representing the densest area in the human genome by harboring >150 genes in an area of approximately 3500 kbp. 60% of those genes are being expressed, and most of them serve immunological functions.[4] HLA-class I molecules are heterodimeric transmembrane molecules consisting of the invariant β_2-microglobulin together with either of the HLA-class I heavy chains encoded by the polymorphic HLA-A, -B, or -C genes. Around 4500 allelic HLA-class I gene variants have been described so far, including HLA-B as the most polymorphic human gene. HLA-class II molecules are also heterodimers consisting of either a nonpolymorphic (DRα) or a polymorphic (DQα, DPα) alpha chain that pairs with a polymorphic beta chain encoded by the HLA-DRβ, -DQβ, and -DPβ genes. Thus, being expressed at the surface level, approximately 80% of HLA-class II molecules are represented by HLA-DR heterodimers. If an HLA-class I or -class II molecule is mentioned below, the respective heterodimer is meant (e.g. DR15 as the dimer of DRα and the beta chain encoded by DRB1*15:01); if a specific allele is referred to, the gene designation will be mentioned in parentheses (e.g. DRB1*15:01 as the DR-β1 chain gene number 1501). To give an impression of the complexity of HLA-class II allelic polymorphisms, there are currently 126 different HLA-DRB1*04 alleles listed by the American Society of Histocompatibility and Immunogenetics (ASHI), and there are hundred more polymorphic DR alleles. Most HLA-class II haplotypes encode for two DR heterodimers, one encoded by DRα together with the DRB1* gene product and the other by DRα paired with the DRB3*-, -B4*, or -B5* gene product. Hence, in humans each chromosome 6 codes for a set of three different class I molecules (HLA-A, -B, -C), and up to four different HLA-class II molecules (two different DR-, one DQ-, and one DP-molecule). If the individual is heterozygous, full HLA-typing will show six HLA-class I and eight class II molecules, and, because HLA genes are expressed codominantly, all corresponding HLA molecules are expressed as proteins on the cell membrane. This enormous complexity explains the difficulties in finding an unrelated organ transplant donor who fits in all HLA loci. For details on HLA typing and nomenclature, the reader is referred to the periodic update of the International HLA Workshops or the ASHI website (http://www.ashi-hla.org or http://www.ebi.ac.uk/imgt/hla/).

The main functional role of MHC/HLA molecules is to display antigenic peptides to T cells. Under physiological conditions the majority of surface HLA molecules on APC are occupied by self-peptides. Self-peptides in the context of the immune system refers to the peptides derived from proteins of the host organism in distinction from peptides that are derived from foreign agents such as viruses, bacteria, fungi, or proteins from other organisms. The repertoire of self-peptides that is continuously presented to T cells serves two major roles. During T cell development in the thymus, recognition of complexes of self-MHC that display a broad selection of self-peptides ensures that the emerging T cell repertoire is tolerant to self-proteins. As mentioned above, this is achieved by processes referred to as positive and negative selection.[2] T cells expressing TCR with high affinity for self-MHC/self-peptide complexes would pose the risk of T cell reactivity against the body's own tissue and organs, and hence they are deleted by activation-induced cell death. T cells with very low affinity for self-MHC/self-peptide complexes fail to be sufficiently stimulated in the thymus to undergo further maturation, and as a result they die of neglect. Only T cells recognizing self-MHC/self-peptide complexes within a range of intermediate affinities are positively selected,[2] mature, and leave the thymus to populate the peripheral lymphoid compartments. Although these rules generally apply, there are peptides from, for example, splice variants of certain proteins such as proteolipid protein (PLP), a major myelin component, that are not expressed in the thymus.[17] As a result, relatively

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higher numbers of T cells with specificity for such peptides can be found in the periphery, and these T cells also express higher-affinity TCR. Beyond thymic T cell development, the constant exposure of peripheral T cells to self-MHC/self-peptide complexes guarantees a degree of low-level stimulation required for homeostatic proliferation that increasingly shapes and replenishes the peripheral repertoire during physiological thymic involution of the maturing immune system or also during lymphopenia. Therefore, the recognition of self-MHC/self-peptide complexes in the thymus will select for a broad set of T cells that do not cause harm to the host's own tissues in the periphery. However, these T cells are ready to be activated as soon as the complexes of self-MHC with peptides derived from an infectious agent, that is, nonself or foreign peptides, are recognized with higher affinity during, for example, a viral infection. Transplantation of histo-incompatible or allogeneic cells or tissue—cells that express a different set of MHC molecules—also leads to the strong activation of T cells and consequently organ rejection. Thymic selection processes take place in the context of the autologous MHC molecules, and T cells with high-affinity TCR for complexes of foreign MHC and self-peptides are found at high frequencies in the peripheral repertoire. Further, it has recently been stated that the recognition of varying endogenous peptides selected by the foreign MHC molecules, rather than inability to interact with, or react to allogeneic MHC molecules, is the primary determinant of alloreactivity.

CHARACTERISTICS OF PEPTIDES PRESENTED BY MHC-CLASS I

Because of their important role as cytotoxic effector cells during viral infections, CD8+ MHC-class I-restricted T cells have probably received greater attention earlier. MHC restriction was discovered by Rolf M. Zinkernagel when examining virus-specific CD8+ T cells with specificity for lymphocytic choriomeningitis virus.[35] Because of its great importance to the understanding of adaptive immune responses, Zinkernagel, together with Peter C. Doherty, was awarded the Nobel Prize in 1996. As already mentioned, studies of influenza virus-specific cytotoxic T cells led to the observation that T cells recognize short overlapping stretches of viral peptides rather than whole proteins or conformational structures of infectious agents.[32] Subsequently, synthetic peptides rather than whole proteins or transfected genes were used for *in vitro* and *in vivo* immune studies to understand the nature of the peptides that are recognized by T cells in the context of MHC-class I. The observation of the periodic occurrence of amino acids with certain physicochemical characteristics initially led to the assumption that antigenic peptides assume a helical conformation, when they associate with MHC molecules.[28] However, soon thereafter, biochemical studies that sequenced peptides eluted from MHC-class I molecules, and X-ray crystallographic examination of MHC-class I molecules and bound peptide showed that antigenic peptides are embedded in an extended linear conformation in a peptide-binding groove that is formed by a beta-pleated sheet at the bottom of the groove and two flanking alpha helices of the MHC-class I heavy chain.[29,31] Many different cell biology-, molecular biology-, biochemical-, and protein chemistry-techniques have been applied to identify the characteristics of the class-I-associated peptides including direct sequencing of pools of self-peptides eluted from either class I or class II molecules, the sequencing of individual self-peptides eluted from HLA-molecules, the use of truncated or L-ala-substituted peptides that were known to bind to a given allele and also peptide libraries either generated recombinantly in phage or synthetically.[6,12] The following characteristics of peptides that are presented by MHC-class I molecules emerged from these studies. Crystal structure analysis of MHC-class I molecules showed that the class I binding groove is closed at both ends,[13,14] and the groove itself accommodates peptides that are usually octamers or nonamers, sometimes decamers, and less often longer peptides are found that do not fit completely into the groove but partly extrude outside.[25,26] Peptides, as short as pentamers, have been shown to be efficient antigens. Depending on the amino acids that line the groove in individual MHC-class I molecules, certain amino acids with charge, hydrophobicity, or size preferences are accommodated preferentially in separate binding pockets, which are numbered from the N- to C-terminus of the peptide as P1 through P9. In almost every class I molecule, one dominant anchor is located at the C-terminus (P9 in nonamer peptides).[25] The second dominant anchor residue is found at varying positions but most frequently at P2 of the peptide. Peptides that have only dominant anchor residues in common can differ in their binding affinity for the same MHC molecule by four orders of magnitude.[23] Amino acid residues present at other positions in the peptide can have significant positive or negative effects on peptide binding. Nondominant amino acids with positive effects are termed auxiliary anchor positions. Using the preceding strategies, the binding motifs and most important anchor amino acids have now been identified for many different MHC-class I alleles in mice, rats and humans. These anchor motifs and additional *in vitro* generated results have been used to devise prediction algorithms that allow scanning any protein and to predict the presence of areas that bind well to an MHC-class I allele of interest.[23] One very useful and publicly available tool is SYFPEITHI (www.syfpeithi.de)[25] or the International Epitope Database (www.iedb.org). Peptides that are presented in the context of MHC-class I to CD8+ T cells are usually generated from cytosolic proteins, proteolytically degraded by the proteasome involving a number of distinct proteases, and subsequently transported

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into the endoplasmic reticulum, where they are loaded onto nascent class I molecules and subsequently shuttled to the cell surface. This pathway is referred to as the endogenous processing pathway, and it is most important for mounting the T cell responses against infections with intracellular pathogens such as enveloped viruses.[8]

CHARACTERISTICS OF PEPTIDES PRESENTED BY MHC-CLASS II

CD4+ T_H cells recognize peptides that are bound by MHC-class II molecules. The same investigational approaches that have been mentioned above for MHC-class I have also been used to study class II-bound peptides, and many distinct characteristics were found. Different from the MHC-class I binding groove, the one of class II molecules is open at either end and hence accommodates the peptides that are often substantially longer than those bound by class I.[31] Most peptides that have been eluted from MHC-class II molecules were 12–20 amino acids long, but even trimers were found to bind to class II and stimulate T cells. At the other extreme, it was shown that entire proteins such as myelin basic protein (MBP), a polypeptide of 170 amino acids that may be present in unfolded form in solution, can bind to surface MHC-class II. The MHC-class II binding groove is formed by the polymorphic stretches encoded by exon 2 of the class II α- and β-chain genes and typically depicted from the top with the alpha helical parts of the DR/DQ/DP α-chain on top, and the β-chain at the bottom. As for class I, the binding groove accommodates usually 9 amino acids, and the numbering of the putative pockets follows the same order from P1–P9, and different MHC-class II molecules favor distinct anchor amino acids of the peptides in the various pockets, although there is considerable sharing of the requirements at least in some of the main anchors such as P1 and P4 between different alleles. Another important feature of peptide:MHC interaction has been discovered when comparing the limited number of crystallographically analyzed structures. It seems that the overall peptide conformation is more divergent toward the N-terminus, as, for example, in the case of $MBP_{85–99}$ binding to HLA-DR2, where the N-terminal part of the peptide is elevated out of the binding groove, thereby impeding TCR engagement to this portion, and resulting in a reduced accessibility of the peptide:MHC surface that is available for recognition to the TCR. Accordingly, it has been hypothesized that this could enhance the probability of molecular mimicry to occur.[15] Certain other features lead to additional characteristics such as the amino acid in position 86 of exon 2 in the different HLA-DR alleles, which determines the size of pocket P1. All alleles have either a valine or glycine in this position, and accordingly the respective allele can either accommodate a large aromatic residues in P1, if the DR allele expresses G86 or a substantially smaller residues, if a valine flanks P1. Further unique pocket properties, like pocket P6 determining the pH dependency and certain conformational pocket–pocket interactions, emphasize the structural plasticity of peptide binding to MHC molecules. Because class II binding grooves are open at both ends, peptides are not necessarily fixed in one position but can bind in multiple different registers—that is either shifted or bound in an inverse fashion. Because the interface of the MHC–peptide complex to the TCR is affected by such a register shift, it may negatively or positively influence T cell recognition. Further, one needs to take into account not only amino acids in anchor positions that interact with the specificity pockets but also those that contribute negative influences—that is, by the size of the side chain, length of the molecule, proline or charge of amino acids in other positions.[12] Although the unique surface structure of a peptide:MHC complex is mainly determined through the vast number of amino acid combinations, two-thirds of the interaction surface between TCR and peptide:MHC is provided by the conserved α-helices of the MHC backbone structure. Taking peptide-induced conformational changes into account results in an even more diverse and highly plastic peptide:MHC surface configuration, wherein the MHC backbone plays an important role. Antigen processing of peptides that are presented by MHC-class II molecules usually follows the so-called exogenous pathway, which differs from the above class I-associated endogenous processing of antigens. Peptides that are later loaded onto MHC-class II originate from endocytosed or phagocytosed proteins—for example, from a bacterial protein—that are proteolytically degraded in the acidic environment of endolysosomes, where they are loaded onto the newly synthesized MHC-class II molecules. On entering the processing compartment as heterotrimers of class II α- and β-chain complexed with the class II-associated invariant chain (Ii),[8] the Ii-derived class II-associated invariant chain peptide is removed from the antigen presenting groove under the influence of HLA-DM and -DO, that serve as peptide editors and replaced by peptides that are derived from proteolysis of the exogenous protein. Similarly to the main class I-associated processing pathway, the majority of class II-bound peptides are generated through the above exogenous pathway, although different loading compartments and processing paths have been described, which would explain the natural abundance of MHC-class II presented self-peptides from within an APC.

BIOLOGICAL ROLES OF MHC-BOUND PEPTIDES

Defense against Foreign Agents/Vaccines

As just outlined, our T cell repertoire constantly probes the internal environment, which is formed by cells expressing

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self-MHC molecules loaded with self-peptides. As long as this environment does not change substantially in the quality of MHC–peptide complexes or the relative amounts of antigenic peptides displayed, T cells stay alive but are not fully activated. On infection with, for example, an enveloped virus, such as influenza virus, epithelial cells are infected, and viral proteins are produced in relatively large quantities and loaded onto MHC-class I molecules of these virus-infected cells. The display of viral peptides in the context of self-MHC class I is recognized by CD8+ T cells with TCR that are specific for these MHC–peptide complexes, which become rapidly activated, then lyse the virus-infected cells and interrupt viral spread from one infected cell to the next.[32] In the context of other infectious agents, for example, those that replicate extracellulary such as many bacteria, the infectious agent itself or protein breakdown products are taken up by phagocytes and shuttled into the class II-associated presentation pathway. Eventually, peptides derived from the infectious organism are presented on the surface of APC in the context of MHC-class II molecules to CD4+ T_H cells, which, according to their functional phenotype, produce either proinflammatory cytokines such as interferon-gamma or interleukin-4, which supports B cell development. The dynamics of the infection, the site of inflammation, the tropism and other characteristics of the infectious organism, the amount of antigen that is produced, the cell types that are infected or take up antigen, and many other factors determine the relative contribution and role of CD4+ versus CD8+ T cells. In most instances, efficient immune responses will require both, potent activation of cytotoxic cells and also T helper cell functions, including the production of growth factors, such as interleukin-2, which is required for the expansion of both CD4+ and CD8+ cells and also helper function to B cells to mount efficient antibody responses. Because the presentation of peptide antigens from infectious organisms in the context of self-MHC plays such a central role for effective immune responses, many of the above strategies that have been used for deciphering MHC–peptide interactions and for understanding the specificity of T cells are also of interest for vaccine developments. Because of the complexity of some infectious organisms and the observation that numerous antigenic sites of viruses, bacteria, and parasites are rapidly mutated under the pressure of the host's cellular immune system, vaccine design aims at combining multiple immunodominant peptides and variants thereof in vaccines—for example, for human immunodeficiency virus (HIV) or malaria—although many questions as to how to render these effective vaccines remain open.

Transplantation

The existence and importance of MHC were discovered in the context of transplant rejection by George Snell, Baruj Benacerraf, and Jean Dausset, who were awarded the Nobel Prize in 1980 for their discovery. Until now, the rejection of organ and cell transplants by the host's cellular immune system or graft-versus-host responses, for example, after hematopoetic stem cell transplants, remain the major obstacles to transplantation medicine or cell reconstitutive approaches in oncology or any field, where autologous cell sources are not available. The reason for these usually very strong and often lethal allospecific immune responses has already been mentioned briefly. T cell development involves "education" of T cells on self-MHC/self-peptide complexes, and T cells expressing high-affinity TCR for these complexes are eliminated, thus creating a TCR repertoire that is broad and reactive with high affinity to complexes that are different from self-MHC/self-peptide. If an allogeneic organ transplant is introduced into the host, all nucleated cells of this transplant express nonself MHC-class I. Further, because the binding grooves of these molecules bind different self-peptides than the autologous MHC molecules, the T cell repertoire of the host is exposed to a large number of complexes of nonself MHC-class I loaded with a different set of self-peptides than the one that was "educated" on in the thymus. As a result, the frequencies of cells with alloreactivity are usually high in the peripheral blood and allospecific immune responses are fulminant and one of the strongest responses observed in the human body. Consequently, matching donor and recipient carefully for the entire HLA-class I and -class II haplotypes is necessary, although many other aspects need to be taken into account as well.

AUTOIMMUNE DISEASES

T cells not only mediate physiological immune responses against infectious organisms but they may also cause pathological immune reactions.[34] If organs or tissues of the host are affected, the resulting diseases are referred to as autoimmune diseases, and many of these result from damage by T cells themselves or by T cell-dependent immune mechanisms. Some well-known examples are rheumatoid arthritis (RA), insulin-dependent type 1 diabetes mellitus (IDDM), and multiple sclerosis (MS),[30] among numerous others. One piece of evidence that strongly suggests a role of CD4+ T cells in these diseases is the observation that HLA-DR or -DQ genes are the strongest susceptibility-conferring genetic factors in each of them.[11] To give one example, MS in Caucasians, the ethnic group with the highest prevalence of MS, is associated with the DR15 haplotype that includes the two DR-β alleles DRB1*15:01 and DRB5*01:01 and the DQ molecules DQA1*01:02 and DQB1*06:02. The relative risk to develop MS that is contributed by these class II alleles has been estimated to be between 10 and 60% of all the genetic susceptibility factors.[11] It is currently not known as to how the presence of one HLA-class II allele or several that are part of one

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haplotype confer risk at the molecular level. The following major possibilities have been considered and are partly supported by experimental data. (1) It has been speculated that disease-associated MHC-class II molecules preferentially present certain autoantigenic peptides from a tissue, in the case of MS, for example, myelin protein-derived peptides resulting in T cell responses against these peptides. (2) As a variation of possibility 1, certain disease-associated MHC-class II molecules present an overall narrower range of peptides with the result that negative selection for autoreactive T cells in the thymus is less complete, and relatively more autoreactive T cells escape to the periphery. (3) MHC-class II gene expression could be regulated differentially in certain tissues, resulting in overexpression of certain MHC-class II molecules in the target organ. Such a mechanism would be particularly attractive in the brain, where most cells do not express any MHC-class II.[30] There is, however, currently no evidence for this possibility in MS. (4) Involvement of MHC-class II molecules in shaping the peripheral TCR repertoire through mechanisms of homeostatic proliferation, thus creating a more cross or autoreactive T cell repertoire. (5) Interaction between certain HLA alleles acting in *trans*, influencing each other on an epigenetic or functional level. Evidence for this hypothesis has been reinforced by results proving the existence of HLA alleles mediating almost complete disease protection or conferring risk in a multiplicative fashion.

Trying to understand which self-peptides are the most important targets for autoreactive T cells in an autoimmune disease and which foreign agents might trigger their initial activation is a central goal of research in this area. T cell responses against some autoantigenic peptides such as myelin basic protein MBP83-99 are probably among the best examined of all T cell responses.[20,22,24] MBP83-99 has been analyzed systematically with respect to peptide binding to the MS-associated HLA-DR alleles—that is, DRB1*15:01 and DRB5*01:01, and later also cocrystallized with them. Although the crystal structure analysis yielded important new insights, many conclusions that had previously been derived from T cell studies and binding experiments were confirmed. The two preceding MBP83-99/DR complexes have been cocrystallized with two different TCR derived from MS patients, one restricted by DRB1*15:01,[10] the other by DRB5*01:01.[19] Different from the few foreign antigen-specific class II-restricted TCR that had been cocrystallized before, these autoreactive TCRs show either an unusual binding topology on the MHC-class II/peptide complex[10] or a remarkable absence of any salt bridges or hydrogen bonds between TCR and MHC-bound peptide explaining the low affinity of this autoreactive TCR.[19] By contrast, more recent investigations show that autoreactive TCR can also bind in an optimal, "foreign-reactive like" conformation, with structural properties, which are stabilizing and even compensating weak peptide:MHC interactions. These results demonstrate that thymic evasion hypotheses not only have to consider TCR affinity, but also have to take into account that peptide binding affinity can have significant impact on thymic selection kinetics. Despite this remarkable progress, too few crystal structures have been resolved so far to distinguish certain TCR binding patterns, leaving many questions related to T cell reactivity to autoantigens open, especially because there is currently no single autoantigen, which can be assumed to be a general target in all patients suffering from one particular autoimmune disease.

Regarding the foreign agents that may trigger autoreactive T cells, a mechanism referred to, as molecular mimicry appears particularly attractive. Molecular mimicry in the context of T cell responses means that a peptide derived from a foreign agent looks similar to a self-peptide. Seminal studies by Fujinami and Oldstone,[7] who proposed this mechanism, showed that molecular mimicry requires sequence identity over a stretch of 6 or more amino acids—for example, between a hepatitis B virus-derived peptide and a peptide from MBP.[7] They showed that the injection of the viral peptide into rabbit causes an immune-mediated encephalomyelitis and crossreactivity of immune cells against the brain protein MBP. The subsequent dissection of the molecular recognition of peptide by T cells led to a better understanding of the rules of T cell crossreactivity, which has to be a necessary feature of the physiological T cell receptor repertoire. It became clear that some amino acids in an antigenic peptide are particularly relevant for binding to the MHC molecule and others for contacting the TCR. Based on this knowledge, searches for molecular mimics concentrated on the presence of these contact amino acids or structural motifs rather than complete sequence homology between an autoantigen and a peptide from a virus or bacterium.[33] Further, the additional and steadily increasing complexity of MHC alleles poses a major challenge to look for molecular mimics. Based on the diversity of binding pockets, MHC molecules present diverse sets of peptides, and moreover are characterized through differences in peptide:MHC binding flexibility, confirming that both, TCR–peptide:MHC interaction and peptide–MHC interaction are highly flexible. Based on theoretical considerations that the T cell repertoire in a mammalian host is orders of magnitude smaller than the potential spectrum of antigenic peptides that can be encountered, it has been proposed that single T cells must be able to recognize large numbers in the range of up to 10^5 different peptides, and these speculations are supported by data that were mainly generated by probing the specificity repertoire of single T cell clones with systematically arranged peptide mutations or combinatorial peptide libraries.[13] These tools are currently being used to better understand the range of molecular mimicry, the

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relative affinity of single clones for peptides, and other related questions.

THERAPEUTIC USE OF PEPTIDES

Not surprisingly, many investigators quickly began to apply the knowledge about the central role of peptides for T cell recognition during infections, tumor control and autoimmune diseases also for therapeutic purposes. The potential relevance of peptides for vaccines has already been mentioned above, and the authors anticipate that this will be one interesting strategy to counter the occurrence of rapidly mutating immunodominant epitopes and immune evasion that is observed in some infectious diseases, such as HIV and malaria. Tumor immunologists have identified the specific target peptides for tumor-infiltrating lymphocyte CD4+ and CD8+ populations, and in numerous cases, these were "neoantigens," that is, peptides that emerged through mutations in the tumor. There are numerous clinical trials ongoing, in which either dendritic cells pulsed with the tumor peptide, peptide alone, or the former in combination with *in vitro* expanded tumor-infiltrating lymphocytes are used for tumor-specific immunotherapies.[27] In the case of infectious diseases and tumor immunology, the use of peptides or peptide-specific T cells aims at enhancing the respective immune responses. By contrast, under conditions in which immune responses are damaging, such as allergic responses or autoimmune diseases, investigators have tried to modulate the phenotype and reactivity of T cells by modifying autoantigenic peptides or peptides derived from an allergen or administered the peptide in a way that causes immunomodulation. Such therapeutic approaches were spurred by the discovery that systematic amino acid modifications of a peptide that serves as an agonist to a T cell clone, that is, it stimulates a full immune response including proliferation, cytokine release, and cytotoxicity, can result in partial agonists or even TCR antagonists.[9]

In summary, such peptides are described as altered peptide ligands (APL). Partial agonism results from the modifications of peptides that still stimulate some functions in the responding T cell but not others, such as proliferation in the absence of cytokine release. Thereby the antigenic peptide does not only determine if a T cell is activated, but also, regulated by the dose and structural quality of the antigenic peptide, shapes the response profile. Further, the different T cell effector functions—for example, cytokine release, surface marker expression, cytotoxicity, and proliferation—are activated in a clone-specific hierarchy, depending on the early signaling events of the trimolecular complex (TCR–peptide:MHC).[14] In this context, TCR antagonists are peptides that have been modified in a way that they inhibit a T cell response even if the agonist peptide is present at the same time by altering the TCR signaling machinery.[9] Each of these effects could be shown *in vitro* or *in vivo* in several different experimental systems, and APL peptides were also successfully used as therapeutic agents in experimental animal models of MS.[5] One particularly attractive mechanism refers to the generation of T cells on vaccination with the APL that are specific for the APL peptide and express immunomodulatory cytokines that attenuate the proinflammatory autoimmune process. The induced APL-specific T cells cross-react with the native autoantigenic peptide, and hence, whenever tissue is damaged and autoantigen is released, the APL-specific T cell population is stimulated to secrete "beneficial" immune mediators. Despite the elegant *in vitro* and *in vivo* findings, the use of APL in human autoimmune diseases remains problematic. A phase II trial of an APL derived from MBP83-99 in MS resulted in disease exacerbation of some patients on injection of high doses and a trend toward benefit at lower doses. Intriguingly, however, a peptide mixture that was designed to induce encephalomyelitis in the mouse model, evolved to be one of most widely prescribed and best-tolerated drugs in relapsing—remitting multiple sclerosis—namely, glatiramer acetate or copolymer-1. It was first synthesized 30 years ago to mimic the peptidic characteristics of myelin basic protein, one of the major candidates as primary autoantigen in multiple sclerosis. Glatiramer acetate is a random synthetic peptide mixture, consisting of only four amino acids, glutamate (E), alanine (A), lysine (K) and tyrosine (Y), differing in length between 40 and 100 aa, which are only fixed in their molar ratio (1.0:1.5:4.5:3.6). It is administered to patients by daily injections, proving to be efficacious, even though the mechanisms of actions are only poorly understood thus far. One of the proposed mechanisms of glatiramer acetate is the shift toward a more T_H2 dominated cytokine profile, probably mediated through glatiramer-acetate specific T cells. Also effects such as bystander suppression, a function as altered peptide ligand, crossreactivity of glatiramer acetate-specific T_H2 cells, influences on the phenotype of antigen presenting cells, and even neuroprotective effects have been discussed. These observations showed, however, that autoantigens that had been discovered in animal or human *in vitro* studies are indeed relevant targets in the human autoimmune diseases. Future research will have to address how such peptides should be applied, that is, by what route, at which dose, and what other factors need to be considered to further improve and use the full capacity of peptide-based therapies.

CONCLUSIONS

The investigation of T cell specificity for antigenic peptides under physiological and pathological conditions remains a focus of basic research in immunology. Beyond this, the use of antigenic peptides for therapeutic or vaccine purposes

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rapidly gains importance in clinical medicine in infectious disease, tumor immunology, for allergic diseases, and in the field of T-cell-mediated autoimmune diseases.

REFERENCES

1. Arstila TP, Casrouge A, Baron V, Even J, Kanellopoulos J, Kourilsky P. A direct estimate of the human alphabeta T cell receptor diversity. *Science* 1999;**286**:958–61.
2. Ashton-Rickardt PG, Bandeira A, Delaney JR, Van Kaer L, Pircher HP, Zinkernagel RM, et al. Evidence for a differential avidity model of T cell selection in the thymus. *Cell* 1994;**76**:651–63.
3. Bevan MJ. The major histocompatibility complex determines susceptibility to cytotoxic T cells directed against minor histocompatibility antigens. *J Exp Med* 1975;**142**:1349–64.
4. Bodmer WF. Evolution and function of the HLA region. *Cancer Surv* 1995;**22**:5–16.
5. Brocke S, Gijbels K, Allegretta M, Ferber I, Piercy C, Blankenstein T, et al. Treatment of experimental encephalomyelitis with a peptide analogue of myelin basic protein. *Nature* 1996;**379**:343–6.
6. Fleckenstein B, Jung G, Wiesmuller KH. Quantitative analysis of peptide-MHC class II interaction. *Semin Immunol* 1999;**11**:405–16.
7. Fujinami RS, Oldstone MB. Amino acid homology between the encephalitogenic site of myelin basic protein and virus: mechanism for autoimmunity. *Science* 1985;**230**:1043–5.
8. Germain RN, Margulies DH. The biochemistry and cell biology of antigen processing and presentation. *Annu Rev Immunol* 1993;**11**:403–50.
9. Germain RN, Stefanova I. The dynamics of T cell receptor signaling: complex orchestration and the key roles of tempo and cooperation. *Annu Rev Immunol* 1999;**17**:467–522.
10. Hahn M, Nicholson MJ, Pyrdol J, Wucherpfennig KW. Unconventional topology of self peptide-major histocompatibility complex binding by a human autoimmune T cell receptor. *Nat Immunol* 2005;**6**:490–6.
11. Haines JL, Terwedow HA, Burgess K, Pericak-Vance MA, Rimmler JB, Martin ER, et al. Linkage of the MHC to familial multiple sclerosis suggests genetic heterogeneity. The Multiple Sclerosis Genetics Group. *Hum Mol Genet* 1998;**7**:1229–34.
12. Hammer J, Valsasnini P, Tolba K, Bolin D, Higelin J, Takacs B, et al. Promiscuous and allele-specific anchors in HLA-DR-binding peptides. *Cell* 1993;**74**:197–203.
13. Hemmer B, Fleckenstein BT, Vergelli M, Jung G, McFarland H, Martin R, et al. Identification of high potency microbial and self ligands for a human autoreactive class II-restricted T cell clone. *J Exp Med* 1997;**185**:1651–9.
14. Hemmer B, Stefanova I, Vergelli M, Germain RN, Martin R. Relationships among TCR ligand potency, thresholds for effector function elicitation, and the quality of early signaling events in human T cells. *J Immunol* 1998;**160**:5807–14.
15. Jones EY, Fugger L, Strominger JL, Siebold C. MHC class II proteins and disease: a structural perspective. *Nat Rev Immunol* 2006;**6**:271–82.
16. Jorgensen JL, Reay PA, Ehrich EW, Davis MM. Molecular components of T-cell recognition. *Annu Rev Immunol* 1992;**10**:835–73.
17. Klein L, Klugmann M, Nave KA, Tuohy VK, Kyewski B. Shaping of the autoreactive T-cell repertoire by a splice variant of self protein expressed in thymic epithelial cells. *Nat Med* 2000;**6**:56–61.
18. Krogsgaard M, Li QJ, Sumen C, Huppa JB, Huse M, Davis MM. Agonist/endogenous peptide-MHC heterodimers drive T cell activation and sensitivity. *Nature* 2005;**434**:238–43.
19. Li Y, Huang Y, Lue J, Quandt JA, Martin R, Mariuzza RA. Structure of a human autoimmune TCR bound to a myelin basic protein self-peptide and a multiple sclerosis-associated MHC class II molecule. *EMBO J* 2005;**24**:2968–79.
20. Martin R, Howell MD, Jaraquemada D, Flerlage M, Richert J, Brostoff S, et al. A myelin basic protein peptide is recognized by cytotoxic T cells in the context of four HLA-DR types associated with multiple sclerosis. *J Exp Med* 1991;**173**:19–24.
21. Meuer SC, Hussey RE, Hodgdon JC, Hercend T, Schlossman SF, Reinherz EL. Surface structures involved in target recognition by human cytotoxic T lymphocytes. *Science* 1982;**218**:471–3.
22. Ota K, Matsui M, Milford EL, Mackin GA, Weiner HL, Hafler DA. T-cell recognition of an immunodominant myelin basic protein epitope in multiple sclerosis. *Nature* 1990;**346**:183–7.
23. Parker KC, Biddison WE, Coligan JE. Pocket mutations of HLA-B27 show that anchor residues act cumulatively to stabilize peptide binding. *Biochemistry* 1994;**33**:7736–43.
24. Pette M, Fujita K, Wilkinson D, Altmann DM, Trowsdale J, Giegerich G, et al. Myelin autoreactivity in multiple sclerosis: recognition of myelin basic protein in the context of HLA-DR2 products by T lymphocytes of multiple-sclerosis patients and healthy donors. *Proc Natl Acad Sci U S A* 1990;**87**:7968–72.
25. Rammensee HG, Falk K, Rotzschke O. Peptides naturally presented by MHC class I molecules. *Annu Rev Immunol* 1993;**11**:213–44.
26. Reddehase MJ, Rothbard JB, Koszinowski UH. A pentapeptide as minimal antigenic determinant for MHC class I-restricted T lymphocytes. *Nature* 1989;**337**:651–3.
27. Rosenberg SA. Shedding light on immunotherapy for cancer. *N Engl J Med* 2004;**350**:1461–3.
28. Rothbard JB, Taylor WR. A sequence pattern common to T cell epitopes. *EMBO J* 1988;**7**:93–100.
29. Silver ML, Parker KC, Wiley DC. Reconstitution by MHC-restricted peptides of HLA-A2 heavy chain with beta 2-microglobulin, in vitro. *Nature* 1991;**350**:619–22.
30. Sospedra M, Martin R. Immunology of multiple sclerosis. *Annu Rev Immunol* 2005;**23**:683–747.
31. Stern LJ, Wiley DC. The human class II MHC protein HLA-DR1 assembles as empty alpha beta heterodimers in the absence of antigenic peptide. *Cell* 1992;**68**:465–77.
32. Townsend AR, McMichael AJ, Carter NP, Huddleston JA, Brownlee GG. Cytotoxic T cell recognition of the influenza nucleoprotein and hemagglutinin expressed in transfected mouse L cells. *Cell* 1984;**39**:13–25.
33. Wucherpfennig KW, Strominger JL. Molecular mimicry in T cell-mediated autoimmunity: viral peptides activate human T cell clones specific for myelin basic protein. *Cell* 1995;**80**:695–705.
34. Zinkernagel RM. Immune protection vs. immunopathology vs. autoimmunity: a question of balance and of knowledge. *Brain Pathol* 1993;**3**:115–21.
35. Zinkernagel RM, Doherty PC. Restriction of in vitro T cell-mediated cytotoxicity in lymphocytic choriomeningitis within a syngeneic or semiallogeneic system. *Nature* 1974;**248**:701–2.

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Chapter 95

RGD Peptides

Ignacy Z. Siemion, Alicja Kluczyk and Marek Cebrat

ABSTRACT

Peptides containing the RGD (Arg–Gly–Asp) motif and their molecular mimetics bind integrin receptors with high affinity and inhibit cell adhesion by competing with the integrins/matrix interaction leading to anti-inflammatory, anticoagulant, antimetastatic, and antiangiogenic effects. RGD peptides are also involved in bacterial pathogen invasion, tumor and endothelial cell-targeting via the integrin receptors as well as in noninvasive tumor imaging, targeting, and radiation treatment. This review presents various functions of RGD peptides and perspectives of their application as potential drugs.

DISCOVERY OF THE ANTIADHESIVE RGD PEPTIDES

A very useful approach in the search for biologically active peptides is the analysis of appearance of particular sequences in proteins involved in the main physiological processes. The classic example of such a procedure is the use of protein fragments that form the sites of interaction of regulatory proteins as templates for design of inhibitors of cell growth and functions. From this point of view, especially interesting are the "universal signal sequences" of proteins, which could be recognized by their broad distribution among different regulatory proteins of various living species.

In the search for biologically active peptides, the sequential analogs of signal sequences present in the proteins are also of interest. This review concentrates on the Arg–Gly–Asp (RGD) signal motif, crucial for cell adhesion and several other vital phenomena.

In 1982, Pierschbacher determined the complete amino acid sequence of the cell attachment domain of human fibronectin, a 250-kDa eukaryotic extracellular matrix protein which contains an RGD motif and plays crucial roles in cell–cell communication, development, tissue homeostasis, and disease development. Fibronectin orchestrates the functions of other extracellular matrix proteins, promoting cell adhesion, migration, and intracellular signaling including activation of several tyrosine kinases. Pierschbacher and Ruoslahti reported that the tetrapeptide RGDS in module III_{10} plays a big role in the formation of the adhesive site of fibronectin. The Arg (R) and Asp (D) residues present in the tetrapeptide were shown to be of major importance for this activity. The peptides containing the RGD sequence compete with fibronectin for cellular receptors and may act as antiadhesive substances.[8]

The cellular adhesion processes can be divided into two categories: cell–cell and cell–extracellular matrix (ECM) adhesion. In both cases, a major role is played by cell adhesion receptors known as integrins. The integrin glycoproteins are evolutionary old. They appear as obligate heterodimers of noncovalently associated α- and β-subunits. Evolutionary divergence has generated 18 α- and 8 β-subunits. At least, 24 αβ dimeric integrins are known to exist in vertebrates. The two subunits consist of a short cytoplasmic tail, a single transmembrane domain, and a multidomain extracellular portion. The ligand-binding site of integrins is present in the globular head domain of the extracellular part of the molecule and is formed by both integrin subunits. The binding of integrins to their ligands is cation dependent. The history of integrin recognition, their structure, and functions were recently reviewed.[6]

Integrin receptors can interact with matrix proteins such as fibronectin, vitronectin, and laminin, with serum fibrinogen, von Willebrand factor, thrombospondin, osteopontin, different kinds of collagen, and others. The RGD sequence in the native ligands is recognized by 8 out of 24 integrins (α_v integrins and integrin $\alpha_5\beta_1$). In some ECM molecules, such as collagen and certain laminin isoforms, the RGD sequences are exposed on denaturation or proteolytic cleavage. Molecules that contain this sequence or its mimetics compete with the natural ligands for binding. Ligands of the integrin receptors were extensively reviewed by Humphries et al.[14]

The cellular adhesion is important for many fundamental biological processes, such as cell differentiation and growth, tumor metastasis, several immune functions, and tissue repair. They regulate cell movement and migrations, wound healing, and the inflammatory phenomena, as well as embryonal development. There is growing evidence that integrins function as receptors for signal transduction and

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00095-6
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that integrin-mediated signaling can affect cell behaviour and even cell survival. Under certain circumstances, loss of integrin-mediated signaling will induce apoptosis. The crucial role of adhesive interactions of cells with other cells and with matrix proteins within the immune system was reviewed by Mousa.[21]

In sequence databases, such as ELM (The Eukaryotic Linear Motif resource for Functional Sites in Proteins, http://elm.eu.org), the RGD sequence is given a separate chapter, due to its importance in inter- and intracellular events. In other bioactive peptide databases, the RGD fragment can be found in several peptides. The search in PepBank (http://pepbank.mgh.harvard.edu) results in 1217 hits (in August 2011 PepBank database contained 21,691 sequences), although a closer analysis reveals that the database includes in the result set all peptides related to RGD or even mentioned in relation to RGD. More reasonable numbers (22 out of 2599 and 32 out of 10,229 peptide sequences) are found in BIOPEP (http://www.uwm.edu.pl/biochemia/index.php/pl/biopep) and EROP-Moscow (http://erop.inbi.ras.ru) databases, respectively.

The importance of RGD and integrin system and the efforts directed at understanding their functions can also be ascertained from the number and character of publications presenting the results of research on integrins. The search for RGD and integrin concepts in the selected scientific databases shows the increasing number of publications, with nearly 60% of articles published in the last 10 years, and again more than half of them published since the first edition of Handbook of Biologically Active Peptides in 2006 (see Table 1 for details). The differences in the numbers result from the scope of respective databases. It is worth noting that a separate volume (vol. 426) of Methods of Enzymology, edited by David A. Cheresh, was devoted to integrins in 2007.

RGD PEPTIDES IN PLATELET AGGREGATION

Because of the role of the RGD peptides in the pathophysiology of thrombosis, the interactions of adhesive proteins with platelets have gained the attention of many research groups.

Platelet aggregation takes place during the development of such cardiovascular diseases as myocardial infarction, transient ischemic attacks, strokes, etc. A key step in platelet aggregation is the crosslinking of fibrinogen or von Willebrand factor to the GP IIb/IIIa ($\alpha_{IIb}\beta_3$) platelet integrin receptor.

Each of the Aα peptide chains of fibrinogen contains two adhesive sites (sequences RGDF and RGDS) and the HHLGGAKQAGDV fragment responsible for receptor recognition. Six binding sites of a dimeric form of fibrinogen participate in binding to the integrin receptors, thus mediating platelet aggregation. Because the fibrinogen-GP IIb/IIIa interaction is the final major step in platelet aggregation and GP IIb/IIIa is only expressed on platelets, it is clear that the development of GP IIb/IIIa antagonists is an attractive strategy for antiplatelet therapy with an expected strong and specific effect.[32]

Disintegrins, very potent inhibitors of the integrin receptors, are cysteine-rich polypeptides containing the exposed RGD motif, which were originally discovered in snake venoms, but are also produced by blood-sucking arthropods and leaches. Echistatin, ornatins, kistrin, variabilin, ixodegrin, and decorsin represent just a few members of this family. In barbourin and ussuristatin, the RGD motif is changed into the KGD sequence, whereas in disagregin, there is an RED sequence.[10]

Some disintegrins specifically bind to integrin $\alpha_v\beta_3$ and are applied as therapeutic agents for angiogenesis-dependent tumor growth and metastasis. Others, such as

TABLE 1 Publications on RGD and Integrin Topics in the Selected Scientific Databases

Database	Period of publication	Integrin		RGD	
Pubmed	Total	50,003		5253	
	2001–2011	28,252	(57%)	3008	(57%)
	2006–2011	14,210	(28%)	1647	(31%)
Web of Science	Total	39,195		6737	
	2001–2011	25,214	(64%)	4203	(62%)
	2006–2011	13,687	(35%)	2501	(37%)
SciFinder	Total	94,099		12,902	
	2001–2011	62,744	(67%)	8093	(63%)
	2006–2011	36,928	(39%)	4897	(38%)

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contortrostatin, which is a disulfide-linked homodimer of 13.5-kDa containing two RGD sites isolated from the venom of *Agkistrodon contortrix*, can bind $\alpha_v\beta_3$, $\alpha_v\beta_5$, as well as $\alpha_5\beta_1$ and have been shown to inhibit tumor growth and angiogenesis in an orthotopic xenograft model for breast cancer.[28] Variabilin from tick *Dermacentor variabilis* is a 4-cysteine, 5-kDa RGD-containing disintegrin that blocks ADP-induced platelet aggregation and prevents integrin $\alpha_{IIb}\beta_3$ binding to immobilized fibrinogen.

The search for new antithrombotic agents was also oriented at the mimetics of RGD motif (extensively reviewed by Cox et al.[8] and Auzzas et al.[5]). The structural investigations of cyclic venom peptides clearly show that the distance between the guanidine and carboxyl groups is crucial for the activity and specificity of the antithrombotic agents. It should be similar to the respective distance in the RGDS peptide in the extended conformation. Assuming that this distance should be about 10–20 Å, a substituted tyrosine was proposed as a new lead motif in the search for antithrombotic drugs, resulting in development of Tirofiban (compound A, Fig. 1). Tirofiban is a specific nonpeptide antagonist of GP IIb/IIIa that mimics the GP IIb/IIIa recognizing peptide RGD. It is effective in unstable angina and non-Q-wave myocardial infarction and is approved in patients undergoing coronary angioplasty for acute coronary syndromes. Another drug developed from the RGD concept is Eptifibatide, cyclic heptapeptide that mimics the KGD sequence of the snake venom barbourin (compound B, Fig. 1). It effectively and safely reduces the acute adverse outcome of patients undergoing PCI and is also beneficial in unstable angina.

The guanidine group of RGD was frequently replaced by the p-amidinophenyl moiety, leading to several drug candidates, including Orbofiban (compound C, Fig. 1), although in this case the clinical trials were terminated at Phase III.

It was found that the RGD amide is sufficient for platelet aggregation inhibition at a high concentration. Replacement of L-Arg residue by D-Arg, Gly by D-Ala, or L-Asp by D-Asp, results in inactive compounds. The Ser residue in RGDS can by replaced by other amino acids, especially hydrophobic ones, whereas the substitution of the Asp β-carboxyl group by a tetrazole moiety leads to a completely inactive compound. The peptides bearing a primary amine instead of the guanidine group (i.e., with Arg/Lys substitution) exhibit enhanced platelet aggregation potency and $\alpha_{IIb}\beta_3$ selectivity. The introduction of Har (homoarginine) instead of Arg into the cyclic RGD peptides also greatly increases the platelet aggregation inhibitory potency.

BIOLOGICALLY ACTIVE CONFORMATION OF RGD PEPTIDES

Information on the integrin structure is limited to ectodomains of two integrins that lack αI domains, $\alpha_V\beta_3$ and $\alpha_{IIb}\beta_3$ and to fragments of α_I integrins.[33] RGD ligands bind at an interface between the α and β subunits, with the R residue fitting into a cleft in a β-propeller module in the α subunit, and the D residue coordinating a cation bound in a von Willebrand factor A-domain in the β subunit.[14]

The crystal structure of the extracellular part of the $\alpha_v\beta_3$ integrin receptor alone and complexed with the Cilengitide (cyclic pentapeptide: cyclo-(Arg–Gly–Asp–D-Phe–(N-Me) Val)) was resolved by Xiong et al.[34] The ligand is located at the major interface between the α_v and β_3 subunits, binding them together. The guanidine group of Arg forms a bidendate salt bridge to Asp^{218} and Asp^{150} of this receptor and the carboxyl group of Asp participates in a hydrogen bond with the amide carbonyls of Tyr^{122} and Asn^{215}. The distance between the carbon atoms of these two groups equals 13.72 Å, and the Arg and Asp side chains are extended in opposite directions. These results enable a more precise analysis of the interaction of adhesive peptides with integrins, presented in the article of Arnaout et al.[3]

The binding selectivity of RGD tripeptide to specific integrin depends much on the conformation of the RGD sequence and its flanking residues. Various conformations, proposed as the receptor-bound conformations of RGD ligands, have led to many independent approaches to the design of RGD-peptide mimetics. NMR, CD, and molecular modeling studies have been applied in the conformational investigations, with special attention given to semirigid, cyclic RGD-peptide analogs. By chemical modification and conformation restriction of the RGD sequence, many researchers have developed a variety of cyclic RGD-containing peptides not only with diverse secondary structures of the RGD fragment, but also with enhanced binding affinity and bioavailability.[5,19,25]

The IC_{50} values for $\alpha_v\beta_3$ for some of the cyclic RGD peptides clearly demonstrate the importance of proper conformation of RGD fragment

- c(RGDRGD) $\alpha_v\beta_3$ 140 nM
- c(RGDfV) $\alpha_v\beta_3$ 50 nM
- c(RGDf(NMe)V) $\alpha_v\beta_3$ 0.6 nM, $\alpha_{IIb}\beta_3$ 860 nM (Cilengitide).

The involvement of pairs of integrins in various diseases and the attempts at design and synthesis of selective antagonists were recently presented.[23]

The pseudodihedral angle between the Arg Cβ and Asp Cβ atoms can be used as the criterion for evaluation of the structure–activity relationship of RGD-containing peptides. The value of this angle in the active compounds is between +45° and −45°. Activity increases with an increase in the distance between the charged centers and diminishes with the increase in ionic interactions between Arg and Asp.

The conformation of the RGD sequence controls the selectivity between $\alpha_v\beta_3$ and the platelet integrin $\alpha_{IIb}\beta_3$.

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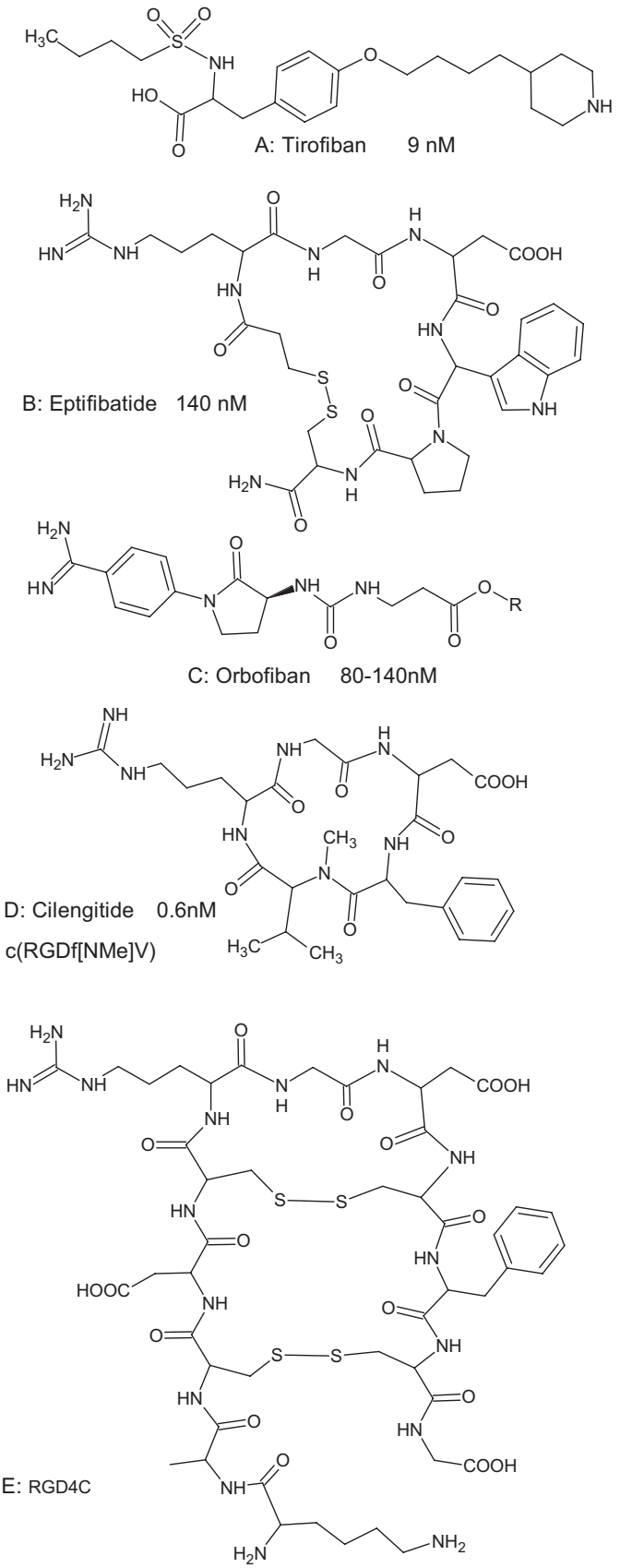

FIGURE 1 Selected integrin antagonists. The IC_{50} values for $\alpha_v\beta_3$ taken from Refs. 5, 7, 25.

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Peptides selective for $\alpha_v\beta_3$ vs. $\alpha_{IIb}\beta_3$ have a turn in Gly region, whereas $\alpha_{IIb}\beta_3$ antagonists prefer an extended conformation of Gly.[11]

RGD PEPTIDES IN IMMUNOLOGICAL PHENOMENA

The role of cell adhesion molecules and extracellular matrix proteins in various pathological processes including angiogenesis, thrombosis, apoptosis, cell migration, and proliferation are well documented. These processes can lead to both acute and chronic disease states such as ocular diseases, metastasis, unstable angina, myocardial infarction, stroke, osteoporosis, a wide range of inflammatory diseases, vascular remodeling, and neurodegenerative disorders. The possibility of an influence of the RGD peptides on such processes is evident.

The homotypic adhesion of T-cells is mediated by intercellular adhesion molecules (ICAM-1), which interact with the leukocyte integrin receptors LFA-1 (leukocyte-function associated antigen-1), and MAC-1, expressed on macrophage cells. MAC-1 mediates the adhesion of granulocytes and monocytes to endothelial cells. LFA-1 contributes also to the binding of killer T-cells to target cells. Natural killer cells constitute a conserved lymphocyte subpopulation that regulates many types of immune response by a rapid secretion of such cytokines as IL-4 and IFN-γ.[2]

ICAM-1 belongs to the group of most intensively studied adhesion molecules of the immune system. It is also the cell surface receptor for human rhinoviruses and red blood cells infected by *Plasmodium falciparum*. Blocking of the cell adhesion signal by inhibition of the ICAM-1/LFA-1 interactions suppresses the allograft rejection and may also induce the apoptosis of antigen-specific T cells. The blockade can be achieved by means of linear and cyclic peptides derived from the ICAM-1 sequence.

The interacting site of ICAM-1 consists of the PSKVIL-PRGGSVLVTG sequence. The PRGGS sequence closely resembles the recognition sequence of fibronectin (GRGDS). Several peptides, peptidomimetics, and small molecules were developed to inhibit ICAM-1/LFA-1 interaction to control immune response in autoimmune diseases and allograft rejection. Several examples, including two potent LFA-1/ICAM-1 interaction inhibitors, cyclic peptides closed by disulfide bridges: cyclo(1,12)-Pen-Pro–Arg–Gly–Gly–Ser–Val–Leu–Val–Thr–Gly–Cys–OH and cyclo(1,12)-Pen–Pro–Ser–Lys–Val–Ile–Leu–Pro–Arg–Gly–Gly–Cys–OH (Pen - L-penicillamine (β,β-dimethyl-L-cysteine)) were reviewed by Anderson et al.[2] As frequently observed, not only in the case of RGD analogs, these cyclic peptides have higher activities than the parental peptide in inhibiting T cell adhesion and mixed leukocyte reaction (MLR). Their structures, especially the β-turn formed by the Pro-Arg-Gly-Gly fragment, are identical to that of ICAM-1. These peptides can also bind to LFA-1 and get internalized into T cells.

Another RGD motif is found in HLA-DQ, the molecule of human leukocyte antigen class II, located on the surface of antigen-presenting cells including macrophages. HLA class II molecules present the peptide fragments of foreign proteins to CD4 T cells. They are composed of two peptide chains (α and β). The thymopentin-like sequence, RGDVY, occupies the 167–171 fragment of the β2-extracellular domain of the β-chain of HLA and is located within the exposed loop of this domain. The synthetic linear peptides containing this sequence suppress cellular immune response, whereas thymopentin (RKDVY) is inactive in this test. The effect of the peptides on humoral immune response depends on the length of the peptide chain; e.g., for the elongated QRGDVY sequence, stimulation of the immune response is observed, but the further elongation of the peptide chain results in suppression of the response.

The presence of the adhesive RGD sequence within the HLA-DQ suggests that the molecule may interact with some integrin receptors, enhancing its binding to the CD4 cell receptor. The synthetic peptides related to the indicated HLA-DQ fragment inhibit the adhesion of platelets to fibrinogen, as well as the adhesion of K562 tumor cells to fibronectin. A disulfide bridged, semirigid CRGDVYC peptide showed a significant increase in inhibitory potency as compared with that of the RGDVY linear pentapeptide.[29] The RGDVF sequence, very similar to the RGDVY motif, appears in the molecule of an adhesive protein, vitronectin.

In HLA-DP and HLA-DR subclasses of HLA II, the RGDVY sequence of HLA-DQ is changed into QGDVY and SGEVY, respectively. A similar sequence, ILDVP, without the basic Arg residues, is the second adhesive site on fibronectin. Therefore, it seems that the indicated mutational changes do not destroy the adhesive properties of the respective HLA-DP and HLA-DQ fragments. The peptides corresponding to these sequences show a distinct immunosuppressory activity in both the cellular and humoral immune responses in mice.

In some proteins, retro-RGD sequences are present. Such a situation appears, for example, in the human CD23 molecule (low affinity receptor for IgE), expressed on a variety of hematopoietic cell types. The (GCY)CPDP-DGRLPTPSAPLHS peptide derived from this protein binds to MHC class II molecules, but not to CD23.[17]

A retro-RGD sequence is found in ubiquitin. Ubiquitin system is involved not only in protein metabolism, but also in the processing of MHC class I antigens. Ubiquitin also augments the production of TNFα in murine macrophages and may also be involved in the modulation of immune response. A series of linear peptides, derived of ubiquitin, were synthesized and investigated for immunomodulatory properties. The 50–59 fragment with retro-RGD motif (LEDGRTLSDY) possesses immunosuppressive activity comparable with that of ubiquitin, and shows conformational resemblance to the respective loop of ubiquitin. A model of the 48–59 loop, a

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cyclic peptide: cyclo-(Glt-QLEDGRTLSDK)-NH_2 (Glt—glutaryl), with the bridge between Glt and the ε-amino group of Lys, was also synthesized.[22]

RGD-containing peptides inhibit cell growth by inducing detachment from extracellular matrix and adhesion-dependent apoptosis, named anoikis. Recent evidence by different investigators has identified an additional mechanism by which RGD peptides participate in cellular internalization, intracellular targeting and direct activation of caspase-3, caspase-8 or caspase-9 in lymphocytes, cardiomyocytes, endothelial cells and chondrocytes, leading to apoptosis most likely by an integrin-independent mechanism.[1]

RGD PEPTIDES AND PATHOGEN INVASIONS

The bacterial and other pathogen invasion into host cells is mediated by adhesive reactions in which the integrin receptors and matrix proteins participate. Attachment to the host tissue is one of the key early steps of the bacterial infection process. Bacterial attachment to surfaces is mediated by surface structures that are anchored in the bacterial outer membrane and covers a broad group of structures, commonly known as adhesins. Many microbial proteins contain the RGD motif that enables binding to human integrins.

Gram-positive bacterial pathogens such as *Streptococcus pneumoniae* and *Streptococcus pyogenes* use vitronectin for effective adhesion to host cells and subsequent internalization. Vitronectin has an RGD sequence for binding the host cell integrin receptors and a separate bacterial-binding domain for pathogens, and thus functions to crosslink bacteria and epithelial cells. Once bacteria are attached to the vitronectin–integrin complex, various host cell-signaling events are activated to promote internalization. RGD-containing peptides and antibodies directed against $\alpha_v\beta_3$ integrin inhibit the bacterial adhesion and internalization. *Candida albicans* yeasts also exploit vitronectin as a tool for cell adhesion. Their binding to vitronectin is strongly inhibited by RGD, but not RGE peptides.[27]

A short review of the role of cell adhesion molecules in the pathogenesis and host defense against microbial pathogens including protozoa, fungi, bacteria, and viruses was presented by Kerr.[16] The role of fibronectin binding in bacterial adherence and the subversion of fibronectin-integrin system by pathogens was presented by Schwarz et al.[24] In particular, the entry of Mycobacteria into leukocytes is mediated by formation of a complex between the bacterial antigen Ag85 and fibronectin. Mycobacteria can invade the cells only in the form of this complex interacting with the fibronectin specific integrin receptor.

It was shown by Siemion and Wieczorek that the GRGDS fibronectin peptide, as well as the RGDVY HLA-DQ peptide and their analogs inhibit the entry of *Mycobacterium kansasii* into leukocytes.[26] The effect consists of the competition of fibronectin and the investigated peptide for the same integrin receptor. Thymopentin (RKDVY) is also a potent inhibitor of Mycobacteria phagocytosis. The exchange of Asp by Glu in splenopentin (RKEVY) results in a decrease of activity. The fragments of such proteins as cecropine, systemine, and BRCT proteins, containing the modified RGD sequences, were also examined for inhibitory activity.

The ω-guanidino acids ($H_2N–C(=N)–NH–(CH_2)_n–COOH$) were used as mimetics of RGD peptides in the Mycobacteria phagocytosis inhibition. Inhibitory activity is seen for amidinohexanoic acid. The compounds with a shortened hydrocarbon chain are practically devoid of any activity, with the exception of the guanidinoacetic acid. *Staphylococcus aureus*, *S. pyrogens*, and several other bacteria also use the binding to fibronectin as a tool for host cell invasion. However, the RGDVY peptide is inactive in *S. aureus* phagocytosis inhibition.[26]

CagL, a 26-kDa protein of the gastric pathogen and type I carcinogen *Helicobacter pylori*, mimics fibronectin in various cellular functions. Like fibronectin, CagL contains an RGD motif and is located on the surface of the bacterial type IV secretion pili. CagL binds to the integrin receptor $\alpha_5\beta_1$ and mediates the injection of virulence factors into host target cells. CagL mimics fibronectin in triggering cell spreading, focal adhesion formation, and activation of several tyrosine kinases in an RGD-dependent manner.[30]

The synthetic peptidomimetics of RGD are used for the inhibition of binding of adenoviruses to integrin receptors. The human adenovirus contains the RGD sequence in the penton base protein.[12] The γ-herpes viruses also use integrin receptors for their entry into the cells via the RGD sequence.[31] Adhesins, such as the *E. coli* antigen 43 and pertactin from *B. pertussis*, bind to integrins via conserved RGD motifs.

Similar to their roles in animal tissue, integrin-like molecules of plants, are involved in connecting the plant cell wall to the plasma membrane, and this interaction can be disrupted by treatment with RGD-containing peptides. Candidate integrin-like RGD-binding receptors have been identified by immunolabeling with integrin-specific antisera, by crosslinking of iodinated RGD-heptapeptides and by affinity chromatography using immobilized RGD-containing peptides. The plant-pathogenic oomycete *Phytophthora infestans* secretes an RGD-containing protein that might bind to a specific receptor protein of *Arabidopsis thaliana*, thus enabling/promoting the infection process via a loosened cellular organization. Subsequently, an RGD-reactive receptor-like kinase with a lectin-like extracellular domain was identified, and it was speculated that

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RGD-dependent protein–protein interactions could play a structural and signaling role at the plant cell surface. Whether bacterial plant pathogens take advantage of RGD motifs for binding to plant cells is still unknown. However, conserved RGD motifs have been found in the monomeric autotransporters XCV2103 and XCV4444 but not in the trimeric autotransporters XadA or XadB. RGD motifs are also present in the FhaB homologs. Even if RGD-mediated binding to integrin-like proteins is involved in attachment to host cells, it is not clear whether these bacterial proteins determine host specificity because of the evolution of new binding specificities. Rather, diversifying selection might lead to host adaptation via escape from detection by the plant's immune system, and these adhesins would thus constitute a further class of pathogen-associated molecular patterns.[20]

RGD PEPTIDES IN OTHER PATHOLOGICAL PHENOMENA

Certain integrins may be important targets for selective antagonists aimed at blocking the pathological processes. The commercial and therapeutic potential of cell adhesion molecules is on the rise. The possibility of use of the $\alpha_4\beta_1$ and $\alpha_4\beta_1$ integrin receptor inhibitors in the treatment of autoimmune disorders was discussed by Jackson.[15]

Changes in the number and identity of integrin receptors are common in cancer cells, resulting in the alteration of the ability of malignant cells to interact with the extracellular matrix, and promoting migration as well as facilitating survival outside the tumor environment. Preclinical studies reveal that integrin antagonists inhibit tumor growth by affecting both tumor cells and tumor-associated host cells, most notably the angiogenic endothelium. Integrin antagonists currently in clinical trials include monoclonal antibodies and RGD peptide mimetics.[8,9]

The development of small-molecule antagonists of β_3 integrins based on the RGD sequence and their potential anticancer applications was reviewed by Sheldrake et al.[25] It is worth mentioning that some integrin antagonists may display agonist-like activity at low doses, including stimulation of angiogenesis. Controlling the concentration of drug in the body is quite difficult in the case of oral administration. Cilengitide (compound D, Fig. 1) shows antiangiogenic and antitumor activity in various animal models and undergoes clinical trials for treatment of glioblastoma (phase III) and several other tumors (phase II) (reviewed by Mas-Moruno et al.[19]). See the Cancer/Anticancer section of this book for other peptides involved in treatment.

The application of turn-inducing motifs allows specific observation of structure–selectivity relationships in RGD analogs in the search for selective $\alpha_v\beta_3$ and $\alpha_v\beta_5$ antagonists, potential antitumor agents. Various analogs of proline have been used to synthesize macrocycles with finely tuned affinity to these integrins.[5]

PERSPECTIVES

The recognition of the RGD-binding motif common to the disintegrins and natural integrin ligands allowed the development of small molecule integrin antagonists, progressing from linear peptides containing the RGD sequence to cyclic peptides with well-defined conformation, and hence to small molecule peptidomimetics with improved pharmacological properties. RGD peptides, and small molecules have become a popular area for the development of new drugs and drug delivery agents.[25]

Many linear and cyclic RGD peptides have been developed as ligands that preferentially bind to integrin $\alpha_v\beta_3$, related α_v forms, or other types of integrins. Among these, cyclic peptides c(RGDfK), c(RGDyK), and RGD4C (compound E, Fig. 1) show high binding affinity and selectivity for integrin $\alpha_v\beta_3$, with Cilengitide undergoing clinical trials for brain tumors. There seems to be a positive synergy for this agent in combination with radiochemotherapy in preclinical studies,[19] which not only encourages further research but also attracts attention to cyclic RGD peptides as vectors for integrin targeted delivery of chemotherapeutics.[7]

Several RGD conjugates, containing relatively small molecules, are used as biological tools. A typical example, c(RGDyK) attached to fluorescent Cy5.5 dye, is used for *in vivo* NIR fluorescence imaging.[18] Radioactive RGD peptides represent an important new class of radiotracers for diagnosis of tumor or thrombosis, detection of inflammatory processes, and monitoring outcomes of therapeutic interventions in patients with cancer, myocardial infarction, and inflammation.[35] [^{18}F]-galacto-c(RGDfK) as well as [^{18}F]-bearing tracers with RGD4C and PEG linker, are being investigated for noninvasive imaging of integrin expression in cancer patients. ^{99m}Tc-apcitide (Fig. 2), a cyclic RGD peptide mimetic that specifically binds to the $\alpha_{IIb}\beta_3$ expressed on fresh thrombi, has been approved by the FDA for the diagnosis of deep vein thrombosis in patients.[5]

Drug delivery systems use RGD sequence to target cancer cells and administer cytotoxic drugs. Conjugating doxorubicin with RGD4C improves the inhibition of tumor growth and spreading of pulmonary metastases in mouse MDA-MB-435 breast cancer model. Moreover, the resulting compound is less toxic to the liver and heart. Because of solubility problems, prodrug development has been attempted (Fig. 2). The conjugation strategy is also applied to RGD-based platinum(IV) complexes and paclitaxel, resulting in the formation of an efficient prodrug with a cleavable linker.

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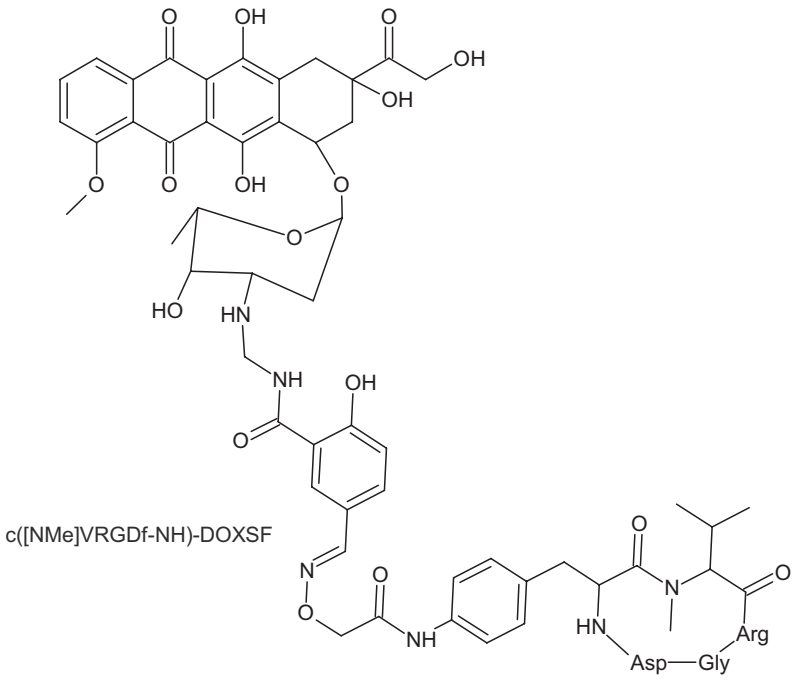

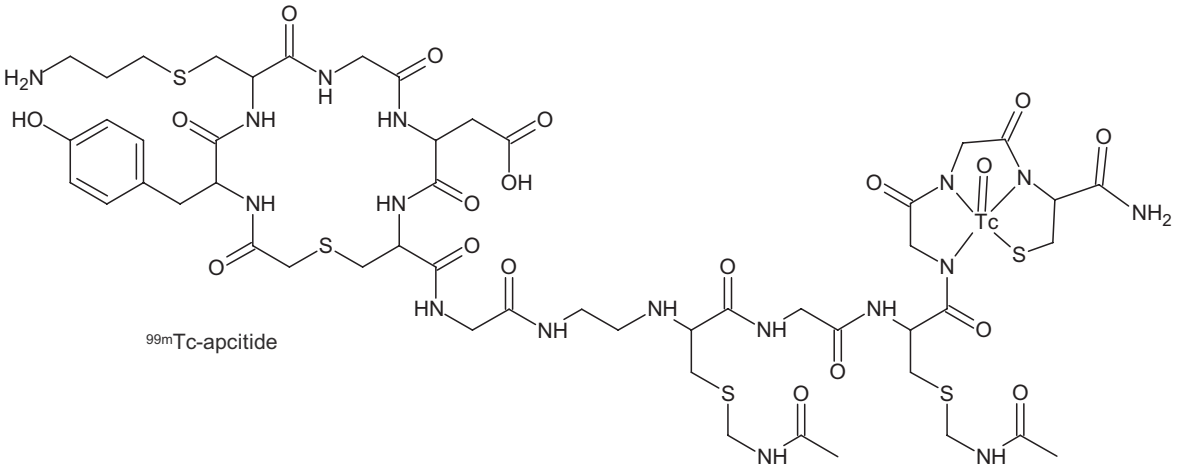

FIGURE 2 RGD conjugates of pharmaceutical interest.[5]

To improve the cellular uptake and accumulation, multimeric systems exploiting the enhanced permeability and retention (EPR) effect of RGD fragment, have been designed.[5,7,18] Dimeric and multimeric constructs, and RGD-decorated liposomes, particulates, and nanoparticles are used not only as drug vesicles but also as tissue-repairing materials, resembling and restoring extracellular matrix.[13] Gold nanoparticles are considered as polyvalent platforms for targeted drug delivery. The cRGD-conjugated gold nanoparticles display an enhanced affinity for $\alpha_V\beta_3$ integrin and *in vitro* target $\alpha_V\beta_3$ overexpressing cells, thus opening the possibility of future application in cancer therapy.[4]

The presented material indicates that besides the well-known role of the RGD peptides in cardiovascular processes, they gain the increasing importance in various areas of modern medicine.

REFERENCES

1. Aguzzi MS, Fortugno P, Giampietri C, Ragone G, Capogrossi MC, Facchiano A. Intracellular targets of RGDS peptide in melanoma cells. *Mol Cancer* 2011;**9**:84–94.
2. Anderson ME, Siahaan TJ. Targeting ICAM-1/LFA-1 interaction for controlling autoimmune diseases: designing peptide and small molecule inhibitors. *Peptides* 2003;**24**:487–501.

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3. Arnaout MA, Goodman SL, Xiong JP. Coming to grips with integrin binding to ligands. *Curr Opin Cell Biol* 2002;**14**:641–51.
4. Arosio D, Manzoni L, Araldi EMV, Scolastico C. Cyclic RGD functionalized gold nanoparticles for tumor targeting. *Bioconjugate Chem* 2011;**22**:664–72.
5. Auzzas L, Zanardi F, Battistini L, Burreddu P, Carta P, Rassu G, et al. Targeting $\alpha_v\beta_3$ integrin: design and applications of mono- and multifunctional RGD-based peptides and semipeptides. *Curr Med Chem* 2010;**17**:1255–99.
6. Barczyk M, Carracedo S, Gullberg D. Integrins. *Cell Tissue Res* 2010;**339**:269–80.
7. Chen K, Chen X. Integrin targeted delivery of chemotherapeutics. *Theranostics* 2011;**1**:189–200.
8. Cox D, Brennan M, Moran N. Integrins as therapeutic targets: lessons and opportunities. *Nat Rev Drug Disc* 2010;**9**:804–20.
9. Desgrosellier JS, Cheresh DA. Integrins in cancer: biological implications and therapeutic opportunities. *Nat Rev Cancer* 2010;**10**:9–22.
10. Francischetti IMB. Platelet aggregation inhibitors from hematophagous animals. *Toxicon* 2010;**56**:1130–44.
11. Frank AO, Otto E, Mas-Moruno C, Schiller HB, Marinelli L, Cosconati S, et al. Conformational control of integrin-subtype selectivity in *iso*DGR peptide motifs: a biological switch. *Angew Chem Int Ed Engl* 2010;**49**:9278–81.
12. Hippenmeyer PJ, Ruminski PG, Rico JG, Lu HS, Griggs DW. Adenovirus inhibition by peptidomimetic integrin antagonists. *Antiviral Res* 2002;**55**:169–78.
13. Huang J, Ding J. Nanostructured interfaces with RGD arrays to control cell–matrix interaction. *Soft Matter* 2010;**6**:3395–401.
14. Humphries JD, Byron A, Humphries MJ. Integrin ligands at a glance. *J Cell Sci* 2006;**119**:3901–3.
15. Jackson DY. Alpha 4 integrin antagonists. *Curr Pharm Des* 2002;**8**:1229–53.
16. Kerr JR. Cell adhesion molecules in the pathogenesis of and host defence against microbial infection. *Mol Pathol* 1999;**52**:220–30.
17. Kijimoto-Ochiai S, Noguchi A. Two peptides from CD23, including the inverse RGD sequence and its related peptide, interact with the MHC class II molecule. *Biochem Biophys Res Commun* 2000;**267**:686–91.
18. Lee S, Xie J, Chen X. Peptides and peptide hormones for molecular imaging and disease diagnosis. *Chem Rev* 2010;**110**:3087–111.
19. Mas-Moruno C, Rechenmacher F, Kessler H. Cilengitide: the first anti-angiogenic small molecule drug candidate. Design, synthesis and clinical evaluation. *Anticancer Agents Med Chem* 2010;**10**:753–68.
20. Mhedbi-Hajri N, Jacques M-A, Koebnik R. Adhesion mechanisms of plant-pathogenic Xanthomonadaceae. In: Linke D, Goldman A, editors. *Bacterial adhesion*. Dordrecht: The Netherlands: Springer; 2011. p. 71–89.
21. Mousa SA. Cell adhesion molecules: potential therapeutic and diagnostic implications. *Mol Biotechnol* 2007;**38**:33–40.
22. Pasikowski P, Cydzik M, Kluczyk A, Stefanowicz P, Szewczuk Z. Ubiquitin fragments: their known biological activities and putative roles. *BioMol Concepts* 2010;**1**:67–83.
23. Perdih A, Sollner Dolenc M. Small molecule antagonists of integrin receptors. *Curr Med Chem* 2010;**17**:2371–92.
24. Schwarz-Linek U, Höök M, Potts JR. The molecular basis of fibronectin-mediated bacterial adherence to host cells. *Mol Microbiol* 2004;**52**:631–41.
25. Sheldrake HM, Patterson LH. Function and antagonism of β_3 integrins in the development of cancer therapy. *Curr Cancer Drug Targets* 2009;**9**:519–40.
26. Siemion IZ, Gawłowska M, Ślepokura K, Biernat M, Wieczorek Z. The mimetics of antiadhesive peptides as the inhibitors of *Mycibacterium kansasii* phagocytosis. *Peptides* 2005;**26**:1543–9.
27. Singh B, Su Y, Riesbeck K. Vitronectin in bacterial pathogenesis: a host protein used in complement escape and cellular invasion. *Mol Microbiol* 2010;**78**:545–60.
28. Swenson S, Ramu S, Markland FS. Anti-angiogenesis and RGD-containing snake venom disintegrins. *Curr Pharm Des* 2007;**13**:2860–71.
29. Szewczuk Z, Wilczynski A, Dyba M, Petry I, Siemion IZ, Wieczorek Z. New conformationally restricted analog of the immunosuppressory mini-domain of HLA-DQ and its biological properties. *Peptides* 2000;**21**:1849–58.
30 Tegtmeyer N, Hartig R, Delahay RM, Rohde M, Brandt S, Conradi J, et al. Small fibronectin-mimicking protein from bacteria induces cell spreading and focal adhesion formation. *J Biol Chem* 2010;**285**:23515–26.
31. Tugizov SM, Berline JW, Palefsky JM. Epstein-Barr virus infection of polarized tongue and nasopharyngeal epithelial cells. *Nat Med* 2003;**9**:307–14.
32. Vyas SP, Vaidya B. Targeted delivery of thrombolytic agents: role of integrin receptors. *Expert Opin Drug Deliv* 2009;**6**:499–508.
33. Xie C, Zhu J, Chen X, Mi L, Nishida N, Springer TA. Structure of an integrin with an αI domain, complement receptor type 4. *EMBO J* 2010;**29**:666–79.
34. Xiong JP, Stehle T, Zhang R, Joachimiak A, Frech M, Goodman SL, et al. Crystal structure of the extracellular segment of integrin $\alpha_v\beta_3$ in complex with an Arg-Gly-Asp ligand. *Science* 2002;**296**: 151–5.
35. Zhou Y, Chakraborty S, Liu S. Radiolabeled cyclic RGD peptides as radiotracers for imaging tumors and thrombosis by SPECT. *Theranostics* 2011;**1**:58–82.

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Chapter 96

Apelin

Xavier Iturrioz and Catherine Llorens-Cortes

ABSTRACT

The APJ receptor was originally isolated as an orphan seven-transmembrane-domain G-protein-coupled receptor in humans. Its endogenous ligand, apelin, was identified in 1998 and is generated from a 77-amino-acid precursor, preproapelin. Apelin and its receptor are expressed in hypothalamic vasopressinergic and oxytocinergic neurons. Apelin acts as a potent aquaretic neuropeptide, counteracting vasopressin actions. Apelin may also act on the kidney to increase diuresis. Central injection of apelin in lactating rats inhibits hypothalamic parvocellular and magnocellular OXY neuron activity in a direct autocrine or paracrine manner, reducing the amount of milk ejected for the pups. Apelin exerts peripheral actions, decreasing mean arterial blood pressure and improving myocardium contractility and load. Altogether these results suggest that apelin may be a key peptide for the fine regulation of functions relating to the maintenance of body fluid homeostasis in certain physiological conditions and plays a crucial role in the regulation of cardiovascular functions.

DISCOVERY

The apelin story began in 1993 with the cloning of the cDNA of the orphan APJ receptor (putative receptor protein related to the type 1 angiotensin receptor) from a human genomic library, which was subsequently cloned in rodents.[6,23,24] The human receptor is 380 amino acids long and is a member of the family of orphan seven-transmembrane-domain G-protein-coupled receptors (GPCRs). Its amino acid sequence was found to be 31% identical to that of the human type 1 angiotensin (AT1) receptor, but it does not bind radiolabeled angiotensin II (Ang II), and exposure of the rat APJ receptor to Ang II does not modify cyclic adenosine monophosphate (cAMP) production, showing that this receptor is not an angiotensin receptor subtype. It therefore remained an orphan GPCR for which the endogenous ligand had to be isolated. The APJ receptor was deorphanized in 1998 when apelin, its endogenous ligand, was isolated from bovine stomach tissue extracts.[34] This ligand was named "apelin" for the endogenous ligand for the APJ receptor.

STRUCTURE OF THE mRNA/GENE

Apelin is a 36-amino-acid peptide (apelin-36) generated from a larger precursor, the 77-amino-acid proapelin (Fig. 1A). The human preproapelin gene is located on chromosome X at locus Xq25–q26.1 and contains three exons, with the coding region spanning exons 1 and 2. The 3′-untranslated region also spans two exons (2 and 3). This may account for the presence of transcripts of two different sizes (~3 and ~3.6 kb) in various tissues.[23]

PROCESSING OF THE PRECURSOR

The alignment of preproapelin amino acid sequences from cattle, human, rat, and mouse shows strict conservation of the C-terminal 17 amino acids, known as apelin-17 or K17F (Fig. 1B). *In vivo*, proapelin gives rise to various molecular forms of apelin of 36, 17, and 13 amino acids, but the mechanism implicated in this processing remains unknown. The presence of pairs of basic residues in proapelin suggests that prohormone convertases could be implicated in this processing. In the brain and plasma of rat, the predominant forms of apelin are the pyroglutamyl forms of apelin-13 (pE13F) and, to a lesser extent, K17F (Fig. 1B).[7] In rat lung, testis, and uterus and bovine colostrum, apelin-36 predominates, whereas, in the rat mammary gland, both apelin-36 and pE13F are present.[10,15] In human heart, pE13F has been identified as the predominant molecular form of apelin,[17] whereas in human plasma, K17F predominates followed by pE13F and to a lesser extent, apelin-36.[1]

DISTRIBUTION OF THE mRNA/PEPTIDE

The apelin precursor, mRNA is expressed in the brain and also in the lung, heart, kidney, adipose tissue, and vascular endothelium.[3,14,16,21,26,27] The development of a polyclonal antibody with high affinity and selectivity for K17F has made it possible to visualize, for the first time, apelin-containing neurons in the central nervous system of rats.[26] The precise neuroanatomical distribution of apelin immunoreactivity shows that apelin-immunoreactive (IR) cell bodies are

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00096-8
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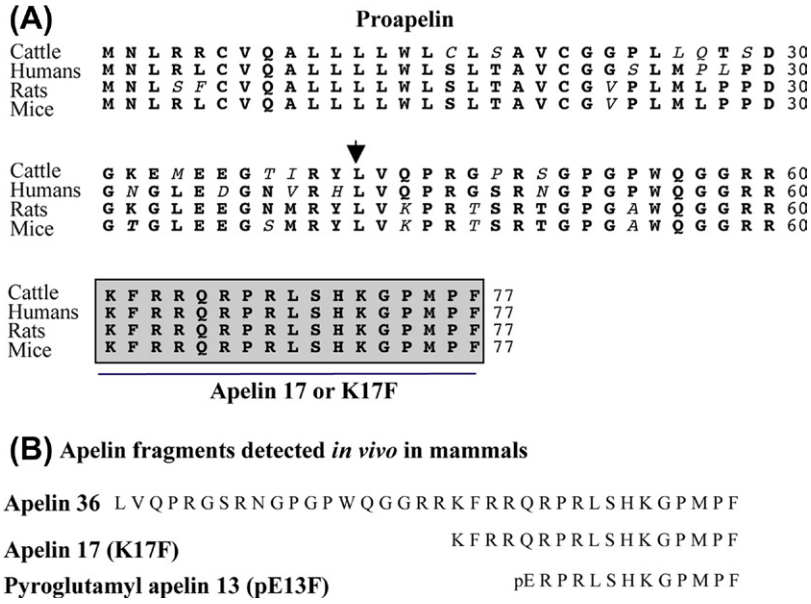

FIGURE 1 (A) Amino acid sequences of the apelin precursor, preproapelin, in cattle, human, rat, and mouse. The first amino acid of apelin-36 is indicated by an arrowhead and the apelin-17 sequence is indicated by a box.[34] (B) Apelin fragments detected *in vivo* in mammals: apelin-36, apelin-17 (K17F), and the pyroglutamyl form of apelin-13 (pE13F).

particularly abundant in the structures of the hypothalamus and medulla oblongata involved in the control of neuroendocrine activities, drinking behavior, and arterial blood pressure (BP), notably in the hypothalamic supraoptic nucleus (SON) and the magnocellular part of the paraventricular nucleus (PVN), the arcuate nucleus, the lateral reticular nucleus, and the nucleus ambiguus (Fig. 2).[26,27] Conversely, apelin-IR nerve fibers are much more widely distributed in many regions of the brain. The density of IR nerve fibers and apelinergic nerve endings is the highest in the inner layer of the median eminence and in the posterior pituitary,[4,27] suggesting that apelin neurons of the SON and PVN, like magnocellular AVP and oxytocin neurons, project into the posterior pituitary. In support of this notion, double immunofluorescence staining showed that apelin colocalizes with AVP[7,29] and oxytocin[2,4] in magnocellular hypothalamic neurons in rats.

Apelin-IR nerve fibers also innervate the median preoptic nucleus, mesencephalon, pons, medulla oblongata, and several circumventricular organs such as the vascular organ of the lamina terminalis (OVLT), the subfornical organ (SFO), the subcommissural organ, and the area postrema.[27]

RECEPTORS AND SIGNALING CASCADES

Distribution of the Apelin Receptor

Like apelin, the apelin receptor mRNA is widely distributed throughout the central nervous system[6,16,21,23] of rats and is also present in peripheral tissues such as heart, vascular endothelium, kidney, adipose tissue, spleen, muscle, and uterus.[8,10,11,14,21] *In situ* hybridization analysis of apelin receptor mRNA expression in the adult rat brain shows intense labeling in the hypothalamus, especially in the SON and PVN and to a lesser extent in the arcuate nucleus. The anterior and intermediate lobes of the pituitary are also strongly labeled, as well as the pineal gland. Labeling is also found in extrahypothalamic structures such as the piriform cortex, the nucleus of the lateral olfactory tract, the central gray matter, the structures containing monoaminergic neuronal cell bodies (pars compacta of the substantia nigra, dorsal raphe nucleus, and locus coeruleus), the entorhinal cortex, the dentate gyrus and Ammon's horn.[6] Further, double-labeling studies combining immunocytochemistry and *in situ* hybridization in rats have shown that in SON and PVN apelin receptors,[23,26] like type 1A and 1B AVP receptors (V1A and V1B), are synthesized by magnocellular AVP neurons suggesting an interaction between AVP and apelin. More recently, Bodineau et al. have shown that apelin receptor mRNA is expressed by magnocellular and parvocellular oxytocin neurons in the SON and PVN, suggesting an interaction between oxytocin and apelin.[2]

Signaling Cascades

The human and rat apelin receptors are 380 and 377 amino acids long, respectively, and belong to class A seven-transmembrane GPCRs. These receptors share high identity (90%) in amino acid sequence. Rat and human apelin receptors are negatively coupled to adenylyl cyclase.[6,21] Indeed, apelin-36, K17F, apelin-13 (Q13F), and pE13F are potent inhibitors of forskolin-induced cAMP production in CHO or HEK 293 cells stably expressing the rat or human receptor.[6,21] Masri et al. have shown that both apelin-36 and pE13F activate Gαi1 and Gαi2 isoforms but not Gαi3 in CHO cells stably

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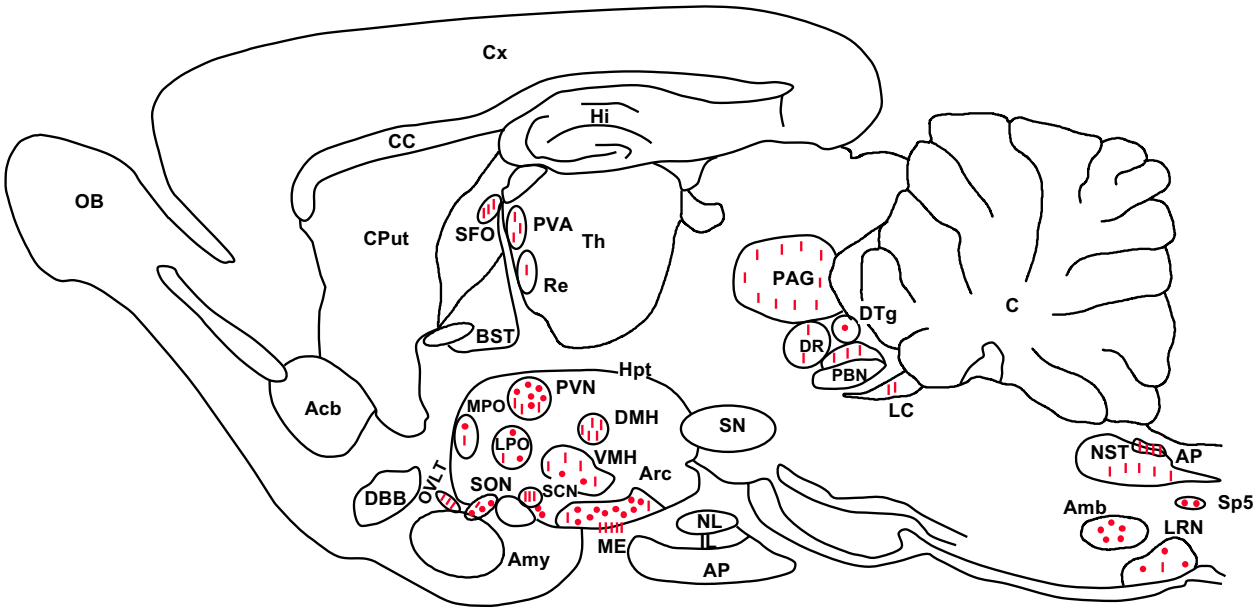

FIGURE 2 Neuroanatomical distribution of apelin-IR cell bodies and nerve fibers on a parasagittal section of adult colchicine-treated rat brain. Apelin-IR cell bodies and nerve fibers are shown as dots and lines, respectively. Abbreviations: Acb, nucleus accumbens; Amb, nucleus ambiguus; Amy, amygdala; AP, anterior pituitary; AP: area postrema; Arc, arcuate nucleus of the hypothalamus; BST, bed nucleus of the stria terminalis; C, cerebellum; CC, corpus callosum; Cput, caudate putamen; Cx, cerebral cortex; DBB, diagonal band of Broca; DMH, dorsomedial nucleus of the hypothalamus; DR, dorsal raphe nucleus; DTg, dorsal tegmental nucleus; Hi, hippocampus; Hpt, hypothalamus; IL, intermediate lobe of the pituitary; LC, locus coeruleus; LPO: lateral preoptic area; LRN, lateral reticular nucleus; ME, median eminence; MPO, medial preoptic nucleus; NL, neural lobe of the pituitary; NST, nucleus of the solitary tract; OB, olfactory bulb; OVLT: vascular organ of the lamina terminalis; PAG, periaqueductal gray; PBN, parabrachial nucleus; PVA, paraventricular thalamic nucleus; PVN, paraventricular nucleus of the hypothalamus; Re, reuniens thalamic nucleus; SCN, suprachiasmatic nucleus; SFO, subfornical organ; SN, substantia nigra; SON, supraoptic nucleus; Sp5, spinal trigeminal nucleus; S, septum; Th, thalamus; VMH, ventromedial nucleus of the hypothalamus. Adapted from Reaux et al.[27]

expressing the murine apelin receptor.[20] Ala-scan of pE13F[21] and N- or C-terminal deletions of K17F [9] showed that the arginine residues in positions 2 and 4 or the leucine residue in position 5 in pE13F play a critical role in binding affinity and inhibition of cAMP production. Apelin-36, K17F, and pE13F also increase intracellular calcium mobilization in several cell lines such as NTera 2 human teratocarcinoma cells or HEK 293 and RBL-2H3 cells stably expressing the human apelin receptor.[5,21] Several kinases are activated by apelin receptor stimulation in different cell types. Masri et al. have shown that pE13F activates extracellular signal-regulated kinases (ERKs) in CHO cells stably expressing the mouse apelin receptor, via a pertussis-toxin-sensitive G protein, a PKC-dependent pathway, and in a Ras-independent pathway.[18] Apelin also activates p70S6 kinase in human umbilical vein endothelial cells (HUVEC) and in CHO cells expressing the mouse apelin receptor via two independent signaling pathways, ERK-dependent and PI3-kinase-dependent pathways.[19] Apelin-36, K17F, and pE13F trigger rat and human apelin receptor internalization.[9,26] Interestingly, the presence of the C-terminal phenylalanine (Phe) of K17F is essential to induce apelin receptor internalization and *in vivo* to decrease arterial blood pressure.[9] The deletion of the C-terminal Phe of K17F (K16P) or its substitution by an alanine (K17A), strongly decreases the ability of the peptide to trigger apelin receptor internalization with no effect on its binding to apelin receptor or its ability to activate Gαi-coupling.[9,12] More recently, Iturrioz et al. by combining molecular modeling and structure–function studies by site-directed mutagenesis of the rat apelin receptor have shown that the C-terminal Phe of pE13F or K17F is embedded in a hydrophobic pocket constituted by the Phe255 and the Trp259 residues in helix VI of the apelin receptor.[12] The substitution of these residues by an alanine abolishes the capacity of the receptor to internalize upon ligand exposure confirming the critical role of the C-terminal Phe residue of pE13F and K17F in inducing apelin receptor internalization.[12]

BIOLOGICAL ACTIONS WITHIN THE BRAIN AND PITUITARY GLAND

Involvement of Apelin in the Regulation of Water Balance

Action of Central Apelin on AVP Neuronal Activity, AVP Release in the Blood Circulation, and Diuresis

Magnocellular neurosecretory neurons release AVP, an antidiuretic vasoconstrictor peptide, in the vicinity of

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the fenestrated capillaries of the posterior pituitary in response to changes in plasma osmolality and volemia or under action of different neurohormones including Ang II or III and natriuretic peptides. The fact that AVP and apelin colocalize in hypothalamic magnocellular neurons and the presence of receptors for AVP and apelin on these neurons (Fig. 3) strongly suggest the existence of an interaction between these two peptides and a potential apelin response to these stimuli. With regard to the involvement of apelin in the regulation of water balance, it is possible that, independently of the feedback control exerted by AVP on its own release, apelin may regulate AVP release. This hypothesis has been tested in lactating rats exhibiting a reinforced phasic pattern of AVP neurons during lactation, thereby facilitating systemic AVP release to maintain body water content for optimal milk production. In this model, icv injection of K17F inhibits the phasic firing activity of AVP neurons, thereby decreasing AVP release into the bloodstream, leading to water diuresis[7] (Fig. 3). Similarly, a marked decrease in systemic AVP release is observed after icv injection of K17F or pE13F in mice deprived of water for 24 h,[26] a condition known to increase AVP neuron activity and AVP release. These results suggest that apelin is probably released from the SON and PVN AVP cell bodies and inhibits AVP release by acting directly on the apelin autoreceptors expressed by AVP/apelin-containing neurons. This mechanism probably involves apelin acting as a natural inhibitor of the antidiuretic effect of AVP. The colocalization and opposite biological actions of these two peptides raise questions concerning how these two peptides are regulated to maintain body fluid homeostasis. This question has been addressed by two different studies focusing on the effects of water deprivation on the neuronal content and release of both apelin and AVP in rodents and on of the effects of hypertonic saline infusion or water loading on plasma AVP and apelin levels in human volunteers.

Opposite Regulation of Vasopressin and Apelin after Water Deprivation

In rats deprived of water for 24 h, a large increase in the hypothalamic apelin content especially in that of the PVN and SON magnocellular neurons[7,29] is mirrored by a decrease in plasma apelin levels,[7] suggesting that, under these conditions, apelin accumulates within AVP neurons rather than being released. The apelin response to dehydration is therefore the opposite of that of AVP, which is released faster than it is synthesized.[7,29] Such a mechanism implies that apelin and AVP which are produced in the same neurons should be released differentially by these neurons. Indeed, double-labeling and confocal microscopy studies have shown that AVP and apelin are mainly present in distinct populations of vesicles which differ in size and distribution in magnocellular neurons[7,29] (Fig. 3).

These opposite regulatory patterns of apelin and AVP suggest that the two peptides act in concert to maintain body fluid homeostasis. During dehydration, an increase in the somatodendritic release of AVP optimizes the phasic activity of AVP neurons, facilitating the release of AVP into the bloodstream while apelin accumulates in these neurons rather than being released into the bloodstream and, probably into the nuclei. Thus, a decrease in the local supply of apelin to SON and PVN AVP cell bodies may facilitate the expression of AVP by AVP neurons and optimize phasic activity by decreasing the inhibitory effects of apelin on these neurons. In addition, apelin gene expression in the brain has been recently reported as a hydration-sensitive gene expression. Indeed, water deprivation in mice decreases brain apelin mRNA transcription and apelin release in the blood circulation.[33] Opposite regulation of apelin and AVP makes it possible to maintain the organism's water balance by preventing additional water loss at the kidney level.

Such a crossregulation of apelin and AVP after osmotic stimuli has also been studied in humans. The relationship between osmolality, apelin, and AVP was investigated in 10 healthy men after infusion of hypertonic saline to increase plasma osmolality or water loading to decrease plasma osmolality.[1] Increased plasma osmolality simultaneously raises plasma AVP concentration and reduces plasma apelin concentration. Conversely, decreased plasma osmolality decreases plasma AVP concentration and rapidly increases plasma apelin concentration. This observation is consistent with plasma osmolality being a major physiologic regulator of plasma apelin levels in humans. Further, the inverse relationship between the regulation of apelin and AVP is consistent with the findings in rats submitted to water deprivation. This strongly suggests that, like AVP, apelin contributes to the maintenance of body fluid homeostasis in humans and rodents.

Involvement of Vasopressin and Apelin in the Maintenance of Water Balance in the Kidney

In addition to a central action, the aquaretic effect of apelin probably involves a renal action because mRNA transcripts for apelin receptor and preproapelin, as well as apelin peptide, have been detected in rat and human kidney.[21,23] Apelin receptor mRNA is present in all renal zones, most abundantly in the inner stripe of the outer medulla.[11] A high expression level is also found in the glomeruli and a moderate expression is observed in all nephron segments, especially in collecting ducts that express vasopressin V2 receptors.[11] Apelin receptor mRNA is expressed in juxtamedullary efferent (EA) and afferent (AA) arterioles, in which K17F induces vasorelaxation by inhibiting the Ang II-induced rise in [Ca^{2+}]. This occurs *via* release of NO through receptors located on the endothelium layer.[11]

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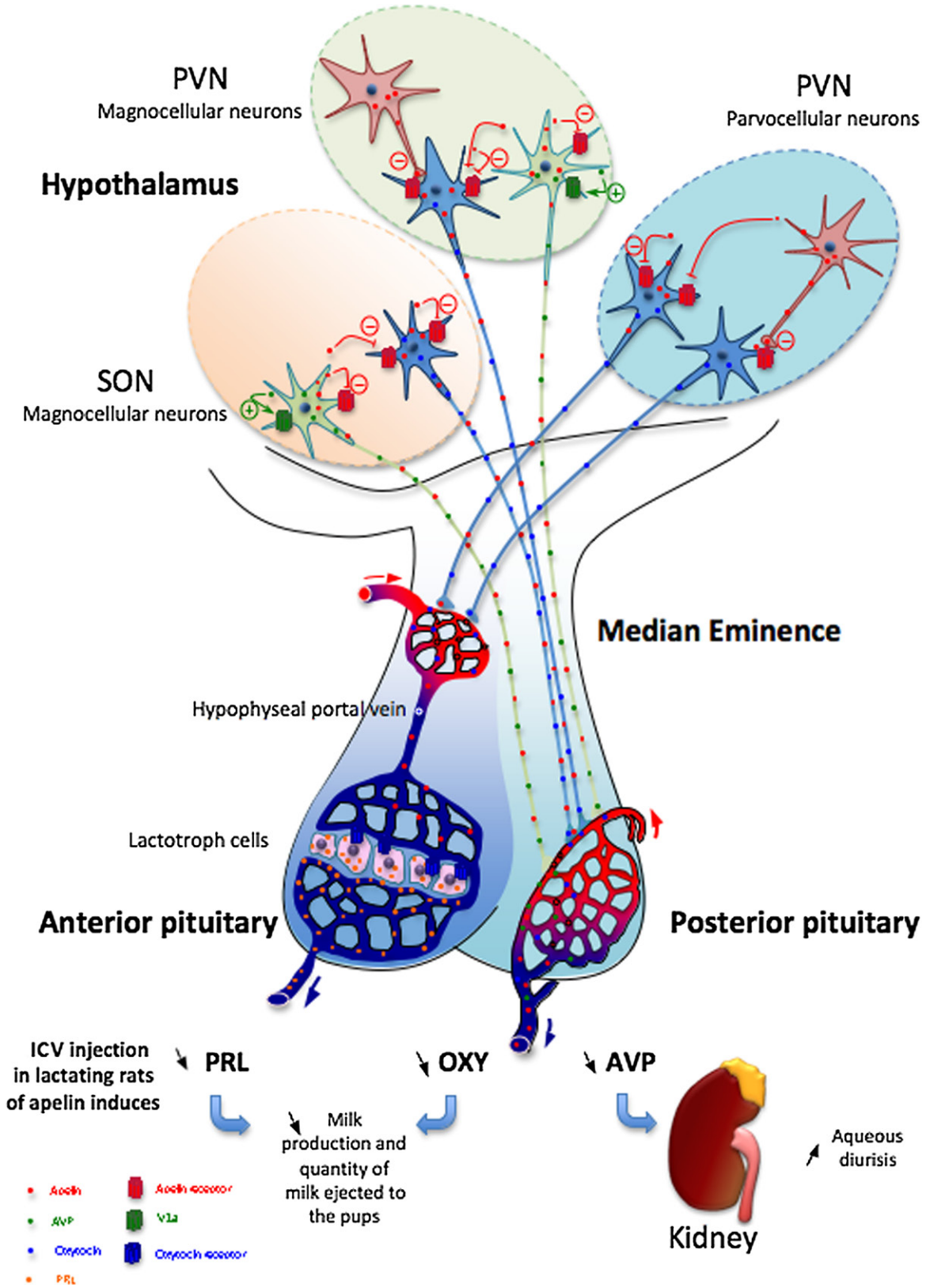

FIGURE 3 Putative schema of apelin of the interactions with AVP and oxytocin. In lactating rats, apelin and its receptor are colocalized with AVP and oxytocin in the SON and PVN magnocellular neurons. Apelin and its receptor are also colocalized with oxytocin in parvocellular neurons. ICV administration of apelin inhibits the phasic electrical activity of AVP and oxytocin neurons. The decrease of AVP neuron activity results in a decrease of AVP secretion into the bloodstream and thereby the increase of aqueous diuresis at the kidney level. In contrast, the decrease of oxytocin neuron activity in magnocellular neurons and in parvocellular neurons would certainly decrease the secretion of oxytocin and PRL in the bloodstream in the anterior pituitary respectively. Adapted from De Mota et al.[7] and Bodineau et al.[2] See color plate 25.

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The Ang II-induced vasoconstriction that is rapidly reversed by apelin in AA and EA, confirms the opposing vascular roles of the apelinergic and renin angiotensin systems.[11] The apelin-dependent vasorelaxation observed in muscular EA, which gives rise to vasa recta, therefore strongly supports a key role of apelin in the control of renal medullary circulation.[11] Further, the expression of apelin receptor mRNA along the nephron and in the collecting duct suggests that apelin acts as an aquaretic peptide through a direct action on this segment. This hypothesis is supported by the observation that intravenous injection of increasing doses of K17F in lactating rats increases diuresis in a dose-dependent manner.[11] This apelin-induced increase in medullary blood flow in addition to a potential direct tubular effect might contribute to increased diuresis, as reported in lactating rats. By contrast, AVP decreases medullary blood flow *via* its action on V_{1A} receptors by inducing vasoconstriction of the outer medullary descending vasa recta, and decreases urinary flow rate *via* its action on V_2 receptors. Apelin therefore seems to exert a complex and fine regulation on renal hemodynamic function through actions on preglomerular and postglomerular microvasculature and probably also on tubular function.

Recent studies have underlined the role of apelin in controlling fluid homeostasis. As previously reported, water deprivation significantly decreases urine volume and increases urine osmolality in wildtype female and male mice, whereas mice deficient for the apelin receptor ($APJ^{-/-}$) are unable to concentrate their urine to the same extent.[30,31] This lack of effect probably does not result from an inability of these mice to increase plasma AVP levels because comparable increases are seen in wildtype and $APJ^{-/-}$ mice after water deprivation, suggesting the presence of an intact central vasopressinergic system in $APJ^{-/-}$ mice. These observations have therefore led these authors to suggest that because the defect in water metabolism observed in $APJ^{-/-}$ mice is probably not resulting from altered AVP neurosecretory function, they have sought the possibility of a deficiency at the kidney level.

Apelin and Drinking Behavior

Consistent with a role of apelin in the regulation of water balance depending not only on the synthesis and release of AVP and on its action at the kidney level but also on the regulation of thirst, apelin-immunoreactive cell bodies and fibers have been detected in brain structures involved in the control of drinking behavior. These include the two circumventricular organs, SFO and OVLT, as well as the median preoptic nucleus.[27] According to this localization, central injection of pE13F (1 nmol) significantly reduces water intake in dehydrated rats.[26] By contrast, Taheri et al. have shown opposite results because icv administration of apelin-13 (3, 10, and 30 nmol) increases water intake in rats with free access to water.[32] Further, $APJ^{-/-}$ mice drink significantly less water than wildtype mice.[30] However, the total absence of apelin receptors during fetal and adult life, could elicit compensatory mechanisms leading to this opposite effect. At present, it is therefore difficult to attribute a clear role for apelin in drinking behavior but whatever its true function is in this process, apelin could contribute to the fine regulation of water balance, and this would vary according to the physiological state.

Apelin Regulates Hypothalamic Oxytocin Neurons in Lactating Rats

Firstly, dual-labeling studies in male and virgin female rats have shown that apelin colocalizes with neurophysin I (a marker of oxytocin-containing neurons) in SON and PVN magnocellular OXY neurons.[4] More recently, double immunofluorescence labeling revealed the colocalization of apelin with OXY in about 20% of the hypothalamic OXY-positive neurons in lactating rats.[2] Finally, a combination of *in situ* hybridization and immunohistochemistry showed the presence of apelin receptor mRNA in hypothalamic OXY neurons in lactating rats.[2] These results suggest an interaction between apelin and oxytocin. Consistent with this hypothesis, infusion of K17F into the lateral ventricle of lactating rats inhibits the neuronal activity in most hypothalamic OXY neurons (i.e., magnocellular and parvocellular neurons) as shown by the measurement of c-fos expression in OXY neurons. The infusion of K17F into the third ventricle also inhibits the electrical activity of magnocellular OXY neurons of the SON displaying a strong rhythmic pattern during lactation. K17F inhibits the basal and bursting activity of these neurons, and this is expected to decrease OXY release from neurohypophyseal axon terminals. These observations suggest that apelin released from OXY or AVP neurons during breastfeeding directly inhibits magnocellular and parvocellular OXY neurons in an autocrine or paracrine manner via the apelin receptors expressed on these neurons. Magnocellular OXY neurons are activated by the suckling pups, leading to the release of OXY into the bloodstream, where OXY acts on the myoepithelial cells of the mammary gland, causing smooth muscle contraction and milk ejection. Parvocellular OXY neurons project to the external layer of the ME and release OXY into the hypophyseal portal circulation to increase directly the release of adenohypophysis prolactin (PRL) from lactotrophs known to express OXY receptors into the bloodstream, participating in milk production (Fig. 3).

Quantification of milk delivery to the pups after icv injection of K17F in conscious lactating rats shows a marked decrease in milk ejection after K17F injection,[2] suggesting that brain apelin, by inhibiting both magnocellular

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and parvocellular neuron activity, decreases the amount of milk delivered to the pups (Fig. 3). The inhibitory control of magnocellular OXY neurons by K17F should decrease the amount of OXY released into the bloodstream, whereas this control on parvocellular OXY neurons would decrease the release of PRL into the bloodstream, both effects decreasing the amount of milk ejected for the pups.

Consistent with the inhibitory effect of apelin on parvocellular OXY neurons, a decrease in plasma PRL levels was previously observed after icv administration of pE13F.[32] The inhibitory effect of apelin on magnocellular OXY neurons is not in agreement with the findings of another study reporting an absence of effect of administration by microdialysis of apelin-13 on the firing rate of these neurons.[35] This difference may result from the physiological status of the animals (lactating rats for Bodineau et al. vs. virgin rats for Tobin et al.), to the mode of administration of apelin (icv route vs. diffusion over the ventral surface of the SON through a dialysis probe) and to the molecular form of apelin used (K17F vs apelin-13).

In conclusion, in lactating rats, the coexistence of apelin receptor mRNA and, to a lesser extent, apelin, with OXY in magnocellular and parvocellular OXY neurons of the PVN and SON, and the inhibitory effect of apelin on OXY neuron activity, markedly decreasing the amount of milk delivered to the pups, provide evidence for the autocrine and paracrine inhibitory regulation of OXY neurons by apelin.

Involvement of Apelin in the Regulation of the Hypothalamus–Adrenal–Pituitary (HPA) Axis

The existence of an apelinergic system within the adult male rat anterior pituitary gland has been reported.[4] Apelin is highly coexpressed in corticotrophs and to a much lower extent in somatotrophs and a high expression of apelin receptor mRNA is also found in corticotrophs.[28] These morphological results suggest a local interaction between apelin and adrenocorticotropin (ACTH). This was confirmed by *ex vivo* anterior pituitary gland perfusion studies, which showed that apelin increases basal or K^+-evoked ACTH release. Together, these results indicate that apelin released from corticotrophs or somatotrophs exerts a direct stimulatory effect in the anterior pituitary gland on ACTH release in an autocrine/paracrine manner.[28] Apelin nerve fibers and apelin receptor mRNA expression were also visualized in the parvocellular part of the PVN.[22] Central administration of apelin significantly increases plasma ACTH[32] and corticosterone[13,32] release, at least in part by stimulating corticotropin-releasing hormone (CRH) release.[32] Apelin has also been shown to stimulate significantly the release of CRH and AVP from hypothalamic explants *in vitro*.[34] These results suggest that apelin stimulates ACTH release not only through a direct effect in the anterior pituitary, but also via indirect effects involving the stimulation of CRH release in the hypothalamus. Consistent with this model, apelin receptor mRNA expression is increased in the parvocellular division of the PVN in rats subjected to acute stress (restraint stress), which is known to increase the activity of the HPA axis.[22]

PERIPHERAL CARDIOVASCULAR ACTIONS

Apelin and its receptor are also present in the cardiovascular system.[25]Apelin receptor mRNA expression has been detected in the endothelial cells of large arteries, coronary vessels, and endocardium of the right atrium. Injection of apelin into the bloodstream decreases arterial BP, via a mechanism dependent on NO production. In normotensive or hypertensive rats, apelin increases the contractile force of the myocardium via a positive inotropic effect. Moreover, APJ knockout mice display an enhanced vasopressor response to systemic Ang II, suggesting a counterregulatory action of apelin on Ang II. This counterregulatory action of apelin on Ang II is also supported by the observation that apelin infusion in ApoE-knockout mice abrogates the formation of atherosclerosis and abdominal aortic aneurysm induced by Ang II. Acute administration of apelin *in vivo* results in a vasodilation-induced decrease in left ventricular preload and afterload, and a potent increase in contractility accompanied by a slight decrease in cardiac output. Conversely, chronic apelin infusion increases cardiac output without causing hypertrophy. In addition, mice deficient for apelin develop progressive impairment of cardiac contractility associated with systolic dysfunction with the age. Moreover, these knockout mice subjected to chronic pressure overload by aortic banding develop severe heart failure. It thus appears that apelin is crucial to maintain cardiac contractility in pressure overload and aging.

Overall, these results suggest that the apelin receptor could constitute a potential therapeutic target in the treatment of heart failure. Indeed, the administration of an agonist of the apelin receptor might improve the contractile performance of the myocardium while reducing peripheral resistance and increasing aqueous diuresis in patients with heart failure.

PATHOPHYSIOLOGICAL IMPLICATIONS

The identification of apelin as the endogenous ligand of the orphan APJ receptor constitutes a major advance, for both fundamental research and, potentially, clinical practice. It shows the validity of the "deorphanization" approach to orphan receptors for the identification of novel bioactive peptides and new therapeutic targets. The experimental results obtained to date show that apelin colocalizes with AVP and

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OXY in hypothalamic vasopressinergic and oxytocinergic neurons and that apelin receptors are expressed by these neurons. Apelin increases aqueous diuresis in lactating rats via its central and renal actions: (i) by inhibiting in an autocrine manner the phasic electrical activity of vasopressinergic neurons and secretion of AVP in the blood circulation, and (ii) by a direct effect on the renal microcirculation and probably on tubule function. Apelin inhibits the activity of OXY neurons in lactating rats through a direct action on apelin receptors expressed by these neurons in an autocrine and paracrine manner. Thus, apelin, which limits the action of OXY during lactation and counteracts the antidiuretic action of AVP, may be an important peptide for the fine regulation of functions related to the maintenance of body fluid homeostasis in certain physiological conditions. Finally, when injected into the bloodstream, apelin decreases blood pressure and improves cardiac contractility, suggesting a crucial role for apelin in regulating cardiovascular functions. Clinically, the development of nonpeptidic agonists of the apelin receptor, based on the knowledge of the structures of apelin and its receptor, could provide new therapeutic tools potentially useful for the treatment of heart failure and states of water and/or electrolyte retention.

REFERENCES

1. Azizi M, Iturrioz X, Blanchard A, Peyrard S, De Mota N, Chartrel N, et al. Reciprocal regulation of plasma apelin and vasopressin by osmotic stimuli. *J Am Soc Nephrol* 2008;**19**:1015–24.
2. Bodineau L, Taveau C, Le Quan Sang HH, Osterstock G, Queguiner I, Moos F, et al. Data supporting a new physiological role for brain apelin in the regulation of hypothalamic oxytocin neurons in lactating rats. *Endocrinology* 2011;**152**(9):3492–503.
3. Boucher J, Masri B, Daviaud D, Gesta S, Guigne C, Mazzucotelli A, et al. Apelin, a newly identified adipokine up-regulated by insulin and obesity. *Endocrinology* 2005;**146**:1764–71.
4. Brailoiu GC, Dun SL, Yang J, Ohsawa M, Chang JK, Dun NJ. Apelin-immunoreactivity in the rat hypothalamus and pituitary. *Neurosci Lett* 2002;**327**:193–7.
5. Choe W, Albright A, Sulcove J, Jaffer S, Hesselgesser J, Lavi E, et al. Functional expression of the seven-transmembrane HIV-1 co-receptor APJ in neural cells. *J Neurovirol* 2000;**6**:S61–9.
6. De Mota N, Lenkei Z, Llorens-Cortes C. Cloning, pharmacological characterization and brain distribution of the rat apelin receptor. *Neuroendocrinology* 2000;**72**:400–7.
7. De Mota N, Reaux-Le Goazigo A, El Messari S, Chartrel N, Roesch D, Dujardin C, et al. Apelin, a potent diuretic neuropeptide counteracting vasopressin actions through inhibition of vasopressin neuron activity and vasopressin release. *Proc Natl Acad Sci USA* 2004;**101**:10,464–9.
8. Devic E, Paquereau L, Vernier P, Knibiehler B, Audigier Y. Expression of a new G protein-coupled receptor X-msr is associated with an endothelial lineage in *Xenopus laevis*. *Mech Dev* 1996;**59**:129–40.
9. El Messari S, Iturrioz X, Fassot C, De Mota N, Roesch D, Llorens-Cortes C. Functional dissociation of apelin receptor signaling and endocytosis: implications for the effects of apelin on arterial blood pressure. *J Neurochem* 2004;**90**:1290–301.
10. Hosoya M, Kawamata Y, Fukusumi S, Fujii R, Habata Y, Hinuma S, et al. Molecular and functional characteristics of APJ. Tissue distribution of mRNA and interaction with the endogenous ligand apelin. *J Biol Chem* 2000;**275**:21,061–7.
11. Hus-Citharel A, Bouby N, Frugiere A, Bodineau L, Gasc JM, Llorens-Cortes C. Effect of apelin on glomerular hemodynamic function in the rat kidney. *Kidney Int* 2008;**74**:486–94.
12. Iturrioz X, Gerbier R, Leroux V, Alvear-Perez R, Maigret B, Llorens-Cortes C. By interacting with the C-terminal Phe of apelin, Phe255 and Trp259 in helix VI of the apelin receptor are critical for internalization. *J Biol Chem* 2010;**285**:32,627–37.
13. Jaszberenyi M, Bujdoso E, Telegdy G. Behavioral, neuroendocrine and thermoregulatory actions of apelin-13. *Neuroscience* 2004;**129**:811–6.
14. Kasai A, Shintani N, Oda M, Kakuda M, Hashimoto H, Matsuda T, et al. Apelin is a novel angiogenic factor in retinal endothelial cells. *Biochem Biophys Res Commun* 2004;**325**:395–400.
15. Kawamata Y, Habata Y, Fukusumi S, Hosoya M, Fujii R, Hinuma S, et al. Molecular properties of apelin: tissue distribution and receptor binding. *Biochim Biophys Acta* 2001;**23**:2–3.
16. Lee DK, Cheng R, Nguyen T, Fan T, Kariyawasam AP, Liu Y, et al. Characterization of apelin, the ligand for the APJ receptor. *J Neurochem* 2000;**74**:34–41.
17. Maguire JJ, Kleinz MJ, Pitkin SL, Davenport AP. [Pyr1]apelin-13 identified as the predominant apelin isoform in the human heart: vasoactive mechanisms and inotropic action in disease. *Hypertension* 2009;**54**:598–604.
18. Masri B, Lahlou H, Mazarguil H, Knibiehler B, Audigier Y. Apelin (65–77) activates extracellular signal-regulated kinases via a PTX-sensitive G protein. *Biochem Biophys Res Commun* 2002;**290**:539–45.
19. Masri B, Morin N, Cornu M, Knibiehler B, Audigier Y. Apelin (65-77) activates p70 S6 kinase and is mitogenic for umbilical endothelial cells. *FASEB J* 2004;**22**:22.
20. Masri B, Morin N, Pedebernade L, Knibiehler B, Audigier Y. The apelin receptor is coupled to Gi1 or Gi2 protein and is differentially desensitized by apelin fragments. *J Biol Chem* 2006;**281**:18,317–26.
21. Medhurst AD, Jennings CA, Robbins MJ, Davis RP, Ellis C, Winborn KY, et al. Pharmacological and immunohistochemical characterization of the APJ receptor and its endogenous ligand apelin. *J Neurochem* 2003;**84**:1162–72.
22. O'Carroll AM, Don AL, Lolait SJ. APJ receptor mRNA expression in the rat hypothalamic paraventricular nucleus: regulation by stress and glucocorticoids. *J Neuroendocrinol* 2003;**15**:1095–101.
23. O'Carroll AM, Selby TL, Palkovits M, Lolait SJ. Distribution of mRNA encoding B78/apj, the rat homologue of the human APJ receptor, and its endogenous ligand apelin in brain and peripheral tissues. *Biochim Biophys Acta* 2000;**21**:72–80.
24. O'Dowd BF, Heiber M, Chan A, Heng HH, Tsui LC, Kennedy JL, et al. A human gene that shows identity with the gene encoding the angiotensin receptor is located on chromosome 11. *Gene* 1993;**136**:355–60.
25. Pitkin SL, Maguire JJ, Bonner TI, Davenport AP. International Union of Basic and Clinical Pharmacology. LXXIV. Apelin receptor nomenclature, distribution, pharmacology, and function. *Pharmacol Rev* 2010;**62**:331–42.
26. Reaux A, De Mota N, Skultetyova I, Lenkei Z, El Messari S, Gallatz K, et al. Physiological role of a novel neuropeptide, apelin, and its receptor in the rat brain. *J Neurochem* 2001;**77**:1085–96.
27. Reaux A, Gallatz K, Palkovits M, Llorens-Cortes C. Distribution of apelin-synthesizing neurons in the adult rat brain. *Neuroscience* 2002;**113**:653–62.

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28. Reaux-Le Goazigo A, Alvear-Perez R, Zizzari P, Epelbaum J, Bluet-Pajot MT, Llorens-Cortes C. Cellular localization of apelin and its receptor in the anterior pituitary: evidence for a direct stimulatory action of apelin on ACTH release. *Am J Physiol Endocrinol Metab* 2007;**292**:E7–15.
29. Reaux-Le Goazigo A, Morinville A, Burlet A, Llorens-Cortes C, Beaudet A. Dehydration-induced cross-regulation of apelin and vasopressin immunoreactivity levels in magnocellular hypothalamic neurons. *Endocrinology* 2004;**145**:4392–400.
30. Roberts EM, Newson MJ, Pope GR, Landgraf R, Lolait SJ, O'Carroll AM. Abnormal fluid homeostasis in apelin receptor knockout mice. *J Endocrinol* 2009;**202**:453–62.
31. Roberts EM, Pope GR, Newson MJ, Landgraf R, Lolait SJ, O'Carroll AM. Stimulus-specific neuroendocrine responses to osmotic challenges in apelin receptor knockout mice. *J Neuroendocrinol* 2010;**22**:301–8.
32. Taheri S, Murphy K, Cohen M, Sujkovic E, Kennedy A, Dhillo W, et al. The effects of centrally administered apelin-13 on food intake, water intake and pituitary hormone release in rats. *Biochem Biophys Res Commun* 2002;**291**:1208–12.
33. Tang C, Zelenak C, Volkl J, Eichenmuller M, Regel I, Frohlich H, et al. Hydration-sensitive gene expression in brain. *Cell Physiol Biochem* 2011;**27**:757–68.
34. Tatemoto K, Hosoya M, Habata Y, Fujii R, Kakegawa T, Zou MX, et al. Isolation and characterization of a novel endogenous peptide ligand for the human APJ receptor. *Biochem Biophys Res Commun* 1998;**251**:471–6.
35. Tobin VA, Bull PM, Arunachalam S, O'Carroll AM, Ueta Y, Ludwig M. The effects of apelin on the electrical activity of hypothalamic magnocellular vasopressin and oxytocin neurons and somatodendritic peptide release. *Endocrinology* 2008;**149**: 6136–45.

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Chapter 97

BNP/CNP

Yoshio Takei

ABSTRACT

The natriuretic peptide (NP) family consists of atrial, brain, and C-type NP (ANP, BNP, and CNP) in mammals, of which BNP and CNP were isolated from the brain. However, BNP is a circulating hormone secreted from the heart, and its brain concentration is extremely low in some species. Thus, BNP is now called B-type NP. CNP is a true brain peptide, but its cerebral actions are still emerging. The most prominent phenotype of CNP knockout mice is a short statue resulting from impaired endochondral bone growth. Four CNPs (CNP1–4) are found in teleost fish, of which CNP4 is an ortholog of mammalian CNP. Comparative genomic analyses of the NP family show that CNP4 is an ancestral molecule of the family and that cardiac ANP and BNP are produced by tandem duplication from CNP3 before divergence of ray-finned fishes and lobe-finned fishes (tetrapods).

DISCOVERY

Natriuretic peptides (NPs) have a common intramolecular ring structure consisting of 17-amino-acid residues and N-terminal and C-terminal extensions of variable lengths (Figures 1 and 2). After the report of a potent diuretic/natriuretic and hypotensive factor in the rat atria in 1981, the factor (atrial natriuretic peptide; ANP) was isolated in 1983.[19,28] Subsequently, the second NP was isolated from the porcine brain in 1988 and named "brain" NP (BNP).[19] In contrast to pigs, however, BNP and its gene transcript are practically undetectable in the brain of rodents and human, while BNP is synthesized abundantly in the heart, particularly in the failing ventricle. Thus, much attention has been paid to BNP in the diagnosis and treatment of cardiac failure.[19] Therefore, only the basic structure and function of BNP are described here, and details are provided in the Cardiovascular Peptides section of this volume. BNP is also present in the heart of birds, amphibians, and fishes.[11,29] Therefore, the B of BNP is now recognized as "B-type."

In 1990, a third NP was isolated from the brain of mammals, amphibians, and fishes.[19,28] In the killifish and eel, it was initially named ANP-like or BNP-like peptide, but C-type NP (CNP) is now generally used in all species. CNP is most abundantly synthesized and stored in the brain, and thus, CNP is the true "brain" peptide of the NP family. CNP has the structural characteristic of lacking the C-terminal extension from the intramolecular ring (Figure 2). CNP has been identified in all vertebrate classes ranging from cyclostomes to mammals.[10]

Phylogenetic studies of the NP family reveal the evolutionary history of the whole family in vertebrates.[10] In ray-finned fishes, four CNPs have been isolated from the brain and named CNP1, 2, 3, and 4 (Figure 2). Synteny analyses show that CNP4 is an ortholog of mammalian CNP. CNP1 is a major brain peptide of teleost fish and thus initially isolated from fish brains. Despite the difference in their origin, mammalian CNP(4) and teleost CNP1 are most similar in sequence with each other at the mature sequence of 22-amino-acid residues (Figure 2). CNP3 is localized in tandem with ANP and BNP on the same chromosome, showing that ANP and BNP are duplicated from CNP3.[11] A novel NP was isolated from the cardiac ventricle of ray-finned fish in addition to ANP and BNP and named ventricular NP (VNP),[28] which is also produced from CNP3 by tandem duplication.[11] Only a single CNP has been isolated from the brain and heart of cartilaginous fish (sharks and rays) and cyclostomes (lamprey), while a single NP with long C-terminal sequence and amidated C-terminus has been found in the brain and heart of another cyclostome (hagfish).[28] Phylogenetic analyses show that shark CNP is a CNP3 type, and lamprey CNP and hagfish NP are CNP4 types.[10] Collectively, an ancestral molecule of the NP family is CNP4, which is duplicated to produce first CNP3 and then CNP1 and CNP2 (Figure 3). ANP, BNP, and VNP are produced by tandem duplication from CNP3. Seven NPs (ANP, BNP, VNP, and CNP1–4) exist in ray-finned fish, but some have disappeared in the tetrapod lineage; ANP, BNP, CNP3, and CNP4 exist in amphibians, BNP, VNP, CNP1, and CNP3 in birds, and ANP, BNP, and CNP4 in mammals (Figure 3). Thus, BNP, not ANP, is the common cardiac NP present throughout vertebrate species.[29]

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00097-X
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STRUCTURE OF THE mRNA/GENE

The cDNAs encoding BNP have been cloned in several species of mammals, chicken, frog, and several species of bony fishes (Table 1).[29] The precursor sequence of BNP is most versatile among the members of the NP family in mammals, but it is conserved in teleosts and other tetrapod species. All BNP mRNA sequences thus far cloned are characterized by the presence of AUUUA repeats in the 3′-untranslated region, which is known to facilitate mRNA degradation.[19,28] The genomic DNA sequences of the BNP gene are also determined in several species of mammals and teleost fish. In the vicinity of the human BNP gene, *cis*-acting elements such as activator protein-1 site, serum responsive element, and the GATA sequence have been identified.

The cDNAs coding for different types of CNPs have been sequenced in several species of tetrapods and fishes (Table 1). The CNP gene is also expressed in the venom gland of snakes and duckbill, an egg-laying mammal.[28] The genomic DNAs of the CNP genes have been sequenced in mammals and teleost fishes (Table 1). A cAMP responsive element-like sequence and GC boxes are localized in the promoter region of the CNP gene.

PROCESSING OF THE PRECURSOR

The BNP prohormone is cleaved by a processing enzyme, furin, to produce mature BNP in the brain[25] (see the chapter on furin in the Peptide Biosynthesis/Processing section of this book). Furin acts on a consensus sequence, Arg-X-X-Arg, so that mature human and porcine BNP have 32-amino-acid residues (BNP-32) and those of rats and mice contain 45 amino acid residues (BNP-45) depending on the location of the sequence. BNP is also processed to BNP-26 in the porcine brain.[33] BNP-32 circulates in the blood of human and pig as a mature hormone. ANP also exists in the brain, and the brain forms have 3–4 amino acids

Atrial Natriuretic Peptide (ANP)

man	SLRRSSCFGGRMDRIGAQSGLGCNSFRY
rat	SLRRSSCFGGRIDRIGAQSGLGCNSFRY
bullfrog	MRRSSDCFGSRIDRIGAQSGMGCGRRF
trout	SKAVSGCFGARMDRIGTSSGLGCSPKRRS
eel	SKSSSPCFGGKLDRIGSYSGLGCNSRK-NH_2
sturgeon	NRGSSGCFGSRIDRIGSMSSMGCGGSRK-NH_2

B-type Natriuretic Peptide (BNP)

man	SPKMVQGSGCFGRKMDRISSSSGLGCKVLRRH
rat	NSKMAHSSSCFGQKIDRIGAVSRLGCDGLRLF
chicken	MMRDSGCFGRRIDRIGSLSGMGCNGSRKN
bullfrog	SNCFGRRIDRIDSVSGMGCNGSRNRYP
trout	KRYSGCFGRRLDRIGSMSSLGCTTVGKYNAKTR
eel	NRYSGCFGRKMDRIGSMSSLGCKTVSKRN
sturgeon	KRYSGCFGRRLDRIGSMSALGCNGGSRLSYKRS

Ventricular Natriuretic Peptide (VNP)

chicken	RHFSGCFGTRMERIGAQTGLGCNHYKARFWRRRRS
trout	KSFNSCFGNRIERIGSWSGLGCNNVKTGNKKRIFGN
eel	KSFNSCFGTRMDRIGSWSGLGCNSLKNGTKKKIFGN
sturgeon	RSMNGCFGNRIERIGSWSSLGCNNSRFGSKKRIF

FIGURE 1 Amino acid sequences of cardiac natriuretic peptides sequenced to date. Brackets show disulfide bonds. Shaded amino acids are conserved in more than half of the species.

C-type Natriuretic Peptide (CNP)

	CNP1	CNP2	CNP3	CNP4
human				GLSKGCFGLKLDRIGSMSGLGC
chicken	AVPRGCFGLKMDRIGAFSGLGC		GLSRSCFGLKLDRIGSMSGLGC	
bullfrog			GYSRGCFGVKLDRIGAFSGLGC	GTSKGCFGLKLDRIGAMSGLGC
green puffer	GWNRGCFGLKLDRIGSMSGLGC	VGGRGCFGMKIDRIGSISGLGC	GGLRSCFGVRLARIGSFSGLGC	SSRSGCFGHKMDRIGTISGMGC
tiger puffer	GWNRGCFGLKLDRIGSMSGLGC	VGGRGCFGMKIDRIGSISGLGC	GGLRSCFGVRLARIGSFSGLGC	SSRSGCFGHKMDRIGTISGMGC
stickleback	GWNRGCFGLKLDRIGSVSGLGC	GGGRGCFGMKMDRIGSISGLGC	GRMRSCFGVRLERIGSFSGLGC	TTRSGCFGHKMDRIGTISGMGC
medaka	GWNRGCFGLKLDRIGSMSGLGC	VAGGGCFGMKMDRIGSISGLGC	GGMRSCFGVRLERIGSFSGLGC	TSRSGCFGHKMDRIGTISGMGC
cod	GWNRGCFGLKLDRIGSMSGLGC		GGHRSCFGVRLERIGSFSGMGC	TARSGCFGHKMDRIGTISGMGC
zebrafish	GWNRGCFGLKLDRIGSMSGLGC	GVARGCFGMKVDRIGVISGLGC	GDLRSCFGVRLERIGSFSGLGC	TARSGCFGHKMDRIGTMSGMGCQPSHYHSINNLS
catfish	GWNRGCFGLKLDRIGSMSGLGC			TRNGCFGHKMDRIGTISGMGC
rainbow trout	GWNRGCFGLKLDRIGSMSGLGC	ATARGCFGMKGDRIGSISGLGC	GGLRSCFGVRLERIGSFSGLGC	AARSGCFGHKMDRIGTISGMGC
Atlantic salmon	GWNRGCFGLKLDRIGSMSGLGC		GGLRSCFGVRLERIGSFSGLGC	AARSGCFGHKMDRIGTISGMGC
pike	GWNRGCFGLKLDRIGSMSGLGC		GGMRSCFGVRLERIGSFSGLGC	RSGCFGQKLDRIGTLSGMGC
eel	GWNRGCFGLKLDRIGSLSGLGC		GGLRSCFGVRLERIGSFSGLGC	PARGGCFGHKLDRISTLSGMGC
bichir	AWSKGCFGLKLDRIGSISGLGC		SGLRSCFGVRLDRIGSMSGLGC	GLSKGCFGLKLDRIGSMSGLGC
houndshark			GPSRGCFGVKLDRIGAMSGLGC	
sea lamprey				GPGKGCFGGKLDRIGALSGLGC
hagfish				STSDGCFGVKMDRIGASTGLGCRGARRRTFS-NH_2

FIGURE 2 Amino acid sequences of C-type natriuretic peptides identified to dates. Tetrapod CNPs were assigned by synteny analyses. Brackets show disulfide bonds. Shaded amino acids are conserved in more than half of the species.

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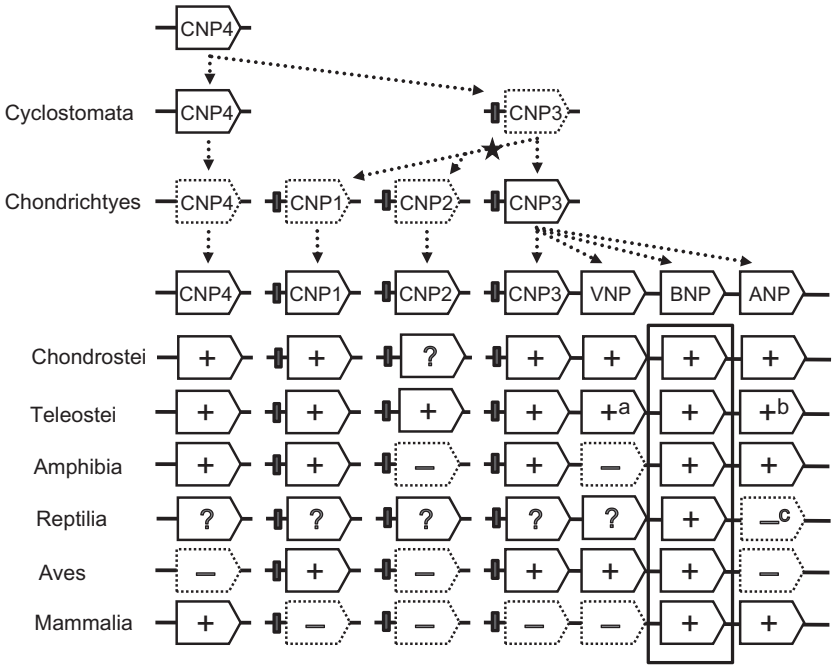

FIGURE 3 Phylogenetic distribution of natriuretic peptides in different classes of vertebrates. Presence and absence are determined by isolation of peptides and cDNAs and by genome and EST database searches. Boxes in dotted line show that the NP may be silenced, but two CNPs are identifiable in the lamprey genome database. Filled square shows linkage with the enolase gene. Asterisk shows the occurrence of an intron shift.[10] +, present; –, absent; ?, not determined. a, absent in pufferfish, medaka and zebrafish genomes; b, absent in medaka genome; c, present in tortoise. Modified from Figure 1 in Ref. 29.

TABLE 1 Accession Numbers of BNP and CNP cDNA/Genomic DNA

Animals	BNP	CNP1	CNP2	CNP3	CNP4
Mammals					
Humans	M25296				AC013435
Rats	M60266				D90219
Mice	AB039044				D28873
Sheep	AF037466				M64710
Pigs	M22477				M64758
Cats	AF253495				AF295101
Birds					
Chicken	E02222	DQ855410			BU417608
Amphibians					
Xenopus	BG346231			BJ069969	AW159572
Fishes					
Eel	AB179820	D88022		AB513621	B513623
Fugu	AB089934	AB089935	AB089936	AB089937	AB089938
Medaka	AB099700	AB081455	AB081456	AB091698	AB091699
Zebrafish	CN023754	BI865725	AL927379	AL919732	BQ092110
A. salmon	CK883047	CA052068	CX258517*	EG920604	G843586

*Rainbow trout.

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deleted from the N-terminus of the mature circulating form. Only BNP prohormone is detected in the porcine atria, but BNP-32 is a major form stored in the human atria.[19] BNP-45 is a major form circulating in the rat blood, and both prohormone and BNP-45 are stored in the rat atria.

Although BNP exists in several nonmammalian species, their mature, circulating forms have not been determined yet. There is a furin-processing signal in the prosegment of chicken BNP, but the signal is absent in most other species.[28] Instead, two consecutive basic amino acids for cleavage by typical proprotein convertases exist in the prosegment of nonmammalian BNP. BNP has been identified in all species of bony fishes, amphibians, reptiles, birds, and mammals thus far examined, whereas ANP and its cDNA are undetectable in most species of birds and reptiles and some species of bony fishes.[29] All BNP sequences are characterized by the presence of two consecutive basic amino acids at positions 4 and 5 of the intramolecular ring.

Two molecular forms of CNP have been isolated from the porcine brain, CNP-22 and CNP-53.[16] CNP-22 is processed after dibasic amino acid residues, whereas CNP-53 is cleaved by furin. Both forms have similar biological activities and have been identified in the brain of all mammalian species. Teleost CNP4 is an ortholog of mammalian CNP, and it is the only teleost CNP that has a furin processing signal.[10] Lamprey CNP(4) and hagfish NP (CNP4) also have furin processing signals to form mature peptide. CNP1 is a major teleost CNP in the brain and most similar to mammalian CNP, but it lacks a furin-processing signal. Elasmobranch CNP(3) is highly similar to mammalian CNP but lacks furin processing signal. Thus, all CNP4-type molecules thus far known have furin-processing signals. High sequence similarity of mature mammalian CNP(4), teleost CNP1 and elasmobranch CNP(3) (Figure 2) indicates convergent evolution, probably because of the binding to their specific receptor (see below).

DISTRIBUTION OF THE mRNA/PEPTIDE

Although BNP was initially isolated from the pig brain, its concentration in the brain is much lower than in the heart in pig and other species.[19] BNP is even less abundant than ANP in the brain of rodents and human. Therefore, BNP is not a brain peptide but is a cardiac hormone like ANP. In the pig brain, BNP is localized preferentially in the striatum, hypothalamus, medulla-pons region, and spinal cord when each brain region is measured by specific radioimmunoassay.[33] *In situ* hybridization histochemistry of the rat brain showed that BNP mRNA is not detectable, but ANP mRNA is expressed in the anterior olfactory nuclei, limbic cortices, dorsal endopiriform nucleus, hippocampal subfield CA1, cortical amygdaloid nuclei, medial habenula, anteroventral periventricular and arcuate nuclei, periventricular stratum, zona incerta, mammillary nuclei, inferior olive, nucleus ambiguus, and pontine paragigantocellular nuclei.[15] In rodents and human, ANP seems to play a more significant role in brain function than BNP. In bony fishes, BNP mRNA expression is also sparse in the brain and abundant in the heart.[11] BNP has not been localized by histochemical methods in the brain of nonmammals yet.

CNP peptide and its mRNA are more abundant in the brain than in the periphery in all mammalian species thus far examined.[19] Immunoreactive CNP and its gene transcripts are ubiquitously distributed in the whole brain, with the highest concentration in the olfactory bulb. In the rat and mouse, relatively high expression of the CNP gene is detected in the tegmentum, as determined by RNase protection assay.[27] More detailed mapping of CNP mRNA by *in situ* hybridization revealed that CNP gene transcripts are localized in the olfactory nuclei, limbic cortices, dorsal endopiriform nucleus, hippocampal subfields CA1–3, anteroventral periventricular and arcuate nuclei, and numerous brainstem regions including pontine, lateral reticular, solitary tract, prepositus hypoglossal, and spinal trigeminal nuclei (Figure 4).[15] Colocalization of CNP and ANP in the same brain nuclei suggests their cooperative function. In the hypothalamus, both CNP and ANP gene transcripts are abundantly expressed in the anteroventral preoptic area and arcuate nucleus, which is consistent with their role in the regulation of neuroendocrine functions and appetite regulation.[8] Only CNP mRNA is detectable in the vasopressin-producing magnocellular paraventricular nucleus and supraoptic nucleus. Interestingly, water deprivation decreases and salt loading (2% NaCl for drinking water) increases CNP gene expression in the olfactory region of the rat brain as shown by northern blot and *in situ* hybridization.[4] CNP is also detected in the pineal gland of the dog.

From a comparative viewpoint, all CNPs thus far obtained are expressed abundantly in the brain in all species examined to date including cyclostomes, cartilaginous fishes, bony fishes, amphibians, and birds, although CNP is also expressed abundantly in the heart of cyclostomes and cartilaginous fishes where CNP is the only peptide of the NP family.[28] Four CNP genes identified in bony fishes are all expressed briskly in the fish brain. CNP3, from which cardiac ANP, BNP, and VNP are derived, is also expressed in the heart of the medaka, *Oryzias latipes*.[11] However, a detailed localization of CNP or its transcripts in the brain by immunohistochemistry or *in situ* hybridization has not yet been examined in nonmammalian species.

RECEPTORS AND SIGNALING CASCADES

BNP and ANP bind with high affinity to the A-type NP receptor (NPR-A or GC-A) that possesses guanylyl cyclase and kinase-like domains intracellularly in its

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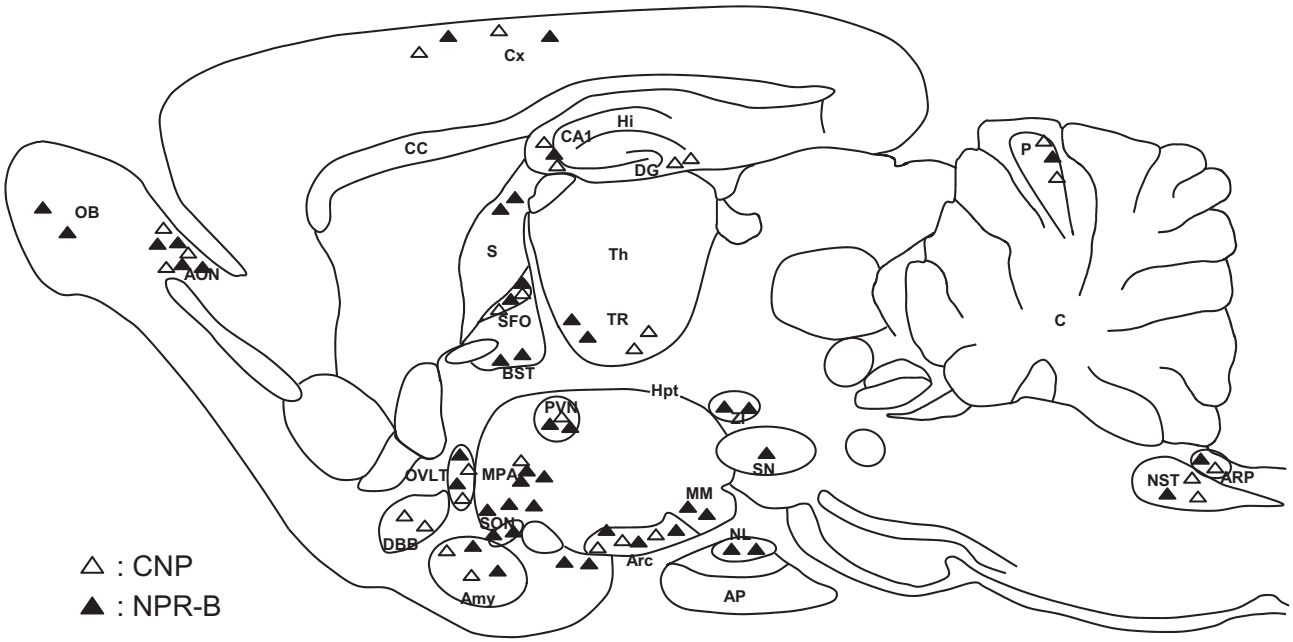

FIGURE 4 Distribution of CNP and NPR-B gene transcripts in the rat brain as revealed by *in situ* hybridization.[7,15] Amy, amygdala; AON, anterior olfactory nucleus; AP, anterior pituitary; Arc, arcuate nucleus; ARP, area postrema; BST, bed nucleus of stria terminalis; C, cerebellum; CA1, CA1 of hippocampus; CC, corpus callosum; Cx, cerebral cortex; DBB, diagonal band of Broca; DG, dentate gyrus; Hi, hippocampus; Hpt, hypothalamus; RN, lateral reticular nucleus; MM, mammilary nucleus; MPA, medial preoptic area; NL, neural lobe of pituitary; NST, nucleus of solitary tract; OB, olfactory bulb; OVLT Organum vasculosum of the lamina terminalis; P, Purkinje cell layer of the cerebellum; PVN, paraventricular nucleus; S, septum; SFO, subfornical organ; SN, substantia nigra; SON, supraoptic nucleus; Th, thalamus; TR, Nucleus reticularis thalami; ZI, Zona incerta.

molecule.[20] CNP, however, binds specifically to cognate B-type NP receptor (NPR-B or GC-B). After ligand binding to the receptors, guanylyl cyclase is activated with the help of ATP to produce cGMP. Increased cGMP acts as a second messenger to activate G-kinase, resulting in phosphorylation of various target proteins.[20] In addition, both BNP and CNP bind with high affinity to a C-type NP receptor (NPR-C) that has only a short (27 amino acids) intracellular domain and exists ubiquitously in various tissues. Judging from the rapid internalization after ligand binding and subsequent degradation of the ligand in the cell, NPR-C was initially believed to be a clearance-type receptor that regulates NP concentration in plasma and tissues. However, physiological functions have been suggested later for this receptor through an interaction with Gi proteins.[18]

In situ hybridization histochemistry shows that significant levels of NPR-A mRNA are expressed in the restricted cell populations of the rat brain that also contain high levels of ANP.[7,21] These include the olfactory bulb and circumventricular organs such as the subfornical organ and area postrema. By contrast, NPR-B mRNA is widely expressed throughout the brain, which coincides well with the wide distribution of CNP in the brain and suggests its paracrine/autocrine action (Figure 4). Autoradiography of the rat brain using ^{125}I-labeled ANP, CNP, or cANF-(4–23) that binds only to NPR-C shows that NPR-A is localized in the olfactory bulb, nucleus tractus solitarius, and circumventricular organs such as the subfornical organ and area postrema, as well as the choroid plexus.[3,19] However, this autoradiographic study could not detect CNP-specific NPR-B.

In addition to NPR-A, NPR-B, and NPR-C, another NP receptor devoid of the guanylyl cyclase domain was identified in the eel and named NPR-D.[28] The relative abundance of NPR-D in the brain suggests some biological function by an intracellular messenger other than cGMP like NPR-C. Two types of NPR-A and NPR-B have been cloned in the medaka fish. In other nonmammalian species, NPR-A has been cloned in the bullfrog and NPR-B in the dogfish where only CNP3 exists in the brain and heart.[28] It is interesting to note that medaka CNP1, 2, and 4 bind with high affinity to NPR-B, but medaka CNP3, from which ANP, BNP, and VNP are originated, binds to a medaka homolog of NPR-A.[11] Our immunohistochemical study using eel-specific NPR antisera shows that immunoreactive NPR-A is detected in the area postrema, glossopharyngeal vagal motor complex, reticular formation, and commissural nucleus of Cajal (equivalent to the nucleus tractus solitarius) in the eel brain, whereas immunoreactive NPR-B is distributed widely in the vascular smooth muscle cells of whole brain.[32]

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BIOLOGICAL ACTIONS WITHIN THE BRAIN AND PITUITARY

As mentioned above, BNP is undetectable in the rodent brain by immunohistochemistry or *in situ* hybridization, and BNP concentration is much lower than ANP concentration in the human and rat brains. Thus, the biological action of ANP in the brain has been characterized in the rat, which will be reviewed here.

ANP is implicated in the central regulation of body fluid homeostasis in rat.[1] It has been shown that water deprivation reduces ANP content in several discrete regions of the brain such as the neural lobe of the pituitary, organum vasculosum lamina terminalis, supraoptic and suprachiasmatic nuclei, but not in other regions.[22] In fact, infusion of rat ANP-(4–28), a major brain form, into the third ventricle inhibits drinking induced by water deprivation or by central infusion of angiotensin II in normally hydrated rats.[9] Interestingly, intravenous infusion of ANP at similar doses equally inhibits drinking induced by these dipsogenic treatments, suggesting that ANP acts on a central site devoid of a blood–brain barrier such as the circumventricular organs. Similarly, central infusion of ANP causes a dose-dependent and long-lasting inhibition of sodium appetite in sodium-deplete rats.[9] In nonmammalian species, direct injection of eel ANP into the area postrema inhibits drinking, and lesioning of the area postrema abolishes the antidipsogenic action of peripheral ANP.[32] Thus, ANP may act on this circumventricular structure for inhibition of drinking caused by reflex swallowing in this aquatic animal.

Central ANP also influences hypothalamic and pituitary hormone secretion in mammals. The hormones that are regulated include vasopressin, GnRH, and CRH secreted from the neural tissues, and prolactin, growth hormone, ACTH, and luteinizing hormone secreted from the anterior pituitary.[22] Vasopressin, whose secretion is induced by angiotensin II or dehydration, is inhibited by ANP *in vivo*, or administered directly onto the supraoptic or paraventricular nuclei in brain slices *in vitro*. ANP also inhibits sympathetic tone through its action on the nucleus tractus solitarius and inhibits the release of catecholamines from sympathetic nerve endings. Noradrenergic projections from the nucleus tractus solitarius to the anterior hypothalamus play pivotal roles in the central cardiovascular control in mammals. ANP has been shown to act on both sites and to decrease arterial pressure via inhibition of norepinephrine release at the anterior hypothalamic area. The deletion of the BNP/ANP gene and/or the NPR-A gene does not induce changes related to their central actions but to cardiovascular actions. Knockout of the gene coding for BNP induces cardiac fibrosis, and mice with deletion of the NPR-A gene exhibit ventricular hypertrophy and fibrosis.[15] Because mice with targeted disruption of the ANP gene are free from cardiac fibrosis, BNP and ANP seem to play differential roles in the cardiovascular regulation through NPR-A.[15]

In contrast to the many studies on the central action of ANP, studies on CNP are still limited, although CNP is a true brain peptide of the NP family. CNP is generally less potent than ANP and BNP as exemplified by neuroendocrine actions such as inhibition of vasopressin[34] and luteinizing hormone (LH) secretion.[31] CNP is produced in rat pituitaries and cultured gonadotrope cells but fails to stimulate cell proliferation and LH release, although it stimulates cGMP production.[31] With respect to body fluid regulation, CNP weakly stimulates, rather than inhibits, water drinking in rat when administered into the cerebral ventricle.[28] There has been no report on the effect of CNP on sodium appetite. Similar opposing actions are suggested on prolactin secretion: stimulation by CNP and inhibition by ANP.[23] In teleost fish (tilapia), CNP stimulates growth hormone secretion from the isolated pituitary, but ANP has no effect. Both CNP and ANP have no effects on prolactin secretion.[29]

CNP affects neurons and brain development. NPR-B gene transcripts are most abundant 1 day after birth in the brain of developing mice, suggesting a role of CNP/NPR-B system in neonatal brain maturation.[17] In sensory neurons, CNP is synthesized abundantly in the dorsal root ganglion of the spinal cord and regulates axonal development via NPR-B and cGMP.[24] Thus, CNP functions as a bifurcation factor of sensory neurons responsible for axonal branching. It has also been suggested that CNP and NPR-B expressed in the rat hippocampus are involved in the regulation of long-term potentiation and depression in the area CA1.[5]

Mice lacking the CNP gene show severe dwarfism as a result of impaired endochondral ossification.[15] Further, most of them die early during postnatal development. Targeted expression of the CNP gene in growth plate chondrocytes rescues the skeletal defect of CNP-deficient mice and prolongs their survival. Thus, their short life results in part from skeletal abnormalities. Disruption of NPR-B also induces profound dwarfism and early death in mice.[15] NPR-B knockout also impairs female, but not male, reproductive organs.[30] The knockout mice exhibit self-clasping and priapism, showing severe neuronal disorder, although no histological abnormalities are detectable in the brain and spinal cord. This result coincides with the perinatal peak expression of the NPR-B gene in the developing mouse.[17] In humans, autosomal recessive skeletal dysplasia is shown to have mutations in the NPR-B gene, and obligate carriers of the mutations are shorter than matched controls.[2] A genomewide association study shows that polymorphism in CNP and its receptor genes plays an important role in determining human body height in the tall populations of northeastern Europe.[6] These results show that the major role of CNP/NPR-B is in the regulation of skeletal growth, although the peptide is most abundant in the brain.

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In relation to the prominent dwarf phenotype of CNP or NPR-B knockdown mice, the role of CNP/NPR-B in skeletal growth has been investigated intensively. These studies show that liver-targeted CNP transgenic mice with high plasma CNP concentrations exhibit skeletal overgrowth in proportion to the plasma CNP concentrations and increased CNP in plasma rescues the dwarf phenotype of CNP knockout mice.[35] Thus, endochondral ossification can be stimulated by circulating CNP and locally produced CNP. CNP also stimulates growth by regulating food intake and energy expenditure as CNP knockout mice with cartilage-specific CNP overexpression still exhibit impaired skeletal growth.[19] The regulatory role of appetite is consistent with CNP gene expression in the arcuate nucleus and paraventricular nucleus, which are important appetite-regulating sites. The CNP/NPR-B system may also be involved in the regulation of adipogenesis.[13] The dwarf phenotype of NPR-B gene-mutant mice may result in part from the lack of gastrointestinal motility.[26] As a result of various central and peripheral actions, CNP seems to maintain normal growth during development.

PATHOPHYSIOLOGICAL IMPLICATIONS

BNP secretion is profoundly augmented from the heart, particularly from the ventricle, in patients with congestive heart failure and acute myocardial infarction, and the increase is correlated with the enlargement, decreased contractility, and increased stiffness of the left ventricle, suggesting its role in ventricular remodeling. Indeed, mice with targeted disruption of the BNP gene show cardiac fibrosis defined as a proliferation of interstitial fibroblasts and biosynthesis of extracellular matrices in the cardiac ventricle.[14] More information on clinical relevance of BNP and ANP is described in the Renal, Cardiovascular, and Endocrine Peptides sections of this book.

CNPs are ancient peptides of the NP family and CNP4, an ortholog of mammalian CNP, is the ancestral molecule of the NP family.[11] Consistent with the phylogenetic ancestry, the genes of CNP and its receptor, NPR-B, are expressed early during development in the brain and seem to play important roles in neurogenesis and axonal branching of neurons. Thus, it is possible that CNP seems to play important roles in neural development in patients with CNP mutations. More promising is a replacement therapy of CNP for retarded skeletal development in CNP mutation diseases such as chondrodysplasia, because direct administration of CNP to the endochondral plate restores normal bone growth.[20] Systemic administration of CNP can also have a therapeutic potential against impaired growth of chondrodysplasias.[35] It seems that CNP acts as an important regulator in the brain for food intake and energy metabolism to regulate body size in cooperation with the peripheral CNP/NPR-B system.[19,12]

REFERENCES

1. Antunes-Rodrigues J, McCann SM, Rogers LC, Samson WK. Atrial natriuretic factor inhibits dehydration- and angiotensin II-induced water intake in the conscious, unrestrained rat. *Proc Natl Acad Sci USA* 1985;**82**:8720–3.
2. Bartels CF, Bukulmez H, Padayatti P, Rhee DK, van Ravenswaaij-Arts C, Pauli RM, et al. Mutations in the transmembrane natriuretic peptide receptor NPR-B impair skeletal growth and cause acromesomelic dysplasma, type Maroteaux. *Am J Hum Genet* 2004;**75**: 27–34.
3. Brown J, Zuo Z. C-type natriuretic peptide and atrial natriuretic peptide receptors of rat brain. *Am J Physiol* 1993;**264**:R513–23.
4. Cameron VA, Cumming SA, Espiner EA, Nicholls G, Richards M. C-type natriuretic peptide expression in olfactory regions of rat brain is modulated by acute water deprivation, salt loading and central angiotensin II. *Neuroendocrinology* 2001;**73**:46–53.
5. Decker JM, Wójtowicz AM, Bartsch JC, Liotta A, Brauewell KH, Heinemann U, et al. C-type natriuretic peptide modulates bidirectional plasiticity in hippocampal area CA1 in vitro. *Neuroscience* 2010;**169**: 8–22.
6. Eatrada K, Krawczak M, Schreiber S, van Duijn K, Stolk L, van Meurs JBJ, et al. A genome-wide association study of northwestern Europeans involves the C-type natriuretic peptide signaling pathway in the etiology of human height variation. *Human Mol Genet* 2009;**18**:3516–24.
7. Herman JP, Doglas CM, Rucker D, Langub Jr MC. Localization of natriuretic peptide-activated guanylate cyclase mRNAs in the rat brain. *J Comp Neurol* 1996;**369**:165–87.
8. Herman JP, Langub Jr MC, Watson Jr RE. Localization of C-type natriuretic peptide mRNA in rat hypothalamus. *Endocrinology* 1993;**133**:1903–6.
9. Imura H, Nakao K, Itoh H. The natriuretic peptide system in the brain: implications in the central control of cardiovascular and neuroendocrine functions. *Frontiers Neuroendocirinol* 1992;**13**: 217–49.
10. Inoue K, Naruse K, Yamagami S, Mitani H, Suzuki N, Takei Y. Four functionally distinct C-type natriuretic peptides found in fish reveal new evolutionary history of the natriuretic system. *Proc Natl Acad Sci USA* 2003;**100**:10,079–84.
11. Inoue K, Sakamoto T, Yuge S, Iwatani H, Yamagami S, Tsutsumi M, et al. Structural and functional evolution of three cardiac natriuretic peptides (ANP, BNP and VNP) revealed in the teleost lineage. *Mol Biol Evol* 2005;**22**:2428–34.
12. Inuzuka M, Tamura N, Yamada N, Katsuura G, Oyamada N, Taura D, et al. C-type natriuretic peptide as a new regulator of food intake and energy expenditure. *Endocrinology* 2010;**151**:3633–42.
13. Katafuchi T, Gerbers DL, Albanesi JP. CNP/GC-B system: a new regulator of adipogenesis. *Peptides* 2010;**31**:1906–11.
14. Kuhn M. Cardiac and intestinal natriuretic peptides: insights from genetically modified mice. *Peptides* 2005;**26**:1078–85.
15. Langub Jr MC, Watson Jr RE, Herman JP. Distribution of natriuretic peptide precursor mRNA in the rat brain. *J Comp Neurol* 1995;**356**:183–99.
16. Minamino N, Aburaya M, Kojima M, Miyamoto K, Kangawa K, Matsuo H. Distribution of C-type natriuretic peptide and its messenger RNA in rat central nervous system and peripheral tissue. *Biochem Biophys Res Commun* 1993;**197**:326–35.

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17. Müller D, Hida B, Guidon G, Speth RC, Michurina TV, Enikolopov G, et al. Expression of guanylyl cyclase (GC)-A and GC-B during brain development: evidence for a role of GC-B in perinatal neurogenesis. *Endocrinology* 2009;**150**:5520–9.
18. Murthy KS, Teng B-Q, Zhou H, Jin J-G, Grider JR, Makhlouf GM. G_{i-1}/G_{i-2}-dependent signaling by single-transmembrane natriuretic clearance receptor. *Am J Physiol* 2000;**278**:G974–80.
19. Nishikimi T, Kuwahara K, Nakao K. Current biochemistry, molecular biology, and clinical relevance of natriuretic peptide. *J Cardiol* 2011;**57**:131–40.
20. Potter LR. Regulation and therapeutic targeting of peptide-activated receptor guanylyl cyclases. *Pharmacol Therap* 2011;**130**:71–82.
21. Quirion R. Receptor sites for atrial natriuretic factors in brain and associated structures: an overview. *Cell Mol Neurobiol* 1989;**9**:45–55.
22. Samson WK. Central nervous system actions of atrial natriuretic factor. *Brain Res Bull* 1988;**20**:831–7.
23. Samson WK, Huang FL, Fulton RJ. Opposing neuroendocrine actions of the natriuretic peptides: C-type and A-type natriuretic peptides do not interact with the same hypothalamic cells controlling prolactin secretion. *J Neuroendocrinol* 1995;**7**:759–63.
24. Schmidt H, Stonkute A, Jütter R, Koesling D, Friebe A, Rathjen FG. C-type natriuretic peptide (CNP) is a bifurcation factor for sensory neurons. *Proc Natl Acad Sci USA* 2009;**106**:16847–52.
25. Seidah NG, Chretien M. Proprotein and prohormone convertases: a family of subtilases generating diverse bioactive polypeptides. *Brain Res* 1999;**848**:45–62.
26. Sogawa C, Abe A, Tsuji T, Koizumi M, Saga T, Kunieda T. Gastrointestinal tract disorder in natriuretic peptide receptor B gene mutant mice. *Am J Pathol* 2010;**177**:822–8.
27. Stepan H, Leitner E, Siems W-E, Maul B, Waltner T. mRNA quantification of C-type natriuretic peptide in brain areas of rodents. *Peptides* 1999;**20**:1243–5.
28. Takei Y. Structural and functional evolution of the natriuretic peptide system in vertebrates. *Int Rev Cytol* 2000;**194**:1–66.
29. Takei Y, Inoue K, Trajanovska S, Donald JA. B-type natriuretic peptide (BNP), but not ANP, is the basic cardiac natriuretic peptide in vertebrates. *Gen Comp Endocrinol* 2010;**171**:258–66.
30. Tamura N, Doolittle LK, Hammer RE, Shelton JM, Richardson JA, Garbers DL. Critical roles of the guanylyl cyclase B receptor in endochondral ossification and development of female reproductive organs. *Proc Natl Acad Sci USA* 2006;**101**:17300–5.
31. Thompson LR, Chand AN, Jonas KC, Burrin JM, Steinhelper ME, Wheeler-Jones CP, et al. Molecular characterization and functional interrogation of a local natriuretic peptid system in rodent pituitaries, αT3-1 and LβT2 gonadotroph cells. *J Endocrinol* 2009;**203**:215–29.
32. Tsukada T, Nobata S, Hyodo S, Takei Y. Area postrema, a brain circumventricular organ, is the site of antidipsogenic action of circulating atrial natriuretic peptide in eels. *J Exp Biol* 2007;**210**:3970–8.
33. Ueda S, Minomino N, Sudoh T, Kangawa K, Matsuo H. Regional distribution of immunoreactive brain natriuretic peptide in porcine brain and spinal cord. *Biochem Biophys Res Commun* 1988;**155**:733–9.
34. Yamamoto S, Morimoto I, Yanagihara N, Kangawa K, Inenaga K, Eto S, Yamashita H. C-type natriuretic peptide suppresses arginine-vasopressin secretion from dissociated magnocellular neurons in newborn rat supraoptic nucleus. *Neurosci Lett* 1997;**229**:97–1000.
35. Yasoda A, Nakao K. Translational research of C-type natriuretic peptide (CNP) into skeletal dysplasias. *Endocrine J* 2010;**57**:659–66.

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Chapter 98

CART

Csaba Fekete and Ronald M. Lechan

ABSTRACT

Cocaine- and amphetamine-regulated transcript (CART) peptides are widely expressed in the central nervous system and in the pituitary. ProCART peptides are directed to secretory granules by a signal sequence and further processed by prohormone convertases into smaller biologically active peptides. These peptides have an important role in the regulation of a number of physiological processes in the central nervous system including reward, feeding, neuroendocrine function, pain, cardiovascular function, and gastrointestinal motility.

DISCOVERY

Cocaine- and amphetamine-regulated transcript (CART) was discovered in 1995 as a striatal transcript induced by psychostimulant administration.[9] As revealed by differential display PCR, the acute administration of either cocaine or amphetamine is capable of inducing an approximately 5-fold increase in CART mRNA in the striatum but not in other regions of the brain.[9] It was later recognized that a somatostatin-like peptide fragment isolated in 1981[25] is identical to the N-terminal portion of CART 55–102 peptide.

STRUCTURE OF THE CART PRECURSOR mRNA AND CART GENE

CART transcript is present in rat tissues as an RNA doublet.[7] An alternate polyA site used in the 3′ noncoding region generates two CART mRNAs approximately 700 and 900 bases long.[7] In addition, alternate splicing generates even further mRNA species resulting from the presence or absence of an in-frame 39 base insert in the coding region.[7] This results in the production of two proCART peptides with 102 or 89 amino acid residues, respectively.[7] Approximately one-third of the CART mRNA content in the rat brain contains the 39 nucleotide insert.[7] Although multiple polyA sites are also present in the human CART gene, only one site is used, resulting in an approximately 900-base-long CART mRNA.[7] In addition, human CART mRNA does not contain the 39-base-long insert observed in the rat.[7] Two different CART mRNAs are also present in the goldfish, resulting in the production of two different proCART polypeptides with 117 and 120 amino acid residues, respectively. However, in this species, the two different CART mRNA are products of two different CART genes.[7]

The chromosomal location of the CART gene has been identified in humans on chromosome 5 and in mice on chromosome 13.[7] Comparison of the cDNA sequence to that of the genomic DNA in different species reveals that in all instances, mature CART mRNA is encoded by three exons.[7]

The proximal promoter (340-nucleotides upstream of the transcription initiation site) of the mouse CART gene that has >80% identity with the human sequences contains multiple putative transcription factor binding sites.[7] The predicted transcription factor binding sites are as follows: TATA box, STAT, cAMP response element (CRE), AP1, SP1, AP2, and E-box. A Pit-1 site is found in a more distal promoter region.[7] Among these sites, the canonical CRE site has been functionally validated. This CRE site binds phosphorylated CREB *in vivo* and *in vitro*, and the absence of this site prevents forskolin responsiveness of the promoter in an *in vitro* promoter assay.[7] Pit-1 responsiveness of the CART promoter has also been shown, but the exact binding site is unknown.[7] Although CART mRNA was believed to be initiated 19 nucleotide upstream of the AUG codon, four additional CART mRNAs have been identified with different 5′-untranslated region lengths (63, 105, 132, and 151 nucleotides upstream of the translation initiation site),[7] suggesting that the mouse CART promoter may lack a functional TATA box.[7]

PROCESSING OF CART PEPTIDES

CART peptides derive from two precursor polypeptides of 129 and 116 amino acid residues that arise via alternative splicing of a single CART gene in rats and mice.[9] The longer form of the CART precursor (rlCART) contains a 13-amino acid insert compared with the short precursor (rsCART).[9] In contrast to rodents, only the shorter CART precursor exists in humans.[8] Both forms of prepro-CART begin with

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00098-1
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a 27-amino acid signal peptide motif, suggesting that CART peptides are targets for posttranslational processing.[9] Cleavage of the signal peptide yields proCART peptides of either 102 or 89 amino acids, which are further processed by protein convertases.[6] The long form of proCART gives rise to an intermediate fragment rlCART 33–102 that is further processed into two bioactive peptides, rlCART 55–102 and rlCART 62–102.[6] Similarly, the shorter form is processed into an intermediate residue, CART 10–89, and two bioactive forms, rlCART 55–102 and rlCART 62–102.[6] (The nomenclature of CART peptides is based on the amino acid sequence of the two forms of the CART precursor (rlCART and rsCART).)

The two main enzymes involved in processing of CART are PC1/3 and PC2 protein convertases[6] (see the chapters on proconvertases in Peptide Biosynthesis/Processing section of this book). PC1/3 is much more potent than PC2 in producing intermediate CART residues. It cleaves the long form of the CART precursor to result in CART 33–102[6] and is exclusively responsible for the production of CART 10–89 from the short CART precursor.[6] By contrast, PC2 is the primary enzyme responsible for the production of CART 55–102, and is exclusively responsible for the generation of CART 62–102.[6] Both intermediate forms and the two bioactive forms of CART peptides are present in the brain.[6] By contrast, only CART 62–102 is present in the neurointermediate lobe where both PC1/3 and PC2 are present, whereas CART 55–102 is present in the anterior lobe, which expresses only PC1/3.[6] Experiments using RNAi-mediated knock down of PC5/6 in GH3 cells have also demonstrated an important role for this enzyme in the processing of CART peptides. The PC5/6A isoform of the enzyme has similar cleaving properties as PC2, cleaving the pairs of basic residues within the proCART isoforms.[29] A fourth enzyme implicated in the processing of proCART is carboxypeptidase E (CPE) (see the chapter on this enzyme in the Peptide Biosynthesis/Processing section of this book). Mature forms of CART are undetectable in CPE KO mice, while the levels of intermediate and high molecular mass CART molecules are increased, indicative of abnormal processing.[2]

DISTRIBUTION OF CART mRNA AND PEPTIDES

CART mRNA is expressed in discrete neuronal groups in the brain, and it is also present in the anterior lobe of the pituitary gland (Fig. 1).[9] The highest concentration of the CART mRNA is found in the hypothalamus, and in particular, neurons of the arcuate, paraventricular, supraoptic and dorsomedial nuclei, periventricular region, zona inzerta, and lateral hypothalamus.[9] Lower concentrations of CART mRNA are also present in the ventral and medial premamillary nuclei and in the lateral aspect of the supramamillary nucleus.[9] The pituitary gland contains as high a concentration of CART mRNA as the hypothalamus,[3] expressed in 80% of the gonadotrophs but not in GH-, TSH-, ACTH-, or prolactin-producing cells.[19] In the telencephalon, CART mRNA is localized to the olfactory tubercle, nucleus accumbens, primary somatosensory, and piriform area of the cortex, bed nucleus of the stria terminalis, dentate gyrus, and amygdaloid complex.[9] The brainstem also contains CART-expressing

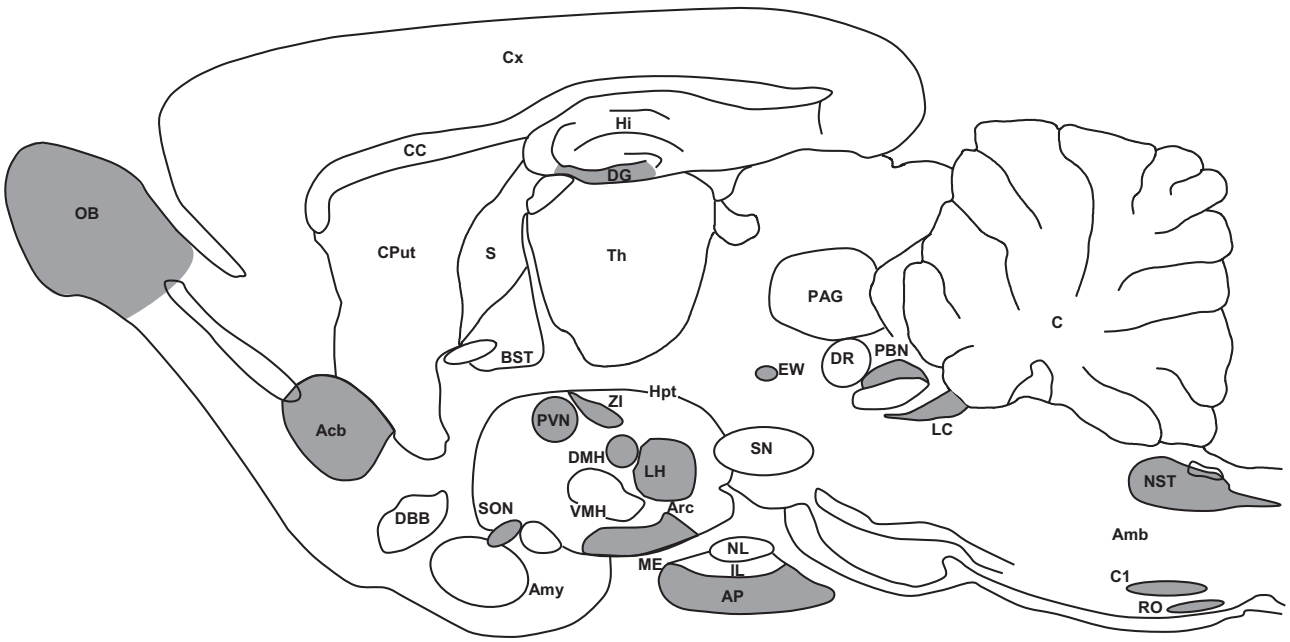

FIGURE 1 Schematic illustration showing the location of main populations of CART-synthesizing neurons.

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cell groups, including the Edinger–Westphal nucleus, locus ceruleus, parabrachial nucleus, nucleus of tractus solitarius, A1/C1 region, and raphe nuclei.[9]

The distribution of CART-immunoreactive (IR) perikarya shows a pattern similar to that observed for CART mRNA-containing cells. However, there are some discrepancies. Although in situ hybridization identifies numerous CART-expressing neurons in the hypothalamic dorsomedial (DMN) and ventral premamillary nuclei, only a few CART-IR cells can be detected in these regions.

The projection fields of CART neurons have been described in detail by Koylu et al.[16,17] Of particular importance is the observation that CART fibers densely innervate vegetative centers of the central nervous system, including the PVN, DMN, arcuate nucleus, nucleus tractus solitarius, C1-3 adrenergic cell groups, and the intermediolateral column of the spinal cord.[16,17] Furthermore, a dense CART-IR fiber network is found in the median eminence and in the posterior lobe of the pituitary arising from the CART expressing hypophysiotropic neurons and magnocellular neurosecretory neurons of the PVN and supraoptic nuclei, respectively.[17]

RECEPTORS AND SIGNALING CASCADES

Until now, little has been known about CART receptors and the intracellular signaling cascades mediating the effects of CART. Even though CART was discovered 16 years ago, CART receptors have not yet been identified and characterized. Tremendous effort was made to map CART binding sites in the brain, but without success. Saturable binding sites were detected, however, on AtT20 cells and on PC12 pheocromocytoma cells, and the membrane preparations of these cells. Furthermore, CART binding was observed in primary culture cells originating from the nucleus accumbens.

Despite the failure to identify even one receptor, it is conceivable that CART may act on more than one receptor: the two active CART peptide fragments show different relative activities in different testing paradigms, but the differences are not always in the same directions in the various tests.[31] In addition, CART signaling would seem to use multiple second messenger pathways. CART inhibits voltage-dependent intracellular $Ca(2^+)$ signaling in the hippocampus, probably through the inhibition of L-type voltage-gated $Ca(2^+)$ channel activity via a G protein-dependent pathway.[31] Sarkar et al.,[26] however, found that CART induces phosphorylation of cyclic-AMP response element binding protein (CREB) in corticotropin-releasing hormone (CRH)-synthesizing neurons of the PVN, suggesting that the cAMP–CREB signaling pathway may be involved in the mediation of CART effects. In addition, CART can rapidly induce ERK phosphorylation in AtT20 cells, mediated through pertussis toxin-sensitive G protein coupled receptors.[31]

BIOLOGICAL ACTIONS OF CART PEPTIDES WITHIN THE BRAIN AND PITUITARY

During the processing of proCART, several different CART fragments are produced. The two biologically active, naturally occurring CART peptides are rlCART 55–102 and 62–102. As the biological activity of N-terminal fragments remains controversial, we focus here only on the most studied biological effects of rlCART 55–102 and 62–102 including their role in the central regulation of reward, feeding, energy metabolism, neuroendocrine regulation, pain, cardiac function, and gut motility.[25] Each action will be briefly summarized below.

Reward

One of the first recognized actions of CART in the CNS is its involvement in reward processes, based on the regulation of CART gene expression in the striatum by cocaine and amphetamine and the presence of CART peptides in the mesolimbic reward circuit including the nucleus accumbens, lateral hypothalamic area, and ventral tegmental area (VTA).[25] In the striatum, CART is synthesized in neurons of the nucleus accumbens, where the majority of CART-expressing cells are GABA-ergic inhibitory neurons and synthesize substance P, the marker of striatal neurons projecting to the substancia nigra.[23] Amphetamine upregulates CART gene expression in the nucleus accumbens, and this is mediated via dopaminergic signaling. CART-containing neurons are innervated by dopaminergic terminals, and CART gene expression is upregulated by dopamine in these cells. Conversely, CART administration into the nucleus accumbens reduces the effect of amphetamines and dopamine on locomotor activity and inhibits amphetamine-induced behavioral sensitization. It is hypothesized, therefore, that CART has a homeostatic function in the nucleus accumbens by blunting the effects of the drug-induced increase in dopamine release.[25] Indeed, CART peptides reduce the rewarding effects of cocaine and inhibits the context induced reinstatement of reward seeking.[15,23]

In the VTA of rodents, CART peptides are present in axon terminals, but CART mRNA is not expressed in this brain region. CART administration into the VTA induces psychostimulant like effects including increase of locomotor activity and induction of conditional place preference.[23] CART innervation of the VTA primarily arises from lateral hypothalamic and perifornical areas, where CART-containing neurons are GABA-ergic and coexpress melanin-concentrating hormone (MCH), a peptide known to influence reward processes.[23] CART neurons in the lateral hypothalamus also project to the nucleus accumbens, but it is not known whether they contribute to reward processes. The strong anatomical interaction of lateral hypothalamic CART neurons with the nucleus

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accumbens and VTA and the well-known role of the lateral hypothalamus in the regulation of reward, however, raise the possibility that the lateral hypothalamic CART has an important role.[23] CART peptides in the amygdala and the prefrontal cortex have also been implicated in the regulation of reward processes.

Feeding and Energy Metabolism

The synthesis of CART peptides in specific neuronal groups that have been implicated in the central control of energy homeostasis has suggested an important role for CART in the regulation of feeding and energy metabolism. These neuronal groups include alpha-melanocyte–stimulating hormone (α-MSH)-producing neurons of the hypothalamic arcuate nucleus, MCH neurons of the lateral hypothalamus, neurons of the DMN and NTS and epinephrine-synthesizing neurons of the C1-3 regions of the brainstem.[12,32] Along these lines, an acute injection of CART into the CSF induces c-fos in the above-mentioned feeding-related regions, decreases feeding, and inhibits the orexinogenic effects of NPY, whereas chronic administration induces significant weight loss.[25] Conversely, the central administration of CART antiserum increases food intake.[25]

In anorexigenic neurons of the arcuate nucleus, the synthesis of CART is highly regulated by circulating levels of leptin.[25] Exogenous administration of leptin or elevated leptin levels induced by high fat diet-induced obesity increases CART mRNA in the arcuate nucleus.[25] Conversely, CART gene expression is decreased in the arcuate nucleus of leptin resistant, fa/fa Zucker rats and leptin deficient ob/ob mice. In the latter animal model, CART mRNA level can be normalized by leptin replacement.[25]

The actions of leptin on CART gene expression is believed to be exerted directly on CART/α-MSH-producing neurons of the arcuate nucleus, as these neurons express leptin receptors and show evidence of SOCS-3 expression following leptin administration.[25] In contrast to POMC gene expression, however, CART does not seem to be regulated by insulin, suggesting that the two anorexigenic peptides of the anorexigenic arcuate nucleus neurons (α-MSH and CART) may have highly different functions in the regulation of energy metabolism.[11]

CART-synthesizing arcuate nucleus neurons project directly to the PVN[25] and the intermediolateral column of the spinal cord,[25] thereby coordinating the effects of CART to simultaneously regulate food intake and energy metabolism.[25]

Hypothalamic CART populations also have connections with the nucleus accumbens shell. Focal injection of CART into the accumbens shell decreases food intake in *ad libitum* fed animals and attenuates the orexigenic effect of GABA administration into the accumbens shell, suggesting that CART may influence the rewarding effect of food. The accumbens shell receives CART innervation from the arcuate nucleus, the perifornical area, and from the accumbens shell, itself. As fasting downregulates CART mRNA level in all three regions, it is probable that hypothalamic CART-synthesizing neurons and local interneurons act together in the accumbens shell to inhibit food intake.[35]

CART also influences food intake through effects on brainstem feeding centers, as administration of CART into the fourth ventricle inhibits food intake.[25]

Although there is general agreement that icv. administration of CART has anorexigenic effects, some discrepancies have been reported. Wang et al.[33] observed inhibition of food intake when CART is injected directly into the PVN, whereas Abbott et al.[1] observed increased food intake after focal injection of CART into either the PVN or arcuate nucleus. In addition, in contrast to the rodent findings, CART is absent from anorexigenic α-MSH neurons and present in the orexigenic AGRP/NPY neurons in the human infundibular nucleus, the analog of the rodent arcuate nucleus.[21] Nevertheless, consistent with the role of CART as an anorexic peptide, CART deficient transgenic mice develop obesity when placed on a high caloric diet,[25] and polymorphisms of the 5' flanking region of the CART promoter are associated with obesity.

Neuroendocrine Regulation of Hypothalamic Hypophysiotropic Neurons

CART has a number of important actions on hypophysiotropic neurons of the hypothalamic–pituitary axes as indicated by the synaptic association of CART-containing axon terminals with CRH-, TRH-, and GnRH-producing hypothalamic neurons. The CART-IR innervation of TRH and CRH neurons originates primarily from two sources: adrenergic C1–3 regions of the medulla and the hypothalamic arcuate nucleus. The central administration of CART activates CRH neurons, and its stimulatory effect on ACTH and corticosterone secretion can be blocked by the CRH antagonist, astressin B.[25] CART also exerts stimulatory effects on hypophysiotropic TRH neurons in the PVN, increasing TRH content and secretion from hypothalamic cells[10] and preventing the fasting-induced decline in TRH gene expression in the PVN.[10] Although these actions may be involved in the mediation of the action of leptin via projections from CART/α-MSH neurons of the arcuate nucleus,[10] dense innervation of TRH neurons by CART/IR cells of the brainstem indicates that CART may also be involved the regulation of the hypophysiotropic TRH neurons by other physiological processes.

CART also seems to play a crucial role in mediating the effects of leptin on the central regulation of gonadal function.[22] LHRH pulse amplitude is similarly increased by both leptin and CART in female rats.[22] Further, immunoneutralization of CART prevents the leptin induced increase of LHRH pulse amplitude in hypothalamic explants.[22]

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In addition to regulation of hypophysiotropic neurons through afferent inputs, CART is also coexpressed in hypophysiotropic neurons that synthesize TRH, somatostatin, vasopressin, and oxytocin, presumably to regulate pituitary function. CART has a direct inhibitory effect on prolactin secretion *in vitro*,[19] and can completely block the stimulatory effects of TRH on prolactin secretion at even low concentrations (10^{-8} to 10^{-10} M).[24] CART has no effect, however, on either basal or stimulated TSH, LH, or FSH secretion. As noted previously, CART has been identified in gonadotrophs in the rat anterior pituitary gland,[19] suggesting the additional possibility that it may be involved in paracrine regulation of prolactin secretion.[19]

Pain

CART peptides 55–102 and 49–89, when administered icv or intrathecally, have been reported to produce significant antinociceptive effects as measured by hindpaw withdrawal latency in the hot-plate test and to enhance antinociceptive effects of morphine in the tail flick test.[25] CART has also been shown to reduce neurophatic hyperalgesia.[4] It is presumed that the major analgesic actions of CART are exerted in the spinal cord, consistent with the presence of CART in peptidergic, nociceptive, primary afferents in the superficial laminae of the dorsal horn of spinal cord.[18]

Vegetative Functions

Intracerebroventricular and intracisternal administration of CART increases mean arterial pressure and heart rate in rats and rabbits,[25] and CART is a potent vasoconstrictor in the cerebral circulation.[25] Because a relatively high dose of CART is necessary to influence cardiovascular function when administered intrathecally,[25] the brain may be the primary site of the action for these physiologic effects of CART. At least two different regions of the brainstem have been identified as mediating these responses, including the rostral ventrolateral medulla, a critical pressor area where intracisternal CART administration induces c-fos expression, and the nucleus tractus solitarius, where local administration of CART attenuates phenylephrine-induced bradycardia.[27] However, CART, alone, is relatively ineffective in influencing the cardiovascular system if administered intrathecally, but even low doses of CART are sufficient to potentiate the stimulatory effects of intrathecally administered glutamate on cardiovascular function.[28] It is presumed that this effect is exerted through the intermediolateral column of the spinal cord,[28] which is heavily innervated by CART-synthesizing vasomotor neurons of the rostral ventrolateral medulla.

CART also acts in the CNS to inhibit gastric acid secretion and gastric motility, and stimulate colonic motility.[25] It is presumed that these actions are mediated in the brain by CRH-synthesizing neurons, as the effects of CART on gastrointestinal function can be inhibited with CRH antagonists.[25]

Neuroprotection (also see Neurotrophic Peptides section of this book)

CART expression is induced in the cerebral cortex after focal cerebral ischemia in the rat *in vivo* and after oxygen-glucose deprivation (OGD) in cultured cortical neurons.[20] This increase is further enhanced by estradiol administration.[20] Because immunoneutralization of CART attenuates estradiol-induced neuroprotection, and administration of CART peptide *in vivo* reduces infarct size after middle cerebral artery occlusion,[20] it is suggested that CART plays important role in neuroprotection. The neuroprotective mechanism of action of CART may be linked to the preservation of mitochondrial function and prevention of energy failure after ischemia–reperfusion injury.[20]

PATHOPHYSIOLOGICAL IMPLICATIONS

To date, little is known about the role of CART in pathological conditions, but an association has been suggested by genetic linkage studies in the development of obesity, alteration in lipid profile, bone remodeling, and alcoholism. Polymorphism in the 5′ flanking region of the CART gene (−156) is significantly associated with an increased BMI,[34] and a missense mutation at codon 34 in the N-terminal region cosegregates with a severe obese phenotype.[5] Subjects bearing the −3608 C allele have a plasma lipid profile protective against atherogenesis[30] and increased bone mineral density in postmenopausal woman.[13] Jung et al.[14] also found that an AvaII polymorphism in intron I predisposes to alcoholism but not with depression or schizophrenia.[14]

REFERENCES

1. Abbott CR, Rossi M, Wren AM, Murphy KG, Kennedy AR, Stanley SA, et al. Evidence of an orexigenic role for cocaine- and amphetamine-regulated transcript after administration into discrete hypothalamic nuclei. *Endocrinology* 2001;**142**:3457–63.
2. Cawley NX, Yanik T, Woronowicz A, Chang W, Marini JC, Loh YP. Obese carboxypeptidase E knockout mice exhibit multiple defects in peptide hormone processing contributing to low bone mineral density. *Am J Physiol Endocrinol Metab* 2010;**299**:E189–97.
3. Couceyro PR, Koylu EO, Kuhar MJ. Further studies on the anatomical distribution of CART by in situ hybridization. *J Chem Neuroanat* 1997;**12**:229–41.
4. Damaj MI, Zheng J, Martin BR, Kuhar MJ. Intrathecal CART (55-102) attenuates hyperlagesia and allodynia in a mouse model of neuropathic but not inflammatory pain. *Peptides* 2006;**27**:2019–23.
5. del Giudice EM, Santoro N, Cirillo G, D'Urso L, Di Toro R, Perrone L. Mutational screening of the CART gene in obese children: identifying a mutation (Leu34Phe) associated with reduced resting energy expenditure and cosegregating with obesity phenotype in a large family. *Diabetes* 2001;**50**:2157–60.

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6. Dey A, Xhu X, Carroll R, Turck CW, Stein J, Steiner DF. Biological processing of the cocaine and amphetamine-regulated transcript precursors by prohormone convertases, PC2 and PC1/3. *J Biol Chem* 2003;**278**:15007–14.
7. Dominguez G. The CART gene: structure and regulation. *Peptides* 2006;**27**:1913–8.
8. Douglass J, Daoud S. Characterization of the human cDNA and genomic DNA encoding CART: a cocaine- and amphetamine-regulated transcript. *Gene* 1996;**169**:241–5.
9. Douglass J, McKinzie AA, Couceyro P. PCR differential display identifies a rat brain mRNA that is transcriptionally regulated by cocaine and amphetamine. *J Neurosci* 1995;**15**:2471–81.
10. Fekete C, Mihaly E, Luo LG, Kelly J, Clausen JT, Mao Q, et al. Association of cocaine- and amphetamine-regulated transcript-immunoreactive elements with thyrotropin-releasing hormone-synthesizing neurons in the hypothalamic paraventricular nucleus and its role in the regulation of the hypothalamic–pituitary–thyroid axis during fasting. *J Neurosci* 2000;**20**:9224–34.
11. Fekete C, Singru PS, Sanchez E, Sarkar S, Christoffolete MA, Riberio RS, et al. Differential effects of central leptin, insulin, or glucose administration during fasting on the hypothalamic–pituitary–thyroid axis and feeding-related neurons in the arcuate nucleus. *Endocrinology* 2006;**147**:520–9.
12. Fekete C, Wittmann G, Liposits Z, Lechan RM. Origin of cocaine- and amphetamine-regulated transcript (CART)-immunoreactive innervation of the hypothalamic paraventricular nucleus. *J Comp Neurol* 2004;**469**:340–50.
13. Guerardel A, Tanko LB, Boutin P, Christiansen C, Froguel P. Obesity susceptibility CART gene polymorphism contributes to bone remodeling in postmenopausal women. *Osteoporos Int* 2006;**17**:156–7.
14. Jung SK, Hong MS, Suh GJ, Jin SY, Lee HJ, Kim BS, et al. Association between polymorphism in intron 1 of cocaine- and amphetamine-regulated transcript gene with alcoholism, but not with bipolar disorder and schizophrenia in Korean population. *Neurosci Lett* 2004;**365**:54–7.
15. King BJ, Furlong TM, McNally GP. Cocaine and amphetamine related transcript (CART) inhibits context induced reinstatement of reward seeking. *Behav Neurosci* 2010;**124**:423–7.
16. Koylu EO, Couceyro PR, Lambert PD, Kuhar MJ. Cocaine- and amphetamine-regulated transcript peptide immunohistochemical localization in the rat brain. *J Comp Neurol* 1998;**391**:115–32.
17. Koylu EO, Couceyro PR, Lambert PD, Ling NC, DeSouza EB, Kuhar MJ. Immunohistochemical localization of novel CART peptides in rat hypothalamus, pituitary and adrenal gland. *J Neuroendocrinol* 1997;**9**:823–33.
18. Kozsurek M, Lukacsi E, Fekete C, Wittmann G, Rethelyi M, Puskar Z. Cocaine- and amphetamine-regulated transcript peptide (CART) is present in peptidergic C primary afferents and axons of excitatory interneurons with a possible role in nociception in the superficial laminae of the rat spinal cord. *Eur J Neurosci* 2007;**26**:1624–31.
19. Kuriyama G, Takekoshi S, Tojo K, Nakai Y, Kuhar MJ, Osamura RY. Cocaine- and amphetamine-regulated transcript peptide in the rat anterior pituitary gland is localized in gonadotrophs and suppresses prolactin secretion. *Endocrinology* 2004;**145**:2542–50.
20. Mao P, Ardeshiri A, Jacks R, Yang S, Hurn PD, Alkayed NJ. Mitochondrial mechanism of neuroprotection by CART. *Eur J Neurosci* 2007;**26**:624–32.
21. Menyhert J, Wittmann G, Lechan RM, Keller E, Liposits Z, Fekete C. Cocaine- and amphetamine-regulated transcript (CART) is colocalized with the orexigenic neuropeptide Y and agouti-related protein and absent from the anorexigenic alpha-melanocyte-stimulating hormone neurons in the infundibular nucleus of the human hypothalamus. *Endocrinology* 2007;**148**:4276–81.
22. Parent AS, Lebrethon MC, Gerard A, Vandersmissen E, Bourguignon JP. Leptin effects on pulsatile gonadotropin releasing hormone secretion from the adult rat hypothalamus and interaction with cocaine and amphetamine regulated transcript peptide and neuropeptide Y. *Regul Pept* 2000;**92**:17–24.
23. Philpot K, Smith Y. CART peptide and the mesolimbic dopamine system. *Peptides* 2006;**27**:1987–92.
24. Raptis S, Fekete C, Sarkar S, Rand WM, Emerson CH, Nagy GM, et al. Cocaine- and amphetamine-regulated transcript co-contained in thyrotropin-releasing hormone (TRH) neurons of the hypothalamic paraventricular nucleus modulates TRH-induced prolactin secretion. *Endocrinology* 2004;**145**:1695–9.
25. Rogge G, Jones D, Hubert GW, Lin Y, Kuhar MJ. CART peptides: regulators of body weight, reward and other functions. *Nat Rev Neurosci* 2008;**9**:747–58.
26. Sarkar S, Wittmann G, Fekete C, Lechan RM. Central administration of cocaine- and amphetamine-regulated transcript increases phosphorylation of cAMP response element binding protein in corticotropin-releasing hormone-producing neurons but not in prothyrotropin-releasing hormone-producing neurons in the hypothalamic paraventricular nucleus. *Brain Res* 2004;**999**:181–92.
27. Scruggs P, Dun SL, Dun NJ. Cocaine- and amphetamine-regulated transcript peptide attenuates phenylephrine-induced bradycardia in anesthetized rats. *Am J Physiol Regul Integr Comp Physiol* 2003;**285**:R1496–503.
28. Scruggs P, Lai CC, Scruggs JE, Dun NJ. Cocaine- and amphetamine-regulated transcript peptide potentiates spinal glutamatergic sympathoexcitation in anesthetized rats. *Regul Pept* 2005;**127**:79–85.
29. Stein J, Steiner DF, Dey A. Processing of cocaine- and amphetamine-regulated transcript (CART) precursor proteins by prohormone convertases (PCs) and its implications. *Peptides* 2006;**27**:1919–25.
30. Vasseur F, Guerardel A, Barat-Houari M, Cottel D, Amouyel P, Froguel P, et al. Impact of a CART promoter genetic variation on plasma lipid profile in a general population. *Mol Genet Metab* 2007;**90**:199–204.
31. Vicentic A, Lakatos A, Jones D. The CART receptors: background and recent advances. *Peptides* 2006;**27**:1934–7.
32. Vrang N. Anatomy of hypothalamic CART neurons. *Peptides* 2006;**27**:1970–80.
33. Wang C, Billington CJ, Levine AS, Kotz CM. Effect of CART in the hypothalamic paraventricular nucleus on feeding and uncoupling protein gene expression. *Neuroreport* 2000;**11**:3251–5.
34. Yamada K, Yuan X, Otabe S, Koyanagi A, Koyama W, Makita Z. Sequencing of the putative promoter region of the cocaine- and amphetamine-regulated-transcript gene and identification of polymorphic sites associated with obesity. *Int J Obes Relat Metab Disord* 2002;**26**:132–6.
35. Yang SC, Shieh KR, Li HY. Cocaine- and amphetamine-regulated transcript in the nucleus accumbens participates in the regulation of feeding behavior in rats. *Neuroscience* 2005;**133**:841–51.

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Chapter 99

CCK

Margery C. Beinfeld

ABSTRACT

CCK is one of the most abundant and widely expressed peptides in the brain. The major form found in the brain is CCK 8 amide. It is present in both local and long projecting neurons. It is colocalized with several classical neurotransmitters. Both the CCK receptors are found in the brain but have different distributions. CCK functions as an excitatory neurotransmitter and modulates the release and function of other neurotransmitters. The precise physiological role of brain CCK has not been established, but it seems to be involved in satiety, anxiety, analgesia, learning, memory, and dopamine-mediated behaviors.

The discovery of the substance that is now called cholecystokinin (CCK) dates to the beginning of the 20th century. Bayliss and Starling showed that intestinal acidification caused gall bladder contraction. They correctly surmised that this treatment was causing something to be released into circulation from the intestine, which caused the gall bladder to contract. The release of CCK and other peptides after this treatment was shown later, and administration of CCK was found to cause gall bladder contraction. Porcine intestinal CCK 33 was isolated and sequenced by Mutt and Jorpes in the 1960s.[20] The name CCK/gastrin arose in 1975 with the detection of CCK-like immunoreactivity in the brain with a gastrin antiserum that crossreacted with CCK.[27] Based on its biological activity, chromatographic profile, and amino acid sequencing, it soon became clear that this material was not gastrin but CCK 8.[9] Small amounts of gastrin are found in the brain and pituitary of several species.

CCK and gastrin share the same five carboxyl-terminal amino acids, and gastrin is believed to have evolved from CCK by gene duplication. CCK has a long evolutionary history. Caerulein, a frog skin peptide that is related to CCK and gastrin, is described in the Amphibian/Skin Peptides section of the book.

The final processed form of both CCK and gastrin has a sulfated tyrosine residue. CCK is completely sulfated, whereas the degree of sulfation of gastrin varies in a tissue-specific manner. The functional and evolutionary relationships between CCK and gastrin are described in more detail in the Gastrointestinal Peptides section of the book.

STRUCTURE OF THE PRECURSOR mRNA/GENE

The CCK cDNA was first cloned using mRNA from a rat thyroid medullary carcinoma, which expressed very high levels of CCK. The cDNAs from many species were subsequently cloned. The transcriptional unit of CCK spans 7 kb and is interrupted by two introns. The promoter is found within 144 bp of the transcription start site.[8] The complex regulation of CCK expression has been studied in detail, reviewed in Ref. 13. The CCK promoter contains cyclic AMP and estrogen response elements. It also contains an E-box element. CCK expression is stimulated synergistically by cAMP and fibroblast growth factor.

DISTRIBUTION OF CCK

CCK is one of the most abundant and widely distributed peptides in the brain. There are numerous CCK mRNA-positive cell bodies in the olfactory bulb (OB), cerebral cortex (Cx), hypothalamus (Hpt), thalamus (Th), hippocampus (Hi), amygdala (Amy), various nuclei in the mesencephalon, ventral tegmental area (VTA), and substantia nigra (SN).[4,23] The distribution of CCK cell bodies and their projections is shown in Fig. 1. One fascinating aspect of the anatomy of CCK in the Cx and Hi is the overlap between neurons, which express CCK, GABA, and cannabinoid CB1 receptors.[11]

PROCESSING OF THE PRECURSOR (SEE PEPTIDE BIOSYNTHESIS/PROCESSING SECTION OF THIS BOOK)

The processing of proCCK occurs mainly at single Arg or Lys residues (Fig. 2). The sequence and structure of proCCK and gastrin are similar, and they both display tissue-specific processing. CCK 8 is the major form in brain, whereas the intestine has larger forms. CCK and gastrin in the gut are discussed in the Ingestive and Gastrointestinal Peptides section of this book.

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00099-3
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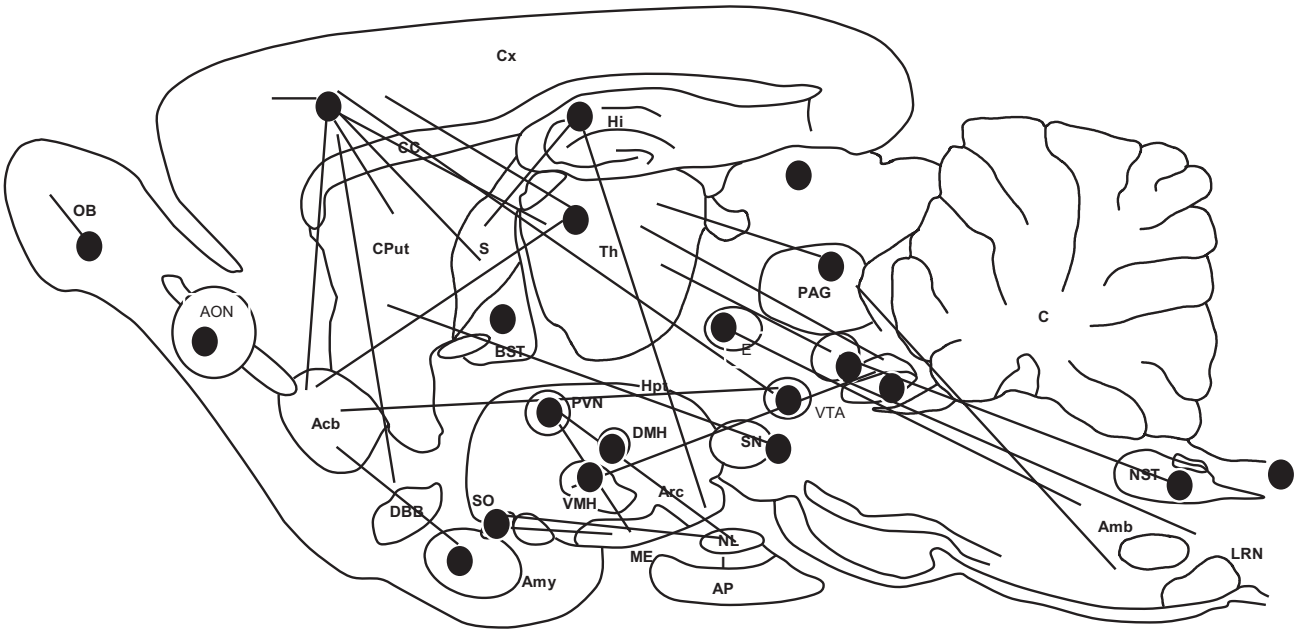

FIGURE 1 Distribution of CCK neurons and their major projections in the rat brain. CCK cell bodies are indicated by dots and their projections by solid lines.

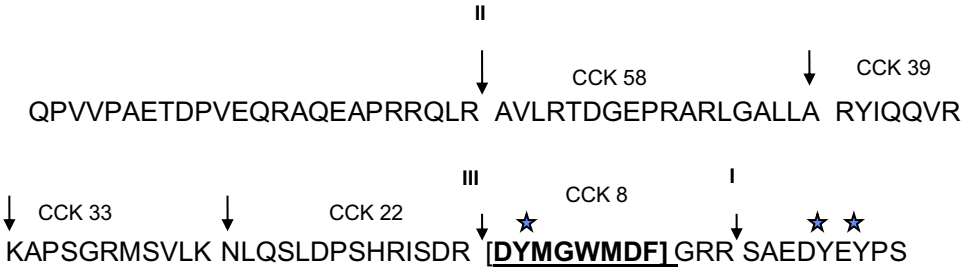

FIGURE 2 Sequence of mouse proCCK written in the single amino acid code. The endoproteolytic cleavage sites are indicated by spaces and downward arrows, the CCK 8 sequence is underlined. CCK peptides are numbered backward from the carboxyl-terminal of CCK 8. The amino terminal end of the major forms of amidated CCK peptides is indicated by the downward arrows. Stars mark the location of the sulfated tyrosine residues. The temporal order of endoproteolytic cleavages for the production of CCK 8 is indicated by the roman numerals.

A strict temporal order of cleavages has been shown for a number of prohormones, including CCK. It is believed that the first site where cleavage occurs is the most accessible. After cleavage of the first site, the remaining prohormone changes structure, making additional sites available for cleavage in a specific order.

The order of proCCK processing to CCK 22 and CCK 8 is indicated in Fig. 2. It is based on products identified in normal and *fat/fat* mouse brain and on site-directed mutagenesis studies in endocrine tumor cells.[28]

Signal Peptide Cleavage

The signal sequence of preproCCK allows it to be inserted into the lumen of the endoplasmic reticulum (ER). The signal sequence is removed by the signalase enzyme located on the luminal surface of the ER membrane.

Tyrosine Sulfation

Three out of four tyrosine residues in proCCK are sulfated by protein tyrosine sulfotransferase in the trans-Golgi network (TGN). The tyrosines that are sulfated have adjacent acidic residues that constitute the consensus sequence for sulfation.

Sorting into Secretory Granules

In the TGN, sulfated proCCK is sorted into secretory granules with the enzymes responsible for its processing.

Cleavage 1 on the Carboxyl-terminal of CCK 8 Gly Arg Arg (GRR)

Mutation of the GRR site on the carboxyl-terminal of CCK 8 to Gly Ala Ala blocks the production of amidated CCK.[28]

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Cleavage 2 at RA (Arg Ala) at CCK 58 (Removal of the Propeptide)

Mutation of the RA cleavage site to Ala Ala completely prevents subsequent production of amidated CCK 22 or CCK 8. CCK 58 GRR does not accumulate in the brain or in cell lines because it is efficiently cleaved to smaller forms.[28]

Cleavage 3 at CCK 22 Lys Asn and Arg Asp CCK 8 to Produce CCK 22 GRR and CCK 8 GRR

Removal of these cleavage sites by mutation prevents their cleavage, but it otherwise has a minor impact on any other aspect of the processing.[28]

Removal of Carboxyl-terminal Arginine Residues by Carboxypeptidase E (CPE)

Conversion of CCK 22 G and CCK 8 G to CCK 22 Amide and CCK 8 Amide by the Amidation Enzyme

The last step in CCK biosynthesis is the amidation of glycine-extended CCK peptides by peptidyl glycine-α-amidating monooxygenase.

Enzymology of ProCCK Processing (see Peptide Biosynthesis/Processing section of this book)

Tyrosyl Protein Sulfotranferase

Two Golgi enzymes (TSP1 and TSP2) have been identified that are believed to be responsible for the sulfation of the three tyrosine residues in proCCK.

Endoproteases

The most probable candidates enzyme(s) responsible for the endoproteolytic cleavages in CCK during its processing are the prohormone convertases (PCs),[25] most notably PC1, PC2, PC5, and PC7. Evidence has *also* been obtained that cathepsin L may *also* be involved in CCK processing.

They are widely colocalized with CCK in the brain[5,6] and present in cell lines that express CCK mRNA. A number of studies have shown that they have the correct catalytic activity to cleave proCCK. Antisense and RNAi studies in cell lines also support a role for these enzymes in CCK processing.

PC1,2, PC7, and cathepsin L knockout mice have decreased levels of CCK in specific brain regions, although considerable CCK is still detected. This is strong evidence that they are involved in CCK processing. In no situation was CCK completely eliminated, indicating that there are multiple enzymes that can compensate for the loss of a single enzyme and continue to provide some CCK.

Carboxypeptidases

In adult *fat/fat* mice that lack carboxypeptidase E (CPE), the levels of amidated CCK in brain are decreased by 85% showing that this enzyme is required for CCK processing. The fact that some CCK remains indicates that other carboxypeptidases are acting in the absence of CPE. There is also a large accumulation of glycine and arginine extended CCK 8, the immediate precursor of CCK 8. This also shows that CCK 33 or CCK 22 amide cannot be the immediate precursor of CCK 8 amide in brain.

Amidating Enzyme

Although C-terminal amidation is a widespread modification, glycine-extended CCK is abundant in rodent brain, intestine and CCK-expressing endocrine cells. This enzyme is dependent upon copper and ascorbate and at least in some cell lines, the quantity of glycine-extended CCK is decreased by adding ascorbate.[2] It is unclear whether this glycine-extended CCK has a function or is simply the precursor of amidated CCK.

Receptors and Signaling Cascades

Two CCK receptors have been cloned. The CCK 1 subtype is relatively specific for sulfated CCK 8, while unsulfated CCK 8, CCK 4, and gastrin are 2–3 orders of magnitude less potent. For the CCK 2 subtype (identical to the gastrin receptor), this difference in potency is about one order of magnitude. The CCK 2 receptor is more abundant and widely distributed in the brain than the CCK 1 receptor, although distinct populations of the CCK 1 receptor are present. The CCK 1 receptor is present on soma and fibers in the SN, VTA, nucleus accumbens (Acb), and dorsal striatum but not in the prefrontal Cx, Amy, or Hi. The CCK 2 receptor is present on soma and fibers in the SN, VTA, Cx, Abc, dorsal striatum, Hi, and Amy.

What happens after CCK activates its receptors has been extensively studied. The model that has been studied in the most detail is the rodent pancreatic acinar cell, which expresses mainly CCK 1 receptors. The AR42 J pancreatic cell line and adrenal chromaffin cells, which express the CCK 2 receptor have also been studied.[32]

In pancreatic acinar cells, CCK receptor activation recruits the heterotrimeric G proteins G_q and G_{11} and results in increased levels of inositol triphosphate, increased intracellular calcium and activation of protein kinase C. At higher concentrations, CCK can activate adenylyl cyclase, increasing cAMP levels. CCK also activates three mitogen-activated protein kinase cascades leading to ERK, JNK and p38 MAPK. CCK also activates the NF-κB pathway and the P13 K-PKB-m-TOR pathway, which regulates protein synthesis.

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Activation of the CCK 2 receptor in gastric cancer cells activates protein kinase D2,[32] and in human adrenal chromaffin cells, it increases adenylyl cyclase and cAMP.[26]

Active and/or Solution Conformation

Biophysical measurements of sulfated and nonsulfated CCK 8 show that these peptides are found mainly in folded forms with beta and gamma turns around the sequence Gly-Trp-Met-Asp and Met-Asp-Phe-NH_2, respectively.[10] NMR spectroscopy and molecular modeling methods have shown that CCK 15 assumes a helical conformation.[1]

Biological Actions within the Brain

The actions of CCK in the brain are diverse and have been widely studied. Progress has been aided by the development of specific agonists and antagonists and the discovery or creation of rodent models that lack the receptors or the peptide.

Satiety

CCK released from the intestine by ingestion of food was shown to have a major influence on food intake even before CCK was detected in the brain.[12] Central CCK seems to have a distinct effect on food intake in the hypothalamus, which may be mediated in part by its interactions with NPY and CART (cocaine- and amphetamine-regulated transcript peptide). The role of CCK in satiety is discussed in the Ingestive and Gastrointestinal Peptides section of this book.

Analgesia

CCK acting through the CCK 2 receptor has antiopiate activity (see Opioid Peptide section of this book). Opiate analgesia is mainly mediated by neurons in the rostral ventromedial medulla (RVM). CCK exerts its inhibitory effect on morphine analgesia by inhibiting activation of pain inhibiting output neurons of the RVM.[14] Acute administration of CCK into the RVM causes acute tactile and thermal hypersensitivity that is antagonized by the CCK 2 receptor antagonist or lesion of the dorsolateral funiculus. Continuous administration of morphine produces sustained tactile and thermal hypersensitivity that is reversed by the CCK 2 antagonist. Continuous morphine treatment results in a fivefold increase in basal CCK levels in the RVM relative to controls.[34] Activation of the endogenous CCK system by repeated morphine treatment may be partially responsible for the observed hypersensitivity. The opiate antagonist effect of CCK appears to be particularly evident in neuropathic pain where increased CCK release as well as changes in expression of CCK receptors has been reported.[31]

CCK Modulation of Dopamine Neurotransmission, Dopamine-Mediated Reward and Psychostimulant Sensitization

CCK is colocalized with dopamine in the VTA,[15] and these neurons project to medial and posterior subdivisions (shell) of the Acb, the olfactory tubercle, and prefrontal Cx.[35] CCK 1 receptors in the Acb shell display DA agonist-like activities (increased dopamine release and enhancement of dopamine-mediated behaviors), whereas in the Acb (core), CCK 2 receptors display DA antagonist-like activities (decreased dopamine release and inhibition of dopamine-mediated behaviors). CCK infusion into Acb shell increases dopamine release while core infusion decreases dopamine release.

Treatment of rats with psychostimulants and stress increases CCK release and augments their locomotor behavior (sensitization). The CCK 1 antagonist blocks expression of psychostimulant sensitization while the CCK 2 antagonist blocks acquisition of this behavior. OLETF rats lacking the CCK 1 receptor display much less behavioral sensitization to repeated cocaine than wild type LETO rats.[3]

CCK 2 receptor knockout mice behave as if they have augmented dopamine neurotransmission. They display more locomotor activity than the wildtype[7] and more locomotor activity in response to a single high dose of amphetamine.[17] This implies that the CCK 1 receptor is required for full expression of cocaine sensitization while the CCK 2 receptor is tonically inhibiting dopamine release.

Anxiety, Learning, Memory, and Sexual Behavior

CCK is very abundant in the Cx, Amy, and Hi, so it is not surprising that it plays a role in these behaviors through modulation of the expression or release of other peptides and classical neurotransmitters. CCK is known to modulate the release of glutamate and GABA. CCK increases the release of glutamate in the hippocampus by increasing the release probability and the number of readily releasable vesicles.

CCK-positive basket cells in the hippocampus express the CB1 cannabinoid receptor on their axon terminals and CB1 antagonists are able to block the CCK-induced suppression of GABA release. Similar results have been seen in other brain areas, including the midbrain periaqueductal gray.

CCK acting through the CCK 2 receptor in the Hi is involved in spatial memory.[24] Other memory tasks that CCK facilitates involve a dopaminergic projection from the VTA to the Nac, central Amy, and Hi.[33] CCK improves spatial memory in elderly rats[29] and in rats whose dopamine has been depleted by 6 hydroxy-dopamine.[22] CCK acting through the CCK 1 receptor is involved in maternal

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behavior and in infant–maternal recognition in sheep[21] and in appetitive learning in infant rats.[30] CCK released by a meal activates peripheral CCK 1 receptors to initiate these behaviors. CCK interacting with estrogen and endogenous opiates in the Hpt is involved in regulating sexual behavior in female rodents.[19]

A large amount of literature describes the ability of CCK to trigger panic attacks in human subjects and to produce anxiety-related behaviors in rodents and primates. This is mediated through the CCK 2 receptor. The CCK 2 receptor seems to act synergistically with CRF 1 receptor in producing anxiety-like behavior induced by immobilization stress.

Pathophysiological Implications

There is currently no CCK-based drug in clinical practice. CCK and its receptors are widely distributed and have many different physiological functions in the brain and the periphery. Selectively targeting CCK receptors in a single organ will not be easy.

Satiety

Although CCK does cause short-term inhibition of food intake, this activity has not been used therapeutically. Interestingly, the CB1 antagonist Rimonabant (Acomplia) was in clinical trials as a satiety agent but was withdrawn because it caused anxiety. Perhaps that is related to its effect on the CCK system.

Analgesia

CCK 2 antagonists have been tested in humans where they improve pain management when given with morphine.[18] It is possible that CCK 2 antagonist treatment might be promising particularly in neuropathic pain to increase morphine efficacy and reduce tolerance. Novel ligands incorporating an opiate peptide with a CCK 2 antagonist have been designed, which might be useful in the treatment of pain.[16]

Anxiety

CCK 2 antagonists have not been shown to be beneficial in the treatment of anxiety, but questions remain about whether these antagonists get into the brain.

REFERENCES

1. Albrizio S, Carotenuto A, Fattorusso C, Moroder L, Picone D, Temussi PA, et al. Environmental mimic of receptor interaction: conformational analysis of CCK-15 in solution. *J Med Chem* 2002;**45**:762–9.
2. Beinfeld MC, Perloff MD, Venkatakrishnan K. Identification of glycine-extended CCK peptides in endocrine cells and modulation of CCK amide and CCK Gly content and secretion from endocrine tumor cells by an inhibitor of amidation. *Peptides* 1998;**19**:1393–8.
3. Beinfeld MC, Connolly K, Pierce RC. OLETF (Otsuka Long-Evans Tokushima Fatty) rats that lack the CCK 1 (A) receptor develop less behavioral sensitization to repeated cocaine treatment than wild type LETO (Long Evans Tokushima Otsuka) rats. *Peptides* 2001;**22**:1285–90.
4. Burgunder J-M, Young I WS. Cortical neurons expressing the cholecystokinin gene in the rat: distribution in the adult brain, ontogeny, and some of their projections. *J Comp Neurol* 1990;**300**:26–46.
5. Cain B, Connolly K, Blum A, Vishnuvardhan D, Marchand JE, Beinfeld MC. The distribution and co-localization of cholecystokinin with the prohormone convertase enzymes PC1, PC2 and PC5 in rat brain. *J Comp Neurol* 2003;**467**:307–25.
6. Cain BM, Connolly K, Blum AC, Vishnuvardhan D, Marchand JE, Zhu X, et al. Genetic inactivation of prohormone convertase (PC1) causes a reduction in cholecystokinin (CCK) levels in the hippocampus, amygdala, pons and medulla in mouse brain that correlates with the degree of colocalization of PC1 and CCK mRNA in these structures in rat brain. *J Neurochem* 2004;**89**:307–13.
7. Dauge V, Sebret A, Beslot F, Matsui T, Roques BP. Behavioral profile of CCK2 receptor-deficient mice. *Neuropsychopharmacology* 2001;**25**:690–8.
8. Deschenes RJ, Haun RS, Funckes CL, Dixon JE. A gene encoding rat cholecystokinin. *J Biol Chem* 1985;**260**:1280–6.
9. Dockray GJ, Gregory RA, Hutchinson JB. Isolation, structure, and biological activity of two cholecystokinin octapeptides from sheep brain. *Nature* 1978;**264**:568–70.
10. Fournie-Zaluski MC, Belleney J, Lux B, Durieux C, Gerard D, Gacel G, et al. Conformational analysis of cholecystokinin CCK26-33 and related fragments by 1H NMR spectroscopy, fluorescence-transfer measurements, and calculations. *Biochemistry* 1986;**25**:3778–87.
11. Freund TF. Interneuron diversity series: rhythm and mood in perisomatic inhibition. *Trends Neurosci* 2003;**26**:489–95.
12. Gibbs J, Young SN, Smith GP. Cholecystokinin elicits satiety in rats with open gastric fistulas. *Nature* 1973;**245**:323–5.
13. Hansen TO. Cholecystokinin gene transcription: promoter elements, transcription factors and signaling pathways. *Peptides* 2001;**22**:1201–11.
14. Heinricher MM, McGaraughty S, Tortorici V. Circuitry underlying antiopioid actions of cholecystokinin within the rostral ventromedial medulla. *J Neurophysiol* 2001;**85**:280–6.
15. Hokfelt T, Skirboll L, Rehfeld JF, Goldstein M, Markey K, Dann O. A subpopulation of mesencephalic dopamine neurons projecting to limbic areas contain a cholecystokinin-like peptide:evidence from immunocytochemistry combined with retrograde tracing. *Neurosci* 1980;**5**:2093–124.
16. Hruby VJ, Agnes RS, Davis P, Ma SW, Lee YS, Vanderah TW, et al. Design of novel peptide ligands which have opioid agonist activity and CCK antagonist activity for the treatment of pain. *Life Sci* 2003;**73**:699–704.
17. Koks S, Volke V, Veraksits A, Runkorg K, Sillat T, Abramov U, et al. Cholecystokinin2 receptor-deficient mice display altered function of brain dopaminergic system. *Psychopharmacology (Berl)* 2001;**158**:198–204.
18. McCleane G. Cholecystokinin antagonists a new way to improve the analgesia from old analgesics? *Curr Pharm Des* 2004;**10**:303–14.
19. Micevych P, Sinchak K. Estrogen and endogenous opioids regulate CCK in reproductive circuits. *Peptides* 2001;**22**:1235–44.

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20. Mutt V. Historical perspectives on cholecystokinin research. *Ann NY Acad Sci* 1994;**713**:1–0.
21. Nowak R, Breton G, Mellot E. CCK and development of mother preference in sheep: a neonatal time course study. *Peptides* 2001;**22**:1309–16.
22. Rex A, Fink H. Cholecystokinin tetrapeptide improves water maze performance of neonatally 6-hydroxydopamine-lesioned young rats. *Pharmacol Biochem Behav* 2004;**79**:109–17.
23. Schiffmann SN, Vanderhaeghen J-J. Distribution of cells containing mRNA encoding cholecystokinin in the rat central nervous system. *J Comp Neurol* 1991;**304**:219–33.
24. Sebret A, Lena I, Crete D, Matsui T, Roques BP, Dauge V. Rat hippocampal neurons are critically involved in physiological improvement of memory processes induced by cholecystokinin-B receptor stimulation. *J Neurosci* 1999;**19**:7230–7.
25. Seidah NG, Day R, Marcinkiewicz M, Chrétien M. Precursor convertases: an evolutionary ancient, cell-specific, combinatorial mechanism yielding diverse bioactive peptides and proteins. *Ann NY Acad Sci* 1998;**839**:9–24.
26. Sturany S, Van Lint J, Gilchrist A, Vandenheede JR, Adler G, Seufferlein T. Mechanism of activation of protein kinase D2(PKD2) by the CCK(B)/gastrin receptor. *J Biol Chem* 2002;**277**:29431–6.
27. Vanderhaeghen JJ, Signeau JC, Gepts LO. New peptide in the vertebrate CNS reacting with gastrin antibodies. *Nature* 1975;**257**:604–5.
28. Vishnuvardhan D, Beinfeld MC. Biosynthesis and post-translational processing of site-directed endoproteolytic cleavage mutants of Pro-CCK in AtT-20 cells. *Biochemistry* 2002;**41**:570–8.
29. Voits M, Hasenohrl RU, Huston JP, Fink H. Repeated treatment with cholecystokinin octapeptide improves maze performance in aged Fischer 344 rats. *Peptides* 2001;**22**:1325–30.
30. Weller A, Tsitolovskya L, Gispan IH, Rabinovitz S. Examining the role of cholecystokinin in appetitive learning in the infant rat. *Peptides* 2001;**22**:1317–23.
31. Wiesenfeld-Hallin Z, Xu XJ, Hokfelt T. The role of spinal cholecystokinin in chronic pain states. *Pharmacol Toxicol* 2002;**91**: 398–403.
32. Williams JA, Sans MD, Tashiro M, Schafer C, Bragado MJ, Dabrowski A. Cholecystokinin activates a variety of intracellular signal transduction mechanisms in rodent pancreatic acinar cells. *Pharmacol Toxicol* 2002;**91**:297–303.
33. Winnicka MM, Wisniewski K. Dopaminergic projection to the central amygdala mediates the facilitatory effect of CCK-8US and caerulein on memory in rats. *Pharmacol Res* 1999;**39**:445–50.
34. Xie JY, Herman DS, Stiller CO, Gardell LR, Ossipov MH, Lai J, et al. Cholecystokinin in the rostral ventromedial medulla mediates opioid-induced hyperalgesia and antinociceptive tolerance. *J Neurosci* 2005;**25**:409–16.
35. Zaborszky L, Alheid GF, Beinfeld MC, Eiden LE, Heimer L, Palkovits M. Cholecystokinin innervation of the ventral striatum: a morphological and radioimmunological study. *Neuroscience* 1985;**14**:427–53.

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Chapter 100

CGRP/Adrenomedullin

Denise Wootten, Nanda Tilakaratne, Arthur Christopoulos and Patrick M. Sexton

ABSTRACT

Calcitonin-gene-related peptide (CGRP) and adrenomedullin (AM), two structurally related peptides, are synthesized and secreted by many mammalian tissues, including the central nervous system. The two peptides, which were initially described as vasodilators, are now considered as multifunctional regulatory agents. Their cellular actions are mediated by pharmacologically distinct receptors, each comprising two subunits: a classic 7 transmembrane spanning G protein-coupled receptor (GPCR) and one of 3 single transmembrane spanning accessory receptor activity modifying proteins (RAMP). Two GPCRs—the calcitonin receptor (CTR) and calcitonin-receptor-like receptor (CLR)—contribute to CGRP/AM receptor phenotypes. In the brain, CGRP and AM are widely expressed and, in addition to their vasodilator effect, activate several autonomic centers involved in the regulation of fluid and electrolyte balance. Additionally, they have modulatory effects on the synthesis and release of several other neuropeptides and have neuroprotective roles. The potent vasodilator activity of CGRP in cerebral microvasculature is possibly relevant to the pathology of migraine.

DISCOVERY

Discovered in 1983, calcitonin-gene-related peptide (CGRP) was so named because the peptide is a product of the same gene, calcitonin-related polypeptide alpha (CALCA), that encodes calcitonin (CT)—a thyroidal hormone that regulates bone metabolism. Rosenfeld et al. found that the CT gene transcript, which yields CT in thyroidal "C" cells, is alternately spliced in brain cells to produce CGRP.[26] CGRP is a 37-amino-acid peptide that shares 55% amino acid sequence identity with CT. This peptide was later named CGRP-α (or CGRP-I), to distinguish it from another related peptide CGRP-β (or CGRP-II). CGRP-α and CGRP-β differ from each other by only a single amino acid in rats and by 3 residues in humans. CGRP-β is encoded by a gene distinct from the CALCA gene. The following discussion will be limited to CGRP-α.

In 1993, Kitamura et al. discovered a peptide that was able to stimulate cyclic AMP (cAMP) production in blood platelets.[15] The 52-amino-acid peptide was isolated from an extract of a pheochromocytoma—a tumor of the adrenal medulla. Hence, the peptide was named adrenomedullin (AM). However, AM is also produced by healthy adrenal medulla and a wide range of other tissues including vascular endothelium, vascular smooth muscle, and the central nervous system.

Although AM and CGRP share only 30% sequence identity, the two structures required for biological activity—a C-terminal aromatic amide and a ring structure made up of six amino acids linked by disulfide-bonded cysteines—are well conserved between the two peptides. These features are also present in the related peptides, CT, amylin, and intermedin (AM2), and there is a degree of crossreactivity of the peptides across their target receptors.[25]

STRUCTURE OF THE PRECURSOR mRNA/GENE

The CT/CGRP cDNAs have been cloned from several species. In humans, the genomic copy is localized to chromosome 11 (11p15.2-p15.1; Fig. 1). The gene spans a region of about 6.3 kilo bases (kb), and comprises 6 exons. As mentioned previously, the primary transcript undergoes tissue-specific alternate splicing. In the brain, the transcript is principally spliced to yield CGRP mRNA; it is formed by the splicing of exon I (5′ noncoding) with exons II, III, and V that form the coding region, and exon VI (3′ noncoding) (Fig. 1). In normal thyroid, the splicing involves exons I, II, III, and IV to form the CT mRNA. The calcitonin-related polypeptide beta gene encoding CGRP-β is also localized to chromosome 11.[33]

Human AM is encoded by a gene also localized to chromosome 11 (11p15.4). The gene spans approximately 2.1 kb DNA and consists of 4 exons and 3 introns (Fig. 1). The 5′ flanking region contains several elements such as TATA, CAAT, and GC boxes, required for its functional expression. There are also consensus transcription factor binding sites for activator protein-2 (AP-2) and a cAMP-regulated

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00100-7
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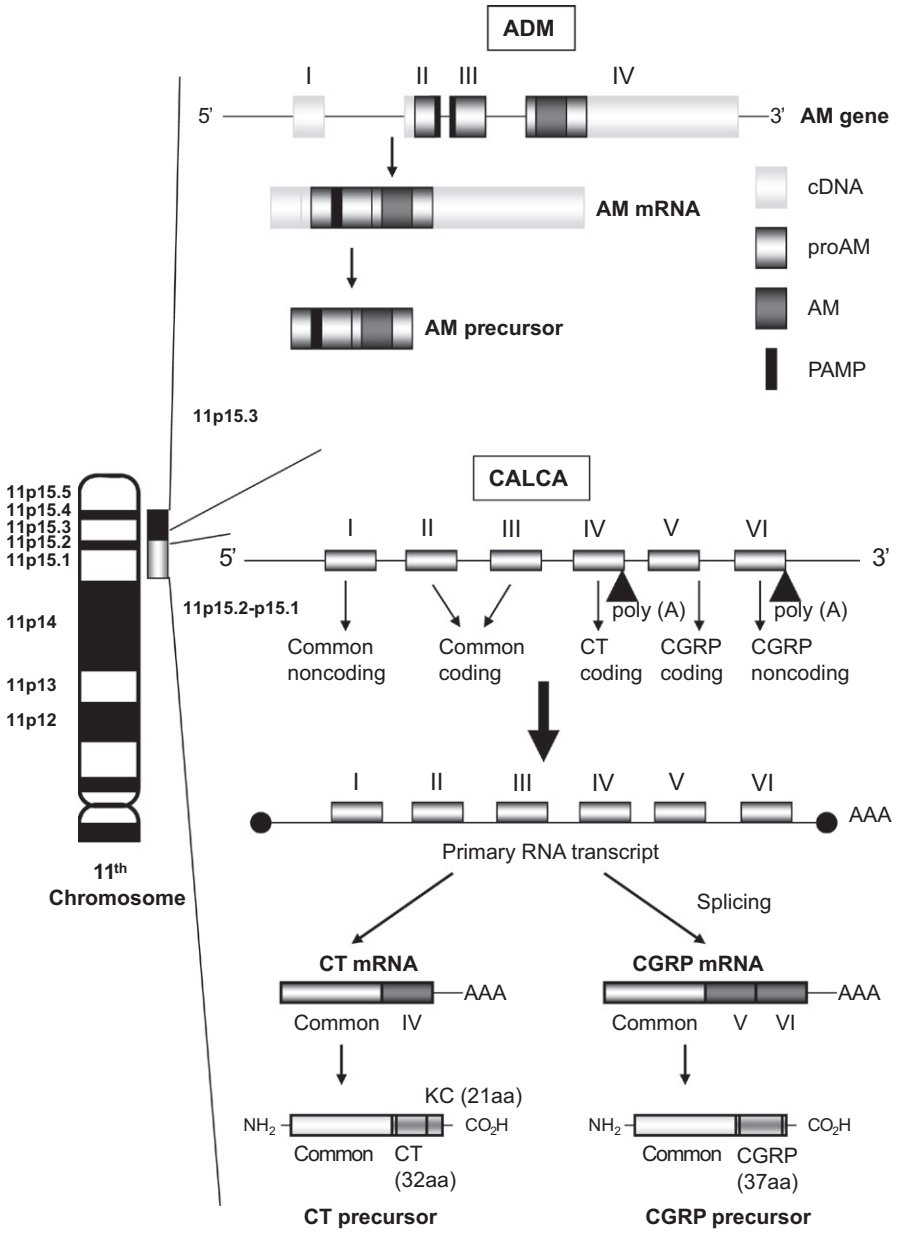

FIGURE 1 Schematic representation of the AM and CALCA genes and their processing to yield the biologically active peptides AM and CGRP. In addition to these peptides, alternative splicing of the CALCA primary transcript leads to the generation of the calcitonin (CT) precursor that yields CT and katacalcin (KC), whereas processing of the AM precursor yields an additional bioactive peptide, PAMP.

enhancer element suggesting that gene expression may be subject to regulation by protein kinase C (PKC) and cAMP.[13] The AM precursor has the potential to generate two bioactive peptides, AM and proadrenomedullin-N-terminal 20 peptide (PAMP), which are released from a common precursor protein (discussed below; Fig. 1).

DISTRIBUTION OF THE mRNA/PEPTIDE

CGRP and AM are widely expressed in many regions of the mammalian brain. This has been revealed by several studies that detected both mRNA (by in-situ hybridization) and the peptide (by immunohistochemistry).

CGRP

Neurons and fibers that contain CGRP mRNA and are immunoreactive to anti-CGRP antibodies are widely but unevenly distributed in the central nervous system (CNS). CGRP-positive cell bodies are observed in the preoptic area and hypothalamus (medial preoptic, periventricular, anterior hypothalamic nuclei, perifornical area, medial forebrain

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bundle), premamillary nucleus, medial amygdala, hippocampus, dentate gyrus, central gray, and the ventromedial nucleus of the thalamus. In the midbrain, a large cluster of CGRP-positive cells is found in the peripeduncular area. The cholinergic motor nuclei (III, IV, V, VI, VII, XII) in the hindbrain also exhibit CGRP immunoreactivity. CGRP is also detected in cell bodies in the parabrachial nuclei, the ventral tegmental nucleus, superior olive, and nucleus ambiguus, and fibers in the central amygdala, caudal caudate putamen, sensory trigeminal area, and substantia gelatinosa (Fig. 2A). Other areas containing CGRP-immunoreactive fibers include the septal area, bed nucleus of the stria terminalis, preoptic and other hypothalamic nuclei (e.g. medial preoptic, periventricular, dorsomedial) median eminence, medial forebrain bundle, central gray, medial geniculate body, peripeduncular area, interpeduncular nucleus, cochlear nucleus, parabrachial nuclei, superior olive, nucleus of the solitary tract, and in the confines of clusters of cell bodies (Fig. 2A). The neuroanatomical localization of CGRP is reviewed more comprehensively elsewhere.[16,29,31]

There is also enrichment of CGRP in the cerebral microvasculature, in both vascular endothelial cells and nerve fibers that are closely associated with blood vessels.

Adrenomedullin

Although initial studies suggested that AM was poorly expressed in the brain, more recent work indicates that it has a widespread distribution and that it is dynamically regulated by events such as ischemia and/or glucose deprivation. Several autonomic centers including the hypothalamic paraventricular nucleus (PVN), hypothalamic supraoptic nucleus (SON), locus coeruleus, and the ventrolateral medulla express high levels of AM, with low to moderate AM immunoreactivity found in many other regions including multipolar neurons and pyramidal cells of layers IV–VI of the cerebral cortex, parts of the thalamus, and cerebellar Purkinje cells and cerebellar nuclei (Fig. 2B). Immunoblotting of brain extracts has shown that the fully processed peptide is the predominantly expressed form, with a relatively smaller amount of the propeptide. The detailed distribution of AM in brain is reviewed elsewhere.[27,31]

AM and PAMP are also expressed in the pituitary, with particularly high levels in the adenohypophysis and neural lobe, and relatively lower expression in the intermediate lobe of the pituitary. The role of AM in the pituitary is discussed elsewhere in this book.

PROCESSING OF THE PRECURSOR

As described above, the precursor for CGRP is derived from alternately spliced mRNA from the CALCA gene. The CGRP precursor in humans consists of 128 amino acids with a molecular mass of 13.87 kDa. The N-terminal region that is encoded principally by exons II and III incorporates a signal peptide from residues 1–21; it is cleaved at the dibasic motif KR (residues 81–82) to yield the biologically active 37-aa CGRP polypeptide together with a short C-terminal fragment. The glycine at residue 120 of the precursor provides the amide for amidation of the C-terminal phenylalanine (aa. 119 in the precursor) (Fig. 1).

The primary polypeptide product of the human AM mRNA is 185 amino acids in length and is called preproAM. The N-terminal signal sequence of 21 residues is cleaved to yield proAM. Further processing of proAM yields two peptides: PAMP derived from residues 22 to 41 and AM (a 52-aa peptide) composed of the amino acid residues 95–146 of preproAM. Glycine 147 provides the amide for amidation of tyrosine 52 (aa. 146 of the precursor) of the mature AM.

Some investigators have reported the presence of two forms (a and b) of the AM mRNA. Form A is devoid of introns and results in a prohormone containing both AM and PAMP. Form B retains the third intron, which introduces a premature stop codon, producing a shorter prohormone with only PAMP. Treatments that modify AM expression, such as hypoxia, have been shown to change the B/A ratio and the relative levels of AM and PAMP, indicating that the splicing mechanism is subject to modification and is physiologically relevant.[19]

RECEPTORS AND SIGNALING CASCADES

CGRP Receptors

CGRP receptors are widely distributed in the brains of many species, and there has been extensive work mapping the distribution of these receptors using ^{125}I-CGRP, particularly in rat brain.[28,31,33] High densities of CGRP-binding sites are present in regions including, accumbens nucleus, *organum vasculosum of the lamina terminalis*, ventral caudate putamen, median eminence, much of the hypothalamus, including enrichment in the arcuate nucleus and premammillary area, various amygdaloid nuclei, the superior and inferior colliculi, pontine nuclei, cerebellar cortex, nucleus of the solitary tract, inferior olivary nucleus, hypoglossal complex, and the vestibular and cochlear nuclei, along with lower levels of binding to many other brain regions. CGRP receptors are also present on most of the cerebral vasculature. The sites labeled by ^{125}I-CGRP are heterogeneous, and there are multiple subtypes of "CGRP" receptor.

AM Receptors

Although membrane-based radioligand binding studies have demonstrated the existence of significant levels of AM receptors in the brain, there is only limited information on the anatomical distribution of AM receptors in this tissue.

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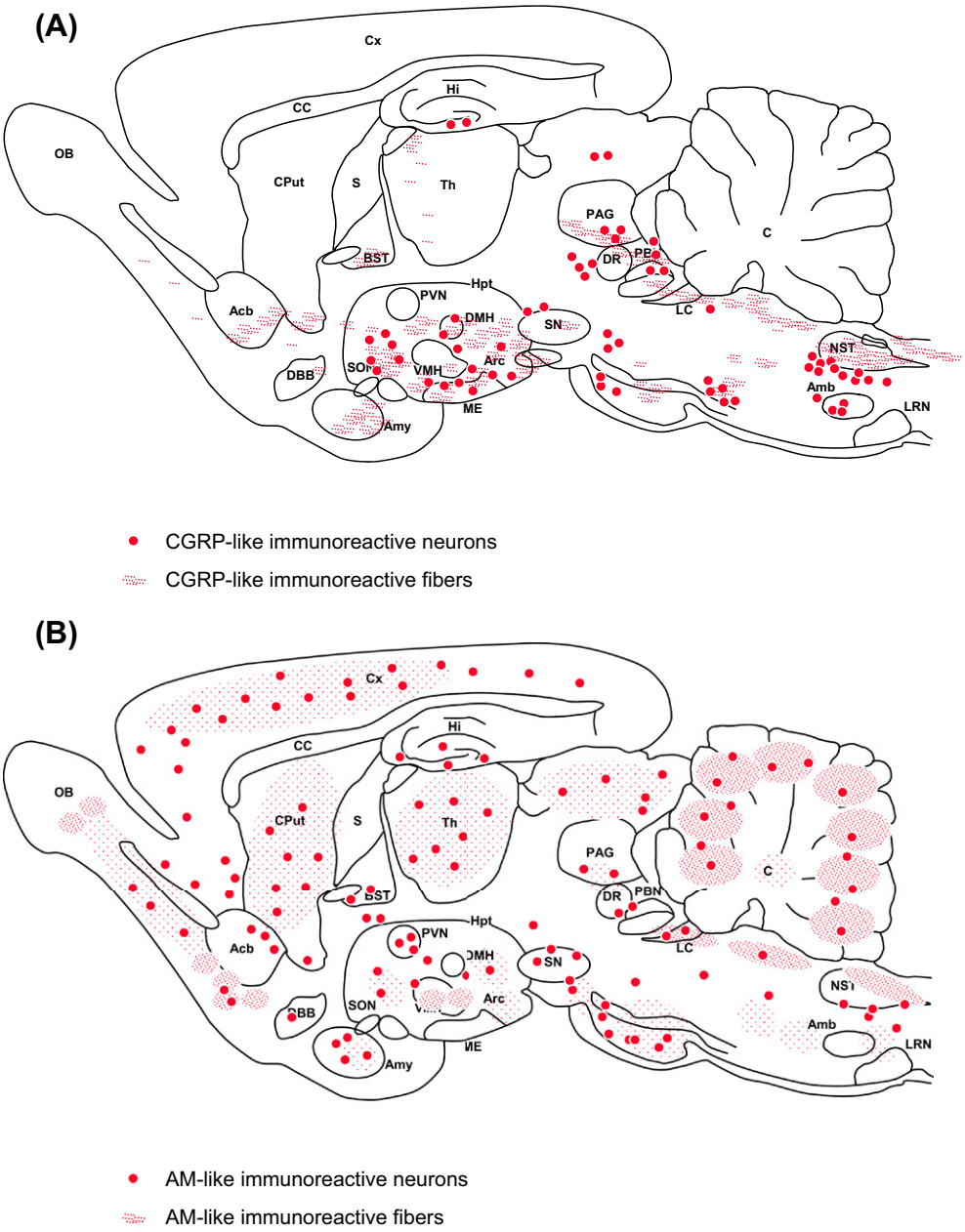

FIGURE 2 Schematic representation of (A) the distribution of CGRP-like immunoreactive fibers (dots) and cell bodies (circles) summarized from Refs. 13, 24, 26, 27 and (B) The distribution of AM-like immunoreactive fibers (dots) and cell bodies (circles) summarized from Ref. 23. Data are indicative of location of immunoreactivity and are not meant to reflect the quantitative levels of neurons or fibers.

Using ^{125}I-AM$_{13–52}$, Juaneda and colleagues observed high levels of AM binding in regions including choroid plexus, the ependymal linings of the cerebral ventricles, cerebellum, and parts of the amygdala, together with the neural lobe of the pituitary gland.[2,14] Elsewhere, only low levels of binding were observed, although this may reflect the sensitivity of the methodology and radioligand probe, as there is evidence for more widespread distribution of the constituent components of the AM receptor complexes (see below).[23] AM receptors are also heterogeneous, with two high affinity receptors characterized.

Molecular Basis of CGRP and AM Receptor Phenotype

The molecular basis of CGRP and AM receptor phenotype(s) is complex; the ligand-binding domains of the receptors comprise two subunits: a classic 7 transmembrane spanning

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GPCR and one of 3 single transmembrane spanning accessory receptor activity modifying proteins (RAMP).[9,25] Two GPCRs, namely, the calcitonin receptor (CTR) and calcitonin-receptor-like receptor (CLR), contribute to CGRP/AM receptor phenotypes. The CLR/RAMP1 heterodimer is a classic $CGRP_1$ receptor and has high affinity for CGRPs but lower affinity for related peptides. CLR/RAMP2 is a high-affinity AM receptor (termed AM_1 receptor) and only weakly interacts with CGRP. CLR/RAMP3 is also a high affinity AM receptor (termed AM_2 receptor), but it may respond with moderate potency to CGRP in a system-dependent manner. In addition to CLR-based receptors, a high-affinity CGRP receptor is also formed by heterodimerization of CTR/RAMP1. This receptor, like other CTR/RAMP-based receptors, exhibits high affinity for the peptide amylin and is termed the AMY_1 receptor (previously also known as a "C3" receptor). Other CTR/RAMP receptors have only low affinity for CGRP, and all CTR/RAMP receptors have very low affinity for AM. Signaling via CLR-based receptors also involves an additional protein, termed receptor component protein (RCP), which is required for the efficient coupling of CLR/RAMP heterodimers to at least $G_{\alpha s}$ proteins.[6]

CLR, RAMPs, and CTR are all expressed within the brain, although with often discrete patterns of distribution. The distribution of the mRNA of the molecular components for CGRP and AM receptors generally correlates well with the distribution of radioligand binding sites in regions including the hypothalamus, caudate putamen, and cerebral vasculature, supporting the heterodimer as the basic unit for these receptors.

An additional receptor, termed the $CGRP_2$-subtype has also been proposed. $CGRP_2$-subtype receptors are defined as having low affinity for $CGRP_1$ receptor antagonists, such as $CGRP_{8-37}$ and BIBN4096BS, but can be activated with reasonable potency by linear CGRP analogs.[31] Recent results suggest that most of the pharmacology ascribed to "$CGRP_2$" receptors can be attributed to one or other of the CTR/RAMP1, CTR/RAMP3, or CLR/RAMP3 complexes.[8,17]

The possible existence of additional non-RAMP modulated CGRP receptors has been suggested. This comes principally from early expression data in the cerebellum, which showed abundant CGRP binding sites that are pharmacologically similar to $CGRP_1$ receptors. However, there is some controversy about their molecular identity because there are reports indicating no expression of CLR or RAMP1 mRNA in this tissue, whereas others suggest that low expression of these mRNA does indeed occur. A more recent study using immunohistochemistry to examine the distribution of CGRP and its receptor components in the rat cerebellum detected CGRP intracellularly in cerebellar Purkinje cell bodies and both CLR and RAMP1 at the surface of the Purkinje cell bodies and in their processes, suggesting the presence of functional CGRP receptors.[4] The cerebellum is also particularly enriched in RCP, and this may contribute to efficient and/or stable expression of the CLR and RAMP protein components in a high affinity complex.[9]

Signaling

CGRP and AM receptors are family B GPCRs; these receptors pleiotropically couple to multiple G proteins, with prominent coupling to $G_{\alpha s}$. CGRP and AM receptors strongly activate $G_{\alpha s}$, and many of their actions are downstream of cAMP generation. This has been confirmed by several studies in various tissues, primary cultures, or transfected cells. As described above, an efficient coupling of the CLR-based receptors to $G_{\alpha s}$ requires the presence of RCP. However, the receptors can also activate pertussin-toxin-sensitive G proteins and probably also $G_{\alpha q/11}$ proteins, the latter leading to Ca^{2+} mobilization. Only limited functional studies have been performed in nerve tissues, primarily in neuronal tumor cells or primary cultures of astrocytes and glial cells. Rat AM stimulates cAMP production in rat astrocytes and NG108-15 neuroblastoma/glial hybrid cells, in a dose-dependent manner. This effect is not blocked by a $CGRP_1$ receptor antagonist, indicating that activation of selective AM receptors is involved. Similarly, CGRP-induced cAMP production has been found in human glioblastoma A172 cells and SKNMC neuroblastoma cells. The effects of AM on Ca^{2+} signaling mechanisms have also been investigated in neuroblastoma and glial tumor cell lines, because endothelial nitric oxide (NO) has been implicated in AM-mediated vasodilation and there is a well-known link between intracellular Ca^{2+} and activation of NO synthase (NOS).[10,27] CGRP-stimulated Ca^{2+} signaling has also been shown, with CGRP triggering responses from intracellular stores in cultured mouse astrocytes and glial cells from isolated cerebellar slices.[21] In addition, both CGRP and AM facilitate Ca^{2+} currents from L-type voltage-dependent calcium channels in the *nucleus tractus solitarius*, with CGRP responses involving $G_{\alpha s}$ and protein kinase A, whereas AM-facilitated responses are via $G_{\alpha i}$ and mitogen-activated protein kinases.[11]

PEPTIDE CONFORMATION

Nuclear magnetic resonance and circular dichroism studies have indicated that CGRP can adopt an α-helical secondary structure in a solvent-dependent manner. Organic solvents such as tetrafluoroethylene promote the formation of the α-helix, and this is believed to more closely resemble the active conformation at the receptor. Under such conditions, *nuclear magnetic resonance (*NMR) studies indicate that an amphipathic α-helix is formed between residues 8 and 18, two β-turn structures between amino acids 17 and 21 and amino acids 29 and 34 together with random coil "structure" from amino acids 23–29. It is believed that the

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disulfide-bonded N-terminal region (aas. 1–7) is important in the stabilization of the helix but that residues 19–37 do not contribute significantly.[31]

AM is disordered in water; however, under certain experimental conditions, residues 22–34 have a tendency to adopt an α-helical structure. The 3-dimensional structure of AM in a membrane mimicking environment has recently been solved using NMR spectroscopy.[29] Under these conditions, AM had an α-helical region spanning approximately one-third of its total length, flanked by two disordered segments at both the N- and C-termini. AM and CGRP likely adopt a similar secondary structure when bound to their receptor(s). The N-terminal 14-amino-acid extension seen in AM, but not in other related peptides, does not seem to be important for biological activity and may not directly interact with the receptor.

BIOLOGICAL ACTIONS WITHIN THE BRAIN

CGRP and AM are best known for their hypotensive effects through their vasodilatory action. However, because the peptides and their receptors are widely distributed in the brain, outside the cerebral vasculature, there is interest in their potential roles in other biological actions within the brain. There is evidence that some of the peripheral effects of both these peptides result from their actions within the brain, particularly on neurons in the autonomic centers.

CGRP

Consistent with its widespread distribution in the brain, CGRP has been implicated in a wide range of centrally mediated functions. As with peripheral vascular beds, CGRP is a potent dilator of cerebral and pial vessels, and via this action, contributes to the pain of migraine headache (see below). Central injection of CGRP elicits multiple effects including hypothermia, catalepsy and reduced motor activity, as well as actions on the hypothalamic–pituitary axis including modulation of growth hormone, luteinizing hormone, and prolactin secretion. CGRP potentiates haloperidol-induced catalepsy and decreases apomorphine-induced hypermotility. Central CGRP can also inhibit thalamic neuronal firing evoked by peripheral noxious mechanical stimuli (at least in rat) and can modulate pain responses at the level of the spinal cord. It is also reported to have a role in learning and memory processing and can increase the production of insulin-like growth factor-1. This has beneficial effects on cognitive function by increasing synaptic transmission and inducing angiogenesis and neurogenesis in the mouse hippocampus.[7] In addition, CGRP has protective effects and can reduce blood–brain barrier injury, brain infarct volume, and cerebral edema after focal cerebral ischemia.[18] CGRP can decrease feeding via receptors in the hypothalamus and also the area postrema (reviewed in more detail elsewhere in this book), but has a more prominent effect on diuresis and natriuresis. It can also decrease gastric acid secretion and gastric motility. In contrast to the hypotensive effect of peripherally administered CGRP, centrally administered CGRP increases the mean arterial pressure and heart rate through increased sympathetic outflow. The coincident distribution of CGRP fibers and receptors in functionally linked nuclei involved in auditory, gustatory, and visual processing suggests that CGRP may also play a role in these systems. The central biological actions of CGRP are reviewed in more detail elsewhere.[2,28,31,33]

Adrenomedullin

The most characterized physiological role of AM is in the regulation of circulation and control of fluid and electrolyte homeostasis (see the chapters on this peptide in Cardiovascular Peptides and Renal Peptides sections of this book). This function of AM is mediated via both peripheral and central actions. Effects of AM in the brain have been studied mostly by AM infusion into various brain regions. Action sites of AM in the rat brain have been mapped by *c-fos* immediate-early gene expression. Intracerebroventricular (icv) injection in conscious rats results in *fos* expression in supraoptic nucleus, paraventricular nucleus, locus coeruleus, and area postrema. This suggests that centrally administered AM causes activation of a neural network in the hypothalamus and brain stem. In the PVN, mostly the corticotropin-releasing hormone (CRH)- and nitric oxide (NO)-producing cells are activated. The *c-fos* induction is blocked by pretreatment with the AM receptor antagonist $hAM_{22–52}$. These observations suggest that AM stimulates the activity of the hypothalamic–pituitary axis, the sympathetic nervous system, and the hypothalamic NO system (see the chapter on this peptide in Endocrine Peptides section of this book). Icv injection of AM in rats also augments tyrosine hydroxylase gene expression in the locus coeruleus and plasma oxytocin levels. Thus, AM activates the neurosecretory cells of the PVN and SON via selective AM receptors, and stimulates oxytocin (OT) secretion by activating hypothalamic-OT producing cells in response to altered plasma sodium levels. Not only does this stimulate OT production but also OT acting within the CNS inhibits further NaCl consumption thus maintaining sodium homeostasis.[27,32] AM can also regulate arginine vasopressin release from the hypothalamus in response to hypovolemia, thereby acting as a physiological regulator of fluid homeostasis.[30]

Investigations have been extended to specific populations of CNS neurons. Patch-clamp studies on dissociated rat area postrema (AP) neurons revealed AM concentration-dependent

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neuronal activity, effects that were mediated via specific ion channel activity. The results have implications to the cardiovascular actions of AM because the AP is a CNS site implicated in cardiovascular control. AM also has protective effects against ischemic/hypoxic injury to neurons. AM expression is upregulated in cultured neurons subjected to hypoxic conditions. AM gene transfer via a viral vector could protect experimentally induced ischemic brains of rats from developing cerebral infarcts, an effect that was abolished by previous treatment with an AM receptor antagonist.[34] In addition, brain-specific conditional AM (and PAMP) KO mice were less resistant to hypobaric hypoxia and displayed a significant increase in infarct size after 48 h of middle cerebral permanent occlusion compared with wild-type mice. Protein and mRNA levels of several markers of nitrosative stress, inflammation, and brain damage were also increased in the brain AM KO mice.[12] Thus, there is evidence to suggest that AM can act as a neurotransmitter, neuromodulator, or a neurohormone in brain autonomic pathways involved in the maintenance of homeostasis, and also a cytoprotective factor in ischemic/hypoxic conditions, in addition to its vasodilator role in the brain microvasculature.

AM has functions at each level of the hypothalamic–pituitary–adrenal axis, suggesting that the peptide plays a role in the organization of neuroendocrine responses to stress. Icv injection into the rat brain causes secretion of corticosterone and prolactin but not growth hormone.

PATHOPHYSIOLOGICAL IMPLICATIONS

Both CGRP and AM have been linked to a broad range of clinical conditions, most of them being inflammatory conditions of the cardiovascular system. In regard to those associated with the brain, the strongest link has been established for a role for CGRP with the physiopathology of headache, particularly migraine-type headache. The intracranial extracerebral blood vessels (e.g. middle meningeal artery and dural arterioles) are believed to dilate in response to CGRP and as a consequence stimulate perivascular sensory nociceptive nerve fibers, producing a pain sensation. Intravenous infusion of CGRP exacerbates headache pain, and CGRP levels are increased in external jugular venous blood, proportionally with pain intensity, during migraine attacks.[3] $CGRP_1$-receptor-specific antagonists BIBN4096BS (Olcegepant) and MK0974 (Telcagepant) demonstrate clinical efficacy in the treatment of migraine pain.[5] Studies with $CGRP^{-/-}$ mice have provided evidence that CGRP is important for the generation of opiate withdrawal symptoms[22] and may therefore contribute to this behavior in humans. An involvement of CGRP in psychiatric disorders has also been suggested by altered levels of CGRP in the cerebrospinal fluid of patients with dementia and depression. In addition, rats treated with either classical or atypical antipsychotic drugs had altered CGRP levels, suggesting that CGRP is involved in CNS functions related to psychiatric disorders.[20]

AM has also been implicated as having a role in the pathogenesis of several psychiatric disorders such as schizophrenia, bipolar disorder, and depression. It is believed that the effect of AM occurs through its NO-generating action. There are also suggestions that AM may have a role in autism.[35] However, the best evidence is for a protective role in ischemic episodes, which is also seen in peripheral tissue.[1]

ACKNOWLEDGMENTS

P.M. Sexton is a Principal, and A. Christopoulos a Senior, Research Fellow of the National Health and Medical Research Council of Australia (NHMRC) and is supported by project (1002180) and program (519461) grant funding from the NHMRC.

REFERENCES

1. Bunton DC, Petrie MC, Hillier C, Johnston F, McMurray JJ. The clinical relevance of adrenomedullin: a promising profile? *Pharmacol Ther* 2004;**103**:179–201.
2. Dumont Y, Chabot JG, Quirion R. Receptor autoradiography as mean to explore the possible functional relevance of neuropeptides: focus on new agonists and antagonists to study natriuretic peptides, neuropeptide Y and calcitonin gene-related peptides. *Peptides* 2004;**25**:365–91.
3. Edvinsson L. Blockade of CGRP receptors in the intracranial vasculature: a new target in the treatment of headache. *Cephalalgia* 2004;**24**:611–22.
4. Edvinsson L, Eftekhari S, Salvatore CA, Warfvinge K. Cerebellar distribution of calcitonin gene-related peptide (CGRP) and its receptor components calcitonin receptor-like receptor (CLR) and receptor activity modifying protein 1 (RAMP1) in rat. *Mol Cell Neurosci* 2011;**46**:333–39.
5. Eftekhari S, Edvinsson L. Possible sites of action of the new calcitonin gene-related peptide receptor antagonists. *Ther Adv Neurol Disord* 2010;**3**:369–78.
6. Evans BN, Rosenblatt MI, Mnayer LO, Oliver KR, Dickerson IM. CGRP-RCP, a novel protein required for signal transduction at calcitonin gene-related peptide and adrenomedullin receptors. *J Biol Chem* 2000;**275**:31438–43.
7. Harada N, Narimatsu N, Kurihara H, Nakagata N, Okajima K. Stimulation of sensory neurons improves cognitive function by promoting the hippocampal production of insulin-like growth factor-I in mice. *Transl Res* 2009;**154**:90–102.
8. Hay DL, Christopoulos G, Christopoulos A, Poyner DR, Sexton PM. Pharmacological discrimination of calcitonin receptor: receptor activity-modifying protein complexes. *Mol Pharmacol* 2005;**67**:1655–65.
9. Hay DL, Poyner DR, Sexton PM. GPCR modulation by RAMPs. *Pharmacol Ther* 2006;**109**:173–97.
10. Hayakawa H, Hirata Y, Kakoki M, Suzuki Y, Nishimatsu H, Nagata D, et al. Role of nitric oxide-cGMP pathway in adrenomedullin-induced vasodilation in the rat. *Hypertension* 1999;**33**:689–93.

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11. Hosokawa S, Endoh T, Shibukawa Y, Tsumura M, Ichikawa H, Tazaki M, et al. Calcitonin gene-related peptide- and adrenomedullin-induced facilitation of calcium current by different signal pathways in nucleus tractus solitarius. *Brain Res* 2010;**1327**:47–55.
12. Hurtado O, Serrano J, Sobrado M, Fernandez AP, Lizasoain I, Martinez-Murillo R, et al. Lack of adrenomedullin, but not complement factor H, results in larger infarct size and more extensive brain damage in a focal ischemia model. *Neuroscience* 2010;**171**:885–92.
13. Ishimitsu T, Kojima M, Kangawa K, Hino J, Matsuoka H, Kitamura K, et al. Genomic structure of human adrenomedullin gene. *Biochem Biophys Res Commun* 1994;**203**:631–9.
14. Juaneda C, Dumont Y, Chabot JG, Quirion R. Autoradiographic distribution of adrenomedullin receptors in the rat brain. *Eur J Pharmacol* 2001;**421**:R1–2.
15. Kitamura K, Kangawa K, Kawamoto M, Ichiki Y, Nakamura S, Matsuo H, et al. Adrenomedullin: a novel hypotensive peptide isolated from human pheochromocytoma. *Biochem Biophys Res Commun* 1993;**192**:553–60.
16. Kresse A, Jacobowitz DM, Skofitsch G. Detailed mapping of CGRP mRNA expression in the rat central nervous system: comparison with previous immunocytochemical findings. *Brain Res Bull* 1995;**36**:261–74.
17. Kuwasako K, Cao YN, Nagoshi Y, Tsuruda T, Kitamura K, Eto T. Characterization of the human calcitonin gene-related peptide receptor subtypes associated with receptor activity-modifying proteins. *Mol Pharmacol* 2004;**65**:207–13.
18. Liu Z, Liu Q, Cai H, Xu C, Liu G, Li Z. Calcitonin gene-related peptide prevents blood-brain barrier injury and brain edema induced by focal cerebral ischemia reperfusion. Regul Pept.
19. Martinez A, Hodge DL, Garayoa M, Young HA, Cuttitta F. Alternative splicing of the proadrenomedullin gene results in differential expression of gene products. *J Mol Endocrinol* 2001;**27**:31–41.
20. Mathe AA, Agren H, Wallin A, Blennow K. Calcitonin gene-related peptide and calcitonin in the CSF of patients with dementia and depression: possible disease markers. *Prog Neuropsychopharmacol Biol Psychiatry* 2002;**26**:41–8.
21. Morara S, Wang LP, Filippov V, Dickerson IM, Grohovaz F, Provini L, et al. Calcitonin gene-related peptide (CGRP) triggers Ca2+ responses in cultured astrocytes and in Bergmann glial cells from cerebellar slices. *Eur J Neurosci* 2008;**28**:2213–20.
22. Muff R, Born W, Lutz TA, Fischer JA. Biological importance of the peptides of the calcitonin family as revealed by disruption and transfer of corresponding genes. *Peptides* 2004;**25**:2027–38.
23. Oliver KR, Kane SA, Salvatore CA, Mallee JJ, Kinsey AM, Koblan KS, et al. Cloning, characterization and central nervous system distribution of receptor activity modifying proteins in the rat. *Eur J Neurosci* 2001;**14**:618–28.
24. Perez-Castells J, Martin-Santamaria S, Nieto L, Ramos A, Martinez A, de Pascual-Teresa B, et al. Structure of micelle-bound adrenomedullin, a first step towards the analysis of its interactions with receptors and small molecules. *Biopolymers*. 2012.
25. Poyner DR, Sexton PM, Marshall I, Smith DM, Quirion R, Born W, et al. International Union of Pharmacology. XXXII. The mammalian calcitonin gene-related peptides, adrenomedullin, amylin, and calcitonin receptors. *Pharmacol Rev* 2002;**54**:233–46.
26. Rosenfeld MG, Mermod JJ, Amara SG, Swanson LW, Sawchenko PE, Rivier J, et al. Production of a novel neuropeptide encoded by the calcitonin gene via tissue-specific RNA processing. *Nature* 1983;**304**:129–35.
27. Serrano J, Alonso D, Fernandez AP, Encinas JM, Lopez JC, Castro-Blanco S, et al. Adrenomedullin in the central nervous system. *Microsc Res Tech* 2002;**57**:76–90.
28. Sexton PM. Central nervous system binding sites for calcitonin and calcitonin gene-related peptide. *Mol Neurobiol* 1991;**5**:251–73.
29. Skofitsch G, Jacobowitz DM. Calcitonin gene-related peptide: detailed immunohistochemical distribution in the central nervous system. *Peptides* 1985;**6**:721–45.
30. Taylor MM, Baker JR, Samson WK. Brain-derived adrenomedullin controls blood volume through the regulation of arginine vasopressin production and release. *Am J Physiol Regul Integr Comp Physiol* 2005;**288**:R1203–10.
31. van Rossum D, Hanisch UK, Quirion R. Neuroanatomical localization, pharmacological characterization and functions of CGRP, related peptides and their receptors. *Neurosci Biobehav Rev* 1997;**21**:649–78.
32. White MM, Samson WK. A possible relationship between brain-derived adrenomedullin and oxytocin in the regulation of sodium balance. *J Endocrinol* 2009;**203**:253–62.
33. Wimalawansa SJ. Amylin, calcitonin gene-related peptide, calcitonin, and adrenomedullin: a peptide superfamily. *Crit Rev Neurobiol* 1997;**11**:167–239.
34. Xia CF, Yin H, Borlongan CV, Chao J, Chao L. Adrenomedullin gene delivery protects against cerebral ischemic injury by promoting astrocyte migration and survival. *Hum Gene Ther* 2004;**15**:1243–54.
35. Zoroglu SS, Yurekli M, Meram I, Sogut S, Tutkun H, Yetkin O, et al. Pathophysiological role of nitric oxide and adrenomedullin in autism. *Cell Biochem Funct* 2003;**21**:55–60.

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Chapter 101

CRH Family

David A. Lovejoy

ABSTRACT

The corticotropin-releasing hormone (CRH) family of peptides consists of four distinct paralogs including CRH, urocortin (Ucn)/urotensin-I (UI), urocortin 2 (Ucn2), and urocortin 3 (Ucn3). The peptides range between 38 and 41 amino acids in length and exist predominantly as amphiphilic alpha helices. Each gene is located on separate chromosomes, and most possess a similar gene structure consisting of two exons and an intron of variable length. CRH is responsible for the hypothalamic activation of the hypothalamic–pituitary adrenal axis, but all paralogs seem to play roles in the regulation of stress and energy homeostasis. The CRH family of peptides has been linked pathologically to affective and neurodegenerative disorders.

DISCOVERY

The first evidence of a hypothalamic corticotropin-releasing substance was reported in 1955. However, the first family member of the CRH family was found not in the hypothalamus but rather in the skin of a frog.[12,20] This peptide, called 'sauvagine,' consists of 40 amino acids and is effective in releasing ACTH from rat pituitary cells (Fig. 1). About the same time, a 41-amino acid peptide, called urotensin-I (UI), from the caudal neurosecretory system (urophysis) of carp was identified. However, the actual structure of CRH was reported by Vale et al. in 1981.[33] Over the next 10 years, the discovery of CRH in fish and amphibians indicated that UI and sauvagine are homologs of CRH.[22] The rat and human orthologs of UI were eventually cloned by the Vale and Sawchenko laboratories and were termed urocortin (Ucn).[10,35] Ucn, like sauvagine, is 40 amino acids long, in contrast to the 41 residue sequence that characterizes CRH and UI (Fig. 1) Neuroanatomical observations[6] and phylogenetic studies[22] suggested that as many as four CRH/UI-like peptides were found in chordates. Then, in 2001, the presence of two additional CRH-related peptides was reported by the Vale and Sawchenko laboratories that called the peptides urocortin 2 (Ucn2) and urocortin 3 (Ucn3),[19,30] and independently by Hsu and Hseuh[15] who named the peptides stresscopin and stresscopin-related peptide on the basis of their anxiolytic effects (Fig. 1). The apparent sequence dissimilarity of Ucns 2 and 3 to CRH and Ucn/UI suggested, however, that the former were not Ucns, per se, but rather the product of a gene duplication early in CRH ancestry. Three sets of independent observations support the hypothesis that the Ucn2 and 3 sequences resemble the ancestral version of chordate CRH. First, the Ucn2/3 sequences possess a greater sequence similarity than the CRH/UI/Ucn lineage, to the insect orthologs of the CRH family.[25] Second, they also retain key amino acid motifs found in a newly discovered calcitonin-like peptide in insects,[25] confirming a close evolutionary relationship between the CRH and calcitonin peptide families. Finally, the characterization of the single CRH-like peptide found in the tunicate, *Ciona intestinalis*, indicates a sequence possessing both Ucn2/3 and insect diuretic peptide structural attributes.[23] Taken together, these observations imply that the CRH/UI (including Ucn and sauvagine) clade of peptides represents a more derived version of the CRH peptide family in chordates and the physiological functions of the Ucn2/3 peptides may resemble the ancestral functions of this peptide family to a greater degree. The highly conserved nature of CRH in chordates may, therefore, be the result of the use of this paralog for the primary control of the hypothalamic–pituitary adrenal/interrenal axis and the subsequent selection constraints imposed on this sequence.[20]

PRECURSOR GENE AND CDNA STRUCTURE

The gene structure of the CRHs and the Ucns support a gene duplication early in chordate ancestry. The basic structure of the CRH and Ucn genes are similar but are located on chromosomes 8q13 and 2p23-p21, respectively, in humans and chromosomes 3 and 13 in mice. There are two exons of unequal sizes where the first encodes for a portion of the 5′ untranslated region (UTR) and exon 2, the remainder of the 5′ UTR, entire preprohormone sequence and the entire 3′ UTR. The intron length varies considerably among species, although the Ucn intron is much smaller compared with that

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00101-9
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Corticotrophin Releasing Hormone	human (NM000756)	SEEPPISLDLTFHLLREVLEM	ARAEQLAQQAHSNRKLMEII-NH2
	Xenopus (S50096)	AEEPPISLDLTFHLLREVLEM	ARAEQIAQQAHSNRKLMDII-NH2
	zebrafish (BC085458)	SEEPPISLDLTFHLLREVLEM	ARAEQMAQQAHSNRKMMEIF-NH2
	trout (AF296672)	SDDPPISLDLTFHMLRQMMEM	SRAEQLQQQAHSNRKMMEIF-NH2
Urocortin/Urotensin-I	human (NM003353) .	DNPSLSIDLTFHLLRTLLEL	ARTQSQRERAEQNRIIFDSV-NH2
	Xenopus (AY596827)	EDPPISIDLTFHILRQMIEI	AKTQNQKQQAEQNRIIFDSV-NH2
	P.sauvageii (AY943910) .	QGPPISIDLSLELLRKMIEI	EKQEKEKQQAANNRLLLDTI-NH2
	dogfish (AAB47080)	PAETPNSLDLTFHLLREMIEI	AKHENQQMQADSNRRIMDTI-NH2
	zebrafish (CAH68941)	NDDPPISIDLTFHLLRNMIEM	ARIENQREQAELNRKYLDEV-NH2
	trout (AJ005264)	NDDPPISIDLTFHLLRNMIEM	ARIESQKEQAELNRKYLDEV-NH2
Urocortin 2	human (BC022096) . . .	IVLSLDVPIGLLQILLEQ	ARARAAREQATTNARILARV-NH2
	chicken (XP425157) . . .	VSLSLDVPTHILRILLDL	AREKELQAKAAANAELMARI-NH2
	pufferfish (see legend) . . .	FALSLDVPTSILSVLIDL	AKNQDMRSKAAANAELMARI-NH2
Urocortin 3	human (NM053049) ...	FTLSLDVPTNIMNLLFNI	AKAKNLRAQAAANAHLMAQI-NH2
	Xenopus (AAT70727) ...	FTLSLDVPTNLMNILFDI	AKAKNIRAKAAANAQLMAQI-NH2
	pufferfish (CAB96535) . . .	LTLSLDVPTNIMNVLFDV	AKAKNLRAKAAENARLLAHI-NH2
CRH Like	tunicate (see legend)	PALGRNPFLSLDVPLSFLSKLMEVKRREEQRKIAKIKSVHNSSLMKRI-NH2	

FIGURE 1 Primary sequence of CRF superfamily orthologs and paralogs. Dark gray regions indicate amino acids that have been conserved in all orthologs and paralogs. Light gray regions indicated the residue identity relative to human CRH. The accession number is given for each sequence. The pufferfish sequence was obtained as a predicted translated protein product from the pufferfish genome database (accession number: lcl SINFRUP00000059907) and the tunicate sequence was obtained from Ref. 21.

of CRH (Fig. 2). Although the CRH cDNA structure remains fairly consistent among orthologs, there is considerable variability among the Ucns and UI. The UI mRNA is 3–5 times longer in fishes than the mammal Ucn mRNA. This length difference results from a much shorter gene-associated peptide in the Ucns and extended 3′ UTR in fish UI, particularly in rainbow trout. In this species, there are multiple polyadenylation sites that correspond to the differential mRNA processing that occurs in the urophysis and brain.[3] The CRH-like peptide gene of the tunicate is unique in that only a single exon is present.[23] It is not clear what the ancestral condition was, however because as many as five exons are found in the insect homologs of CRH, it is probable that at least two or possibly three exons were inherited by the protochordate and chordate ancestor.[25] Thus, the loss of the first exon probably occurred during the extensive genomic reorganization of the urochordate, or tunicate lineage.

The gene structure of the Ucns 2 and 3 also reflects a structural relationship to each other, although these genes have undergone considerable change. Both Ucn2 and 3 genes possess two exons similar to the arrangement for urocortin and CRH (Fig. 2). Ucn2 is found on human chromosome 3p21.3 and on mouse chromosome 9. In both cases, the start of the Ucn2 gene is only a few hundred bases from the terminal exon of type VII α1 collagen. Ucn3, on the other hand possesses an extended intron of >8000 bases. The position of the 3′ UTR of the mRNA is unclear. The gene is found on human chromosome 10p15.1 and on mouse chromosome 5.

Taxon-specific selection pressure has induced a number of alternative expression patterns of the CRH family genes. For example, in the elephant shark, the Ucn2 gene seems to be a pseudogene, although genes for CRH, UI and Ucn3 are present. Unusually, there are two CRH paralogs unique from UI in this species, an observation that is currently unique in chordates.[29] Although there are two CRH paralogs in a number of teleost fish,[22] these peptides are almost identical and reflect a phylogenetically late genome duplication, and probably possess the same function. However, in the sequence differences between the two CRH paralogs in the elephant shark are highly divergent indicating a gene duplication early in this lineage's 400 million year evolution. The retention of the same precursor structure in the second paralog suggests that this peptide is functional as a CRH but possesses a different function and expression pattern in this species. Interestingly, like the elephant shark, the chimpanzee, also possesses a Ucn2 gene that is a pseudogene as the result of a premature stop codon. Moreover, in humans, this gene lacks the amidation signal that is required for full activity, although despite this, numerous studies indicate that this peptide is functional in humans.[7,17] Thus, in light of the close sequence similarity between Ucn2 and 3, it seems that the Ucn2 gene and its peptide function is being lost in some taxa and its function is being replaced by Ucn3.[7,14] A similar situation occurs with the loss of gonadotropin-releasing hormone (GnRH)-II in mammals.[7]

DISTRIBUTION AND EXPRESSION OF mRNA

In mammals, the regulation of pituitary ACTH by CRH occurs primarily via projections from the parvocellular

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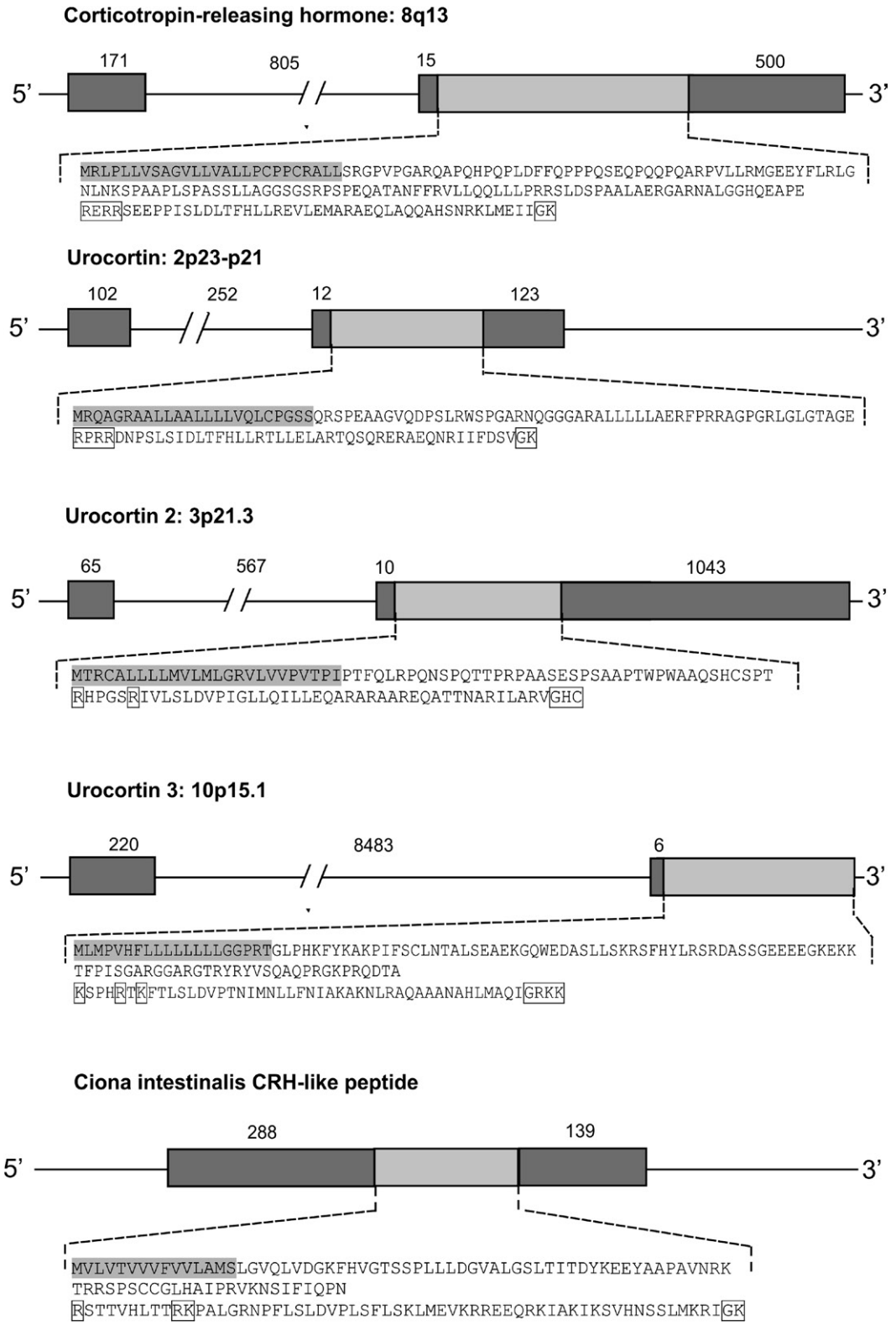

FIGURE 2 Gene and cDNA structure of CRF family members. For each paralog, a schematic of the gene structure (not to scale) is shown along with its position on the relevant human chromosome. Boxes represent exons, and lines indicate intronic regions. The number of bases of the 5′ and 3′ untranslated regions, and introns, are shown above the relevant regions. The translated region of the gene is indicated by the human preprohormone sequence corresponding to the light gray box. The mature peptide is shown bracketed by the cleavage and amidation motifs shown in clear boxes.

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division of the paraventricular nucleus (PVN) in the hypothalamus to the fenestrated capillaries of the median eminence. Its role as a hypothalamic releasing factor for ACTH has been conserved in both sarcopterygian and actinopterygian lineages. Indeed, its presence in the nucleus preopticus and nucleus lateralis tuberalis of elasmobranches indicates that its role as a hypothalamic releasing factor evolved early in vertebrate ancestry.[22,31] In many teleost species, CRH is also synthesized in the nucleus preopticus and is transported along neurosecretory processes to the neurohypophysis where it is released near the pituitary corticotropes. However, CRH is expressed widely within the brain of all vertebrates, where it plays a neuromodulator role. CRH mRNA expression occurs predominantly in rostral regions of the brain. In the rat forebrain, there are clusters of expression in the bed nucleus of the stria terminalis (BNST), central nucleus of the amygdala (CeA), septal regions, olfactory lobe and olfactory tract as well in isolated expression throughout the cortex. In caudal regions, the adrenergic cell groups, A1 and A5, and the parabranchial nucleus are sites of CRH expression (Fig. 3).[6,31]

It is difficult to determine how homologous sites of CRH expression in fishes are to those in mammals, as few in situ hybridization studies in the former have been performed. Immunohistochemical studies suggest that the general pattern of CRH expression in the rostral regions is essentially retained, although the topology of the teleost forebrain differs considerably from that of mammals. For example, in the tilapia, the greatest concentration of immunoreactive (ir) CRH occurs in the lateral part of the ventral telencephalon where it may be released into the systemic circulation.[27] In the brown ghost fish, irCRH was not observed in regions caudal to the posterior diencephalon,[35] and many species show reduced irCRH in caudal regions. Amphibians have caudal CRH expression patterns similar to that found in mammals. Thus, CRH expression patterns may have evolved differently among actinopterygian and sarcopterygian lineages.[22]

In comparison with CRH, Ucn has a much more caudal distribution. The primary site for Ucn mRNA expression in mammals is the Edinger–Westphal nucleus although there are small regions of Ucn expression in the lateral hypothalamic

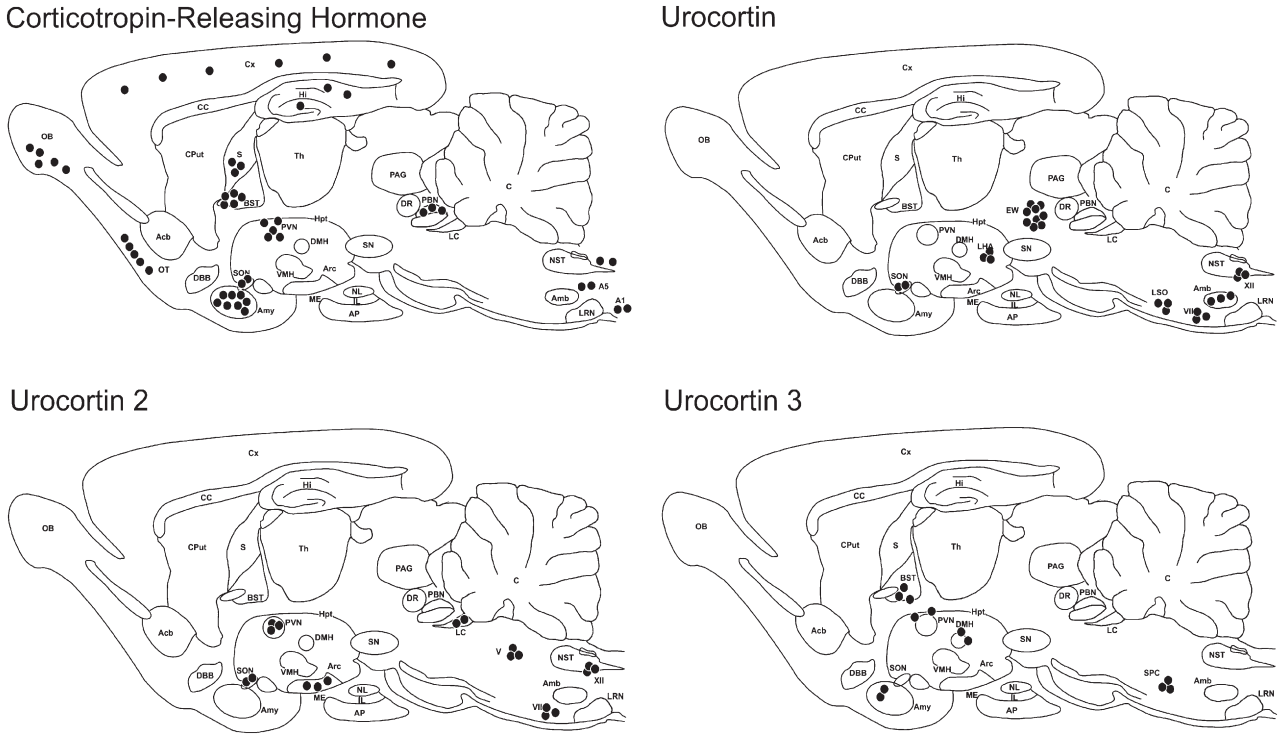

FIGURE 3 Neuroanatomical localization of CRF family member mRNA in rat brain. Regions of immunoreactivity not confirmed by in situ hybridization histochemistry are not shown. See text for further description. Abbreviations: A1, A5, adrenergic cell groups; Acb, nucleus accumbens; Amb, nucleus ambiguous; Amy, amygdala; AP, anterior pituitary; Arc, arcuate nucleus of the hypothalamus; BST, bed nucleus of the stria terminalis; C, cerebellum; CC, corpus callosum; Cput, caudate putamen; Cx, cerebral cortex; DBB, diagonal band of Broca; DMH, dorsomedial nucleus of the hypothalamus; DR, dorsal raphe nucleus; EW, Edinger–Westphal nucleus; Hi, hippocampus; Hpt, hypothalamus; IL, intermediate lobe of the pituitary; LC, locus coeruleus; LRN, lateral reticular nucleus; LSO, lateral superior olive; ME, median eminence; NL, neural lobe of the pituitary; NST, nucleus of the solitary tract; OB, olfactory bulb; OT, olfactory tract; PAG, periaqueductal gray; PBN, parabrachial nucleus; PVN, paraventricular nucleus of the hypothalamus; S, septum; SN, substantia nigra; SON, supraoptic nucleus; Th, thalamus; V, trigeminal motor nucleus; VII facial motor nucleus; VMH, ventromedial nucleus of the hypothalamus; XII hypoglossal motor nucleus.

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area, and the supraoptic nucleus (SON) of the hypothalamus.[34] Ucn is also expressed in a number of brainstem sites (Fig. 3). UI is expressed in the nucleus of the medial longitudinal fasiculus in fishes. However, the majority of UI expression in fishes actually occurs in the urophysis.[4] In phylogenetically older vertebrates and actinopterygians, in general, UI expression is predominant in the spinal cord and brainstem sites where CRH does not seem to be present.[22] In amniotes, however, CRH expression is associated with nuclei that contribute descending autonomic fibers.

Ucns 2 and 3 have a restricted pattern of expression in comparison with CRH and urocortin (Fig. 3). In the rat hypothalamus, Ucn2 mRNA has been detected in the PVN, SON, and arcuate nucleus (Arc). In the brainstem, Ucn2 mRNA is present in the locus coeruleus (LC) and in the trigeminal (V), facial (VII) and hypoglossal (VII) motor nuclei.[30] The main region of Ucn3 expression occurs as clusters of cells associated with the columns of the fornix and throughout the rostral hypothalamus. Regions of expression are also detected in the posterior aspects of the BNST and lateral hypothalamic regions, lateral to the PVN and in dorsal and lateral regions around the dorsomedial hypothalamic nucleus. A few Ucn3-expressing cells have also been detected in the anterodorsal part of the medial amygdaloid nucleus and near the anterior periventricular nucleus and retrochiasmatic area. In the brainstem, the superior paraolivary nucleus is one of the few sites of Ucn3 expression.[18]

PROCESSING OF PREPROHORMONE

In all CRH paralogs, the prohormone consists of a signal peptide, a gene-associated peptide, and a mature peptide (Fig. 2). The comparative simplicity of the preprohormone and conservation of the general structure among paralogs suggest that the peptide is processed in a similar manner regardless of where the gene is expressed. All CRH paralogs generally possess a well-defined signal peptide with a basic residue within a few residues from the initial methionine. Human Ucn2 lacks this basic residue, however. The signal peptides consist of <30 residues with a strong hydrophobic core. The gene-associated peptide, or cryptic peptide, spanning the region between the signal peptide and the mature peptide is highly variable where there is little consensus among paralogs. CRH and Ucn3 possess the longest gene-associated peptides, and Ucn and Ucn2, the shortest. There may be a relationship between the length of the gene-associated peptide and the physiological constraints on the peptide as CRH and Ucn3 are the most highly conserved whereas the Ucn and Ucn2 paralogs are the least conserved among the vertebrates.

The mature peptide is flanked by prohormone convertase (PC) cleavage sites (Fig. 2; see PC chapters in the Peptide Biosynthesis/Processing section of the book). The organization of the PC cleavage sites vary between the CRH/Ucn lineage, and the Ucn2/3 lineage. CRH and Ucn possess an R-X2-R motif, consistent with recognized PC cleavage motifs of R/K-Xn-R/K where X is any amino acid and typically varies between 2, 4, and 6 residues.[32] Human Ucn2 has the motif R-X4-R, but in the mouse and the rat, there are 7 residues between the two basic residues. The number of residues between the basic residues also varies among species at the Ucn3 cleavage. In most, if not all the cases, the mature peptide is amidated. Human Ucn2 has a peculiar motif of GHC instead of the usual G-R/K-R/K; thus, it is not clear whether this version of the peptide is amidated or if it exists as a larger disulfide-bridged cyclic polypeptide.[30] All peptides are amphiphilic and exist predominantly as α helices.

RECEPTORS AND BINDING PROTEINS

The first human CRH receptor (CRH_1) was isolated by expression cloning of cDNA from a human Cushing's corticotropic adenoma.[8] This discovery was followed by the characterization of receptor orthologs in numerous tetrapod and actinopterygian species. The CRH_1 receptor also exists in a series of splice variants including, a frameshift mutation in the rat, a 29 amino acid insertion in the human form, a 133-bp deletion in the sheep isoform and variant possessing a deletion in the seventh transmembrane domain expressed in human myometrium. A paralogous CRH receptor system (CRH_2) was discovered shortly after that of the CRH_1 and exists in at least three different functional splice variants in humans and two in rats although there are no major pharmacological differences among them.[8] Both CRH_1 and CRH_2 receptors belong to the family of G protein-coupled receptors and are structurally related to the calcitonin and the CRH-like insect diuretic hormone receptors.[13] In the brain, stimulatory coupling the CRH receptors to adenylate cyclase is the primary signal transduction system, although a number of other pathways have been associated with CRH signaling systems in nonneural tissues and cells.[8] The CRH_1 receptor is predominant in the brain, whereas the CRH_2 receptor is more prevalent in peripheral tissues. The mammal CRH_1 receptor binds CRH and Ucn with similar affinity but shows little affinity for Ucn2 and 3. However, there are a number of species differences in the relative affinities among the various CRH paralogs.

In addition to the receptors, a high-affinity and selective soluble CRH-binding protein is present in the brain and pituitary gland.[28] The function of the CRH-binding protein is not entirely understood. It shows the greatest affinity for Ucn, less affinity for CRH, and little affinity for Ucns 2 and 3. This protein may act as a sequestering mechanism to attenuate abrupt changes in CRH release, to reduce the CRH/Ucn response after the peptide release, or it may provide ligand specificity in loci where multiple

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CRH family peptides are released. The basic structure of the CRH-binding protein has evolved early in metazoan history as evidenced by the presence of CRH-binding protein ortholog in insects.[16] However, the ligand specificity of the CRH-binding protein may have changed in the chordates, relative to the insects. The mammal CRH shows little binding affinity for Ucn2 and 3, yet these peptides possess a number of significant sequence similarities with the only CRH ortholog in insects. Insects do not seem to possess an analogous hypothalamic–pituitary–adrenal/interrenal (HPA/I) axis;[20,25] thus, the CRH-binding protein may have been selected for CRH/Ucn/UI specificity once these peptides became associated with the stress axis. CRH also has a second soluble binding protein that is a splice variant of the CRH_2 receptor; however, the physiological significance of this binding protein is not known, and also whether or not it plays a function distinct from the CRH-BP.[5] However, because the CRH_2 receptor binds Ucn 2 and 3, whereas the CRH-BP does not, the soluble CRH_2 receptor may act to modulate the actions of Ucn 2 and 3 in the similar manner that the CRH-BP does for CRH and Ucn.

BIOLOGICAL ACTIONS IN THE CENTRAL NERVOUS SYSTEM

The ancient evolution of CRH and its paralogs indicates that this peptide system has had a significant period of time for selective pressure to allow interaction into a number of diverse physiological systems. In vertebrates and humans, CRH and Ucn elicit behaviors resembling the response to the stress and environmental novelty such as increased arousal decreased food intake, diminished sexual and reproductive activity, and increased grooming. Thus, generally the CRH system acts to integrate the autonomic and behavioral responses to stress through interactions at the CNS level. These mechanisms are achieved by a CRH_1 receptor-mediated activation of the hypothalamic–pituitary–adrenal and sympathetic branches of the stress response. By contrast, the CRH_2 receptor has been associated with anxiolytic effects; thus, Ucns 2 and 3 have been deemed anxiolytic peptides as they bind exclusively to this receptor. CRH and Ucn are generally associated with anxiogenic actions.[7] A number of other neurological actions of CRH family peptides have been described.

Studies in nonmammalian vertebrates identified a number of additional functions of the CRH system. A CRH-mediated role on locomotor activity was established shortly after the peptides discovery and is conserved among vertebrates.[10] In the brainstem of the newt, discharges of neurons of the raphe region of the midbrain, associated with walking and swimming behaviors, could be modified by the injections of CRH into the lateral ventricle.[22] Other actions induced by CRH peptides include vocalization changes, modulation of the reproductive axis, and modulation of circadian-induced locomotor behaviors.[7,8,22] In amphibians, CRH is a potent thyrotropin-releasing factor to regulate synthesis and release of thyroid hormones. Because the thyroid axis plays a primarily role in the regulation of metamorphosis, CRH may act to directly regulate metamorphosis in this taxon.[5]

CRH family peptides have well-described effects on feeding and weight regulation in both mammals and fishes. CRH itself is intrinsically involved in energy metabolism. In vertebrates, CRH ultimately induces the release of adrenal/interrenal glucocorticoids. The metabolic actions of the glucocorticoids are to provide the organism with enough energy to survive a stressful situation. Glucocorticoids possess a number of actions associated with the use and flow of energy. This includes the de novo synthesis of glucose; gluconeogenesis, is increased in the liver after mobilization of proteins from the skeletal muscle and subsequent deamination of amino acids that are released during protein breakdown. However, glycogen can also be deposited in the liver because of a glucocorticoid-stimulated increase in the glycogen synthase reaction. In tandem with this, the breakdown of glycogen is inhibited by glucocorticoids.[24] In rats, Ucn is the most potent peptide at decreasing food intake.[8,22] In fishes, food intake is also reduced significantly after CRH administration.[9] The role for the CRH-like diuretic hormones in insect feeding suggests that this mechanism is phylogenetically ancient[2] (see Diuretic/Antidiuretic chapter in Invertebrate Peptides section of the book). The overexpression of the CRH-BP in a mouse also affects body weight[21] presumably by sequestering free CRH thereby reducing its receptor activation ability in circuits associated with food intake. The CRH family, and particularly that of Ucn2 and 3, play a critical role in energy, and especially, in glucose homeostasis. Ucn2 can increase tissue sensitivity to insulin, whereas Ucn3 may act to increase the secretion of pancreatic insulin. However, these mechanisms appear to involve the integrated actions of both CNS and peripheral targets.[17] Given that Ucn2 and 3 possess a number of ancient structural features of the CRH family, this role in energy metabolism may reflect some of their ancestral functions.

CRH can inhibit reproduction directly through direct input on GnRH secretory terminals at the level of the infundibular nucleus and also indirectly by the inhibitory actions of adrenal glucocorticoids on hypothalamic gonadotropin-releasing hormone (GnRH) and pituitary gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH). Sexual dimorphism of the CRH response in humans and other vertebrates, even after the sex steroid feedback mechanisms have been taken into consideration, has been reported.[3] For example, generally the HPA and autonomic responses tend to be lower in women compared with men

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of the same age. In aged-matched men, a greater ACTH a cortisol increase occurs with either physiological exertion such as exercise or pharmacological administration of CRH. Moreover, during aging, a significant increase in the number of CRH neurons occurs in men but not women.[3] The physiological reasons for this are not clear but may involve the different energy demands between the sexes and in particular how this energy is partitioned for use in reproductive-associated physiology. However, it is also not clear as to how this relationship translates across taxa.

The functional role of Ucn in mammals, only provides a partial understanding of its ortholog, UI, in fishes. Urophyseal UI has been implicated in ion and fluid equilibrium, cardiovascular activity and participates in interrenal tissue glucocorticoid release, all of which are necessary for the adaptability of fishes. In vertebrates, UI probably became specialized for its role in the urophysis in an analogous manner that CRH became associated with the HPA/I axis. The urophysis or caudal neurosecretory system is analogous to the neurophysis in that it is a neurohemal organ where the neurosecretory cells release their constituents into the fenestrated capillaries of the vascular system. The caudal neurosecretory system is found in all fishes but has been reduced in the sarcopterygian line and lost entirely in the tetrapods. Thus, this organ system seems to have evolved in response to the osmoregulatory stress of rapidly-changing ambient conditions that occur in aquatic environments. In addition, because water is a much denser medium than air, integration of mechanoreception with the stress response is advantageous for survival if these compression waves can alert the organism of potential prey or predators. Thus, we might speculate that, whereas the pituitary and hypothalamus has been used by chordates to link the higher integration of some sensory systems with the appropriate neural-humoral responses, the urophysis has evolved to link the peripheral osmoreceptor and mechanoreceptor inputs of fishes to the appropriate stress response. This role of UI with mechanoreceptors may also be reflected by the relationship urocortin has with the regulation of auditory sensory processing in mammals.[20,22]

PATHOPHYSIOLOGICAL IMPLICATIONS

CRH is a critical mediator of fear conditioning and other forms of emotional memory to both aversive and rewarding stimuli. In humans, the most recent investigations of the etiology of depression and mood disorders implicate a central role for CRH. Although an overactivated HPA axis has been implicated in a number of depressive disorders, it remains equivocal whether CRH overactivation, per se, has a direct effect on these pathologies. A number of these conditions may work through a common pathway associated with an overactivated HPA axis, and therefore, a direct cause and effect relationship between CRH and the pathology has been problematic to ascertain. However, the CRH-secreting brain cells receive their primary positive feedback from the amygdala and negative feedback from the hippocampus. The hippocampus and neocortex mediate the cognitive aspects of depression such as memory impairment and feelings of worthlessness, hopelessness, guilt, and suicidal feelings. A number of postmortem studies of suicidal victims have increased levels of CRH and cell numbers in the hypothalamus.[1] Increased CRH is also been implicated in addictive aspects of cocaine and other agents[18] suggesting that a CRH-mediated stress response may exacerbate the addictive potential of a number of agents. Other studies implicate a role for the CRH family peptides in neural development and plasticity and thus in the onset of some neurodegenerative disorders such as Alzheimer's Disease. However, the mechanisms and conditions by which this occurs is not understood. Other studies have indicated that CRH-related peptides can have neuroprotective effects.[8,11,26]

REFERENCES

1. Arato M, Banki CM, Bissette G, Nemeroff CB. Elevated CSF CRF in suicide victims. *Biol Psychiatry* 1989;**25**:355–9.
2. Audsley N, Goldsworthy GJ, Coast GM. Circulating levels of locust diuretic hormone: the effect of feeding. *Peptides* 1997;**18**:59–65.
3. Bao AM, Meynan G, Swaab DF. The stress system in depression and neurodegeneration: focus on the human hypothalamus. *Brain Res Rev* 2008;**57**:531–53.
4. Barsyte D, Tipping DR, Smart D, Conlon JM, Baker BI, Lovejoy DA. Rainbow trout (*Oncorhynchus mykiss*) urotensin-I: structural differences between urotensins-I and urocortins. *Gen Comp Endocrinol* 1999;**115**:169–77.
5. Boorse GC, Denver RJ. Widespread tissue distribution and diverse functions of corticotropin-releasing factor and related peptides. *Gen Comp Endocrinol* 2006;**146**:9–18.
6. Bittencourt JC, Sawchenko PE. Do centrally administered neuropeptides access cognate receptors? An analysis in the central corticotropin-releasing factor system. *J Neurosci* 2000;**20**:1142–56.
7. Chand D, Lovejoy DA. Stress and reproduction: controversies and challenges. *Gen Comp Endocrinol* 2011;**171**:253–7.
8. Dautzenberg FM, Hauger RL. The CRF peptide family and their receptors: yet more partners discovered. *Trends Pharm Sci* 2002;**23**:71–7.
9. De Pedro N, Pinillos ML, Valenciano AI, Alonzo-Bedate M, Delgado MJ. Inhibitory effect of serotonin on feeding behaviour in goldfish: involvement of CRF. *Peptides* 1998;**19**:505–11.
10. Donaldson CJ, Sutton SW, Perrin MP, Corrigan AZ, Lewis KA, Rivier JE, et al. Cloning and characterization of human urocortin. *Endocrinology* 1996;**137**:2167–70.
11. Dunn AJ, Berridge CW. Physiological and behavioral responses to corticotropin-releasing factor administration: is CRF a mediator of anxiety or stress responses?. *Brain Res Rev* 1990;**15**:71–100.
12. Erspamer V, Melchiorri P, Broccardo M, Erspamer GF, Falaschi P, Improta G, et al. The brain–gut–skin triangle: new peptides. *Peptides* 1981;**2**(Suppl. 2):7–16.

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13. Fredricksson R, Lagerstrom L, Lundin LG, Schioth H. The G protein coupled receptors in the human genome form five main families. Phylogenetic analysis, paralogon groups and fingerprints. *Mol Pharmacol* 2003;**63**:1256–72.
14. Ikemoto T, Park MK. Comparative genomics of the endocrine system in humans and chimpanzees with special reference to GnRH 2 and UCN2 and their receptors. *Genomics* 2006;**87**:459–62.
15. Hsu SY, Hseuh AJW. Human stresscopin and stresscopin-related peptide are selective ligands for the type 2 corticotropin-releasing hormone receptor. *Nat Med* 2001;**7**:605–11.
16. Huising MO, Flik G. The remarkable conservation of corticotropin-releasing hormone (CRH)-binding protein in the honeybee (*Apis mellifera*) dates the CRH system to a common ancestor of insects and vertebrates. *Endocrinology* 2000;**146**:2165–70.
17. Kuperman Y, Chen A. Urocortins; emerging metabolic and energy homeostasis perspectives. *Trends Endocrinol Metab* 2008;**19**:122–9.
18. Kupferschmidt D, Lovejoy DA, Rotzinger S, Erb S. Teneurin C-terminal associated peptide (TCAP)-1 blocks the effects of corticotropin-releasing factor (CRF) on the reinstatement of cocaine seeking and expression of cocaine-induced behavioral sensitization. *Brit J Pharmacol* 2011;**163**:574–83.
19. Lewis K, Li C, Perrin MH, Blount A, Kunitake K, Donaldson C, et al. Identification of urocortin III, an additional member of the corticotropin-releasing factor (CRF) family with high affinity for the CRF2 receptor. *Proc Natl Acad Sci U S A* 2001;**98**:7570–5.
20. Lovejoy DA. Structural evolution of urotensin-I: retaining ancestral functions before corticotropin-releasing hormone evolution. *Gen Comp Endocrinol* 2009;**164**:15–9.
21. Lovejoy DA, Aubry JM, Turnbull A, Sutton S, Potter E, Yehling J, et al. Ectopic expression of the CRF-binding protein: minor impact on HPA axis regulation but induction of sexually dimorphic weight gain. *J Neuroendocrinol* 1998;**10**:483–549.
22. Lovejoy DA, Balment RJ. Evolution and physiology of the corticotropin-releasing factor (CRF) family of neuropeptides in vertebrates. *Gen Comp Endocrinol* 1999;**155**:1–22.
23. Lovejoy DA, Barsyte-Lovejoy D. Characterization of a diuretic hormone-like peptide from tunicates: insight into the origins of the vertebrate corticotropin-releasing factor (CRF) family. *Gen Comp Endocrinol* 2010;**165**:330–6.
24. Lovejoy DA, Barsyte D. *Sex, stress and reproductive success*. Chichester UK: Wiley Blackwell; 2011, p. 188.
25. Lovejoy DA, Jahan S. Phylogeny and evolution of the corticotropin releasing factor family of peptides. *Gen Comp Endocrinol* 2006;**146**:1–8.
26. Nemeroff CB, Owens MJ. Treatment of mood disorders. *Nat Neurosci Suppl* 2002:1068–70.
27. Pepels PPLM, Meek J, Wendelaar Bonga SE, Balm PHM. Distribution and quantification of the corticotropin-releasing hormone (CRH) in the brain of the teleost fish *Oreochomis mossambicus* (tilapia). *J Comp Neurol* 2002;**453**:247–68.
28. Potter E, Behan DP, Fischer WH, Linton EA, Lowry PJ, Vale WW. Cloning and characterization of the cDNAs for human and rat corticotropin-releasing factor-binding proteins. *Nature* 1991;**349**:423–6.
29. Nock TG, Chand D, Lovejoy DA. Identification of members of the gonadotropin-releasing hormone (GnRH), corticotropin-releasing factor (CRF) families in the genome of the holocephalan, *Callorhinchus milii* (elephant shark). *Gen Comp Endocrinol* 2011;**171**:237–44.
30. Reyes TM, Lewis K, Vale WW. Urocortin II: a member of the corticotropin-releasing factor (CRF) neuropeptide family that is selectively bound by type 2 CRF receptors. *Proc Natl Acad Sci U S A* 2001;**98**:2843–8.
31. Sawchenko PE, Swanson LW. Localization, colocalization, and plasticity of corticotropin-releasing factor immunoreactivity in rat brain. *Fed Proc* 1985;**44**:221–7.
32. Seidah NG, Chretien M. Proprotein and prohormone convertases: a family of subtilases gene diverse bioactive polypeptides. *Brain Res* 1999;**848**:45–62.
33. Vale W, Spiess J, Rivier C, Rivier J. Characterization of a 41-residue ovine hypothalamic peptide that stimulates secretion of corticotropin and b-endorphin. *Science* 1981;**213**:1394–7.
34. Vaughan J, Donaldson C, Bittencourt J, Perrin MH, Lewis K, Sutton S, et al. Urocortin, a mammalian neuropeptide related to fish urotensin-I and to corticotropin-releasing factor. *Nature* 1995;**378**:287–92.
35. Zupanc GKH, Horschke I, Lovejoy DA. Corticotropin releasing factor in the brain of the gymnotiform fish, *Apteronotus leptorhynchus*: immunohistochemical studies combined with neuronal tract tracing. *Gen Comp Endocrinol* 1999;**114**:349–64.

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Chapter 102

Endozepines

Marie-Christine Tonon, Jerome Leprince, Fabrice Morin, Pierrick Gandolfo, Vincent Compere, Georges Pelletier, Maria M. Malagon and Hubert Vaudry

ABSTRACT

The existence of specific binding sites for benzodiazepines has led to the discovery of endogenous ligands for benzodiazepine receptors that are collectively designated by the term endozepines. In the brain and in peripheral nervous tissues, endozepines are exclusively expressed by glial cells. The release of endozepines from cultured astrocytes is finely regulated by neurotransmitters and neuropeptides. Endozepines exert their effects either through classical central- and peripheral-type benzodiazepine receptors or through *G* protein-coupled receptors. Intracerebroventricular injection of endozepines induces marked effects on anxiety, sleep, and food consumption. The concentration of endozepines in the brain and cerebrospinal fluid is affected in various diseases including depression, Alzheimer's disease, and hepatic encephalopathy.

DISCOVERY

Benzodiazepines (BZDs) are artificial pharmaceutical compounds that are widely used for their sedative, anxiolytic, anticonvulsivant, and myorelaxant activities. The search for endogenous ligands for BZD receptors has led to the discovery of diazepam-binding inhibitor (DBI), a 10-kDa peptide originally identified from the rat brain for its ability to displace diazepam from its binding sites. Subsequently, the purification or the molecular cloning of DBI in different species has shown that the sequence of the protein has been relatively well conserved during evolution from bacteria to mammals (Fig. 1).

STRUCTURE OF DBI mRNA/GENE

In humans, the DBI gene is located on chromosome 2 (q12-21) and is composed of four exons. In addition to the common variant encoding an 86-amino acid protein (Fig. 2), ten different transcripts of the gene have been reported in human. All of them show variable 5′-ends due to an alternative promoter or alternative first exon.[26] These variants encode N-terminal truncated or extended isoforms of DBI. The mammalian DBI promoter displays consensus recognition sites for various known transcription factors, including AP-1/2, SP-1, ETF, Y-box-binding protein, CTF/NF-1, C/EBP, HNF-3, SRE-like sequence, GREs, and PPREs (Fig. 2). Although the DBI gene exhibits several typical features of housekeeping genes (e.g. lack of TATA and CAAT boxes, high GC content, multiple transcription initiation sites, consensus sequence for binding of SP-1 and ETF), its expression is clearly regulated by insulin, glucose, NFκB, and androgens,[26,31] indicating that DBI may serve as a housekeeping protein in certain cell types and as a tissue-specific protein in others.

PROCESSING OF THE DBI PRECURSOR

Endoproteolytic cleavage of $DBI_{1–86}$ generates several biologically active peptides including, the triakontatetraneuropeptide TTN ($DBI_{17–50}$) and the octadecaneuropeptide ODN ($DBI_{33–50}$), which are present in various proportions in the different brain regions.[22] The DBI sequence is devoid of dibasic motifs that are typical cleavage sites for prohormone convertases, but it contains multiple individual lysine residues (Fig. 1), three of which, located at positions 16, 32, and 50, delimit the sequences of TTN and ODN (Fig. 3). However, to date, the enzymes responsible for the processing of DBI have not yet been characterized.

DISTRIBUTION OF DBI mRNA AND ENDOZEPINE IMMUNOREACTIVITY

In situ hybridization histochemical labeling has shown that DBI mRNA is widely distributed in the rat brain, the highest concentrations being found in the cerebellum, the ependyma, and the area prostrema.[1,4] In the cerebellum, the DBI gene is strongly expressed in Bergman (astrocyte-like) cells. High concentrations of DBI mRNA are also present in other glial cell populations, for example, ependymocytes bordering the third ventricle and tanycytes in the median eminence (Fig. 4a). In the pituitary, DBI mRNA occurs in the neural

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00102-0
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                          10         20         30         40         50         60         70         80
Mammals
Human     SQAEFEKAAE EVRHLKTKPS DEEMLFIYGH YKQATVGDIN TERPGMLDFT GKAKWDAWNE LKGTSKEDAM KAYINKVEEL KKKYGI
Rat       •••D•DK••• ••KR•K•Q•T ••••••••S• FK••••••V• •DR••L••LK •K•K••S••K •K•••K•N•• KT•VEK•••• KKK•••
Horse     •••••DR••• ••KN•K•K•A •D••••••S• •K•••••V• ••R••L••LK •K•K•••••A •K•••K•••• K••••K••D• KKK•••
Pig       ••••••K••• ••KN•K•K•A •D••••••S• •K•••••••• ••R••I••LK •K•K•••••G •K•••K•••• K••••K•••• KKK•••
Beef      •••••DK••• ••K••K•K•A ••••••••S• •K•••••••• ••R••••••K •K•K•••••• •K•••K•••• K•••DK•••• KKK•••
Dog       •••••DK••• D•K••K•K•A •D•••Y••S• •K•••••••• ••R••L••LR •K•K•••••Q •K•••K•••• K••V•K••D• KKK•••
Rabbit    ••••••K••• ••KN•K•K•A •A••••••S• •K••••••V• ••R•••••LK •K•K•••••• •K••K•S•• R••VDK•••• KQK•••
Birds
Duck      AE•A•QK••• ••KQ•KSQ•• •Q•••DV•S• •K•••••••• ••R••••••K •K•K•••••A •K•M•K•••• K••VAK•••• KGK•••
Chicken   •E•A•QK••• ••KE•KSQ•T •••••DV•S• •K•T••••V• •DR••••••K •K•K•••••A •K•M•K•••• K••VAK•••• KGK•••
Reptiles
Turtle    •••••DK••• ••KQ•KSQ•T •••••Y••S• FK••••••••• ••R••F•••K •K•K••••DA •K•MAK•E•• K•••AK•••• KGK•••
Amphibians
Frog      SP••D•DK••G D•KK•K•K•T •D•LKEL••L •K•S•••••• I•C•••••LK •K•K•••••L KK•L•K•••• S••VSKAH•• IEK••L
Toad      •••A•EK••• ••KQ•K•T•T •••••ET•AL •K••••••V• •DR••M•••K •K•K•••••• KK•••K•••• AQ•••L••K• KGK••C
Fishes
Carp      •E•••QK••• ••KQ•KAKFT •A•••E••SL •K••••E•V• •ARF•••••• •K•K•E••EA KKQM•KD••• K•••AK•••• KGK•••
Goldfish  •E•••QK••• ••KQ•KAK•A •A•••E••SL •K••••••V• •AR••••••• •K•K••••EA KK•M•K•••V K•••AK•••• KGK•••
Echinodermes
Urchin    •D•-•NT••A ••FD•A•S•N D--L•K••SL FK•V••••C• •DR•Q•••YK •T•K•••••G •K•K•TA••E KE••EL•GQ• KTSCQMK
Insects
Drosophila VSEQ•NA••• K•KS•TKR•• •D•F•QL•AL FK••S•••ND •AK••L••LK •K•K•E•••K QK•K•S•A•Q QE••TF••G• VAK•A
Mosquito  SLDQQ•NE••• K•KTFTKR•• •Q•L•EL•AL FK••S•••NT ••K•••F•LK •K•K•Q••SD KK•I•QDA•K E••VKF•••• SAKCL
Nematodes
C. elegans  TLS•DD••A T•KT•K•S•• ND•L•KL•AL FK•G••••NT •DK•••F•LK •K•K•S••D• KK•LAKD••Q K••VAL•••• IAK••A
Fungi
S. cerevisae VSQL•L•K•K A•NE•P•K•• TD•L•EL•AL •K••••••ND K•K••IFNMK DRYK•EDAEN •K•K•Q•••E KE••AL•DQ• IAK•SS
Plants
A. thaliana GLKE•••EH•• K•NT•TEL•• N•DL•IL••L •K••KF••VD •SR•••FSMK ER•K••••KA VE•K•S•E•• ND••TK•KQ• LEVAASKAST
D. lanata ALKD•••EH•• KAKT•PEST• N•NK•IL••L •K•••••NV• •SR••IFNMK DR•K••••KA VE•K•Q•E•• GE••TK•KQ• CEAATASS
Bacteria
S. aurantiaca ALEDD•RS•Q• R•KA•K•R•• N•RL•EL•SL FK•••E••VQ GKR••I••VK ER•KY•••VG RK•LGR•S•• QQ•VAL••R• LRG
```

FIGURE 1 Comparison of selected DBI sequences of representative species from bacteria to mammals. Dots denote identical amino acids to the human sequence. Basic amino acids are in bold letters. The open boxes indicate acetylation of the N-terminal S residue, and phosphorylation of the T^{41} and/or T^{64} residues.

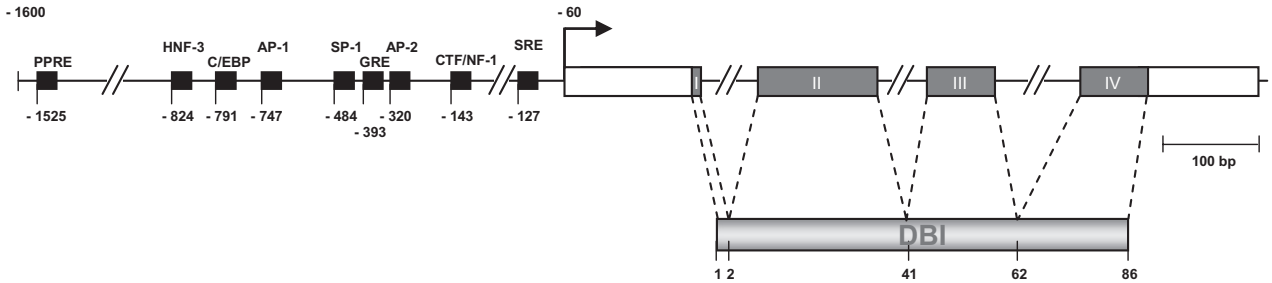

FIGURE 2 Genomic organization of the rat DBI gene. Recognition sites for transcription factors (PPRE, HNF-3, C/EBP, AP-1, SP-1, GRE, AP-2, CTF/NF-1, SRE) are indicated by black boxes. The transcribed regions are boxed and gray boxes (I, II, III, and IV) denote the coding regions. The shaded box represents the DBI sequence.

lobe in adult rat but is expressed only in the anterior and intermediate lobes during embryonic development.[4]

Immunohistochemical labeling has confirmed that in the brain and pituitary of tetrapods including frog, rat, monkey, and human, endozepine immunoreactivity is observed exclusively in glial cells,[18,32,35] that is, astrocytes, Gomori-positive astrocytes (a subset of brain astrocytes most prominent in the arcuate nucleus of the hypothalamus), Bergman cells, ependymocytes, tanycytes, and pituicytes. The density of immunoreactive glial cells is particularly high in the hypothalamus and olfactory bulb (Fig. 4b). Surprisingly, in the fish brain, endozepines are synthesized in a subpopulation of hypothalamic neurons.

DBI mRNA and DBI-like immunoreactivity are also present in numerous peripheral organs including the adrenal, testis, heart, kidney, liver, and intestine.[4] In these organs, endozepines are selectively expressed in specific cell types, notably in steroid-secreting cells and peripheral glial cells.

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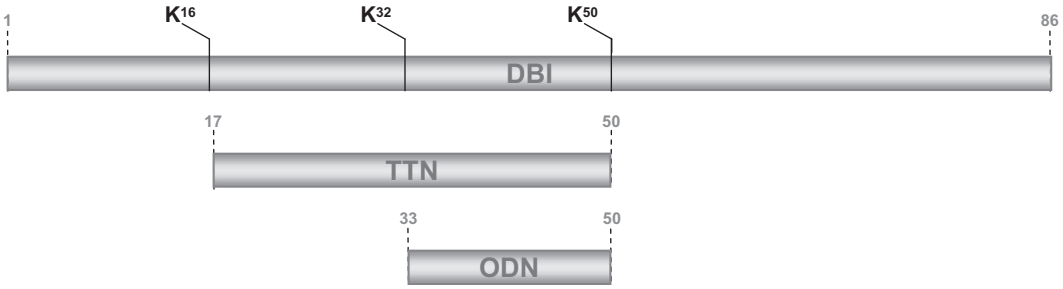

FIGURE 3 Schematic representation of DBI and two of its processing products, TTN and ODN. Cleavage sites, corresponding to individual lysine residues (K), that delimit the sequences of TTN and ODN, are indicated.

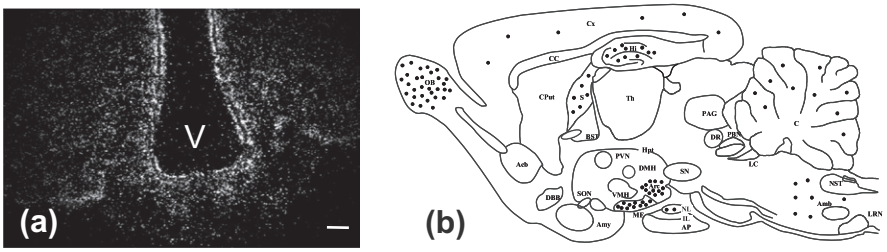

FIGURE 4 Localization of endozepines in the brain. (a) Dark-field photomicrographs showing the expression of DBI mRNA in cells bordering the third ventricule (V) of the hypothalamus. Scale bar = 250 μm (from Ref. 6 © Society for Endocrinology with permission). (b) Schematic parasagittal section through the rat brain depicting the distribution of endozepine-immunoreactive cells. The density of dots (•) is meant to be proportional to the density of endozepine-positive cells. Acb, nucleus accumbens; Amb, nucleus ambiguous; Amy, amygdale; AP, anterior pituitary; Arc, arcuate nucleus of the hypothalamus; BST, bed nucleus of the stria terminalis; C, cerebellum; CC, corpus callosum; Cput, caudate putamen; Cx, cerebral cortex; DBB, diagonal band of Broca; DMH, dorsomedial nucleus of the hypothalamus; DR, dorsal raphe nucleus; Hi, hippocampus; Hpt, hypothalamus; IL, intermediate lobe of the pituitary; LC, locus coeruleus; LRN, lateral reticular nucleus; ME, median eminence; NL, neural lobe of the pituitary; NST, nucleus of the solitary tract; OB, olfactory bulb; PAG, periaqueductal gray; PBN, parabrachial nucleus; PVN, paraventricular nucleus of the hypothalamus; S, septum; SN, substantia nigra; SON, supraoptic nucleus; Th, thalamus; VMH, ventromedial nucleus of the hypothalamus.

CONTROL OF BIOSYNTHESIS AND RELEASE OF ENDOZEPINES

The neuronal mechanisms regulating the secretion of endozepines have been studied in cultured astrocytes and Müller cells (retinal glial cells).[15,20,24,28,34] The release of endozepines is stimulated by pituitary adenylyl cyclase-activating polypeptide (PACAP), acting through the PACAP-specific receptor PAC1-R (see the chapter on PACAP in the Brain Peptides section) positively coupled to adenylyl cyclase and phospholipase C, by β-amyloid peptides acting through formyl peptide receptors, by urotensin II, and by steroid hormones. Endozepine release is inhibited by GABA, acting through $GABA_B$ receptors, and by somatostatin, acting through sst1, sst2, and sst4 receptors, all four receptors being negatively coupled to adenylyl cyclase (see also the chapter on Somatostatin/Cortistatin, in the Brain Peptides section). These factors also regulate DBI gene expression in a coordinated manner. In particular, PACAP and β-amyloid peptides increase, whereas somatostatin reduces DBI mRNA levels in cultured astrocytes. Altogether, these observations indicate that the biosynthesis and release of endozepines are finely regulated by various neuronal factors including classical neurotransmitters and neuropeptides.

RECEPTORS AND SIGNALING CASCADES

Endozepines have been originally characterized as inverse agonists of the central-type benzodiazepine receptor (CBR) that is an intrinsic part of the $GABA_A$ receptor–chloride channel complex. Electrophysiological studies have confirmed that DBI and ODN attenuate GABA-induced Cl^- efflux in neurons and endocrine cells,[3] indicating that endozepines act as negative allosteric modulators of the $GABA_A$ receptor. DBI and TTN can also interact with the peripheral-type benzodiazepine receptor now renamed as translocator protein (TSPO), a five-transmembrane-domain mitochondrial protein of 18kDa. In addition, endozepines can activate TSPO located at the plasma membrane, leading to an influx of Ca^{2+} through voltage-dependent calcium channels in glial cells. Finally, several studies have shown that endozepines can interact with metabotropic membrane receptors. In particular, in rat astrocytes, ODN stimulates phospholipase C through a $G\alpha_{i/0}$ protein-coupled receptor, whereas, in frog adrenocortical cells, TTN activates adenylyl cyclase, which in turn causes Ca^{2+} influx through T-type calcium channels.

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INFORMATION ON SOLUTION CONFORMATION OF ENDOZEPINES

Molecular modeling of bovine DBI_{1-86} under nuclear magnetic resonance (NMR) constraints has shown that the peptide exhibits a characteristic four-helix bundle structure, stabilized by three identified hydrophobic minicores (Fig. 5). The α-helix H1 forms strong interface contacts with α-helix H4, made up of the conserved hydrophobic residues Phe^5, Ala^9, Val^{12}, and Leu^{15} in H1, and of Tyr^{73}, Val^{77}, and Leu^{80} in H4. TTN, which is a selective ligand of TSPO, contains the hydrophobic portion corresponding to H2, whereas ODN, which preferentially binds CBR, fails to adopt an α-helical conformation. A three-dimensional quantitative structure–activity relationship study performed over 130 TSPO ligands supports the hypothesis that two lipophilic regions are essential for interaction with TSPO.[5] The three-dimensional structures of DBI and TTN, derived from NMR data, reveal the existence of a triad of lipophilic amino acids located in the H2 helix (i.e. Met^{46}, Leu^{47}, and Phe^{49}) as likely residues underlying TSPO binding.[5]

Structure–activity relationship studies have shown that the C-terminal octapeptide of ODN (OP, ODN_{11-18}) is the minimum sequence retaining full activity on metabotropic PLC-coupled receptors in astrocytes. The cyclic OP analog $cyclo_{1-8}OP$ is 3 times more potent than OP, whereas the D-Leu^5-substituted analog $cyclo_{1-8}$[D-Leu^5]OP is a full antagonist of this receptor.[17] In water, $cyclo_{1-8}OP$ adopts a single conformation encompassing a γ- and a β-turn (Fig. 6).

BIOLOGICAL ACTIONS OF ENDOZEPINES WITHIN THE BRAIN

Some of the biological effects of endozepines are mediated through CBR or TSPO, whereas others can be accounted for by activation of metabotropic receptors.

Intracerebroventricular (ICV) administration of DBI, TTN, or ODN increases aggressive interactions and induces anxiety and proconflict behavior in rodents.[16] These effects are prevented either by the specific CBR antagonist flumazenil or by the TSPO antagonist PK11195. DBI and ODN, acting through CBR, also shorten pentobarbital-induced sleeping time in mice.[11] ICV injection of ODN markedly reduces drinking and increases the aversive response to 0.9% NaCl in mice, and these effects are mediated through CBR.[23] ODN, acting via CBR, also inhibits apomorphine-induced yawning in rat, suggesting that endozepines can modulate dopaminergic and cholinergic neurotransmission involved in yawning.[14] Incubation of cultured rat astrocytes with picomolar concentrations of endozepines causes a robust increase in DNA synthesis through activation of CBR and TSPO,[13] indicating that endozepines act as autocrine/paracrine factors modulating the proliferation of astroglial cells. On isolated mitochondria from a glioma cell line, endozepines activate the formation of the steroid hormone precursor pregnenolone,[27] suggesting that endozepines also act as intracrine factors stimulating the synthesis of neurosteroids in astrocytes. Because several neuroactive steroids are potent allosteric modulators of the $GABA_A$ receptor, it seems that endozepines can indirectly regulate the activity of the $GABA_A$-benzodiazepine receptor complex through their ability to control the production of neurosteroids.[9]

A single ICV injection of very low doses of ODN (30–100ng) provokes a dose-dependent reduction of food intake in rodents and fishes.[10,25] Chronic ICV infusion of ODN for 15 days inhibits food consumption and causes a significant reduction in body weight in rat and mice. Reciprocally, food

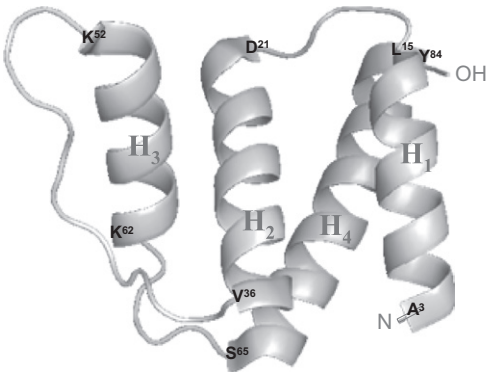

FIGURE 5 Three-dimensional NMR structure of bovine DBI_{1-86}. Helixes are referred to as H1, H2, H3, and H4. Residues limiting α-helixes are indicated. *Adapted from Ref. 5 with permission.*

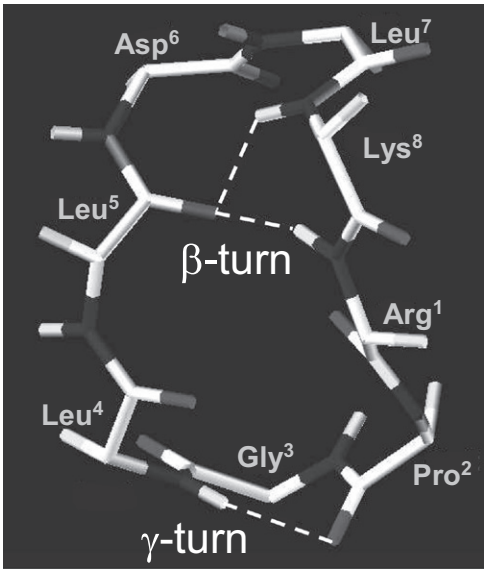

FIGURE 6 Lowest energy conformer of $cyclo_{1-8}OP$ from simulated annealing. The dotted lines indicate hydrogen bonds consistent with NMR data. *From Ref. 17, John Wiley & Sons Ltd with permission.*

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deprivation strongly decreases DBI expression in hypothalamic glial cells,[6] supporting a physiological role for endozepines in the central control of energy homeostasis. Although CBR agonists are known to stimulate food intake, the anorexigenic effect of ODN is not mediated through CBR or TSPO but via metabotropic endozepine receptors. ICV injection of ODN increases proopiomelanocortin (POMC) mRNA level and decreases neuropeptide Y (NPY) mRNA level in the arcuate nucleus of the hypothalamus,[7] suggesting that the effects of ODN on food consumption can be accounted for by stimulation of the production of the anorexigenic POMC-derived peptide α-MSH and inhibition of the production of the orexigenic peptide NPY (see the chapters on Melanocortins and NPY in the Brain Peptides and Ingestive Peptides sections). The fact that insulin stimulates DBI expression in ependymocytes bordering the lateral ventricle[6] suggests that DBI-expressing glial cells relay hormonal signals from the periphery to the central nervous system.

Endozepines also regulate the expression of the genes encoding the hypothalamic hypophysiotropic neurohormones CRH and GnRH and the pituitary adrenocorticotropic hormone precursor POMC.[7,8] Some of these effects are mediated through the activation of metabotropic endozepine receptors.[8]

PATHOPHYSIOLOGICAL IMPLICATIONS OF ENDOZEPINES

GABA is the most important inhibitory neurotransmitter in the central nervous system, and endozepines, by affecting GABA-mediated inhibition of neurotransmission, may be involved in various neurological diseases (e.g. epilepsy) and psychiatric disorders (e.g. anxiety, mood impairment, and psychotic syndromes). Indeed, patients with major depression exhibit significantly higher concentrations of DBI in their cerebrospinal fluid as compared with normal volunteers.[30] Several lines of evidence suggest that anxiety disorders have a strong genetic component. A specific allele corresponding to one single nucleotide polymorphism of the DBI gene (SNP rs8192506, Met70Val) is more frequent in healthy subjects than in patients suffering from panic attacks[33] suggesting an influence of DBI genetic variants in the susceptibility for the development of anxiety disorders. Among the eight common SNPs located in the DBI gene, two of them, SNP rs8192506 and SNP rs2084202 (5′UTR, – 132 A > G), are associated with type-2 diabetes risk,[12] supporting a role of endozepines in the control of energy homeostasis.

An increased level of endozepines in human brain is also observed in other neurological diseases such as hepatic encephalopathy[29] and Alzheimer's disease.[21] In these patients, the high concentration of endozepines is usually associated with an increase in TSPO density or neurosteroid biosynthesis, suggesting that alteration of DBI gene expression could be implicated in various neuronal diseases. In chronic morphine-treated rats, an increase in DBI mRNA level is observed in the CA1 region of the hippocampus, the ventral tegmental area, the nucleus accumbens, the prefrontal cortex, and the amygdala which play a crucial role in addiction, suggesting that endozepines may also be involved in psychological and physical dependence on opiates[19] and possibly other drugs, including nicotine and alcohol. In the rat brain, intense expression of the DBI gene and high concentrations of endozepines occur during embryonic development,[4,22] a period of active neurogenesis. Endozepines are also actively expressed in various human brain tumors and glioblastomas.[2] These latter observations support the view that endozepines could play a role in neuronal cell proliferation and tumorigenesis.

REFERENCES

1. Alho H, Fremeau Jr RT, Tiedge H, Wilcox J, Bovolin P, Brosius J, et al. Diazepam binding inhibitor gene expression: location in brain and peripheral tissues of rat. *Proc Natl Acad Sci U S A* 1988;**85**:7018–22.
2. Alho H, Kolmer M, Harjuntausta T, Helen P. Increased expression of diazepam binding inhibitor in human brain tumors. *Cell Growth Differ* 1995;**6**:309–14.
3. Bormann J. Electrophysiological characterization of diazepam binding inhibitor (DBI) on $GABA_A$ receptors. *Neuropharmacology* 1991;**30**: 1387–9.
4. Burgi B, Lichtensteiger W, Lauber ME, Schlumpf M. Ontogeny of diazepam binding inhibitor/acyl-CoA binding protein mRNA and peripheral benzodiazepine receptor mRNA expression in the rat. *J Neuroendocrinol* 1999;**11**:85–100.
5. Cinone N, Hotje HD, Carotti A. Development of a unique 3D interaction model of endogenous and synthetic peripheral benzodiazepine receptor ligands. *J Comput Aided Mol Des* 2000;**14**:753–68.
6. Compère V, Lanfray D, Castel H, Morin F, Leprince J, Dureuil B, et al. Acute food deprivation reduces expression of diazepam-binding inhibitor, the precursor of the anorexigenic octadecaneuropeptide ODN, in mouse glial cells. *J Mol Endocrinol* 2010;**44**:295–9.
7. Compère V, Li S, Leprince J, Tonon MC, Vaudry H, Pelletier G. Effect of intracerebroventricular administration of the octadecaneuropeptide on the expression of pro-opiomelanocortin, neuropeptide Y and corticotropin-releasing hormone mRNAs in rat hypothalamus. *J Neuroendocrinol* 2003;**15**:197–203.
8. Compère V, Li S, Leprince J, Tonon MC, Vaudry H, Pelletier G. In vivo action of a new octadecaneuropeptide (ODN) antagonist on gonadotropin-releasing hormone gene expression in the male rat brain. *Neuroscience* 2004;**125**:411–5.
9. Do-Rego JL, Leprince J, Luu-The V, Pelletier G, Tonon MC, Vaudry H. Structure-activity relationships of a series of analogs of the endozepine octadecaneuropeptide ($ODN_{11–18}$) on neurosteroid biosynthesis by hypothalamic explants. *J Med Chem* 2007;**50**:3070–6.
10. Do Rego JC, Orta MH, Leprince J, Tonon MC, Vaudry H, Costentin J. Pharmacological characterization of the receptor mediating the anorexigenic action of the octadecaneuropeptide: evidence for an endozepinergic tone regulating food intake. *Neuropsychopharmacology* 2007;**32**:1641–8.

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11. Dong E, Matsumoto K, Tohda M, Watanabe H. Involvement of diazepam binding inhibitor and its fragment octadecaneuropeptide in social isolation stress-induced decrease in pentobarbital sleep in mice. *Life Sci* 1999;**64**:1779–84.
12. Fisher E, Nitz I, Gieger C, Grallert H, Gohlke H, Lindner I, et al. Association of acyl-CoA-binding protein (ACBP) single nucleotide polymorphisms and type 2 diabetes in two German study populations. *Mol Nutr Food Res* 2007;**51**:178–84.
13. Gandolfo P, Patte C, Leprince J, Do-Rego JL, Mensah-Nyagan AG, Vaudry H, et al. The triakontatetraneuropeptide (TTN) stimulates thymidine incorporation in rat astrocytes through peripheral-type benzodiazepine receptors. *J Neurochem* 2000;**75**:701–7.
14. Garcia de Mateos-Verchere J, Leprince J, Tonon MC, Vaudry H, Costentin J. The octadecaneuropeptide ODN inhibits apomorphine-induced yawning in rats. *Eur J Pharmacol* 1998;**357**:121–6.
15. Jarry M, Diallo M, Lecointre C, Desrues L, Tokay T, Chatenet D, et al. The vasoactive peptides urotensin II and urotensin II-related peptide regulate astrocyte activity through common and distinct mechanisms: involvement in cell proliferation. *Biochem J* 2010;**428**:113–24.
16. Kavaliers M, Hirst M. An octadecaneuropeptide (ODN) derived from diazepam binding inhibitor increases aggressive interactions in mice. *Brain Res* 1986;**383**:343–9.
17. Leprince J, Oulyadi H, Vaudry D, Masmoudi O, Gandolfo P, Patte C, et al. Synthesis, conformational analysis and biological activity of cyclic analogs of the octadecaneuropeptide ODN. Design of a potent endozepine antagonist. *Eur J Biochem* 2001;**268**:6045–57.
18. Lihrmann I, Plaquevent JC, Tostivint H, Raijmakers R, Tonon MC, Conlon JM, et al. Frog diazepam-binding inhibitor: peptide sequence, cDNA cloning, and expression in the brain. *Proc Natl Acad Sci U S A* 1994;**91**:6899–903.
19. Liu X, Li Y, Zhou L, Chen H, Su Z, Hao W. Conditioned place preference associates with the mRNA expression of diazepam binding inhibitor in brain regions of the addicted rat during withdrawal. *Mol Brain Res* 2005;**137**:47–54.
20. Loomis WF, Behrens MM, Williams ME, Anjard C. Pregnenolone sulfate and cortisol induce secretion of acyl-CoA-binding protein and its conversion into endozepines from astrocytes. *J Biol Chem* 2010;**285**:21359–65.
21. Luchetti S, Bossers K, Van de Bilt S, Agrapart V, Morales RR, Frajese GV, et al. Neurosteroid biosynthetic pathways changes in prefrontal cortex in Alzheimer's disease. *Neurobiol Aging* 2009http//dx.doi.org/10.1016/j.neurobiolaging.2009.12.0142009.
22. Malagon M, Vaudry H, Van Strien F, Pelletier G, Gracia-Navarro F, Tonon MC. Ontogeny of diazepam-binding inhibitor-related peptides (endozepines) in the rat brain. *Neuroscience* 1993;**57**:777–86.
23. Manabe Y, Toyoda T, Kuroda K, Imaizumi M, Yamamoto T, Fushiki T. Effect of diazepam binding inhibitor (DBI) on the fluid intake, preference and the taste reactivity in mice. *Behav Brain Res* 2001;**126**:197–204.
24. Masmoudi O, Gandolfo P, Leprince J, Vaudry D, Fournier A, Patte-Mensah C, et al. Pituitary adenylate cyclase-activating polypeptide (PACAP) stimulates endozepine release from cultured rat astrocytes via a PKA-dependent mechanism. *FASEB J* 2003;**17**:17–27.
25. Matsuda K, Wada K, Miura T, Maruyama K, Shimakura SI, Uchiyama M, et al. Effect of the diazepam-binding inhibitor-derived peptide, octadecaneuropeptide, on food intake in goldfish. *Neuroscience* 2007;**150**:425–32.
26. Nitz I, Kruse ML, Klapper M, Doring F. Specific regulation of low-abundance transcript variants encoding human Acyl-CoA binding protein (ACBP) isoforms. *J Cell Mol Med* 2010http//dx.doi.org/10.1111/j.jcellmolmed.2010.01055.x2010.
27. Papadopoulos V, Guarneri P, Kreuger KE, Guidotti A, Costa E. Pregnenolone biosynthesis in C6-2B glioma cell mitochondria: regulation by a mitochondrial diazepam binding inhibitor receptor. *Proc Natl Acad Sci U S A* 1992;**89**:5113–7.
28. Qian Z, Bilderback TR, Barmack NH. Acyl coenzyme A-binding protein (ACBP) is phosphorylated and secreted by retinal Muller astrocytes following protein kinase C activation. *J Neurochem* 2008;**105**:1287–99.
29. Rothstein JD, McKhann G, Guarneri P, Barbaccia ML, Guidotti A, Costa E. Cerebrospinal fluid content of diazepam binding inhibitor in chronic hepatic encephalopathy. *Ann Neurol* 1989;**26**:57–62.
30. Roy A. Cerebrospinal fluid diazepam binding inhibitor in depressed patients and normal controls. *Neuropharmacology* 1991;**30**:1441–4.
31. Sandberg MB, Bloksgaard M, Duran-Sandoval D, Duval C, Staels B, Mandrup S. The gene encoding acyl-CoA-binding protein is subject to metabolic regulation by both sterol regulatory element-binding protein and peroxisome proliferator-activated receptor α in hepatocytes. *J Biol Chem* 2005;**280**:5258–66.
32. Slobodyansky E, Kurriger G, Kultas-Ilinsky K. Diazepam binding inhibitor processing in the rhesus monkey brain: an immunocytochemical study. *J Chem Neuroanat* 1992;**5**:169–80.
33. Thoeringer CK, Binder EB, Salyakina D, Erhardt A, Ising M, Unschuld PG, et al. Association of a Met88Val diazepam binding inhibitor (DBI) gene polymorphism and anxiety disorders with panic attacks. *J Psychiatr Res* 2007;**41**:579–84.
34. Tokay T, Hachem R, Masmoudi-Kouki O, Gandolfo P, Desrues L, Leprince J, et al. Beta-amyloid peptide stimulates endozepine release in cultured rat astrocytes through activation of N-formyl peptide receptors. *Glia* 2008;**29**:1380–9.
35. Yanase H, Shimizu H, Yamada K, Iwanaga T. Cellular localization of the diazepam binding inhibitor in glial cells with special reference to its coexistence with brain-type fatty acid binding protein. *Arch Histol Cytol* 2002;**65**:27–36.

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Chapter 103

Galanin and GALP

Andrew L. Gundlach and Philip J. Ryan

ABSTRACT

The galanin family of peptides consists of four members: galanin, galanin message-associated peptide (GMAP), galanin-like peptide (GALP), and alarin. Galanin/GMAP are widely distributed throughout the brain, whereas GALP and alarin have a more restricted expression. Three G protein-coupled receptors, GalR1, GalR2, GalR3 bind galanin with high affinity and are broadly expressed throughout the brain. The native receptors for GMAP, GALP, and alarin are not known. Galanin and related peptides regulate a wide range of neural and physiological functions including neuroendocrine hormone release, reproduction, feeding and metabolism, osmotic regulation, cardiovascular and respiratory control, arousal and sleep, memory, nociception, and neuronal growth and regeneration. Extensive preclinical and clinical research has implicated galanin family peptide and receptor signaling in several neuropathological and psychiatric conditions, including epilepsy, Alzheimer's disease, nerve injury, multiple sclerosis, stroke, anxiety, depression, obesity, and alcohol and drug addiction, suggesting galanin-related treatments hold significant therapeutic potential for such disorders.

DISCOVERY

The galanin family consists of four peptides: galanin, galanin message-associated peptide (GMAP), galanin-like peptide (GALP), and alarin. Galanin is a 29-amino acid neuropeptide (30 in humans), which was first isolated from porcine intestine in 1983 (see galanin chapters in the Ingestive and Gastrointestinal Peptides section of this book), and was named for its N-terminal *g*lycine and C-terminal *ala-nine*[30] (see Ref. 13). Galanin has been isolated in numerous species, and it contains a highly conserved N-terminal 1–15 amino acids. Galanin was initially considered a peptide without a family, but it was subsequently observed that the galanin precursor molecule 'preprogalanin' also, encoded GMAP, a 59- or 60-amino acid peptide, which has distinct biological actions[35] (see Ref. 13).

Galanin-like peptide (GALP) is a 60-amino acid neuropeptide that was isolated from porcine hypothalamus in 1999 and shown to be an endogenous ligand for galanin receptors.[21] GALP residues 9–21 are homologous to galanin residues 1–13. A splice variant of GALP mRNA comprises 25 amino acids and is termed alarin because of its N-terminal *ala*nine and C-terminal se*rine*[25] (see Ref. 13).

Three G protein–coupled receptors for galanin have been identified and characterized - GalR1, GalR2, and GalR3. GalR1 was initially cloned from a human Bowes melanoma cell line, and GalR2 and GalR3 were first identified in rat and subsequently in other species (see Ref. 13). Galanin (porcine [^{125}I]-galanin) has a high affinity [K_D] for each human receptor subtype, 0.15 nM for GalR1, 0.32 nM for GalR2, and 2.23 nM for GalR3, but agonists and antagonists with selectivity for these receptors have been developed that have helped to reveal their respective actions[13] (see Mitsukawa et al. in Ref. 8).

STRUCTURE OF THE mRNA/GENE

Galanin and GMAP

The structure of the 'preprogalanin' gene, which encodes galanin and GMAP, is highly conserved among vertebrates [18] (see Mensah et al. in Ref. 8). The preprogalanin gene is located on human chromosome 11q13.3, rat chromosome 1q42, and mouse chromosome 19A, spanning about 6.5 kb of genomic DNA (http://www.ncbi.nlm.nih.gov/gene/?term=galanin). It comprises 6 exons and 5 introns: exon 1 is noncoding; exon 2 starts with the translation initiation codon of the signal peptide and terminates before the proteolytic site preceding the mature galanin peptide; the mature galanin peptide is encoded on exons 3 and 4; GMAP is encoded by exons 4 to 6; exon 6 also contains the polyadenylation site.

GALP and Alarin

The preproGALP gene encoding GALP and alarin is located on human chromosome 19q13.43, rat chromosome 1q12, and mouse chromosome 7A1, spanning about 11kb of genomic DNA.[3] It comprises six exons, with a structural organization similar to that of galanin: exon 1 is noncoding; exon 2 contains the start codon; the region encoding the mature GALP is contained in exons 2–5; the portion of galanin homology

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00103-2
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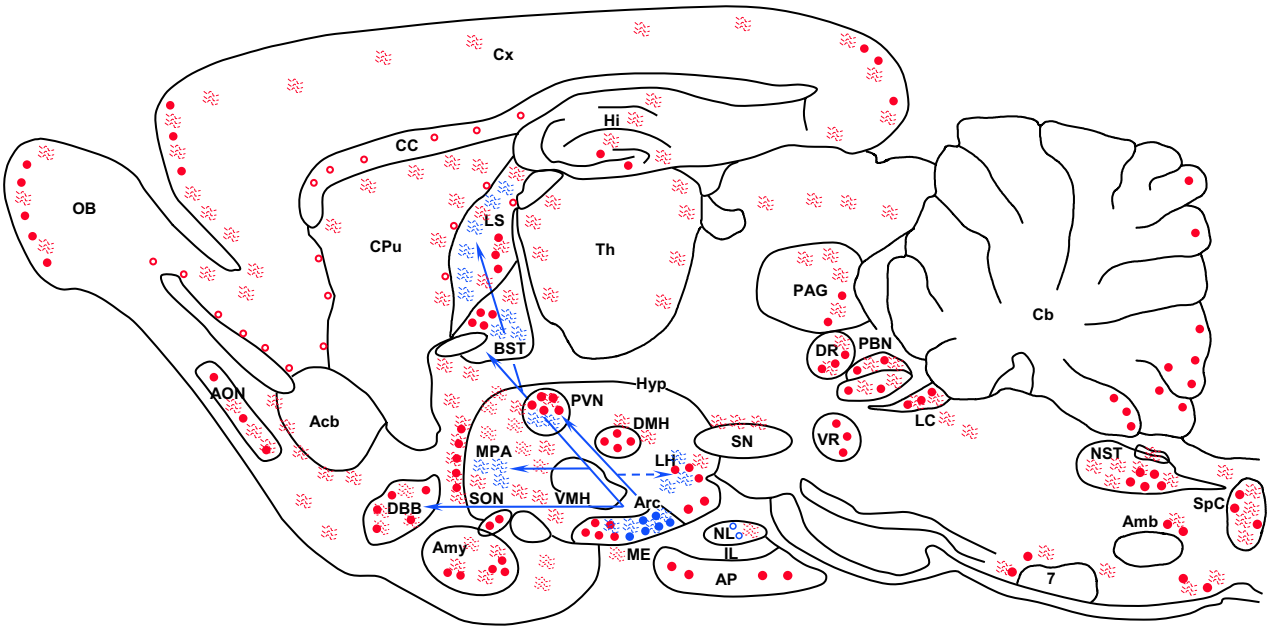

FIGURE 1 Schematic illustration of the contrasting distribution of galanin and GALP positive neurons and their innervations in rat brain. GALP (**blue** dots) are illustrated, along with the approximate localization of their immunoreactive nerve fibers/terminals (small **red** and **blue** lines). Nonneuronal GALP and galanin expression in astrocytes (pituicytes) and oligodendrocyte and other progenitors is also illustrated (**red** and **blue** circles). The two major GALP pathways proposed from the arcuate nucleus to the paraventricular nucleus and to other hypothalamic and limbic regions are indicated by **blue** arrows. The relative topography of the many, widespread galanin pathways has been omitted for clarity. See color plate 26.

[GALP(9–21)] is contained in exon 3, and the polyadenylation site is contained in exon 6.[3] Alarin is formed when post-transcriptional splicing leads to exclusion of exon 3 resulting in a frame shift and a novel precursor peptide.[25]

PROCESSING OF THE PEPTIDE PRECURSOR

Galanin and GMAP

The precursor peptide, preprogalanin, comprises 123–124 amino acids, depending on the species (123 in humans), and consists of a signal peptide, galanin and GMAP. The sequence for galanin consists of amino acids 33–62 of the precursor, and is flanked by two pairs of basic amino acids (lysine and arginine), which are cleavage sites. In most species, the C-terminal *glycine* serves as a donor for amidation; but in humans a *serine* residue replaces the *glycine*, and C-terminal amidation does not occur. Hence, human galanin contains an additional amino acid. The galanin sequence is followed by the sequence for GMAP (see Refs. 13, 18 for a review).

GALP and Alarin

The precursor peptide, preproGALP, comprises 115–120 amino acids, depending on the species (116 in humans), and consists of a signal peptide directly followed by the GALP sequence (i.e. the site of cleavage for the N terminus). The GALP sequence consists of amino acids 25–84 of the precursor peptide and the C terminus is generated by cleavage at residues Ser^{84}-Lys^{85}-Arg^{86}.[21]

The precursor peptide for alarin is 48–49 amino acids (49 in humans), which comprises the signal sequence of preproGALP and the first 5 amino acids of the mature GALP peptide, followed by 19 amino acids that do not display homology to any other protein. A further proteolytic cleavage leads to alarin (1–25).[13,25]

DISTRIBUTION OF THE mRNA/PEPTIDE

Galanin and GMAP

Galanin mRNA and peptide have been mapped in several species, including mouse, rat, primate, and human, and display widespread distribution throughout the CNS, with similar expression patterns among the species currently studied.[10,13,18] Because the rat is the species most frequently mapped its galanin expression pattern is described, and in most cases, a good correlation exists between nuclei expressing galanin precursor mRNA (assessed by in situ hybridization) and those areas containing galanin peptide (assessed by immunohistochemistry) (Fig. 1).[24]

Galanin-positive *cell bodies* are highly abundant in the dorsomedial nucleus of the hypothalamus and in the locus ceruleus, while medium to high densities are observed in the medial septal nucleus, nucleus of the diagonal band, bed nucleus of the stria terminalis, basal nucleus of Meynert (not

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present in equivalent human area), central nucleus of the amygdala, preoptic and lateral hypothalamic areas, hypothalamic paraventricular, periventricular, supraoptic, arcuate and tuberomammillary nuclei, dorsal raphe, raphe pallidus and obscurus, the caudal spinal trigeminal nucleus, nucleus of the solitary tract, and dorsal horn of the spinal cord.[10,13,18] Galanin mRNA is also expressed by cerebellar Purkinje cells in caudal vermis, flocculus and paraflocculus.[24]

Galanin-positive fibers are generally observed in the same areas as galanin cell bodies. Very dense galanin-positive fiber bundles are present in the lateral septal nucleus, the median eminence of the hypothalamus and dorsal horn of the spinal cord. Medium to high densities of galanin fibers are present in the medial prefrontal cortex, hippocampus and dentate gyrus, nucleus of the diagonal band, bed nucleus of the stria terminalis, basal nucleus of Meynert, central nucleus of the amygdala, amygdalohippocampal area, the stria terminalis, thalamic parafascicular and periventricular nuclei, preoptic and lateral hypothalamic areas, hypothalamic paraventricular, periventricular, supraoptic, dorsomedial, arcuate and tuberomammillary nuclei, the locus ceruleus, dorsal and median raphe, raphe pallidus and obscurus, caudal spinal trigeminal nucleus, spinal tract of the trigeminal nucleus, nucleus of the solitary tract, dorsal vagal complex, the area around the central canal of the spinal cord (lamina X) and the intermediolateral column of the spinal cord.[13,18]

Galanin mRNA and immunoreactivity have also been detected in oligodendrocyte precursor cells in the corpus callosum and in immature progenitor cells in the subventricular zone and rostral migratory stream.[27]

Galanin is coexpressed with different neurotransmitters and neuropeptides, including with acetylcholine in septal and basal forebrain neurons projecting to the hippocampus; serotonin in dorsal raphe neurons; histamine in the tuberomammillary nucleus; norepinephrine (NE) in the locus ceruleus and dorsomedial hypothalamic nucleus; GABA in the arcuate nucleus and external layers of the median eminence; vasopressin in the hypothalamic paraventricular nucleus; and cholecystokinin in spinothalamic neurons, particularly in lumbar segments L1–L5[10,13] (see Hökfelt's chapter on neurotransmitters in the General Peptide Topics section of the book).

GMAP has a similar profile of expression to galanin in the rat, although in the retina, cones are reportedly GMAP positive but galanin negative, and prolactin cells in the anterior lobe of the pituitary express high GMAP levels, but low galanin levels, suggesting that differential processing of the preprogalanin precursor occurs in some tissues.

GALP and Alarin

GALP has a more restricted expression than galanin, with GALP-expressing cells found only in the hypothalamic arcuate nucleus, the median eminence and infundibular stalk, and the posterior pituitary of the rat[14,26] (see Refs. 13, 15 for a review). GALP-expressing neurons send projections to various areas of the forebrain, including the lateral septum and bed nucleus of the stria terminalis and medial preoptic area, where they target luteinizing hormone-releasing hormone (LHRH) neurons, as well as various hypothalamic regions, including the periventricular, paraventricular and arcuate nuclei and lateral hypothalamus, where they target orexin- and melanin-concentrating hormone neurons. The leptin receptor is expressed by GALP neurons in the arcuate nucleus and GALP neurons receive orexin and neuropeptide Y projections.[11,29]

Alarin-like immunoreactivity has been comprehensively mapped in the mouse brain, where it was detected in high densities in the accessory olfactory bulb, medial preoptic area, amygdala, and hypothalamic arcuate and ventromedial nuclei of the forebrain, and in the trigeminal complex, locus ceruleus, and ventral cochlear and facial nuclei of the brainstem.[5] In the rat brain, alarin-positive neurons have been described in the locus ceruleus.[32]

RECEPTORS AND SIGNALING CASCADES

The different galanin receptors are coupled to a variety of signal transduction pathways, with both GalR1 and GalR3 acting mainly on inhibitory $G_{i/o}$-proteins, while GalR2 can influence either stimulatory $G_{q/11}$- or inhibitory $G_{i/o}$-proteins, depending on a variety of factors, such as the host cell, and the G-protein repertoire of the cell. GalR1 can influence inwardly rectifying K^+ channels (GIRK) and activate MAP kinase activity; GalR2 most commonly stimulates inositol phosphate hydrolysis, mobilizing intracellular Ca^{2+} and activating Ca^{2+} dependent Cl^- channels; and GalR3 stimulates GIRKs (see Ref. 13). Ferré and colleagues[19] showed that GalR1 can form heteromers with the D1 dopamine receptor in transfected mammalian cells and *in vivo* in the hippocampus, where the D1-GalR1 heteromers integrated dopamine and galanin signaling to modulate cholinergic transmission.

The distribution of the three galanin receptors has been mapped by in situ hybridization and is described here for the rat brain (e.g. Ref. 2; see Ref. 13). GalR1 is widely distributed in the hypothalamus, amygdala, ventral hippocampus, thalamus, medulla, locus ceruleus, lateral parabrachial nucleus, and dorsal horn of spinal cord. GalR2 is moderately to highly expressed in olfactory bulb/tubercle, piriform cortex, hypothalamus (especially preoptic, arcuate, dorsal nuclei), mammillary nuclei, hippocampus, amygdala, substantia nigra pars compacta, cerebellar cortex and sensory trigeminal nucleus. GalR3 expression is highest in the hypothalamus and pituitary gland, and is also expressed in the olfactory bulb, cerebral cortex, medulla, caudate putamen, cerebellum and spinal cord. Some species-based differences in distribution have been identified.

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Although galanin-like peptide (GALP) was originally shown to bind to GalR1 and GalR2 *in vitro*, and GalR3 *in vivo*, it seems that GALP does not act via galanin receptors *in vivo*. GALP retains its actions in both GalR1 and GalR2 knockout (KO) mice, and some evidence indicates that *endogenous* GALP is unlikely to activate GalR3. This suggests that GALP may have its own receptor(s), which has not yet been identified.[15] Similarly, the native receptors for GMAP and alarin have not been identified.[32,35]

BIOLOGICAL ACTIONS WITHIN THE BRAIN AND PITUITARY

Galanin and GMAP

Galanin has an established and/or putative involvement in a range of brain functions. Strong, independent, and consistent evidence supports a role for galanin signaling in the control of aspects of feeding and metabolism, reproduction, memory and cognition, nociception, and neural growth and injury response. There is also experimental evidence for modulatory functions associated with cardiovascular and respiratory control, arousal and sleep, and stress and affective behavior. Galanin is also strongly implicated in neuroplasticity associated with a range of pathological conditions, and galanin expression is highly inducible in a number of these situations.

Feeding and Metabolism

Central (icv) administration of galanin strongly activates feeding behavior in satiated rats. A similar effect has been demonstrated following targeted injections of galanin into various feeding-related hypothalamic nuclei, including the paraventricular nucleus (PVN), and lateral and ventromedial hypothalamus (see Refs. 7, 13; Barson et al. in Ref. 8 for a review). Galanin also increases food intake after injection into the amygdala, via direct interaction with the stress-related transmitter NE, which has led to the hypothesis that galanin may mediate stress-induced overconsumption. Early reports suggested that rats injected with galanin intra-PVN displayed a preference for fat intake over other macronutrients, and although other studies have not replicated this finding specifically, galanin-induced feeding is stronger and more prolonged in rats maintained on a high-fat diet, and in rat strains that naturally prefer fat.

Galanin KO and galanin overexpressing (OE) mice do not display an obese or lean phenotype, or any strong alterations in 'normal' feeding behavior, but galanin KO mice consume less fat than wildtype controls under macronutrient choice conditions and when only fat is available, and galanin OE mice consume more fat and alcohol than controls. Initial studies of GalR1 and GalR2 KO mice revealed no marked phenotype related to difference in feeding behavior or body weight, but a more recent study on GalR1 KO mice suggests that this receptor might mediate the adjustments in food intake and energy levels in response to acute changes in dietary fat.

Thus, although galanin may not play an 'essential' role in food intake and body weight control, it has robust effects on the intake of dietary fat (and alcohol) and acts by modulating the activity of several other key peptides/transmitters/hormones particularly under conditions of altered diet and stress.

Release of Pituitary Hormones

Galanin seems to play a modulatory role in various neuroendocrine systems.[18] For example, in the hypothalamic–pituitary–gonadal (HPG) axis, galanin increases LH secretion and enhances LHRH binding to its receptors in the pituitary. Populations of hypothalamic galanin and LHRH neurons display reciprocal connections and galanin is coexpressed with LHRH in a sexually dimorphic manner in species such as rats, mice, and sheep (higher levels in mature females than males), but not in other species such as humans, suggesting sex- and species-based differences in the reproductive role of galanin. Other evidence that galanin plays a major role in reproduction is the ability of galanin antagonists and galanin antisera to block the preovulatory surge and ovulation in rats; although galanin KO mice are fertile. In addition, the amount of galanin in LHRH neurons is negatively regulated by estrogen in mice, but positively regulated in rats, suggesting that thorough studies of different species are required to properly determine the role and relative importance of galanin for reproduction. The estrogen-dependent presence of galanin in populations of kisspeptin neurons that innervate LHRH neurons further strengthens its role in central regulation of LHRH/LH and reproduction.[12]

Galanin and its receptors are present at each level of the hypothalamic–pituitary–adrenal (HPA) axis, including the hypothalamus (PVN/SON), anterior pituitary and adrenal gland. In the absence of stress, galanin can stimulate the HPA axis and increase corticotropin-releasing hormone (CRH) and adrenocorticotropic hormone (ACTH) release, thereby increasing glucocorticoid secretion from the adrenal cortex, but after activation of the HPA axis by stress, galanin acts to decrease responses.[22] For example, stress-induced hyperthermia, which is accompanied by elevated ACTH/corticosterone levels, is attenuated by central administration of a high dose of galanin (although a low dose enhances the hyperthermic response). Galanin lowers levels of ACTH/cortisol in humans after intravenous injection, and together these results suggest important species and 'site of action' related differences in galanin responses.

Similarly, galanin can increase circulating prolactin (PRL) levels, probably by the inhibition of dopamine release

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from tuberoinfundibular neurons. In galanin KO mice, PRL expression is reduced in adult females, indicating that galanin acts as a growth factor for lactotrophs, particularly under conditions where estrogen levels are elevated, such as pregnancy. Galanin can also modulate other hormones, with reports of increased growth hormone (GH) levels and reduced thyroid-stimulating hormone (TSH).

Arousal and Sleep

Galanin is highly expressed in several arousal and sleep-related brain areas including histamine and serotonin neurons in the tuberomammillary and dorsal raphe nuclei that are functionally related to the control of 'arousal.' Sleep-active neurons are located in subregions of the preoptic area, with a high density in the median preoptic nucleus (MnPN) and the ventrolateral preoptic area (vlPOA) and the latter contain GABA and galanin. Anatomical studies reveal projections from vlPOA and MnPN to multiple monoaminergic arousal regulatory systems in the posterior/lateral hypothalamus and the brainstem. Cumulative evidence indicates that preoptic area neurons promote sleep onset and maintenance by inhibitory modulation of multiple arousal systems and are involved in homeostatic regulation of both rapid eye movement (REM) and non-REM sleep.[28] Notably, sleep-deprived rats express higher levels of galanin mRNA in preoptic area neurons and NE neurons in the locus ceruleus; and galanin (iv) alters sleep patterns in healthy male volunteers.[20]

Memory and Cognition

Various 'global' and site-specific effects of galanin on memory have been reported. Central (icv) and intraseptal and intrahippocampal administration of galanin impairs memory acquisition in rats, as shown in the Morris water maze and other learning and memory tasks[6] (see Crawley in Ref. 8). Galanin is postulated to alter the balance of acetylcholine release from septal afferents in the hippocampus, and galanin decreases long-term potentiation in hippocampal slices by inhibiting cholinergic Schaffer collateral fibers. Galanin receptor antagonists have been tested for their role in memory enhancement. A nonselective peptide antagonist, M40, does not alter the performance of normal rats in a working memory task (delayed nonmatching to position), but does block the effects of galanin. M40 also potentiates the memory enhancing actions of a cholinergic agonist in a working memory task in rats with a 'cholinergic' lesion. The nonselective antagonist M35 facilitates spatial learning in the Morris water maze.

Galanin OE mice have been investigated in paradigms of learning and memory. In one strain, the galanin gene is linked to a dopamine β-hydroxylase (DβH) promoter, which restricts galanin overexpression to epinephrine (E) and NE neurons; in another strain, the galanin gene is linked to the platelet-derived growth factor β (PDGF) promoter, which produces a more widespread galanin expression in the brain, including the hippocampus and cortex. Learning and memory tasks reveal learning impairments with both transgenic strains. For example, DβH-galanin OE mice display deficits in the more difficult components of several learning and memory tasks, including on the probe trial test of the Morris water maze (spatial memory), impaired learning of social transmission of food preference (olfactory memory), and reduced fear conditioned freezing on the trace-fear conditioning task (emotional memory). The PDGF-galanin OE mice display impairment in learning acquisition in the Morris water maze, but only at an older age (19 months).[23] Similarly, galanin KO mice display small learning impairments in a spatial object recognition task and in the Morris water maze at an older age, suggesting too little or too much galanin can cause learning deficits. GalR1 and GalR2 KO mice display no significant differences from wildtype in models of learning and memory, except for some impairment on trace-cued fear conditioning in GalR1 KO mice. Therefore, while galanin-related drugs may eventually be tested for the treatment of aged-related memory deficits, perhaps as an adjunct to other treatments, the ambiguous nature of the current evidence suggests further preclinical testing is warranted to elucidate the precise dose- and global/site-related effects and associated mechanisms of action of galanin receptor activation and inhibition.

Nociception

Galanin modulates pain in the spinal cord, where it is expressed along with its three receptors, particularly in dorsal root ganglion neurons and spinal cord sensory neurons (see Ref. 13; Xu et al. in Ref. 8). Endogenous galanin seems to inhibit spinal nociception to some degree under normal conditions and more strongly after peripheral nerve injury when galanin synthesis is increased in sensory neurons. Thus, independent experimental evidence suggests intrathecal administration of galanin induces a biphasic response in many pain models, with low galanin doses escalating pain via GalR2 activation, and high doses suppressing pain via GalR1 activation. Intrathecal galanin administration reduces neuropathic pain in rat models and the galanin receptor antagonist M35 produces a small enhancement of pain sensitization. Both galanin and GalR1 KO mice display moderately decreased response thresholds in tests of heat nociception, whereas mice that overexpress galanin in sensory neurons display increased pain thresholds and decreased neuropathic pain-like behaviors after nerve injury. Further testing of galanin-related drugs is warranted to determine if they have therapeutic potential as analgesics in humans.

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Neuronal Growth and Regeneration

Galanin expression increases dramatically in a number of different types of neurons after injury and, on the basis of this and related findings, is proposed to play a trophic role in neuronal growth and regeneration. Galanin signaling may also play a role in neuronal development, with probably an important role for GalR2 in these processes (see Ref. 2; Hobson et al. in Ref. 8).

For example, during development, galanin is highly expressed in the dorsal root ganglion (DRG) from day 14 of gestation till shortly after birth, and seems to play a role in the survival of sensory neurons, as galanin and GalR2 KO mice have reduced numbers (>10–20%) of small diameter DRG neurons. Galanin is expressed in <5% of adult DRG neurons (predominantly in small diameter nociceptors) and GalR2-, but not GalR1-, KO mice have a reduction in adult DRG neurons. However, after nerve (sciatic) injury, galanin levels can rise by up to 120-fold, to promote nerve regeneration and outgrowth of neurites. Galanin KO mice have a 35% reduction in nerve regeneration compared to wildtype controls, associated with long-term sensorimotor deficits and decreased neurite number and length.

Galanin also seems to play a role in neuronal survival and neuroprotection after injury in the central nervous system (CNS). During development, galanin KO mice display a loss of one-third of their total number of cholinergic septohippocampal neurons, and galanin KO mice display a significantly higher degree of cell death in the hippocampus after peripheral injection of the neuroexcitatory convulsant, kainic acid, compared to that in wildtype mice, whereas galanin OE mice display less hippocampal death. GalR2 KO mice also display more injury-induced cell death than wildtype mice that cannot be rescued by exogenous galanin, implicating GalR2 in this effect. These changes have functional implications as galanin and GalR2 KO mice display markedly reduced behavioral symptoms under conditions of neuropathic and inflammatory pain; and galanin KO mice display decreased cognitive performance, possibly due to a developmentally related decrease in numbers of cholinergic septohippocampal neurons.

In contrast to galanin, far less is known about the CNS role of GMAP, but fragments of GMAP injected intrathecally facilitate the spinal nociceptive flexor reflex.[35]

GALP and Alarin

Similarly, the precise biological roles of GALP and alarin are not as well characterized as galanin, but anatomical data and independent and consistent experimental evidence support a role for GALP in the control of aspects of feeding and metabolism and reproduction, and GALP expression is regulated by other peptides/hormones associated with metabolic state including leptin, insulin, and thyroid hormone and by blood glucose levels.[15] Alarin (icv) increases feeding over a 0.5- to 4-h period.[32]

Feeding and metabolism

GALP administration modulates food intake and energy expenditure, although its actions are complex and depend on the species studied and the experimental conditions.[15] In rats, central GALP injection acutely increases food intake (over 1h), as does injection directly into the medial preoptic, paraventricular, and dorsomedial hypothalamic nuclei, but over the 24-h period after administration, food intake and body weight decrease. Icv GALP also increases core body temperature in rats for up to 8h. By contrast, in normal mice, icv GALP has no acute stimulatory effect on feeding, and it results in a decrease in food intake and body weight over 24h. Chronic injections of GALP in leptin-deficient obese (*ob/ob*) mice produced prolonged reductions in body weight, relative to lesser effects in C57BL/6 control mice. In control mice, icv GALP produces a rapid, transient drop in body temperature before a period of increased body temperature of approximately 8h, whereas in obese *ob/ob* mice, chronic GALP produces a sustained increase in body temperature. Lastly, in mice but not in rats, central GALP treatment acutely impairs locomotion; but over several days of chronic treatment, mice become hyperactive.

GALP KO mice display no marked differences in feeding, body weight or temperature compared to wildtype mice, but they consume less food after a fast and gain less weight on a high-fat diet, indicating that while GALP is not essential to 'normal' energy balance, it may play a modulatory role in maintenance of metabolic homeostasis under altered dietary conditions.[4]

Reproduction

GALP icv stimulates an increase in LHRH release, which in turn increases LH secretion in male rats. GALP icv also produces a marked stimulation of male sex behaviors in both intact and castrated male rats.[15] Alarin does not affect sexual behavior in intact male rats, but it does increase LH levels in castrated rats.[32]

PATHOPHYSIOLOGICAL IMPLICATIONS

Experimental evidence collected over two decades has highlighted the probable role of galanin in the pathophysiology of a range of neurological and neuropsychiatric conditions.

Seizures and Epilepsy

Administration of galanin receptor agonists into brain areas involved in the initiation and propagation of epileptic

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activity attenuates seizures in animal models of epilepsy, and pharmacological blockade of galanin receptors is proconvulsant (see Lerner et al. in Ref. 8). For example, exogenous galanin decreases the severity of picrotoxin-kindled convulsions in rats when injected icv or into the hippocampus. Galanin seems to produce this effect by inhibiting glutamate, as galanin receptor agonists inhibit depolarization-induced glutamate release from rat hippocampal slices *in vitro*.

Transgenic mice provide further evidence for the involvement of galanin in seizures. Galanin KO mice display a higher susceptibility to seizures, a shorter latency to seizure induction and increased seizure severity. In contrast, mice that overexpress galanin in E/NE pathways (DβH-galanin OE mice), display enhanced resistance to seizure induction, while PDGF-galanin OE mice with a more widespread galanin expression pattern are resistant to kindling. Furthermore, rats injected centrally with an adeno-associated virus (AAV) carrying the galanin gene and an associated fibronectin sequence to ensure active galanin secretion into the extracellular space, display significantly decreased seizures induced by electrical stimulation of the brain.

GalR1 KO mice display spontaneous seizures, and although GalR2 KO mice do not display spontaneous seizures or altered responses to induced seizures, when GalR2 is downregulated in the hippocampus by an antisense approach, seizures produced by hippocampal stimulation are dramatically increased in severity and duration. These results suggest that GalR1 and GalR2 activation can promote the inhibition of seizures and that galanin-related peptides are attractive candidates as antiepileptic agents. Several ligands have been developed that penetrate the blood-brain barrier, including an analog of galanin with preferential affinity for GalR2 that is a potent anticonvulsant after systemic administration in mice.

Alzheimer's Disease (AD)

Galanin and galanin receptors are overexpressed in the basal forebrain of patients with AD, and increased galanin fibers and terminals innervate the surviving cholinergic neurons of the basal nucleus of Meynert (see Counts et al. in Ref. 8). Galanin can reportedly inhibit acetylcholine release in the hippocampus and decrease cognitive performance in rodents, suggesting that increased galanin may worsen the cholinergic deficit seen in AD. However, galanin protects the hippocampus from excitotoxic damage and cholinergic forebrain neurons from amyloid toxicity, suggesting the peptide might promote survival of cholinergic and other neurons in regions undergoing neurodegeneration and so improve AD symptoms. Therefore, further preclinical studies may be required to differentiate these possibilities and provide a clearer idea of the therapeutic potential of galanin receptor modulation for the treatment of AD.

Stress and Anxiety

Galanin modulates the response to stressors, as well as anxiety- and depressive-like behavior (see Ref. 9; Mitsukawa et al. in Ref. 8). Galanin is expressed in various stress-related brain regions, including the paraventricular nucleus of the hypothalamus (PVN), where it coexists with CRH and vasopressin, which are critical regulators of the HPA or 'stress' axis. Galanin is also highly expressed in the locus ceruleus, in NE neurons, which increase arousal and alertness, particularly under stressful conditions. Various stressors increase expression of galanin in the locus ceruleus, amygdala and hypothalamus, including social stress, exercise, cold, pain, and immobilization. Hypothalamic galanin injections reduce stress-induced activation of the HPA axis, suggesting a negative feedback mechanism, whereas galanin injected systemically increases activation of the HPA axis.

In the rat, galanin (icv) reduces anxiety-like behavior in the Vogel conflict test, while in contrast, galanin administered into the amygdala increases anxiety-like behavior in this test. In the elevated plus maze, injection of the galanin antagonist M40 into the central nucleus of the amygdala does not alter anxiety-like behavior in the rat suggesting no significant role for endogenous galanin under nonstressed conditions. However, with increased stress, endogenous galanin release in the amygdala is believed to decrease anxiety-like behavior (and 'balance' the anxiogenic effect of coreleased NE). Thus, galanin signaling can increase or decrease anxiety-like behavior depending on the experimental test, the baseline conditions, and whether the role of endogenous or exogenous peptide is being examined.

However, transgenic mice have helped clarify our understanding somewhat. The DβH-galanin OE strain display normal anxiety-like behaviors under baseline conditions, but notably, do not increase anxiety-like behavior after treatment with the anxiogenic agent, yohimbine, suggesting that increased galanin causes resistance to anxiety-provoking agents. PDGF-galanin OE mice, which overexpress galanin more widely (although not in NE neurons), display normal anxiety in a range of behavioral tests and conditions. These results suggest that under high stress conditions where high levels of NE are released, galanin may be specifically recruited and prevent increased anxiety-like behavior.

The varying effects of galanin observed on anxiety-like behavior may also result from actions at different galanin receptors. GalR1 is believed to mediate galanin modulation of NE activity in the locus ceruleus, as the mixed GalR1/GalR2 agonist AR-M961 is more effective at inhibiting locus ceruleus firing than the GalR2 agonist AR-M1896. GalR1 (and GalR2) KO mice display increased anxiety-like behavior in tests such as the elevated plus maze, but not in

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others such as the light/dark box, or open field; but in GalR1 KO mice, a high dose of galanin fails to induce c-Fos in locus ceruleus and alter stress hormone levels (ACTH/corticosterone), as observed in wildtype mice. GalR3 selective antagonists have been reported to display activity in some anxiety models (stress-induced hyperthermia and punished drinking) and not others (elevated plus maze). Overall, these results reinforce the view that, rather than a unified, consistent role in modulating anxiety, effects of galanin can be facilitatory or attenuating, and are region-, context-, and response- specific.

Importantly though, a polymorphism in the galanin gene is associated with the severity of panic disorder symptoms in female patients, suggesting galanin may play a role in this clinical anxiety condition.[31]

Depression

Exogenous and endogenous galanin seems able to modulate depressive-like behavior, particularly via an interaction with the monoamines serotonin and NE (see Ref. 9; Kuteeva et al. in Ref. 8). Galanin is coexpressed with NE in the locus ceruleus and with serotonin in the dorsal raphe nucleus in the rat, although not in the mouse. Galanin inhibits serotonin neuron activity, probably via multiple receptor subtypes. For example, treatment with a GalR3 antagonist blocks the inhibitory action of galanin on serotonin cell firing. Galanin seems to modulate $5HT_{1A}$ receptor function, which is implicated in depressive-like behaviors, with icv galanin causing a reduction in affinity (K_D values) of $5HT_{1A}$, an increase in the number of $5HT_{1A}$ receptors, and a decrease in $5HT_{1A}$ mRNA levels in the dorsal raphe. Furthermore, a recent clinical genomewide association study (GWAS) suggests a possible role for galanin in relation to major depressive disorder and serotonin signaling.[34]

Galanin injected icv, or directly into the ventral tegmental area, increases depressive-like behavior, reflected by increased immobility time in the forced swim test, and this effect is blocked by coadministration of nonselective galanin receptor antagonists. By contrast, other studies have reported that increased galanin may decrease depressive-like behavior, as peripheral administration of nonpeptide galanin agonists, galmic and galnon, decreases immobility time. A preliminary study in humans reports that intravenous galanin has an antidepressant-like effect, reflected by a suppression of REM sleep in healthy male volunteers. Thus, the site of administration and any resultant differential activation of central or peripheral receptors seem to be important for the outcomes observed in these different studies.

Transgenic mice studies also indicate a role for galanin in depressive-like behavior. PDGF-galanin OE mice display increased 'depressive-like' behavior, with increased immobility in the forced swim test; in contrast, DβH-galanin OE mice, display 'normal' responses in the tail suspension test, another test of depressive-like behavior. Importantly, PDGF-galanin OE mice, but not DβH-galanin OE mice, exhibit elevated NE and serotonin release in the hippocampus after swim stress, suggesting that PDGF-galanin OE mice have a maladaptive response to uncontrollable stress, linked to monoamine release.

GalR1 and GalR2 KO mice display no significant changes in either the forced swim test or tail suspension test, although a meta-analysis combining results of GalR2 KO mice from several studies, suggests increased depressive-like behavior; and GalR2 KO mice display increased depressive-like responses in the learned helplessness paradigm. GalR2 OE mice display a response in the forced swim test that suggests a role for GalR2 in controlling depressive-like behavior.[16]

Furthermore, studies with peptides selective for individual galanin receptors suggest GalR1 and GalR3 signaling may increase, while GalR2 signaling decreases depressive-like behavior. For example, the GalR1 agonist M617 increases immobility in the forced swim test, and the GalR3 antagonist decreases depressive-like behaviors in several tests; while the GalR2 agonist AR-M1896 decreases, and the GalR2 antagonist M871 increases, depressive-like behavior (immobility) in the forced swim test.

Addiction

Galanin seems to act via GalR1 as a negative regulator of opiate (and possibly cocaine) reward (see Picciotto in Ref. 8). Central galanin administration attenuates place preference for morphine in mice, whereas galanin KO mice display enhanced place preference and increased locomotor responses to morphine. Galanin also seems to decrease signs of opiate withdrawal, with the nonspecific galanin receptor agonist, galnon, attenuating opiate withdrawal, while galanin KO mice show increased signs of withdrawal.

Galanin seems to increase intake of alcohol when injected icv into the hypothalamic PVN, which is proposed to be due to the increased caloric content of alcohol. Single-nucleotide polymorphisms (SNPs) in the galanin and the GalR3 genes have also been associated with alcoholism, suggesting endogenous galanin may play a role in this condition.[1] Variations in the GalR1 gene is associated with tobacco craving in smokers attempting to quit, suggesting a role for galanin in nicotine dependence.[17]

Multiple Sclerosis

There is increasing evidence that galanin expression is increased in glial cells and neurons after injury or insult[33] (see Butzkueven and Gundlach in Ref. 8). Notably, galanin is upregulated in oligodendrocytes in experimental autoimmune encephalomyelitis (EAE), a mouse model of multiple sclerosis

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(MS). Galanin KO mice display significantly more severe EAE-related symptoms with an earlier peak of severity. In addition, GalR2 KO mice show a marked increase in severity of EAE symptoms, suggesting an important role of GalR2 in the response. In line with a neuroprotective role of the peptide, galanin OE mice display a marked improvement in EAE symptoms compared with wildtype mice. In addition, a study of postmortem brain tissue from five MS patients reveals a marked upregulation of galanin in ~30% of cells in chronic active MS lesions and in remyelinated shadow plaques.

Stroke

Galanin and galanin receptor upregulation occurs after ischemic brain damage (see Shen and Gundlach in Ref. 8). Focal ischemia induced by middle cerebral artery occlusion in rats increases galanin and GalR1 mRNA in the penumbral and undamaged adjacent areas on days 1 and 2 postinfarct, and increases GalR2 mRNA on day 2; consistent with the proposed neuroprotective role of galanin signaling in other injury models.

Therapeutic Potential

It is now established that most transmitter systems in the brain can release a cocktail of signaling molecules including classical transmitters and peptides and that neuropeptides are mainly recruited after severe stress or aversive/pathological events to modulate transmission and promote plasticity and repair. Human studies have also shown that dysregulation of neuropeptides is probably important for neurodegenerative and neuropsychiatric disorders. Overall, available evidence provides substantial support for the further exploration of galanin-related ligands with differing receptor selectivity as treatments for the pathology or associated physiological/behavioral symptoms of epilepsy, nerve injury and chronic pain, AD, MS and stroke; and stress-related anxiety and depression, alcohol and other addictions, and cognitive impairments associated with many of these disorders.

[This review is respectfully dedicated to the late Kazuhiko Tatemoto, who died in late 2011].

REFERENCES

1. Belfer I, Hipp H, Bollettino A, McKnight C, Evans C, Virkkunen M, et al. Alcoholism is associated with GALR3 but not two other galanin receptor genes. *Genes Brain Behav* 2007;**6**:473–81.
2. Burazin TCD, Larm JA, Ryan MC, Gundlach AL. Galanin-R1 and -R2 receptor mRNA expression during the development of rat brain suggests differential subtype involvement in synaptic transmission and plasticity. *Eur J Neurosci* 2000;**12**:2901–17.
3. Cunningham MJ, Scarlett JM, Steiner RA. Cloning and distribution of galanin-like peptide mRNA in the hypothalamus and pituitary of the macaque. *Endocrinology* 2002;**143**:755–63.
4. Dungan Lemko HM, Clifton DK, Steiner RA, Fraley GS. Altered response to metabolic challenges in mice with genetically targeted deletions of galanin-like peptide. *Am J Physiol Endocrinol Metab* 2008;**295**:E605–12.
5. Eberhard N, Mayer C, Santic R, Navio RP, Wagner A, Bauer HC, et al. Distribution of alarin immunoreactivity in the mouse brain. *J Mol Neurosci*, 2012;**46**:18–32.
6. Elvander E, Schött PA, Sandin J, Bjelke B, Kehr J, Yoshitake T, et al. Intraseptal muscarinic ligands and galanin: influence on hippocampal acetylcholine and cognition. *Neuroscience* 2004;**126**:541–57.
7. Gundlach AL. Galanin/GALP and galanin receptors: role in central control of feeding, body weight/obesity and reproduction? *Eur J Pharmacol* 2002;**440**:255–68.
8. Hökfelt T, editor. *Galanin, experientia supplementum*. **vol. 102**: Springer Basel AG; 2010, 280 pp.
9. Holmes A, Picciotto MR. Galanin: a novel therapeutic target for depression, anxiety disorders and drug addiction? *CNS Neurol Disord Drug Targets* 2006;**5**:225–32.
10. Jacobowitz DM, Kresse A, Skofitsch G. Galanin in the brain: chemoarchitectonics and brain cartography—a historical review. *Peptides* 2004;**25**:433–64.
11. Kageyama H, Kita T, Toshinai K, Guan JL, Date Y, Takenoya F, et al. Galanin-like peptide promotes feeding behaviour via activation of orexinergic neurones in the rat lateral hypothalamus. *J Neuroendocrinol* 2006;**18**:33–41.
12. Kalló I, Vida B, Deli L, Molnár CS, Hrabovszky E, Caraty A, et al. Co-localisation of kisspeptin with galanin or neurokinin B in afferents to mouse GnRH neurones. *J Neuroendocrinol*, 2012;**24:**464–76.
13. Lang R, Gundlach AL, Kofler B. The galanin peptide family: receptor pharmacology, pleiotropic biological actions, and implications in health and disease. *Pharmacol Ther* 2007;**115**:177–207.
14. Larm JA, Gundlach AL. Galanin-like peptide (GALP) mRNA expression is restricted to arcuate nucleus of hypothalamus in adult male rat brain. *Neuroendocrinology* 2000;**72**:67–71.
15. Lawrence C, Fraley GS. Galanin-like peptide (GALP) is a hypothalamic regulator of energy homeostasis and reproduction. *Front Neuroendocrinol* 2011;**32**:1–9.
16. Le Maître TW, Xia S, Le Maitre E, Dun XP, Lu J, Theodorsson E, et al. Galanin receptor 2 overexpressing mice display an antidepressive-like phenotype: possible involvement of the subiculum. *Neuroscience* 2011;**190**:270–88.
17. Lori A, Tang Y, O'Malley S, Picciotto MR, Wu R, Conneely KN, et al. The galanin receptor 1 gene associates with tobacco craving in smokers seeking cessation treatment. *Neuropsychopharmacology* 2011;**36**:1412–20.
18. Merchenthaler I, Lopez F, Negro-Vilar A. Anatomy and physiology of central galanin-containing pathways. *Prog Neurobiol* 1993; **40**:711–69.
19. Moreno E, Vaz SH, Cai NS, Ferrada C, Quiroz C, Barodia SK, et al. Dopamine-galanin receptor heteromers modulate cholinergic neurotransmission in the rat ventral hippocampus. *J Neurosci* 2011;**31**: 7412–23.
20. Murck H, Antonijevic IA, Frieboes RM, Maier P, Schier T, Steiger A. Galanin has REM-sleep deprivation-like effects on the sleep EEG in healthy young men. *J Psychiatr Res* 1999;**33**:225–32.
21. Ohtaki T, Kumano S, Ishibashi Y, Ogi K, Matsui H, Harada M, et al. Isolation and cDNA cloning of a novel galanin-like peptide (GALP) from porcine hypothalamus. *J Biol Chem* 1999;**274**:37041–5.

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22. Picciotto MR, Brabant C, Einstein EB, Kamens HM, Neugebauer NM. Effects of galanin on monoaminergic systems and HPA axis: potential mechanisms underlying the effects of galanin on addiction- and stress-related behaviors. *Brain Res* 2010;**1314**:206–18.
23. Pirondi S, D'Intino G, Gusciglio M, Massella A, Giardino L, Kuteeva E, et al. Changes in brain cholinergic markers and spatial learning in old galanin-overexpressing mice. *Brain Res* 2007;**1138**: 10–20.
24. Ryan MC, Gundlach AL. Localization of preprogalanin messenger RNA in rat brain: identification of transcripts in a subpopulation of cerebellar Purkinje cells. *Neuroscience* 1996;**70**:709–28.
25. Santic R, Fenninger K, Graf K, Schneider R, Hauser-Kronberger C, Schilling FH, et al. Gangliocytes in neuroblastic tumors express alarin, a novel peptide derived by differential splicing of the galanin-like peptide gene. *J Mol Neurosci* 2006;**29**:145–52.
26. Shen J, Larm JA, Gundlach AL. Galanin-like peptide mRNA in neural lobe of rat pituitary. Increased expression after osmotic stimulation suggests a role for galanin-like peptide in neuron-glial interactions and/or neurosecretion. *Neuroendocrinology* 2001;**73**:2–11.
27. Shen PJ, Larm JA, Gundlach AL. Expression and plasticity of galanin systems in cortical neurons, oligodendrocyte progenitors and proliferative zones in normal brain and after spreading depression. *Eur J Neurosci* 2003;**18**:1362–76.
28. Szymusiak R, Gvilia I, McGinty D. Hypothalamic control of sleep. *Sleep Med* 2007;**8**:291–301.
29. Takatsu Y, Matsumoto H, Ohtaki T, Kumano S, Kitada C, Onda H, et al. Distribution of galanin-like peptide in the rat brain. *Endocrinology* 2001;**142**:1626–34.
30. Tatemoto K, Rökaeus Å, Jornvall H, McDonald TJ, Mutt V. Galanin—a novel biologically active peptide from porcine intestine. *FEBS Lett* 1983;**164**:124–8.
31. Unschuld PG, Ising M, Roeske D, Erhardt A, Specht M, Kloiber S, et al. Gender-specific association of galanin polymorphisms with HPA-axis dysregulation, symptom severity, and antidepressant treatment response. *Neuropsychopharmacology* 2010;**35**:1583–92.
32. Van Der Kolk N, Madison FN, Mohr M, Eberhard N, Kofler B, Fraley GS. Alarin stimulates food intake in male rats and LH secretion in castrated male rats. *Neuropeptides* 2010;**44**:333–40.
33. Wraith DC, Pope R, Butzkueven H, Holder H, Vanderplank P, Lowrey P, et al. A role for galanin in human and experimental inflammatory demyelination. *Proc Natl Acad Sci USA* 2009;**106**:15466–71.
34. Wray NR, Pergadia ML, Blackwood DH, Penninx BW, Gordon SD, Nyholt DR, et al. Genome-wide association study of major depressive disorder: new results, meta-analysis, and lessons learned. *Mol Psychiatry* 2012;**17**:36–48.
35. Xu X-J, Andell S, Bartfai T, Wiesenfeld-Hallin Z. Fragments of galanin message-associated peptide (GMAP) modulate the spinal flexor reflex in rat. *Eur J Pharmacol* 1996;**318**:301–6.

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Chapter 104

Ghrelin

Masayasu Kojima, Yuki Nakamura, Takahiro Sato and Kenji Kangawa

ABSTRACT

Small synthetic molecules called growth hormone secretagogs (GHSs) stimulate the release of growth hormone (GH) from the pituitary via the GHS-R. Using a reverse pharmacology method in a stable cell line expressing GHS-R, an endogenous ligand for GHS-R was purified from rat stomach; this molecule was named "ghrelin." The characteristic structure of ghrelin contains an acyl modification at the third amino acid, a conserved serine (threonine in frogs). All ghrelin peptides isolated so far have been modified at this position with a medium-chain fatty acid, typically *n*-octanoic acid. This modification is essential for the biological activity of ghrelin. The primary production site of ghrelin is the stomach, but the peptide's major active sites are in the brain and the pituitary. Currently, ghrelin is the only known circulating orexigenic hormone that is secreted from the stomach under fasting conditions, it transmits a hunger signal from the periphery to the central nervous system, and it acts on the hypothalamic arcuate nucleus, a region involved in the regulation of appetite.

DISCOVERY

Ghrelin was identified in stomach tissues and shown to act as an endogenous ligand for the growth hormone secretagog receptor (GHS-R).[21,23] The name "ghrelin" is based on "ghre," a word root in Proto-Indo-European languages meaning "grow," and refers to the ability of this peptide to stimulate growth hormone (GH) release.

In 1976, C.Y. Bowers found that some opioid peptide derivatives had weak GH-releasing activity. After this observation, many peptide derivatives and nonpeptide compounds with potent GH-releasing activity were synthesized. These compounds are termed growth hormone secretagogs (GHS).[3] In 1996, GHS-R was identified by expression cloning.[17] After the identification of the GHS-R, a search for its endogenous ligand was actively undertaken, using the orphan receptor strategy. Finally, in 1999, the ligand was discovered in an unexpected tissue: the stomach.[21]

The structure of ghrelin is unprecedented. Ghrelin is the first known bioactive peptide modified by a fatty acid: ghrelin is modified at its Ser3 by *n*-octanoic acid, and this modification is essential for ghrelin's activity.[21]

In rat stomach, a second type of ghrelin peptide has been purified and identified as des-Gln^{14}-ghrelin.[15] Except for the deletion of Gln^{14}, des-Gln^{14}-ghrelin is identical to ghrelin, even retaining the *n*-octanoic acid modification. Des-Gln^{14}-ghrelin has the same biological activities and potency as canonical ghrelin. The deletion of Gln^{14} in des-Gln^{14}-ghrelin arises from the use of a CAG codon to encode Gln; this trinucleotide is sometimes recognized as a splicing signal. However, des-Gln^{14}-ghrelin is only present at low levels in the stomach, indicating that ghrelin is the major active form.

In the course of purifying human ghrelin from the stomach, several minor forms of ghrelin peptide were also isolated.[16] These could be classified into four groups by the type of acylation observed at Ser^{3}: nonacylated, octanoylated (C8:0), decanoylated (C10:0), and decenoylated (C10:1). All peptides found were either 27 or 28 amino acids in length, with the shorter peptides lacking the C-terminal Arg^{28} and are derived from the same ghrelin precursor via two alternative pathways. As was the case in the rat, the major active form of human ghrelin is a 28-amino acid peptide with octanoylated Ser^{3}. Synthetic octanoylated and decanoylated ghrelins stimulate the increase of intracellular Ca^{2+} in GHS-Rexpressing cells, and stimulate GH release in rats, to a similar degree (Fig. 1).

Figs. 2 and 3 show sequence comparisons of the identified vertebrate ghrelins.[22]

STRUCTURE OF THE GHRELIN GENE, mRNA, AND PRECURSOR PROTEIN

The human ghrelin gene is located on chromosome 3p25–26. The human ghrelin receptor gene is also on chromosome 3, at position q26–27.

The human ghrelin gene, like the mouse gene, contains four exons (Fig. 4). Another short exon that contains only 20 bp, encoding part of the 5′-untranslated region, is transcribed as a minor species of ghrelin mRNA.[20]

The 28 amino acids of the functional ghrelin peptide are encoded in exons 1 and 2. In the rat and mouse ghrelin genes, the codon for Gln^{14} (CAG) serves as an alternative

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00104-4
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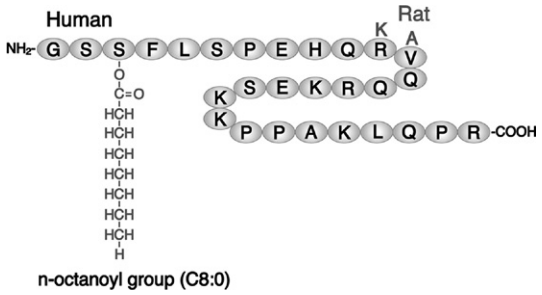

FIGURE 1 Structures of human and rat ghrelins. Both human and rat ghrelins are 28-amino-acid peptides, in which Ser[3] is modified by a lipid, primarily *n*-octanoic acid. This modification is essential for ghrelin's activity.

Mammalian ghrelin

	*
American bison	GSSFLSPEHQKLQ-RKEPKKPSGRLKPR
Bos taurus indicus	GSSFLSPEHQKVQ-RKESKKPPAKLQPR
Cat	GSSFLSPEHQKLQ-RKEAKKPSGRLKPR
Cow	GSSFLSPEHQKLQ-RKEAKKPSGRLKPR
Dog	GSSFLSPEHQKLQQRKESKKPPAKLQPR
Giant panda	GSSFLSPEHQKVQQRKESKKPPAKLQPR
Goat	GSSFLSPEHQKLQ-RKEPKKPSGRLKPR
Golden hamster	GSSFLSPEHQKAQQRKESKKPQAKLQPR
Hamadryas baboon	GSSFLSPEHQRAQQRKESKKPPAKLQPR
Horse	GSSFLSPEHHKVQHRKESKKPPAKLKPR
House shrew	GSSFLSPEHQKGP-KKDPRKPP-KLQPR
Human	GSSFLSPEHQRVQQRKESKKPPAKLQPR
Mongolian Gerbil	GSSFLSPEHQKTQQRKESKKPPAKLQPR
Moose	GSSFLSPDHQKLQ-RKEPKKPSGRLKPR
Mouse	GSSFLSPEHQKAQQRKESKKPPAKLQPR
Mule deer	GSSFLSPEHQKLQ-RKEPKKPSGRLKPR
Pig	GSSFLSPEHQKVQQRKESKKPAAKLKPR
Pronghorn	GSSFLSPEHQKLQ-RKEPKKPSGM----
Pygmy sperm whale	GSSFLSPEHQKLQ-RKEAKKPSGRLKPR
Rabbit	GSSFLSPEHQKVQQRKESKKPAAKLKPR
Rat	GSSFLSPEHQKAQQRKESKKPPAKLQPR
Reindeer	GSSFLSPEHQKLQ-RKEPKKPSGRLKPR
Rhesus Monkey	GSSFLSPEHQRAQQRKESKKPPAKLQPR
Sheep	GSSFLSPEHQKLQ-RKEPKKPSGRLKPR
Spinifex hopping mouse	GSSLFSPEHQKAQQRKESKKPPAKLQPR
Tammar wallaby	GSSFLSPEHPKTQ-RKESKKP-AKLQPR
Wapiti	GSSFLSPEHQKLQ-RKEPKKPSGRLKPR
Water buffalo	GSSFLSPEHQKLQ-RKEPKKPSGRLKPR
White-tailed deer	GSSFLSPEHQKLQ-RKEPKKPSGRLKPR

FIGURE 2 Sequence comparison of mammalian ghrelins. Identical amino acids in each mammal are shaded. The asterisks indicate acyl-modified third amino acids.

splicing signal, resulting in the generation of two different ghrelin mRNAs: one encoding the ghrelin precursor, and the other encoding the des-Gln[14]-ghrelin precursor.[15] As noted above, des-Gln[14]-ghrelin is identical to ghrelin, except for the deletion of Gln[14].

Complementary DNA analyses indicated that des-Gln[14]-ghrelin cDNA is also present in human stomach (GenBank Accession number: AB035700). However, the number of human des-Gln[14]-ghrelin cDNA clones is low, and des-Gln[14]-ghrelin peptides have not yet been isolated from human stomach tissue. Two cDNA clones encoding for human des-Gln[14]-ghrelin, obtained from a human fetus library, have been deposited in the NCBI nucleotide database (AI338429 and

Bird ghrelin

	*
Chicken	GSSFLSPTYKNIQQQKDTRKPTARLH
Duck	GSSFLSPEFKKIQQQNDPTKTTAKIH
Emu	GSSFLSPDYKKIQQRKDPRKPTTKLH
Goose	GSSFLSPEFKKIQQQNDPAKATAKIH
Japanese quail	GSSFLSPAYKNIQQQKNTRKPAARLH
Turkey	GSSFLSPAYKNIQQQKDTRKPTARLHPR

Fish ghrelin

	*
Rainbow Trout 1	GSSFLSPSQKPQ**VRQ**GKGK-PPRV
Rainbow Trout 2	GSSFLSPSQKPQGKGK-PPRV
European sea bass	GSSFLSPSQKPQSRGK-SSRV
Black sea bream	GSSFLSPSQKPQNRGK-SSRV
Channel catfish	GSSFLSPTQKPQNRGDRKPPRV
Goldfish	GTSFLSPAQKPQ--GRRPPRM
Japanese eel	GSSFLSPSQRPQGKDKKPPRV
Tilapia	GSSFLSPSQKPQNKVK-SSRI
Zebrafish	GTSFLSPTQKPQ--GRRPPRV
Shark (Carcharhinus melanopterus)	GVSFHPRLKEKDDNSSGNTRKFSP
Shark (Sphyrna lewini)	GVSFHPRLKEKDDNSSGNSRKSNP

Amphibian ghrelin

	*
Bullfrog	GLTFLSPADMQKIAERQSQNKLRHGNMN
Edible frog	GLTFLSPADMRKIAERQSQNKLRHGNMN

Reptile ghrelin

	*
Red-eared slider turtle	GSSFLSPEYQNTQQRKDPKKHTKLN

FIGURE 3 Sequence comparison of nonmammalian ghrelins. Identical amino acids in each species of bird, fish, and frog are colored. The asterisks indicate acyl-modified third amino acids. N-terminal cores with acyl-modification sites are well conserved among all ghrelins.

BY149645). There are two types of porcine ghrelin cDNA, which encode ghrelin and des-Gln[14]-ghrelin, and are present at an approximate ratio of 1:1 (GenBank Accession number: AB035703 and AB035704). In the cow, only one ghrelin mRNA exists, and it encodes a 27-amino-acid ghrelin.

DISTRIBUTION OF GHRELIN IN THE CENTRAL NERVOUS SYSTEM

Ghrelin mRNA is mainly expressed in the stomach; lower levels of ghrelin expression are also observed in the small and large intestines.[9] In contrast to the high concentration of ghrelin in the gastrointestinal tissues, the ghrelin content of the central nervous system is low. By means of immunohistochemical analysis, ghrelin-responsive neuronal cells can be identified in the hypothalamus.[6,21,29] Peptide extracts from hypothalamus were analyzed and characterized by RP-HPLC and ghrelin-specific RIAs. Two ghrelin forms (*n*-octanoyl ghrelin and des-acyl ghrelin) were identified in the hypothalamus, as seen in gastric tissue.[29] These results indicate that acyl modification is not specific to stomach tissue and that it occurs in the hypothalamus.

Ghrelin-immunoreactive neurons are also found in the internuclear space between the lateral hypothalamus, the arcuate nucleus (ARH), the ventromedial nucleus

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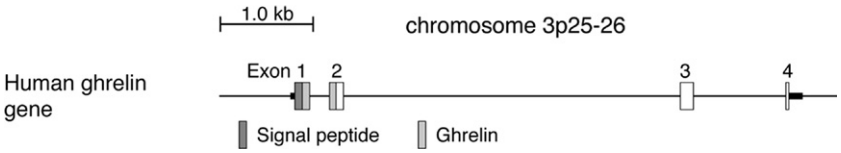

FIGURE 4 Genome structure of human ghrelin. The human ghrelin gene comprises four exons. The first exon encodes the signal peptide and the N-terminal half of ghrelin peptide. The second exon encodes the C-terminal half of ghrelin peptide.

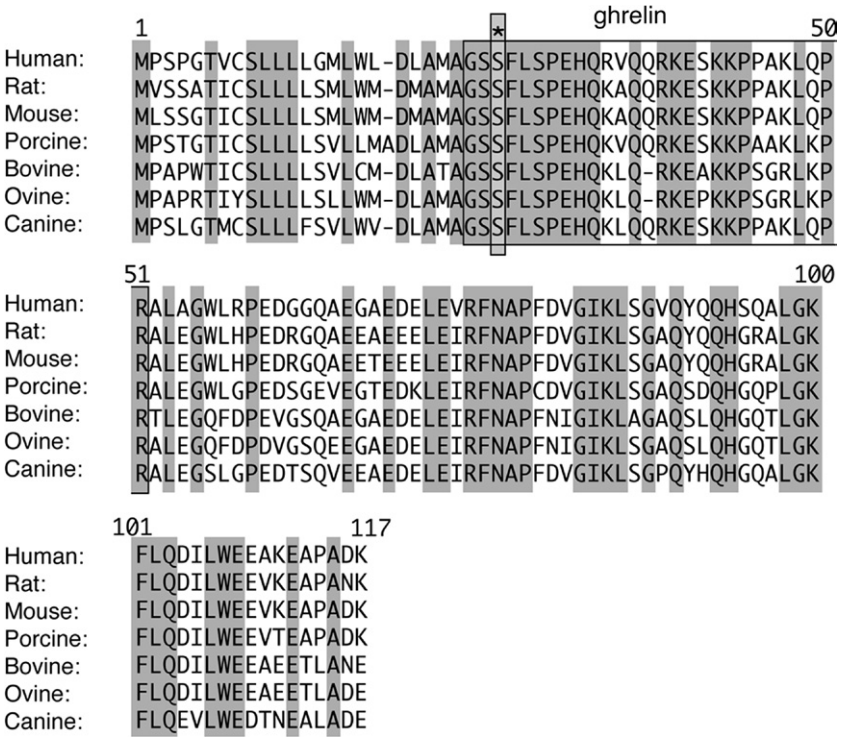

FIGURE 5 Amino-acid sequences of mammalian ghrelin precursors. A sequence comparison between mammalian ghrelin precursors is shown. Identical amino acids are shaded. The asterisk shows the position of the acyl-modified Ser^3. Note that the amino acid sequences of mammalian ghrelin precursors are well conserved; in particular, the N-terminal 10 amino acids of all of these active ghrelin peptides, each of which contains an acyl-modified serine in its active core, are identical.

(VMN), the dorsomedial nucleus (DMN), the paraventricular nucleus (PVN) and the ependymal layer of the third ventricle.[6]

Intravenous injection of ghrelin stimulates NPY/AgRP neurons in the hypothalamus.[27] Immunohistochemical analysis shows that ghrelin neuron fibers directly contact NPY/AgRP neurons.[6] These results indicate that ghrelin exerts its orexigenic activity by stimulating NPY/AgRP neurons in the hypothalamus, to promote the production and secretion of NPY and AgRP peptides.

PROCESSING OF THE GHRELIN PRECURSOR

The amino acid sequences of mammalian ghrelin precursors are highly conserved (Fig. 5).[22] The rat and human ghrelin precursors both consist of 117 amino acids.[21] In these precursors, the 28-amino-acid active ghrelin sequence immediately follows the signal peptide. The cleavage site for the signal peptide is the same in all mammalian ghrelins. Although propeptides are usually processed at dibasic amino acid sites by prohormone convertases, the C-terminus of the ghrelin peptide sequence is processed at an uncommon ProArg recognition site. In knockout mice lacking the prohormone convertases PC1/3, PC2, and PC5/6A, Zhu et al. showed that proghrelin was processed in all but the PC1/3 knockout mouse.[35] Moreover, via immunohistochemical studies, PC1/3 and ghrelin were shown to colocalize in the same cells of the stomach. Thus, PC1/3 is probably a processing protease responsible for proghrelin processing in the stomach. Because PC1/3 is expressed in the brain, it may also be the processing enzyme for ghrelin in the brain, just as in gastric tissue (see the PC1/3 chapter in the Peptide Biosynthesis/Processing section of this book).

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Acyl modification is an important processing step for ghrelin, because ghrelin requires this modification (in particular, *n*-octanoyl modification) to exhibit full activity.[21] The enzyme that catalyzes this acyl transfer to ghrelin was identified in February 2008, when two independent groups reported the identification of ghrelin *O*-acyltransferase (GOAT).[34]

One group, led by Brown and Goldstein at the University of Texas Southwestern Medical Center, searched through the genome database and identified several orphan acyltransferases, that is, enzymes whose functions had not been determined. The authors expressed both ghrelin and candidate enzyme cDNAs in a cell culture system and examined the production of acyl-modified ghrelin. Ultimately, they identified MBOAT4 (Membrane-Bound *O*-Acyltransferase) as the enzyme that acyl modifies the ghrelin peptide.[34]

Another group, at Eli Lilly, identified several candidate acyltransferases in the genome database and suppressed their expression by siRNA in TT cells, a thyroid medulla carcinoma cell line in which acyl-modified ghrelin is endogenously expressed. They also identified MBOAT4 as the enzyme responsible for ghrelin acyl modification.[14] The authors then created MBOAT4 knockout mice and confirmed that ghrelin is not acyl modified in the absence of this gene.

Thus, MBOAT4 is the acyltransferase that catalyzes the transfer of *O*-acyl groups to the hydroxyl moiety of the Ser3 in ghrelin and hereafter called ghrelin *O*-acyltransferases (GOAT). GOAT, a membrane-bound enzyme with multiple transmembrane domains, is a member of an acyltransferase family that includes at least 16 acyltransferase enzymes. Among these, only GOAT shows the ability to acyl-modify ghrelin. Like the existence of ghrelin in all vertebrate species, GOAT is also found in mammals, birds, and fish. The distribution of GOAT is similar to that of ghrelin: GOAT is predominantly found in gastrointestinal organs, particularly the stomach; it is expressed at lower levels in the brain (see the GOAT chapter in the Peptide Biosynthesis/Processing section of this book).

Interestingly, ingestion of either medium-chain fatty acids (MCFAs) or medium-chain triacylglycerols (MCTs) specifically increases the production of acyl-modified ghrelin without changing the total ghrelin level (acyl- and des-acyl-).[28] When mice ingest either MCFAs or MCTs, the acyl group attached to nascent ghrelin molecules corresponds to that of the ingested MCFAs or MCTs. Moreover, *n*-heptanoyl (C7:0) ghrelin, an unnatural form of ghrelin, is produced in the stomach of mice after the ingestion of *n*-heptanoic acid or glyceryl triheptanoate. These findings indicate that ingested fatty acids are directly used for acyl modification of ghrelin.

GHRELIN RECEPTOR IN THE BRAIN

Ghrelin receptor, or GHS-R, is a typical G-protein-coupled receptor with seven-transmembrane domains (7-TM). Two distinct ghrelin receptor cDNAs have been isolated.[17] The first, GHS-R type 1a, encodes a 7-TM GPCR with binding and functional properties consistent with its role as ghrelin's receptor. Another GHS-R cDNA, type 1b, is produced by an alternative splicing mechanism. The GHS-R gene consists of two exons; the first exon encodes TM-1 to TM-5, and the second exon encodes TM-6 and TM-7. Type 1b is derived from only the first exon and encodes only five of the seven predicted TM domains. The type 1b receptor is thus a C-terminal truncated form of the type 1a receptor, and is pharmacologically inactive.

Ghrelin receptor mRNA is prominently expressed in the arcuate (ARC) and ventromedial nuclei (VMN) and in the hippocampus.[13] Ghrelin receptor (GHS-R) mRNA is also detected in multiple hypothalamic nuclei, in the pituitary, as well as in the dentate gyrus, CA2, and CA3 regions of the hippocampus, the substantia nigra, the ventral tegmental area, and the dorsal and median raphe nuclei.

BIOLOGICAL ACTIONS OF GHRELIN WITHIN THE BRAIN

Stimulation of GH Release

Intravenous injection of ghrelin induces potent GH release, in both rat and humans.[21,30] When anaesthetized rats were injected intravenously with ghrelin, an increase in plasma GH concentration was observed. The release of GH peaks approximately 5–15 min after ghrelin injection and returns to basal levels 1 h later. Intracerebroventricular administration of ghrelin also increased rat plasma GH concentrations in a dose-dependent manner.[10] Ghrelin specifically stimulates GH release from cultured pituitary cells in a dose-dependent manner, indicating that ghrelin acts directly on the pituitary.[21] Together, these *in vivo* and *in vitro* assays have confirmed that ghrelin is a specific GH-releasing peptide.

Appetite Stimulation

Immunohistochemical analyses indicate that ghrelin-containing neural cells are localized to the arcuate nucleus of the hypothalamus, a region involved in the regulation of appetite. In fact, when ghrelin is injected into the cerebral ventricles of rats, their food intake is potently stimulated.[27] At least part of this orexigenic effect of ghrelin is exerted by ghrelin's activation of genes encoding two potent appetite stimulators, agouti-related peptide and neuropeptide Y (NPY), whose expression is located primarily in the arcuate nucleus.

Injection of ghrelin icv induces Fos expression in NPY-expressing neurons and increases the amount of NPY mRNA in the arcuate nucleus.[27] Moreover, icv ghrelin injection increases the AgRP mRNA level in the hypothalamus.

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An antagonist of NPY receptor 1 blocks the appetite-stimulating effects of ghrelin. Injections of an AgRP inhibitor icv, anti-NPY IgG, and anti-AgRP IgG inhibit the appetite-stimulating effects of ghrelin. Intravenous injection of ghrelin also stimulates NPY/AgRP neurons in the hypothalamus. Immunohistochemical analysis demonstrated that ghrelin-receptor-expression neuron fibers directly contact NPY/AgRP-expression neurons.[6] These results indicate that ghrelin exerts its feeding activity by stimulating NPY/AgRP neurons in the hypothalamus to promote the production and secretion of NPY and AgRP peptides. Studies with knockout mice lacking NPY, AgRP, or both genes have confirmed these results: although deletion of either NPY or AgRP causes a modest effect (or no effect) on the orexigenic activity of ghrelin, the double knockout mice completley lacked such activity.[4]

Molecular Mechanism of Appetite Stimulation by Ghrelin

AMP-activated protein kinase (AMP-K) has been shown to be involved in hypothalamic appetite regulation.[32] Injection of 5-amino-4-imidazole carboxamide riboside (AICAR), an activator of AMP-K, significantly increases food intake. Administration of ghrelin *in vivo* increases AMP-K activity in the hypothalamus.[1] In contrast, injection of leptin decreases hypothalamic AMP-K activity.

Lipid metabolism in the hypothalamus is believed to be important for appetite regulation.[26] After binding of ghrelin, the ghrelin receptor activates hypothalamic AMP-K (AMP-activated protein kinase), and activated AMP-K then suppresses lipid synthesis. In such experiments, malonyl-CoA level in the hypothalamus decreases while the activity of carnitine palmitoyltransferase 1 (CPT1) is increased. Activated CPT1 then accelerates lipid transport into mitochondria, where lipids are catabolized.

UCP2 in the hypothalamus may be the key target for appetite stimulation by ghrelin, because ghrelin regulates mitochondrial oxidation in hypothalamic cells via UCP2.[2] Ninety percent of UCP2-expressing cells in the hypothalamus also express the ghrelin receptor. In isolated hypothalamic synaptosomes, ghrelin increases oxygen consumption and total respiration. Ghrelin administration also increases the number of mitochondria and UCP2 expression in NPY/AgRP-expressing cells of the hypothalamic arcuate nucleus (Fig. 6).

The orexigenic effect of ghrelin is exerted via the upregulation of NPY mRNA levels in the arcuate nucleus. However, in UCP2-deficient mice, ghrelin administration does not increase NPY level, and the number of c-Fos protein positive cells in the arcuate nucleus NPY/AgRP-expression neurons is decreased. Moreover, in the UCP2-deficient mice, AMP-K activity is not changed by ghrelin administration. AICAR stimulates mitochondrial respiration in the hypothalamus and increases appetite, whereas UCP2-deficient mice show no effects in response to AICAR. By contrast, the AMP-K inhibitor compound C suppresses appetite stimulation by ghrelin; however, in UCP2-deficient mice, appetite suppression by compound C is not observed. These results suggest that AMP-K activation by ghrelin is

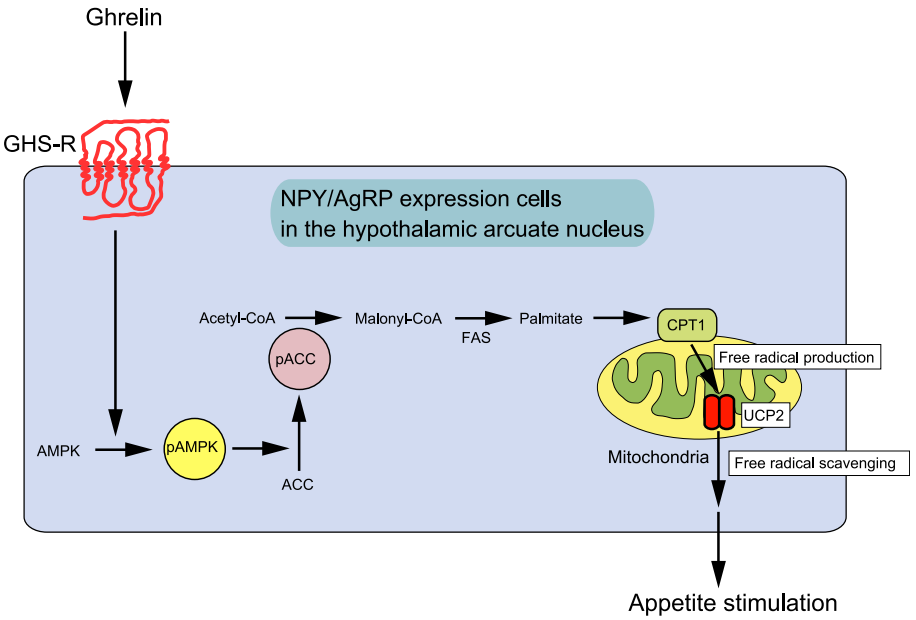

FIGURE 6 Intracellular action of ghrelin in NPY/AgRP-expression cells in the hypothalamic arcuate nucleus. In NPY/AgRP-expression cells in the hypothalamic arcuate nucleus, ghrelin stimulates AMP-K and regulates fatty acid metabolism, leading to CPT1 activation. Ghrelin's effects on mitochondrial respiration are dependent on the function of UCP2 and hence result in an increase of food intake. See color plate 27.

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upstream of UCP2. In other words, ghrelin stimulates UCP2 through AMP-K.

By lipid consumption in the hypothalamic mitochondria, free-radical production was increased. The free radicals thereby produced are scavenged by UCP2. In UCP2-deficient mice, ghrelin increases free-radical production relative to wild type.

Taken together, these results suggest that lipid metabolism and mitochondrial free-radical production in the hypothalamic neurons may be key factors involved in the regulation of appetite by ghrelin.

Pathway of the Ghrelin Signal: From Peripheral Tissues to the Central Nervous System

Circulating ghrelin stimulates hypothalamic neurons and stimulates food intake. In general, circulating peptides do not pass the bloodbrain barrier. In fact, the rate at which circulating ghrelin passes the barrier is very low. Thus, peripheral ghrelin must activate the appropriate hypothalamic regions via an indirect pathway.

The detection of ghrelin receptors on vagal afferent neurons in the rat nodose ganglion suggests that ghrelin signals from the stomach are transmitted to the brain via the vagus nerve.[11] Moreover, the observation that ICV administration of ghrelin induces c-Fos in the dorsomotor nucleus of the vagus, and stimulates gastric-acid secretion, indicates that ghrelin activates the vagus system.

Vagotomy inhibits the ability of ghrelin to stimulate food intake and GH release.[11] A similar effect was also observed when capsaicin, a specific afferent neurotoxin, was applied to vagus nerve terminals to induce sensory denervation. However, the basal level of ghrelin concentration is not affected after vagotomy. In contrast, fasting-induced elevation of plasma ghrelin is completely abolished by subdiaphragmatic vagotomy or atropine treatment.[31]

Moreover, peripheral ghrelin signaling, which travels to the nucleus tractus solitarius (NTS) via the vagus nerve, increases noradrenaline (NA) in the arcuate nucleus of the hypothalamus. Bilateral midbrain transections rostral to the NTS, or toxin-induced loss of neurons in hindbrain neurons expressing dopamine beta-hydroxylase (an NA-synthetic enzyme), abolished ghrelin-induced feeding. Thus, the noradrenergic system is necessary in the central control of feeding behavior by peripherally administered ghrelin. These results indicate that the response of ghrelin to fasting is transmitted through vagal afferent transmission.

Ghrelin Enhances Learning and Memory Processes Through Serotonin System

The ghrelin receptor is expressed in the hippocampus, implicating ghrelin in learning and memory.[13] Several reports indicate that ghrelin enhances learning and memory processes. Neuronal innervation by serotonergic neurons from the dorsal raphe nucleus (DRN) is necessary for the enhancement of learning processes in the hippocampus. These results are confirmed by the observations that direct ghrelin injection into DRN enhances memory retention, and pretreatment of a selective serotonin uptake inhibitor (fluoxetine) decreases it. Moreover, ghrelin promotes dendritic spine synapse formation, whereas ablation of ghrelin decreases the number of spine synapses in the hippocampal CA1 region.[12]

Ghrelin Regulates Emotional Behavior Through Activating Dopaminergic Neurons

Dopaminergic neurons from the ventral tegmental area (VTA) are involved in reward and motivated behaviors. Ghrelin is believed to activate this dopaminergic neural pathway and regulate reward and motivated behaviors.

The ghrelin receptor, GHS-R, is localized in the dopaminergic neurons of the VTA. Direct injection of ghrelin into the VTA region increases food and alcohol intakes dose dependently.[18] In contrast, ghrelin-KO mice, ghrelin receptor knockout or injection of GHS-R antagonists both decrease alcohol intake and suppresses alcohol-induced locomotor activity and accumbal dopamine release. Ghrelin also suppresses hyperactivity resulting from cocaine or amphetamine intake.

In humans, an SNP in the GHS-R gene is associated with heavy alcohol consumption.[24] Moreover, another SNP in GHS-R, as well as haplotypes of both the ghrelin and GHS-R genes, are associated with body mass in heavy consumers of alcohol. Thus, the ghrelin signal in the VTA may be a candidate target for the treatment of alcohol and drug abuse.

Neuroprotective Effects of Ghrelin

Using animal models of neuronal diseases, it has been shown that ghrelin has neuroprotective activity. Ghrelin administration improved symptoms in animal models, including MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine)-induced Parkinson's disease models,[19] neural ischemia models or recirculation models for cerebellar ischemia.[5] Moreover, ghrelin inhibits progress of autoimmune encephalomyelitis in an animal model of multiple sclerosis.

In addition, the ghrelin receptor is expressed in spinal cord, and ghrelin stimulates functional recovery after spinal injuries.[25] The mechanism of ghrelin's neuroprotective effects is not yet fully understood. However, it has been postulated that inhibition of apoptosis, suppression of reactive oxygen species production, and/or mitochondrial neogenesis may be involved in the neuroprotective role of ghrelin.

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Thus, ghrelin may be applicable to clinical use in treatment of neuronal diseases.

Ghrelin and Sleep Regulation

The sleepwake cycle is closely associated with GH release and food intake rhythms, which are regulated partly by ghrelin. The basic sleepwake mechanism is not impaired in ghrelin-KO mice; however, ghrelin increases non-REM sleep and decreases REM sleep. During sleep, ghrelin concentrations increase in the early part of the night and decrease in the morning. Sleep deprivation blunts the increase of ghrelin during night. Thus, the nocturnal increase of ghrelin levels is probably induced by sleep-associated processes. Interestingly, the activity of orexin neurons is stimulated by ghrelin, indicating that fasting-induced ghrelin activates orexin neurons to arouse animals for foraging.[33]

Pathophysiological Implications

Plasma ghrelin concentrations are low in obese people and high in lean people. Anorexia nervosa (AN) is a syndrome often seen in young women; it is characterized by a combination of weight loss, amenorrhea, and behavioral changes. Some of these changes are reversible after weight gain. Plasma ghrelin concentrations in patients with AN are high and return to control levels after weight gain after renutrition. Patients with AN often show markedly elevated GH concentrations, which may result from high circulating levels of ghrelin. Moreover, high ghrelin increases ACTH, prolactin, and cortisol concentrations in humans, which may explain the amenorrhea and behavioral changes observed in patients with AN.

Patients who undergo gastric bypass lose weight, and their ghrelin levels decrease.[8] Changes in ghrelin concentration associated with food intake are diminished in these patients. Plasma ghrelin concentrations are also decreased in patients with short bowel syndrome, probably due to the loss of ghrelin-producing tissues.

High plasma ghrelin concentrations are observed in Prader Willi syndrome (PWS) patients.[7] PWS is a complex genetic disorder characterized by mild mental retardation, hyperphagia, short stature, muscular hypotonia, and distinctive behavioral features. Excessive appetite in PWS causes progressive severe obesity, which in turn leads to an increase in cardiovascular morbidity and mortality. The PWS genotype is characterized by a loss of one or more paternal genes in region q11–13 on chromosome 15. It has been suggested that this genetic alteration leads to dysfunction of several hypothalamic areas, including those involved in the regulation of appetite. The mean plasma concentration of ghrelin was 3- to 4-fold higher in PWS patients than in a reference population. Thus, ghrelin may be responsible, at least in part, for the hyperphagia seen in PWS. It is unclear, however, what underlies the increased ghrelin concentrations in these patients.

REFERENCES

1. Andersson U, Filipsson K, Abbott CR, Woods A, Smith K, Bloom SR, et al. AMP-activated protein kinase plays a role in the control of food intake. *J Biol Chem* 2004;**279**:12,005–8.
2. Andrews ZB, Liu ZW, Walllingford N, Erion DM, Borok E, Friedman JM, et al. UCP2 mediates ghrelin's action on NPY/AgRP neurons by lowering free radicals. *Nature* 2008;**454**:846–51.
3. Bowers CY. Growth hormone-releasing peptide (GHRP). *Cell Mol Life Sci* 1998;**54**:1316–29.
4. Chen HY, Trumbauer ME, Chen AS, Weingarth DT, Adams JR, Frazier EG, et al. Orexigenic action of peripheral ghrelin is mediated by neuropeptide Y and agouti-related protein. *Endocrinology* 2004;**145**: 2607–12.
5. Chung H, Kim E, Lee DH, Seo S, Ju S, Lee D, et al. Ghrelin inhibits apoptosis in hypothalamic neuronal cells during oxygen-glucose deprivation. *Endocrinology* 2007;**148**:148–59.
6. Cowley MA, Smith RG, Diano S, Tschop M, Pronchuk N, Grove KL, et al. The distribution and mechanism of action of ghrelin in the CNS demonstrates a novel hypothalamic circuit regulating energy homeostasis. *Neuron* 2003;**37**:649–61.
7. Cummings DE, Clement K, Purnell JQ, Vaisse C, Foster KE, Frayo RS, et al. Elevated plasma ghrelin levels in Prader Willi syndrome. *Nat Med* 2002;**8**:643–4.
8. Cummings DE, Weigle DS, Frayo RS, Breen PA, Ma MK, Dellinger EP, et al. Plasma ghrelin levels after diet-induced weight loss or gastric bypass surgery. *New Engl J Med* 2002;**346**:1623–30.
9. Date Y, Kojima M, Hosoda H, Sawaguchi A, Mondal MS, Suganuma T, et al. Ghrelin, a novel growth hormone-releasing acylated peptide, is synthesized in a distinct endocrine cell type in the gastrointestinal tracts of rats and humans. *Endocrinology* 2000;**141**:4255–61.
10. Date Y, Murakami N, Kojima M, Kuroiwa T, Matsukura S, Kangawa K, et al. Central effects of a novel acylated peptide, ghrelin, on growth hormone release in rats. *Biochem Biophys Res Commun* 2000;**275**: 477–80.
11. Date Y, Murakami N, Toshinai K, Matsukura S, Niijima A, Matsuo H, et al. The role of the gastric afferent vagal nerve in ghrelin-induced feeding and growth hormone secretion in rats. *Gastroenterology* 2002; **123**:1120–8.
12. Diano S, Farr SA, Benoit SC, McNay EC, da Silva I, Horvath B, et al. Ghrelin controls hippocampal spine synapse density and memory performance. *Nat Neurosci* 2006;**9**:381–8.
13. Guan XM, Yu H, Palyha OC, McKee KK, Feighner SD, Sirinathsinghji DJ, et al. Distribution of mRNA encoding the growth hormone secretagogue receptor in brain and peripheral tissues. *Brain Res Mol Brain Res* 1997;**48**:23–9.
14. Gutierrez JA, Solenberg PJ, Perkins DR, Willency JA, Knierman MD, Jin Z, et al. Ghrelin octanoylation mediated by an orphan lipid transferase. *Proc Natl Acad Sci U S A* 2008;**105**:6320–5.
15. Hosoda H, Kojima M, Matsuo H, Kangawa K. Purification and characterization of rat des-Gln14-Ghrelin, a second endogenous ligand for the growth hormone secretagogue receptor. *J Biol Chem* 2000;**275**: 21995–2000.

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16. Hosoda H, Kojima M, Mizushima T, Shimizu S, Kangawa K. Structural divergence of human ghrelin. Identification of multiple ghrelin-derived molecules produced by post-translational processing. *J Biol Chem* 2003;**278**:64–70.
17. Howard AD, Feighner SD, Cully DF, Arena JP, Liberator PA, Rosenblum CI, et al. A receptor in pituitary and hypothalamus that functions in growth hormone release. *Science* 1996;**273**:974–7.
18. Jerlhag E, Egecioglu E, Landgren S, Salome N, Heilig M, Moechars D, et al. Requirement of central ghrelin signaling for alcohol reward. *Proc Natl Acad Sci U S A* 2009;**106**:11,318–23.
19. Jiang H, Li LJ, Wang J, Xie JX. Ghrelin antagonizes MPTP-induced neurotoxicity to the dopaminergic neurons in mouse substantia nigra. *Exp Neurol* 2008;**212**:532–7.
20. Kanamoto N, Akamizu T, Tagami T, Hataya Y, Moriyama K, Takaya K, et al. Genomic structure and characterization of the 5'-flanking region of the human ghrelin gene. *Endocrinology* 2004;**145**:4144–53.
21. Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H, Kangawa K. Ghrelin is a growth-hormone-releasing acylated peptide from stomach. *Nature* 1999;**402**:656–60.
22. Kojima M, Ida T, Sato T. Structure of mammalian and nonmammalian ghrelins. *Vitam Horm* 2008;**77**:31–46.
23. Kojima M, Kangawa K. Ghrelin: structure and function. *Physiol Rev* 2005;**85**:495–522.
24. Landgren S, Jerlhag E, Zetterberg H, Gonzalez-Quintela A, Campos J, Olofsson U, et al. Association of pro-ghrelin and GHS-R1A gene polymorphisms and haplotypes with heavy alcohol use and body mass. *Alcohol Clin Exp Res* 2008;**32**:2054–61.
25. Lee JY, Chung H, Yoo YS, Oh YJ, Oh TH, Park S, et al. Inhibition of apoptotic cell death by ghrelin improves functional recovery after spinal cord injury. *Endocrinology* 2010;**151**:3815–26.
26. Lopez M, Lage R, Saha AK, Perez-Tilve D, Vazquez MJ, Varela L, et al. Hypothalamic fatty acid metabolism mediates the orexigenic action of ghrelin. *Cell Metab* 2008;**7**:389–99.
27. Nakazato M, Murakami N, Date Y, Kojima M, Matsuo H, Kangawa K, et al. A role for ghrelin in the central regulation of feeding. *Nature* 2001;**409**:194–8.
28. Nishi Y, Hiejima H, Hosoda H, Kaiya H, Mori K, Fukue Y, et al. Ingested medium-chain fatty acids are directly utilized for the acyl modification of ghrelin. *Endocrinology* 2005;**146**:2255–64.
29. Sato T, Fukue Y, Teranishi H, Yoshida Y, Kojima M. Molecular forms of hypothalamic ghrelin and its regulation by fasting and 2-deoxy-d-glucose administration. *Endocrinology* 2005;**146**:2510–6.
30. Takaya K, Ariyasu H, Kanamoto N, Iwakura H, Yoshimoto A, Harada M, et al. Ghrelin strongly stimulates growth hormone release in humans. *J Clin Endocrinol Metab* 2000;**85**:4908–11.
31. Williams DL, Grill HJ, Cummings DE, Kaplan JM. Vagotomy dissociates short- and long-term controls of circulating ghrelin. *Endocrinology* 2003;**144**:5184–7.
32. Xue B, Kahn BB. AMPK integrates nutrient and hormonal signals to regulate food intake and energy balance through effects in the hypothalamus and peripheral tissues. *J Physiol* 2006;**574**:73–83.
33. Yamanaka A, Beuckmann CT, Willie JT, Hara J, Tsujino N, Mieda M, et al. Hypothalamic orexin neurons regulate arousal according to energy balance in mice. *Neuron* 2003;**38**:701–13.
34. Yang J, Brown MS, Liang G, Grishin NV, Goldstein JL. Identification of the acyltransferase that octanoylates ghrelin, an appetite-stimulating peptide hormone. *Cell* 2008;**132**:387–96.
35. Zhu X, Cao Y, Voogd K, Steiner DF. On the processing of proghrelin to ghrelin. *J Biol Chem* 2006;**281**:38,867–70.

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Chapter 105

GHRH

Maria M. Malagon, Rafael Vazquez-Martinez, Antonio J. Martinez-Fuentes, Francisco Gracia-Navarro and Justo P. Castaño

ABSTRACT

Growth hormone-releasing hormone (GHRH), a 44 amino acid peptide primarily secreted by hypothalamic neurons of the arcuate nucleus, is the main regulator of pituitary somatotrophs. GHRH binds to its G protein-coupled receptor (GHRH-R) to activate diverse signaling pathways predominantly involving cAMP and Ca^{2+}, and thereby stimulates GH secretion, GH gene transcription, and somatotroph proliferation. These actions, coupled to hypothalamic GHRH interactions with somatostatin- and ghrelin-producing neurons underlie pulsatile GH secretion. GHRH production is markedly dependent upon age, sex, and metabolic status, and is also involved in sleep and feeding regulation. Defects in GHRH/GHRH-R severely alter somatotroph function and GH production, thereby causing abnormal growth.

DISCOVERY

The original evidence for the existence of a hypothalamic factor that stimulated secretion of growth hormone (GH) from pituitary somatotrophs was followed by a long, unfruitful search through classic purification and bioassay methods using large amounts of hypothalamic extracts, which ended unexpectedly in 1982, when two independent teams isolated and characterized human GH-releasing hormone (GHRH) from two pancreatic tumors that caused acromegaly.[24] Two of the three GHRH forms of 44 and 40 amino acids found in these tumors are identical to the human hypothalamic GHRH forms.[24] Subsequently, GHRH was isolated and identified in a number of vertebrates, from rodents to fish, including a protochordate.[14,32] GHRH is a member of a growing superfamily of brain-gut peptides, which also includes, among others, pituitary adenylate cyclase-activating polypeptide (PACAP), glucagon, secretin, and vasoactive intestinal polypeptide (VIP),[32] as discussed in this and other Sections.

STRUCTURE OF THE GHRH mRNA/GENE

The human pancreatic tumor samples that enabled isolation of GHRH also provided the first cDNA sequences, whose cloning and characterization revealed that GHRH mRNA codes for a pre-pro-GHRH of 108 amino acids composed of a typical signal peptide, the 44 amino acid GHRH, and a 31 amino acid C-terminal peptide with no known function [GHRH-related peptide (GHRH-RP)] (Fig. 1).[22] A second isoform coding for a 107 amino acid precursor lacking the Ser-103 in the C-peptide was also found. Subsequently, the cDNAs and/or genes were isolated and characterized in rat, mouse, and other mammals as well as in birds, amphibians, and fish.[32] In non-mammalian species, GHRH was believed to be encoded together with PACAP by a common precursor gene.[32] However, it has been recently proposed that, at least in fish and amphibians, the previously identified GHRH-like peptides are homologs of mammalian PACAP-related peptides.[14] Indeed, data mining of the genomic sequences from several vertebrates has enabled the identification of genomic sequences encoding for putative GHRHs from amphibians and fish, which share a higher degree of sequence identity with mammalian GHRH than the previously identified GHRH-like peptides.[14]

The primary structure of GHRH is highly variable among species, especially at the C-terminus. In fact, rat GHRH, which is 43 amino acids, shares 68% homology with the human peptide, yet the N-terminal two thirds of the molecule, corresponding to the bioactive core of the peptide ($GHRH_{1-29}$), are more conserved. Likewise, human GHRH shares 81.5% and 74.1% sequence identity with goldfish/zebrafish and *X. laevis* peptides, respectively.[14] However, GHRH sequences are almost identical in the eight fish species wherein this peptide has been identified by in silico analysis.[14] In most species, but not in rodents, GHRH is amidated at its C-terminus because of the presence of a Gly residue.[24,32]

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00105-6
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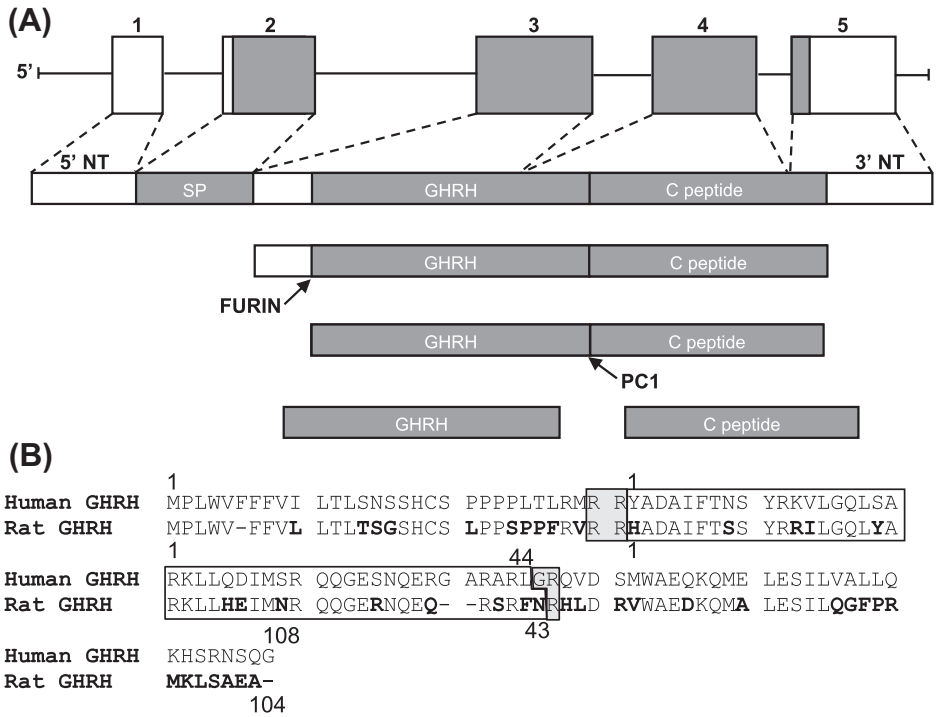

FIGURE 1 (A) Organization of the *GHRH* gene and posttranslational processing of its protein product by the sequential participation of the proprotein convertases furin and PC1. The 5′ nontranslated region (5′-NT), the signal peptide (SP), GHRH, the C-terminal peptide, and the 3′ nontranslated region (3′-NT) are indicated. (B) Alignment of human and rat GHRH precursor sequences. Bold letters illustrate mismatched amino acids in the rat GHRH sequence when compared with the human sequence. Open boxes encompass the amino acid sequence of GHRH. Gray boxes encompass amino acid residues involved in the processing of the precursor protein.

The human and rat genomes contain a single GHRH gene that maps to chromosomes 20 and 3, respectively. In both species, GHRH gene spans about 10 kb and includes five exons: exon 1 contains the 5′-untranslated region and exons 2–4 encode the pre-pro-GHRH together with exon 5, which also contains the 3′-untranslated region (Fig. 1). In fish and amphibians, GHRH genes have four exons and the mature peptides are located in exon 3.[14] The 5′-splice donor sites of intron 4 in human and rat genes differ, which explains differences between the C-terminal portion of human and rat GHRH precursors. In the human gene, differential use of two consensus splice-acceptor sites at the 5′-end of exon 5, which are also present in the rat gene sequence, gives rise to the two distinct types of mRNAs coding for the 108 and 107 amino acid precursor forms.[22] The 5′-flanking region of human and rat GHRH genes includes consensus TATA and CAT box sequences. Analysis of the 5′-promoter region of the rat gene has revealed consensus sites for the transcription factor Gsh-1, which seems to be required for both development of GHRH neurons and tissue-specific regulation of GHRH gene,[25] as well as for the nuclear factor of activated T cells (NFAT), which appears to be involved in the acute regulation of GHRH gene transcription.[3]

PROCESSING OF THE GHRH PRECURSOR

In vitro analysis of GHRH precursor processing in rat hypothalamic neurons initially demonstrated that, after removal of the signal peptide, pro-GHRH (10.5 kDa, 104 amino acids) is first processed to an 8.8-kDa intermediate form that is cleaved to yield GHRH (5.2 kDa) and the 3.6-kDa C-peptide.[29] Later, transfection studies of several neuroendocrine cell types with different members of the subtilisin-like group of proprotein convertases revealed that this process primarily involves the sequential participation of two of these enzymes, furin and PC1 (see Peptide Biosynthesis/Processing Section of this book), which cleave pro-GHRH at two typical recognition sites for these enzymes located at the N- and C-terminus of GHRH, respectively (Fig. 1).[29] Specifically, processing of pro-GHRH is initiated by furin to give rise to the 8.8-kDa intermediate form, which is subsequently cleaved by PC1 to yield mature GHRH and GHRH-RP.

DISTRIBUTION OF THE GHRH mRNA/PEPTIDE

Shortly after isolation of GHRH, the distribution of the peptide was reported in human brain, wherein

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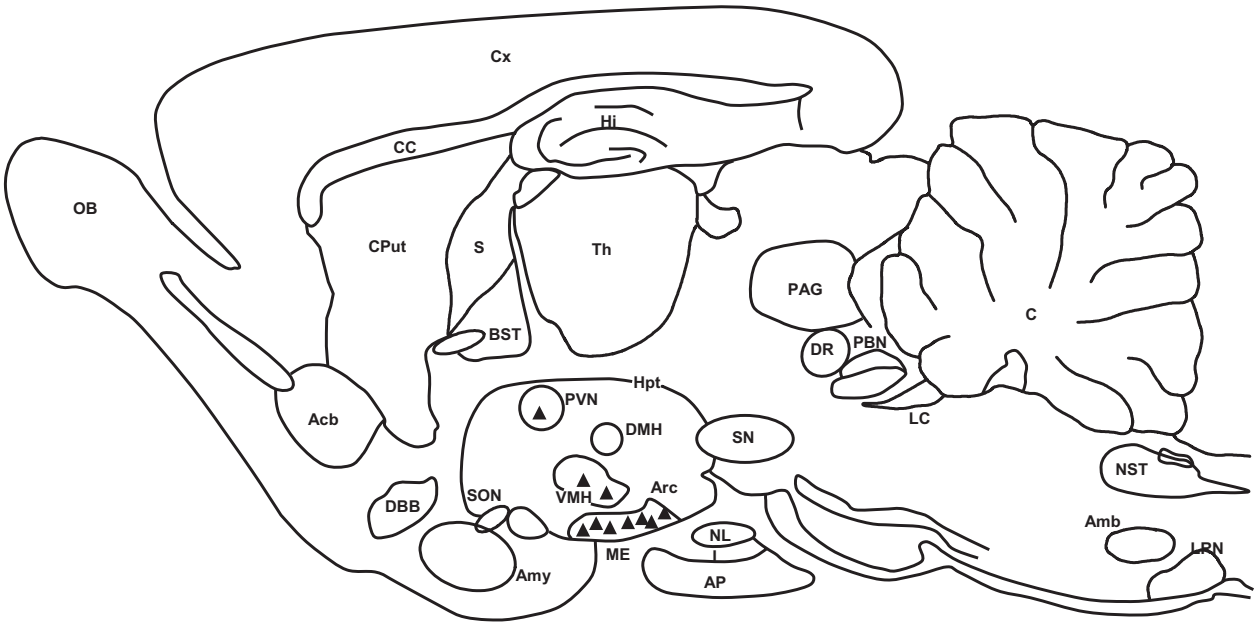

FIGURE 2 Schematic parasagittal section of the rat brain illustrating the distribution of GHRH expression. Black triangles represent GHRH mRNA-containing cell bodies. In rat, the highest GHRH mRNA levels in the brain are found in the ventrolateral part of the arcuate nucleus (Arc).

GHRH-immunoreactivity is mainly associated with neuronal cell bodies in the medial hypothalamus (Hpt), especially in the arcuate nucleus (Arc).[24] In addition, some GHRH-immunopositive cells can be found in the lateral hypothalamus (i.e. nucleus lateralis tuber) and the ventromedial nucleus. GHRH-containing fibers are present in the median eminence and in various hypothalamic nuclei including the arcuate and ventromedial nuclei.[24] In rat, most GHRH-immunoreactive cell bodies are found in the ventrolateral part of the Arc, which also exhibits the highest GHRH mRNA levels in the brain (Fig. 2).[22,24] As in the human brain, these cells project their fibers toward the external layer of the ME, wherein GHRH-immunoreactive terminals are found around the capillaries of the portal vessels. In addition to the Arc GHRH-containing neurons, GHRH mRNA can be detected in some scattered perikarya in the dorsolateral region of the ventromedial nucleus, basolateral Hpt and, to a lesser extent, in the paraventricular nucleus. This distribution correlates well with that of GHRH immunoreactivity, which has been also localized to a few cells in the dorsal part of the dorsomedial nucleus, ventral premammillary nucleus, as well as in the medial perifornical region, zona incerta and the hypothalamic portion of the stria terminalis. GHRH-immunoreactive fibers from these cells extend through the periventricular region of the Hpt and adjoining parts of the basal telencephalon. Nevertheless, the presence of the peptide in extrahypothalamic regions is quite limited as only the cortex and brain stem have been reported to contain detectable amounts of GHRH mRNA.[21]

In rat, GHRH mRNA-containing neurons in the Arc appear at E16 and acquire the adult-like distribution pattern at E20.[24] Transcript levels increase gradually over the course of maturation both in males and females, although GHRH expression is higher in the male than the female from P10 onward.[24] In human fetuses, GHRH-immunoreactive neurons are detected between the 18th and 29th week, while immunoreactive fibers are detectable in the median eminence after the 31st week.[24] Finally, both GHRH mRNA levels and the number of immunoreactive Arc neurons and median eminence fibers decrease in aged rats.[24]

GHRH RECEPTOR AND SIGNALING CASCADES

Earlier studies reported specific GHRH binding sites with high-affinity and low-capacity in membrane extracts or intact cells from pituitary.[11,23,24] However, detailed knowledge of GHRH-R was only achieved after its cloning, first in rat, then in mouse, human, and pig, and more recently in other vertebrate species, including birds and fish.[11,14,23,28] GHRH-R belongs to the G protein-coupled receptor (GPCR) B-II subfamily, which also includes receptors for PACAP/VIP/secretin/glucagon family of peptides (Fig. 3).[23,24] Contrary to other receptors of this family, GHRH-R is highly selective for its cognate ligand, showing subnanomolar affinity.[11] Specificity is conferred by the N-terminal extracellular region, transmembrane domains and associated extracellular loops and also C-terminal intracellular tail.[11]

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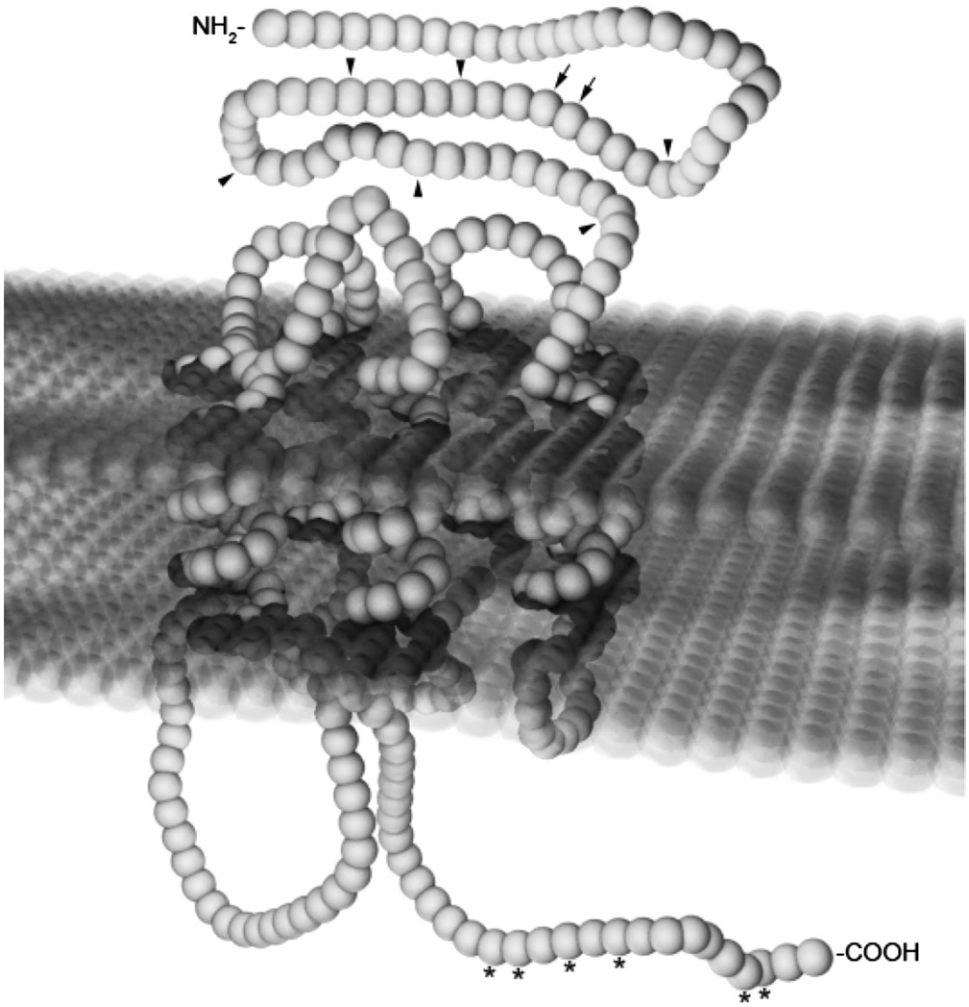

FIGURE 3 Amino acid sequence and membrane topology of the rat GHRH-R. Arrow heads indicate the six Cys residues in the NH_2-terminal domain. Asterisks represent several potential phosphorylation sites and arrows illustrate two N-glycosylation sites.

The GHRH-R gene is localized to human chromosome 7p14-15 and rat chromosome 4q24. The human GHRH gene spans over 15 kb comprising 13 exons separated by introns of variable size, which thereby enable alternative mRNA splice generating receptor isoforms.[11,23] The main form of GHRH-R in most mammals is a 423 amino acid protein,[23] whereas the chicken receptor contains 419 amino acids.[28] Additional, less abundant isoforms bearing C-terminus with distinct length and structure have been reported in the pituitary of several species including human (normal and tumoral), rat, and pig.[23] Although the precise functional significance of these GHRH-R variants is as yet unclear, they show distinct binding and/or cAMP signaling properties and may modulate signaling by the predominant receptor.[23] The N-terminal extracellular domain of GHRH-R contains a site for N-glycosylation as well as six cysteine residues and an aspartate residue that are conserved in this receptor family, while the third intracellular loop and C-intracellular domain contain several potential phosphorylation sites, which may regulate signaling and receptor internalization.[11,23]

Human and rat GHRH-R gene promoters lack a canonical TATA box motif, but contain putative binding sites for a number of transcription factors including Pit-1, Oct-1, enhancer activator protein-1 (AP-1) and AP-4, as well as consensus sequences for nuclear factor 1 (NF-1), upstream regulatory factor (USF), NF-κB, cAMP response element binding protein (CREB), glucocorticoid receptor element (GRE), and estrogen-responsive element (ERE).[20] Studies in dwarf mice and humans indicate that GHRH-R expression is strictly and reciprocally dependent upon Pit-1 gene expression.[11,23] Accordingly, GHRH-R is predominantly expressed in the pituitary in a temporal and spatial pattern corresponding to GH gene expression and therefore essentially restricted to somatotrophs.[24] In addition, GHRH-R mRNA is present in periventricular, Arc, and ventromedial nuclei, and the anterior hypothalamic area as well as in some

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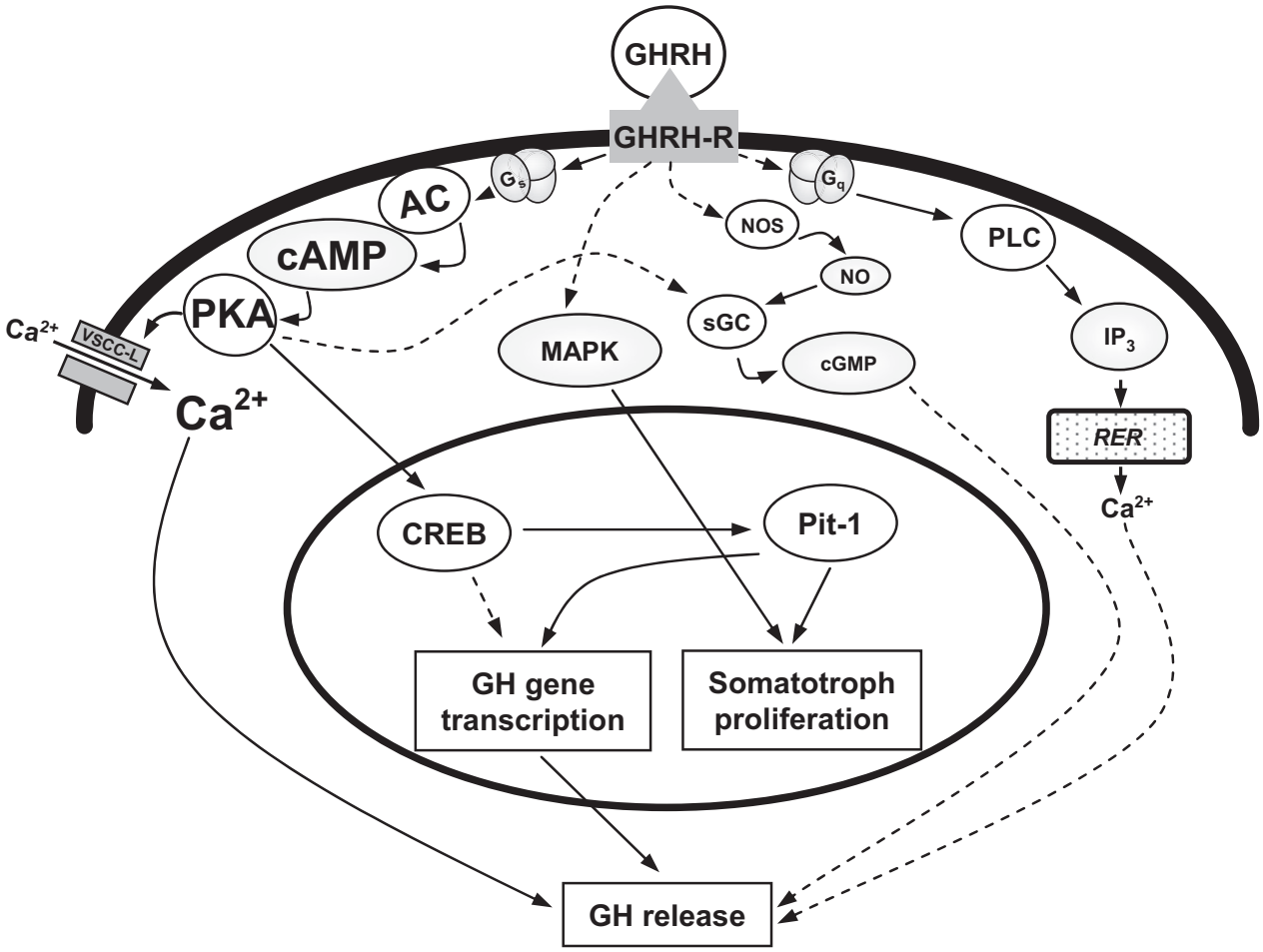

FIGURE 4 Schematic diagram summarizing the main signaling pathways involved in the response of somatotrophs to GHRH. Abbreviations: AC, adenylyl cyclase; CREB, cAMP response element (CRE)-binding protein; IP_3, inositol 1,4,5-trisphosphate; MAPK, mitogen-activated protein kinase; NO, nitric oxide; NOS, nitric oxide synthase; PKA, protein kinase A; PLC, phospholipase C; RER, rough endoplasmic reticulum; sGC: soluble guanylyl cyclase; VSCC-L, L-type voltage-sensitive Ca^{2+} channels.

peripheral tissues (e.g., placenta, kidney, testis and gastrointestinal tract).[23,24] Regulation of GHRH-R is exerted at different levels. Rapid desensitization occurs upon ligand binding, through internalization of the GHRH-R.[11,23] More lasting regulatory effects are exerted on GHRH-R expression by a number of signals including GHRH, which evokes a dual, time-dependent effect: an acute decrease in GHRH-R expression probably mediated via cAMP[11,18,23] and a long-term up-regulation of mRNA levels.[11,23] Acute inhibition of expression is also caused by ghrelin and somatostatin.[18,28] Consistent with the presence of corresponding binding sites in the GHRH-R promoter, and with its sexually dimorphic expression in pituitary, estrogen inhibits GHRH-R transcription.[23,24] By contrast, GHRH-R expression is positively regulated by glucocorticoids and thyroid hormone.[11,23,24]

Upon GHRH binding, GHRH-R activates diverse signaling cascades, which primarily involve two second messengers, cAMP and Ca^{2+} (Fig. 4).[23,24] GHRH increases cAMP levels by stimulating adenylyl cyclase (AC) through a Gs protein. Subsequently, increased cAMP levels induce activation of protein kinase A (PKA) which, in turn, activates several proteins by phosphorylation, including ion channels.[23,24] Likewise, GHRH increases cytosolic free Ca^{2+} concentration levels in somatotrophs through a mechanism essentially dependent on extracellular Ca^{2+} influx through voltage-sensitive Ca^{2+} channels, a process that involves K^+ and Na^+ channels and PKA-dependent phosphorylation.[5] There is also evidence that GHRH activates and requires additional signaling components in somatotrophs.[24] Thus, GHRH-induced GH release is also partially dependent on the phospholipase C (PLC)/inositol phosphate (IP) pathway and/or mobilization of Ca^{2+} from intracellular stores in somatotrophs.[30] In addition, the signaling cascade involving nitric oxide and cGMP also participates in mediating GHRH-induced GH release, and may represent a subsequent, interlinked step in the AC/cAMP/PKA signaling cascade, via PKA-mediated phosphorylation of soluble guanylyl cyclase.[19,24] GHRH-R signaling also activates the

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MAP kinase pathway, which may mediate the effects of the peptide on GH synthesis and somatotroph proliferation.[11,23] Finally, the signaling properties conveyed by GHRH-R may be modulated by other G protein-coupled receptors such as that for ghrelin/GH secretagogs.[6]

BIOLOGICAL ACTIONS OF GHRH WITHIN THE PITUITARY AND THE BRAIN

It is now well established that GHRH is the primary hypothalamic stimulus for GH secretion from pituitary somatotrophs. Indeed, the remarkable ability of GHRH to induce GH release specifically, both *in vivo* and *in vitro*, in mammals, birds and amphibians (and, to a lesser extent, in fish) with a marginal effect on the release of other pituitary hormones, constitutes the major role for this peptide.[23,24] Moreover, GHRH acts as a true somatotrophic factor in that it also increases intracellular GH content by activating GH gene transcription and GH mRNA levels, and stimulates somatotroph proliferation, this latter function being fundamental for somatotroph development during pituitary organogenesis.[20,24]

Consistent with the well-known pulsatile pattern of GH release in plasma, GHRH has been shown to be released in an episodic manner into portal blood at the median eminence in various species.[24] Initial studies in male rats based on measurements of GHRH and somatostatin in portal plasma and of GH in peripheral plasma led to a valuable model wherein GH pulses were the result of alternating episodes of GHRH and somatostatin release from Hpt, which would stimulate and suppress, respectively, GH release.[24] Yet, subsequent studies revealed that this model did not satisfactorily explain GH secretory patterns in human and other species in diverse pathophysiological conditions (e.g., GH pulsatility is maintained in the absence of GHRH input resulting from a mutation in GHRH-R[31]). The available evidence led Veldhuis and coworkers to postulate, in the context of highly complex and elaborated mathematical models, that the generation of a given physiological pattern of pulsatile GH release is the combined result of intricate time-delimited interactions among GHRH, somatostatin, and GH autofeedback, as well as a new major player, ghrelin, which are exerted at both hypothalamic and pituitary levels.[9] In this scenario, GHRH acts on the pituitary to stimulate GH release and synthesis, but would also act by stimulating Arc somatostatin neurons, as suggested by the tight neuroanatomical interplay between these factors. In turn, GHRH expression and secretion from Arc neurons is negatively regulated by hypothalamic somatostatin neurons as well as by GH feedback, whereas ghrelin would act upon GHRH neurons stimulating its release. The components of this cycle are discussed elsewhere in this book.

There is ample evidence that GH levels and secretory patterns are markedly affected by development, sex, aging, and diverse pathological conditions both in human and other species, and that, accordingly, hypothalamic GHRH production is strictly and distinctly regulated by these factors.[24] Indeed, the sexually dimorphic pattern of pulsatile GH secretory profiles in both human and rat, which underlies the distinct (higher in males) gender-dependent growth pattern, probably results from the higher number and mRNA content of hypothalamic GHRH neurons, and presumably, their distinct neuroanatomical connectivity with other neurons (e.g., somatostatin) in the male compared with the female. In this regard, it has been recently shown that the percentage of ventromedial hypothalamic-arcuate GHRH neurons bearing the somatostatin receptor sst2A is higher in female than in male mice and that testosterone reduces the distribution of sst2A on these neurons.[4] Finally, basal and pulsatile GH release is markedly influenced by age, with a decline in GH during the life span in human and animal models, which seems to be paralleled by, and significantly dependent upon, a decreased production of hypothalamic GHRH and reduced somatotroph secretory responsiveness to this peptide, together with an increased somatostatinergic tone.[24]

In addition to controlling somatotroph function, GHRH also possesses extrapituitary central effects. Thus, it has been shown that GHRH promotes sleep (see last Section of this book) and, accordingly, it has been proposed to provide the subcortical mechanism synchronizing sleep and GH secretion.[26] Specifically, non-REM sleep increases in human, rat and rabbit after GHRH administration, whereas sleep is reduced in rats when GHRH action is inhibited by a specific antagonist or by GHRH antibodies. This sleep-promoting effect of GHRH has been documented to occur through a direct action of the peptide on GABAergic neurons in the ventrolateral and median preoptic nuclei.[27] Additionally, GHRH also stimulates REM sleep, although this action seems to depend on GH.

GHRH has been also proposed to be involved in the central regulation of feeding and probably contributes to the circadian pattern of food intake.[24] In support of this, central administration of GHRH stimulates food intake in rat and sheep by a mechanism that was initially proposed to involve GHRH-mediated effects on the suprachiasmatic nucleus/medial preoptic area.[24] A role for orexin A as integrator of the somatotropic axis with the nutritional status, through its inhibitory actions on GHRH neurons in the paraventricular nucleus in relation to the sleep–wake cycle, has been recently proposed.[17]

PATHOPHYSIOLOGICAL IMPLICATIONS

Because of the direct involvement of GHRH in somatotroph proliferation, defects in tandem GHRH/ GHRH-R profoundly alter the morphological and functional

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characteristics of the pituitary, provoking generalized GH secretion disorders. Thus, excessive GHRH release into the median eminence induces somatotroph hyperplasia and an abnormal enlargement of the pituitary.[10] In rodents, symptomatology of GHRH excess has been extensively studied since Mayo and coworkers established a strain of transgenic mice expressing a mouse metallothionein I/human GHRH fusion gene, an animal model of acromegaly.[24] In these mice, chronic GHRH production leads to development of enormous pituitary glands as result of uncontrolled proliferation of somatotrophs that secrete large amounts of GH, an effect that is GHRH-specific and not mediated by GH or IGF-I.[13] In humans, GHRH-dependent somatotroph hyperplasia is very rare; it is limited to certain cases of extrapituitary GHRH overproduction and sporadic examples of gigantism.[12] Moreover, mutations in the GHRH gene have never been described, contrary to that observed in the GHRH-R gene, where a variety of spontaneous punctal mutations affecting GHRH-R activity have been reported.[20] One of these mutations (or gene polymorphism) is produced by a single base change in the GHRH-R gene in human somatotropinomas that confers hypersensitivity to GHRH binding.[20]

On the other hand, deficient GHRH secretion and/or activity is generally associated with somatotroph hypoplasia, subsequent low GH release and, consequently, development of dwarfism. These observations have been obtained from rodent (i.e., *little* mouse) or humans with inactivating mutations in the GHRH-R[20] since, as mentioned earlier, mutations in the GHRH gene have not been reported to date, neither in humans nor in rodents. Despite this, rodent models with GHRH deficiency secondary to deletion of other genes (Gsh-1[16]; PC1[35]) or caused by targeted expression of the human GH transgene in the Hpt[24] have been already reported. Certainly, the recent development of GHRH knockout mice[2,15] has provided valuable information on the precise role of GHRH in the regulation of normal and pathophysiological somatotroph function. GHRH-KO mice have also served to characterize a potent GHRH analog (i.e., CJC-1295) that exhibits a longer half-life and duration of action than the natural peptide because of its selective and covalent binding to endogenous albumin.[1] This analog has been successfully used in clinical trials, which highlights its potential utility as a therapeutic agent.[33] Indeed, since the discovery of the sequences crucial for GHRH somatotrophic activity, much effort has been invested in generating synthetic analogs with extended half-life and duration of action. Thus, besides CJC-1295, PEGylated or cyclic GHRH analogs as well as fatty body-GHRH analogs with improved biological activity have been designed.[7,34] Recently, GHRH analogs have been assayed with the aim of augmenting GH secretion in individuals with low endogenous GH levels and excess visceral obesity, including patients with HIV receiving highly active antiretroviral therapy.[8] Certainly, the growing development of potent and long-acting GHRH analogs will pave the way to prevent or ameliorate the pathological states associated to GHRH deficiency.

REFERENCES

1. Alba M, Fintini D, Sagazio A, Lawrence B, Castaigne JP, Frohman LA, et al. Once-daily administration of CJC-1295, a long-acting growth hormone-releasing hormone (GHRH) analog, normalizes growth in the GHRH knockout mouse. *Am J Physiol Endocrinol Metab* 2006;**291**:E1290–4.
2. Alba M, Salvatori R. A mouse with targeted ablation of the growth hormone-releasing hormone gene: a new model of isolated growth hormone deficiency. *Endocrinology* 2004;**145**:4134–43.
3. Asai M, Iwasaki Y, Yoshida M, Mutsuga-Nakayama N, Arima H, Ito M, et al. Nuclear factor of activated T cells (NFAT) is involved in the depolarization-induced activation of growth hormone-releasing hormone gene transcription in vitro. *Mol Endocrinol* 2004;**18**:3011–9.
4. Bouyer K, Faivre-Bauman A, Robinson IC, Epelbaum J, Loudes C. Sexually dimorphic distribution of sst2A receptors on growth hormone-releasing hormone neurones in mice: modulation by gonadal steroids. *J Neuroendocrinol* 2008;**20**:1278–87.
5. Chen C, Xu R, Clarke IJ, Ruan M, Loneragan K, Roh SG. Diverse intracellular signalling systems used by growth hormone-releasing hormone in regulating voltage-gated Ca^{2+} or K channels in pituitary somatotropes. *Immunol Cell Biol* 2000;**78**:356–68.
6. Cunha SR, Mayo KE. Ghrelin and growth hormone (GH) secretagogues potentiate GH-releasing hormone (GHRH)-induced cyclic adenosine 3′,5′-monophosphate production in cells expressing transfected GHRH and GH secretagogue receptors. *Endocrinology* 2002;**143**:4570–82.
7. Dubreuil P, Désévaux C. Growth hormone-releasing factor: structural modification or protection for more potent analogs. *Comb Chem High Throughput Screen* 2006;**9**:171–4.
8. Falutz J, Potvin D, Mamputu JC, Assaad H, Zoltowska M, Michaud SE, et al. Effects of tesamorelin, a growth hormone-releasing factor, in HIV-infected patients with abdominal fat accumulation: a randomized placebo-controlled trial with a safety extension. *J Acquir Immune Defic Syndr* 2010;**53**:311–22.
9. Farhy LS, Veldhuis JD. Deterministic construct of amplifying actions of ghrelin on pulsatile growth hormone secretion. *Am J Physiol Regul Integr Comp Physiol* 2005;**288**:R1649–63.
10. Frohman LA, Kineman RD, Kamegai J, Park S, Teixeira LT, Coschigano KT, et al. Secretagogues and the somatotrope: signaling and proliferation. *Recent Prog Horm Res* 2000;**55**:269–90:discussion 290–1.
11. Gaylinn BD. Growth hormone releasing hormone receptor. *Receptors Channels* 2002;**8**:155–62.
12. Horvath E, Kovacs K, Scheithauer BW. Pituitary hyperplasia. *Pituitary* 1999;**1**:169–79.
13. Kineman RD, Teixeira LT, Amargo GV, Coschigano KT, Kopchick JJ, Frohman LA. The effect of GHRH on somatotrope hyperplasia and tumor formation in the presence and absence of GH signaling. *Endocrinology* 2001;**142**:3764–73.

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14. Lee LT, Siu FK, Tam JK, Lau IT, Wong AO, Lin MC, et al. Discovery of growth hormone-releasing hormones and receptors in nonmammalian vertebrates. *Proc Natl Acad Sci USA* 2007;**104**:2133–8.
15. Le Tissier PR, Carmignac DF, Lilley S, Sesay AK, Phelps CJ, Houston P, et al. Hypothalamic growth hormone-releasing hormone (GHRH) deficiency: targeted ablation of GHRH neurons in mice using a viral ion channel transgene. *Mol Endocrinol* 2005;**19**:1251–62.
16. Li H, Zeitler PS, Valerius MT, Small K, Potter SS. Gsh-1, an orphan Hox gene, is required for normal pituitary development. *EMBO J* 1996;**15**:714–24.
17. López M, Nogueiras R, Tena-Sempere M, Diéguez C. Orexins (hypocretins) actions on the GHRH/somatostatin-GH axis. *Acta Physiol (Oxf)* 2010;**198**:325–34.
18. Luque RM, Kineman RD, Park S, Peng XD, Gracia-Navarro F, Castano JP, et al. Homologous and heterologous regulation of pituitary receptors for ghrelin and growth hormone-releasing hormone. *Endocrinology* 2004;**145**:3182–9.
19. Luque RM, Rodriguez-Pacheco F, Tena-Sempere M, Gracia-Navarro F, Malagon MM, Castano JP. Differential contribution of nitric oxide and cGMP to the stimulatory effects of growth hormone-releasing hormone and low-concentration somatostatin on growth hormone release from somatotrophs. *J Neuroendocrinol* 2005;**17**:577–82.
20. Martari M, Salvatori R. Diseases associated with growth hormone-releasing hormone receptor (GHRHR) mutations. *Prog Mol Biol Transl Sci* 2009;**88C**:57–84.
21. Matsubara S, Sato M, Mizobuchi M, Niimi M, Takahara J. Differential gene expression of growth hormone (GH)-releasing hormone (GRH) and GRH receptor in various rat tissues. *Endocrinology* 1995;**136**:4147–50.
22. Mayo KE, Godfrey PA, Suhr ST, Kulik DJ, Rahal JO. Growth hormone-releasing hormone: synthesis and signaling. *Recent Prog Horm Res* 1995;**50**:35–73.
23. Mayo KE, Miller LJ, Bataille D, Dalle S, Goke B, Thorens B, et al. International Union of Pharmacology. XXXV. The glucagon receptor family. *Pharmacol Rev* 2003;**55**:167–94.
24. Muller EE, Locatelli V, Cocchi D. Neuroendocrine control of growth hormone secretion. *Physiol Rev* 1999;**79**:511–607.
25. Mutsuga N, Iwasaki Y, Morishita M, Nomura A, Yamamori E, Yoshida M, et al. Homeobox protein Gsh-1-dependent regulation of the rat GHRH gene promoter. *Mol Endocrinol* 2001;**15**:2149–56.
26. Obal Jr F, Krueger JM. GHRH and sleep. *Sleep Med Rev* 2004;**8**: 367–77.
27. Peterfi Z, McGinty D, Sarai E, Szymusiak R. Growth hormone-releasing hormone activates sleep regulatory neurons of the rat preoptic hypothalamus. *Am J Physiol Regul Integr Comp Physiol* 2010; **298**:R147–56.
28. Porter TE, Ellestad LE, Fay A, Stewart JL, Bossis I. Identification of the chicken growth hormone-releasing hormone receptor (GHRH-R) mRNA and gene: regulation of anterior pituitary GHRH-R mRNA levels by homologous and heterologous hormones. *Endocrinology* 2006;**147**:2535–43.
29. Posner SF, Vaslet CA, Jurofcik M, Lee A, Seidah NG, Nillni EA. Stepwise posttranslational processing of progrowth hormone-releasing hormone (proGHRH) polypeptide by furin and PC1. *Endocrine* 2004;**23**:199–213.
30. Ramirez JL, Castano JP, Torronteras R, Martinez-Fuentes AJ, Frawley LS, Garcia-Navarro S, et al. Growth hormone (GH)-releasing factor differentially activates cyclic adenosine 3′,5′-monophosphate- and inositol phosphate-dependent pathways to stimulate GH release in two porcine somatotrope subpopulations. *Endocrinology* 1999; **140**:1752–9.
31. Roelfsema F, Biermasz NR, Veldman RG, Veldhuis JD, Frolich M, Stokvis-Brantsma WH, et al. Growth hormone (GH) secretion in patients with an inactivating defect of the GH-releasing hormone (GHRH) receptor is pulsatile: evidence for a role for non-GHRH inputs into the generation of GH pulses. *J Clin Endocrinol Metab* 2001;**86**:2459–64.
32. Sherwood NM, Krueckl SL, McRory JE. The origin and function of the pituitary adenylate cyclase-activating polypeptide (PACAP)/glucagon superfamily. *Endocr Rev* 2000;**21**:619–70.
33. Teichman SL, Neale A, Lawrence B, Gagnon C, Castaigne JP, Frohman LA. Prolonged stimulation of growth hormone (GH) and insulin-like growth factor I secretion by CJC-1295, a long-acting analog of GH-releasing hormone, in healthy adults. *J Clin Endocrinol Metab* 2006;**91**:799–805.
34. Youn YS, Lee KC. Site-specific PEGylation for high-yield preparation of Lys(21)-amine PEGylated growth hormone-releasing factor (GRF) (1-29) using a GRF(1-29) derivative FMOC-protected at Tyr(1) and Lys(12). *Bioconjug Chem* 2007;**18**:500–6.
35. Zhu X, Zhou A, Dey A, Norrbom C, Carroll R, Zhang C, et al. Disruption of PC1/3 expression in mice causes dwarfism and multiple neuroendocrine peptide processing defects. *Proc Natl Acad Sci U S A* 2002;**99**:10293–8.

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Chapter 106

GnRH (LHRH)

R.P. Millar

ABSTRACT

Gonadotropin-releasing hormone (GnRH) is a decapeptide produced in the hypothalamus and secreted into portal vessels to engage the GnRH receptor (a G protein-coupled receptor) in pituitary gonadotrophs, where it recruits a diversity of signaling pathways to culminate in the biosynthesis and secretion of the gonadotropins, luteinizing hormone (LH), and follicle-stimulating hormone (FSH), which stimulate gamete production and sex steroid hormone production. The predominant signaling pathway is mediated Gq/11 and mobilization of intracellular Ca^{2+} and activation of protein kinase Cs (PKCs). A wide range of GnRH analogs have been developed and extensively used for the treatment of hormone-dependent diseases, as well as for controlling the ovulatory LH surge in the induction of ovulation in *in vitro* fertilization. High continuous doses of GnRH agonists inhibit gonadotropin secretion through the desensitization of the gonadotroph, whereas GnRH antagonists do so by directly competing with endogenous GnRH. In addition to the GnRH and its receptor regulating the pituitary, there are isoforms with diverse other functions.

DISCOVERY AND BACKGROUND

Gonadotropin-releasing hormone (GnRH) is the central initiator of the reproductive hormonal cascade and was first isolated from mammalian hypothalami as the decapeptide (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly·NH_2).[1] GnRH is processed in specialized neurons of the hypothalamus from a precursor polypeptide by enzymatic processing and packaged in storage granules that are transported down axons to the external zone of the median eminence. GnRH is released as synchronized pulses from the nerve endings of about 1000 neurons into the hypophyseal portal system every 30–120 min to stimulate the biosynthesis and secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from pituitary gonadotrophs.[4] Each GnRH pulse stimulates a pulse of LH, but FSH pulses are less clear. The frequency of pulses is the highest at the ovulatory LH surge and lowest during the luteal phase of the ovarian cycle. Differential LH and FSH release, necessary for the ovarian cycle, results from changes in GnRH pulse frequency, modulating effects of gonadal steroid and peptide hormones on FSH and LH responses to GnRH, and differences in the half lives of the two hormones. Moreover, although LH is stored and largely dependent on GnRH for secretion, FSH tends to be constitutively secreted and more dependent on biosynthesis for secretion.

STRUCTURE OF GnRHs AND THEIR PRECURSORS

Primary Structures

Mammalian hypothalamic GnRH was believed to be a unique structure that was conserved among vertebrates. However, it became apparent that diverse forms exist in vertebrates.[12] This led to the structural identification of 13 different forms in vertebrates and another 9 in protochordates (Fig. 1).[3] It also became apparent that GnRHs are distributed in a wide range of tissues in vertebrates, where they apparently have diverse functions in addition to their primary role in gonadotropin secretion. These include other neuroendocrine functions, paracrine and autocrine functions in GnRH neurons, gonads, immune cells, breast, and prostatic cancer cells, and neurotransmitter/neuromodulatory roles in the central and peripheral nervous system. Although a single GnRH is theoretically capable of serving these different roles, it is evident that at least two, and usually three, forms of GnRH are differentially expressed in various tissues as regulators. The most ubiquitous GnRH in vertebrates is chicken GnRH II (first isolated from chicken brain), which is totally conserved from bony fish to human[6] and is probably the earliest evolved form. This form has been designated GnRH II, whereas the hypophysiotropic form is designated GnRH I. In many vertebrate species, a third form of GnRH occurs (localized to the forebrain in fish) and is designated GnRH III. Analysis of the genes encoding the GnRHs supports this general classification.[16]

Three Dimensional Structures

The amino acid sequences of the structural variants of GnRHs from vertebrate and protochordate species (Fig. 1) reveal that there has been conservation in the NH_2 terminus

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00106-8
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		1	2	3	4	5	6	7	8	9	10	
Jawed Vertebrates	Mammal	pGlu	His	Trp	Ser	Tyr	Gly	Leu	Arg	Pro	Gly	NH_2
	Guinea Pig	pGlu	Tyr	Trp	Ser	Tyr	Gly	Val	Arg	Pro	Gly	NH_2
	Chicken I	pGlu	His	Trp	Ser	Tyr	Gly	Leu	Gln	Pro	Gly	NH_2
	Rana d.	pGlu	His	Trp	Ser	Tyr	Gly	Leu	Trp	Pro	Gly	NH_2
	Seabream	pGlu	His	Trp	Ser	Tyr	Gly	Leu	Ser	Pro	Gly	NH_2
	Salmon	pGlu	His	Trp	Ser	Tyr	Gly	Trp	Leu	Pro	Gly	NH_2
	Medaka	pGlu	His	Trp	Ser	Phe	Gly	Leu	Ser	Pro	Gly	NH_2
	Catfish	pGlu	His	Trp	Ser	His	Gly	Leu	Asn	Pro	Gly	NH_2
	Herring	pGlu	His	Trp	Ser	His	Gly	Leu	Ser	Pro	Gly	NH_2
	Dogfish	pGlu	His	Trp	Ser	His	Gly	Trp	Leu	Pro	Gly	NH_2
	Chicken II	pGlu	His	Trp	Ser	His	Gly	Trp	Tyr	Pro	Gly	NH_2
Jawless Fish	Lamprey III	pGlu	His	Trp	Ser	His	Asp	Trp	Lys	Pro	Gly	NH_2
	Lamprey I	pGlu	His	Tyr	Ser	Leu	Glu	Trp	Lys	Pro	Gly	NH_2
Protochordates/ Tunicates	Chelyosoma I	pGlu	His	Trp	Ser	Asp	Tyr	Phe	Lys	Pro	Gly	NH_2
	Chelyosoma II	pGlu	His	Trp	Ser	Leu	Cys	His	Ala	Pro	Gly	NH_2
	Ciona I	pGlu	His	Trp	Ser	Tyr	Ala	Leu	Ser	Pro	Gly	NH_2
	Ciona II	pGlu	His	Trp	Ser	Leu	Ala	Leu	Ser	Pro	Gly	NH_2
	Ciona III	pGlu	His	Trp	Ser	Asn	Gln	Leu	Thr	Pro	Gly	NH_2
	Ciona IV	pGlu	His	Trp	Ser	Tyr	Glu	Phe	Met	Pro	Gly	NH_2
	Ciona V	pGlu	His	Trp	Ser	Tyr	Glu	Tyr	Met	Pro	Gly	NH_2
	Ciona VI	pGlu	His	Trp	Ser	Lys	Gly	Tyr	Ser	Pro	Gly	NH_2
	Ciona VII	pGlu	His	Trp	Ser	Asn	Lys	Leu	Ala	Pro	Gly	NH_2

FIGURE 1 Primary amino acid sequences of naturally occurring decapeptide GnRH structural variants spanning approximately 600 million years of evolution. The boxed regions show the conserved amino- and carboxyl-terminal residues that are involved in receptor binding and activation. The GnRHs are named according to the species in which they were first discovered, and they may be represented in more than one species. For example, mammalian GnRH is widely conserved in amphibians and primitive bony fish, and chicken GnRH II is present in most vertebrate species, including humans. An octopus GnRH and an additional Ciona GnRH comprising 12 and 16 amino acids, respectively, but retaining the conserved amino and carboxy terminal domains are not shown. From Ref. 6 with permission.

(pGlu-His-Trp) and the COOH terminus (Pro-Gly·NH_2), over 600 million years of evolution, indicating that these features are critically important for receptor binding and activation (Fig. 2). This is borne out by structure–activity data from thousands of analogs that were developed largely on an empirical basis.[5–20] Position 8 is the most variable amino acid (Arg, Gln, Ser, Asn, Leu, Try, Lys, Ala, Trp, Thr, Met), suggesting that virtually any residue is tolerated in this position. However, this is not the case for the mammalian pituitary type I receptor, which requires Arg in position 8,[20] and work on cloned nonmammalian receptors also indicates certain specificities for the amino acid in this position. Thus, this residue seems to play an important role in ligand selectivity of the different GnRH receptors and in modulating intracellular signaling at the same receptor.[12]

The conserved NH_2- and COOH-terminal domains of GnRH are closely apposed when mammalian GnRH binds its receptor, resulting from a β-II-type turn involving residues 5–8 (Fig. 2).[5,12,20] This partly results from intramolecular interactions with the side chain of Arg^8. Trp fluorescence studies, computer simulations using the technique of conformational memories, and nuclear magnetic resonance (NMR) studies have shown that substitution of Arg^8 results in a less compact structure, with a loss of predominance of the folded conformers and a low biological activity.[3,12,20] Yet these extended forms (e.g. Gln^8 in chicken GnRH I) exhibit high activity in many nonmammalian GnRH receptors, despite their low activity at the mammalian receptor. The β-II-type turn conformation of GnRH also seems to be induced in part by the interaction of Arg^8 with an acidic residue (D302) in extracellular loop 3 (EC3) of the mammalian receptor (Fig. 5). Substitution of a D-amino acid for Gly^6 enhances the β-II-type conformation and increases the activity of GnRH about tenfold, and this feature is used in enhancing receptor-binding

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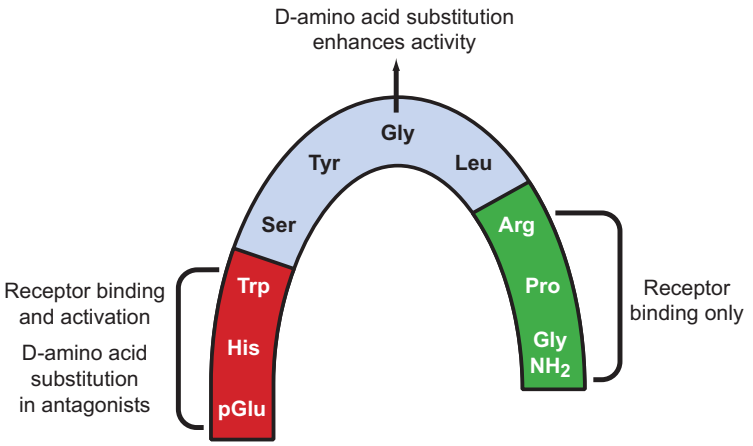

FIGURE 2 Schematic representation of mammalian GnRH in the folded conformation in which it is bound to the GnRH pituitary receptor. The molecule is bent around the achiral glycine in position six. Substitution with D-amino acids in this position stabilizes the folded conformation, and increases binding affinity and decreases metabolic clearance. This feature is incorporated in all agonist and antagonist analogs. The NH_2 (dark grey) and COOH (mid grey) termini are involved in receptor binding. The NH_2 terminus alone is involved in receptor activation, and substitutions in this region produce antagonists. From Ref. 12 with permission.

affinity in agonists and antagonists.[5,12,20] The amino-terminal residues of GnRH are involved in receptor activation, and modification of these residues in GnRH produces analogs with antagonistic properties (Fig. 2).

GnRH DISTRIBUTION

The diversity in molecular structure, function, and anatomical distribution of GnRHs raises the question as to whether GnRH neurons expressing the different GnRHs are embryonically derived from a single precursor population, or have multiple origins. The neuroanatomical organization of GnRH neurons in different vertebrates has been reviewed.[6,17] As discussed earlier, two or three different forms of GnRH are expressed in most vertebrate species. The GnRH I cell bodies regulating the brain–pituitary–gonad axis are located principally in the medial septum and anterior preoptic area in mammals, and give rise to the major GnRH axonal projections through the ventral hypothalamus to the median eminence. GnRH III cell bodies are also present in the terminal nerve, associated with the olfactory system and GnRH II is present in the ventral forebrain, and in several regions of the posterior diencephalon and midbrain. GnRH fibers project to almost all areas of the central nervous system, the vasculature, and the ventricles.

A model for the development of GnRH neuronal systems has been proposed by Muske:[5,17] (a) GnRH neurons have at least two embryonic origins: the olfactory placode, which gives rise to the terminal nerve-septo-preoptic system, and a second, nonplacodal structure, which gives rise to the posterior diencephalon/anterior midbrain system. (b) The two embryonically distinct populations express different molecular forms of GnRH. The posterior neurons express GnRH II, whereas the terminal nerve-septo-preoptic neurons express a different form(s) of GnRH, which varies in different species. Immunocytochemical studies of brain regions reveal many similarities among vertebrate species in their cytoarchitectonic organization, suggesting that, like the peptides, the neuronal systems are phylogenetically ancient. The GnRH neuronal system in amphibians is typical of that described in species from other vertebrate classes (Fig. 3). The terminal nerve–septo–preoptic system is the prime regulator of gonadotropin release in most vertebrates and the neurons originate in the embryonic olfactory placode and migrate centrally during development. The ontogeny of the terminal nerve–septo–preoptic system is thought to reflect its evolutionary origin as a peripheral endocrine organ associated with the olfactory system. The second GnRH system, which arises from nonplacodal precursors, comprises cell bodies in periventricular regions of the posterior diencephalon and midbrain. Evidence suggests that these cells served as the ancestral brain GnRH system, and are the cellular locus of the early-evolved GnRH II peptide. Although GnRH I and GnRH II are both present and have distinct neural distribution patterns in primates, GnRH II has been deleted from the laboratory mouse and rat genome.[14,16] It seems that GnRH I has taken over the role of GnRH II in neurons in which this peptide was previously expressed. In vertebrate species, it is widely established that the different roles of GnRHs are as a releaser of pituitary gonadotropins, and as a neurotransmitter or neuromodulator in extrahypothalamic areas of the brain.

PROCESSING OF THE GnRH PRECURSOR

Initial studies claimed a nonribosomal biosynthesis of GnRH as had been established for the antibiotic small peptides,

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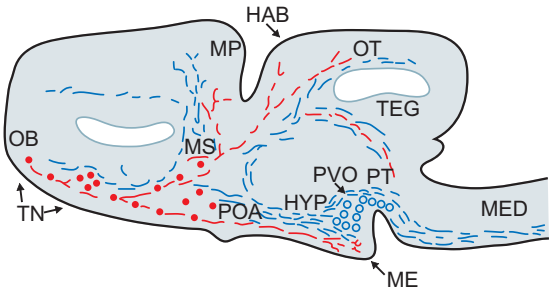

FIGURE 3 GnRH-immunoreactive neuronal systems in an amphibian *Taricha granulosa*. Mammalian GnRH antiserum labeled cell bodies (red circles) in the anterior forebrain, including terminal nerve, medial septum, and anterior preoptic area, and a restricted set of projections to the medial pallium, habenula, and median eminence (red lines). GnRH II antiserum-labeled cell bodies (blue circles) in the paraventricular organ and posterior tubercle and a diffuse system of fibers to most brain area (blue lines). Abbreviations: HAB, habenula; HYP, hypothalamus; ME, median eminence; MED, medulla; MP, medial pallium; MS, medial septum; OB, olfactory bulb; OT, optic tectum; POA, preoptic area; PT, posterior tubercle; PVO, paraventricular organ; TEG, midbrain tegmentum; TN, terminal nerve. Adapted from Muske.[17] See color plate 28.

but other studies provided evidence for biosynthesis from a precursor of a molecular weight of about 5000 through tryptic and carboxypeptidase B cleavage of a dibasic site.[13] These researchers proposed that the product was further processed by the cyclization of Gln to the N-terminal pyro Glu and amidation of the C-terminal glycine using the adjacent glycine as NH_2 donor (Fig. 4). These proposals were supported by the subsequent revelation of the GnRH cDNA sequence.[21] The 56 amino acid sequence following that of GnRH was claimed to affect prolactin and gonadotropin secretion, but this has never been substantiated.

GnRH RECEPTORS AND SIGNALING CASCADES

The amino acid sequence of the GnRH receptor was first deduced for the mouse receptor in the αT3 gonadotroph cell line,[23] and this provided the basis for the cloning of GnRH pituitary receptors from the rat, human, sheep, cow, and pig, which share >80% amino acid identity. Subsequently, GnRH receptors have been cloned from marsupials, birds, and several fish and amphibian species.[12,16]

A second form of GnRH receptor (type II) was identified in humans[10] and subsequently cloned from primates.[11,19] However, the human homolog seems to be a nonfunctional pseudogene whose function has been taken over by the type I receptor.[14,16] In some amphibian and fish species, a third GnRH receptor exists.[12,16] The findings suggest an early evolution of the three GnRH receptor subtypes in vertebrates that parallels that of the GnRH ligands.[12] The conservation of amino acids during evolution from bony fish to mammals probably indicates those residues crucial to GnRH receptor function (Fig. 5).

The GnRH receptor has the characteristic features of G protein-coupled receptors (GPCRs) with an N-terminal domain, followed by seven putative α-helical transmembrane (TM) domains that are connected by three extracellular (EC) loop domains and three intracellular (IC) loop domains (Fig. 5). The EC domains and superficial regions of the TMs are usually involved in the binding of peptide hormones such as GnRH. The highly conserved TMs are believed to be involved in conforming the receptor, and conformational change is associated with signal propagation (receptor activation). The intracellular domains are involved in interacting with G proteins for signal transduction. A unique feature of the mammalian GnRH receptor is the absence of a C-terminal tail, which is present in all other GPCRs and in all the nonmammalian GnRH receptors, and is responsible for rapid desensitization. The structures of the genes encoding the mouse and human GnRH receptors have been elucidated. In both genes, the three exons are separated by introns in the coding regions of TM4 and IC3, and there are numerous splice variants whose function may be regulatory, as some truncated receptor variants affect the expression of the functional receptor. The promoters of the rat and human receptors have been analyzed, and the genes are highly regulated by GnRH, and by gonadal, steroid, and peptide hormones.

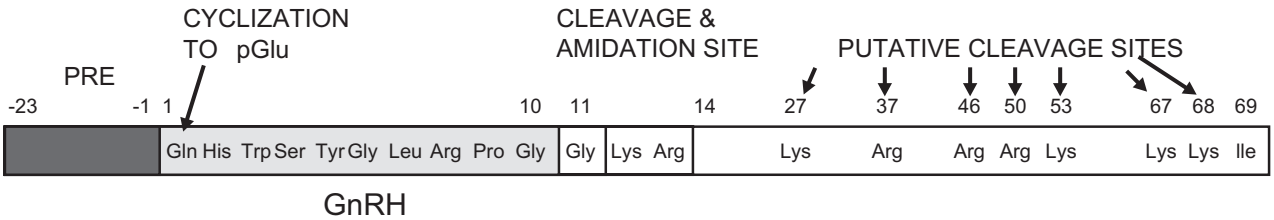

FIGURE 4 Schematic diagram of the structure of the human GnRH precursor. The precursor consists of a signal sequence (PRE) of 23 amino acids followed immediately by the GnRH decapeptide sequence. Cleavage of the signal peptide reveals an NH_2-terminus Gln that cyclizes (enzymatically or spontaneously) to pyro-Glu. The GnRH sequence is followed by a Gly, which is the donor for the COOH-terminal amide of GnRH, and Lys-Arg, which is a conventional dibasic amino acid cleavage site. This is followed by a 56 amino acid peptide sequence. The positions of pairs and single basic amino acid residues present in the 14–69 sequence are shown as potential sites of further processing.

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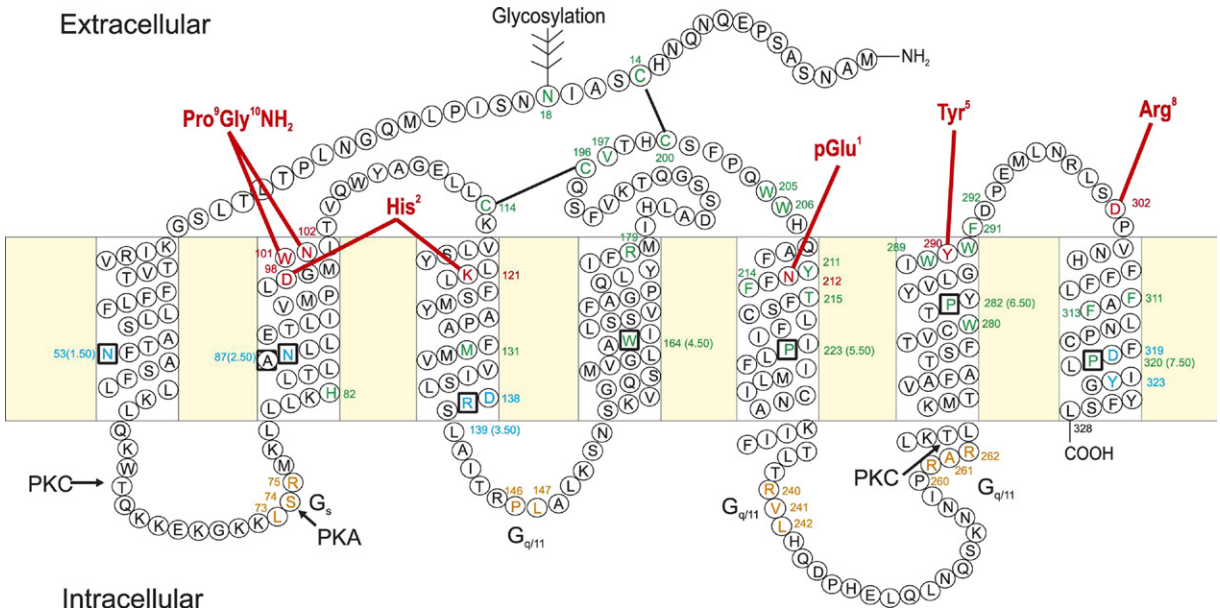

FIGURE 5 Two-dimensional representation of the human GnRH receptor: the 7-(TM) α-helical domains (boxed) are connected by three extracellular loops (ECLs) and three intracellular loops (ICLs). Residues in squares are ones that are highly conserved throughout the rhodopsin family of GPCRs. Ligand-binding residues (red) and residues believed to be important in receptor structure or binding pocket configuration (green) are shown. These include residues involved in disulfide bond formation (black lines) and a glycosylation site. Residues involved in receptor activation are shown in blue. Residues involved in coupling to G proteins are shown in orange. Putative PKC and protein kinase A (PKA) phosphorylation sites are indicated. The intermolecular interactions between GnRH I residues and the receptor are indicated with red lines. Residues shown to alter selectivity for relative binding affinities of GnRH I and GnRH II are shown in bold circles. Figure modified from Ref. 12 with permission. See color plate 29.

Insights Into the Tertiary Structure of the GnRH Receptor

A knowledge of the three-dimensional structure of the GnRH receptor is essential for a complete understanding of its molecular functioning. Recently, crystal structures have been solved for rhodopsin, the B_2-adrenergic, B_1-adrenergic, and adenosine A_{2A}. Molecular models of the GnRH receptor have been generated based on the alignment with these structures. This structure forms the basis for mutagenesis experimental studies designed to test hypotheses and refine the model. The positioning and orientation of the TM helices are therefore crucial. In this regard, an unusual feature of the GnRH receptor has allowed the identification of interhelical interactions. Two residues that are highly conserved in GPCRs, aspartate in TM2 and asparagine in TM7, seem to have undergone reciprocal mutation to Asn^{87} and Asp^{319} (Fig. 5). This suggests that the two residues interact with each other. Mutation of Asn^{87} in TM2 to aspartate abolished receptor function, but a second mutation of Asp^{319} to asparagine in TM7, recreating the arrangement found in other GPCRs, regenerated ligand binding.[7] This restoration of binding by reciprocal mutation suggests that the side chains of two residues in helixes TM2 and TM7 have complementary roles in maintaining the structure of the receptor and occupy the same microenvironment within the receptor.

The 7 TM helical domains are known from the crystal structures of GPCRs to be arranged in a tight bundle enclosing a hydrophilic pocket and surrounded by the hydrophobic membrane environment. The evolutionary conservation of residues along a distinct face of the TM domains in the various GnRH receptors is clearly evident in Fig. 5. This suggests that the conserved, more hydrophilic faces are orientated toward the hydrophilic pocket formed by the helical bundle.

GnRH Agonist Binding to Its Receptor

In the course of the systematic and targeted mutation of more than one-third of all the amino acid residues of the GnRH receptor, considerable advances in identifying ligand contact sites in the mammalian GnRH receptor have been made (Fig. 5). Four putative ligand interaction sites have been identified in the mammalian GnRH receptor. These are $pGlu^1$ with N^{212}, His^2 with K^{120} and D^{98}, Trp^3 with F^{178}, Tyr^5 with Y^{290}, Arg^8 with D^{302}, and $Gly.NH_2^{10}$ with N^{102} [12] (Fig. 5). All these sites have been conserved in vertebrate pituitary GnRH receptors cloned from bony fish, amphibian, and bird species. For details on GnRH binding,

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activation, and desensitization of its receptors, the reader is referred to Ref. 12.

Intracellular Signaling Pathways

The coupling of the GnRH receptor to intracellular signaling pathways has been intensely investigated and comprehensively reviewed.[7,9,18] Although a wide range of intracellular signaling pathways have been implicated in GnRH action, there is a general consensus that the primary pathway is the activation of phospholipase Cβ (PLCβ) through the Gq/11 small guanine nucleotide-binding protein (Fig. 6). PLCβ hydrolyzes phosphatidylinositol-4-5 bisphosphate (PIP_2) to inositol-1-4-5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 binds to receptors in the endoplasmic reticulum (ER) to release Ca^{2+} transiently from these intracellular stores, and this elicits a rapid spike of LH release. DAG activates protein kinase Cs (PKCs). These phosphorylate proteins such as the mitogen-activated protein kinases (MAPKs) that include ERK, JNK, and P38, which are involved in the more sustained release of LH and in gonadotropin biosynthesis. A second major effect of GnRH binding to its receptor is the activation of L-type voltage-operated Ca^{2+} channels (VOCCs), which results in the influx of extracellular Ca^{2+} required for recharging intracellular stores and the prolonged (second phase) of LH release (Fig. 6).

GnRH stimulates DAG production biphasically in pituitary cells, and at a high molar ratio relative to IP_3. This suggests that GnRH stimulates DAG production from a second source independent of PLCβ action on PIP_2. Phospholipase D (PLD) is activated in a gonadotroph cell line a few minutes after PLC activation. Because its substrate phosphatidylcholine is present at almost 100 times higher concentrations in the plasma membrane than that of PIP_2, GnRH stimulation of PLD may be the main source of sustained DAG production.

Another potential signaling pathway of GnRH action is through phospholipase A_2 (PLA_2), which produces arachidonic acid (AA) and its 5-lipoxygenase products, the leukotrienes (LTs), in pituitary cells some minutes after the increase in IP_3. Although PLA_2 may be activated directly by the GnRH receptor, activation is probably by MAPKs, which are known to phosphorylate and activate

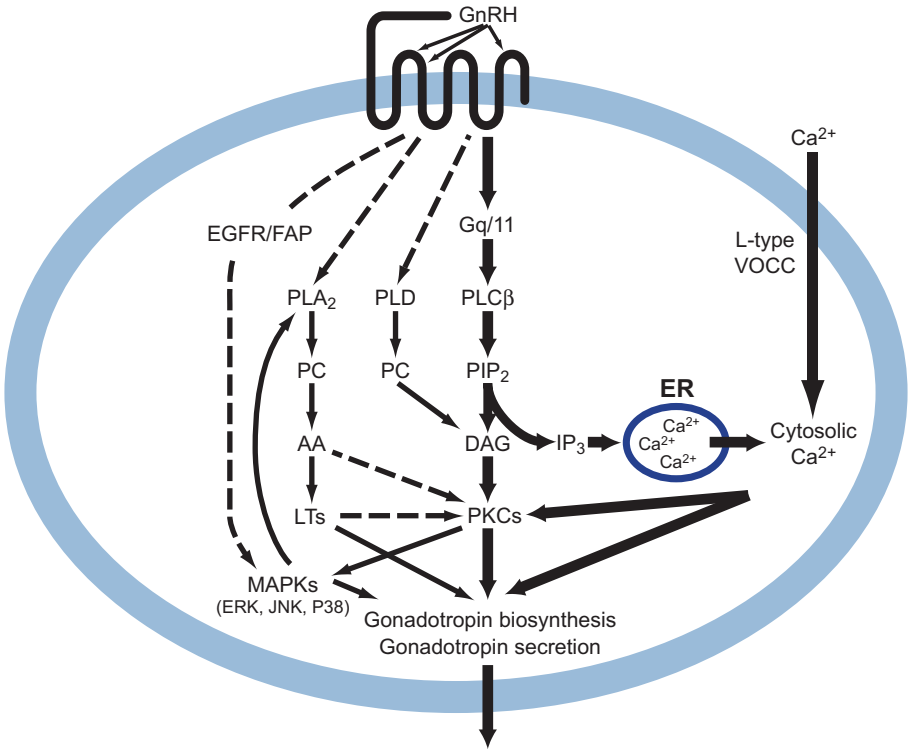

FIGURE 6 Signaling pathways of mammalian, GnRH type I receptor. The primary pathway shown by heavy arrows involves the activation of PLCβ via the Gq/11 G protein and the hydrolysis of phosphatidylinositol-1-4 bisphosphate (PIP_2) to DAG, which activates PKC, and to IP_3, which stimulates Ca^{2+} release from the ER. A second major effect is the entry of extracellular Ca^{2+} through VOCCs. The GnRH receptor also activates PLD, which hydrolyzes phosphatidylcholine (PC) to DAG, and PLA_2, which generates AA and LTs from PC. PLA_2 activation may be mediated via a primary stimulation of MAPK. DAG activates PKC which in turn can activate MAPKs. MAPK isoforms may also be stimulated via FAPs and EGFR and monomeric G proteins such as ras. The products of the diverse pathways affect gonadotropin biosynthesis and secretion. Note that many of the signaling events occur in the plasma membrane, where enzymes (e.g. PLCβ, PLD, PLA_2) and substrates (e.g. PIP_2) are anchored. For simplicity, this is not shown.

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PLA_2. Several studies have shown that GnRH indirectly activates MAPKs in pituitary cells and gonadotroph cell lines via PKCs phosphorylation of Raf-1. MAPKs may also be activated by focal adhesion protein (FAP) complexes involving integrins and by transactivation of the epidermal growth factor receptor (EGFR).

All the signaling pathways outlined above can result in the activation of PKC isoforms (Fig. 6). However, there are distinct differences in the timing and the PKC isoform activated, such that a sequential, coordinated and sustained activation of PKCs occurs. The early DAG and Ca^{2+} generated by PLCβ action might activate Ca^{2+}-dependent PKCs, while late DAG generated by PLD might activate Ca^{2+} independent PKCs. Similarly, the more slowly generated products of PLA_2 such as AA and LTs are known to activate specific PKC isoforms.[7,9,18]

GnRH Regulation of Gonadotropin Gene Expression

The distal targets of the signaling pathways, PKC isoforms, Ca^{2+} calmodulin, MAPKs, and LTs are all variously involved in gonadotropin gene expression, biosynthesis, and exocytosis.[7,18] The mobilization of Ca^{2+} and activation of PKCs resulting from the primary GnRH signaling pathway have both been reported to increase α-, LHβ- and FSHβ-subunit mRNA levels and PKC response elements have been identified in the promoters of these subunits. The more protracted activation of PKCs resulting from the products of the PLD and PLA_2 pathways presumably contributes to the increased gonadotropin subunit mRNA levels. GnRH activation of MAPKs also stimulates the expression of subunit mRNA, and since PKCs can activate MAPKs part, or all, of the PKCs effects may be via MAPKs. These effects may be mediated via distinct GnRH-response elements in the gene promoters.

The relative stimulation of gonadotropin subunits in the rat pituitary is influenced by GnRH pulse frequency. High pulse frequencies stimulate LHβ mRNA levels more that FSβ, whereas the converse is true at low pulse frequencies. Thus, the combination of alterations in pulse frequency together with different phasing and duration of the various signaling pathways provides the potential for fine regulation of gonadotropin secretion. Feedback by gonadal steroid and peptide hormones at the gonadotroph provides additional modulation and the means for asynchronous LH and FSH secretion in response to GnRH, which is crucial for normal ovarian function.

Desensitization

Exposure of the gonadotroph to continuous high doses of GnRH agonists results in the phenomenon of desensitization.[9,12] Gonadotroph desensitization comprises many contributing phenomena, including GnRH receptor down-regulation, receptor uncoupling from cognate G proteins, additional downstream uncoupling (e.g. PKC, Ca^{2+} stores, inositol trisphosphate receptor), inhibition of gonadotropin synthesis, and alterations in the glycosylation of gonadotropins rendering them biologically inactive or even antagonistic. At the level of the GnRH receptor, agonist activation leads to two potential mechanisms of desensitization. For many GPCRs, agonist stimulation results in the activation of protein kinases, which phosphorylate IC domains, resulting in uncoupling from G proteins and internalization of the receptor. GnRH receptors have several putative phosphorylation sites in the IC domains that may serve this function (Fig. 5). However, the mammalian GnRH receptors lack the carboxy-terminal IC domain, which is the prime target for phosphorylation by G protein-coupled receptor kinases and protein kinases. Phosphorylation in this region facilitates the docking of arrestins, thus inducing G protein uncoupling and receptor internalization. Hence, the absence of the carboxy-terminal tail in the mammalian GnRH receptors is associated with a lack of rapid desensitization in inositol phosphate production and an arrestin-independent slow rate of receptor internalization, 7-fold less than that of the chicken GnRH receptors, which have carboxy-terminal tails. Removal of the carboxy-terminal tail from the chicken receptor greatly reduces the rate of internalization to one similar to that of the human receptor. Addition of the carboxy-terminal tail of a nonmammalian GnRH receptor to a mammalian GnRH receptor conveys both rapid desensitization of inositol phosphate production and a more rapid internalization.

It seems that physiological requirements have selected for removal of the carboxy-terminal tail during the evolution of the mammalian GnRH receptor, to prevent rapid desensitization and internalization. A possible driving force is the physiological need of a prolonged LH surge for ovulation in mammals. Thus, although pharmacological doses of GnRH can produce desensitization of the gonadotroph, the GnRH receptor is less susceptible to ligand-mediated desensitization than other GPCRs and may not undergo desensitization under physiological conditions. Nevertheless, pharmacological desensitization occurs with high doses of superactive GnRH agonists, and this has important therapeutic applications (see below).

GnRH Receptor Genes

The structures of the genes encoding GnRH receptors from many mammalian, amphibian, bird, and fish species have been elucidated.[16] In all genes, introns exist in the coding regions of TM4 and IC3. There are numerous splice variants

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whose function may be regulatory, as some truncated receptor variants affect the expression of the functional receptor. The promoters of the rat and human receptors have been analyzed, and the genes are highly regulated by GnRH and downstream signaling molecules resulting from GnRH receptor activation, and also by gonadal, steroid, and peptide hormones.

PHYSICAL STRUCTURE OF GnRH

GnRH is highly flexible in solution, and a crystal structure has yet to be accomplished. However, structure–activity relations and molecular modeling had suggested that GnRH interacts with its receptor in a folded conformation incorporating a βII' bend around Gly^6 [5,12,20] and Section 22. This conformation is supported by NMR studies and approaches combining molecular modeling and ion mobility mass spectrometry.[3]

BIOLOGICAL ACTIONS

GnRH was believed to function exclusively as a stimulator of gonadotropin release from the vertebrate pituitary, but it soon emerged that the peptide has been recruited for diverse functions in vertebrates and protochordates during evolution. This conclusion arose from revelations that

(1) Multiple forms of GnRH are present in tissues of single species;
(2) GnRH is expressed in extrahypothalamic regions of the nervous system (e.g. midbrain, spinal cord, sympathetic ganglia) and in nonneural tissue (e.g. gonads, placenta, breast);
(3) GnRH receptors are present in extrapituitary tissues and GnRH affects cell activity in these tissues;
(4) GnRH stimulates the release of other pituitary hormones (e.g. growth hormone in fish);
(5) GnRH is present in organisms that do not have a pituitary but is still able to stimulate reproduction.

The isolation of several forms of GnRH in neural tissue of tunicates, which represent chordate progenitors, and their activation of the gonads suggest that direct regulation of the gonads was an early-evolved function, and that the neuroendocrine role in regulating the pituitary was a later evolutionary development. The presence of GnRH and GnRH receptors in the gonads of various vertebrate species may reflect this early function. The stimulation of sexual reproduction in yeast by the α-mating factor, which seems to be structurally related to GnRH, may point to an even more ancient example of the direct effects of GnRH on reproduction. It is plausible, therefore, that GnRH peptides were originally involved in cellular communication and in sexual reproduction of simple unicellular and multicellular organisms. Later, they were recruited for expression in nerve cells, to translate external and internal signals into activation of reproduction, initially by acting directly on germ cells, and subsequently via pituitary gonadotroph activation. Although the peptide has been coopted as a regulator at a number of levels in the reproductive system (hypothalamus, gonad, breast, and placenta), there is apparently considerable plasticity in also recruiting it as a regulator in non-reproductive tissues (e.g. adrenal, extrahypothalamic brain, the immune system, retina, and pancreas).

The second form of GnRH originally identified from chicken brain (chicken GnRH II (Fig. 1)) is ubiquitous in vertebrates from primitive bony fish to humans. This complete conservation of structure over 500 million years suggests that the GnRH II has an important function that is mediated through a discriminating receptor that has selected against any structural change in the ligand. The wide distribution of GnRH II in the central and peripheral nervous systems suggests a neurotransmitter/neuromodulatory role. This has been thoroughly demonstrated by the inhibition of M-currents in the bullfrog sympathetic ganglion, which sensitizes neurons to depolarization. GnRH II is present in amphibian sympathetic ganglia, and the receptors are highly selective for the peptide.[6,12]

GnRH has direct effects on sexual arousal in vertebrates, and GnRH II is localized in brain areas associated with reproductive behavior, suggesting that this may be a role for the peptide. GnRH II and an analog are potent stimulators of reproductive behavior in ring doves and song sparrows.[6,12,20] The cognate receptor for GnRH II was cloned from the marmoset[11] and found to be distributed in those areas of primate brain associated with reproductive behaviors. In both the female marmoset[2] and musk shrew,[22] central administration of GnRH II is much more effective than GnRH I in stimulating reproductive behavior. However, the type II GnRH receptor is nonfunctional in humans,[14,16] but cellular context can alter the pharmacology of the GnRH I receptor so that GnRH II can induce different signaling from that of GnRH I.[8]

THERAPEUTIC APPLICATION OF GnRH ANALOGS

Clinical applications of GnRH and its agonist and antagonist analogs are summarized in Fig. 7.[23] Low doses of GnRH (pg/ml) delivered in a pulsatile fashion equivalent to that found in the hypothalamic portal vessels restore fertility in hypogonadal men and women, and are also effective in the treatment of delayed puberty. However, high doses of GnRH or agonist analogs desensitize the gonadotroph, with a resultant decrease in LH and FSH and a decline in ovarian and testicular function (see the earlier section). This

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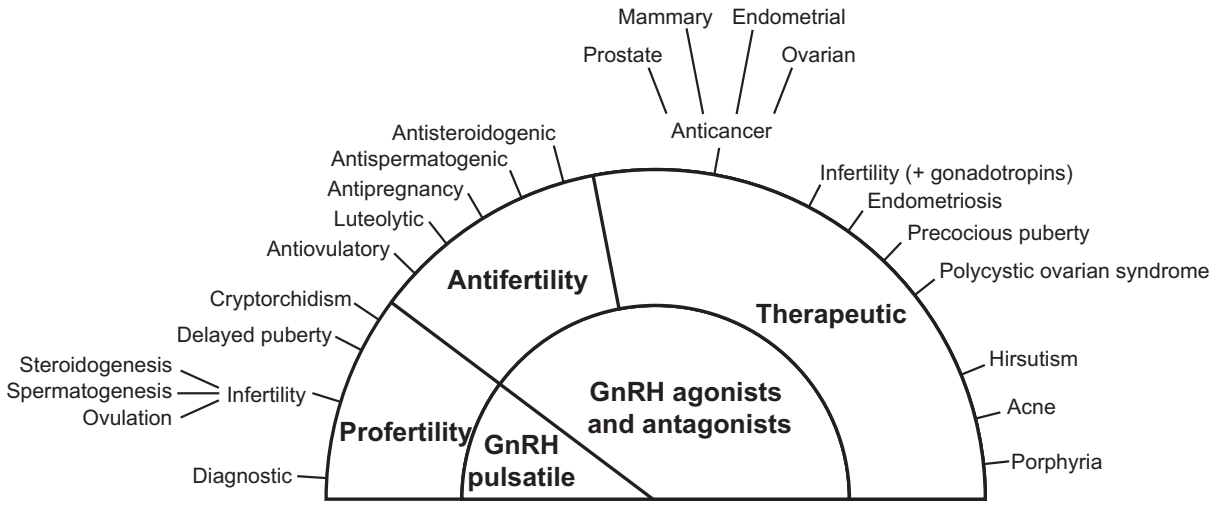

FIGURE 7 Clinical applications of GnRH and GnRH agonists and antagonists. Modified from Ref. 15.

desensitization phenomenon is extensively applied in clinical medicine for the treatment of a wide range of diseases (Fig. 7). GnRH peptide antagonists also inhibit the reproductive system but through competition with endogenous GnRH. The doses required are much higher than the desensitizing agonist doses (mg/day compared with μg/day), and antagonists are currently less extensively used. However, they have the advantage of inducing an immediate inhibition in contrast to agonists which initially (7–14 days) stimulate gonadotropin before the desensitization phenomenon sets in. The development of nonpeptide, orally active GnRH antagonists[12] will probably lead to the replacement of agonists, as they avoid the undesirable stimulation that precedes desensitization, dosage can be more easily modified than for injectable peptides, and treatment may be rapidly withdrawn when required.

FUTURE PERSPECTIVES

GnRH neurons are regulated by novel neuropeptides, kisspeptin, neurokinin B, and dynorphin; all colocalizing in neurons in the arcuate nucleus that has long been recognized as the site of the GnRH pulse generator. The discovery of kisspeptin and neurokinin B as major regulators of the GnRH neuron arose from the observation that inactivating mutations in these genes or their receptors result in a failure to advance through puberty in humans. Intriguingly, these neurons express sex steroid receptors and receptors for metabolic and inflammatory mediators. They thus represent "whole body sensors" for relaying diverse inputs into the regulation of GnRH and provide novel opportunities for the developments of more subtle therapeutic regulation of reproductive hormone secretion.

REFERENCES

1. Baba Y, Matsuo H, Schally AV. Structures of the porcine LH-and FSH-releasing hormone. II. Confirmation of the proposed structure by conventional sequential analyses. *Biochem Biophys Res Commun* 1971;**44**:459–63.
2. Barnett DK, Bunnell TM, Millar RP, Abbott DH. Gonadotropin-releasing hormone II stimulates female sexual behavior in marmoset monkeys. *Endocrinology* 2006;**147**:615–23.
3. Barran PE, Roeske RW, Pawson AJ, Sellar R, Bowers MT, Morgan K, et al. Evolution of GnRH receptor requirement for constrained ligand conformation. *J Biol Chem* 2005;**280**:38569–75.
4. Fink G. Gonadotropin secretion and its control. In: Knobil E, Neill J, editors. *The physiology of reproduction*. New York: Raven Press; 1988. p. 1349–77.
5. Karten MJ, Rivier JE. Gonadotropin-releasing hormone analog design. Structure-function studies toward the development of agonists and antagonists: rationale and perspective. *Endocr Rev* 1986;**7**: 44–66.
6. King JA, Millar RP. Co-ordinated evolution of GnRHs and their receptors. In: Parhar IS, Sakuma Y, editors. *GnRH neurons: gene to behavior*. Tokyo: Brain Shuppan; 1997. p. 51–77.
7. Kraus S, Naor Z, Seger R. Intracellular signaling pathways mediated by the gonadotropin-releasing hormone (GnRH) receptor. *Arch Med Res* 2001;**32**:499–509.
8. Maudsley S, Davidson L, Pawson AJ, Chan R, de Maturana RL, Millar RP. Gonadotropin-releasing hormone (GnRH) antagonists promote proapoptotic signaling in peripheral reproductive tumor cells by activating a $G\alpha_i$-coupling state of the type I GnRH receptor. *Cancer Res* 2004;**64**:7533–44.
9. McArdle CA, Franklin J, Green L, Hislop JN. Signalling, cycling and desensitization of gonadotrophin-releasing hormone receptors. *J Endocrinol* 2002;**173**:1–1.
10. Millar RP, Conklin D, Lofton-Day C, Hutchinson E, Troskie B, Illing N, et al. A novel human GnRH receptor homolog gene: abundant and wide tissue distribution of the antisense transcript. *J Endocrinol* 1999;**162**:117–26.

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11. Millar RP, Lowe S, Conklin D, Pawson A, Maudsley S, Troskie B, et al. A novel mammalian receptor for the evolutionarily conserved type II GnRH. *Proc Natl Acad Sci U S A* 2001;**98**:9636–41.
12. Millar RP, Lu Z, Pawson AJ, Flanagan CA, Morgan K, Maudsley SR. Gonadotropin-releasing hormone receptors. *Endocr Rev* 2003;**25**(2):235–75.
13. Millar RP, Aehnelt C, Rossier G. Higher molecular weight immunoreactive species of luteinizing hormone releasing hormone: possible precursors of the hormone. *Biochem Biophys Res Commun* 1977;**79**:720–31.
14. Millar RP. GnRH II and type II GnRH receptors. *Trends Endocrinol Metab* 2003;**14**:35–43.
15. Millar RP, King JA, Davison JS, Milton RC. Gonadotropin-releasing hormone—diversity of functions and clinical applications. *S Afr Med J* 1987;**72**:748–55.
16. Morgan K, Millar RP. Evolution of GnRH ligand precursors and GnRH receptors in protochordate and vertebrate species. *Gen Comp Endocrinol* 2004;**139**:191–7.
17. Muske LE. Evolution of gonadotropin-releasing hormone (GnRH) neuronal systems. *Brain Behav Evol* 1993;**42**:215–30.
18. Naor Z, Bernard O, Seger R. Activation of MAPK cascades by G-protein-coupled receptors: the case of gonadotropin-releasing hormone receptor. *Trends Endocrinol Metab* 2000;**11**:91–9.
19. Neill JD, Duck LW, Sellers JC, Musgrove LC. A gonadotropin-releasing hormone (GnRH) receptor specific for GnRH II in primates. *Biochem Biophys Res Commun* 2001;**282**:1012–8.
20. Sealfon SC, Weinstein H, Millar RP. Molecular mechanisms of ligand interaction with the gonadotropin-releasing hormone receptor. *Endocr Rev* 1997;**18**:180–205.
21. Seeburg PH, Mason AJ, Stewart TA, et al. The mammalian GnRH gene and its pivotal role in reproduction. *Recent Prog Haorm Res* 1987;**43**:690–8.
22. Temple JL, Millar RP, Rissman EF. An evolutionarily conserved form of gonadotropin-releasing hormone co-ordinates energy and reproductive behavior. *Endocrinology* 2003;**144**:13–9.
23. Tsutsumi M, Zhou W, Millar RP, Mellon PL, Roberts JL, Flanagan CA, et al. Cloning and functional expression of a mouse gonadotropin-releasing hormone receptor. *Mol Endocrinol* 1992;**6**:1163–9.

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Chapter 107

Gonadotropin-Inhibitory Hormone

Kazuyoshi Tsutsui and Takayoshi Ubuka

ABSTRACT

Gonadotropin-inhibitory hormone (GnIH) is a hypothalamic neuropeptide that was discovered in quail as an inhibiting factor for gonadotropin release. GnIH inhibits gonadotropin synthesis and release in birds through actions on gonadotropin-releasing hormone (GnRH) neurons and gonadotrophs, mediated via GPR147. Subsequently, GnIH orthologs were identified in other vertebrate species from fish to humans. As in birds, mammalian and fish GnIH orthologs inhibit gonadotropin release, indicating a conserved role for this neuropeptide in the control of the hypothalamic–pituitary–gonadal (HPG) axis across species. Discovery of GnIH, a novel endogenous inhibitor of reproduction, has opened up new vistas for understanding the regulation of reproduction.

DISCOVERY

Gonadotropin-inhibitory hormone (GnIH) was first discovered in the brain of the Japanese quail *Coturnix japonica* in search of a novel RFamide peptide that has an Arg-Phe-NH_2 motif at its C-terminus. RFamide peptides were first isolated in invertebrate species in the late 1970s. The initial RFamide peptide, Phe-Met-Arg-Phe-NH_2 (FMRFamide), is a cardioexcitatory molecule isolated from the ganglia of the venus clam *Macrocallista nimbosa*.[20] After this discovery, numerous RFamide peptides that act as neurotransmitters, neuromodulators, and peripheral hormones have been identified in various invertebrate phyla, including cnidarians, nematodes, annelids, molluscs, and arthropods. Immunohistochemical studies in vertebrates suggested the presence of RFamide peptides in the nervous system. Some of the FMRFamide-immunoreactive (-ir) neurons projected close to the pituitary gland suggest a role in the regulation of pituitary function. Tsutsui et al.[26] thus hypothesized that avian hypothalamus may also possess a novel RFamide peptide that regulates anterior pituitary function.

In 2000, Tsutsui et al.[26] successfully isolated a novel RFamide peptide from 500 Japanese quail brains by high-performance liquid chromatography (HPLC) and a competitive enzyme-linked immunosorbent assay using an antibody developed against the dipeptide Arg-Phe-NH_2. The isolated peptide has a previously unreported dodecapeptide structure, SIKPSAYLPLRFamide (Table 1). Its C-terminus is identical to chicken LPLRFamide that was reported to be the first RFamide peptide isolated in vertebrates,[8] which may be a degraded fragment of the dodecapeptide. The isolated peptide was localized in the quail hypothalamo–hypophysial system by immunohistochemistry and shown to decrease gonadotropin release from cultured quail anterior pituitaries.[26] This RFamide peptide was thus named gonadotropin-inhibitory hormone (GnIH).[26]

After the isolation of GnIH in the quail brain, the precursor polypeptide for GnIH was examined.[22] A cDNA that encodes GnIH precursor polypeptide was identified by a combination of 3' and 5' rapid amplification of cDNA ends (3'/5' RACE).[22] The GnIH precursor consists of 173 amino acid residues that encode one GnIH and two GnIH-related peptides (GnIH-RP-1 and GnIH-RP-2) possessing an LPXRFamide (X = L or Q) sequence at their C-termini. These peptide sequences are flanked by a glycine C-terminal amidation signal and a single basic amino acid on each end as an endoproteolytic site. GnIH-RP-2 was also identified as a mature peptide by mass spectrometry[22] (Table 1). GnIH was further isolated as mature peptides in European starlings[29] and zebra finch within the class of birds (Table 1).

In mammals, cDNAs that encode LPXRFamide peptides similar to GnIH have been investigated by a gene database search.[10] The cDNAs identified from human and bovine brains encode three RFamide-related peptides (RFRP-1, -2, and -3).[10] RFRP-1 and -3 are both LPXRFamide peptides, but RFRP-2 is not an LPXRFamide peptide. RFRP-1 and -3

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00107-X
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TABLE 1 Amino Acid Sequences of GnIH and its Orthologs (LPXRFamide Peptides) in Vertebrates

Animal	Name	Sequence	Reference
Mammal			
Human	RFRP-1	MPHSFAN**LPLRFa**	Ubuka et al., 2009[31]
	RFRP-3	VPN**LPQRFa**	
Macaque	RFRP-1*	MPHSVTN**LPLRFa**	Ubuka et al., 2009[30]
	RFRP-3	SGRNMEVSLVRQVLN**LPQRFa**	
Bovine	RFRP-1	SLTFEEVKDWAPKIKMNKPVVNKMPPSAAN**LPLRFa**	Fukusumi et al., 2001;
	RFRP-3	AMAHLPLRLGKNREDSLSRWVPN**LPQRFa**	Yoshida et al., 2003
Ovine	RFRP-1*	SLTFEEVKDWGPKIKMNTPAVNKMPPSAAN**LPLRFa**	Clarke et al., 2008[7]
	RFRP-3*	VPN**LPQRFa**	
Rat	RFRP-1*	SVTFQELKDWGAKKDIKMSPAPANKVPHSAAN**LPLRFa**	Ukena et al., 2002
	RFRP-3	ANMEAGTMSHFPS**LPQRFa**	
Hamster	RFRP-1	SPAPANKVPHSAAN**LPLRFa**	Ubuka et al., 2011[28]
	RFRP-3	TLSRVPS**LPQRFa**	
Bird			
Quail	GnIH	SIKPSAY**LPLRFa**	Tsutsui et al., 2000[26];
	GnIH-RP-1*	SLNFEEMKDWGSKNFMKVNTPTVNKVPNSVAN**LPLRFa**	Satake et al., 2001[22]
	GnIH-RP-2	SSIQSLLN**LPQRFa**	
Chicken	GnIH*	SIRPSAY**LPLRFa**	Ikemoto et al., 2005
	GnIH-RP-1*	SLNFEEMKDWGSKNFLKVNTPTVNKVPNSVAN**LPLRFa**	
	GnIH-RP-2*	SSIQSLLN**LPQRFa**	
Sparrow	GnIH*	SIKPFSN**LPLRFa**	Osugi et al., 2004[18]
	GnIH-RP-1*	SLNFEEMEDWGSKDIIKMNPFTASKMPNSVAN**LPLRFa**	
	GnIH-RP-2*	SPLVKGSSQSLLN**LPQRFa**	
Starling	GnIH	SIKPFAN**LPLRFa**	Ubuka et al., 2008[29]
	GnIH-RP-1*	SLNFDEMEDWGSKDIIKMNPFTVSKMPNSVAN**LPLRFa**	
	GnIH-RP-2*	GSSQSLLN**LPQRFa**	
Zebra finch	GnIH	SIKPFSN**LPLRFa**	Tobari et al., 2010
	GnIH-RP-1*	SLNFEEMEDWRSKDIIKMNPFAASKMPNSVAN**LPLRFa**	
	GnIH-RP-2*	SPLVKGSSQSLLN**LPQRFa**	
Amphibian			
Frog	fGRP/R-RFa	SLKPAAN**LPLRFa**	Koda et al., 2002[14];
	fGRP-RP-1	SIPN**LPQRFa**	Chartrel et al., 2002[4];
	fGRP-RP-2	YLSGKTKVQSMAN**LPQRFa**	Ukena et al., 2003[33]
	fGRP-RP-3	AQYTNHFVHSLDT**LPLRFa**	
Newt	nLPXRFa-1	SVPN**LPQRFa**	Chowdhury et al., 2011
	nLPXRFa-2	MPHASAN**LPLRFa**	
	nLPXRFa-3	SIQPLAN**LPQRFa**	
	nLPXRFa-4	APSAGQFIQTLAN**LPQRFa**	
Fish			
Goldfish	gfLPXRFa-1*	PTHLHAN**LPLRFa**	Sawada et al., 2002
	gfLPXRFa-2*	AKSNIN**LPQRFa**	
	gfLPXRFa-3	SGTGLSAT**LPQRFa**	

**Putative peptides. LPXRFamide (X = L or Q) carboxyl peptide consensus sequences are shown in bold.*

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having LPXRFamide sequences were purified from bovine hypothalami (Table 1). Rat RFRP-3, Siberian hamster RFRP-1 and -3,[28] macaque RFRP-3,[30] and human RFRP-1 and -3[31] were also identified as mature peptides in mammals (Table 1). Subsequently, it was shown that human RFRP-3, rat RFRP-3, and Siberian hamster RFRP-3 also inhibit gonadotropin synthesis and/or release in mammals.[7,11,17,21,28] Accordingly, a mammalian LPXRFamide peptide (RFRP-3) acts as a functional ortholog of GnIH in terms of reducing gonadotropin secretion.

In amphibians, a GnIH ortholog that consists of 12 amino acids (SLKPAANLPLRFamide) with a C-terminal LPLRFamide motif was identified in the bullfrog hypothalamus[14] (Table 1). This neuropeptide in frog, named fGRP, has a high sequence homology with GnIH.[22,26] The fGRP precursor also encodes one fGRP and three fGRP-related peptides (fGRP-RP-1, -2, and -3), later identified as mature LPXRFamide peptides.[33] At the same time, fGRP and fGRP-RP-1 were independently purified from the European green frog (R-RFa; 4) and bullfrog (fNRP), respectively. Subsequently, from the goldfish brain, a cDNA that encodes three fish GnIH orthologs (gfLPXRFa-1, -2, and -3) was characterized and gfLPXRFa-3 was identified as a mature peptide (for reviews, see Refs 24, 25; Table 1). Recently, the evolutionary origin of LPXRFamide peptides was discovered in the brain of lamprey, one of the most ancient vertebrate species (for reviews, see Refs 24, 25; also Osugi et al. (2012) as recently reported in *Endocrinology*).

As summarized in Table 1, GnIH and its orthologs have now been documented in a variety of vertebrates from fish to mammals, including humans: RFRPs in mammals, GnIH and GnIH-RPs in birds, fGRP, fGRP-RPs, nLPXRFamides in amphibians, and gfLPXRFamides in teleosts. All the identified GnIH and its orthologs possess a common C-terminal LPXRFamide motif. Tsutsui et al. therefore designated these peptides as LPXRFamide peptides, which form a new group in the RFamide peptide family (for reviews, see Refs 24, 25) (Fig. 1). Extensive studies over the past decade have shown that vertebrate brains produce a variety of RFamide peptides. To date, five groups in the RFamide peptide family have been documented, that is, PQRFamide (NPFF) group, LPXRFamide peptide (GnIH) group, PRL-releasing peptide (PrRP) group, kisspeptin group, and pyroglutamylated RFamide peptide (QRFP)/26RFamide group (Fig. 1).

STRUCTURE OF THE mRNA/GENE

The mRNA encoding for GnIH precursor polypeptide was identified in the quail brain.[22] The GnIH precursor consisted of 173 amino acid residues that encoded one GnIH and two GnIH-RPs that have a C-terminal LPXRFamide motif. These peptide sequences are flanked by a glycine C-terminal amidation signal and a single basic amino acid on each end as an endoproteolytic site. GnIH precursor cDNAs were also cloned in white-crowned sparrow,[18] European starling,[29] zebra finch, and chicken in birds, and they all had similar structures (for reviews, see Refs 24, 25).

Fig. 2A shows the organization of the human RFRP gene, mRNA and posttranslational processing of RFRP precursor polypeptide. The mRNAs encoding for precursor polypeptides of RFRPs, mammalian GnIH orthologs have been reported by a gene database search.[10] Although human and bovine mRNAs encode three RFRPs (RFRP-1, -2, and -3), only RFRP-1 and RFRP-3 possess a C-terminal LPXRFamide structure, and human and bovine RFRP-2 have C-terminal RSamide and RLamide sequences, respectively (Fig. 2B). On the other hand, rodents (rat and hamster) completely lost the RFRP-2 sequence in their precursors (Fig. 2B). The regions that encode RFRP-1/GnIH-RP-1 or RFRP-2/GnIH in the precursor polypeptides are conserved between mammals (only RFRP-1 in rodents) and birds (Fig. 2B). However, the positions of RFRP-3 and GnIH-RP-2 in the precursor polypeptides are different between mammals and birds (Fig. 2B).

Tsutsui et al. also cloned cDNAs that encode GnIH orthologs from bullfrog and newt brains.[14,33] The frog and newt GnIH ortholog precursors consist of 221 and 233 amino acid residues, respectively, both encoding four LPXRFamide peptides (in frog: fGRP, fGRP-1, -2, and -3; in newt: nLPXRFa-1, -2, -3, and -4) (Table 1). The goldfish precursor of GnIH orthologs consists of 197 amino acid residues that encode three LPXRFamide peptides (gfLPXRFa-1, -2, and -3) (for reviews, see Refs 24, 25) (Table 1).

PROCESSING OF THE PRECURSOR

Three and two mature LPXRFamide peptides are possibly cleaved from their precursor polypeptides in birds and mammals, respectively. Fig. 2A shows the posttranslational processing of human RFRP precursor polypeptide. Fig. 2B shows the alignment of precursors of GnIH and GnIH orthologs in human, bovine, rat, Siberian hamster, Japanese quail, white-crowned sparrow, European starling, and zebra finch as representative precursors encoding RFRP-1 and RFRP-3 in mammals, GnIH-RP-1, GnIH, and GnIH-RP-2 in birds. The LPXRFamide motif at their C-termini is followed by glycine as an amidation signal and arginine or lysine as endoproteolytic basic amino acids. Endogenous LPXRFamide peptides can be cleaved at basic amino acids at their N-termini. However, there are some exceptions in the cleavage site at the N-terminal, such as that of Siberian hamster RFRP-1 (Fig. 2B). There is an LPLRSamide peptide sequence encoded in the RFRP-2 position of the human precursor polypeptide (Fig. 2B), although this mature peptide has not yet been isolated.[31] The bovine RFRP-2 with LPLRLamide sequence may not

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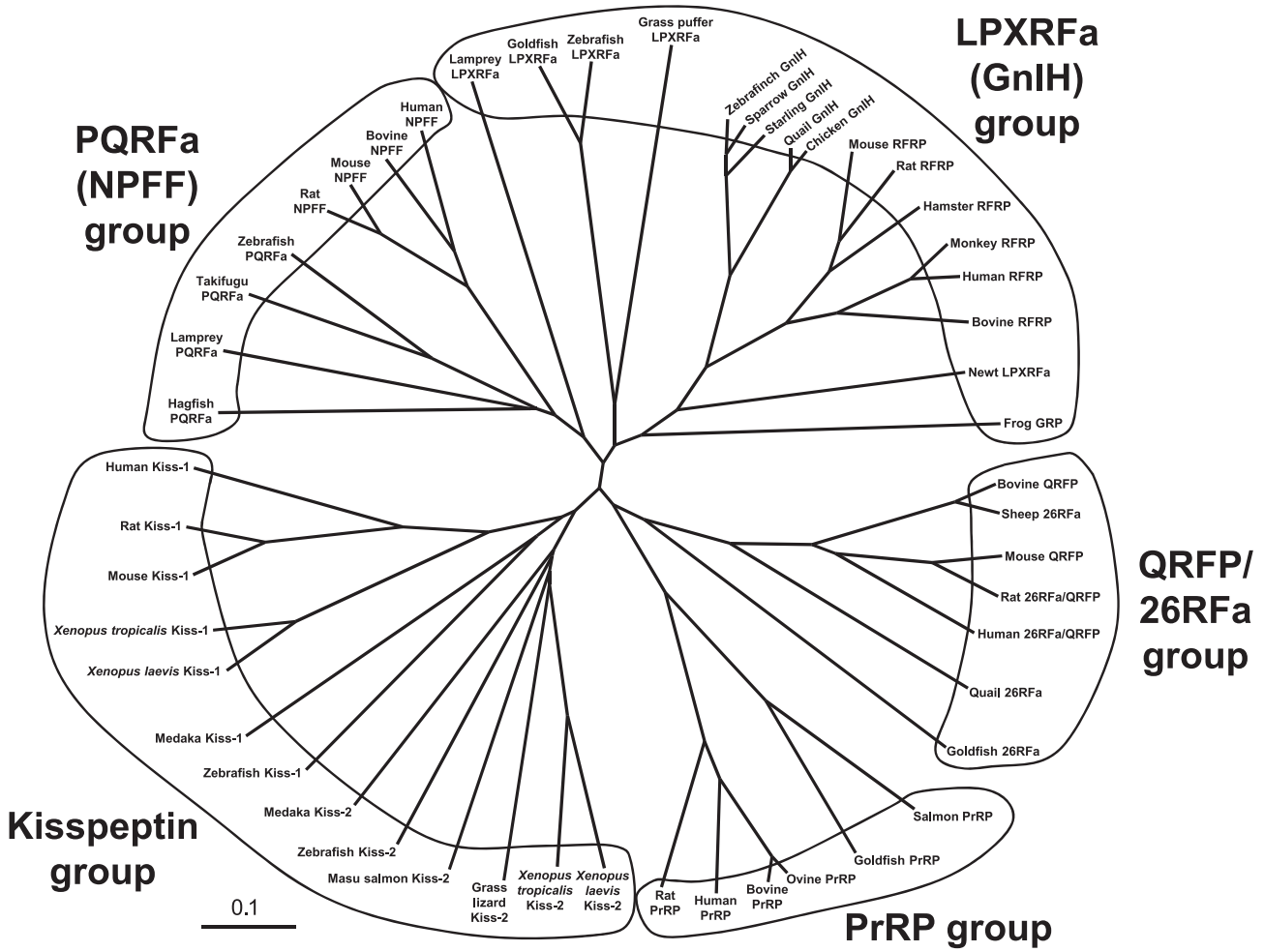

FIGURE 1 Phylogenetic tree of the RFamide peptide family. Vertebrate brains produce a variety of RFamide peptides. To date, five groups in the RFamide peptide family have been documented as follows: PQRFamide peptide (NPFF) group, LPXRFamide peptide (GnIH) group, PRL-releasing peptide (PrRP) group, kisspeptin group, and pyroglutamylated RFamide peptide (QRFP)/26RFamide group. Scale bar refers to a phylogenetic distance of 0.1 amino acid substitutions per site among the precursors.

be processed because this sequence is included within the mature bovine RFRP-3 peptide sequence (Fig. 2B).

DISTRIBUTION OF THE mRNA/PEPTIDE

After cloning GnIH precursor mRNA in the quail brain, its location was first investigated by Southern blot analysis of the RT-PCR products of GnIH precursor cDNA. Within the samples from telencephalon, diencephalon, mesencephalon, and cerebellum, GnIH precursor mRNA is only expressed in the quail diencephalon.[22] In situ hybridization for GnIH precursor mRNA further showed that the cells expressing GnIH mRNA are clustered in the paraventricular nucleus (PVN) in the hypothalamus.[34] Immunohistochemistry with a GnIH antibody has revealed that clusters of GnIH-ir neurons are expressed in the PVN in the quail.[26,34] GnIH expressing cell bodies are also clustered in the PVN in other birds.[2,18,29] In contrast to the highly clustered cell bodies in the PVN, GnIH-ir neuronal fibers are widely distributed in diencephalic and mesencephalic regions in birds.[2,29,34]

In mammals, expression of precursor mRNA of RFRPs, mammalian GnIH orthologs, is only detected in the dorsomedial hypothalamic area (DMH) in the mouse and hamster, investigated by in situ hybridization.[15,24,25,28] In the rat brain, RFRP precursor mRNA is expressed in the periventricular nucleus (PerVN), and the portion between the dorsomedial nucleus (DMN) and the ventromedial nucleus (VMN) of the hypothalamus[10,24,25] (Fig. 3). The majority of RFRP mRNA expression is localized in the intermediate periventricular nucleus (IPe) of the hypothalamus in the macaque,[30] and in the DMN and PVN in the sheep.[7] RFRP-ir neuronal fiber processes and terminals occur widely in the central nervous system. Abundant RFRP-ir fibers are in the median eminence (ME) of sheep,[7] macaque,[30] and

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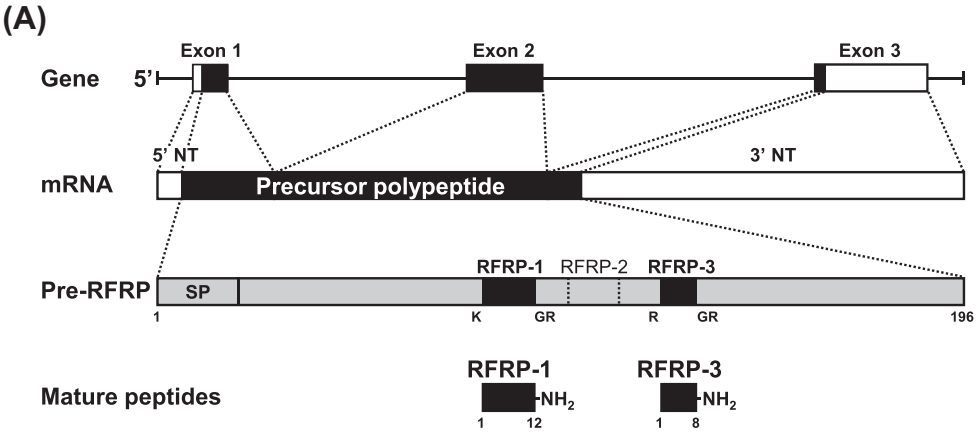

(B)

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Human          MEIISSKLFILLTLATSSLLTSNIFCADELVMSNLHSKE-NYDKYSEPRGYP--KGERSL  57
Bovine         MEIISLKRFILLMLATSSLLTSNIFCTDESRMPNLYSKK-NYDKYSEPRGDLGWEKERSL  59
Rat            MEIISSKRFILLTLATSSFLTSNTLCSDELMMPHFHSKE-GYGKYYQLRGIPKGVKERSV  59
Hamster        MEIISSKRFILLTLATSSLLTSNIFCTEELMMPHFHSKE-KEDKYSQPTGISKGEKERSV  59
Quail          MEIISTQKFILLTLATVAFLTPHGACLDELMKSSLESREDDDDKYYETKDSILEEKQRSL  60
Sparrow        MKVISTKKFILFALATVVFLTSNSMCLNEAMKSRLQSREDNDDKDYEIKDNILEEKQRSL  60
Starling       MKVILTQKFILFALAAVVLLTSNSMCLNEPMKPRLQSREDNDDKYYEIKDNILEEKQRSL  60
Zebra finch    MKVISNKKLILFALATVVFLTSNSMCLNEPMKSRLQSREDNDDKYYEIKDNILEEKQRSL  60

                                                RFRP-1              RFRP-2
Human          NFEELKDWGPKNVIKMSTPAVNKMPHSFANLPLRFGRNVQEERSAGATANLPLRSGRNME 117
Bovine         TFEEVKDWAPK--IKMNKPVVNKMPPSAANLPLRFGRNMEEERSTRAMAHLPLRLGKNRE 117
Rat            TFQELKDWGAKKDIKMSPAPANKVPHSAANLPLRFGRNIEDRRSP--------RARANME 111
Hamster        SFQEVKDWGAKNVIKMSPAPANKVPHSAANLPLRFGRTLEEDRST--------RARTNME 111
Quail          NFEEMKDWGSKNFMKVNTPTVNKVPNSVANLPLRFGRSNPEERSIKPSAYLPLRFGRAFG 120
Sparrow        NFEEMEDWGSKDIIKMNPFTASKMPNSVANLPLRFGRNYPEERSIKPFSNLPLRFGRAFG 120
Starling       NFDEMEDWGSKDIIKMNPFTVSKMPNSVANLPLRFGRNYPEERSIKPFANLPLRFGRAFG 120
Zebra finch    NFEEMEDWRSKDIIKMNPFAASKMPNSVANLPLRFGRNYPEERSIKPFSNLPLRFGRAFG 120
                                           GnIH-RP-1              GnIH
                       RFRP-3
Human          VSLVRRVPNLPQRFGRTTTAKSVCRMLSDLCQGSMHSPCANDLFYSMTCQHQEIQNPDQK 177
Bovine         DSLSRWVPNLPQRFGRTTTAKSITKTLSNLLQQSMHSPSTNGLLYSMACQPQEIQNPGQK 177
Rat            AGTMSHFPSLPQRFGR-TTARRITKTLAGLPQKSLHSLASSELLYAMTRQHQEIQSPGQE 170
Hamster        ARTLSRVPSLPQRFGR-TTARSIPKTLSHLLQRFLHSMATSEVLNAMTCQHGEIQSPGGK 170
Quail          ESLSRRAPNLSNRSGRSPLARSSIQSLLNLPQRFGKSVPIS------LSQGVQESEPGM- 173
Sparrow        ENTPNHSPRVSHRLERSPLVKGSSQSLLNLPQRFGKSLAVN------LPRDIQEFEPGI- 173
Starling       ENIPNHAPKVLHRLGRSPLLKGSSQSLLNLPQRFGKSLVFN------LPQDIQESEPGI- 173
Zebra finch    ENIRNHAPKVSHRLERSPLVKGSSQSLLNLPQRFGKSLPVN------LPRDVEEFEPGI- 173
                                           GnIH-RP-2

Human          QSRRLLFKKIDDAELKQEK--------------                            196
Bovine         NLRRRGFQKIDDAELKQEK--------------                            196
Rat            QPRKRVFTETDDAERKQEKIGNLQPVLQGAMKL                            203
Hamster        QPRRQAFMETDDEEGKHEK--------------                            189
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FIGURE 2 **A**. Organization of the human *RFRP* gene, mRNA and posttranslational processing of RFRP precursor polypeptide. The 5′ and 3′-nontranslated region (5′-NT, 3′-NT), the signal peptide (SP), RFRP-1, and RFRP-3 are indicated. The coding position of the putative RFRP-2 is also indicated. **B**. The alignment of RFRP or GnIH precursor polypeptides in human (NM_022150), bovine (NM_174168), rat (NM_023952), Siberian hamster (JF727837), Japanese quail (AB039815), white-crowned sparrow (AB128164), European starling (EF486798), and zebra finch (AB522971). Identified mature peptides are underlined. Endoproteolytic basic amino acid (**R** or **K**) at the N-termini are shown in bold. A broken line indicates the putative human RFRP-2 sequence.

human,[31] consistent with a role for GnIH orthologs in pituitary gonadotropin regulation. On the other hand, relatively few or no immunoreactive fibers are found in the ME in rodents[15,28] (for reviews, see Refs 24, 25).

In lower vertebrates, amphibian GnIH orthologs, such as fGRP, fGRP-RPs, and nLPXRFamides, are expressed in the cell bodies in the suprachiasmatic nucleus (SCN) of the hypothalamus in the frog[14] and the newt. Abundant immunoreactive fibers are also present in the diencephalon, mesencephalon, and rhombencephalon in these species.[14] In the goldfish, expression of precursor mRNA of gfLPXRFamides, fish GnIH orthologs, is localized in the nucleus posterioris periventricularis (NPPv) of the hypothalamus (for reviews, see Refs 24, 25).

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RECEPTORS AND SIGNALING CASCADES

The receptor for GnIH (GnIH-R) is considered to be GPR147. Bonini et al.[3] have identified two G protein-coupled receptors (GPCRs) for NPFF, and designated them as NPFF1 (identical to GPR147) and NPFF2 (identical to GPR74). Hinuma et al.[10] have also reported a specific receptor for RFRPs, mammalian GnIH orthologs, named OT7T022, which is also identical to GPR147. Other groups have shown that HLWAR77, which corresponds to GPR74, is a receptor for NPFF. The binding affinities for GPR147 and GPR74 and the efficacies on signal transduction pathway were examined with various analogs of RFRPs and NPFF. RFRPs have a higher affinity for GPR147, whereas NPFF had potent agonistic activity for GPR74.[3,16] Taken together, GPR147 (NPFF1, OT7T022) was suggested to be the receptor for RFRPs, mammalian GnIH orthologs (for reviews, see Refs 24, 25). In situ hybridization, autoradiography, and immunohistochemistry in the rat brain have shown that GPR147 is expressed in various brain regions including the limbic system of telencephalon, diencephalon, mesencephalon and the pituitary,[3,16] (See Son et al. (2012) recently reported in *Endocrinology*) (Fig. 3).

To elucidate the mode of action of GnIH in birds, Tsutsui et al. identified GnIH-R in the quail diencephalon and characterized its expression and binding activity.[35] First, a cDNA encoding a putative GnIH-R was cloned by a combination of 3′/5′ RACE using PCR primers designed from the sequence of the receptor for RFRPs (GPR147). The crude membrane fraction of COS-7 cells transfected with the putative GnIH-R cDNA specifically bound GnIH and GnIH-RPs in a concentration-dependent manner, indicating that GPR147 is GnIH-R.[35] GnIH-R is also widely expressed in the brain and the pituitary in birds (for reviews, see Refs 24, 25).

Since GPR147 couples with $G\alpha_i$ to inhibit adenylyl cyclase (AC),[10] GPR147 (GnIH-R) activation may reduce intracellular cAMP levels, and reduce the activities of cAMP-dependent protein kinase (PKA) and mitogen-activated protein kinase (MAPK) signaling cascade. On the other hand, GnRH receptor (GnRH-R) couples with $G\alpha_{q/11}$ to activate phospholipase C (PLC), which results in the production of inositol triphosphate (IP3) and diacylglycerol (DAG). In turn, IP3 and DAG increase intracellular Ca^{2+} and activate the protein kinase C (PKC) pathway and MAPK signaling. GnRH-R also couples with $G\alpha_s$ to stimulate AC/cAMP/PKA pathway (for reviews, see Refs 24, 25). The inhibitory action of GnIH and RFRPs on gonadotropin gene expression was suggested to be mediated by an inhibition of AC/cAMP/PKA-dependent extracellular signal-regulated kinase (ERK) pathway (see Son et al. (2012) as reported in *Endocrinology*). In the sheep, RFRP-3, a GnIH ortholog, can inhibit both GnRH-induced intracellular Ca^{2+} increase and ERK phosphorylation, impacting GnRH-induced gonadotropin release and synthesis.[21] In the chicken, activation of GnRH-R can activate AC and stimulate cAMP responsive element (CRE) binding protein.[23] GnIH may partly inhibit this GnRH-induced CRE activation, thus impacting gene transcription[23] (for reviews, see Refs 24, 25).

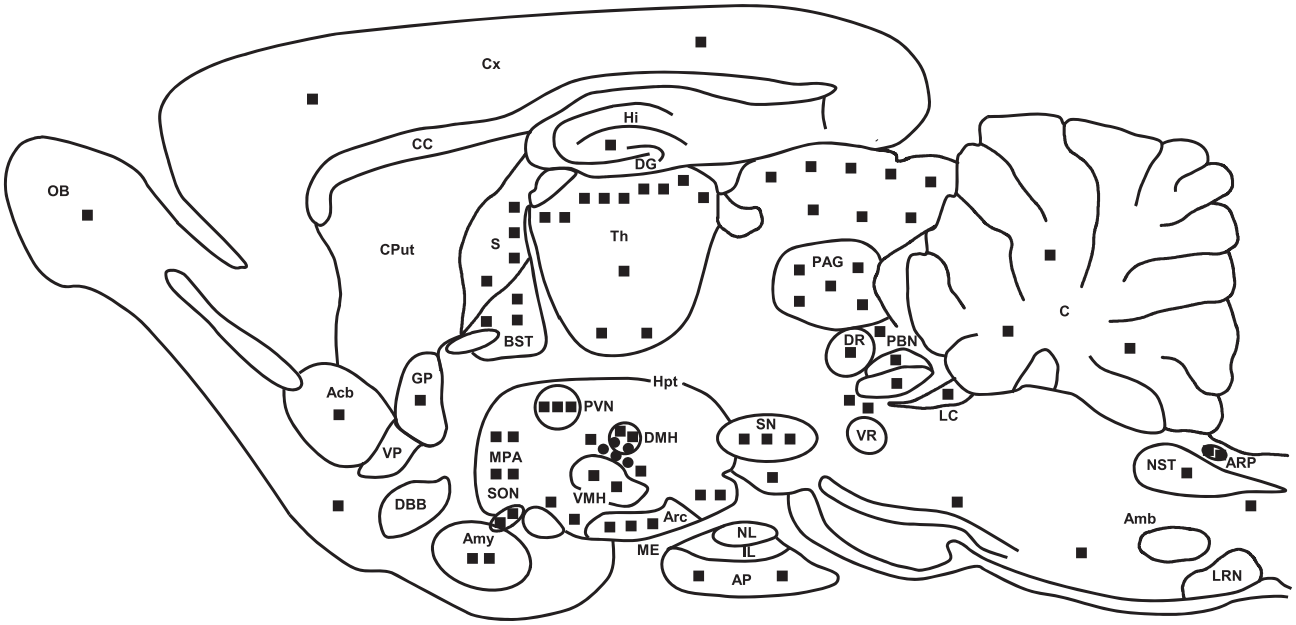

FIGURE 3 Location of GnIH mRNA (●) and GnIH receptor mRNA and protein (■) in the rat brain.

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BIOLOGICAL ACTIONS ON THE PITUITARY AND WITHIN THE BRAIN

Dense population of GnIH-ir fibers at the ME in quail[26,34] and in other birds[2,18,29] suggested a role for GnIH in the regulation of pituitary function in birds. That GnIH inhibits synthesis and/or release of gonadotropins from cultured quail and chicken anterior pituitary provides strong support for this function[6,26] (Table 2). Peripheral administration of GnIH also inhibits gonadotropin synthesis and/or release in birds[18,32] (Table 2). In mammals, abundant RFRP-ir fibers were observed in the ME of sheep,[7] macaque,[30] and humans.[31] As in birds, RFRP-3, a mammalian GnIH ortholog, inhibits gonadotropin synthesis and/or release

TABLE 2 Physiological Functions of GnIH and its Orthologs (LPXRFamide Peptides) in Vertebrates

Animal	Name	Physiological Function	Reference
Mammal			
Human	RFRP-1	Stimulation of prolactin release (i.c.v.)	Hinuma et al., 2000[10]
Bovine	RFRP-3*	Inhibition of GnRH-elicited gonadotropin release (*in vitro*) Inhibition of gonadotropin release (i.v.)	Kadokawa et al., 2009[12]; Kadokawa et al., 2009[12]
Ovine	RFRP-3*	Inhibition of GnRH-elicited gonadotropin release (*in vitro*) Inhibition of gonadotropin release (i.v.) Inhibition of GnRH-elicited gonadotropin synthesis and release (*in vitro*)	Clerke et al., 2008[7]; Clerke et al., 2008[7]; Sari et al., 2009[21]
Rat	RFRP-1* RFRP-3	Stimulation of adrenocorticotropic hormone, oxytocin release (i.c.v.) Stimulation of adrenocorticotropic hormone, oxytocin release (i.c.v.) Inhibition of gonadotropin release (i.c.v.) Inhibition of gonadotropin release (i.v.) Inhibition of GnRH-elicited gonadotropin release (*in vitro*) Inhibition of reproductive behavior (i.c.v.) Stimulation of feeding behavior (i.c.v.) Stimulation of feeding behavior (i.c.v.)	Kaewwongse at al., 2011 Kaewwongse at al., 2011; Johnson et al., 2007[11]; Murakami et al., 2008[17]; Murakami et al., 2008[17]; Johnson et al., 2007[11]; Johnson et al., 2007[11]; Murakami et al., 2008[17]
Hamster	RFRP-1 RFRP-3	Inhibition of gonadotropin release (i.c.v.) Inhibition of gonadotropin release (i.c.v.) Inhibition of gonadotropin release (i.p.)	Ubuka et al., 2011[28] Ubuka et al., 2011[28]; Kriegsfeld et al., 2006[15]
Bird			
Quail	GnIH	Inhibition of gonadotropin release (*in vitro*) Inhibition of gonadotropin synthesis and release (i.p.)	Tsutsui et al., 2000[26]; Ubuka et al., 2006[32]
Chicken	GnIH* GnIH-RP-1* GnIH-RP-2*	Inhibition of gonadotropin synthesis and release (*in vitro*) Stimulation of feeding behavior (i.c.v.) Stimulation of feeding behavior (i.c.v.) Stimulation of feeding behavior (i.c.v.)	Ciccone et al., 2004[6]; Tachibana et al., 2005 Tachibana et al., 2005 Tachibana et al., 2005
Sparrow	GnIH*	Inhibition of GnRH-elicited gonadotropin release (i.v.) Inhibition of gonadotropin release (i.v.) Inhibition of gonadotropin release (i.c.v.) Inhibition of reproductive behavior (i.c.v.)	Osugi et al., 2004[18]; Osugi et al., 2004[18]; Bentley et al., 2006[1]; Bentley et al., 2006[1]
Amphibian			
Frog	fGRP fGRP-RP-2	Stimulation of GH release (*in vitro*, i.p.) Stimulation of GH/PRL release (*in vitro*, i.p.)	Koda et al., 2002[14] Ukena et al., 2003[33]
Fish			
Goldfish	gfLPXRFa-1* gfLPXRFa-2* gfLPXRFa-3	Stimulation of gonadotropin and GH release (*in vitro*) Stimulation of gonadotropin and GH release (*in vitro*) Stimulation of gonadotropin and GH release (*in vitro*)	Amano et al., 2006 Amano et al., 2006 Amano et al., 2006
Zebrafish	zfLPXRFa-3*	Inhibition of gonadotropin release (i.p.)	Zhang et al., 2010

**Putative peptides*. i.c.v.*: intracerebroventricular administration;* i.v.*: intravenous administration;* i.p.*: intraperitoneal administration.*

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from cultured pituitaries in sheep[21] and cattle.[12] Peripheral administration of RFRP-3 also inhibits gonadotropin release in sheep[7] and cattle.[12] It was further shown that GnIH-R mRNA is expressed in gonadotrophs in the human pituitary.[31] Taken together, it is likely that GnIH and RFRP-3 directly act on the pituitary to inhibit gonadotropin secretion from the pituitary at least in these avian and mammalian species (Table 2). It is believed that RFRP-3 does not act directly on the pituitary in rodents, because there are relatively few or no RFRP-IR fibers in the ME.[15,28] However, there are also several studies indicating that RFRP-3 can directly inhibit gonadotropin release from the pituitary[17] (for reviews, see Refs 24, 25). More detailed studies, analyzing peptide release into the hypophysial portal blood, fiber projections by retrograde labeling and other techniques are needed to elucidate the hypophysiotropic action of GnIH and its mammalian ortholog, RFRP-3 (for reviews, see Refs 24, 25).

Immunohistochemical studies using light and confocal microscopy indicate that neuronal axon terminals containing GnIH and RFRP-3 are in probable contact with GnRH neurons in birds[2,29] and mammals.[15,28,30,31] GnIH-R also is expressed in GnRH neurons in starlings[29] and hamsters.[28] Together with the observation that central administration of GnIH or RFRP-3 inhibits the release of gonadotropin in white-crowned sparrows,[1] hamsters,[15,28] and rats,[11] GnIH and RFRP-3 may inhibit the secretion of gonadotropins by decreasing GnRH neuronal activity in addition to regulating the gonadotroph directly (Table 2, Fig. 4) (for reviews, see Refs 24, 25). Direct application of RFRP-3, a mammalian GnIH ortholog, to GnRH cells in cultured mouse brain slices decreases firing rate in a subpopulation of cells, further indicating a direct action of RFRP-3 on GnRH neurons. In addition, RFRP-3 inhibits firing of kisspeptin-activated vGluT2 (vesicular glutamate transporter 2)-GnRH neurons and of kisspeptin-insensitive GnRH neurons (for reviews, see Refs 24, 25). Later results confirmed a role for RFRP-3 by use of an antagonist, RF9, against GnIH-R. Central administration of RF9 to rats and mice led to marked increases in gonadotropin concentrations, providing a pronounced role of RFRP-3 as a key regulator of the reproductive axis in mammals.[19]

More recently, an inhibitory action of zebrafish GnIH ortholog (zfLPXRFa-3) was also reported in goldfish (for reviews, see Refs 24, 25) (Table 2). In general, GnIH and its orthologs seem to act similarly across vertebrate species to regulate reproduction (for reviews, see Refs 24, 25) (Table 2). However, another study found that goldfish GnIH orthologs (gfLPXRFa-1, -2, and -3) stimulate the release of gonadotropins and growth hormone (GH) but not prolactin (PRL) *in vitro* (Table 2). In a marked departure from the actions of GnIH and its mammalian ortholog, RFRP-3, frog GnIH orthologs (fGRP and fGRP-RP-2) stimulate the release of GH and/or PRL in amphibians both *in vitro* and *in vivo*[14,33] (Table 2). Therefore, further studies are necessary to clarify the generality of GnIH function on gonadotropin release in lower vertebrates.

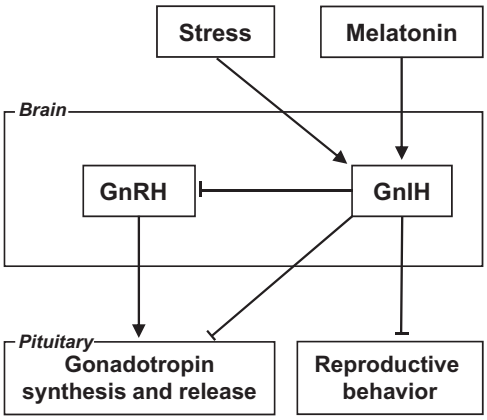

FIGURE 4 GnIH actions within the brain and pituitary

Furthermore, central administrations of GnIH and RFRP-3 inhibit reproductive behavior of female white-crowned sparrows[1] and male rats,[11] respectively. GnIH neurons are also in contact with the second form of GnRH (GnRH-II) neurons in birds,[29] and GnIH-R is also expressed in GnRH-II neurons.[29] Because GnRH-II can stimulate copulation solicitation in female birds, it is possible that GnIH inhibits reproductive behavior of female birds by inhibiting GnRH-II neurons.[1,2,29] On the other hand, central administrations of GnIH and RFRP-3 stimulate feeding behavior in chicken and rats,[11,17] respectively. There is also a recent report that central administration of RFRP-3 can stimulate adrenocorticotropic hormone and oxytocin release in rats (Table 2). It was shown that RFRP-ir fibers project to various neurons in the brain, such as dopamine and/or proopiomelanocortin neurons in the rat, sheep, and macaque,[30] indicating multiple functions of GnIH and its orthologs in the brain of birds and mammals (Fig. 4).

PATHOPHYSIOLOGICAL IMPLICATIONS

Identification of the regulatory mechanisms governing expression of GnIH and its orthologs is important in understanding the physiological role of the GnIH system. A nocturnal hormone, melatonin, seems to act directly on GnIH neurons through its receptor to induce expression and release of GnIH in quail.[5,27] Recently, a similar, but opposite, action of melatonin on the inhibition of expression of mammalian GnIH orthologs, RFRPs, was shown in hamsters, a photoperiodic mammal.[28] These results in quail and hamsters show that expression of GnIH and its orthologs are photoperiodically modulated via a melatonin-dependent process. Stress can also inhibit reproduction in birds and

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mammals. It was shown that immobilization stress induces expressions of GnIH and its orthologs in birds and rats.[13] GnIH and its orthologs may therefore be a mediator of stress-induced reproductive disruption in birds and mammals (for reviews, see Refs 24, 25) (Fig. 4).

The role of RFRP-3, a mammalian GnIH ortholog, in regulating ovulatory function was investigated in female hamsters.[9] The cellular activity of RFRP neurons was suppressed at the time of the luteinizing hormone (LH) surge, suggesting removal of negative feedback by RFRP-3 at this time.[9] The SCN, the master circadian clock triggering ovulation in rodents, projects to a large proportion of RFRP neurons, providing a mechanism for timing removal of negative drive on the GnRH system.[9] Activities of the SCN, GnRH, and GnIH system are coordinated with ovulation.[9]

The identification of human GnIH orthologs, RFRP-1 and -3, in the hypothalamic-hypophysial system[31] and the demonstration that human RFRP-3 has potent inhibitory actions on gonadotropin secretion in sheep[7,21] have opened up new vistas for understanding the regulation of reproduction. Many factors, such as stress, anorexia, diabetes, obesity, and photoperiod, inhibit gonadotropin secretion. The possible role of GnIH in mediating these effects has been implicated in animal models.[5,9,13,27] In view of its potent inhibition of gonadotropin secretion, GnIH has the potential of an alternative or adjunct therapeutic agent to inhibit endogenous levels of gonadotropins and steroid hormones. Thus, GnIH has therapeutic potential in the treatment of hormone-dependent diseases, such as precocious puberty, endometriosis, uterine fibroids, benign prostatic hyperplasia, and prostatic and breast cancers.

REFERENCES

1. Bentley GE, Jensen JP, Kaur GJ, Wacker DW, Tsutsui K, Wingfield JC. Rapid inhibition of female sexual behavior by gonadotropin-inhibitory hormone (GnIH). *Horm Behav* 2006;**49**:550–5.
2. Bentley GE, Perfito N, Ukena K, Tsutsui K, Wingfield JC. Gonadotropin-inhibitory peptide in song sparrows (*Melospiza melodia*) in different reproductive conditions, and in house sparrows (*Passer domesticus*) relative to chicken-gonadotropin-releasing hormone. *J Neuroendocrinol* 2003;**15**:794–802.
3. Bonini JA, Jones KA, Adham N, Forray C, Artymyshyn R, Durkin MM, et al. Identification and characterization of two G protein-coupled receptors for neuropeptide FF. *J Biol Chem* 2000;**275**:39324–31.
4. Chartrel N, Dujardin C, Leprince J, Desrues L, Tonon MC, Cellier E, et al. Isolation, characterization, and distribution of a novel neuropeptide, Rana RFamide (R-RFa), in the brain of the European green frog *Rana esculenta*. *J Comp Neurol* 2002;**448**:111–27.
5. Chowdhury VS, Yamamoto K, Ubuka T, Bentley GE, Hattori A, Tsutsui K. Melatonin stimulates the release of gonadotropin-inhibitory hormone by the avian hypothalamus. *Endocrinology* 2010;**151**:271–80.
6. Ciccone NA, Dunn IC, Boswell T, Tsutsui K, Ubuka T, Ukena K, et al. Gonadotrophin inhibitory hormone depresses gonadotrophin alpha and follicle-stimulating hormone beta subunit expression in the pituitary of the domestic chicken. *J Neuroendocrinol* 2004;**16**:999–1006.
7. Clarke IJ, Sari IP, Qi Y, Smith JT, Parkington HC, Ubuka T, et al. Potent action of RFamide-related peptide-3 on pituitary gonadotropes indicative of a hypophysiotropic role in the negative regulation of gonadotropin secretion. *Endocrinology* 2008;**149**:5811–21.
8. Dockray GJ, Reeve Jr JR, Shively J, Gayton RJ, Barnard CS. A novel active pentapeptide from chicken brain identified by antibodies to FMRFamide. *Nature* 1983;**305**:328–30.
9. Gibson EM, Humber SA, Jain S, Williams 3rd WP, Zhao S, Bentley GE, et al. Alterations in RFamide-related peptide expression are coordinated with the preovulatory luteinizing hormone surge. *Endocrinology* 2008;**149**:4958–69.
10. Hinuma S, Shintani Y, Fukusumi S, Iijima N, Matsumoto Y, Hosoya M, et al. New neuropeptides containing carboxy-terminal RFamide and their receptor in mammals. *Nat Cell Biol* 2000;**2**:703–8.
11. Johnson MA, Tsutsui K, Fraley GS. Rat RFamide-related peptide-3 stimulates GH secretion, inhibits LH secretion, and has variable effects on sex behavior in the adult male rat. *Horm Behav* 2007;**51**:171–80.
12. Kadokawa H, Shibata M, Tanaka Y, Kojima T, Matsumoto K, Oshima K, et al. Bovine C-terminal octapeptide of RFamide-related peptide-3 suppresses luteinizing hormone (LH) secretion from the pituitary as well as pulsatile LH secretion in bovines. *Domest Anim Endocrinol* 2009;**36**:219–24.
13. Kirby ED, Geraghty AC, Ubuka T, Bentley GE, Kaufer D. Stress increases putative gonadotropin inhibitory hormone and decreases luteinizing hormone in male rats. *Proc Natl Acad Sci U S A* 2009;**106**:11324–9.
14. Koda A, Ukena K, Teranishi H, Ohta S, Yamamoto K, Kikuyama S, et al. A novel amphibian hypothalamic neuropeptide: isolation, localization, and biological activity. *Endocrinology* 2002;**143**:411–9.
15. Kriegsfeld LJ, Mei DF, Bentley GE, Ubuka T, Mason AO, Inoue K, et al. Identification and characterization of a gonadotropin-inhibitory system in the brains of mammals. *Proc Natl Acad Sci U S A* 2006;**103**:2410–5.
16. Liu Q, Guan XM, Martin WJ, McDonald TP, Clements MK, Jiang Q, et al. Identification and characterization of novel mammalian neuropeptide FF-like peptides that attenuate morphine-induced antinociception. *J Biol Chem* 2001;**276**:36961–9.
17. Murakami M, Matsuzaki T, Iwasa T, Yasui T, Irahara M, Osugi T, et al. Hypophysiotropic role of RFamide-related peptide-3 in the inhibition of LH secretion in female rats. *J Endocrinol* 2008;**199**:105–12.
18. Osugi T, Ukena K, Bentley GE, O'Brien S, Moore IT, Wingfield JC, et al. Gonadotropin-inhibitory hormone in Gambel's white-crowned sparrow (*Zonotrichia leucophrys gambelii*): cDNA identification, transcript localization and functional effects in laboratory and field experiments. *J Endocrinol* 2004;**182**:33–42.
19. Pineda R, Garcia-Galiano D, Sanchez-Garrido MA, Romero M, Ruiz-Pino F, Aguilar E, et al. Characterization of the potent gonadotropin-releasing activity of RF9, a selective antagonist of RF-amide-related peptides and neuropeptide FF receptors: physiological and pharmacological implications. *Endocrinology* 2010;**151**:1902–13.
20. Price DA, Greenberg MJ. Structure of a molluscan cardioexcitatory neuropeptide. *Science* 1977;**197**:670–1.

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21. Sari IP, Rao A, Smith JT, Tilbrook AJ, Clarke IJ. Effect of RF-amide-related peptide-3 on luteinizing hormone and follicle-stimulating hormone synthesis and secretion in ovine pituitary gonadotropes. *Endocrinology* 2009;**150**:5549–56.
22. Satake H, Hisada M, Kawada T, Minakata H, Ukena K, Tsutsui K. Characterization of a cDNA encoding a novel avian hypothalamic neuropeptide exerting an inhibitory effect on gonadotropin release. *Biochem J* 2001;**354**:379–85.
23. Shimizu M, Bédécarrats GY. Activation of the chicken gonadotropin-inhibitory hormone receptor reduces gonadotropin releasing hormone receptor signaling. *Gen Comp Endocrinol* 2010;**167**:331–7.
24. Tsutsui K. A new key neurohormone controlling reproduction, gonadotropin-inhibitory hormone (GnIH): biosynthesis, mode of action and functional significance. *Prog Neurobiol* 2009;**88**:76–88.
25. Tsutsui K, Bentley GE, Bedecarrats G, Osugi T, Ubuka T, Kriegsfeld LJ. Gonadotropin-inhibitory hormone (GnIH) and its control of central and peripheral reproductive function. *Front Neuroendocrinol* 2010;**31**:284–95.
26. Tsutsui K, Saigoh E, Ukena K, Teranishi H, Fujisawa Y, Kikuchi M, et al. A novel avian hypothalamic peptide inhibiting gonadotropin release. *Biochem Biophys Res Commun* 2000;**275**:661–7.
27. Ubuka T, Bentley GE, Ukena K, Wingfield JC, Tsutsui K. Melatonin induces the expression of gonadotropin-inhibitory hormone in the avian brain. *Proc Natl Acad Sci U S A* 2005;**102**(8):3052–7.
28. Ubuka T, Inoue K, Fukuda Y, Mizuno T, Ukena K, Kriegsfeld LJ, et al. Identification, expression, and physiological functions of Siberian hamster gonadotropin-inhibitory hormone. *Endocrinology* 2012;**153**: 373–85.
29. Ubuka T, Kim S, Huang YC, Reid J, Jiang J, Osugi T, et al. Gonadotropin-inhibitory hormone neurons interact directly with gonadotropin-releasing hormone-I and -II neurons in European starling brain. *Endocrinology* 2008;**149**:268–78.
30. Ubuka T, Lai H, Kitani M, Suzuuchi A, Pham V, Cadigan PA, et al. Gonadotropin-inhibitory hormone identification, cDNA cloning, and distribution in rhesus macaque brain. *J Comp Neurol* 2009;**517**: 841–55.
31. Ubuka T, Morgan K, Pawson AJ, Osugi T, Chowdhury VS, Minakata H, et al. Identification of human GnIH homologs, RFRP-1 and RFRP-3, and the cognate receptor, GPR147 in the human hypothalamic pituitary axis. *PLoS One* 2009;**4**:e8400.
32. Ubuka T, Ukena K, Sharp PJ, Bentley GE, Tsutsui K. Gonadotropin-inhibitory hormone inhibits gonadal development and maintenance by decreasing gonadotropin synthesis and release in male quail. *Endocrinology* 2006;**147**:1187–94.
33. Ukena K, Koda A, Yamamoto K, Kobayashi T, Iwakoshi-Ukena E, Minakata H, et al. Novel neuropeptides related to frog growth hormone-releasing peptide: isolation, sequence, and functional analysis. *Endocrinology* 2003;**144**:3879–84.
34. Ukena K, Ubuka T, Tsutsui K. Distribution of a novel avian gonadotropin-inhibitory hormone in the quail brain. *Cell Tissue Res* 2003;**312**:73–9.
35. Yin H, Ukena K, Ubuka T, Tsutsui K. A novel G protein-coupled receptor for gonadotropin-inhibitory hormone in the Japanese quail (*Coturnix japonica*): identification, expression and binding activity. *J Endocrinol* 2005;**184**:257–66.

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Chapter 108

Hypocretins (Orexins)

Luis de Lecea and J. Gregor Sutcliffe

ABSTRACT

The hypocretins (Hcrts, also called the orexins) are two C-terminally amidated neuropeptides of related sequence. They are produced from a common precursor whose expression is restricted to a few thousand neurons of the rat dorsolateral hypothalamus. Two G-protein-coupled receptors for the hypocretins have been identified, and these have different distributions within the CNS and differential affinities for the two hypocretins. Hypocretin-containing neurons project to areas within the posterior hypothalamus that are implicated in feeding behaviors and hormone secretion. Hypocretin fibers also project to diverse targets in other brain regions and the spinal cord, including several areas implicated in cardiovascular function and sleep–wake regulation. The peptides are generally neuroexcitatory. Administration of the hypocretins stimulates food intake, affects blood pressure, hormone secretion, and locomotor activity, and increases wakefulness while suppressing rapid eye movement (REM) sleep. Inactivating mutations in the hypocretin receptor 2 gene in dogs result in narcolepsy. Mice whose hypocretin gene has been inactivated exhibit a narcolepsy-like phenotype. Most human patients with narcolepsy have greatly reduced levels of hypocretin peptides in their cerebral spinal fluid and no or barely detectable hypocretin neurons in their hypothalami, suggestive of autoimmune attack.

DISCOVERY

An open-system search for undiscovered hypothalamic regulatory peptides provided the first glimpse of the hypocretins. Gautvik and colleagues[9] used subtractive hybridization to identify messenger RNA species restricted to discrete nuclei within the rat hypothalamus. Among these was a species whose expression, as detected by *in situ* hybridization, was restricted to a few thousand neurons that were bilaterally distributed within the dorsolateral hypothalamus.[8]

A large collaborative study to identify endogenous ligands for orphan G-protein-coupled receptors discovered the peptides independently.[23] This group referred to the peptides as orexins because they stimulated acute food intake when administered to rats during the daytime. In this chapter, we will refer to the peptides by their first-used name, the hypocretins, but both names have been used extensively in the vast literature that has developed around the peptides.

STRUCTURE OF THE PRECURSOR mRNA/GENE

The mouse hypocretin gene, *HCRT*, is located on chromosome 11, and the human *HCRT* gene maps to Chromosome 17q21-q24. Genes that encode conserved preprohypocretins have been detected in pufferfish, zebrafish, frog, and chicken species, suggesting that the gene arose early in the chordate lineage. Sequence similarities with various members of the incretin family, especially secretin, suggest that the preprohypocretin gene was formed from the secretin gene by three genetic rearrangement;[2] first, a duplication of the secretin gene; second, deletions of the N-terminal portion of the 5′ duplicate and the C-terminal portion of the 3′ duplicate to yield a secretin with its N- and C- termini leap frogged (circularly permuted); and third, a further duplication of the permuted gene, followed by modifications, to form a secretin derivative that encoded two related hypocretin peptides.

The human preprohypocretin gene consists of two exons and one intron distributed over 1432 bp. The 143-bp first exon includes the 5′-untranslated region and a small part of the coding region that encodes the first seven residues of the secretory signal sequence. The second exon contains the remaining portion of the open reading frame and 3′-untranslated region. The 3.2 kilobase pairs of the 5′-upstream region from a cloned human preprohypocretin gene promoter is sufficient to direct the expression of the *E. coli* β-galactosidase (lacZ) gene in transgenic mice to neurons in the lateral hypothalamic area and adjacent regions.[24]

DISTRIBUTION OF THE mRNA

The rat preprohypocretin mRNA migrates in northern blots at approximately 700 nt in samples obtained from brain.[8] A few thousand neurons highly positive for Hcrt mRNA and immunoreactivity are located between the rat fornix and the mammillothalamic tracts.[8,21] These are first detected at embryonic day E18. Beginning at E20, hypocretin antisera detect a prominent network of axons that project from these cells to other neurons in the perifornical and posterior hypothalamus. Both mRNA and peptide expression dimin-

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00108-1
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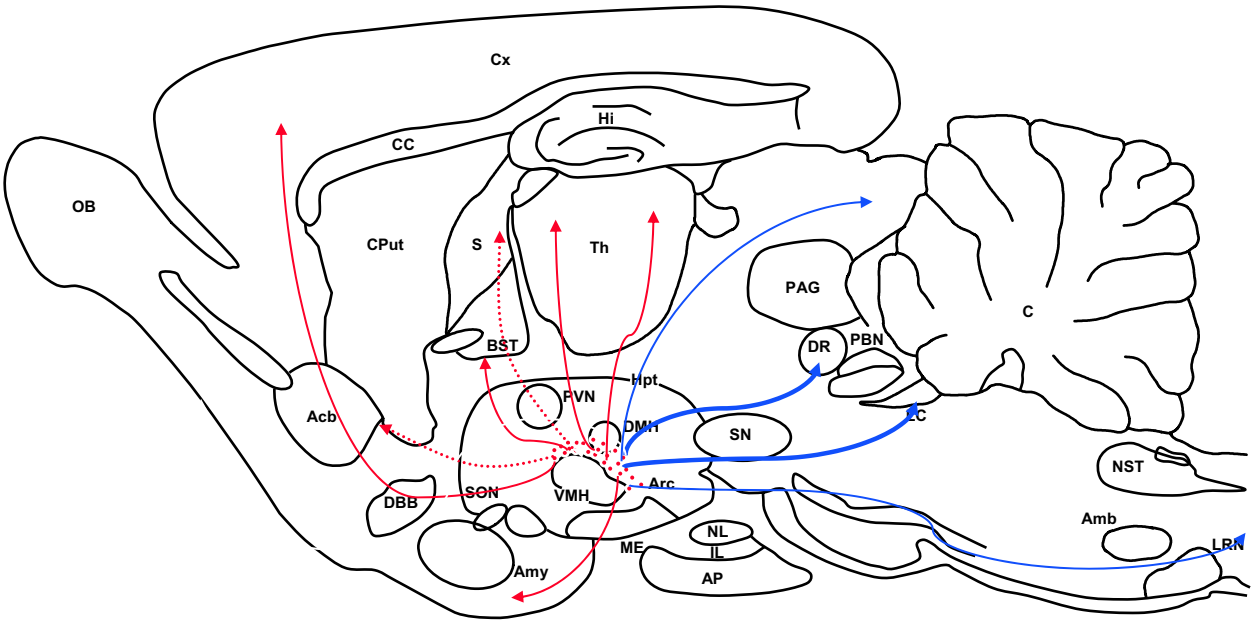

FIGURE 1 Schematic of the distribution of Hcrt projections originating in the lateral hypothalamus. A very dense intrahypothalamic network of Hcrt fibers can be detected by immunohistochemistry in the DMH, VMH, Arc, and PVN. Another set of fibers can be followed along the medial thalamus (Th). An ascending projection connects Hcrt with the ventral forebrain and amygdalar structures (Amy) and diffuse sparse innervation of neocortical areas (Cx). Descending efferent areas include the main monoaminergic nuclei such as the noradrenergic locus coeruleus (LC), serotonergic median and dorsal raphe (DR), and dopaminergic fibers passing through the ventral tegmental area. A direct monosynaptic connection to the spinal cord has also been described.DMH–dorsomedial hypothalamus; VMH–ventromedial hypothalamus; Arc–Arcuate nucleus; PVN–Paraventricular hypothalamic nucleus. See color plate 30.

ish after 1 year of age. The human lateral hypothalamus contains 50,000–80,000 hypocretin neurons. Hcrt neurons are 20–30 μm in diameter and are multipolar or fusiform in shape, with 2–4 primary dendrites bearing few spines. In addition to rats, mice, and humans, Hcrt neurons with a similar restricted hypothalamic distribution have been detected in monkey, hamster, cat, sheep, pig, chicken, various amphibians, and zebrafish. Glutamate, the excitatory amino acid transporter EAAT3, and the vesicular glutamate transporters VGLUT1 and VGLUT2 are expressed by hypocretin neurons; thus, hypocretin-containing neurons are likely to be glutamatergic. Other proteins detected in hypocretin neurons include dynorphin, $GABA_A$ receptor epsilon subunit, 5-HT_{1A} receptor, mu opioid receptor, pancreatic polypeptide Y4 receptor, adenosine A1 receptor, leptin receptor, transcription factor Stat-3, and the neuronal pentraxin Narp, implicated in clustering of ionotropic glutamate receptors.

There are several reports of Hcrt mRNA and Hcrt receptor mRNA expression in the periphery, including the enteric nervous system pancreas, kidney, stomach, and ileum. There are also several reports of Hcrt expression in the human anterior pituitary and adrenal gland. One study showed Hcrt-immunoreactivity in amacrine cells in the human retina.

PROCESSING OF THE PRECURSOR

Preprohypocretin encodes a 130-residue putative secretory protein with an apparent signal sequence and two additional phylogenically conserved sites for potential proteolytic maturation followed by modification of the carboxy-terminal glycines by peptidylglycine α-amidating monooxygenase.[8] These features suggested that the product of this hypothalamic mRNA served as a preprohormone for two C-terminally amidated, secreted peptides. One of these, hypocretin 2 (Hcrt2 or OxB), was, on the basis of the putative preprohormone amino-acid sequence, predicted to contain precisely 28 residues. The other, hypocretin 1 (Hcrt1 or OxA), had a defined predicted amidated C terminus but, because of uncertainties as to how the amino terminus might be proteolytically processed, an undefined N-terminal extent.[8] The C-terminal 19 residues of these two putative peptides shared 13 amino-acid identities, suggesting that the peptides had related structures and functions. This region of Hcrt2 contained a seven-amino-acid match with secretin. Antisera generated against synthetic peptides corresponding to regions of the deduced prohypocretin sequence and to bacterially expressed preprohypocretin have been generated.[8,21] The antisera are specific for the hypocretin-related peptides and have been used extensively to characterize the protein in both anatomical and ELISA-type studies.

The detection of the two hypocretin peptides within the brain allowed the exact structures of these endogenous peptides to be determined by mass spectroscopy.[23] The sequence of endogenous Hcrt2, RPGPPGLQGRLQRLLQAN GNHAAGILTM-amide, was the same as that predicted from the cDNA sequence. The N-terminus of Hcrt1 was found to correspond to a genetically encoded glutamine

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that was derivatized as pyroglutamate. Hcrt1 (33 residues: *EPLPDCCRQKTCSCRLYELLHGAGNHAAGILTL-amide) contains two intrachain disulfide bonds. Human Hcrt1 is identical to the rodent peptide, whereas human Hcrt2 differs from rodent Hcrt2 at two residues.[23]

The enzyme that cleaves preprohypocretin and yields the mature peptides has not been determined. Anatomical evidence suggests that prohormone convertase 1 colocalizes with in the lateral hypothalamus and may be involved in the processing of the precursor.

RECEPTORS AND SIGNALING CASCADES

Sakurai and collaborators[23] prepared transfected cell lines stably expressing each of 50 orphan G-protein-coupled receptors (GPCRs) and then measured calcium fluxes in these cell lines in response to fractions from tissue extracts. One of these transfected cell lines responded to a substance in a brain extract. Mass spectroscopy showed that this substance was a peptide whose sequence was later identified as that of endogenous Hcrt1. The initial orphan GPCR, Hcrtr1 (also referred to as OX1R), bound Hcrt1 with high affinity, but Hcrt2 with 100- to 1000-fold lower affinity. A related GPCR, Hcrtr2 (OX2R), sharing 64% identity with Hcrtr1, was identified by searching database entries with the Hcrtr1 sequence and had a high affinity for both Hcrt2 and Hcrt1.[23] These two receptors are highly conserved (95%) across species. Radioligand-binding studies and calcium flux measurements have shown Hcrt1 to have equal affinity for Hcrtr1 and Hcrtr2, whereas Hcrt2 has an approximately 10-fold greater affinity for Hcrtr2 than Hcrtr1 has.

The mRNAs that encode the two hypocretin receptors and the receptor proteins themselves, detected by immunohistochemistry, are both enriched in the brain and moderately abundant in the hypothalamus but have different distributions within the brain.[18] Hcrtr1 mRNA is prominent in the prefrontal cortex, hippocampus, paraventricular thalamic nucleus, ventromedial hypothalamus, dorsal raphe, locus coeruleus, laterodorsal tegmentum (at a higher density than that of Hcrtr2), pontine raphe, dorsal motor vagal complex, and spinal cord. Additional forebrain regions expressing moderate-to-high levels of Hcrtr1 mRNA are the tenia tecta, bed nucleus of the stria terminalis, diagonal band, and medial amygdala. Immunoreactive terminals have been detected in the LH. This receptor has also been detected in the retina.

Hcrtr2 mRNA (there are 2 alternative splice forms, each encoding proteins with slightly different structure and anatomical distribution, but no demonstrated functional differences) is detected in the neocortex, septum, hippocampus, thalamus, dorsal raphe, and various nuclei of the hypothalamus, including the tuberomammillary nucleus, paraventricular hypothalamic nucleus, and dorsomedial hypothalamus. Periaqueductal gray and midbrain reticular formation are also positive. Hcrtr2 mRNA is prominent in the septum, diagonal band, and central amygdala.

Both receptors are detected in the dorsal raphe, ventral tegmental area, and midline thalamic nuclei. In the cortex, Hcrtr1 mRNA is expressed primarily in layers II, III, and V, whereas Hcrtr2 mRNA is found at higher density in layers II and VI and more diffusely in other layers. In the hippocampus, Hcrtr1 is expressed mainly in the CA2 region and medial dentate gyrus, whereas Hcrtr2 was most abundant in CA3.

The distribution of Hcrt receptors is largely consistent with Hcrt axon innervation patterns (Fig. 1). The composite distribution of the two Hcrt receptors strongly resembles the distribution of the MCH receptor.[13] In the LC, amygdala, and other brainstem noradrenergic groups, MCH receptor mRNA distribution is similar to that of the Hcrtr1. In regions such as the septum, hypothalamus, and much of the brainstem, the distribution of MCH receptor mRNA resembles that of the Hcrtr2.[13]

The Hcrt receptors are not restricted to the central nervous system: in the periphery, they are widely expressed, especially in endocrine tissues. Hcrtr1 or Hcrtr2 have been detected in the pituitary, adrenal gland, testis, gastrointestinal tract, pancreas, and pineal gland.

Both Hcrt1 and Hcrt2 evoke increases in Ca^{2+}, as measured by fura-2 imaging, in about one-third of hypothalamic neurons, probably by opening a TRPC-type calcium channel.[16] Responses to Hcrt peptides are completely blocked by the protein kinase C-specific inhibitors and by phospholipase C (PLC) inhibitors,[28] suggesting that the hypocretins work through a family of GTP-binding proteins (G_q) that activate protein kinase C (PKC) and mobilization of intracellular calcium. G_q activated signaling cascades result in phosphorylation of Ca^{2+} channels, which can increase Ca^{2+} conductance and neuronal excitability. The nonamidated forms of the peptides are not electrophysiologically active. In the SN, hypocretin stimulation was sensitive to an inhibitor of protein kinase A, which mediates effects of cAMP, but insensitive to blockers of PKC, implicating a role of the G_s system.

INFORMATION ON ACTIVE AND/OR SOLUTION CONFORMATION

The structures of both Hcrt1 and Hcrt2 in solution have been determined by nuclear magnetic resonance.[14] Consistent with the hypothesis that the hypocretins and secretin are phylogenically related, portions of their three-dimensional solution structures are similar despite their leap-frogged primary sequence, consisting of two adjacent α-helices (6–7 and 9–14 amino acids long) separated by a short 2-3-amino-acid turn.[14] The longer helix corresponds to the region of identity between the two peptides.

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BIOLOGICAL ACTIONS WITHIN THE BRAIN

Administration of the hypocretin peptides to experimental animals stimulates food intake, affects autonomic and endocrine parameters, and increases arousal. These are discussed below in that order, but because the disease of the hypocretin system is the sleep disorder narcolepsy, the arousal aspects are the most important and will be discussed in a later section of the chapter and in the Sleep Peptides chapter in the last Section of this book.

Feeding and Metabolism

Sakurai and colleagues[23] found that intracerebroventricular (ICV) administration of either Hcrt1 or Hcrt2 increased short-term food consumption in rats. Further, rats that had been deprived of food for 48 h showed increased concentrations of preprohypocretin mRNA and peptides in the hypothalamus.[23] Feeding responses can be elicited by local administration of Hcrt1 to the paraventricular nucleus, the dorsomedial nucleus, the lateral hypothalamus, or the perifornical area. ICV administration of Hcrt2 also increases food intake in sheep and goldfish Many observations leave little doubt that the hypocretin system influences and is influenced by primary nutritional homeostasis circuits. For instance, Hcrt neurons are sensitive to glucose, leptin, and triglyceride concentration. Hcrt neurons are contacted by NPY-containing neurons in the arcuate nucleus,[12] and much of the food intake increase elicited by Hcrt1 seems to be mediated by NPY.

Other findings suggest that the hypocretins are not critical players in feeding activities, but rather play roles in increasing arousal and motivation levels so that feeding can take place. Continuous administration of Hcrt1 for seven days in rats does not significantly alter daily food intake, body weight, blood glucose, total cholesterol, or free fatty acid levels, suggesting that many of the effects of hypocretin may be limited to short-term, immediate stimulation of feeding behavior due to increased wakefulness. If hypocretin has a direct role in food intake, one would expect a lean phenotype in Hcrt-deficient mice, as has been described for melanin concentrating hormone. Hcrt knockout mice show very modest,[30] if significant, differences in food intake. Hypocretin-ataxin 3 mice, which are genetically depleted of Hcrt neurons and would be expected to be lean, show obesity and hypolocomotion,[11] and this effect seems to be dependent on diet and genetic background. During fasting, Hcrt1 accumulation in the cerebrospinal fluid (CSF) does not exceed concentrations normal for the waking period. Also, Hcrt ataxin-3 mice, unlike wild-type animals, do not show increase in locomotor activity after fasting.[31] All these data suggest that some of the food-uptake effect may result from arousal rather than direct feeding pressure.

Autonomic and Endocrine Effects

Hypocretin neurons receive input from brainstem areas that are associated with cardiovascular function, and project to the ventrolateral medulla, the locus coeruleus, the lateral paragigantocellular nucleus, the nucleus of the solitary tract, the pre-Botzinger region of the ventrolateral medulla, phrenic motoneurons, and other areas that have been implicated in the regulation of blood pressure, heart rate, and breathing. Projections to the arcuate nucleus also suggest a role in the regulation of hormone release. In the ovine hypothalamus, there are hypocretin terminals on the neurons that produce gonadotropin-releasing hormone, suggesting that hypocretin might particularly modulate reproductive endocrinology. In addition, projections to the raphe magnus and subcoeruleus suggested a role for hypocretins in the regulation of body temperature. The dense hypocretinergic projections to the ventrolateral preoptic area, tuberomammillary nucleus, pontine reticular formation, pedunculo pontine area, and locus coeruleus suggested involvement in states of arousal.[21] Very strong hypocretin-immunoreactive projections have been described in regions of the spinal cord that are related to modulation of pain,[27] and hypocretin-like immunoreactivity has also been detected in the intestinal epithelium.

In accordance with the wide distribution of hypocretin terminals, ICV administration of the hypocretins affects not only feeding but also several other functions. Both Hcrt1 and Hcrt2 elevate mean arterial blood pressure, heart rate (both suppressed by the hypocretin receptor 1 antagonist SB334867), and oxygen consumption.[25] Hcrt1 increases body temperature independent of peripheral thermogenesis, at least in part via clozapine-sensitive pathways in the cerebral cortex. Hcrt1 also increases water consumption and stimulates gastric acid secretion in the gut and increases locomotor activity and wakefulness, while decreasing slow wave and depressing REM sleep.[3]

The peptides also stimulate the secretion of luteinizing hormone in ovarectomized and proestrus female rats (suppressed by central administration of SB334867) and hypothalamic explants of male pituitaries. Consistent with these findings, in humans, LH concentration in serum and pulsatile LH secretion are lower in male narcolepsy patients (who lack hypocretin function; see below) than in unaffected controls. Hcrt concentrations are elevated during proestrus in cycling female rats but not in noncycling middle-aged rats and in pregnant rats, and c-*fos* is increased in Hcrt neurons in lactating mice. In male rats, Hcrt1 increased basal testosterone secretion. Hcrt1 decreases the concentrations of circulating growth hormone and prolactin, while increasing corticosterone, ACTH, and insulin levels. Hypoglycemia-induced elevation of pancreatic nerve firing is antagonized by SB334867 microinjection in the dorsal motor nucleus of

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the vagus, indicative of a central circuit in this pathway. Hcrt2, but not Hcrt1, increases circulating thyroid-stimulating hormone and has direct effects on the pituitary, adrenal, and pineal glands.

Both peptides depolarize neurons containing corticotropin releasing-hormone in the paraventricular hypothalamic nucleus and increase c-*fos,* and arginine vasopressin mRNA concentrations in the paraventricular, thus exerting clear effects on the hypothalamo–pituitary–adrenal axis and stress-related physiology; various stress paradigms increase c-*fos* expression by Hcrt neurons. Hcrt2 is directly excitatory on superficial dorsal horn neurons of the spinal cord[27] and exhibits an analgesic effect in models of pain.

Motivation and Addiction

The Hcrt system has recently been shown to be a key component in the brain reward circuitry.[4] Rats can be trained to turn a wheel to deliver electrical current to the lateral hypothalamus (LH): LH self-stimulation (LHSS). LHSS thresholds measure brain reward systems; lower thresholds represent increased reward. Most drugs of abuse lower LHSS thresholds. LHSS is believed to be rewarding in part because it activates cholinergic neurons in the laterodorsal tegmentum that consequently activate dopaminergic neurons in the ventral tegmental area. Hypocretins excite brainstem cholinergic neurons both directly and indirectly acting synergistically with glutamatergic afferents[5] to drive dopamine release in the nucleus accumbens by exciting dopaminergic neurons in the ventral tegmental area. Thus, the Hcrt system acts as a modulator of brain reward function. Interestingly in this regard, naltrexone injected into the accumbens suppresses the Hcrt1-induced acute feeding response, suggesting that the response is mediated by opioidergic pathways involved in motivation.

Pain and Anesthesia

In models of pain elicited by noxious stimuli or chronic constriction, Hcrts administered intrathecally or microinjected into the posterior hypothalamus decreased pain parameters. Hypocretin knockout mice (see below) exhibited greater hyperalgesia and less stress-induced analgesia than did wild-type mice. Hcrt1 decreases barbiturate anesthesia time in rats by 15–40%, an action reversed by SB-334867. *In vitro*, barbiturates inhibit Hcrt-induced norepinephrine release, although they do not interact directly with Hcrt receptors. In isoflorane-anesthetized animals, Hcrt1 elicits arousal without cardiovascular activation, in contrast to its effect on awake animals. Pain and stress activate Hcrt neurons, which respond by inhibiting pain pathways. Some of these effects may be due to the interaction with the nociceptin system.

PATHOPHYSIOLOGICAL IMPLICATIONS

Narcolepsy affects around 1 in 2000 adults, appears between the ages of 15–30 years, and exhibits four characteristic symptoms: (i) excessive daytime sleepiness with irresistible sleep attacks during the day; (ii) cataplexy (brief episodes of muscle weakness or paralysis precipitated by strong emotions such as laughter or surprise); (iii) sleep paralysis, a symptom considered to be an abnormal episode of REM sleep atonia, in which the patient suddenly finds himself unable to move for a few minutes, most often on falling asleep or waking up; and (iv) hypnagogic hallucinations, or dream-like images that occur at sleep onset. These latter symptoms have been proposed as pathological equivalents of REM sleep. The disorder is considered to represent a disturbed distribution of sleep states rather than an excessive amount of sleep.

Hypocretin neurons seem to control the boundaries between vigilance states through direct and indirect mechanisms. Hcrt neuronal activity at frequencies >5 Hz is sufficient to elicit sleep-to-wake transitions,[1] and acute optogenetic inhibition of Hcrt correlates with behavioral arrests. However, stimulation of Hcrt neurons does not seem to affect sleep or wake maintenance directly. The neuronal targets of this state boundary control are still unknown, but they are probably related to brain structures that express Hcrt 2 receptors. Because optogenetic high frequency stimulation of locus coeruleus neurons leads to behavioral arrests that resemble narcolepsy attacks,[6] it is tempting to speculate that Hcrt r2-expressing cells that modulate the excitability of noradrenergic neurons in the LC are critical for the onset of cataplexy.

Canine Narcolepsy

Both sporadic and heritable narcolepsies are observed in dogs, and the symptoms resemble those exhibited by human narcoleptics. The first link between the hypocretins and narcolepsy came from genetic linkage studies in a colony of Doberman Pinschers in which narcolepsy was inherited as an autosomal recessive, fully penetrant phenotype. Fine mapping and cloning of the defective canine narcolepsy gene, showed it to be the gene that encodes the hypocretin receptor, Hcrtr2.[17] The mutation in the Doberman lineage is an insertion of a short interspersed repeat (SINE element) into the third intron of *HCRTR2* that causes aberrant splicing of the Hcrtr2 mRNA (exon 4 is skipped) and results in a truncated receptor protein. In cells that have been transfected with the mutant gene, the truncated Hcrtr2 protein does not properly localize to the membrane and, therefore, does not bind its ligands. Analysis of a colony of narcoleptic Labradors revealed that their *HCRTR2* gene contained a distinct mutation that resulted in the skipping of exon 6, also leading to a truncated receptor protein. A third family of narcoleptic Dachshunds carries a

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point mutation in *HCRTR2* that results in a receptor protein that reaches the membrane but cannot bind the hypocretins.

Mouse Knockout Mutants

Continuous recording of the behavior of knockout mice in which the hypocretin gene was inactivated by homologous recombination in embryonic stem cells revealed periods of ataxia, which were especially frequent during the dark period.[7] EEG recordings showed that these episodes were not related to epilepsy, and that the mice suffered from cataplectic attacks, a hallmark of narcolepsy. In addition, the mutant mice spent almost twice as much time in REM sleep during the dark period as did their wild-type littermates, and their EEGs showed episodes of direct transition from wakefulness to REM sleep, another event that is unique to narcolepsy. Mice with an inactivated *HCRTR2* gene have a milder narcoleptic phenotype than did the *HCRT* knockouts; *HCRTR1* knockouts exhibit only a sleep fragmentation phenotype, whereas double *HCRTR1* and *HCRTR2* mutants recapitulate the full *HCRT* knockout phenotype,[29] suggesting that signaling through both receptors contributes to normal arousal, although the role of *HCRTR2* is greater than that of *HCRTR1*.[22]

Human Narcolepsy

Nishino and colleagues[19] studied hypocretin concentrations in the CSF of normal controls and patients with narcolepsy by radioimmunoassay. In control CSF, hypocretin concentrations were highly clustered, suggesting that tight regulation of the substance is important. However, of nine patients with narcolepsy, only one had a hypocretin concentration within the normal range. One patient had a greatly elevated concentration, whereas seven patients had no detectable circulating hypocretin. In an expanded study, hypocretin was undetectable in 37 of 42 narcoleptics and in a few cases of Guillain-Barré syndrome. CSF hypocretin was in the normal range for most neurological diseases, but was low, although detectable, in some cases of CNS infections, brain trauma, and brain tumors.

Peyron,[20] Thannikal,[26] and their groups of collaborators found that, in the brains of narcolepsy patients, they could detect few or no hypocretin-producing neurons. Whether the hypocretin neurons are selectively depleted, as is most likely, or only no longer expressing hypocretin is not yet known, although one report showed some indications of gliosis.[26] The codistributed neurons expressing melanin-concentrating hormone (MCH) were unaffected. Further, a single patient with a non-HLA-linked narcolepsy carries a mutation within the hypocretin gene itself. The mutation results in a dominant negative amino-acid substitution in the secretion signal sequence that sequesters both the mutant and heterozygous wild-type hypocretin nonproductively to the smooth endoplasmic reticulum.[20]

These findings leave no doubt as to the central role of the hypocretin system in this sleep disorder. Because most cases are sporadic, mutations in the hypocretin gene or those for its receptors can account for no more than a small subset of the human narcolepsies. The HLA association, loss of neurons with signs of gliosis, and age of disease onset are consistent with autoimmune destruction of the hypocretin neurons accounting for the majority of narcolepsy,[17] although a non-immune-mediated degenerative process has not been ruled out. The precipitating factor for the development of the autoimmunity is also unknown, but recent studies have suggested a link between certain cases of narcolepsy with lower expression of purinergic receptors in CD8+ cells and with the T cell receptor alpha locus.[10,15] The narcolepsies as a group are probably a collection of disorders that are caused by defects in the production or secretion of the hypocretins or in their signaling, and these could have numerous genetic, traumatic, viral, and/or autoimmune causes.

REFERENCES

1. Adamantidis A, Zhang F, Aravanis A, Deisseroth K, de Lecea L. Neural substrates of awakening probed with optogenetic control of hypocretin neurons. *Nature* 2007;**450**:420–4.
2. Alvarez CE, Sutcliffe JG. Hypocretin is an early member of the incretin gene family. *Neurosci Lett* 2002;**324**:169–72.
3. Bourgin P, Huitron-Resendiz S, Spier AD, Fabre V, Morte B, Criado JR, et al. Hypocretin-1 modulates rapid eye movement sleep through activation of locus coeruleus neurons. *J Neurosci* 2000;**20**:7760–5.
4. Boutrel B, Cannella N, de Lecea L. The role of hypocretin in driving arousal and goal-oriented behaviors. *Brain Res* 2010;**1314**:103–11.
5. Burlet S, Tyler CJ, Leonard CS. Direct and indirect excitation of laterodorsal tegmental neurons by Hypocretin/Orexin peptides: implications for wakefulness and narcolepsy. *J Neurosci* 2002;**22**:2862–72.
6. Carter ME, Yizhar O, Chikahisa S, Nguyen H, Adamantidis A, Nishino S, et al. Tuning arousal with optogenetic modulation of locus coeruleus neurons, *Nat Neurosci* 2010;**13**:1526–33.
7. Chemelli RM, Willie JT, Sinton CM, Elmquist JK, Scammell T, Lee C, et al. Narcolepsy in orexin knockout mice: molecular genetics of sleep regulation. *Cell* 1999;**98**:437–51.
8. de Lecea L, Kilduff TS, Peyron C, Gao X, Foye PE, Danielson PE, et al. The hypocretins: hypothalamus-specific peptides with neuroexcitatory activity. *Proc Natl Acad Sci U S A* 1998;**95**:322–7.
9. Gautvik KM, de Lecea L, Gautvik VT, Danielson PE, Tranque P, Dopazo A, et al. Overview of the most prevalent hypothalamus-specific mRNAs, as identified by directional tag PCR subtraction. *Proc Natl Acad Sci U S A* 1996;**93**:8733–8.
10. Hallmayer J, Faraco J, Lin L, Hesselson S, Winkelmann J, Kawashima M, et al. Narcolepsy is strongly associated with the T-cell receptor alpha locus. *Nat Genet* 2009;**41**:708–11.
11. Hara J, Beuckmann CT, Nambu T, Willie JT, Chemelli RM, Sinton CM, et al. Genetic ablation of orexin neurons in mice results in narcolepsy, hypophagia, and obesity. *Neuron* 2001;**30**:345–54.

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12. Horvath TL. The hardship of obesity: a soft-wired hypothalamus. *Nat Neurosci* 2005;**8**:561–5.
13. Kilduff TS, de Lecea L. Mapping of the mRNAs for the hypocretin/orexin and melanin- concentrating hormone receptors: networks of overlapping peptide systems. *J Comp Neurol* 2001;**435**:1–5.
14. Kim HY, Hong E, Kim JI, Lee W. Solution structure of human orexin-A: regulator of appetite and wakefulness. *J Biochem Mol Biol* 2004;**37**:565–73.
15. Kornum BR, Kawashima M, Faraco J, Lin L, Rico TJ, Hesselson S, et al. Common variants in P2RY11 are associated with narcolepsy. *Nat Genet* 2011;**43**:66–71.
16. Kukkonen JP, Akerman KE. Orexin receptors couple to Ca^{2+} channels different from store-operated Ca^{2+} channels. *Neuroreport* 2001;**12**:2017–20.
17. Lin L, Faraco J, Li R, Kadotani H, Rogers W, Lin X, et al. The sleep disorder canine narcolepsy is caused by a mutation in the hypocretin (orexin) receptor 2 gene. *Cell* 1999;**98**:365–76.
18. Marcus JN, Aschkenasi CJ, Lee CE, Chemelli RM, Saper CB, Yanagisawa M, et al. Differential expression of orexin receptors 1 and 2 in the rat brain. *J Comp Neurol* 2001;**435**:6–25.
19. Nishino S, Ripley B, Overeem S, Lammers GJ, Mignot E. Hypocretin (orexin) deficiency in human narcolepsy. *Lancet* 2000;**355**:39–40.
20. Peyron C, Faraco J, Rogers W, Ripley B, Overeem S, Charnay Y, et al. A mutation in a case of early onset narcolepsy and a generalized absence of hypocretin peptides in human narcoleptic brains. *Nat Med* 2000;**6**:991–7.
21. Peyron C, Tighe DK, van den Pol AN, de Lecea L, Heller HC, Sutcliffe JG, et al. Neurons containing hypocretin (orexin) project to multiple neuronal systems. *J Neurosci* 1998;**18**:9996–10015.
22. Sakurai T. The neural circuit of orexin (hypocretin): maintaining sleep and wakefulness. *Nat Rev Neurosci* 2007;**8**:171–81.
23. Sakurai T, Amemiya A, Ishii M, Matsuzaki I, Chemelli RM, Tanaka H, et al. Orexins and orexin receptors: a family of hypothalamic neuropeptides and G protein-coupled receptors that regulate feeding behavior. *Cell* 1998;**92**:573–85.
24. Sakurai T, Moriguchi T, Furuya K, Kajiwara N, Nakamura T, Yanagisawa M, et al. Structure and function of human prepro-orexin gene. *J Biol Chem* 1999;**274**:17771–6.
25. Samson WK, Gosnell B, Chang JK, Resch ZT, Murphy TC. Cardiovascular regulatory actions of the hypocretins in brain. *Brain Res* 1999;**831**:248–53.
26. Thannickal TC, Moore RY, Nienhuis R, Ramanathan L, Gulyani S, Aldrich M, et al. Reduced number of hypocretin neurons in human narcolepsy. *Neuron* 2000;**27**:469–74.
27. van den Pol AN. Hypothalamic hypocretin (orexin): robust innervation of the spinal cord. *J Neurosci* 1999;**19**:3171–82.
28. van den Pol AN, Gao XB, Obrietan K, Kilduff TS, Belousov AB. Presynaptic and postsynaptic actions and modulation of neuroendocrine neurons by a new hypothalamic peptide, hypocretin/orexin. *J Neurosci* 1998;**18**:7962–71.
29. Willie JT, Chemelli RM, Sinton CM, Tokita S, Williams SC, Kisanuki YY, et al. Distinct narcolepsy syndromes in Orexin receptor-2 and Orexin null mice: molecular genetic dissection of Non-REM and REM sleep regulatory processes. *Neuron* 2003;**38**:715–30.
30. Willie JT, Chemelli RM, Sinton CM, Yanagisawa M. To eat or to sleep? Orexin in the regulation of feeding and wakefulness. *Annu Rev Neurosci* 2001;**24**:429–58.
31. Yamanaka A, Beuckmann CT, Willie JT, Hara J, Tsujino N, Mieda M, et al. Hypothalamic orexin neurons regulate arousal according to energy balance in mice. *Neuron* 2003;**38**:701–13.

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Chapter 109

Kisspeptins

Manuel Tena-Sempere

ABSTRACT

KISS1 was originally identified as a metastasis suppressor gene that encodes a number of related peptides including kisspeptin-54 (formerly, metastin) and shorter active fragments which, acting through the G protein-coupled receptor GPR54, seem capable of inhibiting tumor spread. The neuroendocrine dimension of the Kiss1/GPR54 system was disclosed by the observation, reported in late 2003, that loss-of-function mutations of the GPR54 gene are associated with the absence of puberty and hypogonadotropic hypogonadism. This seminal finding underscored a pivotal role of the Kiss1 system in the control of reproduction and boosted intense research efforts in an attempt to characterize the neuroendocrine physiology of kisspeptins. Indeed, in the past years, kisspeptins have emerged as major physiological regulators of GnRH neurons and, hence, of the reproductive axis. The major features of the Kiss1 system are comprehensively described in this chapter.

DISCOVERY OF THE KISS1 SYSTEM

Identification of *KISS1*, the gene encoding kisspeptins, occurred in late 1996 in the context of tumor biology. By the use of subtractive hybridization in melanoma cell lines with different metastatic capacity, the *KISS1* gene was originally reported to be selectively overexpressed in metastasis-suppressed tumor cells.[22,25] This original finding was expanded in the following years by assessing *KISS1* gene expression and/or the biological actions of kisspeptins in additional tumor specimens and cell lines. Those results led to the conclusion that *KISS1* has potent antimetastasis activity in several tumors, such as papillary thyroid carcinoma, breast carcinoma, melanoma, and pancreatic cancer cells, whereas the loss of *KISS1* gene expression was cataloged as a bad-prognosis factor for tumor progression and metastasis in esophageal squamous cell carcinoma, gastric carcinoma, and bladder cancer, among others.[25]

Besides its role in tumor progression, fragmentary information already showed several years ago additional roles for *Kiss1*-derived peptides in the endocrine/paracrine control of specific biological functions. Notably, the expression of the *KISS1* gene in normal human tissues is maximum in placenta, which probably accounts for the dramatic increase in serum kisspeptin levels during pregnancy, a phenomenon of as yet unknown endocrine relevance.[25,31] In terms of paracrine regulation, placental *Kiss1*-derived peptides have been suggested to play a role in the physiological regulation of trophoblast invasion.[25] In addition, some preliminary evidence pointed out already in 2001 that Kiss1 peptides might participate in the regulation of specific neuroendocrine systems, such as oxytocin release.[13] Indeed, at that time, expression of *Kiss1* and/or the putative kisspeptin receptor (see below) was found in a variety of normal tissues, including not only placenta but also different brain areas, spinal cord, pituitary, pancreas, testis, small intestine, and human plasma,[13,15,23] which strongly suggested additional, as yet unknown, physiological roles of the Kiss1 system.

However, it was not until late 2003 when the neuroendocrine facet of such system was fully recognized by the striking observation that some forms of isolated hypogonadotropic hypogonadism (iHH) are associated with inactivating mutations of the gene encoding the putative receptor for kisspeptins (GPR54).[7,26] Such a hypogonadal phenotype was reproduced in mouse models where the *Gpr54* or *Kiss1* genes were knocked out.[22,25] These clinical and experimental findings boosted an extraordinary interest among reproductive physiologists, who endeavor to characterize the physiological roles of the Kiss1 system in the neuroendocrine control of the gonadotropic axis.

As an introductory note, in this chapter, we will focus on the mammalian Kiss1 system, where the terms *KISS1* and *Kiss1* will be used to name the primate and nonprimate genes, respectively, whereas their peptide products will be termed kisspeptins, with a numeric extension (if needed) indicating the number of amino acid residues, following previous literature.[10] In addition, the kisspeptin receptor will be named here GPR54 (or Gpr54, if specific mention to studies in nonprimate species is made), to keep the homogeneity with previous and current literature. Yet, we acknowledge that the use of the term Kiss1R is fully valid as well.[10] Finally, because most of the experimental results reviewed herein have been obtained in nonprimate mammalian species, the

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00109-3
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term Kiss1 will be used for global reference to the ligand–receptor system.

Structure of the *Kiss1* Gene

In the human genome, the *KISS1* gene has been mapped to chromosome 1q32.[34] The original report suggested that this gene comprises four exons and three introns, with the first and second exons being nontranslated and the last two exons encoding the 145 amino acid precursor peptide. This genomic organization was later challenged by an alternative proposal, in which the human *KISS1* gene contains 3 exons: the first exon consists of 111 bases and it is not translated, whereas exon 2 contains 141 nucleotides, with the start codon at position 39, and exon 3 consists of 322 translated nucleotides, followed by the translational stop codon and the polyadenylation sequence. The deduced sequence of the precursor KISS1 protein in humans consists of 145 amino acids.

The mouse *Kiss1* gene has been mapped to chromosome 1, whereas in rat, the gene is located in chromosome 13q13. The cDNA sequences and the genomic organization of the rat and mouse *Kiss1* genes seem to be highly homologous. Thus, the mouse *Kiss1* gene has been proposed to contain only two translated exons, which correspond to the last two exons of the human gene.[33] These two exons are highly homologous to the corresponding exons 2 and 3 of the human sequence, with analogous splicing sites and a similar spacing intron of approximately 2 kb. In addition, mouse and rat cDNA sequences share 90% identity. Of note, comparison of human and mouse genomic sequences at www.ensembl.org suggest a structure of the mouse gene that is strikingly similar to that proposed for the human, with three exons: a noncoding exon 1 of 102 bases, an exon 2 with a start codon at base 41 and a final exon 3 harboring the stop codon and polyadenylation site. Likewise, although in the rat gene only two exons, corresponding to human exons 2 and 3, have been annotated, a blast search with the human exon 1 sequence and its corresponding region in the mouse detects a highly similar sequence in the rat genome, located at approximately 7500 bp upstream from the corresponding *Kiss1* gene, which conserves all putative splicing signals. Assuming that such a sequence actually corresponds to the first, noncoding exon of rat *Kiss1*, the structure of human, mouse, and rat genes would be essentially identical. Moreover, in human and rodent species, there is a TATA-box-like sequence located 15–18 bp upstream from the predicted 5′-end of the putatively common exon 1.

Despite the fact that this chapter will mostly focus on the mammalian Kiss1 system, it is stressed here that the analysis of the genomic organization of *Kiss1* in different phyla has revealed the presence of a second form of *Kiss* gene, termed *Kiss2*, which exists in the genome of fish and amphibians, as well as in nonplacental mammals.[1,8,33] In fact, several fish species do have two different *Kiss* genes in their genomes, whereas in some of them, only the *Kiss2* gene has been retained. Moreover, Kiss2-derived peptides have been suggested to play a more prominent role than Kiss1 peptides in the control of the reproductive axis in some fish species.[8] These observations illustrate the complexity and functional diversity of the molecular evolution of kisspeptins in different animal groups.

Processing of the Kiss1 Precursor: Kisspeptins

In the human, the *KISS1* gene encodes a 145 amino acid precursor, which contains a putative 19 amino acid signal sequence, two potential dibasic cleavage sites (at amino acids 57 and 67), and one putative site for terminal cleavage and amidation (at amino acids 121–124).[13,22,25] These structural features predicted the generation of secretory product(s). Indeed, through several proteolytic steps, the full-length KISS1 precursor has been shown to give rise to a number of structurally related, secreted peptides, globally termed kisspeptins.[13,22,25] Among these, the major product of the human *KISS1* gene appears to be a 54-amino acid peptide, largely secreted by the placenta, initially termed metastin or kisspeptin-54.[13,22,23,25] In addition, other derivatives of the KISS1 precursor, such as kisspeptin-14, kisspeptin-13, and kisspeptin-10, have been also identified.[13] Yet, there is an ongoing debate on whether these smaller peptide products are endogenously generated by differential proteolytic processing of the kisspeptin precursor or arise from unspecific degradation of the major product, kisspeptin-54. Notwithstanding, it has been conclusively shown that the shorter 10-amino acid fragment at the C-terminus (kisspeptin-10) retains maximal activity at the receptor level. All kisspeptins share the C-terminal region of the kisspeptin-54 molecule, and harbor an Arg-Phe-NH_2 motif distinctive of the RF-amide peptide family.[13,22,25] Other prominent members of this family have been recently involved in relevant neuroendocrine functions, as reviewed elsewhere in this book (e.g. chapters on ***GnIH/RFRPs*** and ***PrRP***). In rodent species, the overall structure of the Kiss1 precursor and derived kisspeptins is roughly similar. Nonetheless, rat and mouse kisspeptin contains 52 amino acids, where the terminal RF-amide signature is substituted by an Arg-Tyr-NH_2 motif.[22,25]

Distribution of *Kiss1* mRNA/Protein within the Brain

Upon identification of kisspeptins as endogenous ligands for the previously orphan GPR54, expression analyses were conducted to characterize the pattern of *Kiss1* (and *GPR54*) gene expression in normal tissues. Although not totally overlapping, these screening analyses show prominent expression of the *KISS1* gene in human placenta and,

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at lower levels, in the testis and small intestine. Expression of *KISS1* mRNA is also detected in the human brain, with a scattered distribution throughout the central nervous system, including the basal ganglia and the hypothalamus.[15,23] Similar initial screening was conducted in rodent tissues. Thus, in the rat, the highest levels of *Kiss1* mRNA are in the colon and cecum, with moderate to low expression levels in different peripheral tissues including placenta.[32] In addition, *Kiss1* gene expression occurs is in rat brain and specifically in the hypothalamic area.[16,32] These expression data in the CNS were further extended by in situ hybridization assays that revealed *Kiss1*-expressing neurons at different levels along the rostral–caudal extent of the hypothalamus in the mouse, including the arcuate nucleus (Arc) and the anteroventral periventricular nucleus (AVPV).[29] In addition, *Kiss1* mRNA expressing cells are also detected in the anterodorsal preoptic nucleus, the medial amygdala and the bed nucleus of the stria terminalis, among other areas of the rodent CNS (Fig. 1)[22,25] yet, the physiological relevance of such extrahypothalamic expression remains largely unknown.[25] The expression of *Kiss1* mRNA has been also detected in the Arc/infundibular region of the hypothalamus of primates and humans, and in the Arc in the sheep, just to mention several relevant species.[25,27] Studies in a transgenic mouse line genetically engineered to express the markers green fluorescent protein (GFP) or β-galactosidase selectively in Kiss1 neurons have replicated previous results of *Kiss1* mRNA distribution in the mouse brain, with prominent expression in the Arc and preoptic area, and in different extrahypothalamic sites.[6]

Initial immunohistochemical analyses revealed a wide pattern of distribution of kisspeptin immunoreactivity (Kp-IR) within the CNS, with detectable Kp-IR cell bodies and fibers in different brain areas.[2] Yet, those studies were biased by the use of a polyclonal antiserum against kisspeptin-10, which was later found to crossreact with other peptides of the RF-amide superfamily.[14] However, subsequent studies, conducted mainly in rodent and human species, using more specific antisera have allowed confirmation of the presence of a prominent population of Kp-IR neurons in the Arc/infundibular region (Fig. 1).[14] In addition, in rodents (mainly in female mice), the existence of a subset

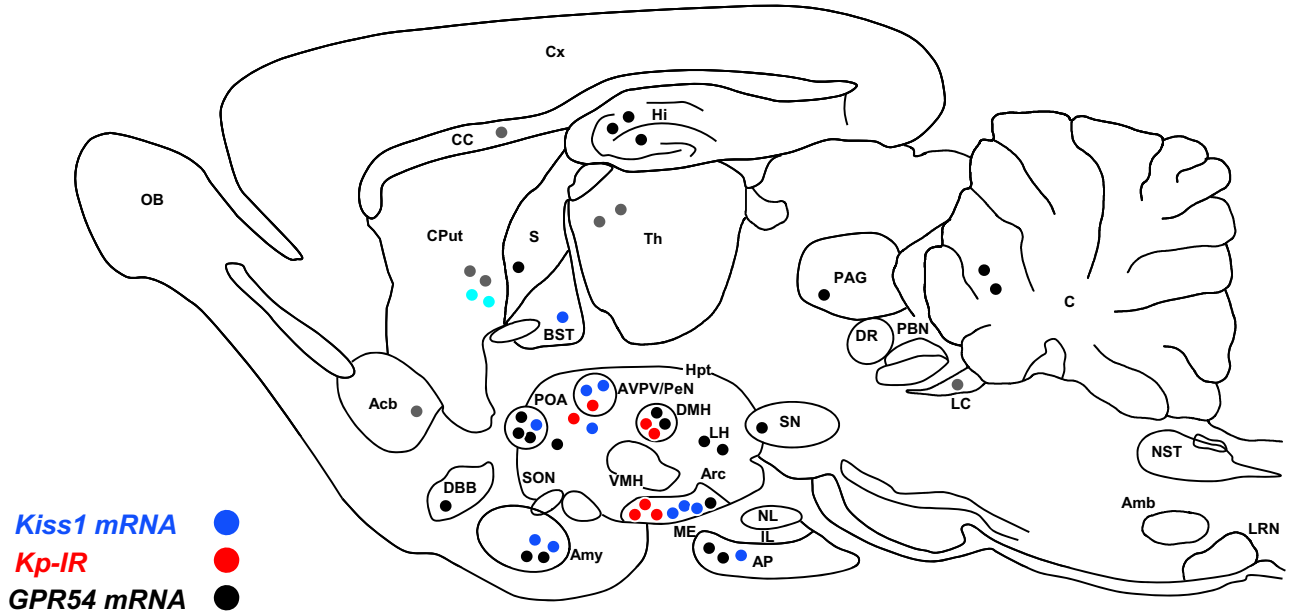

FIGURE 1 Anatomical distribution of *Kiss1* mRNA (*blue dots*), kisspeptin immunoreactivity –Kp-IR- (*red dots*) and *GPR54* mRNA (*black dots*) in the different brain areas of rodent brain. In the mouse and rat, *Kiss1* mRNA expression has been detected in the hypothalamus, mostly in the arcuate nucleus (Arc) as well as in the anteroventral periventricular area (AVPV) and the adjacent periventricular nucleus (PeN). In addition, the presence of *Kiss1* mRNA has been reported in the preoptic area (POA), amygdala (Amy) and bed nucleus of stria terminalis (BST). In addition, the expression of *KISS1* gene has been detected in the basal ganglia in humans (as detected by RT-PCR; denoted by light blue dots). In the rat brain, Kp-IR has been identified mainly in neurons in the Arc and the AVPV/PeN (the latter, especially in the female), with additional signals being detected in the dorsomedial hypothalamic nucleus (DMH), among other CNS areas. In addition, multiple Kp-IR fibers, originating from the Arc and AVPV/PeN populations and projecting to various hypothalamic and extrahypothalamic areas, have been mapped; yet, for the sake of simplicity, these are not specifically depicted in the scheme. Finally, in the rat, *Gpr54* mRNA has been detected in different forebrain areas, including the diagonal band of Broca (DBB), septum, POA, anterior and lateral hypothalamus (LH), and DMH. Likewise, prominent expression of GPR54 has been observed in the hippocampus (Hpt), locus coeruleus (LC), Amy and periaqueductal gray (PAG), among others. The functional relevance of such expression sites remains to be elucidated, except for the POA, where the expression of GPR54 in GnRH neurons has been thoroughly documented. In addition, in the human brain, scattered expression of *GPR54* mRNA has been reported, including basal ganglia –CPut-, Hpt, substantia nigra (SN-) and, at low levels, thalamus (Th-), cerebellum (C-) and corpus callosum. These are denoted by light gray dots. See color plate 31.

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of kisspeptin-positive neurons in the AVPV has been thoroughly documented.[5] Whether this more rostral population exists in other species, including humans, remains contentious. Yet, there is evidence for the presence of a population of Kp-IR neurons in the periventricular area of human hypothalamus.[12] In addition, the patterns of projections of Kp-IR neurons have been characterized in the mouse.[35] Thus, although Arc Kp-IR neurons seem to innervate a wide number of hypothalamic and limbic nuclei, Kiss1 neurons from the AVPV display a narrower pattern of projection that targets a smaller number of medially located hypothalamic nuclei. Among those, AVPV Kiss1 neurons seem to heavily project to the Arc. Although most of the Kp-IR fibers innervating GnRH neurons seem to originate from the AVPV (at least in female rodents), at least 20% of Arc Kiss1 neurons project to the POA,[35] where most GnRH neurons are located, thus providing the neuroanatomical basis for direct communication between these two neuronal populations as well. However, the wide patterns of fiber projection of Kp-IR neurons strongly suggest that Kiss1 neuronal populations are probably involved in the control of various biological functions, including reproductive and nonreproductive systems.[35]

GPR54 as receptor for Kisspeptins: Structure and Signaling Cascades

Using a degenerate PCR strategy and a rat brain cDNA library, Lee and coworkers published in 1999 the identification of an orphan G protein-coupled receptor, with a typical heptahelical conformation and significant identity (>40%) with the transmembrane regions of galanin receptors, which was termed GPR54.[25] The human ortholog of GPR54 was subsequently cloned and named AXOR12 or hOT7T175.[15] In 2001, this receptor was 'deorphanized' as three groups independently reported that kisspeptins are the natural ligands of GPR54.[13,15,23] As was the case for the ligand, expression analyses were conducted to screen the tissue distribution of *GPR54* gene. These showed a rather scattered pattern of expression, with maximal mRNA levels in placenta, pancreas, pituitary, spinal cord, and different brain areas, including hypothalamus, basal ganglia, amygdala, substantia nigra and hippocampus, among others.[25] These observations further suggested the potential involvement of the Kiss1/GPR54 system in the control of diverse biological systems, including different CNS functions. Within the hypothalamus, *GPR54* mRNA was initially reported to be highly expressed in the Arc, the dorsomedial nucleus, and the lateral hypothalamic area (Fig. 1). Interestingly, already in 2004, the expression of *GPR54* mRNA was shown in GnRH neurons in the rat and fish species.[25] In good agreement, a study, in a mouse model that expresses the reporter LacZ gene under the *GPR54* promoter, has confirmed the expression of this receptor in GnRH neurons at the rostral preoptic area.[11] These observations are in keeping with the proposed role of the Kiss1 system as a major gatekeeper of the reproductive axis. Notably, this study also showed that the highest receptor density is located at the dentate gyrus of the hippocampus, with abundant expression, in addition to the preoptic area, at the septum, anteroventral nucleus of the thalamus, posterior hypothalamus, periaqueductal gray, supramammillary and pontine nuclei, and dorsal cochlear nucleus;[11] these are areas where the function of GPR54 signaling remains largely unknown.

Initial analyses of the intracellular signaling systems recruited by GPR54 to convey the biological actions of kisspeptins revealed that, upon ligand–receptor interaction, activation of phospholipase C (PLC) and an increase in PIP_2 hydrolysis take place, which is followed by accumulation of inositol-(1,4,5)-triphosphate, Ca^{2+} mobilization, arachidonic acid release, and phosphorylation of ERK1/2 and p38 MAP kinases.[13] However, those initial signaling studies were conducted in a heterologous system (i.e. Chinese Hamster Ovary K1 cells stably expressing GPR54). Later studies with hypothalamic explants and isolated GnRH neurons confirmed the importance of the above PLC-Ca^{2+} pathway in mediating the biological effects of kisspeptins in a more relevant cellular context in terms of control of reproductive function.[4,25]

Biological Actions of Kisspeptins: Central Control of the Gonadotropic Axis

As indicated in previous sections, the *Kiss1* gene was initially labeled as metastasis suppressor and its major product was originally named metastin because of its proven ability to inhibit tumor metastasis and progression.[23] However, the possibility of additional physiological functions of the Kiss1 system was strongly suggested by the initial reports of the widespread pattern of the expression of *Kiss1* and *GPR54* genes in different peripheral and central tissues. Indeed, based on its biological properties on cell migration and prominent expression in human placenta, locally produced kisspeptins have been proposed to play a role in the physiological regulation of trophoblast invasion.[25] At the CNS, the scattered distribution of *Kiss1* and *GPR54* mRNAs, and of Kp-IR, allowed the speculation of potential roles of kisspeptins in different brain functions, including neuroendocrine regulation.[25] However, up to late 2003, such biological functions remained largely unexplored.

In 2003, two groups independently reported that a number of point mutations and deletions of the *GPR54* gene are found in patients suffering familial (or even sporadic) forms of idiopathic hypogonadotropic hypogonadism,[7,26] a clinical syndrome that was reproduced in mouse models carrying null mutations of the *GPR54* or the *Kiss1* gene.[25] These findings provided conclusive evidence for a major, previously unsuspected, role of the Kiss1 system in the

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regulation of the development and/or function of the hypothalamic–pituitary–gonadal (HPG) axis.[22,25] Moreover, the above observations underpinned the need for a complete characterization of essential aspects of Kiss1 physiology in the neuroendocrine control of reproduction, including its key biological effects, primary site(s) of action, major signaling pathways, and main regulatory mechanisms. Elucidation of such facets of kisspeptin physiology has attracted considerable attention and research efforts during the last years.

Studies conducted in laboratory animals, initially in rodents and primates and later in other mammalian and non-mammalian species,[22,25] have conclusively shown that kisspeptins are extraordinarily potent elicitors of gonadotropin secretion. The secretory effects of kisspeptins on gonadotropins have been particularly well characterized in rodents; studies that have shown that the gonadotropin-releasing action of kisspeptins are observed both after central (intracerebroventricular) and systemic (intravenous, intraperitoneal, subcutaneous) administration of the peptides.[17,18,22,25] The sensitivity of the gonadotropic system to the stimulatory effect of kisspeptins is documented by the finding that doses as low as 1 pmol of kisspeptin-10 injected centrally, or 75 pmol injected systemically, are able to robustly increase serum LH levels.[18] Notably, FSH secretory responses to kisspeptin appear to be approximately 100- to 200-fold less sensitive than those of LH [17] (Fig. 2). Comparison of the releasing effects of kisspeptins with those of other neuropeptides and neurotransmitters indicates that kisspeptins are probably the most potent stimulator of the gonadotropic axis known so far.

Numerous studies have now addressed the mechanisms and site(s) of action whereby kisspeptins conduct the above stimulatory effects. Based on the proven expression of *Kiss1*/Kisspeptin and GPR54 in the hypothalamus, a primary site of action at this level was already suggested in the original reports underscoring the essential role of the Kiss1 system in the control of the reproduction. Indeed, direct stimulatory actions of kisspeptins upon hypothalamic GnRH neurons, as the pivotal element driving the gonadotropic axis, has been well documented in rodents.[22,25] The observation that hypothalamic GnRH content is preserved in GPR54-null mice strongly suggests that kisspeptin signaling is not critically involved in the process of GnRH neuron migration from the olfactory placode that occurs during development, but rather it plays a role in the control of GnRH secretion.[22,25] Such a contention is indirectly supported by the initial observation that the potent LH releasing effect of kisspeptin is blunted by pretreatment with a GnRH antagonist.[22,25] Similarly, the demonstration of GPR54 expression in GnRH neurons and the stimulatory effects of kisspeptin-10 on GnRH release, both *in vivo* and ex vivo, provides further support to the hypothesis that GnRH neurons are direct targets of kisspeptins.[22,25] This contention is in keeping with neuroanatomical results showing close appositions between Kiss1 and GnRH neurons, as documented in rodent and primate species.

In addition to the hormonal tests described above, a wealth of expression and functional analyses has now documented a prominent role of the Kiss1 system in key aspects of the maturation and function of the reproductive axis. Although thorough revision of such aspects of kisspeptin physiology clearly exceeds the scope of this chapter, Kiss1 neurons have been proposed to be involved in virtually all crucial facets of reproductive maturation and function, from brain sexual differentiation to puberty onset and feedback regulation of gonadotropin secretion.[22,25] A few paradigmatic examples are described in detail below.

Hypothalamic expression of Kiss1 gene seems to be developmentally regulated with increased expression at the

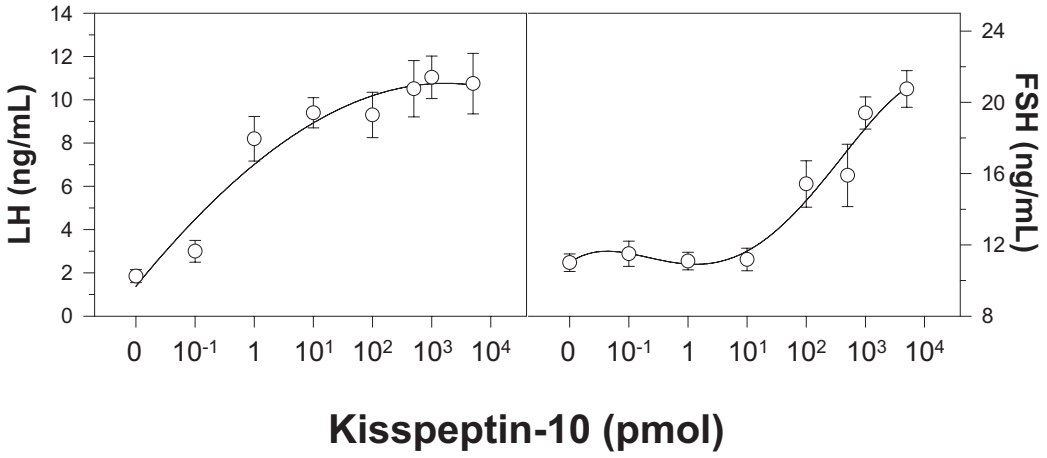

FIGURE 2 Analysis of the effects of central administration of KiSS-1 peptide upon serum LH and FSH levels. A range of doses of KiSS-1 (100-fmol, 1-, 10-, 100- and 500-pmol, 1- and 5-nmol) were tested (icv injection). Calculation of the mean effective dose (ED_{50}), as determined by nonlinear regression, indicates that LH secretion is approximately 100- to 200-fold more sensitive to the stimulatory effect of kisspeptin than FSH.

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time of puberty, in both rats and primates.[16,27] This observation suggests a role of the Kiss1 system in the timing of puberty onset in mammals, in keeping with the original observations of impuberism in humans and mouse models with inactivating mutations of GPR54 (or Kiss1). Indeed, this contention has been corroborated by functional studies that have demonstrated that the administration of kisspeptin-10 is able to induce precocious activation of the reproductive axis in immature female rats,[19] whereas blockade of kisspeptin signaling by the use of a specific antagonist delays it.[24] All in all, these observations highlight the prominent role of the Kiss1 system in the control of the timing of puberty.

Similarly, the expression analyses strongly suggest that the Kiss1 system plays a key role in mediating the classical negative feedback effect of sex steroids on gonadotropin secretion, as hypothalamic expression of the *Kiss1* gene significantly increases in various mammalian species after gonadectomy, whereas testosterone and estradiol replacement fully prevents such a response (as documented in rodents, among other species).[22,25] Interestingly, in situ hybridization assays already showed in 2004 that such a negative regulation of hypothalamic *Kiss1* gene expression takes place in the Arc, an area classically recognized as pivotal for the negative feedback actions of sex steroids.[22] In contrast, in rodents, it is well characterized that at the AVPV, *Kiss1* mRNA decreases after gonadectomy and increases after sex steroid replacement.[29] Considering that the AVPV, which is a sexually dimorphic nucleus with far more *Kiss1*-expressing neurons in the female, has been involved in mediating the positive feedback effect of estrogen upon GnRH and LH surges, it has been proposed that Kiss1 neurons at this nucleus are involved in the generation of the preovulatory gonadotropin surges via positive regulation of GnRH secretion.[22,25] This contention is supported by the observation that antagonism of kisspeptin signaling perturbs the occurrence of the preovulatory surges of LH and FSH.[24] In the same context, the hypothalamic expression of the *Kiss1* gene is also sensitive to the organizing effects of estrogens during critical periods of brain sex differentiation.[16,25] This phenomenon might contribute to the sexually dimorphic content of Kiss1 neurons within the hypothalamus (females >>> males), and opens up the possibility that decreased expression of *Kiss1* at the hypothalamus, as observed after neonatal estrogenization, may be a causative factor for the plethora of developmental and functional defects of the gonadotropic axis that take place after early exposure to pharmacological doses of estrogenic compounds.[16,25]

Although the role of the Kiss1 system as an essential gatekeeper of reproductive function is now undisputed, the eventual interplay of kisspeptins with other central transmitters with proven regulatory effects on the GnRH/gonadotropic axis remains incompletely characterized and is likely to draw considerable attention in the coming years. It is particularly exciting that Kiss1 neurons in the Arc coexpress the neuropeptide neurokinin-B (NKB), and that mutations of the genes encoding either NKB (*TAC3* in humans) or its receptor (*TACR3*) induce a state of HH similar to that of GPR54 inactivation.[21] These observations have prompted the conduction of a number of experimental studies that have documented the ability of the NKB agonist, senktide, to stimulate gonadotropin release (as surrogate marker of GnRH secretion) and that have led to the proposal that NKB participates in the autoregulation of kisspeptin output from Kiss1/NKB neurons onto GnRH neurons, thereby playing a role in the dynamic control of GnRH pulsatility.[20,21]

Peripheral Biological Actions of Kisspeptins: Reproductive and Nonreproductive Roles

The expression of *Kiss1* and *GPR54* at additional levels of the hypothalamic–pituitary–gonadal axis makes it possible that kisspeptins could operate at other (extrahypothalamic) sites to regulate reproductive function.[25] This is the case of the pituitary, where expression of Kiss1/kisspeptins and GPR54 has been documented in various species. However, the assessment of the direct biological effects of kisspeptins at the pituitary level in the control of gonadotropin secretion has received limited attention to date. Nonetheless, studies in rodent, primate, ovine, bovine, and fish species have now documented the ability of kisspeptins to modulate LH secretion directly at the pituitary level,[25] where kisspeptin-10 has been shown to induce calcium responses in gonadotrops both in rat and nonhuman primates. Admittedly, however, other studies in adult rodents have failed to find direct gonadotropin responses to kisspeptins and the physiological relevance of such direct pituitary actions in the generation of the preovulatory surge of LH has been questioned in sheep.[25] Although the causes for the above discrepancies remain unclear, kisspeptins can induce GH secretion directly at the pituitary level in rats and nonhuman primates, thereby suggesting a potential role of kisspeptins in the control of the somatotropic axis, whose physiological relevance is yet to be defined.

In addition to their potential roles at the pituitary, expression of the elements of the Kiss1 system has been also described in the gonads, where kisspeptin may also participate in the direct regulation of specific gonadal functions. This phenomenon has been well documented in the ovary, where the expression of *Kiss1*/*KISS1* and Kp-IR has been shown in rat, monkey, and human.[3,9] Interestingly, *Kiss1* mRNA levels in the rat ovary fluctuate according to the stage of the cycle, with prominent expression during the preovulatory phase that is apparently driven by the ovulatory surge of gonadotropins.[3] In addition, recent analyses in a rat model of ovulatory dysfunction induced by inhibition of prostaglandin synthesis show a marked suppression of ovarian *Kiss1* mRNA levels during the ovulatory period coincident with

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altered ovulatory dynamics.[9] Furthermore, Kp-IR has been documented in various ovarian compartments, including theca cells, corpora lutea and the interstitial gland in rat and primate species. Although all the above evidence strongly suggests a putative role of locally produced kisspeptins in the control of ovarian physiology, the actual relevance, if any, of such local functions remains to be defined.

Finally, besides human and rodent placenta, the expression of Kiss1/kisspeptins and GPR54 has been documented in a number of peripheral tissues including, among others, blood vessels, adrenals, adipose tissue, kidney and pancreas. Although functional studies have suggested potential roles of kisspeptin signaling in these peripheral tissues, such as the control of vasomotor tone and blood pressure, glucose homeostasis and even renal development, the physiological relevance of such peripheral, nonreproductive functions of kisspeptins remains obscure. Similarly, although kisspeptins were initially cataloged as antimetastasis factors, and the loss of *KISS1* expression has been regarded as bad-prognosis factor in several tumors, the actual role of kisspeptins in the prevention of cancer spread needs to be further clarified.

PATHOPHYSIOLOGICAL IMPLICATIONS

As indicated in previous sections, identification and characterization of the Kiss1/GPR54 system as pivotal element in the neuroendocrine network controlling the reproductive axis is an illuminating example of the power of bedside-to-bench research, where the finding of disease-causing mutations in the *GPR54* gene has led to the systematic analysis of different aspects of kisspeptin physiology.[22,25] Because of these features, it is obvious that perturbation of the Kiss1 system poses pathophysiological implications in terms of reproductive health. Thus, initial reports identified several inactivating mutations of the *GPR54* gene associated with the lack of puberty onset and hypogonadism of central (hypothalamic) origin. These included the homozygous deletion of 155 nucleotides around intron IV-exon V junction, the homozygous L148S mutation, and different compound heterozygous (R331X and X399R; C223R, and R297L) mutations.[25] Additional inactivating mutations in the *GPR54* gene reported to date include an insertion at 1001_1002C, with subsequent change in open-reading frame, a L102P mutation, and a combined insertion/deletion mutation at the 3′ splicing acceptor site of exon 2. Likewise, a new homozygous mutation involving a pF272S substitution in the *GPR54* sequence has been recently described in cases of hypogonadotropic hypogonadism. A summary of the inactivating mutations of *GPR54* gene reported to date is provided in Fig. 3. Notably, the first *KISS1* gene mutation causing central hypogonadism has been published very recently, Moreover, more recent studies have described for the first time suspected activating mutations of *KISS1* and *GPR54* genes in humans. Thus, two different

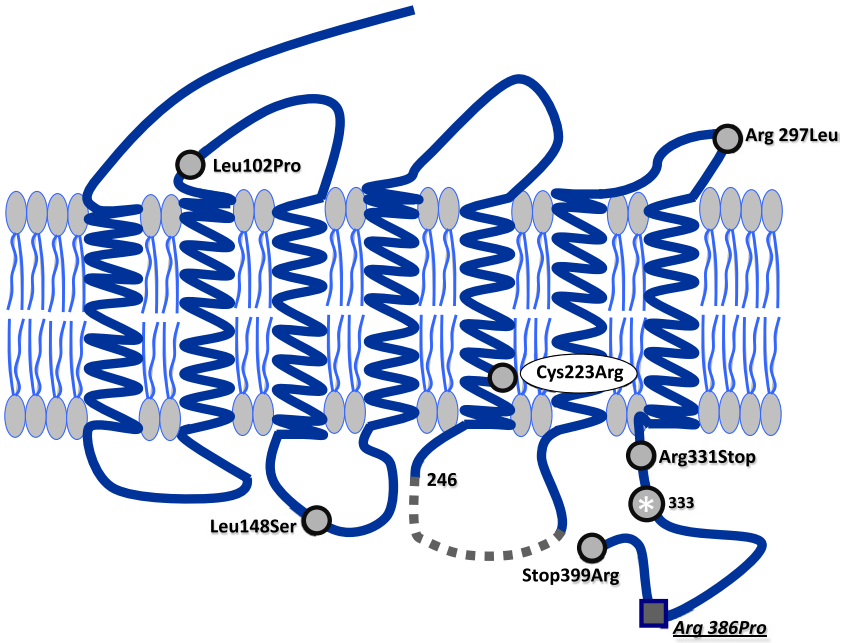

FIGURE 3 Schematic representation of deletions and disease-causing mutations of *GPR54* gene reported so far in humans. Inactivating mutations are indicated as *black dots*, with reference to the reported amino acid substitution. In addition, the location of the predicted deletion reported by de Roux et al. is marked as *gray dots*, while the position of the predicted insertion (1001_1002insC), which results in a shift of the open-reading frame is denoted by an *asterisk*. Finally, the only activating mutation of GPR54 described so far in humans, as potential cause for precocious puberty, is denoted as an open square. See color plate 32.

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missense mutations, causing P74S and H90D substitutions, have been reported in the *KISS1* gene in a cohort of children with central precocious puberty.[28] Yet, whether these mutations are the sole cause for the striking clinical phenotype of the affected individuals is yet to be clarified. On the other hand, the first case of an activating mutation of *GPR54* has been also reported[30] (Fig. 3). This consists of a R386P substitution that does not cause constitutive activity of the receptor but rather prolongs its pattern of intracellular IP response upon stimulation. Again, further studies are needed to conclusively show that such a receptor mutation is fully responsible for the state of precocious puberty of the affected child.

From an epidemiological standpoint, initial analyses already suggested that the frequency of *GPR54* null mutations as a cause for hypogonadotropic hypogonadism is rather low; a contention confirmed now by different genetic studies in various human populations. Nonetheless, besides obvious physiological implications (see previous Sections), identification of inactivating mutations of GPR54 as a causative factor for reproductive failure has helped to expand our knowledge on the molecular basis of some forms of infertility. In this sense, *GPR54* and *KISS1* enlarge the set of genes (such as KAL1, steroidogenic factor 1, DAX-1, GnRH receptor, GnRH and leptin, just to mention but few) whose inactivation had been previously linked to hypogonadotropic hypogonadism. In addition, taking into account the apparent widespread pattern of the expression of GPR54 and kisspeptins in central and peripheral tissues and their proposed biological roles (e.g. control of tumor progression and metastasis), further analyses are needed in affected humans and corresponding null mouse models to evaluate in detail whether mutations of this system can cause additional nonreproductive phenotypes. Overall, complete characterization of the panel of *GPR54*, and eventual *KISS1*, gene mutations inducing clinical alterations of sexual development and function will help to elucidate not only the molecular basis of some forms of reproductive pathology, but also the whole array of physiological roles and pathophysiological implications of this recently discovered, and critically important ligand–receptor system.

ACKNOWLEDGMENTS

The author is indebted to V.M. Navarro, L. Pinilla, E. Aguilar, C. Dieguez, J. Roa and other members of the Physiology Section of the University of Cordoba for helpful discussions during preparation of this manuscript. Experimental work conducted in the author's laboratory was supported by grants BFU 2005-07446, BFU 2008-00984, and BFU 2011-25021 (Ministerio de Ciencia e Innovación, Spain); Project PI042082 (Ministerio de Sanidad, Spain); Project P08-CVI-03788 (Junta de Andalucía, Spain) and EU research contract DEER FP7-ENV-2007-1. CIBER Fisiopatología de la Obesidad y Nutrición is an initiative of Instituto de Salud Carlos III, Ministerio de Sanidad, Spain.

REFERENCES

1. Akazome Y, Kanda S, Okubo K, Oka Y. Functional and evolutionary insights into vertebrate kisspeptin systems from studies of fish brain. *J Fish Biol* 2010;**76**:161–82.
2. Brailoiu GC, Dun SL, Ohsawa M, Yin D, Yang J, Chang JK, et al. KiSS-1 expression and metastin-like immunoreactivity in the rat brain. *J Comp Neurol* 2005;**481**:314–29.
3. Castellano JM, Gaytan M, Roa J, Vigo E, Navarro VM, Bellido C, et al. Expression of KiSS-1 in rat ovary: putative local regulator of ovulation? *Endocrinology* 2006;**147**:4852–62.
4. Castellano JM, Navarro VM, Fernandez-Fernandez R, Castano JP, Malagon MM, Aguilar E, et al. Ontogeny and mechanisms of action for the stimulatory effect of kisspeptin on gonadotropin-releasing hormone system of the rat. *Mol Cell Endocrinol* 2006; **257–258**:75–83.
5. Clarkson J, Herbison AE. Postnatal development of kisspeptin neurons in mouse hypothalamus; sexual dimorphism and projections to gonadotropin-releasing hormone neurons. *Endocrinology* 2006;**147**:5817–25.
6. Cravo RM, Margatho LO, Osborne-Lawrence S, Donato Jr J, Atkin S, Bookout AL, et al. Characterization of Kiss1 neurons using transgenic mouse models. *Neuroscience* 2011;**173**:37–56.
7. de Roux N, Genin E, Carel JC, Matsuda F, Chaussain JL, Milgrom E. Hypogonadotropic hypogonadism due to loss of function of the KiSS1-derived peptide receptor GPR54. *Proc Natl Acad Sci U S A* 2003;**100**:10972–6.
8. Felip A, Zanuy S, Pineda R, Pinilla L, Carrillo M, Tena-Sempere M, et al. Evidence for two distinct KiSS genes in non-placental vertebrates that encode kisspeptins with different gonadotropin-releasing activities in fish and mammals. *Mol Cell Endocrinol* 2009;**312**:61–71.
9. Gaytan F, Gaytan M, Castellano JM, Romero M, Roa J, Aparicio B, et al. KiSS-1 in the mammalian ovary: distribution of kisspeptin in human and marmoset and alterations in KiSS-1 mRNA levels in a rat model of ovulatory dysfunction. *Am J Physiol Endocrinol Metab* 2009;**296**:E520–31.
10. Gottsch ML, Clifton DK, Steiner RA. From KISS1 to kisspeptins: an historical perspective and suggested nomenclature. *Peptides* 2009;**30**:4–9.
11. Herbison AE, de Tassigny X, Doran J, Colledge WH. Distribution and postnatal development of Gpr54 gene expression in mouse brain and gonadotropin-releasing hormone neurons. *Endocrinology* 2010;**151**:312–21.
12. Hrabovszky E, Ciofi P, Vida B, Horvath MC, Keller E, Caraty A, et al. The kisspeptin system of the human hypothalamus: sexual dimorphism and relationship with gonadotropin-releasing hormone and neurokinin B neurons. *Eur J Neurosci* 2010;**31**:1984–98.
13. Kotani M, Detheux M, Vandenbogaerde A, Communi D, Vanderwinden JM, Le Poul E, et al. The metastasis suppressor gene KiSS-1 encodes kisspeptins, the natural ligands of the orphan G protein-coupled receptor GPR54. *J Biol Chem* 2001;**276**:34631–6.
14. Mikkelsen JD, Simonneaux V. The neuroanatomy of the kisspeptin system in the mammalian brain. *Peptides* 2009;**30**:26–33.
15. Muir AI, Chamberlain L, Elshourbagy NA, Michalovich D, Moore DJ, Calamari A, et al. AXOR12, a novel human G protein-coupled receptor, activated by the peptide KiSS-1. *J Biol Chem* 2001;**276**:28969–75.

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16. Navarro VM, Castellano JM, Fernandez-Fernandez R, Barreiro ML, Roa J, Sanchez-Criado JE, et al. Developmental and hormonally regulated messenger ribonucleic acid expression of KiSS-1 and its putative receptor, GPR54, in rat hypothalamus and potent luteinizing hormone-releasing activity of KiSS-1 peptide. *Endocrinology* 2004;**145**:4565–74.
17. Navarro VM, Castellano JM, Fernandez-Fernandez R, Tovar S, Roa J, Mayen A, et al. Effects of KiSS-1 peptide, the natural ligand of GPR54, on follicle-stimulating hormone secretion in the rat. *Endocrinology* 2005;**146**:1689–97.
18. Navarro VM, Castellano JM, Fernandez-Fernandez R, Tovar S, Roa J, Mayen A, et al. Characterization of the potent luteinizing hormone-releasing activity of KiSS-1 peptide, the natural ligand of GPR54. *Endocrinology* 2005;**146**:156–63.
19. Navarro VM, Fernandez-Fernandez R, Castellano JM, Roa J, Mayen A, Barreiro ML, et al. Advanced vaginal opening and precocious activation of the reproductive axis by KiSS-1 peptide, the endogenous ligand of GPR54. *J Physiol* 2004;**561**:379–86.
20. Navarro VM, Gottsch ML, Chavkin C, Okamura H, Clifton DK, Steiner RA. Regulation of gonadotropin-releasing hormone secretion by kisspeptin/dynorphin/neurokinin B neurons in the arcuate nucleus of the mouse. *J Neurosci* 2009;**29**:11859–66.
21. Navarro VM, Tena-Sempere M. Neuroendocrine control by kisspeptins: role in metabolic regulation of fertility. *Nat Rev Endocrinol* 2012;**8**:40–53.
22. Oakley AE, Clifton DK, Steiner RA. Kisspeptin signaling in the brain. *Endocr Rev* 2009;**30**:713–43.
23. Ohtaki T, Shintani Y, Honda S, Matsumoto H, Hori A, Kanehashi K, et al. Metastasis suppressor gene KiSS-1 encodes peptide ligand of a G-protein-coupled receptor. *Nature* 2001;**411**:613–7.
24. Pineda R, Garcia-Galiano D, Roseweir A, Romero M, Sanchez-Garrido MA, Ruiz-Pino F, et al. Critical roles of kisspeptins in female puberty and preovulatory gonadotropin surges as revealed by a novel antagonist. *Endocrinology* 2010;**151**:722–30.
25. Roa J, Aguilar E, Dieguez C, Pinilla L, Tena-Sempere M. New frontiers in kisspeptin/GPR54 physiology as fundamental gatekeepers of reproductive function. *Front Neuroendocrinol* 2008;**29**:48–69.
26. Seminara SB, Messager S, Chatzidaki EE, Thresher RR, Acierno Jr JS, Shagoury JK, et al. The GPR54 gene as a regulator of puberty. *N Engl J Med* 2003;**349**:1614–27.
27. Shahab M, Mastronardi C, Seminara SB, Crowley WF, Ojeda SR, Plant TM. Increased hypothalamic GPR54 signaling: a potential mechanism for initiation of puberty in primates. *Proc Natl Acad Sci U S A* 2005;**102**:2129–34.
28. Silveira LG, Noel SD, Silveira-Neto AP, Abreu AP, Brito VN, Santos MG, et al. Mutations of the KISS1 gene in disorders of puberty. *J Clin Endocrinol Metab* 2010;**95**:2276–80.
29. Smith JT, Cunningham MJ, Rissman EF, Clifton DK, Steiner RA. Regulation of Kiss1 gene expression in the brain of the female mouse. *Endocrinology* 2005;**146**:3686–92.
30. Teles MG, Bianco SD, Brito VN, Trarbach EB, Kuohung W, Xu S, et al. A GPR54-activating mutation in a patient with central precocious puberty. *N Engl J Med* 2008;**358**:709–15.
31. Tena-Sempere M. GPR54 and kisspeptin in reproduction. *Hum Reprod Update* 2006;**12**:631–9.
32. Terao Y, Kumano S, Takatsu Y, Hattori M, Nishimura A, Ohtaki T, et al. Expression of KiSS-1, a metastasis suppressor gene, in trophoblast giant cells of the rat placenta. *Biochim Biophys Acta* 2004;**1678**:102–10.
33. Um HN, Han JM, Hwang JI, Hong SI, Vaudry H, Seong JY. Molecular coevolution of kisspeptins and their receptors from fish to mammals. *Ann NY Acad Sci* 2010;**1200**:67–74.
34. West A, Vojta PJ, Welch DR, Weissman BE. Chromosome localization and genomic structure of the KiSS-1 metastasis suppressor gene (KISS1). *Genomics* 1998;**54**:145–8.
35. Yeo SH, Herbison AE. Projections of arcuate nucleus and rostral periventricular kisspeptin neurons in the adult female mouse brain. *Endocrinology* 2011;**152**:2387–99.

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Chapter 110

MCH

Françoise Presse and Jean-Louis Nahon

ABSTRACT

Melanin-concentrating hormone (MCH) is a cyclic peptide highly conserved in vertebrates, which was originally characterized as a neurohypophyseal skin-paling factor in teleosts. In fishes, MCH participates also in the regulation of the stress response and feeding behavior. Mammalian MCH is a hypothalamic neuropeptide that displays multiple functions, mostly controlling feeding behavior and energy homeostasis and regulating sleep, the stress axis, emotion, and reward processes. The MCH precursor may generate additional putative peptides in the brain and peripheral organs but their roles remain elusive. In mammals, alternative splicing and antisense transcription at the *MCH* gene locus lead to the production of novel proteins, whereas variant *MCH* genes are found only in primates. Two G protein-coupled MCH receptors are found in fish and primates, whereas a single one appears functional in rodents. Transgenic mouse models and pharmacological studies show the importance of the MCH signaling pathway as a potential target to treat appetite disorders and obesity and also anxiety and psychiatric diseases.

DISCOVERY

The existence of a paling factor present in the pars tuberalis of amphibia that would regulate skin pigmentation in response to environmental changes in lower vertebrates was first proposed by Hogben and Slome in 1931.[18] Although this concept never received any experimental support in amphibians, melanin-concentrating activity was firmly established in teleosts with the demonstration of pituitary control of teleost melanophores by a paling neurohypophysial hormone. The development of *in vitro* teleost scale bioassays allowed functional characterization and purification of the hormone. Indeed, based on a Tilapia scale bioassay, Kawachi et al.[19] purified melanin-concentrating hormone (MCH) from pituitary glands of chum salmon (*Oncorhynchus keta*) and characterized its structure. Sequence analysis revealed that MCH is a 17 amino-acid peptide with one disulfide bond (Fig. 1). This seminal work paved the road for immunocytochemical localization, structure–activity studies, and investigation of MCH function and regulation, apart from its pigmentary action, in fish models.[4,5,10,16,32]

Thereafter, the availability of salmon MCH (sMCH) antisera facilitated the characterization of the MCH counterpart in higher vertebrates. Thanks to the strong expression of MCH-like peptide in the rat hypothalamus and using the sMCH RIA, Vaughan et al.[33] isolated the rat MCH and established its sequence. Rat MCH is a cyclic nonadecapeptide that displays strong structural similarity with the fish peptide but an N-terminal extension and four additional substitutions (Fig. 1).

The role of MCH in mammalian systems remained elusive until 1996 when MCH mRNA expression studies revealed striking overexpression in obese leptin-deficient (*Ob/Ob*) mice and after fasting.[29] Intracerebral injections of MCH further revealed an acute orexigenic effect in the rat that was confirmed in several laboratories.[27] The importance of the MCH neuronal system in the regulation of feeding behavior, body weight, and energy homeostasis is by far the most documented. However, MCH is also involved in the control of a broad spectrum of cerebral and peripheral functions.[1,2,5,16,17,20,21,24,27]

Cloning of the cDNAs encoding the MCH precursor in teleost fishes, then in rats and humans, opened the era of molecular characterization of *MCH* gene structure and expression. Most of this research concentrated on mammalian genes and led to the discovery of additional putative peptides generated from the MCH precursor, a spliced variant of the MCH primary transcript, a natural antisense *MCH* gene and a family of "variant" *MCH* genes that arose lately in the Hominidae lineage.[13,17,24] All these *MCH* gene-derived products may have functions, distinct from MCH effects, which are still to be defined.

A long-awaited achievement was the cloning and functional characterization in cellular and animal models of two MCH receptors, both members of the G protein-coupled receptor (GPCR) family.[9,15,17] The first MCH receptor, MCH-R1, was isolated by a reverse-pharmacological strategy and was found in all mammalian species, whereas the second, designated here MCH-R2, was identified by computer data mining and seemed to be absent in rats and mice

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00110-X
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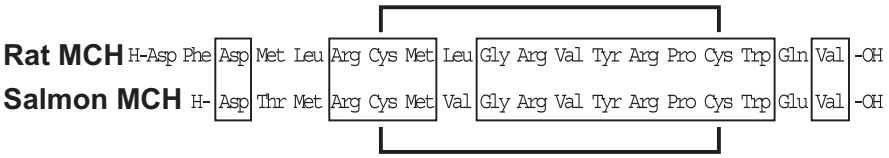

FIGURE 1 Structure of salmon and rat/human MCH. Conserved amino acids are boxed and the disulfide bridge between the two cysteines is indicated.

but present in other mammalian species, including primates. Because MCH-R1 *per se* seems to mediate MCH actions in the regulation of food intake and energy expenditure, several pharmaceutical companies have developed a diverse array of MCH-R1 antagonists. Preclinical studies with MCH-R1 antagonists are still limited but provide some evidence that non-peptidic MCH-R1 antagonists represent attractive molecules for the treatment of obesity, metabolic syndrome, drug addiction, sleep disorder, and anxiety in humans.[11,22,23]

STRUCTURE OF THE PRECURSOR mRNA/GENE

Two *MCH* genes and/or corresponding mRNAs have been originally cloned and sequenced from salmonids, tilapia, bonito, eel, and flounder.[16,24] Both were intronless and displayed the highest level of substitutions when compared within the same species rather than among other species, in agreement with early tetraploidization during teleost fish evolution (Fig. 2A). The salmonid and tilapia/flounder pre-proMCHs are 132- and 135 amino acids long, respectively, with the MCH peptide located at the C-terminus. In contrast with the strong conservation in the MCH region between teleost fish species, the remaining part of the precursor displays marked sequence divergences.[6,10] However, additional putative peptides could be generated from the teleost fish proMCH and named MCH-gene-related peptide (Mgrp) in tilapia, Neuropeptide-E (glutamic acid)-V (valine) (NEV) in chum salmon, or Neuropeptide-A(alanine)-L (leucine) in barfin flounder but their functional importance, if any, remains elusive.[16,24] Recently, a second MCH gene was identified in the zebrafish, fugu, three-spined sticleback, and medaka.[6] This gene, named *Pmch2*, shares striking resemblance with the mammalian *MCH* gene in terms of structure, synteny, and peptide sequence. Two different MCH peptides can be expressed in distinct regions of the zebrafish hypothalamus. Although both MCH peptides could play a role in pigmentation,[35] preliminary functional studies in zebrafish indicate that *Pmch2* could be selectively involved in food intake regulation,[6] as in mammals (discussed later).

The sequence of the single gene encoding the full MCH precursor comprises three exons and two introns and exhibits high conservation in mammalian species (Fig. 2B). In humans, this gene is located on chromosome 12q24 covering about 1.4 kb of genomic DNA. The first exon encodes the 5′-untranslated region of the mRNA and the N-terminal part of proMCH including the signal peptide that allows targeting to the secretory pathway. The second exon consists of the sequence corresponding to putative neuropeptide(N)-glycine(G)-glutamic acid(E) (NGE), neuropeptide(N)-glutamic acid(E)-isoleucine(I) (NEI), and the first three amino acids of MCH. The last 15 amino acids of MCH and the 3′-untranslated amino acids are localized in exon III. Intron B splits the methionine codon with the nucleotides A on exon II and the TG on exon III. This intronic organization is identical in the rat, mouse, and primate *MCH* genes. This unusual intron position for a neuropeptide encoding gene is of primary importance to generate by alternative splicing a short mRNA encoding a protein named MCH-gene-overprinted-polypeptide (MGOP)[3] (Fig. 2C). Rat/mouse MGOP contains 125 amino acid residues and shares an identical N-terminal part with the MCH precursor (encoded by exon I of the *MCH* gene) but lacks NGE and NEI (encoded by exon II of the MCH gene) as well as MCH that is replaced by a new C-terminal part (resulting from a shift in the reading frame within exon III of the MCH gene). Structural analysis of MGOP-IR and distribution in the rat and mouse brain revealed a strict colocalization with MCH in neurons of the zona incerta/lateral hypothalamus and a unique expression of an MGOP-like antigen that was characterized as the prosomatostatin$_{1\text{-}64}$, in neurons of the hypothalamic periventricular nucleus and other brain areas.[3] The role of MGOP in the brain remains elusive.

In addition to the *MCH/MGOP* gene, two other *MCH*-gene-related transcription units were found in mammalian genomes. First, the characterization of high molecular weight transcripts recognized by MCH cDNA/oligoprobes in the pheochromocytoma PC12 cell line and rat tissues revealed the existence of "natural" antisense RNAs transcribed from the genomic region complementary to the *MCH* gene. This new gene was named AROM for Antisense-RNA-Overlapping-MCH.[8] Two major classes of RNAs seem to be produced from the AROM gene: (1) noncoding unspliced RNAs overlapping exon II/exon III and flanking intronic/regulatory sequences of the MCH gene; (2) alternatively spliced mRNAs complementary to the 3′-flanking region of the MCH gene and coding for a new family of RNA/DNA binding proteins. Because of the reciprocal regulation of MCH and AROM gene expression in PC12 cells, it is

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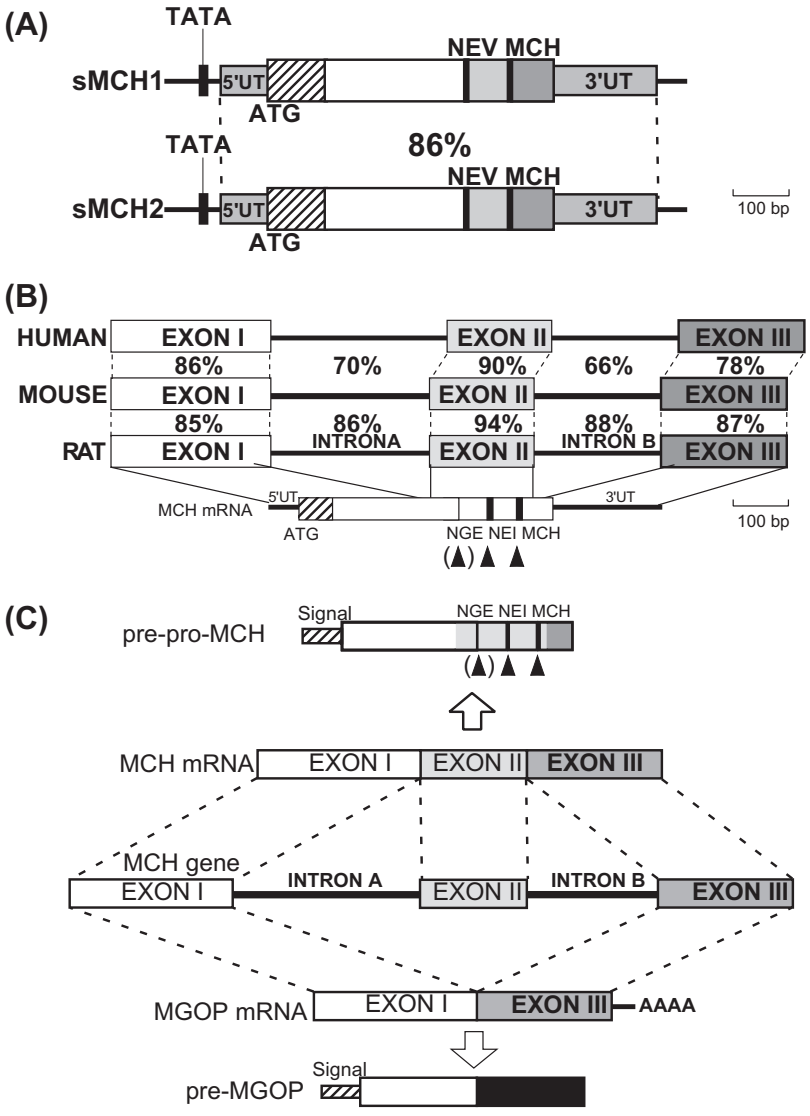

FIGURE 2 The *MCH* genes (A) Comparison of the two *MCH* genes in salmon. TATA, putative TATA box; UT, untranslated region. ATG, first codon of preproMCH. The putative signal peptide is indicated by a striped box. Arrowheads and black bars indicate the basic residues involved at the cleavage sites to generate MCH and the putative NEV. DNA sequence percent identities are noted (B) Comparison of the human, mouse and rat *MCH* genes. Exons and introns are indicated by boxes and lines respectively. The putative signal peptide and the location of peptides NGE, NEI, and MCH are noted as in (A). (C) Structure of the MCH and MGOP mRNA and deduced proteins produced by alternative splicing of the MCH primary transcript. Black box indicates the MGOP-specific C-terminus due to exon II skipping and ORF frameshift in exon III.

tempting to speculate that AROM mRNA and/or proteins may control MCH gene expression, but this remains to be directly tested.

A decade ago, we provided evidence for the emergence in higher primates of two chimeric genes.[13] These genes were named *PMCHL1* and *PMCHL2*, genes based on partial homology with the authentic *MCH* gene, and were found respectively on human chromosome 5p14 and 5q13, that is, located on chromosomal regions clearly distinct from those of the genuine *MCH* gene. Detailed structural expression and phylogenetic analyses showed that the *PMCHL1* gene was created about 25 million years ago (mya) by a complex mechanism of exon-shuffling through retroposition of an antisense *MCH* messenger RNA coupled to *de novo* creation of splice sites. *PMCHL2* arose 5–10 mya by an event of duplication involving a large chromosomal region encompassing the *PMCHL1* locus. Interestingly, both sense and antisense transcripts from the *PMCHL1* gene are expressed in different areas of the developing human brain, whereas the *PMCHL2* gene is totally silent in the central nervous system. Further, one of the PMCHL1 mRNAs, encodes a 72 amino-acid protein carrying a putative nuclear localization signal, suggesting an intranuclear function for this *PMCHL1*-gene-derived protein in primates. However, most of the

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PMCHL1/PMCHL2 mRNAs expressed in primate testis[31] or brain (unpublished data) appear as non-protein-coding RNAs. The *PMCHL1* gene represents a prototype of the so-called "primate specific" genes that are presently under intensive investigation because of their major interest in understanding the basis of human evolution and emergence of novel functions.

PROCESSING OF THE MCH PRECURSOR

Very few studies have attempted to investigate the biosynthesis of the proMCH-derived peptides in teleost precursors.[16] Large molecular weight MCH precursors are produced in the hypothalamus of salmon or trout and are further cleaved to generate intermediate products and mature MCH in the trout pituitary glands. Interestingly, the main intermediate corresponds to the $proMCH_{101–132}$, that is, the NEV-MCH peptide generated after cleavage at a pair of arginine residues at positions 99–100 of the trout MCH precursor.

In the mammalian brain, most of the proMCH-derived peptides are mature MCH and amidated NEI peptides (Fig. 4A). Conversely, fully processed MCH and NEI peptides are lacking or weakly produced in rodent and human peripheral organs (spleen, gut, thymus, and testis) while a large MCH-IR product is found. This large polypeptide was identified as a proMCH intermediate, bearing at least NEI and MCH (Fig. 4B). In this context, the rat $proMCH_{131–165}$ (NEI-MCH peptide) was synthesized and injected icv to investigate the effect on appetite, behavior, and energy metabolism. The NEI-MCH polypeptide exerts long-term inductions in feeding and body weight gain, being much more potent than MCH itself, probably because of better resistance to peptidase degradation.[17] It is attractive to suggest that a similar kind of proMCH intermediate may be either transiently expressed in the brain or produced peripherally under particular physiopathological conditions and may cross the blood–brain barrier to exert sustained MCH-like activity.

The processing of the mammalian MCH precursor by prohormone-convertases (PCs) was examined by recombinant vaccinia virus-infected cellular systems, PC-transfected PC12 cells, and an *in vivo* PC2 KO-mouse model (Fig. 4 B[34]). The main finding was that active PC2 is necessary and sufficient to generate NEI in the mouse brain and in PC12 cells. By contrast, several PCs, including PC1/3, PC2, and PC5/6A, may cleave at the appropriate site to produce MCH either in vaccinia expression systems or in the mouse brain (see the convertase chapters in Peptide Biosynthesis/Processing section of this book). Incidentally, colocalization studies show simultaneous expression of MCH mRNA and PC2 in all MCH-expressing cell bodies of the lateral hypothalamus, whereas only 15–20% of these perikarya contain PC1. Therefore, PC2 is probably the key enzyme that cleaves MCH-precursor to generate MCH and NEI in the brain. The counterpart in peripheral organs remains unknown.

DISTRIBUTION OF THE mRNA/PEPTIDES

The distribution of MCH-immunoreactive (IR) perikarya and projection fields has been established in most vertebrate taxa.[4,16,17,24,32] In fish, MCH neurons are located in three distinct areas, the lateral and posterior parts of the nucleus lateralis tuberis (NLTl and NLTp) and in more dorsal nuclei above the lateral ventricular recess (LVR). The magnocellular MCH expressing neurons of the NLTp predominate in lately evolved actinopterigians, including teleosts, and project massively to the posterior neurohypophysis where MCH is released into the bloodstream to act peripherally as a skin paling hormone. By contrast, the parvocellular MCH neurons of NLTl and LVR are prominent in early evolved vertebrates, including lampreys, and project widely to different brain areas with the exception of the pituitary, suggesting that NLTl/LVR-originating MCH probably acts as a neurotransmitter/neuromodulator. Both MCH neuronal cell groups function differently under white or black environmental challenge in tilapia, rainbow trout, barfin flounder,[4] or medaka.[6,35]

The mapping of MCH-IR neurons in the rat brain revealed two striking features highly conserved in mammals: (1) quite exclusive clustering of MCH neuronal perikarya in the subzona incerta, lateral hypothalamic area (LHA), and posterior hypothalamic area (PHA); (2) extensive distribution of the projections throughout the brain with the notable exception of the cerebellum, external median eminence, and pituitary gland (Fig. 3[7]). Fellmann and Risold's team performed a series of immunocytochemical investigations looking for the differential expression of neurokinin 3 (NK3) receptor and cocaine- and amphetamine-regulated transcript (CART) in MCH neurons and projections.[30] In particular, two MCH cell groups were clearly identified, based on the coexpression of MCH/CART/NK3 (type B neurons) or only MCH (type A neurons). Further, they display very different projection patterns; type A neurons have quite exclusively descending inputs to reach the spinal cord, whereas type B neurons project massively to all known telencephalic areas receiving ascending MCH inputs. Intriguingly, at least four MCH neuronal subpopulations can be distinguished according to their times of genesis during rat diencephalon development, two of them overlapping with type A and type B MCH neurons.

Minor MCH-expressing neuronal groups are also present in extrahypothalamic areas such as the olfactory tubercle and pontine tegmentum in the adult rat (Fig. 3).[7] Transient expression of MCH-containing neurons was identified throughout the embryo development in rats in the lateral

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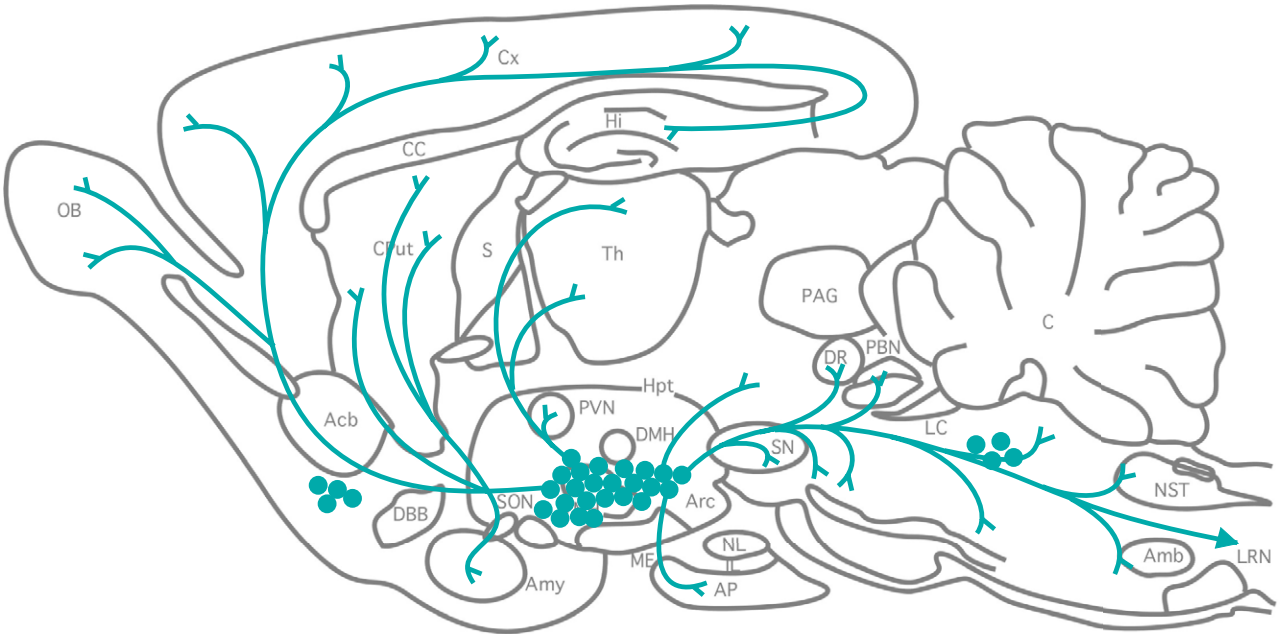

FIGURE 3 Schematic representation of MCH-producing neurons (dots) and projections (lines/arrow) in the rat brain. (from Ref. 7).

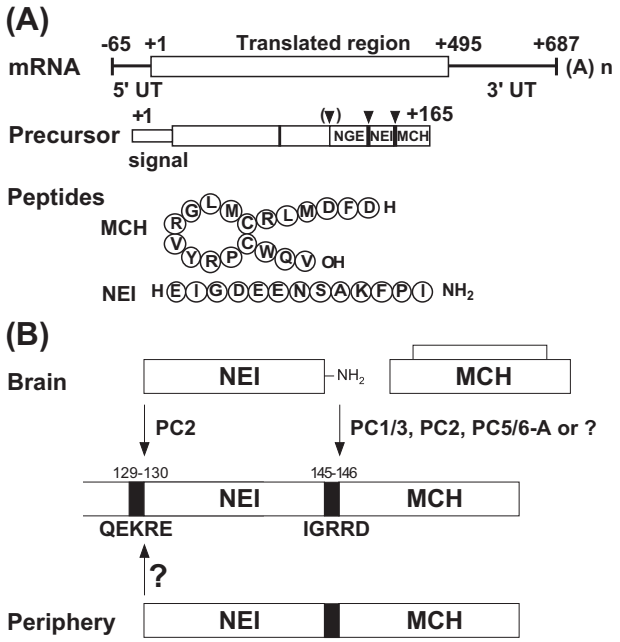

FIGURE 4 Rat MCH precursor processing and peptide production. (A) Rat MCH mRNA structure (as in Fig. 2B) and peptide sequences. (B) ProMCH processing by prohormone-convertases (PCs) in the brain and in peripheral organs. Sequences at the cleavage sites are noted as well as PCs with demonstrated effects on proMCH to generate MCH and NEI. The question mark indicates that the PC involved in MCH precursor processing at the periphery is not yet known.

geniculate nucleus, lateral ZI, PVN, and lateral septum and also in the medial preoptic area and PVN of lactating female rats.[17] The significance of this transient appearance of MCH neurons remains elusive but could be related to similar expression patterns reported for other neuropeptide systems.

RECEPTORS AND SIGNALING CASCADES

The pioneer attempts to characterize MCH receptors were largely based on binding assays with MCH analogs such as (Phe13, Tyr 19)-MCH and allowed the identification of MCH binding sites in different mammalian cell types, including melanoma and neuroblastoma cells.[14] The lack of ligand selectivity in competition experiments, ligand-induced signal transduction pathways associated with these MCH binding sites, and rapid internalization of the radioligand probably through scavenger receptor binding have cast some doubt on the reliability of these putative MCH receptors. However, a functional MCH receptor distinct from the two presently identified MCH receptors (and named MCH-Rpc) can be found in mouse and human melanoma, but its sequence is still unknown.[14]

MCH-R1 STRUCTURE AND SIGNALING

A milestone discovery in the MCH receptor field was the quite simultaneous identification by several laboratories in 1999 of an orphan GPCR called SLC-1 (GPR24) as being a *bona fide* MCH receptor, named here MCH-R1.[9,11,15] This receptor belongs to the class 1 subfamily of GPCRs and displays strong sequence conservation among mammals (91% protein sequence identity between rats and humans). The MCH-R1 gene consists of two exons that encode for a 353 aminoacid protein with seven putative transmembrane

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domains, three N-glycosylation sites in the N-terminal part, that seem to be necessary for cell surface targeting, and multiple phosphorylation sites in the intracellular loops; some of them, located in its C-terminal tail, are involved in the MCH-dependent internalization of MCH-R1. MCH-R1 is widely and strongly expressed in rat, mouse, and primate brains with a distribution that matches with the MCH projection fields, suggesting that this receptor may mediate most MCH actions in mammals.[11,17]

MCH-R1 signaling has been mostly investigated in stably transfected cells and these studies reveal that this MCH receptor couples to a variety of G proteins, resulting in the activation of multiple signaling pathways. Indeed, in MCH-R1 overexpressing CHO or HEK293 cells, MCH inhibits forskolin-induced cAMP synthesis through a PTX-sensitive mechanism, indicating the coupling to Gαi/Gαo. In the same cellular models, MCH also stimulates IP3 production and transiently increases intracellular Ca^{2+} via Gαq interaction with MCH-R1. Finally, MCH-stimulated MAP kinase activity in transfected CHO cells seems to be mediated through both Gαi and Gαq coupled signaling pathways. Intriguingly, a combination of MCH with forskolin potentiates MAP kinase phosphorylation in HEK293 cells stably expressing MCH-R1 and in the various regions of the rat brain suggesting that a complex interaction between MCH-R1 and receptors coupled to Gαs could be the rule rather than the exception in the mammalian brain. In contrast to these results, the signaling pathways that mediate MCH action in mammalian cells endogenously expressing MCH-R1 remain poorly explored except in neuroblastoma or melanoma cell models.[12,14] Interestingly, both p53 and MAP kinase pathways contribute to mediate MCH effect through genuine MCH-R1 on neurite outgrowth in human neuroblastoma cells,[12] suggesting that MCH may play a role in neuronal cell differentiation and plasticity.

MCH-R2 STRUCTURE AND SIGNALING

The second MCH receptor, named here MCH-R2, was identified in the human genome by using in silico data mining (see the chapter on this in Peptide Biosynthesis/Processing section), and its cDNA was subsequently isolated from human brain cDNA libraries.[17] In contrast to the *MCH-R1* gene, which is intronless in its coding region, *MCH-R2* gene encompasses five coding exons and one noncoding exon and may produce receptor variants by alternative splicing that would lead to a truncated version of MCH-R2. It is noteworthy that human MCH-R1 and MCH-R2 display low sequence identity (only 36%), which is rather unusual for a family of GPCRs that bind an identical ligand, suggesting weak evolutionary constraints. In this context, the *MCH-R2* gene is found in teleots (pufferfish, zebrafish), dog, ferret, and primates (including human), but a functional *MCH-R2* gene is selectively lost in the rodent/lagomorph lineage. This proved to be useful for elucidating the physiological importance of the single MCH-R1 in rats and mice and also hampered the functional characterization of MCH-R2 in available animal models.

In mammals, the overall brain expression pattern of MCH-R2 seems quite similar to that of MCH-R1 but with a more restricted expression to the limbic regions, including medial and central amygdala, enthorhinal and temporal cortex, CA1 of the hippocampus, and to a lesser extent in the ventral hypothalamus and claustrum. In monkeys, MCH-R2 is strongly expressed in the arcuate and ventromedial nuclei. The differential distribution of the two MCH receptors, particularly in the human brain, may indicate distinct biological roles in mediating the large spectrum of MCH effects. In the periphery, weak MCH-R2 expression is detected in pituitary, adipose tissue, and pancreas, which also express MCH-R1, but the precise cellular distribution and functional relevance of MCH-R2 in these organs are presently unknown.

The signaling pathways associated with MCH-R2 activation have been studied only in transfected mammalian cells and proved to be much more restricted than those reported for MCH-R1. Indeed, MCH causes an increase in intracellular free Ca^{2+} levels and IP3 production, but has no effect on basal or forskolin-induced cAMP synthesis. This action is not blocked by PTX treatment indicating that MCH-R2 couples mainly to Gαq. The difference in G protein coupling between MCH-R1 and MCH-R2 could be ascribed to the marked sequence difference in the second and third intracellular loops that are essential for specific G-protein interaction in the GPCR family.

ACTIVE STRUCTURE OF THE MCH PEPTIDE AND RECEPTOR CONFORMATION

There have been numerous studies characterizing MCH analogs for their functional activity initially using fish skin bioassays and more recently cellular models expressing cloned mammalian MCH receptors.[9,10,23] All information converges toward the following model for MCH ligand–receptor interactions: (1) The cyclic peptide fragment ring (MCH 5–14 in fishes and MCH 7–16 in mammals; see Fig. 1) is necessary but not sufficient to get maximal activity; indeed the C-terminal part (mainly the Tyr 15 residue) of the fish peptide and additional amino acids flanking the ring structure (Arg6 and Trp17) in the rat/human MCH are required for agonist potency; (2) only Met 8, Arg 11, and Tyr 13 of the mammalian MCH are essential to elicit full and potent responses with both MCH receptors in binding and functional tests; (3) changes in Arg6, Leu 9-Gly 10, or Gln18 of the rat/human MCH generate peptides that are less efficient in binding or activating MCH-R2 than MCH-R1. In

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addition, studies using NMR spectrometry, circular dichroism, and molecular dynamics indicate conformational flexibility of the salmon MCH 5–14 core backbone, whereas mammalian MCH exhibits a rather limited variability in the 4–17 region.

Site-directed mutagenesis of the MCH-R1 and comparative molecular dynamics simulations of both MCH receptors in their free and ligand-bound conformation have highlighted the importance of ionic interactions between Arg 11 and Tyr 13 of rat/human MCH and portions of the receptors close to the E/DRY and NPxxY motifs under the active state.

BIOLOGICAL ACTIONS WITHIN THE BRAIN (AND PITUITARY)

Feeding Behavior and Energy Homeostasis

The dominant expression of MCH in the lateral hypothalamus of mammalian brains, the so-called feeding-drinking center, has early suggested that this peptide may regulate appetite and/or energy homeostasis. The role of MCH and MCH-R1 signaling in the control of feeding behavior and energy balance is now firmly established in rodents.[11,15,17,22,25,27–29]

In this chapter, we focus on the most important results regarding the regulation of feeding behavior and energy expenditure by MCH. First, acute icv injections of MCH (>5 μg per animal) transiently stimulate food intake in rats, an orexigenic effect which is not mediated by NPY, galanin, or orexins (also known as hypocretins), but could be blocked by αMSH (see corresponding peptide chapters in the book). Intrahypothalamic injection studies and electrophysiological characterization further identified the PVN, VMH, and Arc as major targets of MCH action, with differential effects in small-litter and overnourished rats. Interestingly, MCH also stimulates water consumption independently from its feeding and water osmolality actions, consistent with the strong adipsia observed in LHA-lesioned rats. Second, chronic icv infusion of MCH in rats or mice induces hyperphagia and body weight gain, especially on a moderate high-fat diet. The development of obesity is accompanied by an increase in liver and fat weights resulting in the lipogenic activity stimulation of white adipose tissue (WAT) and concomitant reduction of brown adipose tissue (BAT) functions. Further, plasma glucose, insulin and leptin levels are also raised in these animals, all these parameters being associated with obesity in men. Third, although acute or chronic icv injections in rodents of MCH-R1 agonists fully recapitulate the feeding and/or obese phenotype observed with MCH itself, central administration of MCH-R1 antagonists leads to a sustained reduction of appetite, body weight, and fat storage without overt alteration of lean mass. Peripheral MCH-R1 signaling may also be involved in this process through regulation of leptin or insulin secretion. Finally, in agreement with the pharmacological data, genetically modified mouse models also showed an important role of *MCH* and *MCH-R1* genes in appetite and, mainly, energy metabolism. Indeed, transgenic mice overexpressing the *MCH* gene develop hyperphagia, mild obesity, and insulin resistance, when maintained on a high-fat diet. Conversely, MCH gene ablation in mice leads to a leanness phenotype associated with hypophagia and an increase in oxygen consumption, both effects being highly dependent on the genetic background and food diet. In this regard, the reduction in body fat observed in mice combining MCH and leptin deficiencies, by crossing MCH null mice and *ob/ob* mice, does not result from feeding behavior changes (the mice remaining hyperphagic) but improvements in energy expenditure regulation (increased locomotor activity and resting metabolic rate). It is worth noting that targeted disruption of the MCH-R1 gene in mice maintained on regular chow also leads to a lean phenotype associated with both hyperphagia on the one hand and increased energy metabolism on the other hand. In agreement with the hyperactive phenotype, MCH-R1 null mice are resistant to high-fat-diet-induced obesity.

Overall, pharmacological studies and animal models have shown that MCH and MCH-R1 neuronal networks play a major role in the control of energy homeostasis by increasing food intake and mostly regulating energy expenditure and thermogenesis in mammals. Interestingly, MCH1 exerts an anorexigenic action in goldfish, whereas fasting selectively induces an increase in MCH2 mRNA expression in zebrafish,[6] suggesting major differences of action between the two MCH systems in teleosts.[5,6] The relative importance of central, neuroendocrine, and local influences versus sympathetic-innervated peripheral regulatory pathways involved in mediating the MCH effects on energy balance under normal and pathological conditions remain to be determined.

Stress Response and Anxiety

In fish, MCH depresses the stress-induced activity of the hypothalamic–pituitary–adrenal (HPA) axis and repeated moderate stress enhances MCH release in the neurohypophysis and *MCH* gene expression in the NLT or LVR groups of MCH neurons, depending on the stress paradigm. Early modulation of LVR-MCH neurons may be related to a central inhibitory action on the HPA axis, whereas the late response of NLT-MCH neurons may be secondary to their regulation on pigmentation. Interestingly, MCH synthesis and release seem to be enhanced under mild stress, whereas intense stress tends to supress MCH production, a regulatory balance that is under plasma glucocorticoid control.[4,5]

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In mammals, there are conflicting reports regarding the action and regulation of MCH on the HPA axis.[11,17] Indeed, icv injections of MCH in rats either increase or do not modify the basal secretion of ACTH in two separate studies. Results of the intravenous administration of MCH are consistent with a stimulatory effect on the HPA axis. On the contrary, icv-injected MCH inhibits ACTH release after mild stress such as ether or handling; this action can be reversed by the coadministration of NEI or αMSH. In addition, some chronic stressors, such as footshock stress or dehydration, induce a reduction in MCH mRNA expression and/or peptide release, whereas others, such as immobilization or cold stress, do not modify or even stimulate (after fasting for instance) *MCH* gene activity. Similarly, central injections of MCH agonists are either anxiogenic or anxiolytic and MCH-R1 antagonists have antidepressant-like or no anxiolytic effects. Differences in experimental parameters may explain this apparent confusing situation, but we could also consider the diurnal pattern of *MCH* gene expression (and probably peptide secretion) that seems to be higher at night and closely follows plasma glucocorticoid levels and the heterogeneity of the MCH neuronal population (see above). Both parameters could explain differential responses to distinct stimuli or even opposite regulation in MCH subpopulations under a single stress (for instance, a hypertonic saline regimen increases MCH mRNA levels in the ZI and decreases them in the LHA). Finally, MCH may have overt opposite actions in the neuroendocrine systems and in stress-mediated behaviors, a hypothesis that deserves to be carefully explored if MCH antagonists have to be used to treat depression and anxiety disorders.

Reward and Mesolimbic System

MCH neurons are projected massively to the cortex, nucleus accumbens shell (AcSh), and to a lesser extent to the ventral tegmental area (VTA), all major components of the reward control circuit.[7] MCH-R1 was found to be also strongly expressed in neurons containing D1 and D2 dopamine receptors, suggesting that MCH may regulate the mesolimbic system and modulate reward. Accordingly, *MCH-/-* or *MCH-R1-/-* mice carry marked modifications in dopamine release and reuptake and differential sensitization to psychostimulants such as amphetamine or cocaine. An important functional consequence of the alteration of the mesolimbic system in MCH -/- mice is the lack of hyperphagia induced by palatable diet. Administration of MCH-R1 antagonists to the AcSh blocks feeding and induces an antidepressant-like effect while MCH injection leads to opposite actions. Further, MCH signaling blockade with an MCH-R1 antagonist reduces cocaine self-administration and cocaine-induced reinstatement. Collectively, these results strongly suggest that the MCH system is a powerful potentiator of the dopaminergic response in the AsSh neurons and exhibits an important role in the reinforcement and reward processes linked to normal behavior (feeding behavior and stress response) or pathological situations (addiction to drugs).[2,11]

Other Functions

The actions of MCH and/or NEI on a large spectrum of behaviors have been studied in rats or wildtype and transgenic mice. These include sexual behavior, grooming, rearing, aggressive behavior, locomotion, exploratory behavior, learning, and memory formation. It is beyond the scope of this chapter to enumerate these behavioral studies discussed in exhaustive reviews.[1,16,17,24,27] Besides these multiple behavioral effects, evidence suggests that MCH neurons play an important role in sleep–wake regulation.[26] Early studies indicated that icv injection of low doses of MCH (<5 μg per rat) induces paradoxical sleep (PS also termed REM sleep) and slow wave sleep in rats. Reciprocally, MCH neuronal activity (monitored by cFos induction) increases selectively in PS recovery rats. Recently, reciprocal profiles of discharge were found for MCH and orexin neurons across the sleep–wake cycle. Overall, these results suggest a critical role for MCH in the entrance from slow-wave-sleep to paradoxal sleep.

Recent reviews[20,21] consider the local expression and role of MCH and its receptor outside the brain. Indeed, the importance of MCH/MCH-R1 in the digestive tract, adipose and bone tissues, cardiovascular or immune systems, for instance, should be considered in analyzing phenotype alterations in MCH null or overexpressing transgenic mice.

PATHOPHYSIOLOGICAL IMPLICATIONS

Feeding studies and characterization of MCH/MCH-R1 transgenic mice models have boosted interest in MCH as a suitable target for pharmaceutical intervention in the treatment of the obesity syndrome. Most of the efforts focus on the development of small-molecule antagonists for MCH-R1, because of the promising pharmacology of this receptor and the lack of suitable *in vivo* models for testing MCH-R2 -selective antagonists. Initially, a series of peptidic analogs with antagonist activity was generated and proved to counteract the orexigenic effect of MCH agonists but was ineffective alone on feeding behavior. This limited effect and the complications inherent in the use of peptidic analogs as therapeutic tools (blood–brain barrier crossing, sensitivity to peptidase degradation) prompted most of the pharmaceutical and biotech companies working in the obesity field to invest in high throughput and targeting screening for discovering new nonpeptide MCH-R1 antagonists.

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The first orally active MCH antagonist in rats was originally isolated by the Takeda group in 2002 and named T-226,296. This compound binds with high affinity to MCH-R1, suppresses the stimulatory effects of MCH on food intake, and significantly decreases feeding and body weight in rats under high-fat diet by reducing meal size but not meal frequency. The T-226296 molecule does not alter locomotor activity or induce malaise in a one-hour test period. These results provide encouraging clues that T-226296 molecule (and derivatives) may be effective in the treatment of overeating disorders.

The second nonpeptide MCH-R1 antagonist, named SNAP-7941, was characterized in 2002 by the Synaptic group. This molecule is a competitive antagonist of MCH with high affinity for MCH-R1 and displays potent anorectic effects when injected intraperitoneally (ip) alone or in preventing the orexigenic effect of MCH. Chronic ip administration of SNAP-7941 in diet-induced obese (DIO) rats suppresses feeding and provokes a sustained weight loss that is reversible after the termination of drug treatment. This compound does not have apparent toxic or aversive actions. Strikingly, oral administration of SNAP-7941 molecule leads to unexpected anxiolytic and antidepressant effects as shown in the rat forced-swim test, rat social interactions, and guinea pig maternal-separation vocalization tests. The potential antidepressant property of SNAP-7941 was independently replicated at the Institut de Recherche Servier and another MCH-R1 antagonist, SNAP-94847, displayed also anxyolytic properties under acute or chronic administration. Interestingly, different MCH-R1 antagonists developed either at Taisho Pharmaceutical Company (named ATC0065 and ATC0175) or in Lilly Research Laboratories (named GW803430) exhibit also antidepressant and anxiolytic effects in rodents after oral administration, without sedation or motor coordination impairment. These results provide further support for an anxiogenic effect of MCH, as found after MCH injection in the medial preoptic area or AcSh in rats, and offer novel approaches to treat depression and/or anxiety. However, conflicting results were reported regarding the role of MCH in the regulation of mood as found for the regulation of the HPA axis. Furthermore, the specificity of the MCH antagonists needs to be systematically evaluated in MCH-R1 null mouse models to preclude the possibility that the *in vivo* activities of these compounds may be mediated by another receptor. Efforts are also needed to identify MCH-R1 antagonists with low affinity for the hERG channel, precluding major side effects associated with cardiac dysregulations.

Because of these pioneering studies, several companies have synthesized, tested, and patented a variety of nonpeptide MCH antagonists acting at MCH-R1 (close to 60 patent applications are presently registered at http://www.xennexinc.com:8080/GeneIP/). As such, the compounds are claimed to be useful in suppressing appetite and in treating or preventing a wide range of diseases, including diabetes, hyperlipidemia, depression, anxiety, and associated somatic symptoms, neurodegenerative and psychiatric disorders, as well as even more distantly related pathologies such as bacterial, fungal, and viral infections. There is no doubt that, among this huge number of compounds, suitable candidates will be identified and subsequently enter clinical trials. However, major questions remain about the role of MCH and MCH-R1 in the control of energy balance (e.g. phenotype discrepancy between MCH and MCH-R1 null mice) and other behaviors (stress response, sexual activity, sleep, and learning). Further, the MCH physiological functions mediated by MCH-R2 in the human brain deserve thorough investigations and the development of suitable animal models.

REFERENCES

1. Adamantis A, de Lecea L. A role for melanin-concentrating hormone in learning and memory. *Peptides* 2009;**30**:2066–70.
2. Adamantidis A, de Lecea L. Physiological arousal: a role for hypothalamic systems. *Cell Mol Life Sci* 2008;**65**:1475–88.
3. Allaeys I, Bouyer K, Loudes C, Faivre-Bauman A, Petit F, Ortola C, et al. Characterization of MCH-gene overprinted-polypeptide-immunoreactive material in hypothalamus reveals an inhibitory role of pro-somatostatin1-64 on somatostatin secretion. *Eur J Neurosci* 2004;**19**:925–36.
4. Baker B. In: Vaudry H, Eberle A, editors. *Trans. in melanotropic peptides*, vol. 680. New York: New York Academy of Sciences Series; 1993. p. 279–89.
5. Baker BI. Melanin-concentrating hormone: a general vertebrate neuropeptide. *Int Rev Cytol* 1991;**126**:1–47.
6. Berman J, Skariah G, Maro GS, Mignot E, Mourrain P. Characterization of two melanin-concentrating hormone genes in zebrafish reveals evolutionary and physiological links with the mammalian MCH system. *J Comp Neurol* 2009;**517**:695–710.
7. Bittencourt JC, Presse F, Arias C, Peto C, Vaughan J, Nahon JL, et al. The Melanin-Concentrating Hormone system of the rat brain: an immuno- and hybridization histochemical characterization. *J Comp Neurol* 1992;**319**:218–45.
8. Borsu L, Presse F, Nahon JL. The AROM gene: spliced mRNAs encoding new DNA/RNA binding proteins are transcribed from the opposite strand of the melanin-concentrating hormone gene in mammals. *J Biol Chem* 2000;**275**:40576–87.
9. Boutin JA, Suply T, Audinot V, Rodriguez M, Beauverger P, Nicolas JP, et al. Melanin-concentrating hormone and its receptors: state of the art. *Can J Physiol Pharmacol* 2002;**80**:388–95.
10. Cardinaud B, Darré-Toulemonde F, Duhault J, Boutin JA, Nahon JL. Comparative analysis of melanin-concentrating hormone structure and activity in fishes and mammals. *Peptides* 2004;**25**:1623–32.
11. Chung S, Parks G, Lee C, Civelli O. Recent updates on the melanin-concentrating hormone (MCH) and its receptor system: lessons from MCH1R antagonists. *J Mol Neurosci* 2011;**43**:115–21.

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12. Cotta-Grand N, Rovère C, Guyon A, Cervantes A, Brau F, Nahon JL. *Peptides* 2009;**30**:2014–24.
13. Courseaux A, Nahon JL. Birth of two chimeric genes in the *Hominidae* lineage. *Science* 2001;**291**:1293–7.
14. Eberle AN, Mild G, Schlumberger SE, Drozdz R, Hintermann E, Zumsteg U. Expression and characterization of melanin-concentrating hormone receptors on mammalian cell lines. *Peptides* 2004;**25**: 1585–95.
15. Forray C. The MCH-receptor family: feeding brain disorders? *Curr Opin Pharmacol* 2003;**3**:85–9.
16. Griffond B, Baker BI. Cell and Molecular cell biology of melanin-concentrating hormone. *Int Rev Cytol* 2002;**213**:233–77.
17. Hervieu G, Maulon-Feraille L, Chambers JK, Cluderay JE, Wilson S, Presse F, et al. In: Quirion R, Björklund A, Hökfelt T, editors. *Trans. in handbook of chemical neuroanatomy Peptide receptors, part II,* vol. 20. Elsevier Sci.; 2003. p. 31–101.
18. Hogben LT, Slome D. *Trans. in Vol. VI, The dual character of endocrine coordination in amphibian colour change. Ser. B*, vol. 108. London: Proc. Roy. Soc.; 1931. p. 10–6.
19. Kawauchi H, Kawazoe I, Tsubokawa M, Kishida M, Baker BI. Characterization of melanin-concentrating hormone in chum salmon pituitaries. *Nature* 1983;**305**:321–3.
20. Kemp EH, Weetman AP. Melanin-concentrating hormone and melanin-concentrating hormone receptors in mammalian skin physiopathology. *Peptides* 2009;**30**:2071–5.
21. Lakaye B, Coumans B, Harray S, Grisar T. Melanin-concentrating hormone and immune function. *Peptides* 2009;**30**:2076–80.
22. Luthin DR. Anti-obesity effects of small molecule melanin-concentrating hormone receptor1 (MCHR1) antagonists. *Life Sci* 2007;**81**:423–40.
23. MacNeil DJ, Bednarek MA. MCH receptor peptide agonists and antagonists. *Peptides* 2009;**30**:2008–13.
24. Nahon JL. The melanin-concentrating hormone: from the peptide to the gene. *Crit Rev Neurobiol* 1994;**8**:221–62.
25. Nahon JL. The melanocortins and melanin-concentrating hormone in the central regulation of feeding behavior and energy homeostasis. *C R Biol* 2006;**329**:623–38.
26. Peyron C, Sapin E, Leger L, Luppi PH, Fort P. Role of the melanin-concentrating hormone neuropeptide in sleep regulation. *Peptides* 2009;**30**:2052–9.
27. Pissios P, Bradley RL, Maratos-Flier E. Expanding the scales: the multiple roles of MCH in regulating energy balance and other biological functions. *Endocr Rev* 2006;**27**:606–20.
28. Pissios P. Animal models of MCH function and what they tell us about its role in energy balance. *Peptides* 2009;**30**:2040–4.
29. Qu D, Ludwig DS, Gammeltoft S, Piper M, Pelleymounter MA, Cullen MJ, et al. A role for melanin-concentrating hormone in the central regulation of feeding behaviour. *Nature* 1996;**380**:243–7.
30. Risold PY, Croizier S, Leganeux K, Brischoux F, Fellmann D, Griffond B. The development of the MCH system. *Peptides* 2009; **30**:1969–72.
31. Schmeider S, Darré-Toulemonde F, Arguel MJ, Delerue-Audegond A, Christen R, Nahon JL. *BMC Evol Biol* 2008;**8**:330. doi:10.1186/1471-2148-8-330.
32. Vallarino M, Bruzzone F, Vaudry H. Neuroanatomical distribution of MCH in the brain and pituitary of submammalian vertebrates. *Peptides* 2009;**30**:1973–8.
33. Vaughan JM, Fischer WH, Hoeger C, Rivier J, Vale W. Characterization of melanin-concentrating hormone from rat hypothalamus. *Endocrinology* 1989;**125**:1660–5.
34. Viale A, Ortola C, Hervieu G, Furuta M, Barberot P, Steiner DF, et al. Cellular localization and role of prohormone convertases in the processing of Pro-melanin concentrating hormone in mammals. *J Biol Chem* 1998;**274**:6536–45.
35. Zhang C, Song Y, Thompson DA, Madonna MA, Milhauser GL, Toro S, et al. Pineal-specific agouti protein regulates teleost background adaptation. *Proc Natl Acad Sci U S A* 2010;**107**: 20164–71.

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Chapter 111

Melanocortins

Sylvie Jégou, Roger D. Cone, Alex N. Eberlé and Hubert Vaudry

ABSTRACT

Melanocortins are a family of related peptides comprising α-MSH, γ-MSH, and ACTH, which all derive from the precursor protein POMC. In the brain, processing of POMC gives rise essentially to α-MSH and γ-MSH. A prominent group of POMC neurons is located in the arcuate nucleus of the hypothalamus, and these neurons project toward multiple brain regions. Melanocortins exert a wide range of biological activities in the brain, including control of feeding, grooming, hyperalgesia, social and sexual behaviors, central regulation of inflammation, fever, and cardiovascular functions, as well as neurotrophic activities. Five melanocortin receptor subtypes (MC1-R to MC5-R) have been characterized. The central effects of melanocortins are mediated through two types of receptors, namely, MC3-R and MC4-R.

DISCOVERY

The term melanocortins refers to a family of structurally related peptides that includes α-melanocyte-stimulating hormone (α-MSH), β-MSH, γ-MSH, and adrenocorticotrophic hormone (ACTH). α-MSH and β-MSH were initially isolated and characterized from the porcine pituitary by virtue of their ability to induce darkening of frog skin.[15] ACTH was first characterized from the ovine pituitary on the basis of its capacity to stimulate adrenocortical activity.[22] Finally, β-lipotropin (β-LPH), whose sequence encompasses β-MSH, was isolated as a side fraction during the course of purification of ACTH from the ovine pituitary gland. The observation that the α-MSH sequence corresponds to the first third of the ACTH molecule and that the β-MSH and β-endorphin sequences are part of β-LPH suggested the existence of a precursor–product mode of biosynthesis for this family of peptides. This hypothesis was confirmed when Nakanishi et al.[26] reported the nucleotide sequence of a cDNA encoding a protein that encompasses both ACTH and β-LPH. This precursor protein, which has the potential to generate the opioid peptide β-endorphin, three MSH sequences (α-, β-, and γ-MSH), and ACTH, was termed proopiomelanocortin (POMC).

The POMC gene is primarily expressed in corticotroph cells of the pars distalis and melanotroph cells of the pars intermedia of the pituitary.[9] Besides, it has long been known that biologically active MSHs are also present in the hypothalamus[34] and that central administration of α-MSH facilitates learning and memory, elicits stretching, yawning and grooming, and induces antipyretic effects,[2,9] suggesting that melanocortins could also act as authentic neuropeptides. The occurrence of the highly potent opioid peptide β-endorphin within the precursor sequence further supported the notion that POMC could generate various neuroactive peptides. Finally, the cloning of melanocortin receptors (MC-Rs)[24] and the demonstration that some members of the MC-R family are expressed in the brain[12,29] confirmed the existence of a brain melanocortin system.

Structure of the Proopiomelanocortin mRNA/Gene

The POMC genes of all species examined so far exhibit essentially the same structural organization with 3 exons separated by 2 large introns.[9] In mammals, the second exon encodes for the signal peptide and the first 18 amino acid residues, whereas the third exon encodes for all bioactive POMC-derived peptides (Fig. 1). In mammals and amphibians, POMC encompasses four melanocortins namely α-MSH, β-MSH, γ-MSH, and ACTH (Fig. 2). In teleost fish, POMC lacks the γ-MSH region, whereas, in elasmobranch fish, POMC contains an additional MSH peptide named δ-MSH that is located between the ACTH and β-MSH sequences. The sequence of the POMC cDNA has been preserved in the regions coding for pro-γ-MSH/γ-MSH, ACTH/α-MSH, β-MSH, and β-endorphin. The POMC sequence is more variable in the regions corresponding to the joining peptide (JP) and the N-terminal portion of β/γ-LPH. Most of the dibasic motifs that are typical cleavage sites for prohormone convertases are conserved across species, except for the Arg^{63} and Lys^{190} of human POMC that are lacking in the rat and mouse POMC

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00111-1
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sequences, indicating that, in rodents, γ3-MSH and γ-LPH cannot be further processed to generate γ-MSH and β-MSH, respectively. In contrast to other mammals that have only one POMC gene, the mouse also possesses a pseudogene. Several submammalian vertebrates, including xenopus, salmon, trout, and sturgeon, possess two functional POMC genes.

Processing of Proopiomelanocortin

After the removal of the signal peptide from the precursor protein, the first steps of POMC maturation consist of glycosylation of pro-γ-MSH and ACTH, and phosphorylation of ACTH (Fig. 1), two events that apparently do not affect processing or release of the mature peptides.[9] Then, prohormone convertases 1 and 2 (PC1 and PC2) catalyze differential cleavage of POMC at dibasic or tetrabasic sites in a tissue-dependent manner (see Convertase chapters in the Peptide Biosynthesis/Processing section of this book). Thus, in corticotroph cells of the pars distalis, which only express PC1, cleavage occurs at three Lys-Arg sites to generate the large N-terminal fragment pro-γ-MSH, JP, ACTH, and the large C-terminal peptide β-LPH. In melanotroph cells of the pars intermedia and in hypothalamic POMC neurons, which both express PC1 and PC2, processing of the precursor gives rise to the formation of shorter peptides including (Lys-)γ3-MSH, $ACTH_{1-17}$, corticotrophin-like intermediate lobe peptide (CLIP), γ-LPH, and β-endorphin. (Lys-)γ3-MSH can be further processed to yield (Lys-)γ2-MSH. Carboxyterminal basic residues are trimmed from the cleaved peptides by

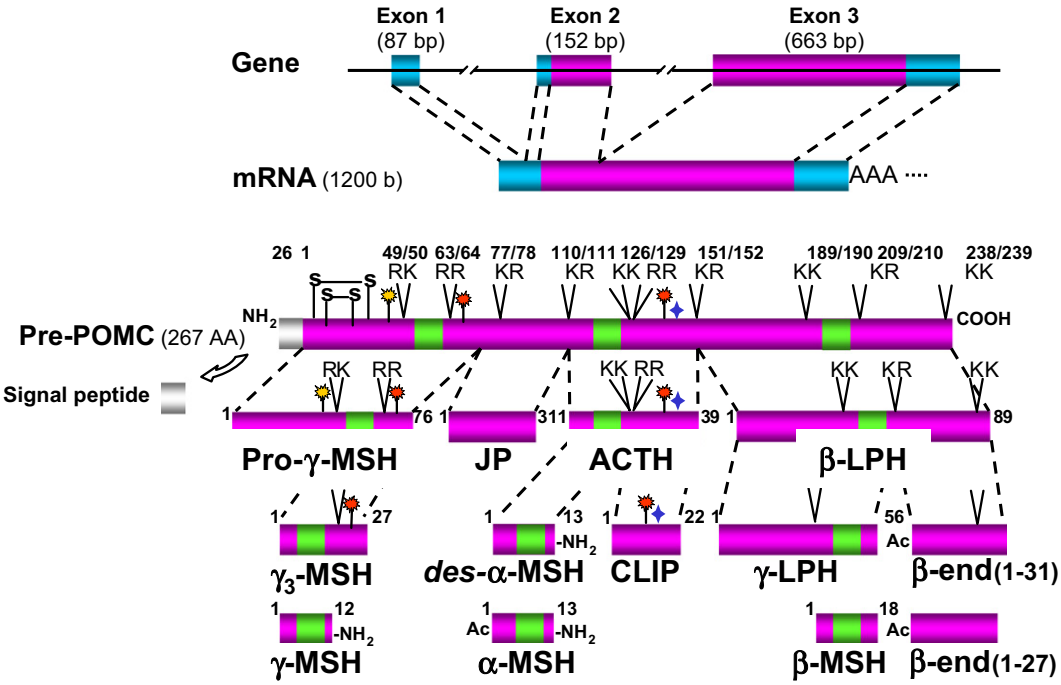

FIGURE 1 Structural organization of the human POMC gene and processing of the precursor in neurons. ⋁: paired basic residue cleavage sites where processsing of POMC occurs to yield various peptide products. The numbers of the amino acid residues contributing to the cleavage site are indicated. K = Lys ; R = Arg, ■: MSH core sequence, ✹: site of N-glycosylation, ✹: site of O-glycosylation, ✦: site of phosphorylation, CLIP : corticotropin-like intermediate lobe peptide, JP : joining peptide. See color plate 33.

ACTH	NH_2-Ser-Tyr-Ser-Met-Glu-*His-Phe-Arg-Trp*-Gly-Lys-Pro-Val-Gly-Lys-Lys-Arg-Arg-Pro-Val-Lys-Val-Tyr-Pro-Asn-Gly-Ala-Glu-Asp-Glu-Ser-Ala-Glu-Ala-Phe-Pro-Leu-Glu-Phe-OH
α-MSH	Ac-Ser-Tyr-Ser-Met-Glu-*His-Phe-Arg-Trp*-Gly-Lys-Pro-Val-NH_2
β-MSH	NH_2-Asp-Glu-Gly-Pro-Tyr-Arg-Met-Glu-*His-Phe-Arg-Trp*-Gly-Ser-Pro-Pro-Lys-Asp-OH
γ1-MSH	NH_2-Lys-Tyr-Val-Met-Gly-*His-Phe-Arg-Trp*-Asp-Arg-Phe-NH_2
γ3-MSH	NH_2-Lys-Tyr-Val-Met-Gly-*His-Phe-Arg-Trp*-Asp-Arg-Phe-Gly-Arg-Arg-Asn-Ser-Ser-Ser-Ser-Gly-Ser-Ser-Gly-Ala-Gly-Gln-OH

FIGURE 2 Amino acid sequences of human melanocortins. All melanocortins share the invariant sequence *His-Phe-Arg-Trp*.

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carboxypeptidase E. Then, $ACTH_{1-14}$ and (Lys-)γ2-MSH are C-terminally amidated by peptidylglycine-α-amidating-monooxygenase (PAM) to form des-Nα-acetyl α-MSH and (Lys)γ1-MSH, respectively. Finally, Nα-acetylation of des-Nα-acetyl α-MSH and β-endorphin may occur, and this last event profoundly affects the biological activity of the two peptides: mono- and diacetylation of the N-terminal serine residue of des-Nα-acetyl α-MSH generally enhances the biological potency of the peptide, whereas acetylation of the N-terminal tyrosine residue of β-endorphin suppresses its analgesic activity.[2]

Distribution of POMC mRNA and Melanocortins in the Brain

The arcuate nucleus (Arc) of the hypothalamus and the nucleus of the solitary tract (NTS) in the medulla are the only two sites of POMC expression in the rodent brain (Fig. 3 left).[18,27] In primates, POMC expression has been found in the infundibular nucleus but has not yet been reported in the NTS. In rat, the predominant POMC neuronal population is located in the Arc. POMC neurons project ventrally to the median eminence, rostrally to the anterior hypothalamic and medial preoptic nuclei, the nucleus of the stria terminalis, and the lateral septum, dorsally to the thalamic periventricular nucleus and the periventricular, paraventricular, dorsomedial, and posterior hypothalamic nuclei, laterally to the amygdala, and caudally to the substantia nigra, the dorsal raphe nucleus, the periaqueductal gray, the parabrachial nucleus, the locus coeruleus, the nucleus ambiguus, the lateral reticular nucleus, and the nucleus of the solitary tract (Fig. 3, left). NTS POMC neurons project within the nucleus solitarius, as well as to the nucleus reticularis gigantocellularis, the A1 group, and all along the spinal cord (Fig. 3, left).

Melanocortin Receptors and Signaling Cascades

Five subtypes of MC-Rs have been cloned in mammals and termed MC1-R to MC5-R.[32] All MC-Rs belong to the G protein-coupled receptors (GPCRs) superfamily and activate the adenylyl cyclase–protein kinase A pathway via coupling to Gs. MC3-R is also coupled to the inositol phospholipid/Ca^{2+}-mediated signaling cascade. In addition, MC3-R and MC4-R activate the mitogen-activated kinase pathway[28,31] and MC4-R inhibits c-Jun N-terminal kinase.[6] In the hypothalamic cell lines GT-7 and GT1-1, MC4-R activates pertussis toxin-sensitive $G_{i/o}$ proteins and increases intracellular Ca^{2+} through Gq/phospholipase C-dependent signaling pathways, respectively.[4] Like many other GPCRs, MC-Rs can form dimeric or oligomeric complexes that confer distinct functions both *in vitro* and *vivo*.[3] Furthermore, MC1-R, MC3-R and MC4-R have been reported to have a constitutive activity.[10,28,31] The MC-R subtypes exhibit distinct anatomical distribution and ligand selectivity (Table 1). Thus, MC1-R is expressed almost exclusively in melanocytes and mediates the effects of melanocortins on skin and coat pigmentation. MC1-R is also expressed at a much lower level in some other peripheral tissues, for example, in human adipose tissue, with elevated expression in obese subjects. MC2-R is expressed primarily in adrenocortical cells and mediates the effects of ACTH on corticosteroid secretion. MC3-R and MC4-R both occur in the brain (Fig. 3, right): MC3-R is expressed primarily within the hypothalamus, with limited expression in the thalamus and brainstem. In particular, MC3-R is abundantly expressed in POMC and agouti-related protein (AgRP)/neuropeptide-Y neurons of the Arc.[17,23] MC3-R is not expressed in the hindbrain and spinal cord. Conversely, MC4-R is widely distributed in the CNS including the autonomic centers of the hindbrain and the spinal cord. MC5-R is primarily expressed in exocrine

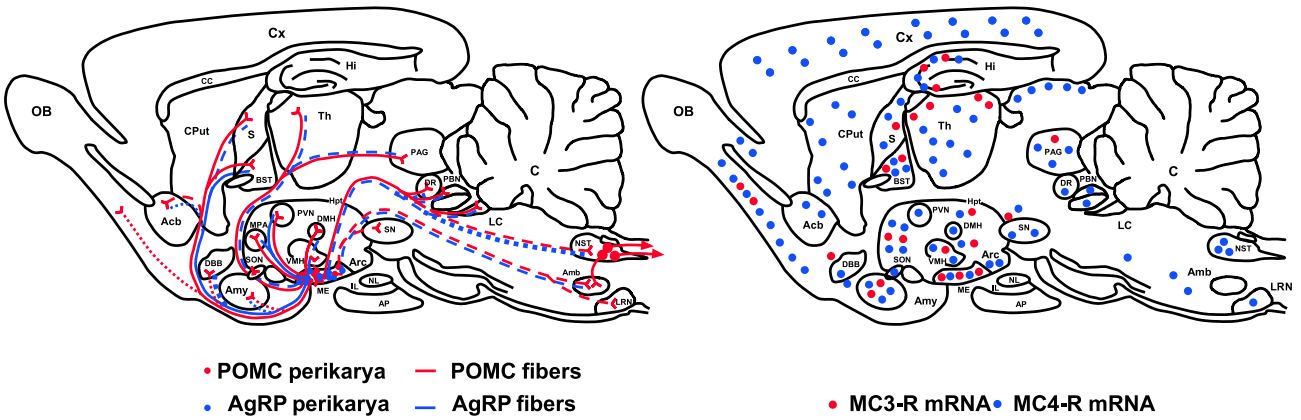

FIGURE 3 Sagittal section of the rat brain depicting the distribution of POMC and AgRP neuronal systems (left). Solid lines denote high density, and dashed lines denote moderate density of fibers. Comparative distribution of MC3-R and MC4-R mRNA expression in the rat brain (right). See color plate 34.

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TABLE 1 Pharmacological Properties and Distribution of Melanocortin Receptor Subtypes

	Agonists potency/endogenous antagonists	Tissue distribution
MC1-R	α-MSH = ACTH > β-MSH >> γ-MSH/Agouti	Melanocytes, keratinocytes macrophages, leukocytes, adipose tissue
MC2-R	ACTH	Adrenal cortex
MC3-R	α-MSH = β-MSH = γ-MSH = ACTH/AgRP	CNS, gut, kidney, testis, ovary, skeletal muscle, placenta, mammary gland
MC4-R	α-MSH = ACTH > β-MSH >> γ-MSH/Agouti, AgRP	CNS
MC5-R	α-MSH > ACTH > β-MSH > γ-MSH	Exocrine glands, muscle, CNS

glands and in skeletal muscle, but low levels of MC5-R mRNA are also found in a few regions of the rat brain notably in the olfactory bulb, cerebral cortex, striatum, and medulla. Two endogenous melanocortin receptor antagonists participate in the control of melanocortin signaling, that is the agouti protein, expressed in dermal papillae, which confers the yellow color typical of agouti mice, and AgRP, mainly expressed in the hypothalamus and adrenal gland, which acts as an orexigenic peptide.[8] AgRP-containing neurons of the Arc project globally to the same hypothalamic and septal regions as POMC-containing neurons (Fig. 3, left), indicating that AgRP can actually modulate melanocortin neurotransmission.

Biological Actions within the Brain

1. Food intake and energy homeostasis

This important function of melanocortins topic is covered in the "Ingestive Peptides" section of this Handbook.

2. Stretching–yawning syndrome and grooming

One of the first reported behaviors induced by intracerebroventricular (icv) injection of melanocortins are the stretching–yawning syndrome (SYS) and excessive grooming.[11,13] The brain regions that respond to local injection of $ACTH_{1–24}$ and α-MSH with SYS and grooming behavior are the hypothalamic periventricular regions surrounding the third ventricle including the paraventricular nucleus, the dorsomedial nucleus, the anterior hypothalamic area, and the ventromedial nucleus.[1] Grooming is also elicited by local injection of $ACTH_{1–24}$ in the periaqueductal gray or the substantia nigra. Excessive grooming is observed when animals are exposed to a novel environment and various studies converge to show the implication of brain endogenous melanocortins in this process. Structure–activity relationships indicate that MC4-R is probably involved in melanocortin-induced SYS[1] and a functional knockout rat model for MC4-R shows that melanocortin-induced grooming is exclusively mediated by MC4-R.[25] Consistent with the findings that melanocortins induce stress-related behaviors in rodents, several pharmacological studies indicate a role of MC4-R in regulation of the activity of the hypothalamic–pituitary–adrenal (HPA) axis. For example, central injection of α-MSH or an MC4-R agonist increases corticotropin-releasing factor expression in the paraventricular nucleus and elevates plasma ACTH and corticosterone levels, and these responses are attenuated by a selective MC4-R antagonist. By contrast, administration of a selective MC3-R agonist has no effect on HPA axis activity.[7]

3. Sexual and social behaviors

Local injection of α-MSH or $ACTH_{1–24}$ in the hypothalamic periventricular region induces penile erection in rats,[1] and MC3-R/MC4-R have been implicated in the modulation of erectile and sexual function.[1,33] Melanocortins also seem to exert their effects in spinal cord as intrathecal administration of the synthetic MC-R agonist MTII induces penile erection in rats. By contrast, icv injection of $ACTH_{1–24}$ or $ACTH_{4–10}$ delays the copulatory behavior in inexperienced male rats, and $ACTH_{1–24}$ reduces sexual performance. In female rats that exhibit a low level of receptivity, α-MSH stimulates lordosis, whereas in receptive females, α-MSH has the opposite effect. Neuroanatomical results provide evidence for a role of hypothalamic MC4-R in the efferent control of rodent clitoris and vagina. Clinical studies show that MC-R agonists can induce transient erections in men with erectile dysfunction and increase sexual desire and arousal in female subjects with sexual disorder.[30,35] The Administration of melanocortins icv generally reduces social interactions and increases aggressive behavior. The same effects are observed when α-MSH or $ACTH_{4–10}$ is injected into the ventromedial hypothalamic nucleus or the septum, respectively. The MC4-R is involved in social interactions and mediates the anxiogenic effect of brain melanocortins. α-MSH in paraventricular and dorsomedial hypothalamic

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nuclei and in the central nucleus of the amygdala might play a role in these processes.[19] In rodents, peripheral and central melanocortins seem to influence sexual activity and social interactions in a coordinate manner, because circulating α-MSH stimulates the release of pheromone from sebaceous and preputial glands, which in turn influence sexual behavior, while ACTH stimulates the secretion of glucocorticoids, which promote aggressive behavior.

4. Antipyretic and anti-inflammatory activities

Central administration of α-MSH provokes marked antipyretic effects in different species.[9,21] In rabbits, α-MSH inhibits cytokine-induced fever and, reciprocally, fever causes an increase in α-MSH concentrations in the septum. In rats, α-MSH reduces lipopolysaccharide-evoked fever, and this effect is antagonized by an MC4-R antagonist. Enhanced febrile responses to interleukin-1 (IL-1) and to endotoxin have been reported in the rabbit and rat after icv injection of either an α-MSH antiserum or an MC3-R/MC4-R antagonist, indicating that endogenous α-MSH actually exerts antipyretic activity. Melanocortins inhibit the hypothalamic production of proinflammatory molecules such as nitric oxide and prostaglandins induced by IL-1β. MC4-R agonists also attenuate brain inflammation and promote neuronal survival.[21]

Melanocortins inhibit different types of inflammatory responses including acute and chronic inflammation and contact hypersensitivity. Injection of α-MSH icv inhibits the response to tumor necrosis factor-α in endotoxin-induced brain inflammation and cutaneous inflammation provoked by the application of cytokines. α-MSH can only reduce inflammation induced in the mouse hind paw when the spinal cord is intact, indicating that α-MSH probably acts through descending neurogenic pathways capable of modulating inflammation in peripheral tissues. A variety of effects of central IL-1β administration are blocked by α-MSH such as activation of the HPA axis and the anxiogenic behavior and memory impairment induced by this cytokine. The protective effect of α-MSH on impairment memory consolidation induced by IL-1β is mediated by MC4-R.

5. Nerve generation and neuroprotection

Perinatal treatment with melanocortins enhances the maturation of the CNS and accelerates maturation of the neuromuscular system. In various peripheral nerve injury models, melanocortins are effective growth factors improving axonal regeneration, muscle reinnervation, and recovery of sensory and motor function.[2,9] In addition, melanocortins stimulate neurite outgrowth in neuroblastoma cells and primary cultures of dorsal root ganglia and spinal cord neurons. The stimulatory effect of α-MSH on Neuro 2A cells is blocked by a specific MC4-R antagonist. Because MC4-R is the only receptor subtype found in the spinal cord and in sympathetic ganglia, MC4-R probably mediates the neurotrophic effects of melanocortins. However, the $ACTH_{4–9}$ analog ORG2766, which fails to activate MC-Rs, also enhances recovery of neuromuscular functions, suggesting that a distinct receptor, which does not belong to the MCR family may be involved. Nerve injury provokes an increase in POMC expression and ACTH/MSH immunoreactivity in mouse spinal cord motoneurons. ACTH/MSH levels are also higher in Wobler mice, which exhibit motoneuron disease, and in diabetic neuropathy. An increase in ACTH binding sites also occurs on diabetic, developing, and dystrophic muscle, suggesting that melanocortins may act as paracrine factors involved in the innervation of muscle, probably through MC5-R which is uniquely expressed in muscle. Melanocortins also exert strong protective effects against damage consequent to global or focal cerebral ischemia in gerbils and rats.[5] Melanocortins act by blocking several ischemia-relayed mechanisms of damage, through the activation of MC4-R, in a broad time therapeutic window and exhibit long lasting effects.

6. Cognitive functions

Melanocortins restore the deficient acquisition of shuttle box avoidance behavior in hypophysectomized rats. ACTH and α-MSH facilitate acquisition and delay extinction of active and passive avoidance responses and of rewarded behavior in intact rats, suggesting that these peptides influence motivation, attention, learning, and memory.[2,9] Although α-MSH and β-MSH have similar positive effects on active and passive avoidance, γ-MSH facilitates extinction of active avoidance and attenuates passive avoidance behavior. The short-chain analog $MSH/ACTH_{4–7}$ is the smallest fragment that exhibits full activity on learning and memory, whereas the minimum sequence required to stimulate MC-Rs is $MSH/ACTH_{6–9}$. Indeed, structure-activity relationship studies strongly suggest that the effects of melanocortins on avoidance behavior are not mediated by a receptor of the MC-Rs family.

7. Cardiovascular effects

Melanocortins exert a variety of effects on the cardiovascular system.[16] Injection of γ2-MSH or $MSH/ACTH_{4–10}$ icv induces a slight pressor effect that is not mediated through any of the identified MC-Rs but may involve FMRFamide-stimulated sodium-gated channel (see the chapter on RFamide peptides in this section of the book), which has affinity for peptides that possess a C-terminal Arg-Phe-NH_2 sequence like γ1-MSH. Microinjection of γ2-MSH into the caudal part of the NTS causes bradycardia and reduces blood pressure through reduction in sympathetic nervous system activity. A depressor effect is also observed when α-MSH is injected in the dorsal motor nucleus of the vagus nerve or the NTS where MC4-R is abundantly expressed, and the

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response is blocked by an MC4-R antagonist indicating that the depressor activity of melanocortins is probably mediated by MC4-R. Stimulation of POMC neurons in the Arc has a depressor effect that is mediated through the dorsal vagal complex, suggesting that endogenous melanocortins may play a role in the control of cardiovascular functions. However, other mechanisms for central cardiovascular regulations by melanocortins are probably involved because icv administration of α-MSH leads to increased mean arterial pressure and heart rate, concomitant with the increase in the lumbar sympathetic nerve activity. Mice lacking MC4-R do not exhibit a cardiovascular response to icv injection of α-MSH, providing evidence for MC4-R implication in these effects. Clinical studies indicate that the prevalence of hypertension is lower in MC4-R-deficient patients than in control subjects with a similar obesity. Administration of a highly selective MC4-R to overweight or obese volunteers leads to an increase in systolic and diastolic blood pressure.[14]

8. Interactions with opiates

The melanocortin and opiate systems have opposite activities in many tests and may be considered as functional antagonists.[2] Central administration of α-MSH and ACTH causes hyperalgesia in various pain models and reverses the analgesic effect of morphine and β-endorphin. The antinociceptive response of morphine is antagonized by an MC-R agonist and potentiated by an MC4-R antagonist. Administration of melanocortins icv in morphine-dependent rats precipitates a typical opiate-withdrawal syndrome, whereas central injection of an MC4-R antagonist prevents morphine tolerance and withdrawal hyperalgesia.[20] Reciprocally, chronic administration of morphine downregulates the expression of POMC in the hypothalamus and MC4-R in the striatum and periaqueductal gray, two regions implicated in drug reward and withdrawal. Melanocortinergic and opioidergic systems also interact in the regulation of food intake. Opioid antagonists can block feeding induced by AgRP, whereas icv β-endorphin-induced feeding is reduced by pretreatment with a melanocortin agonist.

Physiological and Pathophysiological Implications

Cloning of MC-Rs, studies with genetic models, and development of selective MC-R ligands have opened new perspectives for the comprehension of the physiological roles of melanocortins. In particular, the role of α-MSH, MC3-R, and MC4-R in feeding and energy homeostasis as well as sexual activity has received a great deal of attention and has provided a framework to explore the melanocortin system for the treatment of obesity or other metabolic disorders, and sexual dysfunctions. Indeed, an MC-R agonist has proven to be effective in treating erectile dysfunction and increasing sexual desire and arousal in women in clinical trials.[30,35] The recent development of a selective, nonpeptide MC4-R antagonist has revealed that MC4-R plays an important role in stress responses and in the regulation of emotional states, suggesting that blockade of MC4-R may be of therapeutic value for the treatment of stress-related disorders such as anxiety and depression.[7] Moreover, antagonists of MC4-R may be regarded as possible therapeutic modulators of nociception and morphine tolerance.[20] α-MSH acts centrally to inhibit fever and inflammation, and a number of potential therapeutic targets for melanocortins have been proposed based on preclinical investigations in inflammatory disorders, such as acute and chronic inflammation diseases, brain inflammation, neurodegenerative diseases, and systemic host reactions.[5]

REFERENCES

1. Argiolas A, Melis MR, Murgia S, Schioth HB. ACTH- and alpha-MSH-induced grooming, stretching, yawning and penile erection in male rats: site of action in the brain and role of melanocortin receptors. *Brain Res Bull* 2000;**51**:425–31.
2. Bertolini A, Tacchi R, Vergoni AN. Brain effects of melanocortins. *Pharmacol Res* 2009;**59**:13–47.
3. Biebermann H, Krude H, Elsner A, Chubanov V, Gudermann T, Grüters A. Autosomal-dominant mode of inheritance of a melanocortin-4 receptor mutation in a patient with severe early-onset obesity is due to a dominant-negative effect caused by receptor dimerization. *Diabetes* 2003;**52**:2984–8.
4. Büch TR, Heling D, Damm E, Gudermann T, Breit A. Pertussis toxin-sensitive signaling of melanocortin-4 receptors in hypothalamic GT1-7 cells defines agouti-related protein as a biased agonist. *J Biol Chem* 2009;**284**:26411–20.
5. Catania A. Neuroprotective actions of melanocortins: a therapeutic opportunity. *Trends Neurosci* 2008;**31**:353–60.
6. Chai B, Li JY, Zhang W, Wang H, Mulholland MW. Melanocortin-4 receptor activation inhibits c-Jun N-terminal kinase activity and promotes insulin signaling. *Peptides* 2009;**30**:1098–104.
7. Chaki S, Okubo T. Melanocortin-4 receptor antagonists for the treatment of depression and anxiety disorders. *Curr Top Med Chem* 2007;**7**:1145–51.
8. Cone RD. Studies on the physiological functions of the melanocortin system. *Endocr Rev* 2006;**27**:736–49.
9. Eberle AN. *The melanotropins: chemistry, physiology and mechanisms of action*. Basel: Karger; 1988.
10. Eberle AN, Bódi J, Orosz G, Süli-Vargha H, Jäggin V, Zumsteg U. Antagonist and agonist activities of the mouse agouti protein fragment (91-131) at the melanocortin-1 receptor. *J Recept Signal Transduct Res* 2001;**21**:25–45.
11. Ferrari W. Behavioural changes in animals after intracisternal injection with adrenocorticotrophic hormone and melanocyte-stimulating hormone. *Nature* 1958;**181**:925–6.
12. Gantz I, Miwa H, Konda Y, Shimoto Y, Tashiro T, Watson SJ, et al. Molecular cloning, expression, and gene localization of a fourth melanocortin receptor. *J Biol Chem* 1993;**268**:15174–9.

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13. Gispen WH, Wiegant VM, Greven HM, de Wied D. The induction of excessive grooming in the rat by intraventricular application of peptides derived from ACTH: structure-activity studies. *Life Sci* 1975;**17**:645–52.
14. Greenfield JR, Miller JW, Keogh JM, Henning E, Satterwhite JH, Cameron GS, et al. Modulation of blood pressure by central melanocortinergic pathways. *N Engl J Med* 2009;**360**:44–52.
15. Harris JI, Lerner AB. Amino-acid sequence of the alpha-melanocyte-stimulating hormone. *Nature* 1957;**179**:1346–7.
16. Humphreys MH, Ni XP, Pearce D. Cardiovascular effects of melanocortins. *Eur J Pharmacol* 2011;**660**:43–52.
17. Jégou S, Boutelet I, Vaudry H. Melanocortin-3 receptor mRNA expression in pro-opiomelanocortin neurones of the rat arcuate nucleus. *J Neuroendocrinol* 2000;**12**:501–5.
18. Joseph SA, Pilcher WH, Bennett-Clarke C. Immunocytochemical localization of ACTH perikarya in nucleus tractus solitarius: evidence for a second opiocortin neuronal system. *Neurosci Lett* 1983;**38**:221–5.
19. Kokare DM, Dandekar MP, Singru PS, Gupta GL, Subhedar NK. Involvement of alpha-MSH in the social isolation induced anxiety- and depression-like behaviors in rat. *Neuropharmacology* 2010;**58**:1009–18.
20. Kalange AS, Kokare DM, Singru PS, Upadhya MA, Chopde CT, Subhedar NK. Central administration of selective melanocortin 4 receptor antagonist HS014 prevents morphine tolerance and withdrawal hyperalgesia. *Brain Res* 2007;**1181**:10–20.
21. Lasaga M, Debeljuk L, Durand D, Scimonelli TN, Caruso C. Role of alpha-melanocyte stimulating hormone and melanocortin 4 receptor in brain inflammation. *Peptides* 2008;**29**:1825–35.
22. Li CH, Geschwind II, Dixon JS, Levy AL, Harris JE. Corticotropins (ACTH). I. Isolation of alpha-corticotropin from sheep pituitary glands. *J Biol Chem* 1955;**213**:171–85.
23. Mounien L, Bizet P, Boutelet I, Vaudry H, Jégou S. Expression of melanocortin MC3 and MC4 receptor mRNAs by neuropeptide Y neurons in the rat arcuate nucleus. *Neuroendocrinology* 2005;**82**:164–70.
24. Mountjoy KG, Robbins LS, Mortrud MT, Cone RD. The cloning of a family of genes that encode the melanocortin receptors. *Science* 1992;**257**:1248–51.
25. Mul JD, van Boxtel R, Bergen DJ, Brans MA, Brakkee JH, Toonen PW, et al. Melanocortin receptor 4 deficiency affects body weight regulation, grooming behavior, and substrate preference in the rat. *Obesity* 2011;**20**:612–21.
26. Nakanishi S, Inoue A, Kita T, Nakamura M, Chang AC, Cohen SN, et al. Nucleotide sequence of cloned cDNA for bovine corticotropin-beta-lipotropin precursor. *Nature* 1979;**278**:423–7.
27. O'Donohue TL, Dorsa DM. The opiomelanotropinergic neuronal and endocrine systems. *Peptides* 1982;**3**:353–95.
28. Renquist BJ, Lippert RN, Sebag JA, Ellacott KL, Cone RD. Physiological roles of the melanocortin MC_3 receptor. *Eur J Pharmacol* 2011;**660**:13–20.
29. Roselli-Rehfuss L, Mountjoy KG, Robbins LS, Mortrud MT, Low MJ, Tatro JB, et al. Identification of a receptor for gamma melanotropin and other proopiomelanocortin peptides in the hypothalamus and limbic system. *Proc Natl Acad Sci U S A* 1993;**90**:8856–60.
30. Safarinejad MR. Evaluation of the safety and efficacy of bremelanotide, a melanocortin receptor agonist, in female subjects with arousal disorder: a double-blind placebo-controlled, fixed dose, randomized study. *J Sex Med* 2008;**5**:887–97.
31. Tao YX. The melanocortin-4 receptor: physiology, pharmacology and pathophysiology. *Endocr Rev* 2010;**31**:506–43.
32. Tatro JB. Melanocortins receptor expression and function in the nervous system. In: Cone RD, editor. *The melanocortin receptors*. Totowa: Humana Press Inc; 2000. p. 173–207.
33. Van der Ploeg LH, Martin WJ, Howard AD, Nargund RP, Austin CP, Guan x, et al. A role for the melanocortin 4 receptor in sexual function. *Proc Natl Acad Sci U S A* 2002;**99**:11381–6.
34. Vaudry H, Tonon MC, Delarue C, Vaillant R, Kraicer J. Biological and radioimmunological evidence for melanocyte stimulating hormones (MSH) of extrapituitary origin in the rat brain. *Neuroendocrinology* 1978;**27**:9–24.
35. Wessels H, Gralnek D, Dorr R, Hruby VJ, Hadley ME, Levin N. Effect of an alpha-melanocyte stimulating hormone analog on penile erection and sexual desire in men with organic erectile dysfunction. *Urology* 2000;**56**:641–6.

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Chapter 112

MIF-1/Tyr-MIF-1

Abba J. Kastin and Weihong Pan

ABSTRACT

The MIF-1/Tyr-MIF-1 family of peptides has served to introduce several novel concepts into the field of biologically active peptides. The best known of these showed that most peptides can cross the blood–brain barrier in intact form by quantifiable transport systems. Some, like MIF-1 but not Tyr-MIF-1, enter the brain by a saturable process and a few, like Tyr-MIF-1 but not MIF-1, leave the brain by a saturable process. MIF-1 established the existence of antiopioid peptide actions, leading to the substantiated prediction that other peptides would be found with similar antiopioid actions. MIF-1 was also the first hypothalamic peptide shown to act on higher centers of the brain, including clinical effects in Parkinson's disease and mental depression. Thus, this family of small peptides has served a prototypic function.

Discovery of MIF-1: The search for the mammalian hypothalamic factor controlling the release of melanocyte-stimulating hormone (MSH; melanocortin) did not begin until one of us decided to look for the hypothalamic regulation of what was then the only mammalian pituitary hormone whose release was not being actively investigated.[14] After determining that unlike most pituitary hormones, the primary release of MSH was inhibited by the hypothalamus rather than being stimulated, the tripeptide Pro-Leu-Gly-NH_2 was isolated from bovine hypothalamic tissue and named MSH-release inhibiting factor (MIF).[26] Others also found MIF to inhibit MSH release in mammals,[29,31] but since it did not seem to do this robustly in all situations the numeral "1" was added to the abbreviation, resulting in its designation as MIF-1. Essentially no further work was done on this subject until 2009 when more sophisticated mass spectrometric quantification of this tripeptide using triple quadrupole multiple reaction monitoring established the natural occurrence of MIF-1 in the hypothalamus as well as elsewhere in the brain.[25]

Discovery of Tyr-MIF-1 and related peptides: Most radioimmunoassays involve the labeling of a peptide with radioactive iodine, but MIF-1 does not contain easily iodinated amino acids. To facilitate early research with MIF-1, Tyr was added to the N-terminal of MIF-1, resulting in Tyr-MIF-1 (Tyr-Pro-Leu-Gly-NH_2). Surprisingly, use of the resulting assay revealed that Tyr-MIF-1-like immunoreactivity was widely present in the rodent brain. The assay then was used in the isolation of Tyr-MIF-1 from bovine hypothalamus[12] as well as human brain tissue obtained many hours after autopsy.[13] Use of a sample of fresh human brain showed a 500-fold increase in the amount of Tyr-MIF-1 isolated. Tyr-W-MIF-1 (Tyr-Pro-Trp-Gly-NH_2) was first isolated from human frontal cortex,[11] as was Tyr-K-MIF-1 (Tyr-Pro-Lys-Gly-NH_2), and then from bovine hypothalamus.

Precursor/processing of MIF-1: Many years ago it was shown that MIF can be split from oxytocin by an exopeptidase present in a microsomal preparation,[5,32] although a group from Utrecht reported that oxytocin was not the source of MIF-1.[4] More recently, incubation of stable isotope-labeled oxytocin with brain tissue preparations showed that at least some MIF-1 could arise from oxytocin in the hypothalamus, but this probably does not explain the presence of MIF-1 in brain cortex.[25] Moreover, MIF-1 does not arise from Tyr-MIF-1 in blood[19] or brain homogenates.[20] The metabolism of MIF-1 is very unusual; like most peptides, it is rapidly degraded in rat blood, but unlike most peptides, it survives intact in human blood for several days.[19,28,32]

Salzet and Deloffre isolated MIF-1 from invertebrate leech brain in 2000, confirming "the very ancient origin of this peptide." Moreover, it should not be overlooked that essentially all cells in the body can synthesize the tripeptide glutathione *de novo*, but this possible method of synthesis has not been explored for MIF-1.

Precursor/processing of Tyr-MIF-1: Since the amino acid in oxytocin adjacent to MIF-1 is not tyrosine, it is unlikely that oxytocin is the precursor of Tyr-MIF-1. No precursor is known, although there are many possibilities shown by BLAST search. The metabolism of Tyr-MIF-1 differs in the cerebral microvasculature composing the blood–brain barrier obtained from different regions of the brain cortex, showing yet another mechanism by which the blood–brain barrier can regulate communication between the brain and body.[21]

Distribution of MIF-1: Mass spectrometry shows the hypothalamus to contain the highest concentrations of MIF-1 in mouse brain, followed by smaller amounts in the striatum, thalamus, cortex, and hippocampus.[25] After intravenous (iv)

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00112-3
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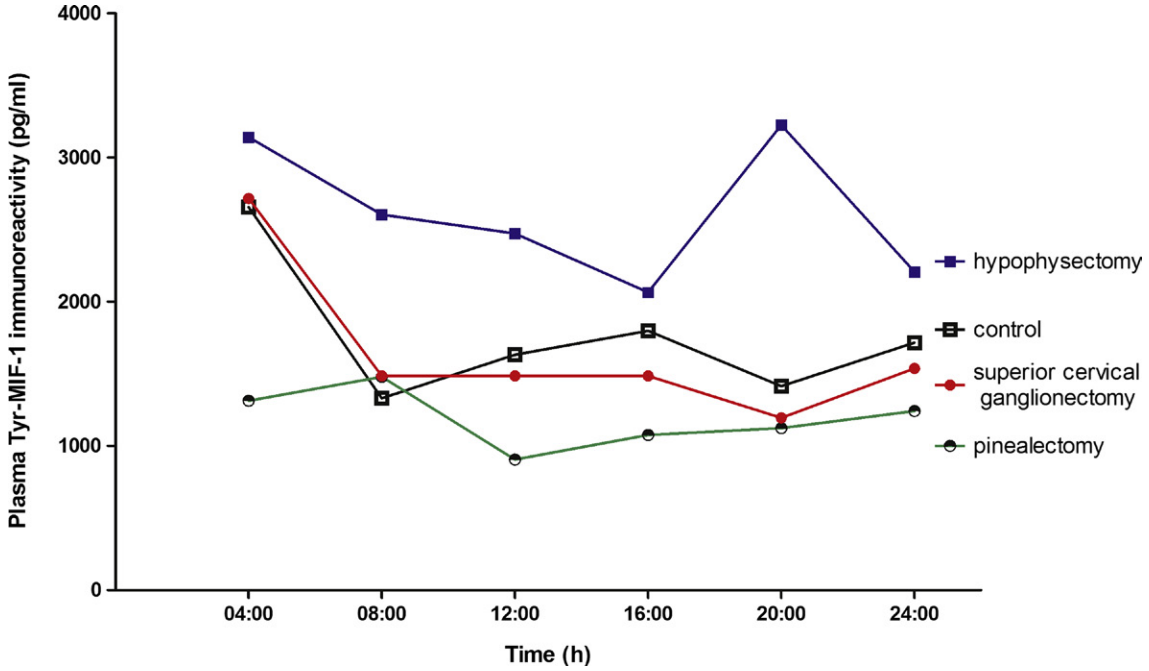

FIGURE 1 Effects of hypophysectomy, pinealectomy, or superior cervical ganglionectomy on the circadian variation of plasma Tyr-MIF-1 immunoreactivity in rats. The control rats showed a peak of Tyr-MIF-1 at 4 am and a nadir at 8 pm. Hypophysectomy changed the acrophase of the circadian rhythm whereas pinealectomy reduced the amplitude of circadian variation as well as changing the acrophase. Modified from Kastin et al.[18]

injection, most MIF-1 is found in the pineal and pituitary, with which MIF-1 interacts,[15] with lesser amounts in cortex, dorsal hippocampus, and hypothalamus.[8] MIF-1 enters the brain rapidly from blood by a saturable transport system, but once there is retained more than most peptides.[2]

Distribution of Tyr-MIF-1: By radioimmunoassay, the highest levels of Tyr-MIF-1 are found in the hypothalamus and pineal, followed by thalamus and striatum.[17] Hypophysectomy increases, and pinealectomy decreases plasma concentrations; circadian evaluation reveals the highest plasma at 0400 (Fig. 1), with the lowest brain concentrations then and at 2400 (Fig. 2).[18]

Tyr-MIF-1 is saturably transported out of the brain into blood faster than most peptides.[1] The system has highly specific structural requirements such that no component, including MIF-1, competes. Yet Met-enkephalin, having little if any structural similarity, saturably exits the brain by the same system. The P-glycoprotein system is not involved in this brain-to-blood transport.[22] Since Tyr-MIF-1 has antiopioid effects whereas Met-enkephalin has opioid effects, it seems intriguing that functional rather structural similarities prevail.

MIF-1 receptors and signaling: The highest concentration of MIF-1 binding sites has been reported to be present in the striatum, followed by the hypothalamus and cortex.[6] MIF-1 does not bind to opiate or Tyr-MIF-1 binding sites. After iv injection of MIF-1, there is a transient increase of pERK but a decrease of pSTAT3. These transient effects precede cFos activation that is highest is the cingulate cortex, nucleus accumbens, paraventricular hypothalamus, and parts of the thalamus.[24]

Tyr-MIF-1 receptors: The highest concentration of Tyr-MIF-1 binding sites is in the cortex, striatum, and amygdala;[33] MIF-1 does not bind to these sites. Tyr-Pro-opioids do bind to the Tyr-MIF-1 site, but ligands for the mu, delta, kappa, and sigma receptors do not.[34] Tyr-K-MIF-1 and Tyr-W-MIF-1 bind to the Tyr-MIF-1 binding site but Tyr-K-MIF-1 has its own binding site to which MIF-1, Tyr-MIF-1, Tyr-W-MIF-1, naloxone, and DAMGO do not interact. Tyr-MIF-1 and Tyr-W-MIF-1 are at least 200-times more selective for mu than delta or kappa opiate receptors, but Tyr-W-MIF-1 has about 20-times higher affinity than Tyr-MIF-1.[35] Single amino acid substitutions of Tyr-W-MIF-1 resulted in the isolation of the endomorphins discussed in the Opioid Peptides Section of this book.

CONFORMATION

MIF-1: R. Kostrzewa and colleagues including F. Bymaster, constructed a CPK (Corey–Pauling–Koltung) space-filling molecular model of MIF-1 and found it resembles that of dopamine in at least three different conformations. The amino group of the prolyl moiety of MIF-1 is separated from the N-terminal glycinamide oxygen atoms by the same distance as the dopamine amino group is separated from one of the catechol oxygen groups. This is compatible with

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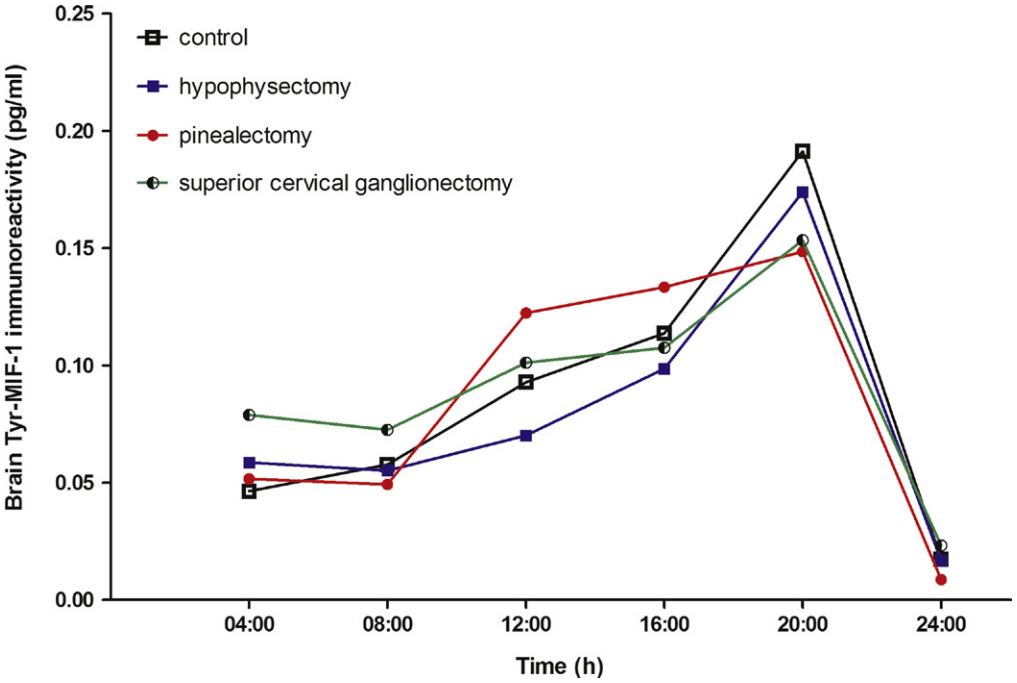

FIGURE 2 Circadian variation of brain Tyr-MIF-1 levels in rats after hypophysectomy, pinealectomy, or superior cervical ganglionectomy, in comparison with the controls. Endogenous Tyr-MIF-1 immunoreactivity peaked at 8 pm and the nadir was at 4 am. This was not changed significantly by surgery. Modified from Kastin et al.[18]

MIF-1 competing with dopamine at the postsynaptic dopamine receptor, or even modifying the receptor to enhance dopamine binding. Although this could explain some of the biological actions of MIF-1, evidence of this binding has only been found by the Utrecht group but not by us. There are conflicting reports on the effects of MIF-1 on turnover of dopamine in the striatum, on striatal tyrosine hydroxylase and dopa decarboxylase, and on enhancement of the actions of apomorphine. R.L. Johnson, R.K. Mishra, and associates have expanded this area with several studies designing and synthesizing MIF-1 analogs with dopamine receptor modulating activity.

Tyr-MIF-1: M. Petersheim and colleagues studied the conformation of Tyr-MIF-1 in aqueous solution by conventional one- and two-dimensional (COSY and NOESY) 1H nuclear magnetic resonance spectroscopy at 300 MHz. They found that Tyr-MIF-1 slowly exchanges between two conformers, probably determined by the cis and trans states of the proline residue. In the major conformation, the aromatic ring of tyrosine is close enough to interact directly with the proline pyrrolidine ring, as indicated by a strong temperature dependence of the proline C beta H, C delta H and C delta H' chemical shifts. By contrast, this interaction of the tyrosine and proline rings is absent in the minor conformation.

Biological actions: As with most chapters of this section, the biological actions of MIF-1 and Tyr-MIF-1 have been discussed in detail elsewhere with inclusive references.[23,27] They are active in animal models of depression and Parkinson's disease and in patients with these disorders. Both of these small peptides have opioid modulating properties, including antiopioid actions, a concept introduced with MIF-1 in 1979.[16]

Opioid actions: Whereas MIF-1 does not exert opioid effects, Tyr-MIF-1 has weak opioid activity. Its opioid potency and selectivity for the mu receptor is greater after the single amino acid substitution found in the isolated tetrapeptide Tyr-W-MIF-1, and additional single amino acid substitutions result in the endomorphins.

Antiopioid actions: The antiopioid actions of MIF-1 and Tyr-MIF-1 have been shown in several types of animals in a wide variety of test situations. Besides reversing the effects of exogenously administered substances like morphine, they can also reverse the effects of endogenous opioids, such as seen in forced warm water swimming, novelty, restraint, aggression, body rotation, and defeat. Some of these antiopioid effects follow an inverted-U shaped dose-response curve, as also seen in some tests of depression and parkinsonism. The antiopioid effects of MIF-1 have even been shown in healthy men.[9] For Tyr-MIF-1, its antiopioid actions are particularly evident when the opioid receptor reserve has been reduced by the administration of β-chlornaltrexamine (CNA) or induction of morphine tolerance.

Behavior: MIF-1 improves performance in the 12-choice Warden maze, extra-dimensional shift, brightness discrimination, and passive avoidance retention. The improved

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learning in some of these tests is consistent with facilitated attention. It also lessens the effect of induced amnesia. Newborn mice injected with MIF-1 have accelerated development, including improved performance in the Lashley II maze when tested as adults.[7]

Temperature: MIF-1 blocks the hyperthermic effect of amphetamine. It also reverses the hypothermic effect of a phenothiazine, reserpine, morphine, and β-endorphin.

Parkinson's disease: As the first hypothalamic peptide to be tested for effects upon higher centers in the brain, MIF-1 is active in several older animal models of parkinsonism. These include the dopa-potentiation, oxotremorine antagonism, and deserpidine antagonism tests. Later it was shown to protect against the symptoms resulting from administration of the neurotoxin MPTP. More than ten clinical studies have shown the beneficial effects of MIF-1 in patients with Parkinson's disease. In a study published in the *Lancet*, Andre Barbeau reported the improvement after MIF-1 "was of such magnitude that it far surpassed the clinical effect of any of the numerous antiparkinson drugs which we have tested in our laboratory over the past 15 years, including levodopa alone."[3] Subsequently, the only clinical studies of MIF-1 in this disorder have been published in Chinese.

Depression: Many older animal models for Parkinson's disease, such as the dopa-potentiation test, are also considered animal models of mental depression. MIF-1 is active in all of these, even in animals lacking a pituitary gland, an observation illustrating the concept that hypothalamic peptides can exert extra-pituitary, extra-endocrine effects "up" on the brain as well as "down" on the pituitary. MIF-1 is also effective in the classic Porsolt test and its Nomura modification for mice with as little as 10 ng/kg body weight given intraperitoneally. A dose of 100 ng/kg MIF-1 is active in the prolonged Katz test of unpredictable chronic stress. In the small numbers of patients tested, the effects of MIF-1 in depression are dramatic. Even in patients with Parkinson's disease, some of the marked improvement in a wide range of symptoms include mental depression. Van der Velde compared oral MIF-1 with the tricyclic imipramine (10 patients each) and concluded that MIF-1 was "at least as effective as imipramine in this study, and that its anti-depressive effect was a rapid and often dramatic one."[30] Unlike depressed patients receiving the currently used antidepressant drugs, patients receiving MIF-1 show rapid and prolonged improvement starting within a few days.[10] It remains puzzling why the clinical actions of MIF-1 in mental depression and Parkinson's disease have not been extended, although the frequently observed U-shaped dose-response curve certainly did not encourage pharma. The potential of MIF-1 in depression is illustrated by the results of orally administered MIF-1 from clinical studies under the direction of Rudolph H. Ehrensing (Fig. 3).

Implications: MIF-1/Tyr-MIF-1 peptides have served to introduce several concepts into the brain peptide field. These include the first demonstration that hypothalamic peptides can exert extra-hypothalamic/extra-pituitary actions, that some of these actions follow an inverted dose-response pattern (illustrated in Fig. 3 below), that peptides can cross the blood-brain barrier, and that brain peptides can exert antiopioid actions. The possibility exists that some of the recently discovered actions of oxytocin may be caused by MIF-1, its C-terminal tail. It is hoped that the beneficial effects of these small peptides will be explored further.

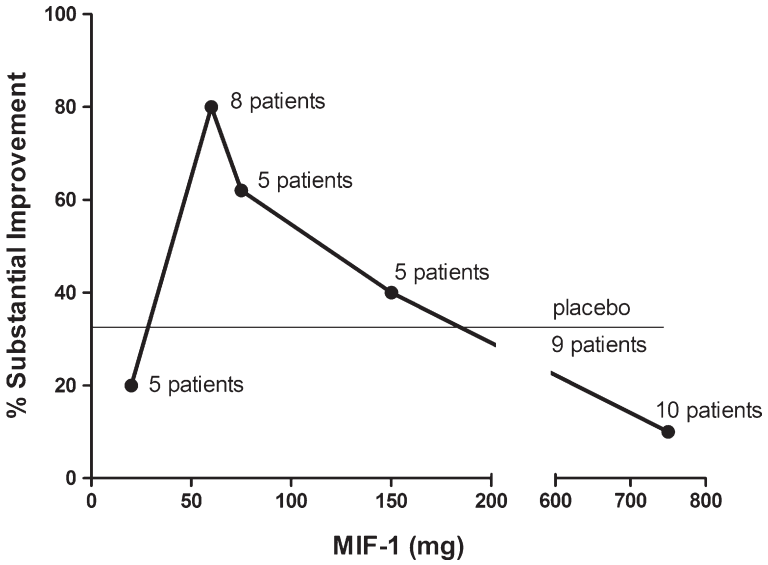

FIGURE 3 Early and dramatic improvement in small numbers of depressed patients at low but not high doses of MIF-1 given by mouth.

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REFERENCES

1. Banks WA, Kastin AJ, Fischman AJ, Coy DH, Strauss SL. Carrier-mediated transport of enkephalins and N-Tyr-MIF-1 across blood–brain barrier. *Am J Physiol* 1986;**251**:E477–82.
2. Banks WA, Kastin AJ. Opposite direction of transport across the blood-brain barrier for Tyr-MIF-1 and MIF-1: comparison with morphine. *Peptides* 1994;**15**:23–9.
3. Barbeau A. Potentiation of levodopa effect by intravenous L-prolyl-L-leucyl-glycine amide in man. *Lancet* 1975 Oct;**2**(7937):683–4.
4. Burbach JP, Lebouille JL. Proteolytic conversion of arginine-vasopressin and oxytocin by brain synaptic membranes. Characterization of formed peptides and mechanisms of proteolysis. *J Biol Chem* 1983 Feb;**258**(3):1487–94.
5. Celis M, Taleisnik S, Walter R. Regulation of formation and proposed structure of the factor inhibiting the release of melanocyte-stimulating hormone. *Proc Natl Acad Sci U S A* 1971;**68**:1428–33.
6. Chiu S, Wong YW, Wan YP, Chiu P, Mishra RK. Are the pharmacological effects of L-prolyl-L-leucyl-glycinamide (PLG) mediated through specific receptor mechanisms? *Prog Neuropsychopharmacol Biol Psychiatry* 1983;**7**(4–6):739–42.
7. D'Amore A, Pieretti S, Palazzesi S, Pezzini G, Chiarotti F, Scorza T, et al. MIF-1 can accelerate neuromotor, EEG and behavioral development in mice. *Peptides* 1990;**11**:527–32.
8. Dupont A, Labrie F, Pelletier G, Puviani R, Coy DH, Schally AV, et al. Distribution of radioactivity in the organs of the rat and mouse after injection of L-(3H)prolyl-L-leucyl-glycinamide. *J Endocrinol* 1975 Feb;**64**(2):243–8.
9. Ehrensing RH, Kastin AJ, Michell GF. Antagonism of morphine analgesia by prolyl-leucyl-glycinamide (MIF-1) in humans. *Pharmacol Biochem Behav* 1984;**21**:975–8.
10. Ehrensing RH, Kastin AJ, Wurzlow GF, Michell GF, Mebane AH. Improvement in major depression after low subcutaneous doses of MIF-1. *J Affect Disord* 1994;**31**:227–33.
11. Erchegyi J, Kastin AJ, Zadina JE. Isolation of a novel tetrapeptide with opiate and antiopiate activity from human brain cortex: Tyr-Pro-Trp-Gly-NH2 (Tyr-W-MIF-1). *Peptides* 1992;**13**:623–32.
12. Horvath A, Kastin AJ. Isolation of tyrosine-melanocyte-stimulating hormone release-inhibiting factor 1 from bovine brain tissue. *J Biol Chem* 1989;**264**:2175–9.
13. Horvath A, Kastin AJ. Evidence for presence of Tyr-MIF-1 (Tyr-Pro-Leu-Gly-NH_2) in human brain cortex. *Int J Peptide Protein Res* 1990;**36**:281–4.
14. Kastin AJ, Ross GT. Melanocyte-stimulating hormone (MSH) and ACTH activities of pituitary homografts in albino rats. *Endocrinology* 1964 Aug;**75**:187–91.
15. Kastin AJ, Viosca S, Nair RM, Shally AV, Miller MC. Interactions between pineal, hypothalamus, and pituitary involving melatonin, MSH release-inhibiting factor and MSH. *Endocrinology* 1972 Nov;**91**(5):1323–8.
16. Kastin AJ, Olson RD, Ehrensing RH, Berzas MC, Schally AV, Coy DH. MIF-1's differential actions as an opiate antagonist. *Pharmacol Biochem Behav* 1979;**11**:721–3.
17. Kastin AJ, Lawrence SP, Coy DH. Radioimmunoassayable N-Tyr-MIF-1-like activity in the rat brain is increased by pinealectomy. *Brain Res Bull* 1981;**7**:697–702.
18. Kastin AJ, Dickson JC. Hypophysectomy increases Tyr-MIF-like immunoreactivity in rat plasma. *Neuroendocrinology* 1987;**45**:177–81.
19. Kastin AJ, Hahn K, Erchegyi J, Zadina JE, Hackler L, Palmgren M, et al. Differential metabolism of Tyr-MIF-1 and MIF-1 in rat and human plasma. *Biochem Pharmacol* 1994;**47**:699–709.
20. Kastin AJ, Hahn K, Zadina JE, Banks WA, Hackler L. Melanocyte-stimulating hormone release-inhibiting factor-1 (MIF-1) can be formed from Tyr-MIF-1 in brain mitochondria but not in brain homogenate. *J Neurochem* 1995;**64**:1855–9.
21. Kastin AJ, Hahn K, Banks WA, Zadina JE. Regional differences in the metabolism of Tyr-MIF-1 and Tyr-W-MIF-1 by rat brain mitochondria. *Biochem Pharmacol* 1998;**55**:33–6.
22. Kastin AJ, Fasold MB, Zadina JE. Endomorphins, Met-Enkephalin, Tyr-MIF-1, and the P-glycoprotein efflux system. *Drug Metab Disp* 2002;**30**:231–4.
23. Kastin AJ, Pan W. Concepts for biologically active peptides. *Curr Pharm Des* 2010 Oct;**16**(30):3390–400.
24. Khan RS, Yu C, Kastin AJ, He Y, Ehrensing RH, Hsuchou H, et al. Brain activation by peptide Pro-Leu-Gly-NH(2) (MIF-1). *Int J Pept* 2010:2010.
25. Kheterpal I, Kastin AJ, Mollah S, Yu C, Hsuchou H, Pan W. Mass spectrometric quantification of MIF-1 in mouse brain by multiple reaction monitoring. *Peptides* 2009 Jul;**30**(7):1276–81.
26. Nair RMG, Kastin AJ, Schally AV. Isolation and structure of hypothalamic MSH release-inhibiting hormone. *Biochem Biophys Res Commun* 1971;**43**:1376–425.
27. Pan W, Kastin AJ. From MIF-1 to endomorphin: the Tyr-MIF-1 family of peptides. *Peptides* 2007 Dec;**28**(12):2411–34.
28. Redding TW, Kastin AJ, Gonzalez-Barcena D, Coy DH, Hirotsu Y, Ruelas J, et al. The disappearance, excretion, and metabolism of tritiated prolyl-leucyl-glycinamide in man. *Neuroendocrinology* 1974;**16**:119–26.
29. Scimonelli T, Celis ME. Inhibition by L-prolyl-L-leucyl-glycinamide (PLG) of alpha-melanocyte stimulating hormone release from hypothalamic slices. *Peptides* 1982 Nov;**3**(6; 1):885–9.
30. van der Velde CD. Rapid clinical effectiveness of MIF-I in the treatment of major depressive illness. *Peptides* 1983 May;**4**(3):297–300.
31. Walter R, Griffiths E, Hooper K. Production of MSH-release-inhibiting hormone by a particulate preparation of hypothalami: mechanisms of oxytocin inactivation. *Brain Res* 1973;**60**:449–57.
32. Walter R, Neidle A, Marks N. Significant differences in the degradation of pro-leu-gly-nH2 by human serum and that of other species. *Proc Soc Exp Biol Med* 1975;**148**:98–103.
33. Zadina JE, Kastin AJ, Krieg EF, Coy DH. Characterization of binding sites for N-Tyr-MIF-1 (Tyr-Pro-Leu-Gly-NH_2) in rat brain. *Pharmacol Biochem Behav* 1982;**17**:1193–8.
34. Zadina JE, Kastin AJ, Ge L-J, Brantl V. Hemorphins, cytochrophins, and human β-casomorphins bind to antiopiate (Tyr-MIF-1) as well as opiate binding sites in rat brain. *Life Sci* 1990;**47**:PL25–30.
35. Zadina JE, Kastin AJ, Ge L-J, Hackler L. *Mu, delta*, and *kappa* opiate receptor binding of Tyr-MIF-1 and of Tyr-W-MIF-1, its active fragments, and two potent analogs. *Life Sci* 1994;**55**:PL461–6.

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Chapter 113

Nesfatin-1

Andreas Stengel and Yvette Taché

ABSTRACT

Nesfatin-1 is a recently described 82 amino acid polypeptide derived from its precursor protein, nucleobindin2 (NUCB2). The peptide is widely distributed throughout the brain suggesting an expanding role in stress response, neuroendocrine regulation, autonomic control of viscera, and also onset of puberty in addition to the initially described inhibitory action on food intake. Although the understanding of nesfatin-1's actions in the brain have markedly advanced since its discovery in 2006, several important mechanisms still remain to be understood encompassing the regulation of NUCB2 processing leading to nesfatin-1 release in the brain as well as the identification of the nesfatin-1 receptor(s) and intracellular signaling pathways.

DISCOVERY

About two decades ago, a protein that promotes DNA antibody production was isolated from a large volume of mouse cell culture supernatant and human cell lines and named nucleobindin.[5] The 55 kDa protein was also termed NEFA (D*N*A binding/*EF*-hand/*a*cidic amino acid rich region) in keeping with features of its functional domains encompassing a basic putative DNA-binding domain, two Ca^{2+}-EF-hand motifs separated by an amino acid-rich leucine/isoleucine region and a leucine zipper motif in the C-terminal region.[5] The nucleobindin family so far comprises two members, nucleobindin 1 (NUCB1 or CALNUC) and nucleobindin2 (NUCB2 or NEFA).[5] The 420 amino acid protein NUCB2 contains a 24 amino acid N-terminal signal peptide and a 396 amino acid sequence highly conserved (>85%) in mouse, rat and human (Fig. 1A) implying a physiological significance across mammals. Nesfatin-1 was identified a few years ago in the rat hypothalamus by a subtraction-cloning strategy as an 82 amino acid peptide derived from NUCB2 and termed after *N*UCB2-*e*ncoded *s*atiety and *fat in*fluencing protein.[22]

STRUCTURE OF THE mRNA/GENE

The NUCB2 gene is located on chromosome 11 in humans, on chromosome 7 in mice and on chromosome 1 in rats, and contains 14 exons and 13 introns in humans.[31] The translation initiation site and putative signal sequence have been localized on exon 3.[31] Nesfatin-1 is encoded in the region between exons 3 and 5, while nesfatin-2 and -3 are encoded in regions between exons 5 and 14. The 3′-untranslated region has been identified in exon 14.[31] The translational start site forms clusters around 246 base pairs upstream of the start codon and the promoter region of human NUCB2 contains a CAAT box and several regulatory elements including nuclear factor 1, cAMP response element-binding protein-binding sites, as well as activator protein-1 and -2.[31]

PROCESSING OF THE PRECURSOR

The NUCB2 protein contains several cleavage recognition sites for prohormone convertases (PC). The proteolytic processing of NUCB2 by PC-1/3 is assumed to generate several products including nesfatin-1 (amino acids 1–82), nesfatin-2 (amino acids 85–163) and nesfatin-3 (amino acids 166–396, Fig. 1B).[22] In their landmark study, Oh-I and colleagues reported the presence of the 82 amino acid peptide in rat cerebrospinal fluid, whereas the mature peptide was not detectable in hypothalamic tissue.[22] Subsequent reports did not detect mature nesfatin-1 (9.7 kDa) in the brain[9,10] or plasma,[29] whereas full length NUCB2 as well as small amounts of exogenous synthetic nesfatin-1 were detectable by Western blot.[29] So far, most studies describing the expression at the protein level did not distinguish between NUCB2 and nesfatin-1 as the antibodies used recognized both molecules. Because of these findings, it remains to be clarified whether nesfatin-1 or full length NUCB2 is the biologically active molecule since NUCB2 also shows biological activity upon third ventricular injection in rats.[22] Among the known cleavage products, only nesfatin-1 influences food intake, whereas nesfatin-2 and nesfatin-3 do not.[22]

DISTRIBUTION OF THE mRNA/PEPTIDE

Initially, Oh-I and colleagues described the localization of NUCB2/nesfatin-1 immunostaining in several rat brain

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00113-5
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(A)

h(1–60)	MRWRTILLQY	CFLLITCLLT	ALEAVPIDID	KTKVQNIHPV	ESAKIEPPDT	GLYYDEYLKQ
m(1–60)	MRWRIIQVQY	CFLLVPCMLT	ALEAVPIDVD	KTKVHNTEPV	ENARIEPPDT	GLYYDEYLKQ
r(1–60)	MRWRTIQARY	CFLLVPCVLT	ALEAVPIDVD	KTKVHNVEPV	ESARIEPPDT	GLYYDEYLKQ
h(61–120)	VIDVLETDKH	FREKLQKADI	EEIKSGRLSK	ELDLVSHHVR	TKLDELKRQE	VGRLRMLIKA
m(61–120)	VIEVLETDPH	FREKLQKADI	EEIRSGRLSQ	ELDLVSHKVR	TRLDELKRQE	VGRLRMLIKA
r(61–120)	VIEVLETDPH	FREKLQKADI	EEIRSGRLSQ	ELDLVSHKVR	TRLDELKRQE	VGRLRMLIKA
h(121–180)	KLDSLQDIGM	DHQALLKQFD	HLNHLNPDKF	ESTDLDMLIK	AATSDLEHYD	KTRHEEFKKY
m(121–180)	KLDALQDTGM	NHHLLLKQFE	HLNHQNPNTF	ESRDLDMLIK	AATADLEQYD	RTRHEEFKKY
r(121–180)	KLDALQDTGM	NHHLLLKQFE	HLNHQNPDTF	ESKDLDMLIK	AATADLEQYD	RTRHEEFKKY
h(181–240)	EMMKEHERRE	YLKTLNEEKR	KEEESKFEEM	KKKHENHPKV	NHPGSKDQLK	EVWEETDGLD
m(181–240)	EMMKEHERRE	YLKTLSEEKR	KEEESKFEEM	KRKHEDHPKV	NHPGSKDQLK	EVWEETDGLD
r(181–240)	EMMKEHERRE	YLKTLSEEKR	KEEEAKFAEM	KRKHEDHPKV	NHPGSKDQLK	EVWEETDGLD
h(241–300)	PNDFDPKTFF	KLHDVNSDGF	LDEQELEALF	TKELEKVYDP	KNEEDDMVEM	EEERLRMREH
m(241–300)	PNDFDPKTFF	KLHDVNNDGF	LDEQELEALF	TRELEKVYNP	QNAEDDMIEM	EEERLRMREH
r(241–300)	PNDFDPKTFF	KLHDVNNDGF	LDEQELEALF	TKELDKVYNP	QNAEDDMIEM	EEERLRMREH
h(301–360)	VMNEVDTNKD	RLVTLEEFLK	ATEKKEFLEP	DSWETLDQQQ	FFTEEELKEY	ENIIALQENE
m(301–360)	VMSEIDNNKD	RLVTLEEFLR	ATEKKEFLEP	DSWETLDQQQ	LFTEDELKEY	ESIIAIQENE
r(301–360)	VMNEIDNNKD	RLVTLEEFLR	ATEKKEFLEP	DSWETLDQQQ	LFTEEELKEY	ESIIAIQESE
h(361–420)	LKKKADELQK	QKEELQRQHD	QLEAQKLEYH	QVIQQMEQKK	LQQGIPPSGP	AGELKFEPHI
m(361–420)	LKKRAEELQK	QKEDLQRQHD	HLEAQKQEYH	QAVQHLEQKK	LQQGIAPSGP	AGELKFEPHT
r(361–420)	LKKKADELQK	QKEELQRQHD	HLEAQKQEYQ	QAVQQLEQKK	FQQGIAPSGP	AGELKFEPHT

(B)

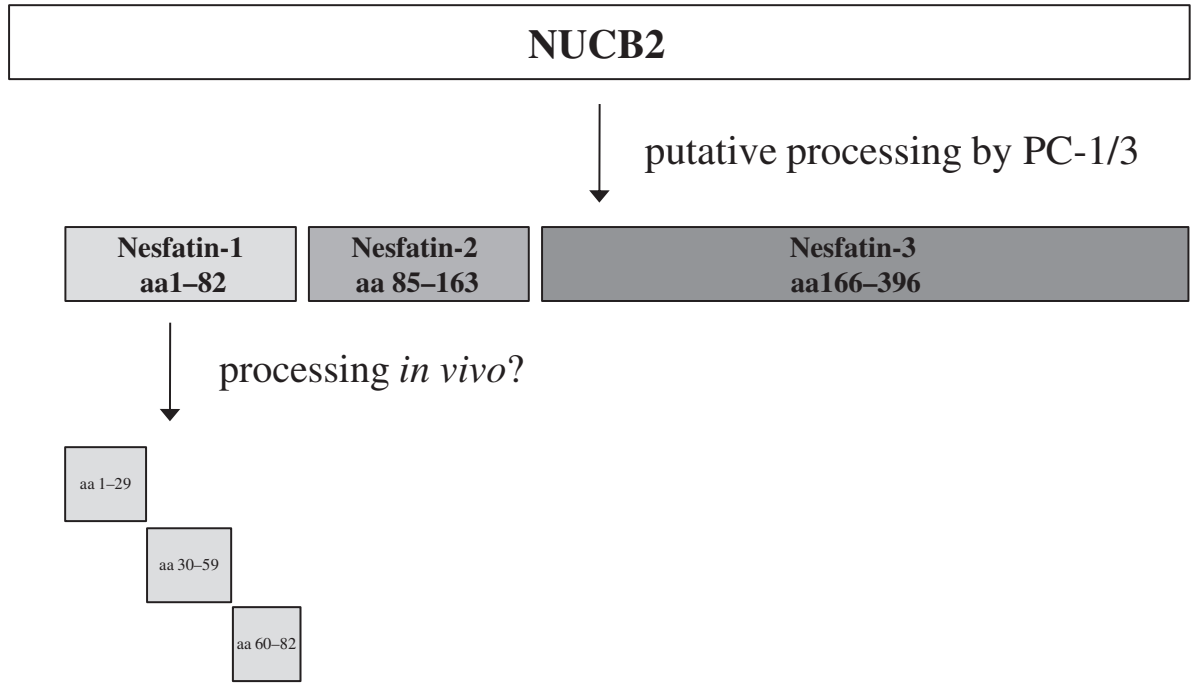

FIGURE 1 A Comparison of the amino acid sequences of human (h), mouse (m) and rat (r) nucleobindin2 (NUCB2). The differences are highlighted. B Processing of NUCB2 to nesfatin-1, nesfatin-2 and nesfatin-3 by prohormone convertase 1/3 and suggested cleavage of nesfatin-1 into the three fragments, $N_{1–29}$, $M_{30–59}$ and $C_{60–82}$. Abbreviations: aa, amino acid; PC-1/3, prohormone convertase 1/3.

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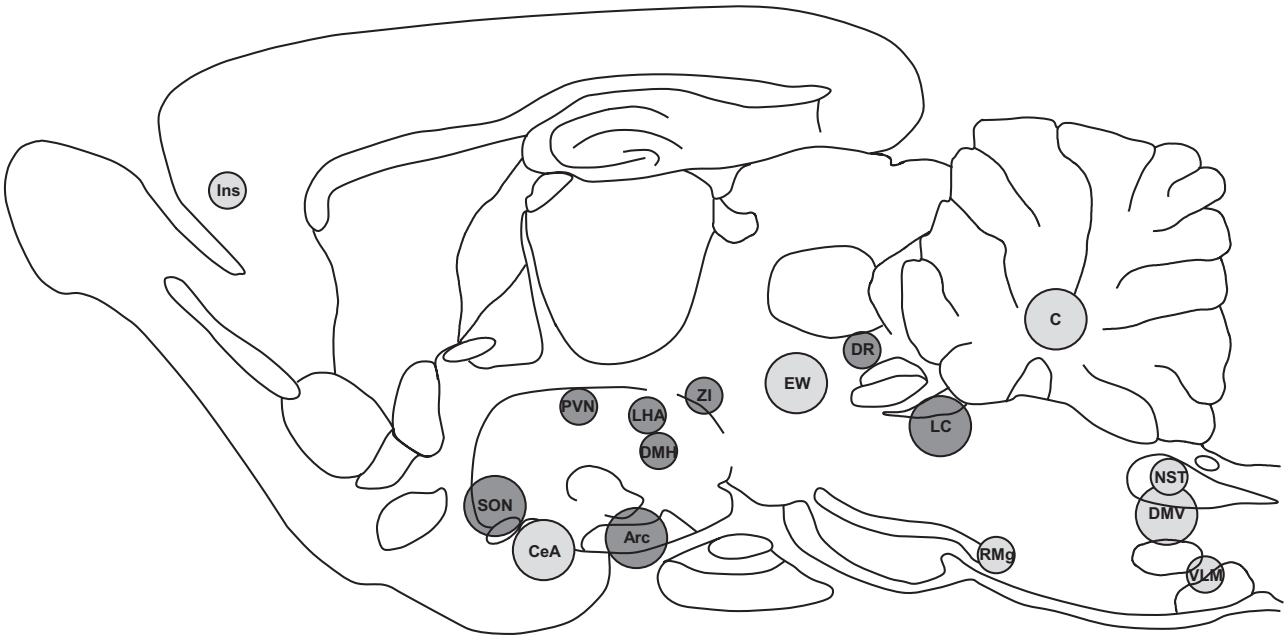

FIGURE 2 Expression of NUCB2/nesfatin-1 immunoreactivity in rat brain nuclei. Large circles represent high density and smaller circles moderate density of NUCB2/nesfatin-1-immunoreactive cells. Dark gray background represents intensive and light gray moderate intensive immunolabeling. Abbreviations: Arc, arcuate nucleus of hypothalamus; C, cerebellum; CeA, central amygdala; DMH, dorsomedial nucleus of hypothalamus; DMV, dorsal motor nucleus of the vagus nerve; DR, dorsal raphe nucleus; EW, Edinger–Westphal nucleus; Ins, insular cortex; LC, locus coeruleus; LHA, lateral hypothalamic area; NST, nucleus of solitary tract; PVN, paraventricular nucleus of hypothalamus; RMg, raphe magnus nucleus; SON, supraoptic nucleus; VLM, ventrolateral medulla; ZI, zona incerta.

nuclei implicated in the regulation of hunger and satiety encompassing the supraoptic nucleus, arcuate nucleus, paraventricular nucleus (PVN), lateral hypothalamic area, zona incerta and the nucleus of the solitary tract (NTS) (Fig. 2).[22] This neuroanatomical localization was confirmed in rats and mice by independent groups and extended to the insular cortex, central amygdaloid nucleus, dorsomedial hypothalamic nucleus, Edinger–Westphal nucleus, locus coeruleus, medullary raphe nuclei, dorsal motor nucleus of the vagus nerve, ventrolateral medulla, cerebellum, as well as preganglionic sympathetic and parasympathetic neurons of the spinal cord (Fig. 2).[8,9,11,12,16] Interestingly, in mice NUCB2/nesfatin-1 immunoreactivity was also detected in a previously undefined region which was named intermediate dorsomedial hypothalamus (Fig. 3).[11] Of note, however, at the cellular level the localization of NUCB2/nesfatin-1 immunoreactivity is confined to the cytoplasm and primary dendrites while not present in axons and terminals which is at variance with other brain neuropeptides.[8,9,12,17,22] This points toward non-axonal signaling, probably involving a paracrine or autocrine mode of action of NUCB2/nesfatin-1.

NUCB2/nesfatin-1-immunoreactive neurons colocalize with various brain neuropeptides in the hypothalamus and midbrain including 70–90% of cocaine- and amphetamine-regulated transcript (CART) or proopiomelanocortin (POMC),[9,15,23] 90% of urocortin-1,[23] 82% of melanin-concentrating hormone,[9] 64% of α-melanocyte-stimulating

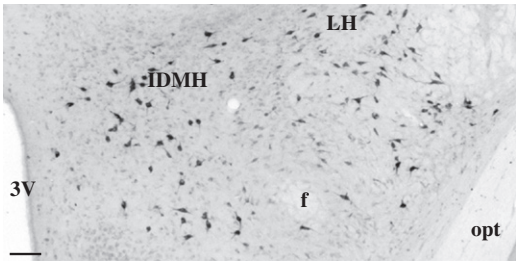

FIGURE 3 NUCB2/nesfatin-1-immunoreactive cells in the mouse intermediate dorsomedial hypothalamus. The scale bar represents 100 μm. Abbreviations: 3V, third ventricle; f, fornix; IDMH, intermediate dorsomedial hypothalamus; LH, lateral hypothalamic area; opt, optic tract. For further description of the nucleus please see.[11]

hormone (α-MSH),[9,15] 59% of mammalian target of rapamycin,[15] 47–59% of vasopressin,[9,16] 40–49% of oxytocin,[9,16] 46% of neuropeptide Y (NPY),[15] 38% of somatostatin,[9] 29% of growth hormone-releasing hormone,[9] 18–27% of thyrotropin-releasing hormone,[9,16] 13–24% of corticotropin-releasing hormone (CRH)[9,16] and 14% of neurotensin.[9] In the brainstem, NUCB2/nesfatin-1 immunoreactivity was colocalized with serotonin-positive neurons in the medullary raphe,[8] POMC/CART or tyrosine hydroxylase neurons of the NTS[8,9] as well as cholinergic neurons in the dorsal motor nucleus of the vagus nerve.[8] The widespread colocalization of NUCB2/nesfatin-1 with several neuropeptides and transmitters involved in the regulation of hunger and satiety, homeostatic circuitries and stress provide neuroanatomic support

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for nesfatin-1's involvement in several biological processes related to energy homeostasis, stress, neuroendocrine and autonomic regulation.

RECEPTORS AND SIGNALING CASCADES

Structure activity relationship studies indicate that full length NUCB2 as well as nesfatin-1 exerts an anorexigenic effect after injection into the brain ventricle while nesfatin-2 and nesfatin-3 do not.[22] Several fragments of nesfatin-1, namely N-terminal nesfatin-$1_{1–29}$, the mid-fragment nesfatin-$1_{30–59}$ and the C-terminal nesfatin-$1_{60–82}$ (Fig. 1B) were also synthesized based on structural analysis of the α-helix of nesfatin-1 and tested for biological activity. Only the mid fragment is able to reproduce the anorexigenic effect of nesfatin-1 after peripheral injection in mice suggesting that this fragment may contain the active core of the peptide interacting with the receptor.[26] However, it remains to be established whether nesfatin-$1_{30–59}$ is generated *in vivo*.

Still to be identified is the receptor to which nesfatin-1 binds. One study showed that nesfatin-1 interacts with a receptor that has pharmacological characteristics of G protein-coupled receptors. This was based on *in vitro* results in cultured rat hypothalamic neurons showing that nesfatin-1 induces a fast rise in $[Ca^{2+}]_i$ which is abolished by pertussis toxin, indicating the involvement of a $G_{i/o}$ protein-coupled receptor and is also reduced by a specific protein kinase A inhibitor indicative of a role of protein kinase signaling.[8] The identification and consecutive pharmacological characterization of the nesfatin-1 receptor will represent an important leap forward to better understand nesfatin-1 signaling and physiology.

BIOLOGICAL ACTIONS WITHIN THE BRAIN (AND PITUITARY)

Effects of Nesfatin-1 on Food Intake

The initially described biological action of nesfatin-1 was the reduction of dark phase food intake after injection into the third brain ventricle in rats.[22] Subsequent studies showed that nesfatin-1 injected into the lateral brain ventricle reduces dark phase food intake with a delayed onset and long duration in rats, mice and goldfish.[13,27] Microstructure analysis using an automated food intake monitoring system in mice established that nesfatin-1 injected intracerebroventricularly reduces dark phase food intake by lowering meal frequency, increasing inter-meal intervals and reducing meal size.[13] Besides its anorexigenic action after injection into the forebrain where the PVN has been identified as a responsive site, nesfatin-1 injected into the hindbrain either into the cisterna magna or the fourth ventricle also reduces dark phase food intake in rats.[27]

The appetite suppressive action of intracerebroventricular injection of nesfatin-1 involves the activation of peptidergic anorexic circuitries as shown by the blockade of nesfatin-1's inhibitory effect by intracerebroventricular injection of the CRH_2 receptor antagonist, astressin$_2$-B,[27] the melanocortin 3/4 receptor antagonist, SHU9119[22] and an oxytocin antagonist.[33] Since the oxytocin and CRH receptor antagonists also block the α-MSH-induced reduction of food intake,[33] the signaling cascade could be nesfatin-1→α-MSH→oxytocin/CRH receptor activation. Lastly, inhibition of the orexigenic NPY pathways may also be involved in nesfatin-1's food intake reducing action based on *in vitro* experiments showing a hyperpolarization of NPY positive neurons by nesfatin-1.[24] By contrast, the anorexigenic action of nesfatin-1 is independent of leptin signaling as the food intake reduction after intracerebroventricular injection of nesfatin-1 is still observed in leptin receptor-deficient Zucker rats.[22]

Conclusive evidence supports the physiological relevance of NUCB2/nesfatin-1's action in the brain to curtail food intake. Expression of the NUCB2/nesfatin-1 mRNA in the PVN and supraoptic nucleus and the hypothalamic NUCB2 protein content are regulated by changes in metabolic status as indicated by the down-regulation after food deprivation for 24 h or chronic undernutrition at puberty in rats.[10,16,22] Conversely, food intake suppressing transmitters such as α-MSH or cholecystokinin increase hypothalamic NUCB2 mRNA expression[22] or activate NUCB2/nesfatin-1-immunopositive neurons in hypothalamic and brainstem nuclei.[20,27] Lastly, injection of an anti-NUCB2 antisense oligonucleotide into the third ventricle increases food intake[22] pointing toward an inhibitory tone on food consumption exerted by NUCB2/nesfatin-1. Nesfatin-1 selectively reduces dark phase food intake in *ad libitum* fed animals, whereas studies performed during the light phase investigating the feeding response after an overnight fast resulted in inconsistent findings and required higher doses to reduce food intake.[18,22,27] This may suggest that nesfatin-1 interferes more prominently with physiological processes of nocturnal feeding than those involved in the hyperphagic response to food deprivation.

Effects of Nesfatin-1 on Gastrointestinal Motility

Centrally acting peptides regulating food intake also affect gastrointestinal motility. Likewise, intracerebroventricular injection of nesfatin-1 delays gastric emptying in rats[27] and mice[2,11] and suppresses the fed pattern of gastroduodenal motility in mice.[2] Such alterations in gastric transit could contribute to the long lasting anorexigenic action of the peptide.

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Involvement of Nesfatin-1 in Stress Responses

NUCB2/nesfatin-1's distribution in stress responsive brain neuronal circuitries including the PVN, central amygdaloid nucleus and locus coeruleus supports a potential role in the stress response. In addition, functional studies indicate that nesfatin-1 injected intracerebroventricularly, although at a higher dose than used for the feeding studies, induces anxiety and fear as indicated by characteristic behavior including less time spent in the open arms of the elevated plus maze test as well as increased startle response and freezing in an emotional response test.[19] It may be hypothesized that these effects are mediated by CRH in the PVN known to play a role in the induction of anxiety and depression. This is supported by the observation that brain injection of nesfatin-1 increases plasma adrenocorticotropin and corticosterone levels and cytosolic Ca^{2+} concentrations in CRH immunoreactive neurons isolated from the PVN.[17,32] Moreover, various stressors activate NUCB2/nesfatin-1 in specific brain nuclei as shown by double labeling with Fos used as a marker of neuronal activation. For instance, abdominal surgery, a physical stressor, induces a robust activation of NUCB2/nesfatin-1-immunoreactive neurons in the magnocellular neuroendocrine system, in the anterior parvicellular part of the PVN, the Edinger–Westphal nucleus, and nuclei of the catecholaminergic and serotonergic system.[28] Similarly, injection of lipopolysaccharide, an immunological stressor, activates NUCB2/nesfatin-1-immunopositive neurons in hypothalamic and hindbrain nuclei.[7] Also, restraint stress as a psychological stressor activates NUCB2/nesfatin-1-immunoreactive neurons in several hypothalamic and hindbrain nuclei.[14,17,23,30,32] Activation of these neurons may be part of mechanisms underlying the reduced food intake observed under these stress conditions.

Onset of Puberty

Hypothalamic nesfatin-1 may play a key role in driving the onset of puberty in female rats.[10] This is substantiated by the increased expression of hypothalamic NUCB2 mRNA and protein content during puberty transition and the delay of puberty by inhibition of NUCB2 synthesis after intracerebroventricular injection of an anti-NUCB2 antisense oligonucleotide.[10] In addition, intracerebroventricular injection of nesfatin-1 stimulates luteinizing hormone and follicle-stimulating hormone release into the circulation only in pubertal but not adult female rats.[10] These observations suggest that NUCB2/nesfatin-1 may be an important regulator of the hypothalamic gonadotropic axis at puberty.

PATHOPHYSIOLOGICAL IMPLICATIONS

Patients with newly diagnosed primary generalized epilepsy have elevated concentrations of NUCB2/nesfatin-1 in the circulation and saliva which decrease after treatment.[4] In addition, directly after a seizure, serum concentrations of NUCB2/nesfatin-1 are significantly increased.[3] Based on these results, nesfatin-1 could be used as a biomarker for the diagnosis of epilepsy, in case of a suspected recent seizure, but also for monitoring treatment outcome of the disease. Moreover, nesfatin-1 plasma concentrations are increased in patients with major depression,[1] a finding that was confirmed in the brain of depressed suicide victims who had higher NUCB2 mRNA levels in the Edinger–Westphal nucleus compared with the brains of control subjects with acute cardiac-respiratory failure or myocardial infarction as causes of death.[6]

In view of its proposed role as a tonic inhibitor of food intake, several clinical studies investigated the association of circulating NUCB2/nesfatin-1 and body weight. A positive correlation between plasma NUCB2/nesfatin-1 concentrations and body mass index (BMI) was reported with lower concentrations under conditions of anorexia nervosa[21] and higher concentrations in obese subjects,[25] indicating that circulating nesfatin-1 may be responsive to sustained changes in adipose tissue mass. In addition, three different single nucleotide polymorphisms in the NUCB2 gene have been identified in a large cohort of men that were associated with obesity[34] possibly representing a genetic cause for the increase of body weight.

ACKNOWLEDGMENTS

This work was supported by NIH R01 DK-33061, NIH Center Grant DK-41301 (Animal Core), VA Research Career Scientist (Y.T.) and Charité University funding (A.S.). We thank Drs. Miriam Goebel-Stengel and Lixin Wang for generously supplying Figure 2.

REFERENCES

1. Ari M, Ozturk OH, Bez Y, Oktar S, Erduran D. High plasma nesfatin-1 level in patients with major depressive disorder. *Prog Neuropsychopharmacol Biol Psychiatry* 2011;**35**:497–500.
2. Atsuchi K, Asakawa A, Ushikai M, Ataka K, Tsai M, Koyama K, et al. Centrally administered nesfatin-1 inhibits feeding behaviour and gastroduodenal motility in mice. *Neuroreport* 2010;**21**:1008–11.
3. Aydin S, Dag E, Ozkan Y, Arslan O, Koc G, Bek S, et al. Time-dependent changes in the serum levels of prolactin, nesfatin-1 and ghrelin as a marker of epileptic attacks young male patients. *Peptides* 2011;**32**:1276–80.
4. Aydin S, Dag E, Ozkan Y, Erman F, Dagli AF, Kilic N, et al. Nesfatin-1 and ghrelin levels in serum and saliva of epileptic patients: hormonal changes can have a major effect on seizure disorders. *Mol Cell Biochem* 2009;**328**:49–56.
5. Barnikol-Watanabe S, Gross NA, Gotz H, Henkel T, Karabinos A, Kratzin H, et al. Human protein NEFA, a novel DNA binding/EF-hand/leucine zipper protein. Molecular cloning and sequence analysis of the cDNA, isolation and characterization of the protein. *Biol Chem Hoppe Seyler* 1994;**375**:497–512.

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6. Bloem B, Xu L, Morava E, Faludi G, Palkovits M, Roubos EW, et al. Sex-specific differences in the dynamics of cocaine- and amphetamine-regulated transcript and nesfatin-1 expressions in the midbrain of depressed suicide victims vs. controls. *Neuropharmacology* 2012;**61**:297–303.
7. Bonnet MS, Pecchi E, Trouslard J, Jean A, Dallaporta M, Troadec JD. Central nesfatin-1 expressing neurons are sensitive to peripheral inflammatory stimulus. *J Neuroinflammation* 2009;**6**:27.
8. Brailoiu GC, Dun SL, Brailoiu E, Inan S, Yang J, Chang JK, et al. Nesfatin-1: distribution and interaction with a G protein-coupled receptor in the rat brain. *Endocrinology* 2007;**148**:5088–94.
9. Foo K, Brismar H, Broberger C. Distribution and neuropeptide coexistence of nucleobindin-2 mRNA/nesfatin-like immunoreactivity in the rat CNS. *Neuroscience* 2008;**156**:563–79.
10. Garcia-Galiano D, Navarro VM, Roa J, Ruiz-Pino F, Sanchez-Garrido MA, Pineda R, et al. The anorexigenic neuropeptide, nesfatin-1, is indispensable for normal puberty onset in the female rat. *J Neurosci* 2010;**30**:7783–92.
11. Goebel-Stengel M, Wang L, Stengel A, Taché Y. Localization of nesfatin-1 neurons in the mouse brain and functional implication. *Brain Res* 2011;**1396**:20–34.
12. Goebel M, Stengel A, Wang L, Lambrecht NW, Taché Y. Nesfatin-1 immunoreactivity in rat brain and spinal cord autonomic nuclei. *Neurosci Lett* 2009;**452**:241–6.
13. Goebel M, Stengel A, Wang L, Taché Y. Central nesfatin-1 reduces the nocturnal food intake in mice by reducing meal size and increasing inter-meal intervals. *Peptides* 2011;**32**:36–43.
14. Goebel M, Stengel A, Wang L, Taché Y. Restraint stress activates nesfatin-1-immunoreactive brain nuclei in rats. *Brain Res* 2009;**1300**:114–24.
15. Inhoff T, Stengel A, Peter L, Goebel M, Taché Y, Bannert N, et al. Novel insight in distribution of nesfatin-1 and phospho-mTOR in the arcuate nucleus of the hypothalamus of rats. *Peptides* 2010;**31**:257–62.
16. Kohno D, Nakata M, Maejima Y, Shimizu H, Sedbazar U, Yoshida N, et al. Nesfatin-1 neurons in paraventricular and supraoptic nuclei of the rat hypothalamus coexpress oxytocin and vasopressin and are activated by refeeding. *Endocrinology* 2008;**149**:1295–301.
17. Konczol K, Bodnar I, Zelena D, Pinter O, Papp RS, Palkovits M, et al. Nesfatin-1/NUCB2 may participate in the activation of the hypothalamic–pituitary–adrenal axis in rats. *Neurochem Int* 2010;**57**:189–97.
18. Maejima Y, Sedbazar U, Suyama S, Kohno D, Onaka T, Takano E, et al. Nesfatin-1-regulated oxytocinergic signaling in the paraventricular nucleus causes anorexia through a leptin-independent melanocortin pathway. *Cell Metab* 2009;**10**:355–65.
19. Merali Z, Cayer C, Kent P, Anisman H. Nesfatin-1 increases anxiety- and fear-related behaviors in the rat. *Psychopharmacology (Berl)* 2008;**201**:115–23.
20. Noetzel S, Stengel A, Inhoff T, Goebel M, Wisser AS, Bannert N, et al. CCK-8S activates c-Fos in a dose-dependent manner in nesfatin-1 immunoreactive neurons in the paraventricular nucleus of the hypothalamus and in the nucleus of the solitary tract of the brainstem. *Regul Pept* 2009;**157**:84–91.
21. Ogiso K, Asakawa A, Amitani H, Nakahara T, Ushikai M, Haruta I, et al. Plasma nesfatin-1 concentrations in restricting-type anorexia nervosa. *Peptides* 2010;**32**:150–3.
22. Oh-I S, Shimizu H, Satoh T, Okada S, Adachi S, Inoue K, et al. Identification of nesfatin-1 as a satiety molecule in the hypothalamus. *Nature* 2006;**443**:709–12.
23. Okere B, Xu L, Roubos EW, Sonetti D, Kozicz T. Restraint stress alters the secretory activity of neurons co-expressing urocortin-1, cocaine- and amphetamine-regulated transcript peptide and nesfatin-1 in the mouse Edinger–Westphal nucleus. *Brain Res* 2010;**1317C**:92–9.
24. Price CJ, Samson WK, Ferguson AV. Nesfatin-1 inhibits NPY neurons in the arcuate nucleus. *Brain Res* 2008;**1230**:99–106.
25. Ramanjaneya M, Chen J, Brown JE, Tripathi G, Hallschmid M, Patel S, et al. Identification of nesfatin-1 in human and murine adipose tissue: a novel depot-specific adipokine with increased levels in obesity. *Endocrinology* 2010;**151**:3169–80.
26. Shimizu H, Oh-I S, Hashimoto K, Nakata M, Yamamoto S, Yoshida N, et al. Peripheral administration of nesfatin-1 reduces food intake in mice: the leptin-independent mechanism. *Endocrinology* 2009;**150**:662–71.
27. Stengel A, Goebel M, Wang L, Rivier J, Kobelt P, Mönnikes H, et al. Central nesfatin-1 reduces dark-phase food intake and gastric emptying in rats: differential role of corticotropin-releasing factor2 receptor. *Endocrinology* 2009;**150**:4911–9.
28. Stengel A, Goebel M, Wang L, Taché Y. Abdominal surgery activates nesfatin-1 immunoreactive brain nuclei in rats. *Peptides* 2010;**31**:263–70.
29. Stengel A, Goebel M, Yakubov I, Wang L, Witcher D, Coskun T, et al. Identification and characterization of nesfatin-1 immunoreactivity in endocrine cell types of the rat gastric oxyntic mucosa. *Endocrinology* 2009;**150**:232–8.
30. Xu L, Bloem B, Gaszner B, Roubos EW, Kozicz T. Stress-related changes in the activity of cocaine- and amphetamine-regulated transcript and nesfatin neurons in the midbrain non-preganglionic Edinger–Westphal nucleus in the rat. *Neuroscience* 2010;**170**:478–88.
31. Yamada M, Horiguchi K, Umezawa R, Hashimoto K, Satoh T, Ozawa A, et al. Troglitazone, a ligand of peroxisome proliferator-activated receptor-{gamma}, stabilizes NUCB2 (Nesfatin) mRNA by activating the ERK1/2 pathway: isolation and characterization of the human NUCB2 gene. *Endocrinology* 2010;**151**:2494–503.
32. Yoshida N, Maejima Y, Sedbazar U, Ando A, Kurita H, Damdindorj B, et al. Stressor-responsive central nesfatin-1 activates corticotropin-releasing hormone, noradrenaline and serotonin neurons and evokes hypothalamic–pituitary–adrenal axis. *Aging (Albany NY)* 2010;**2**:775–84.
33. Yosten GL, Samson WK. The anorexigenic and hypertensive effects of nesfatin-1 are reversed by pretreatment with an oxytocin receptor antagonist. *Am J Physiol Regul Integr Comp Physiol* 2010;**298**:R1642–7.
34. Zegers D, Beckers S, Mertens IL, Van Gaal LF, Van Hul W. Association between polymorphisms of the Nesfatin gene, NUCB2, and obesity in men. *Mol Genet Metab* 2011;**103**:282–6.

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Chapter 114

Neuromedin U

Preeti H. Jethwa, Caroline J. Small, Michael Patterson and Stephen R. Bloom

ABSTRACT

Neuromedin U (NMU) is one of the most abundant neuropeptides and has been found in significant concentrations in both the gastrointestinal tract and the central nervous system (CNS). Within the CNS, NMU cell bodies are found in the rostrocaudal part of the arcuate nucleus of the hypothalamus with more widespread distribution of NMU fibers in the nucleus accumbens, medial thalamus, brainstem, paraventricular nucleus, ventromedial nucleus, and dorsomedial nucleus, areas where the NMU2 receptor is expressed. Recently, NMU has been shown to be a potent anorexigenic peptide, and alterations of NMU signaling can predispose to obesity.

DISCOVERY

Neuromedin U (NMU) was first isolated from porcine spinal cord in 1985 and named for its potent contractile activity on the uterus.[23,24] Two molecular forms were purified; an icosapentapeptide (NMU-25) and an octapeptide (NMU-8) identical to the C-terminal of NMU-25.[23,34] Both these forms of NMU are biologically active. NMU has since been fully sequenced in several species, and most of the NMU peptides that have been isolated from different species are icosapentapeptides with the exception of rat NMU, NMU-23.[4]

STRUCTURE OF NMU mRNA

The human cDNA encoding the precursor protein for NMU has been sequenced.[2] The human NMU is an icosapentapeptide and has no internal dibasic cleavage site for the generation of a shorter version. The comparison of human and rat cDNA encoding NMU reveal about 70% homology between the two sequences suggesting that NMU is synthesized from a 174-amino acid precursor containing the NMU peptide within the C-terminus.[2,22] The precursor contains a hydrophobic signal peptide and several dibasic cleavage sites that give rise to a number of possible secreted peptides, including NMU (Fig. 1). Southern blot analysis of genomic DNA reveals that NMU is a single copy gene.[22]

NMU DISTRIBUTION AND PROCESSING

NMU shows remarkable conservation throughout evolution: the C-terminal pentapeptide (Phe-Arg-Pro-Arg-Asn-NH^2) is conserved across all species, and the heptapeptide (Phe-Leu-Phe-Arg-Pro-Arg-Asn-NH^2) is conserved throughout all mammalian species.[22] This degree of conservation suggests the importance of the carboxyl-terminus sequence for biological activity.

NMU is an abundant peptide, found in significant concentrations in both the gastrointestinal tract[1,3,6] and central nervous system.[3,6,9,14,16,17,31] It is also found in other tissues such as the pituitary gland,[6] the thyroid gland,[5] and the urogenital tract.[6]

In the rat brain, the highest levels of NMU-like immunoreactivity are found in the nucleus accumbens (Acb), hypothalamus, anterior pituitary, and thalamus.[6,7] NMU-immunoreactive cell bodies are found in the rostrocaudal part of the arcuate nucleus of the hypothalamus (Arc) with more widespread distribution of immunoreactive fibers in the Acb, medial thalamus, paraventricular nucleus (PVN), ventromedial hypothalamus (VMH), dorsomedial hypothalamus (DMH), and supraoptic nucleus (SON), and in the brainstem with dense terminals fields primarily in the nucleus of the solitary tract (NST) and parabrachial nucleus (PBN) (Fig. 4A).[3,14,16,31,32] In situ hybridization has shown NMU mRNA to be most abundant in the pituitary pars tuberalis and slightly less in the Arc and median eminence, the caudal brainstem (NST, area postrema (AP), and dorsal motor nucleus of the vagus (DMV), and the spinal cord.[14,16,17,31]

The presence of NMU mRNA in the median eminence was initially disputed, as some studies reported expression is localized to the par turberalis.[17] However, this was clarified when Graham et al.[9] showed that the expression of NMU mRNA is species dependent (Figs 2 and 3). In mice, NMU mRNA is detected in the Arc, DMH, VMH, and suprachiasmatic nucleus (SCN) in contrast to the expression of NMU mRNA in the rat in which it is absent from the median eminence, the VMH and the DMH but highly expressed in the pituitary par tuberalis.[9] In mice, NMU mRNA is located within the same region of the Arc as proopiomelanocortin

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00114-7
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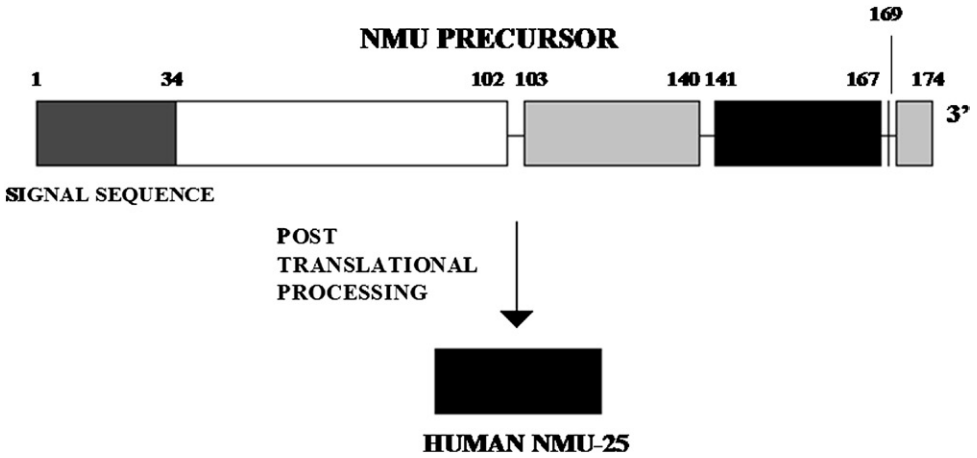

FIGURE 1 Neuromedin U-25 precursor peptide.[2]

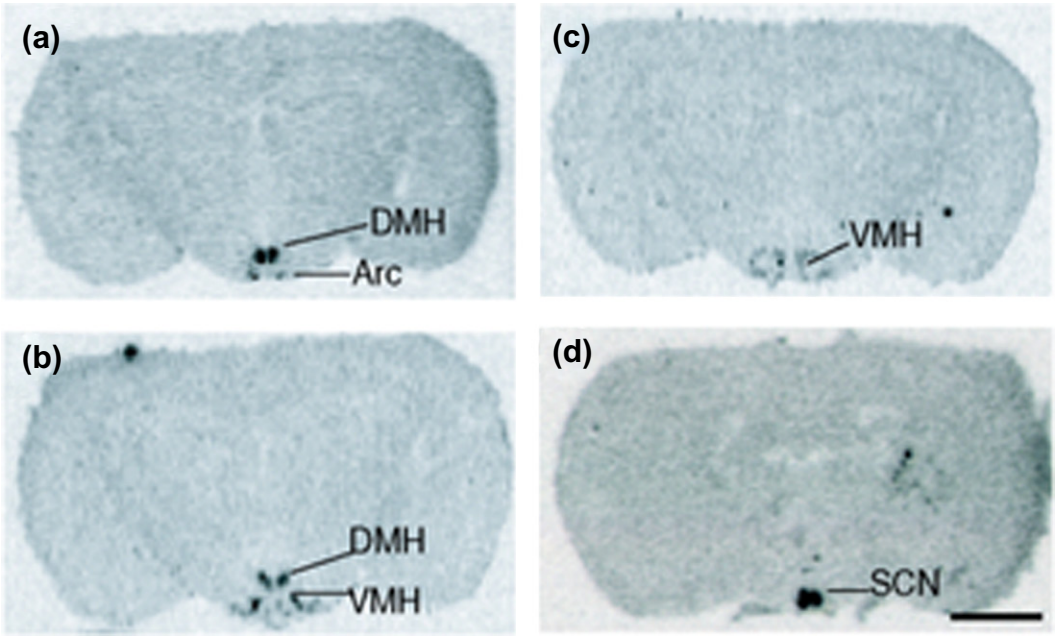

FIGURE 2 NMU expression in the mouse hypothalamus Autoradiographs showing NMU mRNA expression in the mouse hypothalamus. In situ hybridization was conducted using ^{35}S-labeled NMU antisense RNA probe. Specific expression is observed in the (a) DMH and Arc, (b) VMH, (c) DMH and VMH, and (d) SCN. Scale bar in (d) represents 2 mm.[9]

(POMC)/cocaine- and amphetamine-regulated transcript (CART) neurones, which play a key role in energy homeostasis, but there is no colocalization of CART and NMU mRNA.[9]

NMU RECEPTORS

In 1998, a G protein-coupled receptor (GPCR) FM-3 was cloned from human and murine cDNA libraries because of its homology with the growth hormone secretogogue receptor and the neurotensin receptor. Cloning enabled a subsequent "reverse pharmacological" approach for the identification of NMU as an endogenous ligand for the orphan human GPCR, FM-3.[8,13,15,16,30,31,33] NMU binds to and activates human FM3 with sub-nanomolar affinity and potency. Extracts from rat tissue contain the endogenous ligand for FM-3, the brain and small intestine extracts having the highest affinity. A 23-amino acid peptide identical to rat NMU-23 was subsequently chromatographically purified. A number of other potential ligands with homology to NMU were screened for receptor affinity but only NMU peptides activate the receptor. However, later a second high affinity ligand, Neuromedin S (NMS), has been identified.[26]

Simultaneously, a second NMU receptor, a GPCR, FM-4 was identified in humans and rodents.[15,16,30,31] FM-3 and FM-4 are cognate receptors and have been renamed NMU1R and NMU2R, respectively. Both receptors have

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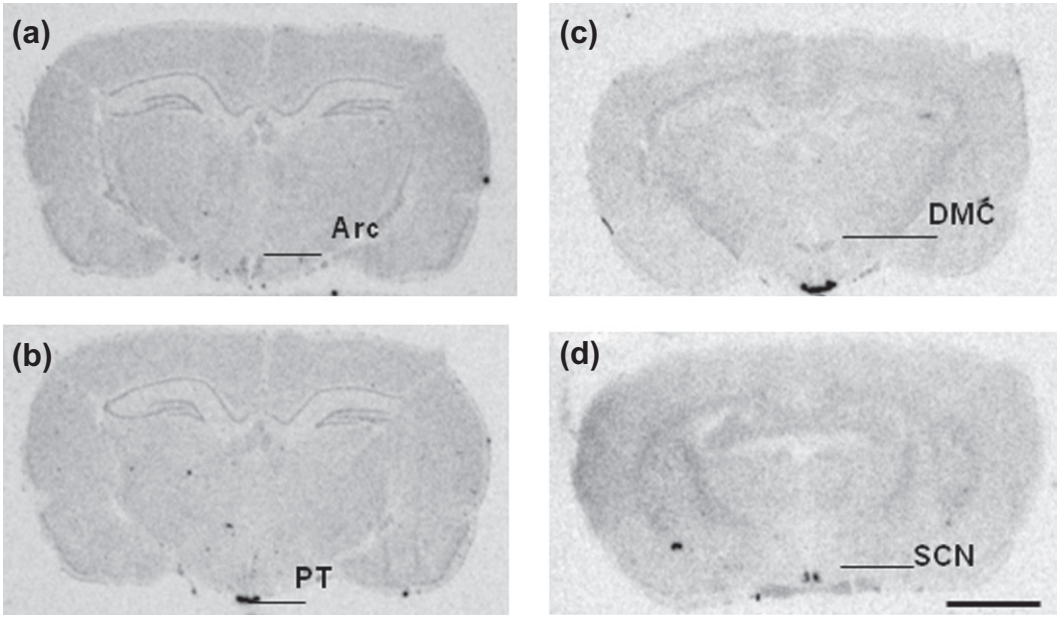

FIGURE 3 NMU expression in the rat hypothalamus Autoradiographs showing NMU mRNA expression in the rat hypothalamus (*ad libitum* fed hooded listers). NMU mRNA was detected in (a) the Arc (−2.8 to Bregma), (b) pars tuberalis (−3.6 to Bregma), (c) Dorsomedial compact zone of the DMH (−3.3 to the bregma), and (d) in the SCN (−1.4 to Bregma). Scale bar in (d) represents 2 mm.[9]

significant abilities to distinguish between different forms of NMU, and have species and tissue selectivity in the biological actions of NMU.[8,13,15,16,30,31,33] NMS is a second endogenous ligand for both receptors. NMU and NMS have a similar affinity for NMU1R, but NMS binds NMU2R with a slightly greater affinity.[26]

NMU1R

The gene for human NMU1R has been mapped to SHGC-33253, which is localized on chromosome 2q34–937[30] and is encoded by two exons.[31] Human NMU1R has a molecular weight of 44979 Da. The mRNA for NMU1R is expressed in a wide variety of tissues, but levels are the greatest in peripheral tissues in particular the small intestines and stomach. NMU1R mRNA is also present in relatively high levels in the pancreas, testis, adrenal cortex, liver, heart, lung, trachea, mammary gland, bone marrow, and peripheral blood leucocytes.[13,15,16,33] A similar distribution pattern exists in the rat with highest levels in the small intestine, lung and bone marrow.[8] NMU1R mRNA expression is also found in approximately 25% of the small/medium diameter neurons within the dorsal root ganglia.[35] There is relatively low expression of NMU1R in human or rodent brain.[8,16]

NMU2R

The gene for human NMU2R has been mapped to SHGC-8848, which is localized to chromosome 5q31.1–q31.1.[30] Human NMU2R has a molecular weight of 47450 Da. NMU2R mRNA in humans is confined predominantly to specific regions within the brain, with the greatest expression observed in the thalamus, substantia nigra, pontine reticular formation, medulla oblongata, and spinal cord. Moderate to high levels are present in the indusium griseum, vascular organ of the lamina terminalis, setophippocampal nucleus, CA1 region of the hippocampus, parafasicular thalamic nucleus, the PVN and the wall of the third ventricle in the hypothalamus and the dorsal raphe nucleus (Fig. 4B).[16] A detailed analysis of NMU2R expression is now complete for the rat.[9] The NMU2R gene is found in the medial postal region of the Arc adjacent to the third ventricle, isolated neurons in other portions of the Arc,[9] and the PVN. NMU2R is less abundant in the bed nucleus of the stria terminalis, subfornical organ, dorsal fornix, dorsal tuberomammillary nucleus, raphe obscurus, raphe pallidus nuclei, cunneate nucleus, and paratrigneinal nucleus.[16]

Peripherally, NMU2R is expressed in the testes with lower levels present in the GI tract, genitourinary tract, liver, pancreas, adrenal gland, thyroid gland, lung, trachea, spleen, and thymus.[16]

BIOLOGICAL ACTIONS OF NMU IN THE BRAIN

The Role of NMU in the Regulation of Energy Balance

Intracerebroventricular (icv) administration of NMU reduces nocturnal food intake and decreases body weight.[17,20,28,34] In addition, icv administration of NMU

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antiserum increases food intake,[20] whereas fasting reduces the levels of NMU mRNA in the VMH.[16] NMU may also play a role in leptin-induced satiety; in rats, icv injection of anti-NMU antiserum partially attenuates the feeding effects of intraperitoneal leptin administration.[18]

Administration of NMU into specific hypothalamic areas potently reduces food intake. Wren et al.[34] showed a dose-dependent reduction of feeding 1 hour after the administration of NMU into the PVN and Arc. In addition to the immediate reduction in food intake observed after NMU administration into the PVN or Arc, NMU has a much delayed inhibitory (4–8 h postinjection) effect after administration into the medial preoptic area (MPOA) of the hypothalamus.[34] The MPOA is involved in the reproductive function and *c-fos* expression, and NPY levels increase after feeding, while αMSH reduces food intake after administration in the MPOA.[19] Thus, NMU may interact with other neuropeptides to mediate the delayed feeding effect.[34]

The decrease in food intake after a single icv injection of NMU is associated with a significant increase in gross locomotor activity, core body temperature, heat production, and oxygen consumption.[11,16,17,28,34] However, NMU does not cause shivering, suggesting that the effects on body temperature are mediated by changes in sympathetic outflow to brown adipose tissue and skeletal muscle. Ivanov et al.[17] proposed that the effects of NMU on feeding and energy expenditure are independent as they observed an increase in core body temperature in the absence of a decrease in food intake in satiated animals.

NMU null (NMU–/–) mice[12] and NMU overexpressing (NMU Tg) mice[21] have been developed. NMU–/– mice show increased body weight and adiposity, hyperphagia, and decreased locomotor activity and energy expenditure. Obese NMU–/– mice develop hyperleptinemia, hyperinsulinemia, late-onset hyperglycemia, and hyperlipidemia.[12] The NMU Tg mice, however, show decreased body weight and adiposity, hypophagia, and a modest increase in respiratory quotient during food deprivation and refeeding. Unlike the NMU–/ mice, the NMU Tg mice also show improved insulin sensitivity on both normal and high fat diet.[21] In addition, many neuropeptides important to the regulation of feeding are altered in these transgenic animals. In the NMU–/– mice, there is a decrease in POMC mRNA, while neuropeptide Y (NPY) and agouti-gene-related peptide (AgRP) mRNA levels remain unchanged.[12] However in the NMU Tg mice, both POMC and NPY mRNA levels are increased, while AgRP remains unchanged.[21] The phenotype of these animals suggests that alterations in the NMU system lead to altered energy metabolism.

The Role of NMU in Gastric Acid Secretion and Emptying

NMU icv reduces gastric acid secretion.[25] This CNS effect of NMU seems to be independent of vagal innervation because NMU significantly suppresses pentagastrin secretion in vagotomized rats. Prostaglandin E_2 reduces gastric acid secretion directly by inhibiting parietal cell secretion and indirectly by inhibiting gastric acid release. Peripheral administration of indomethacin, to block prostaglandin synthesis, prevents the inhibitory action of neuropeptides on gastric secretion. However, administration of NMU suppresses acid secretion in indomethacin-treated rats, suggesting that NMU acts independently of prostaglandin E_2.[25]

The Role of NMU in the Regulation of the HPA Axis and Stress Response

Corticotropin-releasing hormone (CRH) is another hypothalamic peptide located in the PVN, which decreases food intake, increases energy expenditure via stimulation of the sympathetic nervous system, induces stress responses, and increases locomotor and grooming behavior. Icv and intra-PVN administration of NMU increases plasma adrenocorticotropic hormone and corticosterone. In addition, the release of CRH and arginine vasopressin is increased from *ex vivo* hypothalamic explants incubated with NMU.[29] Consistent with these effects, NMU increases *c-fos* expression in CRH-containing neurons in the parvocellular region of the PVN and SON[17,29] and augments CRH mRNA expression in the PVN.[12]

The mechanism by which hypothalamic NMU alters feeding behavior and energy homeostasis is not clear. However, given that the icv effects of CRH appear to be similar to those observed after NMU administration Hanada et al.[10] hypothesized that the changes in feeding behavior and energy homeostasis with NMU may be via CRH. NMU icv does not alter dark phase food intake or fasting-induced feeding in CRH null ($CRH^{-/-}$) mice when compared with that in wild-type mice.[10] In addition, the increase in oxygen consumption, body temperature, and locomotor activity observed after NMU administration are suppressed in the $CRH^{-/-}$ mice. The effects of NMU are also abolished in wild-type mice pretreated with the CRH receptor antagonist alpha-helical-$CRH_{(9–41)}$ when compared with their controls.[11] Further supporting this, Hanada et al.[12] showed that CRH mRNA expression is reduced in the PVN of $NMU^{-/-}$ mice and show a lower plasma corticosterone level. However, surprisingly, NMU Tg mice show no changes in CRH mRNA expression,[21] although this may result from a differing method of detection.

To further support the hypothesis, NMU-induced inhibition of gastric acid secretion is blocked by pretreatment with anti-CRH IgG, suggesting that NMU-induced acid inhibition is mediated by CRH. CRH inhibits gastric acid secretion by activation of the sympathetic, noradrenergic nervous system and not vagal fibers. This finding supports the possibility that CRH mediates NMU-induced acid inhibition through a vagus-independent pathway. Icv administration

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of NMU also inhibits gastric emptying in rats; although peripheral NMU has no effect.[25] This suggests that NMU mediates its effects on food intake and energy expenditure via hypothalamic CRH.

COMPARISON OF THE BIOLOGICAL ACTIONS OF NMU AND NMS

NMS was discovered in 2005 as an alternative ligand for the NMU receptors.[26] NMU and NMS share a common amidated C-terminal hexapeptide. Distribution of NMS seems more limited than NMU; in rats, high levels of NMS mRNA are only detected in the hypothalamus, testis, and spleen.[26] Within the hypothalamus particularly high levels of NMS are detected in the SCN suggesting a possible role in the regulation of circadian rhythms.[26] Like NMU, central administration of NMS suppresses feeding and stimulates the HPA axis. Nakahara et al.[27] compared the effects of NMU and NMS on feeding. When administered icv, both NMS and NMU increase CRH and POMC mRNA levels in the PVN and Arc.[27] Anti-NMS antisera block the feeding effects of leptin but in contrast to previous reports anti-NMU antisera have no effect.[27] Thus, it is probable the NMU system is involved in mediating the anorexi-

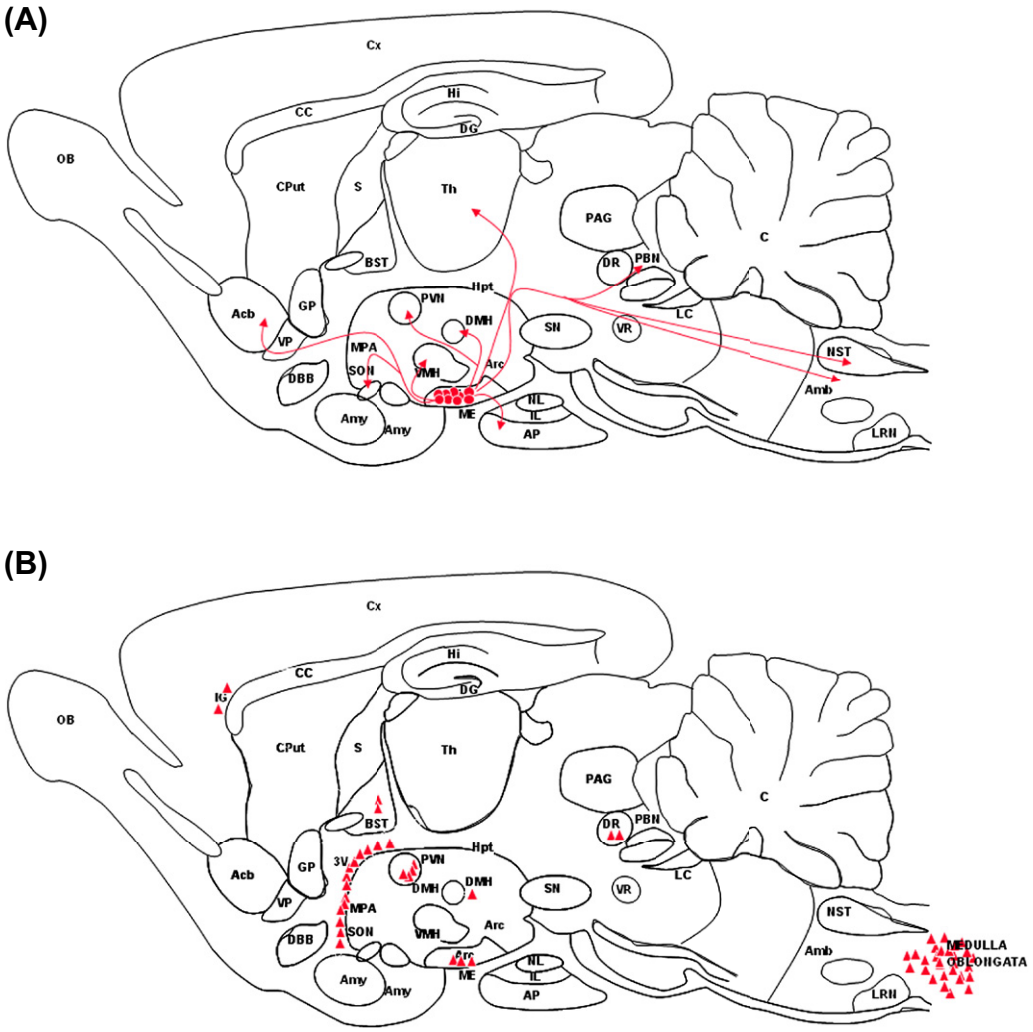

FIGURE 4 Drawing of a parasagittal section of rat brain showing: (A) Distribution of NMU cell bodies ● and immunoreactive fibers ⟶. (B) Distribution of neuromedin U 2 receptor (NMU2R) ▲, this receptor is found predominantly in the brain, unlike the NMU1 receptor (NMU1R), which is found predominantly in peripheral tissues. Acb, nucleus accumbens; Amb, nucleus ambiguus; Amy, amygdala; AP, anterior pituitary; Arc, arcuate nucleus of hypothalamus; ARP, area postrema; BST, bed nucleus of stria terminalis; C, cerebellum; CC, corpus callosum; CPut, caudate putamen; Cx, cerebral cortex; DBB, diagonal band of Broca; DG, dentate gyrus; DMH, dorsomedial nucleus of hypothalamus; DR, dorsal raphe nucleus; GP, globus palidus; Hi, hippocampus; Hpt, hypothalamus; IG, indusium griseum; IL, intermediate lobe of pituitary; LC, locus coeruleus; LRN, lateral reticular nucleus; ME, median eminence; MPA, medial preoptic area; NL, neural lobe of pituitary; NST, nucleus of solitary tract; OB, olfactory bulb; PAG, periaqueductal gray; PBN, parabrachial nucleus; PVN, paraventricular nucleus of hypothalamus; S, septum; SN, substantia nigra; SON, supraoptic nucleus; Th, thalamus; VMH, ventromedial nucleus of hypothalamus; VP, ventral pallidum; VR, ventral raphe nuclei; 3V, 3rd ventricle.

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genic effects of leptin, but the contribution of each peptide requires further clarification. NMU and NMS elicit many of the same actions; however, significant differences in distribution suggest there may be discrepancies in their biological functions.

PATHOPHYSIOLOGICAL IMPLICATIONS

The development of the NMU null ($NMU^{-/-}$) mice[12] and the NMU overexpressing (NMU Tg) mice[21] further supports the role of NMU as an endogenous anorexigenic peptide. The $NMU^{-/-}$ mice develop an obese phenotype, whereas NMU Tg mice that have ubiquitous expression of the NMU transgene develop a lean phenotype. These findings suggest that NMU is an important regulator of energy balance as perturbation of NMU signaling can result in obesity. It is probable that NMU mediates its effects on body weight via alterations in hypothalamic CRH. Although this is an important and interesting hypothalamic circuit, it may not prove a useful antiobesity target. Stimulation would result in weight loss but also an overactive hypothalamic–pituitary–adrenal axis. Thus, NMU may not be useful as a long term antiobesity therapy.

REFERENCES

1. Augood SJ, Keast JR, Emson PC. Distribution and characterisation of neuromedin U-like immunoreactivity in rat brain and intestine and in guinea pig intestine. *Regul Pept* 1988;**20**:281–92.
2. Austin C, Lo G, Nandha KA, Meleagros L, Bloom SR. Cloning and characterization of the cDNA encoding the human neuromedin U (NmU) precursor: NmU expression in the human gastrointestinal tract. *J Mol Endocrinol* 1995;**14**:157–69.
3. Ballesta J, Carlei F, Bishop AE, Steel JH, Gibson SJ, Fahey M, et al. Occurrence and developmental pattern of neuromedin U-immunoreactive nerves in the gastrointestinal tract and brain of the rat. *Neuroscience* 1988;**25**:797–816.
4. Conlon JM, Domin J, Thim L, DiMarzo V, Morris HR, Bloom SR. Primary structure of neuromedin U from the rat. *J Neurochem* 1988;**51**:988–91.
5. Domin J, Al Madani AM, Desperbasques M, Bishop AE, Polak JM, Bloom SR. Neuromedin U-like immunoreactivity in the thyroid gland of the rat. *Cell Tissue Res* 1990;**260**:131–5.
6. Domin J, Ghatei MA, Chohan P, Bloom SR. Neuromedin U—a study of its distribution in the rat. *Peptides* 1987;**8**:779–84.
7. Domin J, Ghatei MA, Chohan P, Bloom SR. Characterization of neuromedin U like immunoreactivity in rat, porcine, guinea-pig and human tissue extracts using a specific radioimmunoassay. *Biochem Biophys Res Commun* 1986;**140**:1127–34.
8. Fujii R, Hosoya M, Fukusumi S, Kawamata Y, Habata Y, Hinuma S, et al. Identification of neuromedin U as the cognate ligand of the orphan G protein-coupled receptor FM-3. *J Biol Chem* 2000;**275**:21068–74.
9. Graham ES, Turnbull Y, Fotheringham P, Nilaweera K, Mercer JG, Morgan PJ, et al. Neuromedin U and Neuromedin U receptor-2 expression in the mouse and rat hypothalamus: effects of nutritional status. *J Neurochem* 2003;**87**:1165–73.
10. Hanada T, Date Y, Shimbara T, Sakihara S, Murakami N, Hayashi Y, et al. Central actions of neuromedin U via corticotropin-releasing hormone. *Biochem Biophys Res Commun* 2003;**311**:954–8.
11. Hanada R, Nakazato M, Murakami N, Sakihara S, Yoshimatsu H, Toshinai K, et al. A role for neuromedin U in stress response. *Biochem Biophys Res Commun* 2001;**289**:225–8.
12. Hanada R, Teranishi H, Pearson JT, Kurokawa M, Hosoda H, Fukushima N, et al. Neuromedin U has a novel anorexigenic effect independent of the leptin signaling pathway. *Nat Med* 2004;**10**:1067–73.
13. Hedrick JA, Morse K, Shan L, Qiao X, Pang L, Wang S, et al. Identification of a human gastrointestinal tract and immune system receptor for the peptide neuromedin U. *Mol Pharmacol* 2000 Oct;**58**(4):870–5.
14. Honzawa M, Sudoh T, Minamino N, Kangawa K, Matsuo H. Neuromedin U-like immunoreactivity in rat intestine: regional distribution and immunohistochemical study. *Neuropeptides* 1990;**15**:1–9.
15. Hosoya M, Moriya T, Kawamata Y, Ohkubo S, Fujii R, Matsui H, et al. Identification and functional characterization of a novel subtype of neuromedin U receptor. *J Biol Chem* 2000;**275**:29528–32.
16. Howard AD, Wang R, Pong SS, Mellin TN, Strack A, Guan XM, et al. Identification of receptors for neuromedin U and its role in feeding. *Nature* 2000;**406**:70–4.
17. Ivanov TR, Lawrence CB, Stanley PJ, Luckman SM. Evaluation of neuromedin U actions in energy homeostasis and pituitary function. *Endocrinology* 2002;**143**:3813–21.
18. Jethwa PH, Small CJ, Smith KL, Seth A, Darch SJ, Abbott CR, et al. et al. Neuromedin U has a physiological role in the regulation of food intake and partially mediates the effects of leptin. *Am J Physiol Endocrinol Metab* 2005 Aug;**289**(2):E301–5.
19. Kim MS, Rossi M, Abusnana S, Sunter D, Morgan DG, Small CJ, et al. Hypothalamic localization of the feeding effect of agouti-related peptide and alpha-melanocyte-stimulating hormone. *Diabetes* 2000;**49**:177–82.
20. Kojima M, Haruno R, Nakazato M, Date Y, Murakami N, Hanada R, et al. Purification and identification of neuromedin U as an endogenous ligand for an orphan receptor GPR66 (FM3). *Biochem Biophys Res Commun* 2000;**276**:435–8.
21. Kowalski TJ, Spar BD, Markowitz L, Maguire M, Golovko A, Yang S, et al. Transgenic overexpression of neuromedin U promotes leanness and hypophagia in mice. *J Endocrinol* 2005;**185**:151–64.
22. Lo G, Legon S, Austin C, Wallis S, Wang Z, Bloom SR. Characterization of complementary DNA encoding the rat neuromedin U precursor. *Mol Endocrinol* 1992;**6**:1538–44.
23. Minamino N, Kangawa K, Honzawa M, Matsuo H. Isolation and structural determination of rat neuromedin U. *Biochem Biophys Res Commun* 1988;**156**:355–60.
24. Minamino N, Kangawa K, Matsuo H. Neuromedin U-8 and U-25: novel uterus stimulating and hypertensive peptides identified in porcine spinal cord. *Biochem Biophys Res Commun* 1985;**130**:1078–85.
25. Mondal MS, Date Y, Murakami N, Toshinai K, Shimbara T, Kangawa K, et al. Neuromedin U acts in the central nervous system to inhibit gastric acid secretion via corticotropin-releasing hormone system. *Am J Physiol Gastrointest Liver Physiol* 2003.
26. Mori K, Miyazato M, Ida T, Murakami N, Serino R, Ueta Y, et al. Identification of neuromedin S and its possible role in the mammalian circadian oscillator system. *EMBO J* 2005;**24**:325–35.

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27. Nakahara K, Katayama T, Maruyama K, Ida T, Mori K, Miyazato M, et al. Comparison of feeding suppression by the anorexigenic hormones neuromedin U and neuromedin S in rats. *J Endocrinol* 2010;**207**: 185–93.
28. Nakazato M, Hanada R, Murakami N, Date Y, Mondal MS, Kojima M, et al. Central effects of neuromedin U in the regulation of energy homeostasis. *Biochem Biophys Res Commun* 2000;**277**:191–4.
29. Ozaki Y, Onaka T, Nakazato M, Saito J, Kanemoto K, Matsumoto T, et al. Centrally administered neuromedin U activates neurosecretion and induction of c-fos messenger ribonucleic acid in the paraventricular and supraoptic nuclei of rat. *Endocrinology* 2002;**143**:4320–9.
30. Raddatz R, Wilson AE, Artymyshyn R, Bonini JA, Borowsky B, Boteju LW, et al. Identification and characterization of two neuromedin U receptors differentially expressed in peripheral tissues and the central nervous system. *J Biol Chem* 2000;**275**:32452–9.
31. Shan L, Qiao X, Crona JH, Behan J, Wang S, Laz T, et al. Identification of a novel neuromedin U receptor subtype expressed in the central nervous system. *J Biol Chem* 2000;**275**:39482–6.
32. Steel JH, Van Noorden S, Ballesta J, Gibson SJ, Ghatei MA, Burrin J, et al. Localization of 7B2, neuromedin B, and neuromedin U in specific cell types of rat, mouse, and human pituitary, in rat hypothalamus, and in 30 human pituitary and extrapituitary tumors. *Endocrinology* 1988;**122**:270–82.
33. Szekeres PG, Muir AI, Spinage LD, Miller JE, Butler SI, Smith A, et al. Neuromedin U is a potent agonist at the orphan G protein-coupled receptor FM3. *J Biol Chem* 2000;**275**:20247–50.
34. Wren AM, Small CJ, Abbott CR, Jethwa PH, Kennedy AR, Murphy KG, et al. Hypothalamic actions of neuromedin u. *Endocrinology* 2002;**143**:4227–34.
35. Yu XH, Cao CQ, Mennicken F, Puma C, Dray A, O'Donnell D, et al. Pro-nociceptive effects of neuromedin U in rat. *Neuroscience* 2003;**120**:467–74.

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Chapter 115

Neuropeptide B/W

Fumiko Takenoya, Haruaki Kageyama, Ki Sung Kang and Seiji Shioda

ABSTRACT

Neuropeptide B (NPB) and neuropeptide W (NPW) have been identified as endogenous ligands for GPR7 (NPBWR1) and GPR8 (NPBWR2), which belong to the orphan G protein-coupled receptor (GPCRs)family. In this chapter, we provide an overview of the distribution and localization of NPBWR-1 and -2, NPB/W mRNA and these transcripts in brain including human, rabbit, and rodent. NPB/W has various functions, such as feeding regulation and behavior, energy homeostasis, neuroendocrine function, and modulation of inflammatory pain. Therefore, the analysis of the functional significance of NPB and NPW may facilitate the development of therapeutic drugs. This chapter also discusses the morphological and physiological relationship between NPW and other neuropeptides in the central nervous system.

INTRODUCTION

In 1995, O'Dowd et al.,[17] by using oligonucleotides based on the opioid receptor and also on the structurally-related somatostatin receptor, identified two genes, GPR7 (NPBWR1) and GPR8 (NPBWR2), which were predicted to encode two GPCRs in the human brain. These two GPCRs share 70% sequence identity with each other and have a significant similarity with the sequences encoding transmembrane regions of the opioid and somatostatin receptors. NPBWR1 gene expression is found in both human and rodent brains, whereas the NPBWR2 gene is detected in human and rabbit brains, but not in rat or mouse brains.

IDENTIFICATION OF NPB/W

In 2002, Shimomura et al.[20] identified the endogenous ligand for NPBWR1 and NPBWR2 by exposing Chinese hamster ovary (CHO) cells to the porcine hypothalamic extracts while monitoring alterations in the level of cAMP. Moreover, when cell lines that expressed either NPBWR1 or NPBWR2 were incubated with the hypothalamic extracts, it seemed that the inhibition of forskolin induced cAMP production. NPBR-1 and -2 are reported to be coupled to the heterotrimeric Gi protein-mediated receptors. Further structural analysis of the ligands responsible for the inhibition of cAMP production led to the identification of a novel peptide NPW. NPW is named after the tryptophan residues (single-letter code W) appearing at both its N- and C-terminals in its two mature forms: NPW-30 (the 30-amino-acid form) and NPW-23 (consisting of 23 amino acids) (Fig. 1). These two peptides are derived from a common precursor peptide, proNPW, by proteolytic processing of two pairs of arginine residues at positions 24 and 25, and 31 and 32, respectively. At the same time, by using similar structural analysis methods, other research groups discovered NPB, which is also a ligand of NPBWR-1 and -2.[1,5,26]

The NPB cDNAs were cloned by Fujii et al.[5] at Takeda Chemical Industries to search for genes encoding proteins with the motif of the secretory signal sequence. They found a gene, NPB, which encoded a novel secretory protein. The purified endogenous ligand contains 29 amino acid residues and was modified with a bromine. The C-6 position of the indole moiety at the N-terminal tryptophan was brominated and therefore was named neuropeptide B. After the discovery of NPB, Tanaka et al.[26] fractionated acid–acetone extracts of bovine hypothalami and identified NPB and NPW. Similarly, Brezillon et al.,[1] using human NPB mRNA obtained by bioinformatic search of the EST database, discovered NPW's amino acid sequence. NPB is a 29-amino acid peptide with a brominated N-terminal tryptophan. In human tissues, a shorter 23-amino acid form (NPB-23) is generated by the proteolytic processing. NPW-23 and NPB-23 share a 61% amino-acid sequence homology, while NPW-30 and NPB-29 share a 66% amino-acid sequence homology.[1,5]

Distribution and Localization of NPB in Brain

In situ hybridization histochemical and immunohistochemical studies have shown that NPB mRNA is expressed in the hippocampus (CA1, CA2, CA3), lateral habenular nucleus (LHb), paraventricular nucleus (PVH), medial parvocellular subdivision (PaMP), Edinger–Westphal nucleus (EW), motor root of the trigeminal nerve (m5), sensory root of the trigeminal nerve (s5), lateral parabrachial nucleus (LPB)

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00115-9
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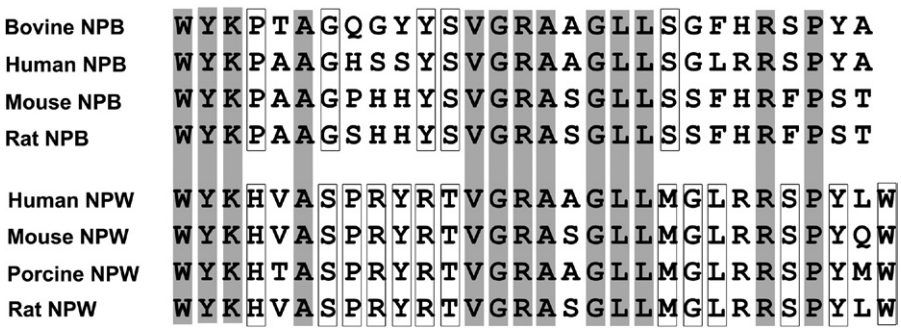

FIGURE 1 Bovine, human, mouse, porcine, and rat amino acid sequences of NPB and NPW. Dark shadow: amino acid identities between NPB and NPW. Light shadow: conserved amino acids within NPB or NPW.

anterior part (Sub CA), locus coeruleus (LC), noradrenergic cell group A5 (A5), and the inferior olive subnucleus B (IOB) in mouse brain.[26] In rat brain, RT-PCR analysis has shown that NPB mRNA is present in the cerebellum, hippocampus, hypothalamus, thalamus, substantial nigra, and in the spinal cord.[5,26] NPB-like immunoreactive (LI) staining is completely abolished by preabsorption of the primary antibody with NPB-29 but not with NPW-30. Dun et al.[3] have reported, using NPW-29 antiserum in an immunohistochemical study, that NPB-LI neurons are widely distributed in rat brain. In the frontal hypothalamic region, NPB-LI neurons are present in the PVH, supraoptic nucleus (SO), and suprachiasmatic nucleus (SCh), these areas being activated as part of the hypothalamic–pituitary–adrenal (HPA) in response to stress. In the caudal hypothalamic area, elevated expression of NPB is observed in cells of the supraoptic retrochiasmatic nucleus (SOR), zona incerta (ZI), lateral hypothalamic area (LH), dorsal hypothalamic area (DA), dorsomedial hypothalamic nucleus dorsal part (DMD), the tuber cinereum area, and caudal arcuate nucleus (ARC). Moreover, NPB-IL nerve fibers are intensely distributed in the median eminence. In the midbrain, NPB-IL cells are located in the EW, substantia nigra reticular part (SNR), lateral part (SNL), and medial part (SNM), as well as in the paranigral nucleus (PN), ventral tegmental area (VTA), and interfascicular nucleus (IF). The NPB mRNA is most abundantly expressed in the EW of the midbrain and many neurons in the EW actually project to the superficial dorsal horn. NPB-LI nerve fibers originating from the EW may be involved in nociceptive processing in the dorsal horn of the spinal cord. Schulz S. et al.[18] carried out a comparative analysis of the distribution of NPBWR1 mRNA, NPB mRNA, and NPB-LI in the rat hypothalamus. These areas show heightened expression in the ventral region of the zona incerta, anterior part of the dorsal thalamic nucleus, ventrolateral hypothalamic nucleus (VLH), and other areas of the ARC and SOR. Several regions in the hypothalamus and midbrain, such as the PVH, ARC, ventromedial nucleus (VMH), and dorsomedial nucleus (DMH) show an overlapping expression of NPB and NPBWR1 mRNAs, with these being important regions for energy and feeding regulation. Moreover, NPBWR1 mRNA and NPB/NPW-LI neurons are located in the SCh and SO, suggesting that the NPB/NPW system may be implicated in the modulation of circadian rhythms. The expression of NPB-LI in rodent brain is summarized in Fig. 2

Distribution and Localization of NPW in Brain

Quantitative RT-PCR results by Brezillon et al.[1] shows that NPW mRNA is highly expressed in the substantia nigra and spinal cord and moderately expressed in the hippocampus, amygdala, hypothalamus, corpus callosum (cc), cerebellum (Cb), and dorsal root ganglia in the human central nervous system (CNS). In rodents, in situ hybridization histochemistry has revealed that NPW mRNA is expressed in a few restricted brain regions including in rat brain of the periaqueductal gray (PAG), EW, dorsal raphe nucleus (DR), and dorsal part of dorsal raphe nucleus (DRD).[1,10,26] Kitamura et al.[10] reported that NPW mRNA in rat brain is confined to the specific nuclei in the midbrain and brainstem including periaqueductal gray and EW. However we have published articles by using RT-PCR analysis that NPW mRNA is expressed in the rat hypothalamic PVH, LH, ARC, and VMH.[23] In the peripheral tissues, NPW mRNA is also expressed in the urogenital system, including kidney, testis, uterus, ovary, and placenta.[5] Moreover, by using RT-PCR analysis, we have also confirmed the presence of NPW mRNA in the pituitary gland, adrenal gland, and stomach.[15,19] NPW may play an important role in the regulation of the endocrine system in response to stress by the activation of the HPA axis.[16,19]

Several immunohistochemical studies of NPW in the brain summarized in Fig. 2 shown that NPW-LI neuronal cell bodies are present in the various brain hypothalamic regions such as the PVH, especially in the parvocellular subdivisions, SO, accessory neurosecretory nuclei, dorsal and LH, perifornical nucleus (Pe), ARC, and anterior and posterior pituitary gland.[4] Moreover, NPW-LI cells seem to

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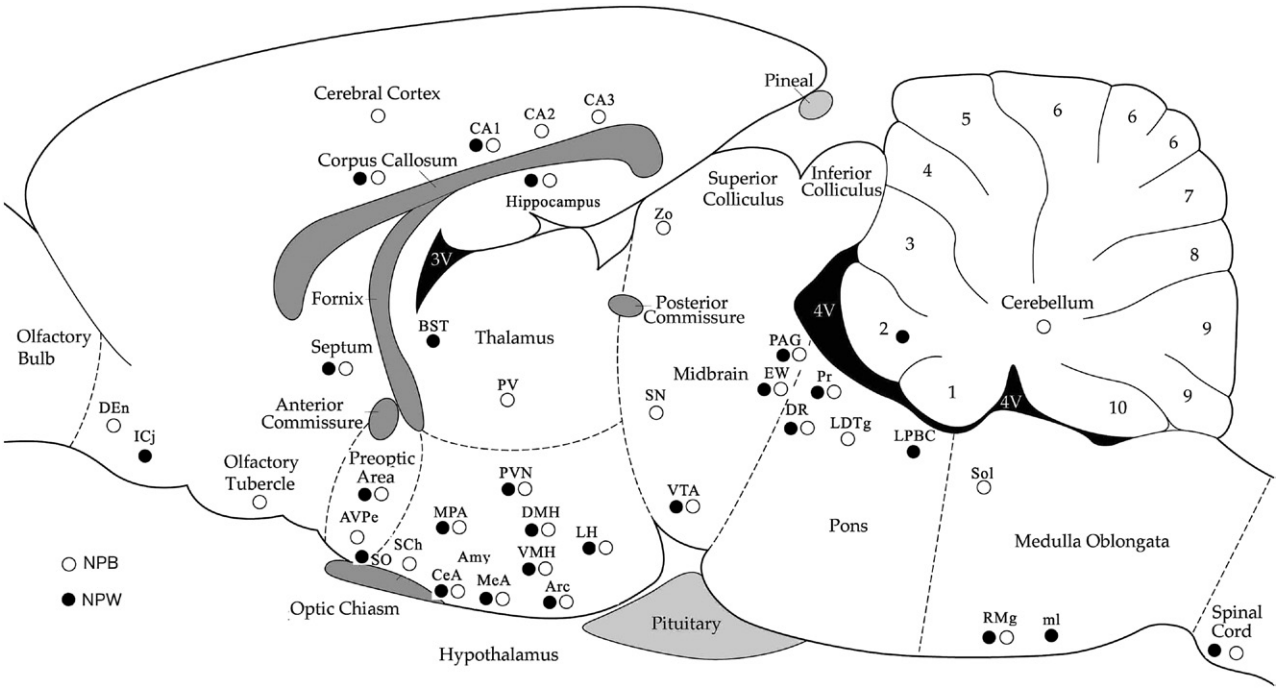

FIGURE 2 Schematic drawing of the distribution of NPW- and NPB-containing neurons throughout rat and mouse brain. The distribution of NPW (black circle), NPB (white circle) dorsal endopiriform nucleus (DEn), islands of Calleja (ICj), hippocampus (CA1), cerebral cortex (Cx), Corpus Callosum, bed nucleus of the stria terminals(BST), Preoptic Aria (POA), anteroventral periventricular nucleus (AVPe), paraventricular thalamic nucleus (PV), Suprachiasmatic nucleus (Sch), supraoptic nucleus (SO), central amygdaloid nucleus (CeA), medial amygdaloid nucleus (MeA), paraventricular hypothalamic nucleus (PVH), arcuate hypothalamic nucleus (Arc), dorsomedial hypothalamic nucleus, dorsal part (DMD), lateral hypothalamic area (LH), Thalamus, zonal layer of the superior colliculus (Zo), substantia nigra (SN), ventral tegmental area (), Edinger-Westphal nucleus(EW), periaqueductal gray matter (PAG), dorsal raphe nucleus(DR), prepositus nucleus (Pr), laterodorsal tegmental nucleus (LDTg), lateral parabrachial nucleus (LPB), solitary tract (sol), raphe magnus nucleus (RMg), Cerebellum, Spinal Cord. *Study by RT-PCR, In situ hybridization, Immunocytochemistry. These data are obtained from Brezillon et al. (2003), Dun et al. (2005), Dun et al. (2003), Fujii et al. (2002), Singh et al. (2004) and Tanaka et al. (2003). Kitamura et al. (2006), Schulz et al.(2007), Takenoya et al. (2009).*

be more numerous in the male hypothalamus as compared with those of female ones.[4] Kitamura et al. have reported a heavy localization of NPW-LI cell bodies in the midbrain including the PAG and EW. However, our recent observation shows that NPW-LI cell bodies are found in many regions in the brain of rats.[23,24] In the forward part of the brain, NPW-LI cell bodies are found in the islands of Calleja (ICj), VLH, anterior parvocellular part (PaAP), medial parvocellular part (PaMP), PVH, DMD, VMH, ARC, and LH. In addition, NPW-LI cell bodies are also detected in the midbrain, and in the dorsal premammillary nucleus (PDM), dorsal tuberomammillary nucleus (DTM), PAG, EW, LPB, prepositus nucleus (Pr), and the raphe nucleus.[23] Moreover, we first identified at the electron microscopic level the presence of NPW-LI cell bodies in the PVH, VMH, and amygdala.[23] NPW-LI neurons in these regions make axosomatic and axodendritic synaptic contacts with unknown axon terminals in the PVH and ARC.[4,23]

NPW-LI nerve fibers have also been identified in the SO, retrochiasmatic nucleus, dorsal and LH, median eminence, amygdala, and posterior pituitary in rat brain,[4] as well as in the septum, BST, dorsomedial and posterior hypothalamus, central amygdaloid nuclei (CeA), CA1 field of the hippocampus, ARC, VMH, inferior colliculus, PBL, facial nucleus, and hypoglossal nucleus.[10] Simultaneously, NPW-LI axon terminals are shown in the POA, bed nucleus of the stria terminalis (BST), amygdala, PVN, ARC, VMH, LH, and PAG.[23] Dense staining of NPW axon terminals are found in the amygdala and BST. Given the abundant distribution of NPW-LI nerve fibers in the midbrain and limbic system such as in the CeA and BST, these observations suggest that NPW may play a role in the regulation of fear and anxiety as well as in feeding behavior.[8,10,23,24]

NPBWR1

The distribution of messenger RNA (mRNA) encoding NPBWR1 and NPBWR2 has been described in anatomical studies by the use of in situ hybridization histochemistry. In human beings, RT-PCR analysis has identified NPBWR1 mRNA located mainly in the hippocampus and amygdala of CNS. As compared with mouse and rat brains, NPBWR1 mRNA is highly expressed in the amygdala, hippocampus, neocortex, and hypothalamus.[11] Additionally, *in vitro*

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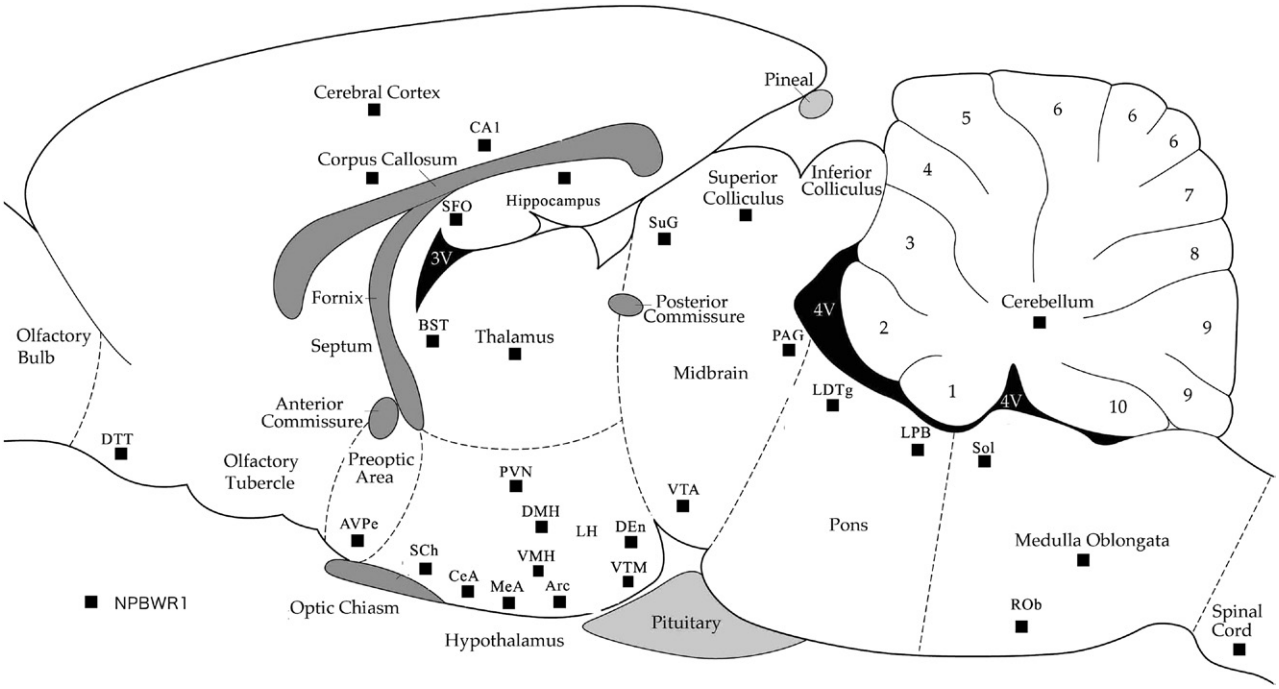

FIGURE 3 A schematic drawing of the distribution of NPBWR1 expression throughout rat and mouse brain (shown by *square*). dorsal tenia tecta (DTT), hippocampus (CA1), cerebral cortex (Cx), corpus callosum (cc), bed nucleus of the stria terminals(BST), Preoptic Aria (POA), anteroventral periventricular nucleus(AVPe), Suprachiasmatic nucleus (Sch), supraoptic nucleus (SO), central amygdaloid nucleus(CeA), medial amygdaloid nucleus (MeA), paraventricular hypothalamic nucleus (PVN), arcuate hypothalamic nucleus (Arc), dorsomedial hypothalamic nucleus, dorsal part (DMD), dorsal endopiriform nucleus (DEn),Thalamus, ventral tegmental area (), superficial gray layer of the superior colliculus (SuG), Superior Colliculus, periaqueductal gray matter (PAG), lateral parabrachial nucleus (LPB), solitary tract (sol) raphe obscurus nucleus (Rob), Cerebellum, Medulla oblongata, Spinal Cord. *Study by RT-PCR, In situ hybridization, Immunocytochemistry. These data are from Brezillon et al. (2003), Fujii et al. (2002), Lee et al. (1999), O'Dowd et al. (1995), Singh et al. (2004) and Tanaka et al. (2003).*

autoradiography study has shown that NPBWR1 mRNA is abundantly expressed in the BST in rat brain, with expression levels yet to be determined in mouse brain. Furthermore, in situ hybridization histochemical studies have revealed that NPBWR1 mRNA is present in the rat hypothalamus, including in the ARC, VMH, PVH, and DMH, which are well known as centers for the regulation of feeding and energy homeostasis. These findings suggest that NPBWR1 receives feeding signals in the feeding-regulating center of the hypothalamus, and it is involved in the regulation of feeding behavior. To this extent, NPBW1 knockout mice show hyperphagia and adult-onset obesity.[9] Moreover, NPBW1R is detected in the midbrain including the LPB, dorsal tenia tecta (DTT), and laterodorsal tegmental nucleus (LDTg).[11,26]

With the aid of [125I]-NPW receptor autoradiography, significant expression of NPBW1 is detected in the amygdala and hypothalamus of the rat brain.[22] Radioactively labeled NPBW1 is also localized in other regions of the rat brain such as the BST, medial preoptic area (MPA), PAG, superficial gray layer of the superior colliculus (SuG), medial amygdaloid nuclei (MeA), and subfornical organ (SFO).[22] In general, NPBWR1 is most commonly expressed in brains at high levels in the amygdala.[10,21,22] Although the BST shows the highest level of NPBWR1 expression in small mammals, this phenomenon has not been shown in human beings. Kitamura et al.[10] have reported that NPBWR1 is most abundantly expressed in the CeA and BST, this may suggest that NPBWR1 is involved in the regulation of stress, emotion, fear, and anxiety. In addition, NPBWR1 is localized in the SCh and SO,[11] suggesting that NPB/W may be involved in the regulation of circadian rhythm via NPBWR1. On the other hand, NPBWR1 and NPBWR2 mRNAs are also localized peripherally in the pituitary and adrenal glands (in both the adrenal cortex and adrenal medulla).[14] These observations suggest that NPBWR-1 and -2 may be responded to stress by the hypothalamic–pituitary–adrenal HPA axis.[14,16] Moreover, NPBWR1 mRNA is expressed in the dorsal horn of the spinal cord, so it may be involved in pain regulation. On the basis of the distribution of NPBWR1, it is possible that species differences between human and rodent's CNS exist in relation to the physiological functions of NPB/NPW. The NPBR1 expression is summarized in Fig. 3.

Ziolkowska et al.[27] examined the expression of that system in cultured rat calvaria osteoblastlike cells (ROB) and its possible role in regulating ROB cell function. The results suggest that the system of NPW, NPB, and NPBWR1

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directly regulates proliferative activity of cultured rat calvaria osteoblastlike cells.

NPBWR2

NPB is present in larger mammals and in rabbits but not in rats or mice. RT-PCR analysis has shown that NPBWR2 mRNA is highly expressed in the amygdala and hippocampus in human brain. Moderate levels of expression are found in the parietal cortex, thalamus, and caudate nucleus, and lower levels in the cerebellum, substantia nigra, and corpus callosum.[1] In the peripheral tissues, NPBWR2 mRNA is moderately expressed in the pituitary gland, adrenal gland, and testis, as well as in the cortical cells in the zona glomerulosa, fasciculata, and reticulata of the adrenal gland in human beings.[14]

NPW and Other Neuropeptides in the Hypothalamus

It is well known that the ARC, LH, and VMH in the hypothalamus are associated with energy homeostasis for feeding regulation. We have examined the neural colocalization of NPW with other feeding-regulating peptides. Typical orexigenic peptides are neuropeptide Y (NPY), orexin, and melanin-concentrating hormone (MCH) in the LH. Our results show very close neuronal interactions between NPW-containing nerve fibers and orexin- or MCH-containing neuronal cell bodies and nerve fibers in the rat brain.[25] On the other hand, using dual immunostaining method, Levine et al.[12] showed that c-Fos expression was induced in orexin-containing neurons in the perifornical region of the LH after intracerebroventricular infusion of NPW. In particular, c-Fos-LI was increased in the perifornical region of the LH after NPW infusion. Orexin is recognized as a peptide[6] that promotes feeding regulation and playing a role in the control of sleep/wakefulness. These findings suggest that the effect of NPW on food intake and sleep/wakefulness may be mediated via activation of the orexin pathway. Hirashima et al.[7] reported that NPB injection decreases the time spent in the waking state and increases the time spent in slow wave sleep during the dark phase. These results suggest that NPB may induce physiological slow wave sleep through NPBWR1 and modulate the occurrence of sleep/wakefulness. However, interactions between NPB- and orexin-containing neurons have not yet been shown by morphological studies.

The VMH is most commonly associated with the satiety center. However, most neurons have not yet been shown to be expressed in the VMH. We identified NPW-LI cell bodies in the VMH in the rat hypothalamus by immunohistochemistry. Leptin acts on the VMH where its receptors are abundantly expressed and reduces food intake via the signal transducer and activator of transcription-3 (STAT3) and suppressors of cytokine signaling (SOCS-3). Changes to the synaptic activity of projections from the VMH to proopiomelanocortin (POMC)-containing neurons in the ARC are known to inhibit food intake and to regulate glucose levels and energy homeostasis. Date et al.[2] have reported that leptin treatment promotes phosphorylation of STAT3 and then induces SOCS-3 in the NPW-containing neurons isolated from the VMH. It is possible that leptin mediates satiety signals via NPW neurons in the VMH. Moreover, using a double immunostaining method, NPW-containing neurons project to POMC-containing neurons in the ARC. Furthermore, treatment with NPW inhibits the firing of NPY neurons and decreases the frequency of spontaneous inhibitory postsynaptic current (sIPSCs) in POMC neurons in the ARC, as shown by loose patch extracellular recording of these neurons (identified by promoter-driven green fluorescent protein expression). These findings strongly suggest that NPW inhibits NPY and activates the POMC neuronal pathway, followed by inducing the suppression of food intake.

Based on the distribution of NPW-LI neurons in rat brain, we speculate that there are other functions of NPW in the brainstem because NPW-LI fibers abundantly project to the nucleus tractus solitarii (NTS) and parabrachial nucleus. Premotor neurons are well known to innervate the salivary glands and tongue. Neuroanatomical studies have shown that descending projections from the LH to the NTS and parabrachial nucleus contain the second- and third-order gustatory neurons, respectively.[13] Therefore, it is possible that NPW may be involved not only in the regulation of food intake but also in salivary secretion. Further studies are required to analyze precisely the neuronal networks underpinning each of these physiological functions.[24]

ACKNOWLEDGMENTS

This study was supported in part by the Ministry of Education, Culture, Sports, Science and Technology of Japan via a Grant-in-Aid for Scientific Research (C) (to FT 23500863) and (to HK 21590222). Support was also received from the Ministry of Education, Culture, Sports, Science and Technology of Japan via a Grant-in-Aid for Exploratory Research to SS (21659059) and by a High-Technology Research Center project grant from the Ministry of Education, Culture, Sports, Science and Technology of Japan (to SS).

REFERENCES

1. Brezillon S, Lannoy V, Franssen JD, Le Poul E, Dupriez V, Lucchetti J, et al. Identification of natural ligands for the orphan G protein-coupled receptors GPR7 and GPR8. *J Biol Chem* 2003;**278**:776–83.
2. Date Y, Mondal MS, Kageyama H, Ghamari-Langroudi M, Takenoya F, Yamaguchi H, et al. Neuropeptide W: an anorectic peptide regulated by leptin and metabolic state. *Endocrinology* 2010;**151**:2200–10.
3. Dun SL, Brailoiu GC, Mizuo K, Yang J, Chang JK, Dun NJ. Neuropeptide B immunoreactivity in the central nervous system of the rat. *Brain Res* 2005;**1045**:157–63.

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4. Dun SL, Brailoiu GC, Yang J, Chang JK, Dun NJ. Neuropeptide W-immunoreactivity in the hypothalamus and pituitary of the rat. *Neurosci Lett* 2003;**349**:71–4.
5. Fujii R, Yoshida H, Fukusumi S, Habata Y, Hosoya M, Kawamata Y, et al. Identification of a neuropeptide modified with bromine as an endogenous ligand for GPR7. *J Biol Chem* 2002;**277**:34010–6.
6. Funahashi H, Hori T, Shimoda Y, Mizushima H, Ryushi T, Katoh S, et al. Morphological evidence for neural interactions between leptin and orexin in the hypothalamus. *Regul Pept* 2000;**92**:31–5.
7. Hirashima N, Tsunematsu T, Ichiki K, Tanaka H, Kilduff TS, Yamanaka A. Neuropeptide B induces slow wave sleep in mice. *Sleep* 2011;**34**:31–7.
8. Hondo M, Ishii M, Sakurai T. The NPB/NPW neuropeptide system and its role in regulating energy homeostasis, pain, and emotion. *Results Probl Cell Differ* 2008;**46**:239–56.
9. Ishii M, Fei H, Friedman JM. Targeted disruption of GPR7, the endogenous receptor for neuropeptides B and W, leads to metabolic defects and adult-onset obesity. *Proc Natl Acad Sci U S A* 2003;**100**:10540–5.
10. Kitamura Y, Tanaka H, Motoike T, Ishii M, Williams SC, Yanagisawa M, et al. Distribution of neuropeptide W immunoreactivity and mRNA in adult rat brain. *Brain Res* 2006;**1093**:123–34.
11. Lee DK, Nguyen T, Porter CA, Cheng R, George SR, O'Dowd BF. Two related G protein-coupled receptors: the distribution of GPR7 in rat brain and the absence of GPR8 in rodents. *Brain Res Mol Brain Res* 1999;**71**:96–103.
12. Levine AS, Winsky-Sommerer R, Huitron-Resendiz S, Grace MK, de Lecea L. Injection of neuropeptide W into paraventricular nucleus of hypothalamus increases food intake. *Am J Physiol Regul Integr Comp Physiol* 2005;**288**:R1727–32.
13. Matsuo R, Shimizu N, Kusano K. Lateral hypothalamic modulation of oral sensory afferent activity in nucleus tractus solitarius neurons of rats. *J Neurosci* 1984;**4**:1201–7.
14. Mazzocchi G, Rebuffat P, Ziolkowska A, Rossi GP, Malendowicz LK, Nussdorfer GG. G protein receptors 7 and 8 are expressed in human adrenocortical cells, and their endogenous ligands neuropeptides B and w enhance cortisol secretion by activating adenylate cyclase- and phospholipase C-dependent signaling cascades. *J Clin Endocrinol Metab* 2005;**90**:3466–71.
15. Mondal MS, Yamaguchi H, Date Y, Tsuruta T, Shimbara T, Toshinai K, et al. Ontogeny of a new enteric peptide, neuropeptide W (NPW), in the developing rat stomach. *Regul Pept* 2008;**145**:141–6.
16. Niimi M, Murao K. Neuropeptide W as a stress mediator in the hypothalamus. *Endocrine* 2005;**27**:51–4.
17. O'Dowd BF, Scheideler MA, Nguyen T, Cheng R, Rasmussen JS, Marchese A, et al. The cloning and chromosomal mapping of two novel human opioid–somatostatin-like receptor genes, GPR7 and GPR8, expressed in discrete areas of the brain. *Genomics* 1995;**28**:84–91.
18. Schulz S, Stumm R, Hollt V. Immunofluorescent identification of neuropeptide B-containing nerve fibers and terminals in the rat hypothalamus. *Neurosci Lett* 2007;**411**:67–71.
19. Seki M, Kageyama H, Takenoya F, Hirayama M, Kintaka Y, Inoue S, et al. Neuropeptide W is expressed in the noradrenalin-containing cells in the rat adrenal medulla. *Regul Pept* 2008;**145**:147–52.
20. Shimomura Y, Harada M, Goto M, Sugo T, Matsumoto Y, Abe M, et al. Identification of neuropeptide W as the endogenous ligand for orphan G-protein-coupled receptors GPR7 and GPR8. *J Biol Chem* 2002;**277**:35826–32.
21. Singh G, Davenport AP. Neuropeptide B and W: neurotransmitters in an emerging G-protein-coupled receptor system. *Br J Pharmacol* 2006;**148**:1033–41.
22. Singh G, Maguire JJ, Kuc RE, Fidock M, Davenport AP. Identification and cellular localisation of NPW1 (GPR7) receptors for the novel neuropeptide W-23 by [125I]-NPW radioligand binding and immunocytochemistry. *Brain Res* 2004;**1017**:222–6.
23. Takenoya F, Yagi M, Kageyama H, Shiba K, Endo K, Nonaka N, et al. Distribution of neuropeptide W in the rat brain. *Neuropeptides* 2010;**44**:99–106.
24. Takenoya F, Kageyama H, Shiba K, Date Y, Nakazato M, Shioda S. Neuropeptide W: a key player in the homeostatic regulation of feeding and energy metabolism? *Ann N Y Acad Sci* 2010;**1200**:162–9.
25. Takenoya F, Kitamura S, Kageyama H, Nonaka N, Seki M, Itabashi K, et al. Neuronal interactions between neuropeptide W- and orexin- or melanin-concentrating hormone-containing neurons in the rat hypothalamus. *Regul Pept* 2008;**145**:159–64.
26. Tanaka H, Yoshida T, Miyamoto N, Motoike T, Kurosu H, Shibata K, et al. Characterization of a family of endogenous neuropeptide ligands for the G protein-coupled receptors GPR7 and GPR8. *Proc Natl Acad Sci U S A* 2003;**100**:6251–6.
27. Ziolkowska A, Rucinski M, Tyczewska M, Malendowicz LK. Neuropeptide B (NPB) and neuropeptide W (NPW) system in cultured rat calvarial osteoblast-like (ROB) cells: NPW and NPB inhibit proliferative activity of ROB cells. *Int J Mol Med* 2009;**24**:781–7.

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Chapter 116

Neuropeptide S

Rainer K. Reinscheid

ABSTRACT

Neuropeptide S (NPS) is one of the "youngest" members among the biologically active peptides. As such, description of its physiological functions and involvements in pathological conditions is still ongoing. NPS and its receptor NPSR1 are primarily expressed in the brain. In rodents, NPS precursor expression is restricted to a few brainstem nuclei, most intriguingly in a neuronal cluster adjacent to the noradrenergic locus coeruleus. NPSR1 transcripts are more widely expressed, with highest levels found in cortex, amygdala, thalamus, hypothalamus, and subiculum. Early preclinical investigations suggest that NPS might regulate arousal states, exploratory and anxiety-like behaviors, learning and memory, drug reward, feeding behavior, and sensory-motor gating. A functional polymorphism in human NPSR1 that changes the receptor's sensitivity for NPS has been associated with panic disorder and fear learning. These early results suggest that the NPS system may be an innovative drug target for the treatment of various mental illnesses.

DISCOVERY

Neuropeptide S (NPS) was first disclosed in a patent filed by Takeda Pharmaceuticals.[27] The patent reported that it had been isolated and purified according to the "orphan receptor strategy" by using its cloned receptor and ligand-induced second messenger activation as a bioassay.[5] The patent also disclosed the sequence of the receptor but contained no further data on tissue distribution, biological effects, or physiological functions. In 2004, we described the pharmacological characterization, tissue distribution and behavioral effects of this novel ligand/receptor system and suggested naming the peptide after the conserved N-terminal serine residue (single letter code: S; Fig. 1).[31] NPS is a 20 amino acid peptide that is found in the genome of all tetrapods but appears to be absent from fish and invertebrate genomes.[24] Recently however, a putative precursor protein encoding a truncated peptide that is identical with NPS 1–7 has been cloned from the cephalochordate *Branchiostoma floridae* (Fig. 1).[11] It is currently unclear if this peptide represents a species ortholog or is the result of convergent evolution. Intriguingly, the same seven terminal amino acid residues of NPS (SFRNGVG) are perfectly conserved across all species examined thus far (Fig. 1),[24] suggesting that the *Branchiostoma* peptide might represent an "early" evolutionary version of NPS. However, the absence of NPS-like sequences in the more complex and evolutionarily younger fish genomes is difficult to explain in this context.

STRUCTURE OF THE mRNA/GENE

NPS is encoded by a rather short, but typical, precursor protein. Including the signal peptide, the human NPS precursor is only 89 amino acid long (Fig. 2). Despite the short coding sequence, the human NPS gene contains at least 3 exons, with exon 1 encoding the 5′-UTR and the first two amino acids of the precursor protein, exon 2 encoding the signal peptide, and exon 3 coding for the remaining precursor sequence and the 3′-UTR. The absolute size of the NPS precursor mRNA has never been determined experimentally in any species, for example by Northern Blot analysis, but a search of the EST database in GenBank yields a bovine EST clone with a length of 669 bp (accession: DN276816) that contains the entire open reading frame and a substantial 3′ sequence. This suggests that the 3′-UTR of the NPS precursor mRNA might be at least 300 bp in size. The unprocessed NPS sequence is found at the C-terminus of the predicted precursor and is preceded by a pair of basic amino acids (Lys–Arg; Fig. 2), which are typically found as proteolytic processing sites in most neuropeptide precursors.

PROCESSING OF THE PRECURSOR

Processing of the NPS precursor protein has not yet been investigated systematically. Full length NPS 1–20 was isolated from rat and mouse brain extracts,[27] suggesting that proteolytic cleavage of the precursor must have occurred at the dibasic Lys–Arg motif immediately preceding immature NPS. A comparison of putative NPS precursor proteins from over 30 tetrapod species revealed that the protein "spacer" between the signal peptide and unprocessed NPS shows little conservation, suggesting that no other biologically active peptides may be encoded by this precursor protein, other than NPS itself.[24]

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00116-0
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SFRNGVGTGMKKTSFQRAKS   Human
SFRNGVGTGMKKTSFRRAKS   Chimpanzee
SFRNGVGSGAKKTSFRRAKQ   Mouse
SFRNGVGSGVKKTSFRRAKQ   Rat
SFRNGVGTGMKKTSFRRARS   Cat
SFRNGVGTGMKKTSFRRAKS   Dog
SFRNGVGTGMKKTSFRRAKS   Cow
SFRNGVGTGMKKTSFRRAKP   Pig
SFRNGVGTGMKKTSFQRAKS   Elephant
SFRNGVGTGMRNTSFQRAKS   Dolphin
SFRNGVGQGIKKTSFRRAKS   Platypus
SFRNGVGSGMKKTSFRRAKS   Opossum
SFRNGVGSGIKKTSFRRAKS   Chicken
SFRNGVGSGIKKNSFRRAKL   Xenopus

SFRNGVG                Branchiostoma floridae
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FIGURE 1 Alignment of NPS sequences from various species. Sequences were deduced from cDNA or genomic DNA sequences as described.[11,24] Amino acids identical to human NPS are shaded.

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                                                   ↓
1    M I S S V K L N L I L V L S L S T M H V F W C Y P V P S S K    human
1    M I G S L K L S F V L A L S L S V M H V L W C Y P V L S S K    mouse
1    M I G S L K F N F I L F L L I S T M H M F W C H P I S S S K    bovine

31   V S G K S D Y F L I L L N S C P T R L D R S K E L A F L K P    human
31   V P G K P D Y F L I L L S S C P A R L E G S D R L A F L K P    mouse
31   V P G K S D Y F V I L L N S C P T R M D R R V G L D F L K P    bovine

61   I L E K M F V K R S F R N G V G T G M K K T S F Q R A K S      human
61   I L E K T S M K R S F R N G V G S G A K K T S F R R A K Q      mouse
61   I L E K T L M K R S F R N G V G T G M K K T S F R R A K S      bovine
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FIGURE 2 Alignment of human, mouse and bovine NPS precursor proteins. The putative signal peptide is underlined and the presumed signal peptide cleavage site is indicated by an arrow. The presumed dibasic proteolytic processing site is indicated by a box. Amino acids different from the human precursor sequence are shaded. Amino acid positions are given on the left.

Indirect evidence about the presumed processing of NPS precursors comes from a recent study of global gene expression in NPS-synthesizing neurons. Using NPS/EGFP-transgenic mice, NPS/EGFP-positive cells were harvested by laser-capture microdissection and mRNA was extracted and prepared for microarray gene expression analysis.[16] The results showed strong expression of the prohormone convertases Pcsk1, Pcsk2, Pcsk6, and nardilysin, that are presumably involved in neuropeptide maturation (see convertase chapters in Peptide Biosynthesis/Processing section of this book).

As a typical neuropeptide, NPS is most probably degraded by extracellular proteases after its synaptic release. The structures of degradation products and enzymes involved in NPS inactivation are, however, unknown at this time.

DISTRIBUTION OF THE mRNA/PEPTIDE

Currently, detailed expression results are only available from rat and mouse brain. Analysis of NPS distribution in rat tissues revealed that the precursor mRNA is strongly expressed in the brain. Peripheral tissues displaying NPS precursor mRNA expression are salivary and mammary glands, and thyroid.[31] NPS precursor transcripts are strongly expressed in three nuclei of the rat brainstem: the locus coeruleus (LC) area, principal sensory trigeminal nucleus (Pr5), and the lateral parabrachial nucleus (LPB) (Fig. 3). A more detailed investigation by double in situ hybridization in the LC region revealed a cluster of NPS-expressing neurons localized ventromedial to the noradrenergic neurons of LC proper that are situated in between the LC and Barrington's nucleus. This region is also referred to as the pericoerulear (periLC) area. The anatomical location of these NPS-producing neurons is unique and defines a previously unclassified cluster of neurons in the periLC region.[31,32] Neurochemical analyses show that the periLC NPS-expressing cells are mostly glutamatergic neurons with a small number of presumed cholinergic neurons. All NPS-producing cells in Pr5 are glutamatergic neurons, whereas in the rat LPB, NPS co-localizes with corticotropin-releasing hormone

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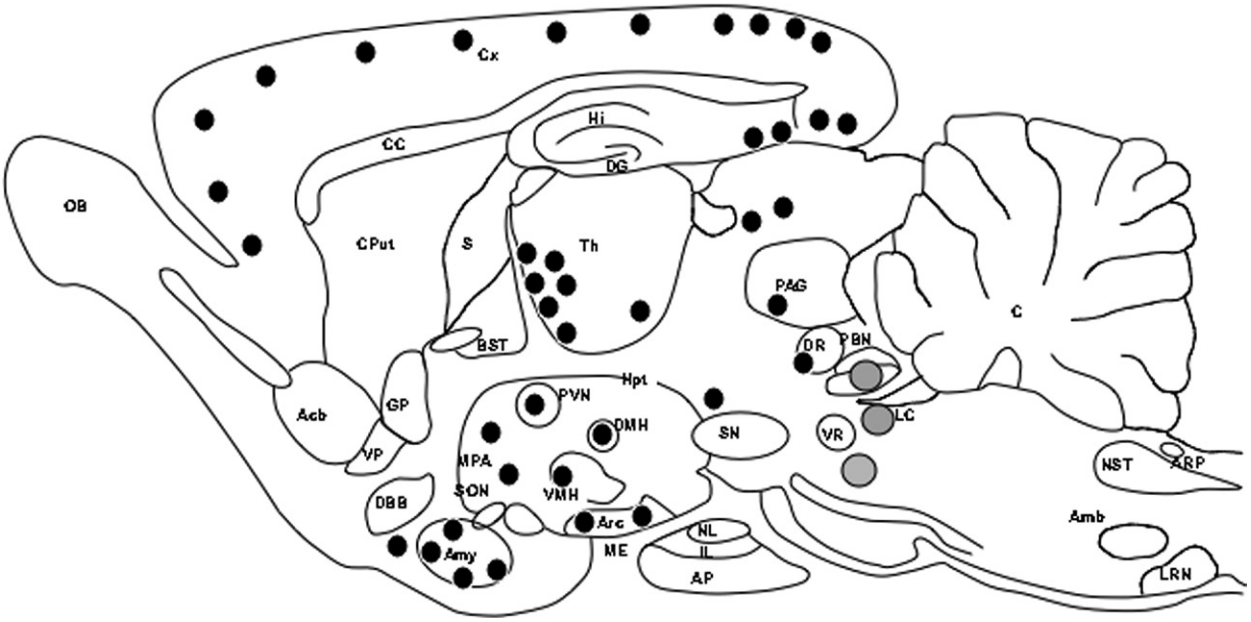

FIGURE 3 Schematic drawing of NPS precursor and NPSR1 transcript distribution in the rat brain. Gray circles depict areas of NPS precursor mRNA expression, while black circles indicate brain regions with high NPSR1 transcript expression.

(CRH).[32] The neurochemical profile of NPS-producing neurons suggests that the NPS system may have an excitatory role since NPS is predominantly found colocalized with excitatory transmitters. Besides the brainstem, only a few isolated cells expressing NPS precursor mRNA are found in the rat amygdala and hypothalamus.

In the mouse brain, strong NPS precursor mRNA expression is found in only two regions of the brainstem: the periLC and the Kölliker-Fuse (KF) nucleus that is located in the lateral parabrachial area.[6] Expression of NPS peptide in periLC and KF nucleus of the mouse brain was also confirmed by immunostaining. In contrast to the rat brain, mouse Pr5 does not appear to express NPS precursor transcripts or protein. Furthermore, no isolated neurons expressing NPS mRNA were found in hypothalamus or amygdala of the mouse brain. The location of periLC NPS-expressing neurons in rat and mouse brain appears to be very similar, indicating that this brainstem region might have equivalent functions in both rodent species. NPS-expressing neurons in the mouse KF nucleus are co-expressing CRH,[16] suggesting that this group of neurons might be the mouse equivalent to the more medially located LPB NPS-producing cells of the rat brain.

Projections of NPS-synthesizing neurons were studied in the mouse brain by immuno-staining with antisera raised against either the N- or C-terminus of NPS.[6] Dense NPS-immunopositive fiber projections were detected in hypothalamic and thalamic regions, while lower fiber densities were found in amygdala and forebrain structures. The distribution of NPS-immunopositive fibers overlaps well with NPSR1 mRNA expression in thalamic and hypothalamic regions. Mismatches between NPSR1 expression and NPS-immunoreactive fiber staining were observed in hippocampal, olfactory and cortical regions (Fig. 3).[6] This might result from insufficient sensitivity of the antisera, or activity-dependent regulation of NPS expression in neurons targeting these brain areas. Cell bodies of NPS-synthesizing neurons in the brainstem were clearly labeled by the antisera.[6,16]

RECEPTORS AND SIGNALING CASCADES

Like many formerly orphan receptors, NPSR1 was known under multiple names (GPR154, GPRA, VRR1) before the identification of its endogenous ligand. Analysis of available genomes suggests that only one NPSR1 gene exists in diploid tetrapods, with no closely related subtypes. NPSR1 is only distantly similar to vasopressin and oxytocin receptors. NPSR1 is however a very complex gene, divided into at least 9 exons and presumably spanning more than 300 kb on human chromosome 7p14-15. For human NPSR1, multiple splice variants (some of which cannot encode functional receptors) have been reported by Vendelin et al., 2005.[9] In addition, several coding polymorphisms have been identified in human NPSR1. In fact, NPSR1 was originally identified as a putative asthma-susceptibility gene using a positional cloning approach.[14] Several studies have independently replicated the association of certain NPSR1 haplotypes with asthma and possibly other forms of allergic immune disorders,[30] while others could not verify

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these findings [e.g. Ref. 2]. Since the expression of NPSR1 in lung tissue has not been firmly established, the pathophysiological mechanism of NSPR1 function in asthma is currently discussed controversially. A recent study however suggests a neurogenic model by which NPSR1 activation may influence respiratory function.[33]

Expression of NPSR1 in HEK 293 cells or CHO cells reveals that NPS dose-dependently induces mobilization of intracellular Ca^{2+}, formation of cAMP, and activation of MAPK phosphorylation.[25,31] Structure-activity relationship studies show that the conserved N-terminal part of NPS (NPS 1-6) is absolutely critical for receptor activation while the C-terminal half of the peptide tolerates truncation or substitutions.[12,25] The glycine residue in position 5 of full-length NPS appears to play a critical role in ligand/receptor interaction, because substitution with bulky side-chain D-amino acids creates molecules with antagonistic profiles.[3] Finally, several studies have reported synthetic small-molecule antagonist structures with medium to high potency and selectivity for NSPR1,[19] for review see Refs. 7,19].

A coding polymorphism (rs324981, A/T) in human NPSR1 changes the amino acid at position 107 from asparagine to isoleucine ($N^{107}I$). Analysis of the pharmacological profile of the two receptor isoforms revealed that NPSR-N^{107} requires 5–10-fold higher concentrations of NPS for half-maximal activation than NPSR-I^{107}, suggesting that I^{107} (encoded by the T allele) represents a gain-of-function genotype.[25] The polymorphism occurs with surprisingly high frequency in the human gene pool and has been recently associated with panic disorder.[18] Interestingly, this polymorphism appears to be primate-specific, as all other tetrapod genomes encode only NPSR-I^{107}, suggesting that the N^{107} variant might confer some selective advantage to primates.

BIOLOGICAL ACTIONS WITHIN THE BRAIN

Since NPS was discovered only a few years ago, the description of its physiological functions is still at a relatively early stage. Nevertheless, a number of interesting brain functions have been identified that appear to be modulated by the NPS system. Since NPS is a peptide, most studies have used intracerebroventricular administration to investigate central functions of NPS. More recently, behavioral studies using NPSR1-selective antagonists or NPSR1 knockout mice are starting to complete the picture of biological effects produced by either NPSR1 over-activation or disrupted NSPR1 signaling.

Most consistently, central administration of NPS was found to increase arousal and produce wakefulness.[3,31] Despite this psychostimulatory effect, NPS was also found to attenuate anxiety-like behaviors.[15,26,31] This combination of anxiolytic and stimulatory effects of NPS implies that synthetic small-molecule agonists targeting NPSR1 could be the long-sought non-sedating anxiolytic drugs that the pharmaceutical industry has hoped to develop for a long time. Other studies suggest that NPS might modulate food intake,[28] although reports of conflicting findings make it currently difficult to interpret the role of NPS in feeding behavior. Several studies also linked activation of central NPS neurotransmission to the effects of rewarding or abused drugs, in particular to reinstatement of drug-seeking behaviors.[4,22] NPS appears to stimulate drug-seeking behaviors and is able to reinstate such behaviors even after extinction. Furthermore, NPS produces protective effects in animal models of pharmacologically induced psychosis: Central NPS administration is able to reverse neurotoxic, biochemical, and behavioral effects produced by the NMDA receptor antagonist MK-801, similar to the atypical antipsychotic clozapine.[20] Analysis of behavioral phenotypes in NPSR1 knockout mice reveals deficits in arousal, circadian activity, and mildly increased anxiety-like behaviors, suggesting that endogenous NPS signaling is required for these brain functions.[10]

A number of recent studies support a prominent role of NPS signaling in cognitive processes, in particular in processing of aversive memories. By enhancing glutamatergic neurotransmission, NPS accelerates fear extinction[13] and modulates expression of conditioned fear.[17] These effects were attributed to specific neuronal circuits within the amygdala and the adjacent endopiriform nucleus. Conversely, a synthetic NPSR1 antagonist is able to attenuate fear extinction.[13] Adding further evidence to the hypothesis that NPS may play an important role in cognition, we recently described a prominent function of NPS signaling in two different animal models of learning and memory.[21] Post-training central NPS administration enhances memory consolidation by interacting with noradrenergic systems in the brain. This pro-cognitive effect of NPS is evident for both aversive and neutral memory contents, and can last for several days after drug administration. Conversely, NPSR1 knockout mice display significant deficits in those memory paradigms, indicating that the endogenous NPS system is required for efficient memory processing.

In the obvious absence of approved synthetic drug molecules targeting NPSR1, no pharmacological studies involving humans are currently available to probe the role of NPS signaling in human brain functions. However, taking advantage of the above mentioned functional polymorphism in human NPSR1 and the hypothesized change in agonist sensitivity of the receptor, a number of recent studies have addressed the question: What functional consequences does attenuated NPS signaling have in humans? A recent study in healthy volunteers reported that NPSR genotypes influence processing and evaluation of fear memories.[23] The study used fear conditioning in humans and found that

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T-carriers (coding for NPSR-I[107]) rated their subjective fear significantly higher than AA homozygotes (coding for NPSR-N[107]), who were expected to display attenuated NPS signaling. Acquisition and extinction of fear memories was accelerated in T-carriers, suggesting that the endogenous NPS system might be involved in these processes. These observations in humans are in line with recent findings in mice, showing that central NPS administration can enhance extinction of fear memories.[13] Neuroimaging by fMRI shows selectively higher activation of the dorsomedial prefrontal cortex and the dorsal anterior cingulate cortex in NPSR T-carriers during acquisition of fear memories,[23] indicating functional control over these two important brain structures by the NPS system. Similar neuroimaging observations were made in two separate studies on panic disorder patients or healthy human controls: T-carriers rate pictures with emotional facial expressions higher than AA homozygotes and showed increased activation of prefrontal cortical and amygdala structures.[8,9] These early results suggest, that, similar to preclinical animal models, the human NPS system might also be involved in processing of acute fear and formation of fear memories.

PATHOPHYSIOLOGICAL IMPLICATIONS

As mentioned above, NPSR1 was originally identified as an asthma-susceptibility gene that may also be associated with other forms of allergic diseases.[14] Numerous studies have replicated the initial findings while others did not. Despite the original report of upregulated expression of NPSR1 in asthmatic human or sensitized mouse lung tissue, subsequent studies could not firmly establish a physiological role for NPSR1 in lung or lung-associated immune cells.[1] A possible explanation for the discordance between strong genetic data implying certain NPSR1 haplotypes in asthma and the absence of functional responses of NPS in lung tissue was recently suggested by a study showing that central administration of NPS increased respiratory frequency and reduced airway responsiveness to methacholine in mice.[33] These new observations imply a neurogenic mechanism by which the NPS system may influence respiratory function and might thus be indirectly involved in the pathophysiology of asthma.

At this time, the most convincing evidence for an involvement of the NPS system in pathophysiological conditions comes from genetic data, linking NPSR1 to panic disorder. As mentioned earlier, T-allele carriers (coding for the more sensitive NSPR-I[107]) are found over-represented among panic disorder patients, as compared with healthy controls, while AA homozygosity appears to be a protective genotype.[18,9] The association was reported to be gender-specific in Japanese males,[18] while the second study used a German case/control cohort and reported no gender effects but a trend toward stronger association in females.[9] The reason for the gender discrepancy between the two studies is currently unclear, but certainly warrants further attention, including independent replication in other ethnic cohorts. Most importantly, another recent study in healthy volunteers may offer a mechanistic explanation for the association of NPSR1 genotypes with panic disorder. T-carriers were reported to over-rate their fear and form fear memories more efficiently, leading to "catastrophizing" fear reactions that involve NPSR genotype-dependent activation of the prefrontal cortex.[23] It is plausible that this effect may contribute to progressively enhanced panic responses.

The many physiological functions that have been attributed to NPS offer numerous therapeutic opportunities for developing drugs that target NPSR1. Thus far however, only synthetic NPSR1 antagonists have been reported[7] and none of them has entered clinical trials at this stage. Preclinical development is certainly still at an early stage since the NPS system was only discovered a few years ago, and it remains to be seen which therapeutic indications will emerge for compounds targeting NPSR1. However, the strong genetic results supporting a role for NPS signaling in panic disorder should make a compelling case for pharmaceutical development.

REFERENCES

1. Allen IC, Pace AJ, Jania LA, Ledford JG, Latour AM, Snouwaert JN, et al. Expression and function of NPSR1/GPRA in the lung before and after induction of asthma-like disease. *Am J Physiol Lung Cell Mol Physiol* 2006;**291**:L1005–17.
2. Blakey JD, Sayers I, Ring SM, Strachan DP, Hall IP. Positionally cloned asthma susceptibility gene polymorphisms and disease risk in the British 1958 Birth Cohort. *Thorax* 2008;**64**:381–7.
3. Camarda V, Rizzi A, Ruzza C, Zucchini S, Marzola G, Marzola E, et al. In vitro and in vivo pharmacological characterization of the neuropeptide S receptor antagonist [D-Cys(tBu)5]neuropeptide S. *J Pharmacol Exp Ther* 2009;**328**:549–55.
4. Cannella N, Economidou D, Kallupi M, Stopponi S, Heilig M, Massi M, et al. Persistent increase of alcohol-seeking evoked by neuropeptide S: an effect mediated by the hypothalamic hypocretin system. *Neuropsychopharmacology* 2009;**34**:2125–34.
5. Civelli O, Nothacker HP, Saito Y, Wang Z, Lin SH, Reinscheid RK. Novel neurotransmitters as natural ligands of orphan G-protein-coupled receptors. *Trends Neurosci* 2001;**24**:230–7.
6. Clark SD, Duangdao DM, Schulz S, Zhang L, Liu X, Xu YL, et al. Anatomical characterization of the neuropeptide S system in the mouse brain by in situ hybridization and immunohistochemistry. *J Comp Neurol* 2011;**519**:1867–93.
7. Dal Ben D, Antonini I, Buccioni M, Lambertucci C, Marucci G, Thomas A, et al. Neuropeptide S receptor: recent updates on nonpeptide antagonist discovery. *Chem Med Chem* 2011;**6**:1163–71.
8. Dannlowski U, Kugel H, Franke F, Stuhrmann A, Hohoff C, Zwanzger P, et al. Neuropeptide-S (NPS) receptor genotype modulates basolateral amygdala responsiveness to aversive stimuli. *Neuropsychopharmacology* 2011;**36**:1879–85.

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9. Domschke K, Reif A, Weber H, Richter J, Hohoff C, Ohrmann P, et al. Neuropeptide S receptor gene—converging evidence for a role in panic disorder. *Mol Psychiatry* 2010 Jul 6http://dx.doi.org.10.1038/mp.2010.81.
10. Duangdao DM, Clark SD, Okamura N, Reinscheid RK. Behavioral phenotyping of neuropeptide S receptor knock-out mice. *Behav Brain Res* 2009;**205**:1–9.
11. Elphick MR. NG peptides: a novel family of neurophysin-associated neuropeptides. *Gene* 2010;**458**:20–6.
12. Guerrini R, Salvadori S, Rizzi A, Regoli D, Calo G. Neurobiology, pharmacology, and medicinal chemistry of neuropeptide S and its receptor. *Med Res Rev* 2010;**30**:751–77.
13. Jüngling K, Seidenbecher T, Sosulina L, Lesting J, Sangha S, Clark SD, et al. Neuropeptide S-mediated control of fear expression and extinction: role of intercalated GABAergic neurons in the amygdala. *Neuron* 2008;**59**:298–310.
14. Laitinen T, Polvi A, Rydman P, Vendelin J, Pulkkinen V, Salmikangas P, et al. Characterization of a common susceptibility locus for asthma-related traits. *Science* 2004;**304**:300–4.
15. Leonard SK, Dwyer JM, Sukoff Rizzo SJ, Platt B, Logue SF, Neal SJ, et al. Pharmacology of neuropeptide S in mice: therapeutic relevance to anxiety disorders. *Psychopharmacology (Berl)* 2008;**197**:601–11.
16. Liu X, Zeng J, Zhou A, Theodorsson E, Fahrenkrug J, Reinscheid RK. Molecular fingerprint of neuropeptide S-producing neurons in the mouse brain. *J Comp Neurol* 2011;**519**:1847–66.
17. Meis S, Bergado-Acosta JR, Yanagawa Y, Obata K, Stork O, Munsch T. Identification of a neuropeptide S responsive circuitry shaping amygdala activity via the endopiriform nucleus. *PLoS One* 2008;**3**:e2695.
18. Okamura N, Hashimoto K, Iyo M, Shimizu E, Dempfle A, Friedel S, et al. Gender-specific association of a functional coding polymorphism in the neuropeptide S receptor gene with panic disorder but not with schizophrenia or attention-deficit/hyperactivity disorder. *Prog Neuropsychopharmacol Biol Psychiatry* 2007;**31**:1444–8.
19. Okamura N, Habay SA, Zeng J, Chamberlin AR, Reinscheid RK. Synthesis and pharmacological in vitro and in vivo profile of 3-oxo-1,1-diphenyl-tetrahydro-oxazolo[3,4-a]pyrazine-7-carboxylic acid 4-fluoro-benzylamide (SHA 68), a selective antagonist of the neuropeptide S receptor. *J Pharmacol Exp Ther* 2008;**325**:893–901.
20. Okamura N, Reinscheid RK, Ohgake S, Iyo M, Hashimoto K. Neuropeptide S attenuates neuropathological, neurochemical and behavioral changes induced by the NMDA receptor antagonist MK-801. *Neuropharmacology* 2010;**58**:166–72.
21. Okamura N, Garau C, Duangdao DM, Clark SD, Jüngling K, Pape HC, et al. Neuropeptide S enhances memory during the consolidation phase and interacts with noradrenergic systems in the brain. *Neuropsychopharmacology* 2011;**36**:744–52.
22. Pañeda C, Huitron-Resendiz S, Frago LM, Chowen JA, Picetti R, de Lecea L, et al. Neuropeptide S reinstates cocaine-seeking behavior and increases locomotor activity through corticotropin-releasing factor receptor 1 in mice. *J Neurosci* 2009;**29**:4155–61.
23. Raczka KA, Gartmann N, Mechias ML, Reif A, Büchel C, Deckert J, et al. A neuropeptide S receptor variant associated with over interpretation of fear reactions: a potential neurogenetic basis for catastrophizing. *Mol Psychiatry* 2010;**15**:1067–74.
24. Reinscheid RK. Phylogenetic appearance of neuropeptide S precursor proteins in tetrapods. *Peptides* 2007;**28**:830–7.
25. Reinscheid RK, Xu YL, Okamura N, Zeng J, Chung S, Pai R, et al. Pharmacological characterization of human and murine neuropeptide s receptor variants. *J Pharmacol Exp Ther* 2005;**315**:1338–45.
26. Rizzi A, Vergura R, Marzola G, Ruzza C, Guerrini R, Salvadori S, et al. Neuropeptide S is a stimulatory anxiolytic agent: a behavioural study in mice. *Br J Pharmacol* 2008;**154**:471–9.
27. Sato S, Shintani Y, Miyajima N, Yoshimura K. Novel G protein-coupled receptor protein and DNA thereof. *World Patent Application* 2002:WO 02/31145 A1.
28. Smith KL, Patterson M, Dhillo WS, Patel SR, Semjonous NM, Gardiner JV, et al. Neuropeptide S stimulates the hypothalamo–pituitary–adrenal axis and inhibits food intake. *Endocrinology* 2006;**147**:3510–8.
29. Vendelin J, Pulkkinen V, Rehn M, Pirskanen A, Räisänen-Sokolowski A, Laitinen A, et al. Characterization of GPRA, a novel G protein-coupled receptor related to asthma. *Am J Respir Cell Mol Biol* 2005;**33**:262–70.
30. Vergara C, Jiménez S, Acevedo N, Martínez B, Mercado D, Gusmão L, et al. Association of G-protein-coupled receptor 154 with asthma and total IgE in a population of the Caribbean coast of Colombia. *Clin Exp Allergy* 2009;**39**:1558–68.
31. Xu YL, Reinscheid RK, Huitron-Resendiz S, Clark SD, Wang Z, Lin SH, et al. Neuropeptide S: a neuropeptide promoting arousal and anxiolytic-like effects. *Neuron* 2004;**43**:487–97.
32. Xu YL, Gall CM, Jackson VR, Civelli O, Reinscheid RK. Distribution of neuropeptide S receptor mRNA and neurochemical characteristics of neuropeptide S-expressing neurons in the rat brain. *J Comp Neurol* 2007;**500**:84–102.
33. Zhu H, Perkins C, Mingler MK, Finkelman FD, Rothenberg ME. The role of neuropeptide S and neuropeptide S receptor 1 in regulation of respiratory function in mice. *Peptides* 2011;**32**:818–25.

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Chapter 117

Neurotensin/Neuromedin N

Paul R. Dobner and Robert E. Carraway

ABSTRACT

Neurotensin (NT) and neuromedin N, two bioactive peptides of brain and gut origin, are processed from a common precursor by prohormone convertases (PCs). Differential processing occurs in some tissues most probably resulting from the tissue-specific expression of distinct PCs, yielding large, N-terminally extended forms of the peptides. Two G protein-coupled receptors show preference for NT but bind both the peptides. NTR-1 signaling elevates intracellular [Ca^{2+}] and can also increase cGMP, cAMP, and arachidonic acid metabolites. NTR-1/sortilin (NTR-3) heterodimers seem to modulate NT signaling and trafficking of internalized receptors, but NT may also signal through sortilin. Intimately associated with dopaminergic systems, NT is most probably an endogenous antipsychotic and has also been implicated in the modulation of psychostimulant responses, pain perception, stress-induced analgesia, appetite control, stress responses, and reproductive functions. The development of NT agonists may provide new pharmaceuticals for the treatment of schizophrenia, drug addiction, and stress-related neuropathic pain syndromes.

DISCOVERY OF NEUROTENSIN

In 1967, Susan Leeman discovered that the extracts of bovine hypothalami induce vasodilation and anaphylactic shock in rats. Using this assay, her laboratory isolated, sequenced, and synthesized the active substance, a 13-residue peptide that they named neurotensin (NT).[6] NT is broadly expressed in the limbic nervous system and intestinal endocrine cells and is also found in various glands and in peripheral nerves.

NT is a member of a family of peptides that are strikingly similar in their C-terminal regions (Table 1). These peptides share C-terminal residues with NT that have been shown to be critical for NT function and display many of the same pharmacological properties for those that have been tested. For instance, the venom of the cone snail contains the NT-like peptide contulakin G, which is a potent analgesic in rats and dogs.[2] Several NT-related peptides are also present in neuronal cells of the protocordate *Ciona intestinalis* (Table 1).

NT most probably has multiple neuronal and endocrine signaling functions, including the modulation of dopamine (DA) signaling, stress-induced analgesia, energy balance, stress, and reproductive functions. This review focuses on the biosynthesis, distribution, receptor signaling, and potential functions of NT in the CNS.

STRUCTURE OF THE NEUROTENSIN/ NEUROMEDIN N PRECURSOR mRNA/GENE

The amino acid sequence of the NT precursor was first inferred from canine cDNA sequences and is highly conserved in vertebrates.[8] The predicted prepro form of the precursor ranges from 165 to170 amino acids in length, with NT and the related hexapeptide neuromedin N (NMN) lying in tandem near the carboxyl terminus of the precursor, bounded and separated by Lys-Arg processing sites (Fig. 1). The analysis of the rat, mouse, and human NT/NMN genes reveal that the coding region is spread over four exons, separated by three introns spanning just over 10 kb.[20] The gene is transcribed to yield two different transcripts (1.0 and 1.5 kb) that differ only in the extent of the 3'-untranslated region, most probably arising from the differential use of alternative polyadenylation signals. The two mRNAs are expressed in about equal amounts in the brain, but the 1.0 kb mRNA greatly predominates in the gastrointestinal tract. The sequences immediately upstream of the transcriptional start site contain a series of response elements (AP-1, CRE, and GRE) that are important for NT/NMN gene induction in response to environmental stimuli in PC12 cells. A variety of stimuli regulate NT/NMN gene expression in a brain region-specific manner.

NT/NMN mRNA EXPRESSION IN BRAIN

NT/NMN mRNA and its peptide products are expressed in many forebrain regions, with higher levels in limbic regions, including hippocampus and subicullum, VP, BNST, medial Acb, and Cput (Fig. 2).[1] NT/NMN mRNA-positive neurons are located mainly in the dorsomedial and ventral aspects of the Cput rostrally but are more evenly distributed in the caudal regions. A continuum of positive cells was observed

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00117-2
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TABLE 1 Peptides of the Neurotensin Family

Peptide	Source	Sequence
NT	Cow, dog, human	<Glu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu*
NT	Chicken	<Glu-Leu-His-Val-Asn-Lys-Ala-Arg-Arg-Pro-Tyr-Ile-Leu*
NT	Green frog	<Glu-Ser-His- Ile-Ser-Asn-Ala-Arg-Arg-Pro-Tyr-Ile-Leu*
NMN	Cow, dog, pig, human	Lys-Ile-Pro-Tyr-Ile-Leu*
LANT-6	Chicken	Lys-Asn-Pro-Tyr-Ile-Leu*
XP	Xenopus	<Glu-Gly-Lys-Arg-Pro-Trp-Ile-Leu
XP	Dog, turkey	Phe-His-Pro-Lys-Arg-Pro-Trp-Ile-Leu
Contulakin G	Cone snail	<Glu-Ser-Glu-Glu-Gly-Gly-Ser-Asn-Ala-Thr-Lys-Lys-Pro-Try-Ile-Leu†,‡
Ci-NTLP-1	*Ciona intestinalis*	<Glu-Leu-His-Val-Pro-Ser-Ile-Leu*
Ci-NTLP-3	*Ciona intestinalis*	Met-Met-Leu-Gly-Pro-Gly-Ile-Leu*

NT, neurotensin; NMN, neuromedin N; LANT-6, [Lys8, Asn9]-NT 8-13; XP, xenopsin; NTLP, neurotensin-like peptide.
**Bordered by double basic cleavage sites in putative precursors.*
†N-terminal double basic cleavage site only.
‡The side chain is O-glycosylated.

stretching from the S through the preoptic area, including the lateral S, DBB, lateral BNST, and lateral Hpt. Steroid hormones and neurotransmitters have been implicated in the regulation of NT/NMN mRNA expression in several regions of the brain, including the medial preoptic area, Arc, PVN, periventricular nucleus, amygdala, Cput, and Acb. In the brainstem, NT/NMN mRNA-positive neurons are most abundant in the PAG and DR and can also be found in adjacent lateral structures (cuneiform nucleus, microcellular tegmental nucleus).

PROCESSING OF THE PRECURSOR

The NT precursor is differentially processed by distinct prohormone convertases (PCs) in the brain and gastrointestinal tract (Fig. 1, reviewed in Ref. 22). PCs are described in detail in the Peptide Biosynthesis/Processing section of this book. In the brain, PC2 results in nearly complete processing of both the peptides, although brain NT levels are only decreased by ~15% in PC2 knockout mice. This is most probably because the precursor can also be processed by PC1 and PC5A, which are colocalized with NT and are capable of generating NT from the precursor. After liberation of the NT sequence from the precursor, the N-terminal Gln residue is converted into pGlu most probably through the action of glutaminyl cyclase. In the gastrointestinal tract, PC1 inefficiently cleaves the Lys-Arg pair amino terminal to NMN, resulting in the accumulation of amino-terminally extended NMN (large NMN in Fig. 1) that stretches from the signal peptide cleavage site to the carboxyl terminus of NMN (amino acids 23–147 in the rat sequence) and NT in gut endocrine cells. Similarly, colon cancer cell lines expressing both NT and PC5A produce predominantly amino-terminally extended forms of both NMN and NT (large NT in Fig. 1), similar to those found in the adrenal medulla, indicating that differential expression of distinct PCs can account for the observed tissue-specific processing of the NT precursor. The secretion of amino-terminally extended forms of NMN and NT may produce longer lasting biological effects because of their enhanced stability in the bloodstream compared with that of the fully processed peptides. A dibasic amino acid pair near the central region of the precursor is inefficiently processed in the brain and not used in the gastrointestinal tract, and complete processing of the NT precursor results in the production of a relatively stable fragment (Fig. 1, amino acids 24–140 of the human NT precursor) that extends from the signal peptide cleavage site through to the amino acid preceding the Lys-Arg amino terminal processing site of NMN.[11] This precursor fragment is detectable in serum from healthy individuals, is stable in both human plasma and serum, and is released into the serum after ingestion of an ordinary meal, opening the possibility that it may serve as a surrogate marker for NT secretion.

RECEPTORS AND SIGNALING CASCADES

Four NT receptors have been identified, two that are 7-transmembrane G protein-coupled receptors (NTR-1, NTR-2) and two primarily intracellular proteins related to yeast sorting receptors (NTR-3/sortilin, NTR-4/SorLA). NTR-1 displays the highest affinity (Kd, 0.1 nM) and seems to mediate NT effects on dopaminergic transmission, hot-plate latency, temperature regulation, and smooth muscle contractility (reviewed in Ref. 21). First described as a lower affinity receptor (Kd, 3 nM) with a differing specificity, NTR-2 has been implicated in the analgesic effects of NT (reviewed in Ref. 29). Although the role of NTR-3 is less clear, one possibility is that through binding and internalizing NT this receptor serves to regulate turnover of the peptide. Evidence supports the involvement of NTR-3 in the growth-promoting effects of NT and in NT-induced migration of brain microglial cells.

Signal transduction studies in transfected cell systems and cancer cell lines indicate that NTR-1 is primarily coupled to $G_{\alpha q}$ and signals via the activation of phospholipase C and the generation of IP_3, causing an elevation in intracellular $[Ca^{2+}]$ (reviewed in Ref. 21). Concomitant generation of DAG leads to the activation of PKC in some cells, stimulating MAP kinases and PI3 kinase via complex mechanisms that can involve EGF receptor transactivation. This

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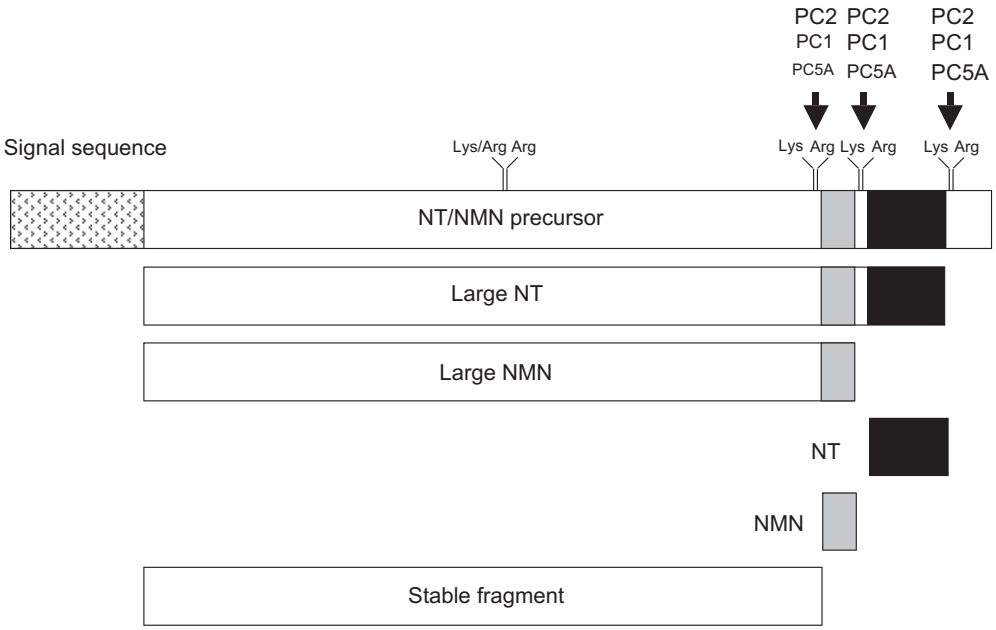

FIGURE 1 Schematic diagram of the NT/NMN precursor and its major processing products. The full length precursor is 165–170 amino acids long depending on the species and comprises 169 amino acids in the rat. The positions of the signal peptide (stippled fill, aa 1–23) and the NMN (gray fill) and NT (black fill) coding domains are indicated. The NMN and NT coding domains are bounded and separated by the indicated Lys-Arg processing sites. An additional Lys-Arg pair is located near the center of the precursor, but the available evidence suggests that this is not a major processing site. The prohormone convertases that have been shown to cleave the Lys-Arg processing sites are indicated and the extent to which they cleave a particular site is indicated by the size of the type, with the most efficient cleavage indicated by larger letters and the least efficient by smaller letters. PC1 cleaves the Lys-Arg pair located at the amino terminal end of NMN inefficiently but cleaves the sites surrounding NT efficiently resulting in the production of NT and Large NMN (intestinal pattern). PC5A cleaves the site at the carboxy-terminal end of NT efficiently, but the upstream sites less efficiently leading to the production of Large NT and Large NMN (adrenal pattern). PC2 cleaves all three sites efficiently resulting primarily in the production of NT and NMN (brain pattern).

signaling can have a feed forward component, in that NT-induced MAP kinase activation leads to the upregulation of EGF receptor. In neuroblastoma cells and rat brain slices, NT can elevate cGMP formation, inhibit cAMP synthesis and stimulate PI turnover, and evidence suggests that the third intracellular loop of NTR-1 is required for interactions with G_q, while the carboxy-terminal tail interacts with $G_{i/o}$ and G_s (reviewed in Ref. 21). Liposomal ceramide has been shown to decrease the interaction of NTR-1 with G_q within structured membrane microdomains, which inhibits the growth stimulatory effects of NT in MDA-MB-231 breast cancer cells. NTR-1 is inducibly palmitoylated on Cys^{381} and Cys^{383} (rat sequence), and inhibition of this process by site-directed mutagenesis and pharmacological strategies diminishes NT-induced ERK phosphorylation and reduces the ability of NT to protect against apoptosis in breast cancer cells.[17] NT activation of DA neurons appears to involve cAMP signaling, and NT potentiates cAMP formation (via NTR-1) in response to G_s-agonists by facilitating the activation of Ca^{2+}-dependent adenylyl cyclases. Perhaps secondary to its effects on $[Ca^{2+}]$, NT also activates PLA2 and DAG lipase, stimulating arachidonic acid release and the formation of bioactive metabolites via the cyclooxygenase and lipoxygenase pathways. This signaling could also be amplified by feed forward mechanisms because the expression of PLA2 is upregulated by the activation of MAP kinase (reviewed in Ref. 7). NT is the most potent substance known to stimulate leukotriene formation in rats, and the possible relevance of this to its central effects is not well investigated.

Signaling mechanisms for NTR-2 and NTR-3 are not yet clear. NTR-2 constitutively activates IP production, and this process is stimulated by the NTR-1 antagonist SR48692 but suppressed by NT (reviewed in Ref. 29). NTR-3 is primarily localized to ER-Golgi membranes but ≅10% is on the cell surface. NTR-3 can form heterodimers with NTR-1, decreasing the potency of NT to stimulate IP formation and to activate MAP kinase. Thus, NTR-3, which cannot promote either response, modulates these effects of NTR-1.

Results obtained by site-directed mutagenesis favor a model for NTR-1 activation in which binding of NT to key residues in EC loop 3 and TM 6 and 7 leads to a shift in IC loop 3 that enhances binding to G_q (reviewed in Ref. 21). The relative instability of NTR-1 in detergents has led investigators to use a systematic mutational approach to identify receptor mutants that display enhanced thermostability, resulting in the identification of an NTR-1 variant that is stabilized in an agonist binding conformation that presumably might be used with a wider range of detergent and buffer conditions for crystallization.[35]

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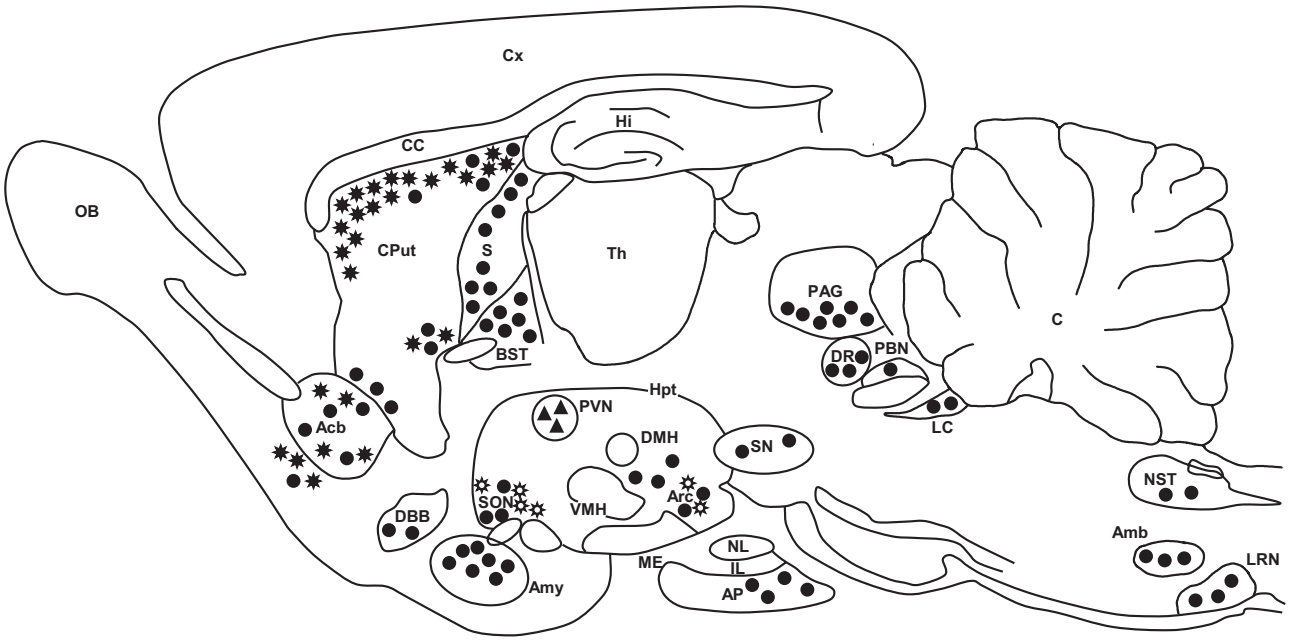

FIGURE 2 Schematic diagram of the distribution of NT-IR neurons in the rat brain. (●) NT-IR neurons detected after colchicine treatment. (▲) NT-IR neurons induced by stressful stimuli that are also detected after colchicine treatment. (✸) NT-IR neurons induced by either psychostimulants (dorsomedial CPu, Acc) or dopamine D_2 antagonists, including typical antipsychotic drugs (dorsolateral CPu, Acc). (✡) Estrogen-inducible NT-IR neurons in the female MPO and arcuate nucleus. NT gene expression in the rostal MPO nucleus is sexually dimorphic. Quantitative analysis of NT content by RIA (pmole/g wet weight tissue) provides a more comprehensive picture of the distribution of the peptide in various brain regions (OB, 3; Cx, 4-5; Acc, 29; CPut, 10; S, 73; BST, 172; Hi, 7; Th, 5-23; Hyp, 76-128; ME, 128; SN, 13; PAG, 42; LC, 54).

CONFORMATION

2D solid-state NMR spectroscopy has been used to study the conformational changes of NT8-13 on interaction with an N-terminally truncated form of rat NTR-1.[27] On binding to the receptor, the peptide rearranges to adopt a β-strand conformation, characterized by a ψ(Pro10) dihedral angle of 146±15°. The NMR spectrum of NT (8–13) in solution is also consistent with the presence of a type II β-turn, whereas the spectrum of a homolog that displays 40-fold less affinity for human NTR-1 does not.[34] Furthermore, a lactam-bridged analog of NT8-13 with ψ(Pro10) angles restricted to an angle (130°) close to the experimentally determined one exhibits ~1000-fold higher affinity for porcine NTR-1 than the stereoisomer (−130°), providing further evidence for the NMR-derived bioactive conformation.

BRAIN FUNCTIONS

Close Alliance between NT and Dopamine

NT has been broadly implicated in the modulation of DA signaling and can produce both psychostimulant- and antipsychotic drug (APD)-like effects after microinjection into different regions of the brain (reviewed in Ref. 5). Most midbrain DA neurons express high levels of NTR-1, and NT can stimulate these neurons to increase striatal DA release in the Acb. NT can also act presynaptically to potentiate DA release in both the Acb and Cput, most probably by antagonizing DA D_2 receptor autoinhibition. Consistent with these observations, NT microinjection in the VTA stimulates locomotor activity and repeated administration results in sensitization to not only NT but also psychostimulant drugs. Prolonged administration with the NT antagonist SR48692 lowers basal DA release in the Acc, consistent with the hypothesis that endogenous NT acts to augment DA release through NTR-1; however, basal and stress-induced DA release in the mPFC are unaffected. NTR-1, but not NTR-2, knockout mice are reported to be hyperdopaminergic with elevated basal DA release in the Cput, although release in the Acc and mPFC was not examined.[26] These differences could reflect regionally distinct roles for NT in the control of DA release, but could also result from either adaptive changes in NTR-1 knockout mice or unanticipated effects of prolonged NT antagonist administration.

NT can also produce neuroleptic-like effects when administered in the Acc, seemingly inconsistent with the evidence cited above indicating that NT acts presynaptically to augment DA signaling. The most probable explanation for this is that NT either acts postsynaptically or on other presynaptic afferents to oppose DA signaling and electron microscopic immunohistochemical analysis indicates that NTR-1 is expressed on both dopaminergic and excitatory afferents- and on postsynaptic neurons in the Acc; however, NTR-1 seems to be expressed primarily on striatal afferents

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in the Cput. NTR-2 knockout mice have lower levels of basal striatal glutamate release and both NTR-1 and NTR-2 knockout mice display muted amphetamine-stimulated striatal glutamate release,[25] consistent with the hypothesis that endogenous NT modulates striatal glutamate release.

NT may be an Endogenous Antipsychotic

Considerable evidence suggests that NT mediates at least a subset of responses to certain APDs. APDs elevate striatal NT expression and NT administration in the Acb suppresses amphetamine-stimulated locomotor activity, similar to APDs, consistent with the idea that increases in NT signaling might be involved in APD actions (reviewed in Ref. 5). Although animal models of schizophrenia are necessarily limited, latent inhibition (LI) and prepulse inhibition (PPI) of the acoustic startle response measure aspects of sensorimotor gating that can be influenced by APDs. Enhanced DA transmission and isolation rearing disrupt PPI and APDs can restore PPI to control levels. In mice, APDs can also increase PPI above basal levels without prior disruption by DA agonists. There is increasing evidence that either pharmacological or genetic disruption of NT signaling can have a profound impact on APD responses in these animal models.

NT has been implicated in both the acquisition of LI and APD enhancement of LI. NT antagonist administration was shown to interfere with the acquisition of LI, and this was observed with both the relatively selective NTR-1 antagonist SR48692 and the NTR-1/NTR-2 antagonist SR142948A, indicating that NT signaling through NTR-1 may be particularly important (reviewed in Ref. 5). The suppressive effects of SR142948A are ameliorated by coadministration of the D_2 antagonist sulpiride, suggesting that NT antagonist treatment may increase DA D_2 transmission in the Acb or other key brain regions involved in LI. NT also seems to play a role in APD enhancement of LI, because SR142948A administration 1 hour before the last of 7 daily injections of haloperidol prevents augmentation of LI. Thus, NT likely plays a role in both basal and APD-enhanced LI, possibly through effects on DA D_2 signaling.

NT also appears to play a role in both basal and APD-enhanced PPI; however, results from NTR-1 and NTR-2 knockout mice suggest a more complicated picture. Preadministration of the NTR-1/NTR-2 antagonist SR142948A blocks the ability of both the typical APD haloperidol and the atypical APD quetiapine to restore PPI deficits in isolation-reared rats, without affecting basal PPI (reviewed in Ref. 5). Furthermore, NT mRNA levels are decreased by ~45% and NT receptor binding is increased by ~21% in the Acb shell in isolation-reared animals, and NT mRNA levels are restored to those observed in socially reared animals 4 hours after quetiapine treatment. Similarly, both haloperidol and quetiapine fail to augment PPI in NT knockout mice, which also have reduced basal PPI, although the response to clozapine is unaffected.[19] The decrease in basal PPI is modest and is not observed in either NTR-1 or NTR-2 knockout mice where it is instead elevated.[13,31]

The indirect DA agonist amphetamine disrupts PPI, but this effect is blocked in NT knockout mice,[19] suggesting that NT may play a crucial role in this process. Similar results have been reported for NTR-1 knockout mice;[31] however, another report indicates that amphetamine disruption of PPI is normal in these mice,[13] as it is in NTR-2 knockout mice.[31] These different results most likely reflect methodological differences, because both used NTR-1 knockout mice from the same source. These results provide a preliminary indication that NT signaling is required for amphetamine disruption of PPI in mice. NT participation in processes that either enhance or reduce PPI most likely reflects compartmental specificity in NT action within the circuits that modulate PPI, particularly the DA circuits. The analysis of amphetamine and haloperidol induction of striatal Fos expression has provided direct evidence for such compartmentalized NT signaling.[9,12]

NT and Psychostimulant Sensitization

As discussed above, NT is capable of modulating DA transmission at different levels. In addition to antipsychotic drug-like effects, NT produces psychostimulant-like effects after direct administration into the VTA and increases DA release in the Acb (reviewed in Ref. 5). Several different laboratories have reported that NT antagonist pretreatment blocks or attenuates amphetamine and can delay cocaine sensitization while sparing the acute locomotor stimulation produced by these drugs. By contrast, both amphetamine (PRD, unpublished observations) and cocaine[16] sensitization are augmented somewhat in NT knockout mice. Several other lines of evidence also suggest that NT acts to limit psychostimulant sensitization, including the observations that peripheral administration of the NT agonist, NT69L, suppresses nicotine sensitization, and that NT antagonist administration potentiates rather than blocks supersensitive locomotor responses to L-DOPA in dopamine-deficient mice. NTR-1 knockout mice show both increased basal and amphetamine-stimulated locomotor activity and striatal DA release, also consistent with the idea that NT acts to limit DA signaling, although psychostimulant sensitization was not examined.[26] However, both basal and cocaine-stimulated locomotor activities are similar to wildtype in NT knockout mice[16] and in rats treated with SR48692 (reviewed in Ref. 5). Thus, gene knockout and several other approaches favor a model in which NT acts mainly to limit psychostimulant sensitization, while experiments with NT antagonists indicate the opposite. Further work will be required to determine whether this discrepancy results from compensatory mechanisms in knockout animals or unanticipated antagonist effects, for instance agonist or partial agonist activity at NTR-2.

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NT and Pain

Given centrally, NT enhances nociceptive responses at lower doses, but produces a potent analgesic response at higher doses that in most studies does not require μ-opioid receptor signaling (reviewed in Ref. 10). NT and NT receptors are expressed in the central descending circuitry involved in pain modulation and in the spinal cord, consistent with a possible role in pain modulation. The evidence to date indicates that endogenous NT plays a relatively minor role in basal nociception but is critical for at least certain aspects of stress-induced antinociception (SIAN). Remarkably, SIAN is totally absent in NT knockout mice, where water avoidance/cold-water swim stress instead results in a hyperalgesic response to colonic distension.[15] Similar results were obtained in rats pretreated with SR48692, indicating that SIAN probably requires NT signaling through NTR-1, and the defects observed in NT knockout mice are not because of developmental or compensatory changes resulting from the lifelong absence of NT signaling.[15] NTR-2 signaling has also been implicated in certain aspects of SIAN. NTR-2 knockout mice display a selective defect in SIAN after cold-water swim stress during the latter part of the second phase of the pain response in both the intraplantar formalin test and the paw withdrawal response to thermal stimuli, while nonstressed responses are comparable with wildtype.[23] Similar results occur in the paw withdrawal test after repeated administration of the NTR-1/NTR-2 antagonist SR142948A, indicating that the differences observed in NTR-2 knockout mice also do not result from developmental defects or compensatory changes resulting from the absence of NTR-2. Furthermore, intrathecal administration of both nonselective and NTR-2-selective NT agonists potentiate cold-water swim stress-induced SIAN in the paw withdrawal test. These results are consistent with the previous observations that NTR-2-selective agonists suppress pain responding in the intraplantar formalin test selectively during the latter part of the second phase of the pain response, influencing predominantly paw lifting while sparing paw licking, biting, and shaking behaviors. These results suggest that endogenous NT signaling through both NTR-1 and NTR-2 is particularly important for SIAN.

In contrast to SIAN, NT seems to play a relatively minor role in basal pain perception, where it seems to facilitate pain responses. Although both NTR-1 and NTR-2 knockout mice respond similarly to wild type in a variety of pain tests, NTR-2 knockout mice display longer jump latencies in the hot-plate test.[28] Viseromotor responses to colorectal distension are also diminished in both NT knockout mice and rats pretreated with SR48692.[15]

The evidence discussed above suggests that NT not only facilitates basal pain responses but it also plays a key role in SIAN and any theory of NT modulation of pain responses must take into account these seemingly contradictory results. Perhaps the simplest explanation is that the direction of pain modulation depends on the intensity of NT signaling, with basal NT signaling facilitating nociception and increased NT signaling being analgesic. The observation that cold-water swim stress increases NT mRNA levels in certain Hpt regions that project to the PAG is consistent with the idea that stress-induced increases in NT signaling to the PAG may underlie SIAN. Increased NT signaling could lead to the selective recruitment of neurons involved in analgesic responses, such as PAG "off" cells.

Appetite Control

NT has been implicated as a possible satiety factor and mediator of the central suppressive effects of leptin on food consumption (reviewed in Ref. 3). Leptin administration suppresses food consumption and weight gain, possibly through the decreased expression of orexigenic peptides and increased NT expression in the hypothalamus. Similarly, NT expression is decreased in specific Hpt regions in response to a high-fat diet that results in obesity. Combined central administration of NT and leptin potentiates both the short-term inhibitory effects of NT on spontaneous food intake and early, but not late (24 h) leptin effects. Because the leptin effect is blocked by pretreatment with either NTR-1 antagonist or NT antiserum, it has been suggested that NT mediates the central effect of leptin. Collectively, these results indicate that dynamic regulation of NT in specific Hpt nuclei in response to alterations in the levels of circulating leptin and perhaps other factors is involved in appetite suppression and body weight balance. Other results, showing that the anoretic effect of NT is antagonized by the H1 receptor blocker pyrilamine and in H1 knockout mice, suggest that the histamine H1 system, which is known to inhibit food intake, could be a final common mediator for the effects of both leptin and NT.[30]

NT and Stress Responses

Stress increases NT expression in several Hpt nuclei, suggesting that NT may modulate stress responses (reviewed in Ref. 14). Centrally administered NT was found to rapidly increase the levels of circulating ACTH and corticosterone and these increases were attenuated by corticotrophin-releasing hormone (CRH) antagonists and bilateral lesions of the PVN. Furthermore, SR 48692 administration in the PVN suppresses stress-induced increases in the plasma levels of ACTH and corticosterone and decreases CRH expression in the PVN. Both basal and stress-induced blood corticosterone levels are lower in NTR-2 knockout compared with those of control mice, consistent with NT playing a role in the control of corticosterone release.[23]

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NT and Reproductive Functions

NT has been implicated in the preovulatory luteinizing hormone (LH) surge and the Hpt control of prolactin (PRL) release in rat (reviewed in Ref. 33). Estrogen increases NT mRNA and peptide expression in several Hpt regions that have been implicated in reproductive functions, and NT expression in these regions is sexually dimorphic. In addition, NT mRNA levels vary throughout the estrus cycle in female rats with NT mRNA-positive cells being ~3-fold more numerous at proestrus than at diestrus. These results coupled with microinjection data indicating that NT can augment LH release have led to speculation that NT might mediate the feedforward effects of estrogen on gonadotropin-releasing hormone (GnRH) release from rostral medial preoptic nucleus GnRH neurons expressing NTR-1 in the rat. However, this system may not operate in the mouse, because even though estrogen increases NT mRNA expression similarly in ovariectomized mice, icv NT administration does not augment LH release in ovariectomized mice treated with estrogen.[24] GnRH neurons are clearly subject to complex peptide control with kisspeptin playing a crucial role, and it remains to be determined how NT interacts with kisspeptin to modulate reproductive function. Kisspeptin neurons apparently express NTR-2 but not NTR-1 or NT.[24]

NT has also been implicated in both positive and negative regulation of PRL secretion from pituitary lactotropes. The evidence suggests that NT directly activates tuberoinfundibular DA (TIDA) neurons and mediates feedback inhibition of PRL release through the activation of these neurons.[18] Estrogen increases NT expression in TIDA neurons and NT is intensely expressed in virtually all TIDA neurons in nursing rats, but decreases markedly after removal of pups. Increased NT release from TIDA neurons in the median eminence would most probably counteract the inhibitory effects of DA on PRL secretion, because NT stimulates pituitary PRL release. NT knockout mice reproduce and successfully rear their young, indicating that NT is not essential for reproductive functions.[9]

PATHOPHYSIOLOGICAL IMPLICATIONS

There is now considerable evidence from animal models that endogenous NT signaling is both required for certain APD actions and that decreased NT signaling may underlie deficits in PPI. Comparison of NT levels in cerebrospinal fluid (CSF) from schizophrenics both before and after APD treatment indicates that low CSF NT levels are associated with more severe psychopathology and that clinical improvement is associated with increased CSF NT (reviewed in Ref. 5). Some NT agonists produce effects similar to those of atypical APDs and the relatively stable NT analog, NT69L, prevents drug-induced disruption of PPI in rats after either acute or chronic administration.[4] These results suggest that the development of NT agonists could provide an important new class of APDs. In the shorter term, there could be some therapeutic benefit associated with the use of NT-dependent (e.g. quetiapine, haloperidol) and NT-independent (e.g. clozapine) APDs in combination.

NT also seems to play an important role in stress-induced analgesia, suggesting that NT agonists could be effective in treating certain chronic pain syndromes associated with abnormalities in stress responses. The observation that cold-water swim/water avoidance stress in NT knockout mice results in hyperalgesia rather than analgesia is particularly interesting in this regard.[15] Stable NT agonists may prove to be particularly effective in treating stress-related and neuropathic pain.[32]

REFERENCES

1. Alexander MJ, Miller MA, Dorsa DM, Bullock BP, Melloni Jr RH, Dobner PR, et al. Distribution of neurotensin/neuromedin N mRNA in rat forebrain: unexpected abundance in hippocampus and subiculum. *Proc Natl Acad Sci USA* 1989;**86**:5202–6.
2. Allen JW, Hofer K, McCumber D, Wagstaff JD, Layer RT, McCabe RT, et al. An assessment of the antinociceptive efficacy of intrathecal and epidural contulakin-G in rats and dogs. *Anesth Analg* 2007;**104**:1505–13.
3. Arora S, Anubhuti. Role of neuropeptides in appetite regulation and obesity—a review. *Neuropeptides* 2006;**40**:375–401.
4. Briody S, Boules M, Oliveros A, Fauq I, Richelson E. Chronic NT69L potently prevents drug-induced disruption of prepulse inhibition without causing tolerance. *Behav Brain Res* 2010;**207**:118–24.
5. Caceda R, Kinkead B, Nemeroff CB. Neurotensin: role in psychiatric and neurological diseases. *Peptides* 2006;**27**:2385–404.
6. Carraway R, Leeman SE. The synthesis of neurotensin. *J Biol Chem* 1975;**250**:1912–8.
7. Carraway RE, Plona AM. Involvement of neurotensin in cancer growth: evidence, mechanisms and development of diagnostic tools. *Peptides* 2006;**27**:2445–60.
8. Dobner PR, Barber DL, Villa-Komaroff L, McKiernan C. Cloning and sequence analysis of cDNA for the canine neurotensin/neuromedin N precursor. *Proc Natl Acad Sci USA* 1987;**84**:3516–20.
9. Dobner PR, Fadel J, Deitemeyer N, Carraway RE, Deutch AY. Neurotensin-deficient mice show altered responses to antipsychotic drugs. *Proc Natl Acad Sci USA* 2001;**98**:8048–53.
10. Dobner PR. Neurotensin and pain modulation. *Peptides* 2006;**27**: 2405–14.
11. Ernst A, Hellmich S, Bergmann A. Proneurotensin 1–117, a stable neurotensin precursor fragment identified in human circulation. *Peptides* 2006;**27**:1787–93.
12. Fadel J, Dobner PR, Deutch AY. Amphetamine-elicited striatal Fos expression is attenuated in neurotensin null mutant mice. *Neurosci Lett* 2006;**402**:97–101.
13. Feifel D, Pang Z, Shilling PD, Melendez G, Schreiber R, Button D. Sensorimotor gating in neurotensin-1 receptor null mice. *Neuropharmacol* 2010;**58**:173–8.
14. Geisler S, Berod A, Zahm DS, Rostene W. Brain neurotensin, psychostimulants, and stress-emphasis on neuroanatomical substrates. *Peptides* 2006;**27**:2364–84.

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15. Gui X, Carraway RE, Dobner PR. Endogenous neurotensin facilitates visceral nociception and is required for stress-induced antinociception in mice and rats. *Neuroscience* 2004;**126**:1023–32.
16. Hall FS, Centeno M, Perona MTGAdair J, Dobner PR, Uhl GR. Effects of neurotensin gene knockout in mice on the behavioral effects of cocaine. *Psychopharmacology* 2012;**219**:35–45.
17. Heakal Y, Woll MP, Fox TSeaton K, Levenson R, Kester M. Neurotensin receptor-1 inducible palmitoylation is required for efficient receptor-mediated mitogenic-signaling within structured membrane microdomains. *Cancer Biol Ther* 2011;**12**:427–35.
18. Hentschel K, Cheung S, Moore KE, Lookingland KJ. Pharmacological evidence that neurotensin mediates prolactin-induced activation of tuberoinfundibular dopamine neurons. *Neuroendocrinology* 1998;**68**:71–6.
19. Kinkead B, Dobner PR, Egnatashvili V, Murrary T, Deitemeyer N, Nemeroff CB. Neurotensin-deficient mice have deficits in prepulse inhibition: restoration by clozapine but not haloperidol, olanzapine or quetiapine. *J Pharmacol Exp Ther* 2005;**315**:256–64.
20. Kislauskis E, Bullock B, McNeil S, Dobner PR. The rat gene encoding neurotensin and neuromedin N: structure, tissue-specific expression, and evolution of exon sequences. *J Biol Chem* 1988;**263**:4963–8.
21. Kitabgi P. Functional domains of the subtype 1 neurotensin receptor (NTS1). *Peptides* 2006;**27**:2461–8.
22. Kitabgi P. Prohormone convertases differentially process pro-neurotensin/neuromedin N in tissues and cell lines. *J Mol Med* 2006;**84**:628–34.
23. Lafrance M, Roussy G, Belleville K, Maeno H, Beaudet N, Wada K, et al. Involvement of NTS2 receptors in stress-induced analgesia. *Neuroscience* 2010;**166**:639–52.
24. Lemko HMD, Naderi R, Adjan V, Jennes LH, Navarro VM, Clifton DK, et al. Interactions between neurotensin and GnRH neurons in the positive feedback control of GnRH/LH secretion in the mouse. *Am J Physiol Endocrinol Metab* 2010;**298**:E80–8.
25. Li Z, Liang Y, Boules M, Gordillo A, Richelson E. Effect of amphetamine on extracellular concentrations of amino acids in striatum in neurotensin subtype 1 and 2 receptor null mice: a possible interaction between neurotensin receptors and amino acid systems for study of schizophrenia. *Neuropharmacology* 2010;**58**:1174–8.
26. Liang Y, Boules M, Li Z, Williams K, Miura T, Oliveros A, et al. Hyperactivity of the dopaminergic system in NTS1 and NTS2 null mice. *Neuropharmacol* 2010;**58**:1199–205.
27. Luca S, White JF, Sohal AK, Filippov DV, van Boom JH, Grisshammer R, et al. The conformation of neurotensin bound to its G protein-coupled receptor. *Proc Natl Acad Sci USA* 2003;**100**:10706–11.
28. Maeno H, Yamada K, Santo-Yamada Y, Aoki K, Sun Y-J, Sato E, et al. Comparison of mice deficient in the high- or low-affinity neurotensin receptors, Ntsr1 or Ntsr2, reveals a novel function for Ntsr2 in thermal nociception. *Brain Res* 2004;**998**:122–9.
29. Mazella J, Vincent J-P. Functional roles of the NTS2 and NTS3 receptors. *Peptides* 2006;**27**:2469–75.
30. Ohinata K, Shimano T, Yamauchi R, Sakurada S, Yanai K, Yoshikawa M. The anorectic effect of neurotensin is mediated via a histamine H1 receptor in mice. *Peptides* 2004;**25**:2135–8.
31. Oliveros A, Heckman MG, del Pilar Corena-McLeod M, Williams K, Boules M, Richelson E. Sensorimotor gating in NTS1 and NTS2 null mice: effects of d-amphetamine, dizocilpine, clozapine and NT69L. *J Exp Biol* 2010;**213**:4232–9.
32. Rossi GC, Matulonis JE, Richelson E, Barbut D, Pasternak GW. Systemically and topically active antinociceptive neurotensin compounds. *J Pharm Exp Ther* 2010;**334**:1075–9.
33. Rostene WH, Alexander MJ. Neurotensin and neuroendocrine regulation. *Front Neuroendocrinol* 1997;**18**:115–73.
34. Seebach D, Lukaszuk A, Patora-Komisarska K, Podwysocka D, Gardiner J, Ebert MO, et al. On the terminal homologation of physiologically active peptides as a means of increasing stability in human serum–neurotensin, opiorphin, B27-KK10 epitope, NPY. *Chem Biodivers* 2011;**8**:711–39.
35. Shibata Y, White JF, Serrano-Vega MJ, Magnani F, Aloia AL, Grisshammer R, et al. Thermostabilization of the neurotensin receptor NTS1. *J Mol Biol* 2009;**390**:262–77.

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Chapter 118

NPY

Yvan Dumont and Rémi Quirion

ABSTRACT

Neuropeptide Y (NPY) is one of the most abundant peptides found in various brain structures and one of the most evolutionary conserved ones. NPY and its homologs (peptide YY and pancreatic polypeptide) have been implicated in several biological processes including food intake, anxiety and depression-related behaviors, neuronal excitability and seizures, circadian rhythms, alcohol consumption, neuroendocrine secretions, nociception, and cardiorespiratory functions. The various effects of NPY in the central nervous system are mediated by the activation of at least five receptor subtypes designated as Y_1, Y_2, Y_4, Y_5, and y_6, the last one not being functionally expressed in human and rat. Clinical and preclinical studies suggest that NPY plays a major role in obesity, epilepsy, anxiety, depression, and cardiovascular disorders.

DISCOVERY OF NPY AND HOMOLOGS

The discovery of this peptide family started with the isolation of a 36 amino acid peptide from the chicken pancreas in 1975 that was termed avian pancreatic polypeptide (aPP). Later on, a strong PP-immunopositive signal was observed in the central nervous system (CNS) of several species. These results were subsequently confirmed by other investigators, suggesting the presence of a PP-like protein in mammalian brains. However, the isolation of genuine PP proteins from the CNS remained unproductive. The nature of the PP-like immunoreactive (ir) materials detected in the CNS was resolved in 1982 with the discovery of two peptides isolated from porcine intestine and brain extracts; the first one was called peptide YY (PYY); while the second became known as neuropeptide Y (NPY).[30] These two peptides contain a tyrosine residue at their N-and C-termini, and 36 amino acids with structural homologies to the PPs. All three peptides are thus included in the same family usually designated as the Y, NPY, or PP family. Further information on this peptide family is provided in the Ingestive and Gastrointestinal Peptides sections.

Antibodies were developed against NPY, and immunohistochemical and radioimmunoassay techniques revealed the presence of very high amounts of NPY-like immunoreactive (ir) materials in the mammalian CNS, including brain, and established that the PP-like ir previously observed in brain tissues resulted from cross-reactivity of the antibody used with NPY.[12] Thus far, NPY has been exclusively found in the central and peripheral nervous systems, while PYY and PPs are mostly restricted to endocrine cells of the intestine and pancreatic gland.

STRUCTURE OF THE PRECURSOR mRNA/GENE

The human gene coding for NPY is located on chromosome 7 and nonisotopic in situ hybridization revealed that it is on 7q15.1 in proximity to the HOXA cluster (7q15-q14). It exists as a single-copy gene.[5] Early analysis of the gene sequence revealed that the transcription unit spans about 8000-base pairs (bp) divided in four exons separated by three introns of about 965-, 4300-, and 2300-bp as well as 5′ and 3′-untranslated domains (Fig. 1). The 5′-nontranscribed region of the NPY gene regulates the expression of the NPY mRNA and contains the sequence ATAAA or TATA box, as well as nucleotide sequences for DNA-binding proteins such as CAAT, AP1, and several SP1.[5] The mRNA transcript includes the first exon, which contains the 5′-untranslated domain; the second exon, which codes for the initiating codon and the main part of the mature NPY sequence up to glutamine 34; the third exon, which codes for the last two amino acids, the glycine amine donor site, the dibasic cleavage site, and the main portion of the carboxy terminal peptide of neuropeptide Y (CPON); and the fourth exon, which includes the end of CPON and the 3′-untranslated region, including the polyadenylation recognition signal AATAAA (Fig. 1). The NPY mRNA consists of 551-bp and translates for a 97 amino acid precursor protein (Fig. 2). Comparable NPY gene organization has been reported in various species.[5]

PROCESSING OF THE NPY PRECURSOR

As for most low-molecular-weight peptides, NPY is synthesized as a precursor protein containing a hydrophobic

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00118-4
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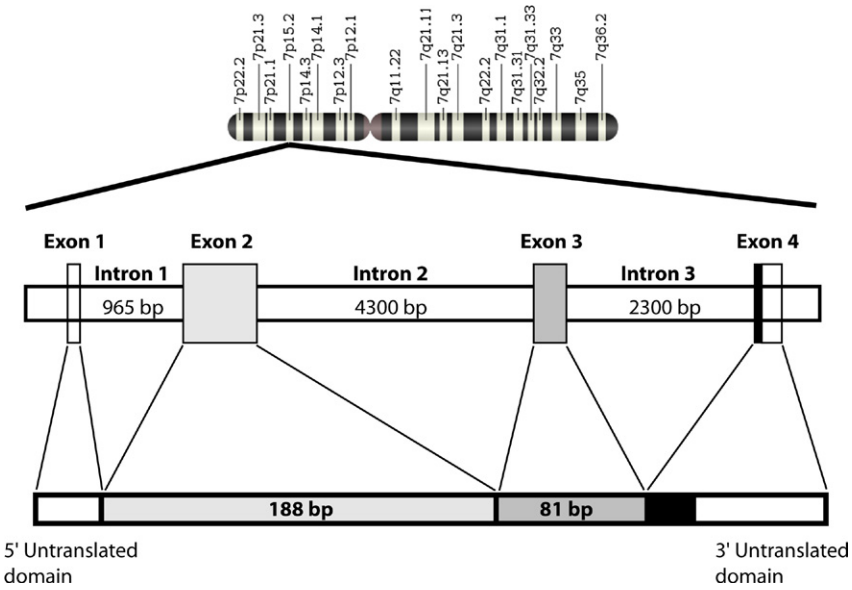

FIGURE 1 Schematic representation of the human NPY gene organization.

signal peptide, the mature peptide NPY, the amidation-proteolytic site, and the carboxy-terminal extension CPON (Fig. 2).[5] The signal sequence has two main functions. In the early stage of translation, as the first amino acid residue of the nascent polypeptide emerges from the large subunit of the ribosome, this region of interacts with the signal recognition particle, a ribonucleoprotein, resulting in slowing down translation in the cytoplasmic space.[5] The complex (ribosome-nascent chain-signal recognition particle) is then targeted to the endoplasmic reticulum, where it interacts with the signal recognition particle receptor. This event reactivates the translation process and ensures that the translation of the polypeptide coding mRNA occurs at the surface of the rough endoplasmic reticulum. The second function of the signal sequence is to facilitate the passage of the nascent polypeptide through the lipid bilayer of the endoplasmic reticulum into the lumen to be packed into secretory vesicles.[5] The 97 amino acid pre-pro-NPY is then cleaved by signal peptidases to generate a 69 amino acid peptide, or pro-NPY, and the signal peptide.[5] The pro-NPY is next processed by enzymatic cleavage to generate a 39 amino acid peptide that contains NPY and the amidation proteolytic site motif (glycine-lysine-arginine) and a 30 amino acid residue peptide usually referred to as CPON.[5] The C-terminal lysine and arginine are removed by a carboxypeptidase from the immature NPY and the glycine residue donates its amino group to generate the amide of the C-terminal tyrosine residue of NPY[5] (see carboxypeptidase chapter in Peptide Biosyntheis/Processing section of this book). NPY and CPON are then stored in vesicles and co-released upon stimulation. In contrast to NPY, the role of CPON is still unknown.

DISTRIBUTION OF NPY mRNA AND PROTEIN

Anatomical studies have demonstrated the presence of important quantities of NPY-like ir material and NPY mRNA in the brain of multiple species including the rat, mouse, and human, and a high degree of similarity in the distribution of NPY-ergic neurons is observed between species.[12] In situ hybridization and immunostaining studies revealed that NPY mRNA and immunoreactive materials are distributed very similarly.[12] In mammalian brain, NPY-ergic neurons are found in interneurons, as well as long projection neurons. While interneurons are mostly located in the forebrain, projection neurons are located, for example, in the arcuate nucleus, lateral geniculate nucleus, and various brain-stem nuclei.[12]

NPY, one of the most abundant brain peptides, is expressed in numerous brain regions where it is often colocalized with either norepinephrine, GABA, somatostatin. or the agouti-related protein. Schematic distribution of NPY-ir is presented in Fig. 3. Enrichment in NPY-ergic neurons varies between different cortical areas, with the highest concentrations found in the cingulate and temporal cortices and the lowest in the occipital lobe. Cortical NPY-positive cells are mostly interneurons.[12] In the hippocampus, large quantities of NPY-containing cells are located in the hilus of the dentate gyrus, striatum, and orien layers of the CA2 and CA3 subfields, and near or within the stratum pyramidale of the CAl subfield.[12] Most of this innervation is from local projections, but some NPY-like ir terminals may have extrahippocampal origins. In the striatum, the majority of NPY-ergic neurons are interneurons with the

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CACCCCATCCGCTGGCTCTCACCCCTCGGAGACGCTCGCCCGACAGCA

TAGTACTTGCCGCCCAGCCACGCCCGCGCGCCAGCCACCATGCTAGGT
                                       MetLeuGly

AACAAGCGACTGGGGCTGTCCGGACTGACCCTCGCCCTGTCCCTGCTC
AsnLysArgLeuGlyLeuSerGlyLeuThrLeuAlaLeuSerleuLeu

GTGTGCCTGGGTGCGCTGGCCGAGGCGTACCCCTCCAAGCCGGACAAC
ValCysLeuGlyAlaLeuAlaGluAlaTyrProSerLysProAspAsn

CCGGGCGAGGACGCACCAGCGGAGGACATGGCCAGATACTACTCGGCG
ProGlyGluAspAlaProAlaGluAspMetAlaArgTyrTyrSerAla

CTGCGACACTACATCAACCTCATCACCAGGCAGAGATATGGAAAACGA
LeuArgHisTyrIleAsnLeuIleThrArgGlnArgTyrGlyLysArg

TCCAGCCCAGAGACACTGATTTCAGACCTCTTGATGAGAGAAAGCACA
SerSerProGluThrLeuIleSerAspLeuLeuMetArgGluSerThr

GAAAATGTTCCCAGAACTCGGCTTGAAGACCCTGCAATGTGGTGATGG
GluAsnValProArgThrArgLeuGluAspProAlaMetTrp***

ATCGTGTAAAACGAGAATCCACCCATCCTACCAATGCATGCAGCCACT

GTGCTGAATTCTGCAATGTTTTCCTTTGTCATCATTGTATATATGTGT

GTTTAAATAAAGTATCATGCATTCAAAAGTGAAAAAAAAAAAA
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FIGURE 2 Representation of the human NPY mRNA and the preproNPY protein. hydrophobic signal peptide (highlighted in light gray), the mature peptide NPY (highlighted in mid-gray), the amidation-proteolytic site (highlighted in black), and the carboxy-terminal extension CPON (highlighted in light gray, italic letter). The 5′- and 3′-untranslated domains are presented in white boxes.

highest densities located in the nucleus accumbens and the bed nucleus of the stria terminalis.[12] The anterior olfactory nucleus, olfactory tubercle, islands of Calleja, lateral septum, and tenia tecta also contain important amounts of NPY-like ir neurons. The hypothalamus is the structure that expresses the highest levels of NPY. NPY-like ir and mRNA cell bodies are mainly found in the arcuate nucleus and lateral hypothalamus,[12] while fibers and terminals spread throughout this region. The suprachiasmatic nucleus is innervated by NPY axons originating from the ventral lateral geniculate nucleus.[12] NPY innervation of the paraventricular nucleus of the hypothalamus arises from the brainstem with shorter projections originating in the arcuate nucleus.[12] The amygdaloid complex is also an area that contains several NPY-like ir cell bodies and numerous fibers. On the other hand, no or very low levels of NPY-like material are expressed in most thalamic nuclei and the cerebellum. The only thalamic nucleus that contains significant amounts of NPY is the lateral geniculate nucleus, with fibers projecting to the suprachiasmatic nucleus. Significant amounts of NPY-like ir neurons are also found in the inferior colliculus, central gray area, medulla oblongata, lateral reticular nucleus, raphe nuclei, and locus coeruleus. The dorsal horn of the spinal cord also expresses significant amounts of NPY, especially under pathological conditions.

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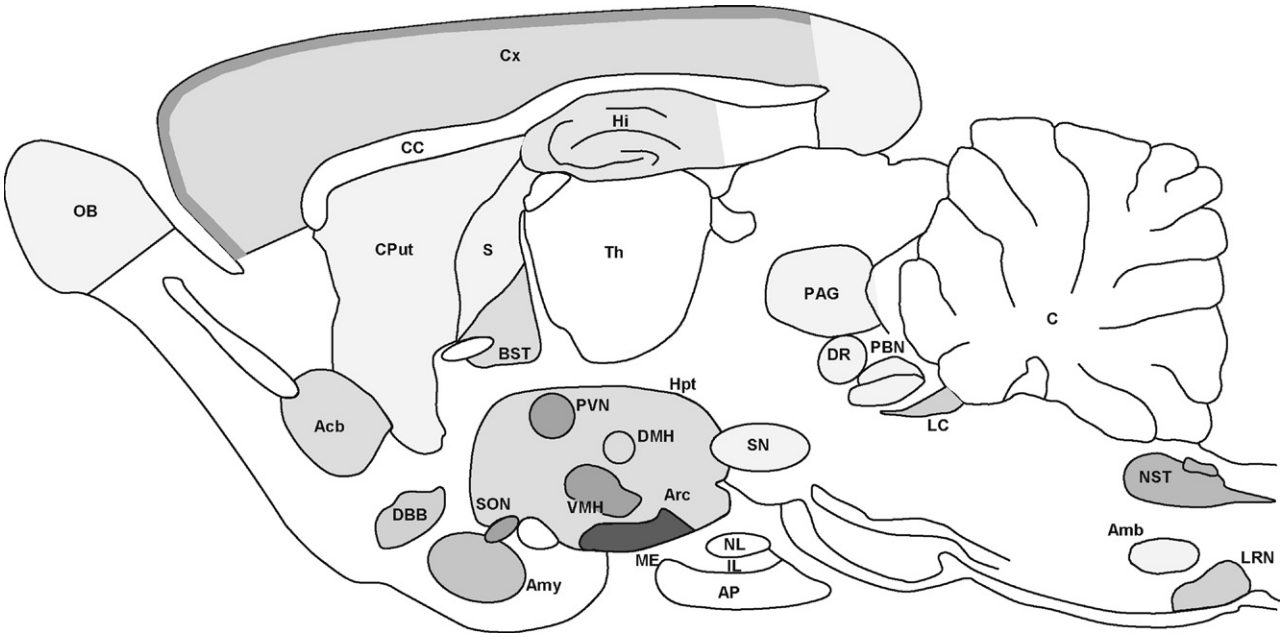

FIGURE 3 Schematic distribution of NPY-ergic neurons. Levels of expression of NPY are expressed in gray. Dark gray represents the highest amount of expression, while light gray shows the lowest ones.

STRUCTURE OF NPY-LIKE PEPTIDES

A few studies have evaluated the secondary and tertiary structures of NPY and homologs. Using a combination of molecular modeling and structural dynamics, comparison of the possible structure of NPY with that of aPP has shown that structural elements found in aPP are maintained in NPY.[2] In the model proposed, N-and C-termini are stabilized by the intramolecular association of the hydrophobic moieties of the polyproline type II helix (residues 1–9) and the amphiphilic α-helix (residues 14–30), these two structures being connected by a type II β-turn.[2] This confers a hairpin-like structure to the NPY molecule, where the tyrosine residue in position 1 and amino acid residues 30–36 are located in close proximity to each other, a structure often referred to as the PP-fold. Structure activity studies have also shown that the central segment of NPY, amino acid residues is important in conferring the helical conformation and ensuring high affinity for various NPY receptor subtypes.[3]

RECEPTORS AND SIGNALING CASCADES

There are 7 receptor subtypes in vertebrates, of which 5 exist in mammals, and these are designated Y_1, Y_2, Y_4, Y_5, and y_6.[22] They all belong to the subfamily of seven transmembrane G protein-coupled receptor type 1.[22] They are expressed in all mammalian brains except for the y_6 subtype which is restricted to only a few species including the mouse, rabbit, and dog.[22] Rather surprisingly, NPY receptors display relatively low sequence homologies between themselves and appear to be among the most divergent receptor families.[19] Some NPY receptor subtypes even have higher homology for other families of G protein-coupled receptors. The detailed CNS distribution of the various NPY receptor subtypes has also been evaluated in various species. Generally, the Y_1 and Y_2 subtypes are the most abundant NPY receptors expressed in the CNS, especially in all cortical areas, thalamic nuclei, hippocampus, and brainstem nuclei while the Y_4 and Y_5 classes are found in much lower amounts.[8]

NPY receptors are usually coupled to Gi/o leading to the inhibition of the production of cyclic AMP (cAMP). However, coupling to other signal transduction pathways can also occur. In HEK293 cells transfected with the human Y_1 receptor cDNA, the receptor couples to a pertussis toxin-sensitive G protein that mediates the inhibition of cAMP accumulation while in CHO cells this same receptor is associated with the elevation of intracellular Ca^{++} levels.[22] Likewise, transfection of different cell types with the human Y_2 receptor cDNA can generate a receptor that can couple to a pertussis toxin-sensitive G protein mediating the inhibition of cAMP production and the elevation of intracellular Ca^{++} levels.[22] Similar findings have been reported for the human and rat Y_2 receptor.[22] It has also been shown that some of the effects of NPY are mediated by protein kinase C (PKC3),[6] and its downstream cascade extracellular signal-regulated kinase (ERK)1/2 and CAMP responsive element binding protein.[29,35]

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BIOLOGICAL ACTIONS

Numerous studies have addressed the biological functions of NPY and homologs in the CNS and shown a broad range of effects after intracerebroventricular (icv) injections of NPY as well as by direct administration of this peptide into specific nuclei including: the paraventricular, perifornical hypothalamic nuclei, hippocampus, and brainstem nuclei. NPY has been reported as the most potent substance known thus far to stimulate feeding behavior and water consumption in various species including rat, mouse, sheep, pig, rabbit, and pigeon. NPY also increases the respiratory quotient indicative of increased carbohydrate utilization as energy substrates, revealing its role in catabolism. NPY can facilitate learning and memory processes, modulate locomotor activities, produce hypothermia, decrease sexual behavior, shift circadian rhythms, modulate cardiorespiratory parameters, produce antinociceptive effects, and generate anxiolytic-like effects; it also has been implicated in bone formation. Additionally, NPY alters secretion of neuroendocrine hormones such as gonadotropin-releasing hormone, and corticotropin-releasing hormone. Furthermore, NPY has been shown to modulate the neuronal activity of glutamatergic, adrenergic, serotonergic, GABAergic and dopaminergic neurons. In amphibians, NPY inhibits α melanocyte-stimulating hormone release. The various effects of NPY have been reviewed in various publications; see for example, Refs. 9, 14, 20, 26, 32, and 33.

PATHOPHYSIOLOGICAL IMPLICATIONS

Based on results obtained by use of NPY antibodies, receptor antagonists, antisense oligonucleotides, siRNA, and genetically modified animals, it has been suggested that NPY could be implicated in various pathophysiological disorders including feeding and satiety, cardiovascular functions, memory impairment and cognition, anxiety- and depression-related behaviors, pain, epilepsy, alcohol consumption, and drug dependence. For example, neutralizing endogenous NPY effects by the administration of NPY antibody into specific brain regions such as the hippocampus induces amnesia in the rat,[27] while NPY antibodies administered in the ventromedial or paraventricular hypothalamic nuclei decrease food intake.[7] Similarly, blocking the synthesis of NPY by icv administration of NPY antisense oligonucleotides results in decreased food intake and attenuation of the progesterone-induced LH surge.[14] Injection of an NPY Y_1 receptor antisense induces marked anxiogenic-like behavior.[11] Moreover, NPY-deficient mice show mild spontaneous seizures, while kainate-induced seizures result in death in these animals, which can be prevented by the exogenous administration of NPY.[33] Furthermore, NPY-deficient mice are alcohol preferring and less sensitive to the sedative effect of alcohol, while transgenic mice overexpressing NPY drink less alcohol.[31] Additionally, young and old transgenic rats are less anxious than wildtype rats.[4] Furthermore, Y_1 knockout mice display highly aggressive behaviors,[16] whereas NPY Y_2 knockout mice show anxiolytic-like behavior and drink less alcohol as compared with wildtype mice.[4] Similar results are observed with Y_2 receptor antagonists.[4,13] More recently, the therapeutic potential of Y_2 receptor antagonist in anxiety and depression-like behaviors has been confirmed.[1,6,23] In fact, evidence suggests that Y_1, Y_2, Y_4 and Y_5 receptor subtypes can be implicated in the pathophysiology of feeding[15,21] and anxiety and depressive disorders.[10,24] In humans, Karvonen and collaborators[17] have identified a 1128 T-C polymorphism that results in substitution of leucine by proline at residue 7 in the peptide part of preproNPY, and its frequency is higher in alcohol-dependent subjects.[18] Additionally, recent studies in humans have suggested that NPY could be implicated in the ability to cope with stressful events. Specifically, research with US Special Forces military personnel exposed to an extremely stressful training experience has shown that effective coping is correlated with higher blood levels of NPY.[25,34] Interestingly, men with combat-related PTSD have low levels of NPY in the cerebrospinal fluid as compared with control subjects.[28] These results point to a potential role for NPY in resilience to stress. Hence, it would appear that NPY and its receptors can be associated with various aberrant CNS behaviors, suggesting their possible relevance in the development of new therapeutic avenues to treat neurological and mental illnesses.

ACKNOWLEDGMENT

Supported by the Canadian Institutes of Health Research (CIHR).

REFERENCES

1. Aydin C, Oztan O, Isgor C. Effects of a selective Y2R antagonist, JNJ-31020028, on nicotine abstinence-related social anxiety-like behavior, neuropeptide Y and corticotropin releasing factor mRNA levels in the novelty-seeking phenotype. *Behav Brain Res* 2011;**222**:332–41.
2. Beck-Sickinger AG. The importance of various parts of the NPY molecule for receptor recognition. In: Grundemar L, Bloom SR, editors. *Neuropeptide Y and drug development*. London, UK: Academic Press; 1997. p. 107–26.
3. Cabrele C, Wieland HA, Langer M, Stidsen CE, Beck-Sickinger AG. Y-receptor affinity modulation by the design of pancreatic polypeptide/neuropeptide Y chimera led to Y5-receptor ligands with picomolar affinity. *Peptides* 2001;**22**:365–78.
4. Carvajal C, Dumont Y, Quirion R. Neuropeptide Y: role in emotion and alcohol dependence. *CNS Neurol Disord Drug Targets* 2006;**5**: 181–95.

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5. Cerda-Reverter JM, Larhammar D. Neuropeptide Y family of peptides: structure, anatomical expression, function, and molecular evolution. *Biochem Cell Biol* 2000;**78**:371–92.
6. Cippitelli A, Rezvani AH, Robinson JE, Eisenberg L, Levin ED, Bonaventure P, et al. The novel, selective, brain-penetrant neuropeptide Y Y2 receptor antagonist, JNJ-31020028, tested in animal models of alcohol consumption, relapse, and anxiety. *Alcohol* 2011;**45**: 567–76.
7. Dube MG, Xu B, Crowley WR, Kalra PS, Kalra SP. Evidence that neuropeptide Y is a physiological signal for normal food intake. *Brain Res* 1994;**646**:341–4.
8. Dumont Y, Chabot JG, Quirion R. Receptor autoradiography as mean to explore the possible functional relevance of neuropeptides: focus on new agonists and antagonists to study natriuretic peptides, neuropeptide Y and calcitonin gene-related peptides. *Peptides* 2004;**25**:365–91.
9. Dumont Y, Martel JC, Fournier A, St Pierre S, Quirion R. Neuropeptide Y and neuropeptide Y receptor subtypes in brain and peripheral tissues. *Prog Neurobiol* 1992;**38**:125–67.
10. Dumont Y, Morales-Medina JC, Quirion R. Neuropeptide Y and its role in anxiety-related disorders. In: Shioda S, Homma I, Kato N, editors. *Transmitters and modulators in health and disease*. Tokyo, Japan: Springer; 2009. p. 51–82.
11. Heilig M. The NPY system in stress, anxiety and depression. *Neuropeptides* 2004;**38**:213–24.
12. Hendry SH. Organization of neuropeptide Y neurons in the mammalina central nervous system. In: Colmers WF, Wahlested C, editors. *The biology of neuropeptide Y and related peptides*. Totawa, NJ: Humana Press, Inc.; 1993. p. 65–156.
13. Ishida H, Shirayama Y, Iwata M, Katayama S, Yamamoto A, Kawahara R, et al. Infusion of neuropeptide Y into CA3 region of hippocampus produces antidepressant-like effect via Y1 receptor. *Hippocampus* 2007;**17**:271–80.
14. Kalra SP, Kalra PS. To subjugate NPY is to improve the quality of life and live longer. *Peptides* 2007;**28**:413–8.
15. Kamiji MM, Inui A. NPY Y2 and Y4 receptors selective ligands: promising anti-obesity drugs? *Curr Top Med Chem* 2007;**7**:1734–42.
16. Karl T, Lin S, Schwarzer C, Sainsbury A, Couzens M, Wittmann W, et al. Y1 receptors regulate aggressive behavior by modulating serotonin pathways. *Proc Natl Acad Sci U S A* 2004;**101**:12742–7.
17. Karvonen MK, Pesonen U, Koulu M, Niskanen L, Laakso M, Rissanen A, et al. Association of a leucine(7)-to-proline(7) polymorphism in the signal peptide of neuropeptide Y with high serum cholesterol and LDL cholesterol levels. *Nat Med* 1998;**4**:1434–7.
18. Lappalainen J, Kranzler HR, Malison R, Price LH, Van Dyck C, Rosenheck RA, et al. A functional neuropeptide Y Leu7Pro polymorphism associated with alcohol dependence in a large population sample from the United States. *Arch Gen Psychiatry* 2002;**59**:825–31.
19. Larhammar D. Structural diversity of receptors for neuropeptide Y, peptide YY and pancreatic polypeptide. *Regul Pept* 1996;**65**:165–74.
20. Lee NJ, Herzog H. NPY regulation of bone remodelling. *Neuropeptides* 2009;**43**:457–63.
21. MacNeil DJ. NPY Y1 and Y5 receptor selective antagonists as anti-obesity drugs. *Curr Top Med Chem* 2007;**7**:1721–33.
22. Michel MC, Beck-Sickinger A, Cox H, Doods HN, Herzog H, Larhammar D, et al. XVI. International Union of Pharmacology recommendations for the nomenclature of neuropeptide Y, peptide YY, and pancreatic polypeptide receptors. *Pharmacol Rev* 1998;**50**:143–50.
23. Morales-Medina JC, Dumont Y, Benoit CE, Bastianetto S, Flores G, Fournier A, et al. Role of neuropeptide Y Y(1) and Y(2) receptors on behavioral despair in a rat model of depression with co-morbid anxiety. *Neuropharmacology* 2012;**62**:200–8.
24. Morales-Medina JC, Quirion R. Role of neuropeptide Y in depression: current status. In: Lopez-Munoz F, Alamo C, editors. *Neurobiology of depression*. New York, USA: CRC Press; 2011. p. 327–41.
25. Morgan CA, Rasmusson AM, Wang S, Hoyt G, Hauger RL, Hazlett G. Neuropeptide-Y, cortisol, and subjective distress in humans exposed to acute stress: replication and extension of previous report. *Biol Psychiatry* 2002;**52**:136–42.
26. Morley JE, Farr SA. Cachexia and neuropeptide Y. *Nutrition* 2008;**24**:815–9.
27. Redrobe JP, Dumont Y, St Pierre JA, Quirion R. Multiple receptors for neuropeptide Y in the hippocampus: putative roles in seizures and cognition. *Brain Res* 1999;**848**:153–66.
28. Sah R, Ekhator NN, Strawn JR, Sallee FR, Baker DG, Horn PS, et al. Low cerebrospinal fluid neuropeptide Y concentrations in posttraumatic stress disorder. *Biol Psychiatry* 2009;**66**:705–7.
29. Sheriff S, Qureshy F, Chance T, Kasckow JW, Balasubramaniam A. Predominant role by CaM kinase in NPY Y(1) receptor signaling: involvement of CREB and ambikaipakan. *Peptides* 2002;**23**:87–96.
30. Tatemoto K, Carlquist M, Mutt V. Neuropeptide Y—a novel brain peptide with structural similarities to peptide YY and pancreatic polypeptide. *Nature* 1982;**296**:659–60.
31. Thiele TE, Sparta DR, Hayes DM, Fee JR. A role for neuropeptide Y in neurobiological responses to ethanol and drugs of abuse. *Neuropeptides* 2004;**38**:235–43.
32. Thorsell A. Brain neuropeptide Y and corticotropin-releasing hormone in mediating stress and anxiety. *Exp Biol Med (Maywood)* 2010;**235**:1163–7.
33. Vezzani A, Sperk G, Colmers WF. Neuropeptide Y: emerging evidence for a functional role in seizure modulation. *Trends Neurosci* 1999;**22**:25–30.
34. Yehuda R, Brand S, Yang RK. Plasma neuropeptide Y concentrations in combat exposed veterans: relationship to trauma exposure, recovery from PTSD, and coping. *Biol Psychiatry* 2006;**59**:660–3.
35. Zhang H, Sakharkar AJ, Shi G, Ugale R, Prakash A, Pandey SC. Neuropeptide y signaling in the central nucleus of amygdala regulates alcohol-drinking and anxiety-like behaviors of alcohol-preferring rats. *Alcohol Clin Exp Res* 2010;**34**:451–61.

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Chapter 119

PACAP

David Vaudry, Tomoya Nakamachi, Magali Basille, Olivier Wurtz, Alain Fournier, Hubert Vaudry and Seiji Shoida

ABSTRACT

Pituitary adenylate-cyclase-activating polypeptide (PACAP) is a 38- or 27-amino-acid peptide belonging to the VIP/secretin/GHRH/glucagon superfamily that was discovered from hypothalamic extracts for its ability to stimulate cAMP formation in anterior pituitary cells. PACAP and its 3 receptor subtypes are widely expressed in the brain, suggesting that this peptide may exert numerous functions in the central nervous system. In the developing brain, PACAP controls progenitor proliferation, migration, and differentiation. In the adult brain, PACAP regulates circadian rhythms, food intake, water drinking, locomotor activity, pain, and stress responses. PACAP is able to protect brain tissue from neurodegenerative diseases and stroke by inhibiting neuronal cell death and by stimulating neurogenesis. The diversity and complex effects of PACAP can be accounted for by the existence of at least 14 splice variants for the PACAP specific receptor, PAC1, which can modulate the coupling to various second messengers.

DISCOVERY

In an attempt to isolate novel hypophysiotropic neuropeptides, Akira Arimura and coworkers screened fractions from an extract of 4300 ovine hypothalami, by monitoring their ability to induce cAMP production in cultured rat anterior pituitary cells. Using this approach, they isolated a 38-amino-acid C-terminally α-amidated peptide that they named pituitary adenylate-cyclase-activating polypeptide [PACAP; Ref. 21]. A shorter form of PACAP encompassing only 27 amino acids (PACAP27) has subsequently been characterized. PACAP and its receptors are widely expressed in the central nervous system (CNS) and in most peripheral organs. In humans, the sequence of PACAP27 shares 68% identity with that of vasoactive intestinal polypeptide (VIP), identifying PACAP as a member of the VIP/secretin/GHRH/glucagon superfamily.[33] The primary structure of PACAP has been extremely well conserved in vertebrates, including mammals, birds, reptiles, amphibians, and fish, suggesting that this peptide may exert vital biological functions.[28] Indeed, PACAP induces vasodilation and bronchodilation, activates intestinal motility, increases insulin and histamine secretion, and modulates immune responses. In the central and peripheral nervous systems, PACAP acts as a neurotransmitter, neuromodulator, and/or neurotrophic factor.[1]

STRUCTURE OF THE mRNA/GENE

PreproPACAP encompasses 176 amino acids in humans, sheep, and mice, and 175 amino acids in rats.[33] The sequence of PACAP is located in the C-terminal region of the precursor and is identical in all mammalian species studied so far. The precursor also contains a 24-amino-acid signal peptide and a 29-amino-acid peptide, exhibiting moderate sequence homology with PACAP27, termed PACAP-related peptide (PRP; Fig. 1).

In humans and mice, the PACAP gene comprises five exons, the sequence of PRP being encoded by exon 4 and that of PACAP by exon 5 (Fig. 2). The overall structural organizations of the PACAP precursor and gene exhibit strong similarities with those of VIP, confirming that they originate from a common ancestral sequence through exon and gene duplications.[28,33] PACAP is constitutively expressed, but its transcription can be enhanced by cAMP, phorbol diester, dexamethasone, progesterone, and even PACAP itself. The promoter region of the mouse PACAP gene (ADCYAP1) is schematically represented in Fig. 2. In humans, the PACAP gene is localized on the P11 region of chromosome 18,[4] which is associated with holoprosencephaly and psychiatric disorders,[25] suggesting that PACAP may be involved in the control of brain development and/or the etiology of psychiatric disorders.[13]

PROCESSING OF THE PRECURSOR

Endoproteolytic cleavage of the PACAP prepro-protein at three dibasic sites (i.e. Arg^{79}-Arg^{80}, Lys^{129}-Arg^{130}, and Arg^{170}-Arg^{171}) by prohormone convertases generates a large PRP form (big PRP) and a glycine-extended form of PACAP38 (Fig. 1). Cleavage at the single Arg^{110}

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00119-6
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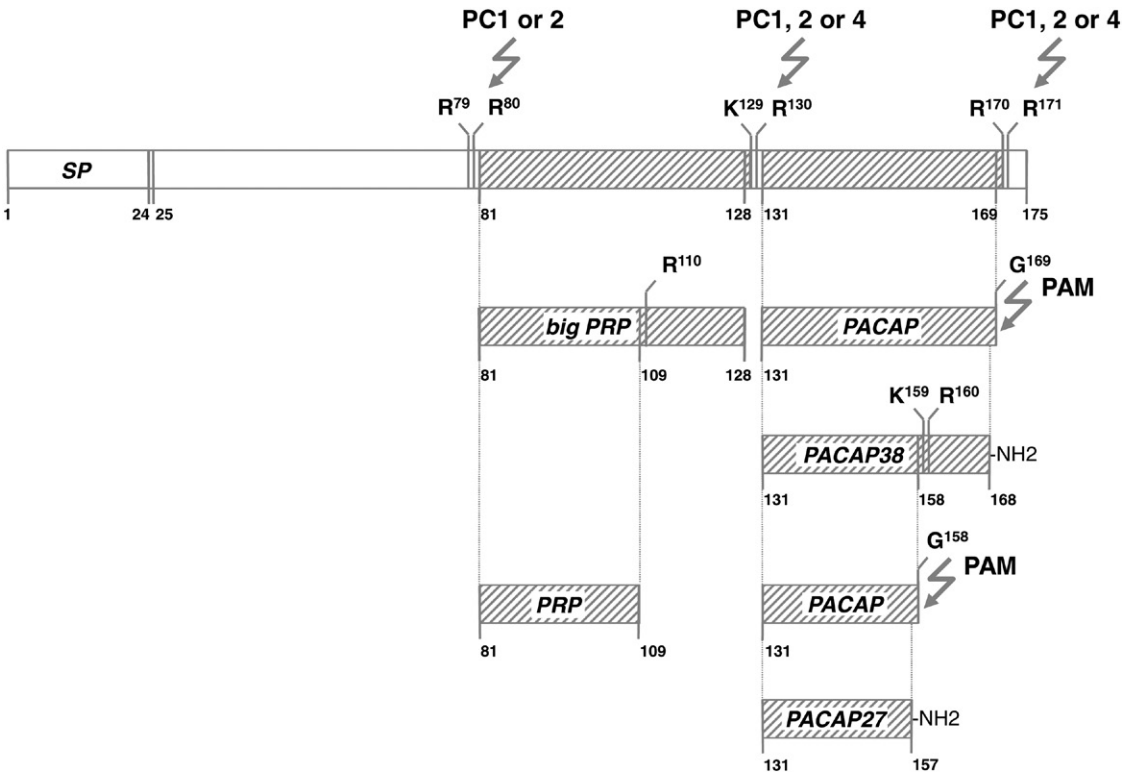

FIGURE 1 Schematic representation of the posttranslational processing of the rat PACAP precursor showing the nature and location of each cleavage and amidation site. PAM, peptidyl glycine α-amidating monooxygenase; PC1, 2, or 4, prohormone convertase 1, 2, or 4; SP, signal peptide; G, Gly; K, Lys; R, Arg. Reproduced from Ref. 33.

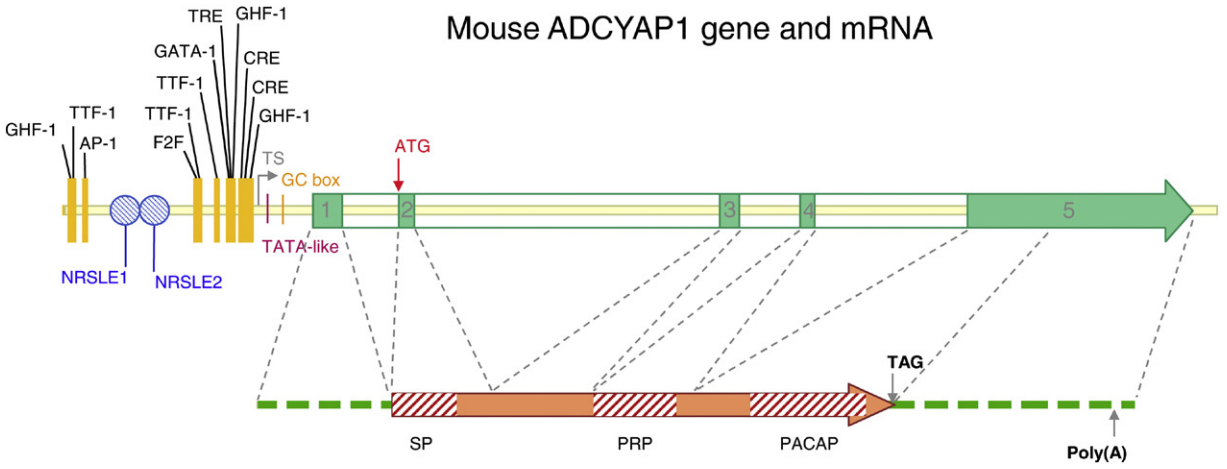

FIGURE 2 Organization of the human PACAP gene and PACAP mRNA. The 5 exons are numbered. The untranslated regions of exons 1 and 5 are denoted by a dashed line. Exons domains encoding SP, PRP, and PACAP are hatched. The locations of binding sites for potential transcriptional factors and polyadenylation have been indicated on the gene. AP-1, CRE, cAMP responsive element; GHF-1, growth hormone transactivator factor-1; Inr-like, initiator-like element; NRSLE, neural-restrictive silencer-like element; SP, signal peptide; TRE, TPA response element; TS, transcription start site; TTF-1, thyroid-specific transcription factor-1. Reproduced from Ref. 33.

followed by hydrolysis of this C-terminal Arg residue by carboxypeptidases (see the Carboxypeptidase chapter in the Peptide Biosynthesis Processing section of this book) generates PRP, and the Gly^{169} residue is used by peptidyl glycine α-amidating monooxygenase (see the PAM chapter in the same section) for the amidation of the Lys^{168} residue at the C-terminal extremity of PACAP38. Finally, the tripeptide Gly^{158}-Lys^{159}-Arg^{160} can be cleaved to generate the α-amidated PACAP27 isoform (Fig. 1). The prohormone convertases involved in the processing of preproPACAP

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seem to include at least PC1, PC2, and PC4, all discussed in the section of the book).[15]

DISTRIBUTION OF THE mRNA/PEPTIDE

The mapping of PACAP-expressing neurons has been investigated by immunocytochemistry and *in situ* hybridization in mammals. The highest densities of PACAP-producing neurons are found in the hypothalamus, especially in the paraventricular and supraoptic nuclei.[33] However, PACAP expression is not restricted to hypothalamic neurons but is widely distributed throughout the brain (Fig. 3). In particular, numerous populations of PACAP-expressing neurons are found in the cerebral cortex, amygdala, hippocampus, pineal gland, substantia nigra, cerebellum, and pons (Fig. 3). A dense network of PACAP-immunoreactive fibers is found in the thalamus and septum (Fig. 3). The overall distribution of PACAP-immunoreactivity is very similar in nonmammalian vertebrates.[33] In all brain structures, PACAP38 is by far the predominant form, PACAP27 representing <10% of the peptide content.[2]

In peripheral organs, the highest concentration of PACAP is found in the rat testis where the total mRNA content is higher than in the whole brain. PACAP is expressed in other endocrine glands such as the pituitary, the adrenal, the parathyroid, and the endocrine pancreas (see the PACAP chapter in the Endocrine Peptides section of the book). PACAP-immunoreactive fibers are also found in nonendocrine tissues such as the urinary bladder, the airways (see the PACAP chapter in the Respiratory Peptides section), and the retina. In peripheral organs, in contrast to the CNS, PACAP, and VIP often seem to be coexpressed by the same cells.

RECEPTORS AND SIGNALING CASCADES

Three PACAP/VIP receptors, which belong to the class B family of seven-transmembrane (TM) G protein-coupled receptors (GPCR), have been cloned and termed, respectively, PAC1, VPAC1, and VPAC2, according to their relative affinity for PACAP and VIP.[11] PAC1 recognizes PACAP27 and PACAP38 with 1000-fold higher affinity than VIP, whereas VPAC1 and VPAC2 exhibit similar affinity for both PACAP and VIP. All three receptors stimulate adenylyl cyclase (AC) through coupling to the Gαs protein, but the receptors have some variations in their signaling cascades and binding affinities.[14]

The PAC1 was first cloned from the rat pancreatic acinar carcinoma cell line AR4–2J, and subsequently in humans, cows, rats, and mice. The human PAC1 cDNA encodes a 525 amino acid GPCR with seven TM helices, which exhibits 30–50% sequence identity with the glucagon, secretin, parathyroid hormone, corticotropin-releasing hormone (CRH), and calcitonin receptor cDNAs. PAC1 has

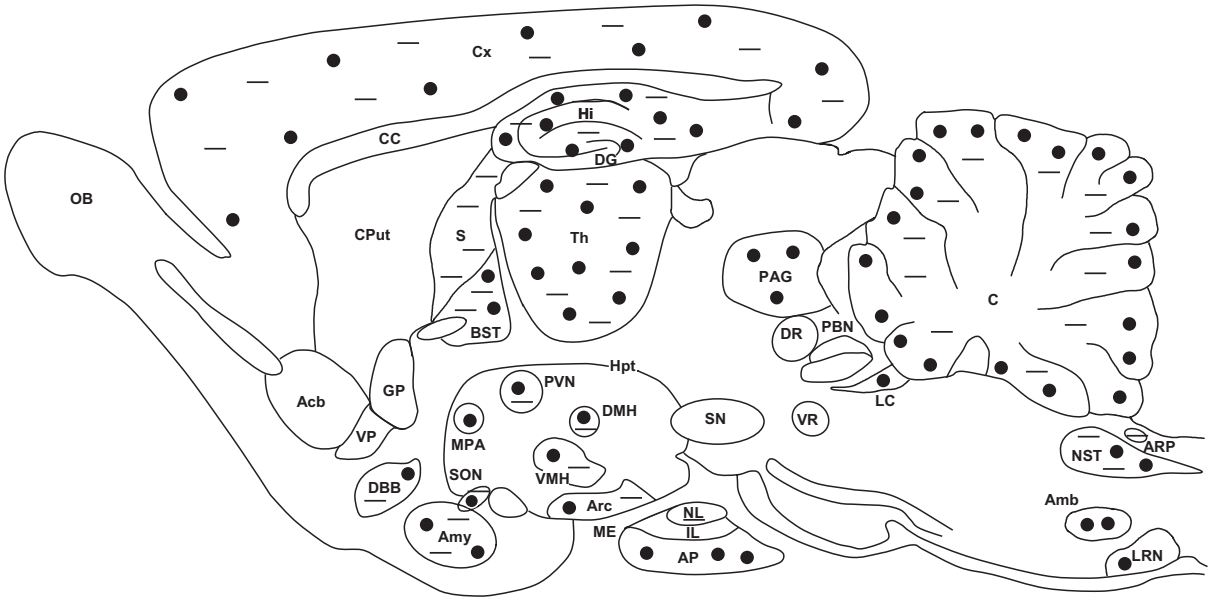

FIGURE 3 Localization of PACAP cell bodies (●) and fibers (–) in the rat brain. Adapted from Ref. 7. Abbreviations: Acb, nucleus accumbens; Amb, nucleus ambiguus; Amy, amygdala; AP, anterior pituitary; Arc, arcuate nucleus of hypothalamus; ARP, area postrema; BST, bed nucleus of stria terminalis; C, cerebellum; CC, corpus callosum; CPut, caudate putamen; Cx, cerebral cortex; DBB, diagonal band of Broca; DG, dentate gyrus; DMH, dorsomedial nucleus of hypothalamus; DR, dorsal raphe nucleus; GP, globus pallidus; Hi, hippocampus; Hpt, hypothalamus; IL, intermediate lobe of pituitary; LC, locus coeruleus; LRN, lateral reticular nucleus; ME, median eminence; MPA, medial preoptic area; NL, neural lobe of pituitary; NST, nucleus of solitary tract; OB, olfactory bulb; PAG, periaqueductal gray; PBN, parabrachial nucleus; PVN, paraventricular nucleus of hypothalamus; S, septum; SN, substantia nigra; SON, supraoptic nucleus; Th, thalamus; VMH, ventromedial nucleus of hypothalamus; VP, ventral pallidum; VR, ventral raphe nuclei.

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various splice variants that can modulate the coupling of the receptor to specific signaling pathways. In particular, splice variants with or without hip and hop cassettes in the third intracellular domain have been identified (Table 1). Both the short and hop variants stimulate AC and PLC. By contrast, PLC activation and subsequent Ca^{2+} response is impaired in the presence of the hip variant, which indicates that the various cassettes are involved in different coupling mechanisms to second messengers.

Besides the hip and hop cassettes, other variants such as the PAC1-TM4 subtype have been identified. This variant exhibits substitution and deletion of two amino acids in the fourth TM domain as well as two other substitutions in the second TM domain and N-terminal region of the receptor. The PAC1-TM4 causes an increase in $[Ca^{2+}]_i$ by enhancing calcium influx through L-type voltage-sensitive calcium channels, but it has no effect on AC or PLC activation. Two very short variants of PAC1 (PAC1-vs1 and PAC1-vs2) with a deletion of 21- or 57-amino acids, respectively, in the N-terminal extracellular domain have also been reported. In contrast to PAC1-s, PAC1-vs variants bind to PACAP38 and PACAP27 with similar affinity and have equal potency to stimulate AC and PLC. Another splice variant of PAC1 (PAC1-3a) with the insertion of 24 amino acids (exon 3) in the N-terminal extracellular domain was cloned. The transcripts for PAC1-3a are preferentially expressed in the developing rat brain and in cultured astrocytes but are undetectable in the adult brain. The PAC1-3a variant has a sixfold higher affinity for PACAP38 than PAC1-s, but the two variants show similar affinity for PACAP27. PAC1-3a is coupled to both cAMP and inositol phosphate signaling pathways although with lower response when compared with PAC1-s (Table 1).

TABLE 1 Splice Variants of the PACAP Receptors and Coupling to the Adenylate Cyclase (AC) and Phospholipase C (PLC)

Receptor subtype	Receptor variant	Signaling pathway	
		AC	PLC
$PAC_{1\text{-}R}$	$PAC_{1\text{-}s}$	+++	+++
	$PAC_{1\text{-}hip}$	+++	++
	$PAC_{1\text{-}hop}$	+++	+++
	$PAC_{1\text{-}hiphop}$	+++	+++
	$PAC_{1\text{-}3a}$	++	+
	$PAC_{1\text{-}\delta5}$	+++	++
	$PAC_{1\text{-}\delta5hip}$	++	-
	$PAC_{1\text{-}\delta5hop}$	+++	++
	$PAC_{1\text{-}\delta5hiphop}$		
	$PAC_{1\text{-}vs(\delta5,6)}$	+++	+++
	$PAC_{1\text{-}vship(\delta5,6hip)}$	++	-
	$PAC_{1\text{-}vshop(\delta5,6hop)}$	+++	+++
	$PAC_{1\text{-}vshiphop(\delta5,6hiphop)}$		
	$PAC_{1\text{-}\delta4,5,6}$	++	-
	$PAC_{1\text{-}\delta4,5,6hip}$		
	$PAC_{1\text{-}\delta4,5,6hop}$		
	$PAC_{1\text{-}\delta4,5,6hiphop}$		
	$PAC_{1\text{-}\delta4,5,6,16,17}$	-	-
	$PAC_{1\text{-}TM4}$	-	-
$VPAC_{1\text{-}R}$	$VPAC_{1\text{-}7TM}$	+++	
	$VPAC_{1\text{-}5TM}$	-	
$VPAC_{2\text{-}R}$	$VPAC_{2\text{-}7TM}$	+++	++
	$VPAC_{2\text{-}5TM}$		

Stimulation of AC or PLC pathway is classified by the EC50 values for PACAP, +++: 5 nM <, ++: 50 nM <, +: 500 nM <, -: 500 nM >.

Lutz and coworkers[16] identified 14 PAC1 splice variants in the human SHSY-5Y neuroblastoma cell line, including the hip and hop cassettes. They found deletions of exon 5, exon 5 and 6, and exons 4–6, which are responsible for shorter N-terminal extracellular loops, called δ5, δ5,6 and δ4,5,6 variants. Deletion of exon 14–17 regions by frame shift also results in the loss of amino acid residues in TM6, extracellular loop 3, TM7, and carboxyl tail named variants δ14,15,16,17. Investigation of the coupling of these variants to cAMP and calcium signaling in COS7 cells revealed that PACAP38 is approximately 30-fold more potent than VIP in activating cAMP and inositol phosphate mobilization in the δ5,6 variants. By contrast, PACAP does not stimulate cAMP level in cell expressing the hPAC1-δ5,6,14,15,16,17 variant (Table 1).

The VPAC1, cloned from rats and humans, consists of 459 and 457 amino acids, respectively. In cells transfected with the VPAC1 cDNA, PACAP and VIP activate AC with similar potency. VPAC2 has been cloned from rat, mouse, and human cDNA libraries. VPAC2 has similar affinity for PACAP and VIP as VPAC1. VPAC2 is known to activate AC and can also in some cases stimulate inositol phosphate production and induce calcium mobilization. Bokaei and collaborators identified novel five-transmembrane (5TM) forms of both human VPAC1 and VPAC2. These VPAC1 and VPAC2 variants lack 88 and 74 amino acid residues in the third intracellular loop, the fourth extracellular loop, and TM6–7, which results in impaired activation of AC. The signaling pathways associated with activation of these various receptor variants are summarized in Table 1.

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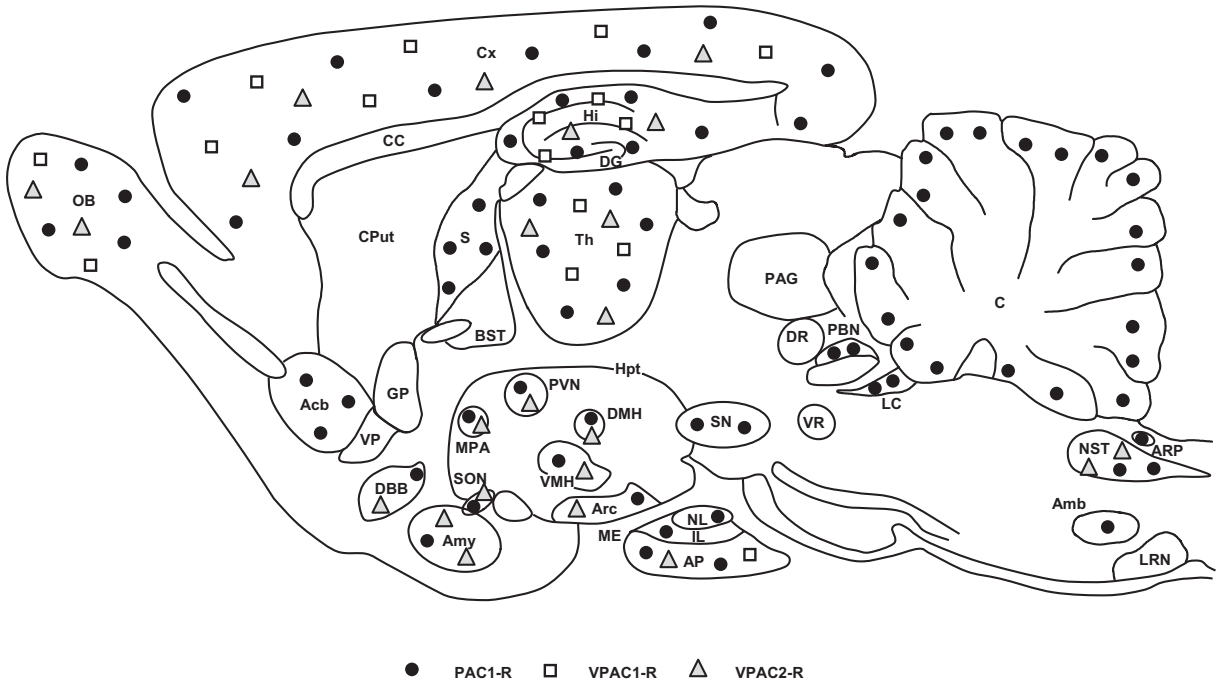

FIGURE 4 Localization of PACAP receptors in the rat brain. Abbreviations: Acb, nucleus accumbens; Amb, nucleus ambiguus; Amy, amygdala; AP, anterior pituitary; Arc, arcuate nucleus of hypothalamus; ARP, area postrema; BST, bed nucleus of stria terminalis; C, cerebellum; CC, corpus callosum; CPut, caudate putamen; Cx, cerebral cortex; DBB, diagonal band of Broca; DG, dentate gyrus; DMH, dorsomedial nucleus of hypothalamus; DR, dorsal raphe nucleus; GP, globus palidus; Hi, hippocampus; Hpt, hypothalamus; IL, intermediate lobe of pituitary; LC, locus coeruleus; LRN, lateral reticular nucleus; ME, median eminence; MPA, medial preoptic area; NL, neural lobe of pituitary; NST, nucleus of solitary tract; OB, olfactory bulb; PAG, periaqueductal gray; PBN, parabrachial nucleus; PVN, paraventricular nucleus of hypothalamus; S, septum; SN, substantia nigra; SON, supraoptic nucleus; Th, thalamus; VMH, ventromedial nucleus of hypothalamus; VP, ventral pallidum; VR, ventral raphe nuclei.

PACAP receptors are widely expressed in the CNS. In the rodent brain, a high density of PAC1 is found in the olfactory bulb, cerebral cortex, hippocampus, thalamus, hypothalamus, substantia nigra, and cerebellum (Fig. 4).[6,17] VPAC1 and VPAC2 are mainly expressed in the olfactory bulb, cerebral cortex, dendate gyrus, pineal gland, and thalamus (Fig. 4).[17]

BIOLOGICAL ACTIONS WITHIN THE BRAIN

PACAP exerts pleiotropic biological activities as a neurotrophic factor, neurotransmitter/neuromodulator, and neurohormone. PACAP regulates both proliferation and differentiation of neurons during development.[29] PACAP and its receptors are expressed in germinative regions of the brain, and PACAP plays an important role in neural tube patterning and neurogenesis.[33] On gestational day 9.5, the pattern of PAC1 expression in the mouse is very similar to that of the transcription factor Math 1 known as a major neural progenitor marker. In rat embryos, on gestational day 10.5, PACAP contributes to neural tube formation by down-regulating sonic hedgehog and gli-1 gene expression. On gestational day 13, PACAP stimulates proliferation of cortical precursor neurons whereas, on gestational day 18, PACAP suppresses cell proliferation and stimulates differentiation of neurons. In the cerebellar cortex, PACAP inhibits granule cell migration.[3] Although the effect of PACAP on cell migration is robust, it lasts for only approximately 2 h because of a desensitization process. The different effects of PACAP on brain development can probably be ascribed to temporal expression of specific splice variants of PAC1 in developing neurons. PACAP promotes the differentiation of embryonic stem cells into neurons and the differentiation of neural stem cells into astrocytes.[22] Taken together, these observations indicate that PACAP contributes to brain development suggesting that selective agonists may prove useful for neuronal regeneration to repair brain lesions.

Indeed, many reports indicate that PACAP exerts neuroprotective effects *in vivo* and *in vitro*.[30,34] PACAP application on cultured neurons prevents damages induced by hypoxia, withdrawal of nerve growth factor, excitotoxicity, oxidative stress, gp120 (from human immunodeficiency virus), and other neurotoxins.[26] The neuroprotective activity of PACAP on neuron/glia cocultures occurs at subpicomolar and subnanomolar concentrations, whereas the antiapoptotic effect of PACAP on PC12 cells and cerebellar granular neurons

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is only seen at nanomolar concentrations.[32,34] These findings suggest that glial cells contribute to the neuroprotective effect observed at very low concentrations of the peptide. The neuroprotective activity of PACAP on cultured granule neurons involves Gαs signaling, coupled to the AC/PKA pathway.[34] PACAP also induces phosphorylation of ERK through Rap1 and Ras activation, which stimulates the expression of the antiapoptotic factor B-cell lymphoma 2 (Bcl-2). This increase of Bcl-2 suppresses cytochrome *c* release from mitochondria, which in turn reduces caspase-9 activation, causing long-lasting inhibition of caspase-3 activity that is responsible for the neuroprotective action of PACAP.[31,34] *In vivo*, the neuroprotective effect of PACAP has been well characterized in rodent models of stroke and Parkinson's disease.[24,26,27] Ischemic death of hippocampal and cerebral cortex neurons can be prevented by intracerebroventricular infusion of low doses of PACAP. Systemic administration of PACAP38 also reduces infarct volume in the brain after transient focal ischemia induced by occlusion of the middle cerebral artery in rats.[27] This indicates that a therapeutic concentration of PACAP can be reached in the brain parenchyma after systemic intravenous administration of the peptide. Despite the relatively large size of the peptide, PACAP38 is translocated from the blood to the brain across the blood brain barrier with a high efficacy transport system in a saturable manner. Indeed, the efficacy of the transport mechanism is 6 times greater for PACAP38 than for morphine. PACAP38 protects hippocampal neurons from apoptosis by inhibiting the c-Jun N-terminal kinase 1 (JNK)/stress-activated protein and p38 signaling pathways. The antiapoptotic effect of PACAP38 after ischemia is indirect and involves IL-6 release.[24] PACAP stimulates the production of various neurotrophic factors in astrocytes such as IL-6 and macrophage inflammatory protein 1 (MIP1), regulates the activation of normal T expressed and secreted (RANTES) and activity-dependent neuroprotective protein (ADNP), and suppresses astrocytic cell death.[22] In Parkinson's disease model, PACAP administration attenuates 6-hydroxydopamine and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced mesencephalic dopaminergic neuronal death.[27] To summarize, PACAP can rescue injured neural cells from apoptosis, suggesting that PACAP derivatives could be useful for the treatment of stroke or neurodegenerative diseases.

The role of PACAP in the regulation of the circadian rhythm has been widely documented in mammals.[20] PACAP stimulates melatonin secretion by the rat pineal gland and cultured pinealocytes through phosphorylation of cAMP-responsive element-binding protein (CREB). The effect of PACAP on CREB phosphorylation culminates in the first part of the dark period of the 24-h cycle together with the peak of PACAP level in the pineal gland. Circadian variations in PACAP content occur in the rat pineal gland, and a high density of PACAP binding sites is present in the pineal, suggesting that PACAP is involved in the regulation of the rhythmicity of melatonin production.[33] The main biological clock center in the brain is the suprachiasmatic nucleus (SCN). PACAP causes phosphorylation of CREB in the SCN during the light period, and the effect of PACAP on CREB is suppressed by melatonin. Similarly, melatonin blocks PACAP-induced stimulation of cAMP production in the rat SCN, indicating that the hypothalamus is a site for functional interaction between PACAP and the pineal hormone melatonin. PACAP interacts with glutamate to induce light resetting of the circadian clock.[9] Thus, in rodents, PACAP enhances the phase delay provoked by glutamate in the early night, and blockade of PACAP neurotransmission inhibits the effect of glutamate. PACAP also activates clock genes such as Clock or Cry1.[10] Taken together, these results indicate that PACAP from the retinohypothalamic tract acts as a neurotransmitter to phase shift the SCN circadian rhythm in a manner similar to light.

Central administration of PACAP induces many behavioral effects in rodents. In particular, there is now clear evidence that PACAP is involved in the control of feeding behavior. Intracerebroventricular injection of PACAP decreases food intake in a dose-dependent manner.[18] In mice, the anorexigenic action of PACAP is mediated through the melanocortinergic system. Injection of PACAP in the vicinity of the perifornical lateral hypothalamus stimulates drinking and, reciprocally, water deprivation causes an increase in PACAP immunoreactivity in the subfornical organ. These observations show that PACAP plays a role in the regulation of feeding and drinking behavior.

The most abundant population of PACAP-containing neurons and the highest density of PACAP binding sites are found in the hypothalamus. In particular, a dense accumulation of PACAP-immunoreactive neurons and PACAP receptors are present in the magnocellular region of the PVN and SON, where the neurosecretory perikarya producing oxytocin and vasopressin are located. Administration of PACAP within the PVN or SON increases the firing rate activity and causes membrane depolarization of magnocellular neurons. Intracerebroventricular and intracisternal injections of PACAP induce a dose-dependent elevation of plasma vasopressin. In the neural lobe of the pituitary, PACAP stimulates the release of oxytocin and vasopressin through the activation of the cAMP/PKA signaling pathway. Collectively, these observations indicate that PACAP is a potent activator of hypothalamic magnocellular neurons.

PACAP modulates the activity of various other hypothalamic neuronal populations. Specifically, intracerebroventricular injection of PACAP provokes a significant increase in GnRH, somatostatin, prolactin, and CRH mRNA expression in the hypothalamus. PACAP injection also stimulates

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Fos expression in CRH neurons in the PVN, leading to a substantial increase in plasma corticosterone concentration and inducing stress-like behavior. In the ovariectomized ewe, infusion of PACAP in the arcuate nucleus reduces plasma prolactin concentration. A single administration of PACAP to neonatal female rats delays the onset of puberty by influencing the GnRH neuronal system through PAC1. Taken together, these results indicate that PACAP may act within the hypothalamus as a neurotransmitter or neuromodulator to regulate the secretion of hypophysiotropic neurohormones (see the PACAP Chapter in the Endocrine Peptides section of this book).

PATHOPHYSIOLOGICAL IMPLICATIONS

Pharmacological experiments support the view that PACAP is involved in various pathophysiological conditions. The PACAP- or PAC1-deficient mouse strains that have been established in different laboratories provide additional evidence for the involvement of PACAP in vital biological functions. For instance, most PACAP-deficient mice die within the first two weeks after birth with abundant lipid droplets in liver, heart, and skeletal muscle cells.[8] Premature death of PACAP-deficient mice occurs when the animals are raised at 21°C and can be prevented by placing the animals at an environmental temperature of 24°C.[8] When 7-day-old PACAP-KO mice are separated from their mother and placed at 21°C, they show a greater loss in body temperature compared with the wild-type littermates, indicating an impaired thermal adaptation in PACAP-deficient mice. Adaptive thermogenesis is mediated by norepinephrine, which is released from sympathetic nerve endings innervating the brown adipose tissue. The importance of (nor)epinephrine in thermogenesis is supported by the finding that mice deficient in dopamine β-hydroxylase, the enzyme that catalyzes the conversion of dopamine to norepinephrine, are highly cold sensitive. These findings provide important information regarding the physiological role of PACAP in the adaptation of the organism to a cold environment, through adaptive thermogenesis by the brown adipose tissue. The adaptive effect to low temperature and the protective action of PACAP to other stresses seem to be mediated by the ability of the peptide to sustain the production of catecholamines in sympathetic nerves and the adrenal medulla.

PACAP-null and heterozygous mice exhibit severer neurological defects, higher mortality rate and larger infarct volume after permanent middle cerebral artery occlusion, compared to wild-type animals.[23] These deficits could be improved by either intracerebroventricular or intravenous administration of PACAP. PACAP acts by inhibiting cytosolic cytochrome *c* release and stimulating Bcl-2 expression as well as STAT3 and ERK phosphorylation. Using an oligo-antisense strategy, Banks and colleagues[5] showed that the peptide transport system-6 (PTS6) was responsible for the efflux from the brain to the blood of PACAP27. They also revealed that accumulation of endogenous PACAP into the brain could reduce the infarct volume and neurological deficit score after ischemia. Collectively, these results indicate that endogenous PACAP acts as a neuroprotective agent in the case of stroke.

Behavioral studies with PACAP- and PAC1-mutant mice provide evidence for the involvement of PACAP in the control of psychomotor behavior. PAC1-deficient mice exhibit increased locomotor activity, reduced anxiety-like behavior, and markedly impaired social behavior, suggesting that PAC1 signaling plays a role in the development and/or function of neural pathways associated with pheromone processing and regulation of social interaction. Similarly, PACAP-deficient mice display behavioral abnormalities, including increased locomotor, exploratory, and explosive jumping activity in the open field.[12] These mice also show deficit in prepulse inhibition of the acoustic startle response, an operational measure of sensorimotor gating. Some behavioral abnormalities found in PACAP-deficient mice are reminiscent of schizophrenia-like behavior, and these abnormalities can be reversed by treatment with the atypical antipsychotic drug risperidone.[13] Noteworthy, cognitive dysfunctions in schizophrenic patients correlate with a reduction of hippocampal volume and a decrease of hippocampal neurogenesis, which occur in the brains of both schizophrenic patients and animal models of the disease, suggesting that the neurogenic dysfunction in the hippocampus contributes to the etiology of schizophrenia. Interestingly, intracerebroventricular administration of PACAP stimulates hippocampal neurogenesis in adult mice[19] and PACAP increases the expression of Disrupted-In-Schizophrenia 1 (DISC1), which is a susceptibility gene for schizophrenia. DISC1 is prominently expressed in the adult hippocampus and acts as a regulator of neurogenesis. These studies imply that PACAP could play a role in schizophrenia, possibly by altering hippocampal neurogenesis.

Targeted deletion of PAC1 alters learning and memory in rodents.[33] Mutant mice harboring either complete or forebrain-specific inactivation of PAC1 suffer from a deficit in contextual fear conditioning, a hippocampus-dependent associative learning paradigm. In contrast, water maze spatial memory is unaffected in PAC1 mutants. Long-term potentiation (LTP) in the dentate gyrus of PAC1-deficient mice and PACAP-heterozygous mice is induced by both suprathreshold and threshold tetanic stimulation. However, the population spike at threshold but not suprathreshold LTP decreases significantly in both PAC1-deficient and PACAP-heterozygous mice. These findings suggest that PACAP, acting through PAC1, may play a pivotal role in learning and memory functions.[33]

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PACAP-deficient mice exhibit significant impairment in the magnitude of response to brief light exposures with altered light-induced phase delays and advances of the circadian system. Light stimulation at early night results in a larger phase delay in PAC1-knockout mice than in wild-type animals.[10] PAC1-knockout mice under a more natural light stimulation regimen disclose a significantly decreased phase delay of the endogenous rhythm at early night.[9] At late night, the phase advance observed after light stimulation is attenuated in PACAP-deficient mice and converted into a phase delay in PAC1-knockout animals. Time-dependent dysfunctions of photic entrainment in PACAP-knockout mice can be counteracted by infusion of PACAP38. Altogether, these observations indicate that PACAP regulates particular nonvisual light responses by conveying parametric light information. Thus, dysfunction of the PACAP/PAC1 system produces selective deficits in the light response, especially during the light-to-dark transition period of the circadian system.

As indicated above, analysis of the phenotype of PACAP- and PACAP receptor-knockout mice indicate that the peptide must exert important physiological and pathophysiological functions *in vivo*. However, some phenotype changes can only be observed under pathological conditions, as PACAP has been shown to be released in the presence of psychogenic and metabolic stressors. The difficulty in some cases to detect phenotypic changes occurring in PACAP-knockout animals also arises from the fact that PACAP belongs to a superfamily of genes, including VIP and secretin, which can have partially overlapping functions. Undoubtedly, future studies will continue to disclose novel and unexpected functions of PACAP, which may lead to further clinical studies and applications for the treatment of various diseases.

REFERENCES

1. Arimura A. Perspectives on pituitary adenylate cyclase activating polypeptide (PACAP) in the neuroendocrine, endocrine, and nervous systems. *Jpn J Physiol* 1998;**48**:301–31.
2. Arimura A, Somogyvari-Vigh A, Miyata A, Mizuno K, Coy DH, Kitada C. Tissue distribution of PACAP as determined by RIA: highly abundant in the rat brain and testes. *Endocrinology* 1991;**129**:2787–9.
3. Cameron DB, Galas L, Jiang Y, Raoult E, Vaudry D, Komuro H. Cerebellar cortical-layer-specific control of neuronal migration by pituitary adenylate cyclase-activating polypeptide. *Neuroscience* 2007;**146**:697–712.
4. Chang E, Welch S, Luna J, Giacalone J, Francke U. Generation of a human chromosome 18-specific YAC clone collection and mapping of 55 unique YACs by FISH and fingerprinting. *Genomics* 1993;**17**: 393–402.
5. Dogrukol-Ak D, Kumar VB, Ryerse JS, Farr SA, Verma S, Nonaka N, et al. Isolation of peptide transport system-6 from brain endothelial cells: therapeutic effects with antisense inhibition in Alzheimer and stroke models. *J Cereb Blood Flow Metab* 2009;**29**:411–22.
6. Gonzalez BJ, Basille M, Mei YA, Vaudry D, Fournier A, Cazin L, et al. Ontogeny of PACAP and PACAP receptors in the rat brain: role of PACAP in the cerebellum during development. *Ann N Y Acad Sci* 1996;**805**:302–13; discussion 13–4.
7. Gonzalez BJ, Basille M, Vaudry D, Fournier A, Vaudry H. [Pituitary adenylate cyclase-activating polypeptide]. *Ann Endocrinol (Paris)* 1998;**59**:364–405.
8. Gray SL, Yamaguchi N, Vencova P, Sherwood NM. Temperature-sensitive phenotype in mice lacking pituitary adenylate cyclase-activating polypeptide. *Endocrinology* 2002;**143**:3946–54.
9. Hannibal J, Brabet P, Fahrenkrug J. Mice lacking the PACAP type I receptor have impaired photic entrainment and negative masking. *Am Journal Physiology Regul Integrative Comparative Physiology* 2008;**295**:R2050–8.
10. Hannibal J, Jamen F, Nielsen HS, Journot L, Brabet P, Fahrenkrug J. Dissociation between light-induced phase shift of the circadian rhythm and clock gene expression in mice lacking the pituitary adenylate cyclase activating polypeptide type 1 receptor. *J Neurosci* 2001;**21**:4883–90.
11. Harmar AJ, Arimura A, Gozes I, Journot L, Laburthe M, Pisegna JR, et al. International Union of Pharmacology. XVIII. Nomenclature of receptors for vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide. *Pharmacol Rev* 1998;**50**:265–70.
12. Hashimoto H, Shintani N, Tanaka K, Mori W, Hirose M, Matsuda T, et al. Altered psychomotor behaviors in mice lacking pituitary adenylate cyclase-activating polypeptide (PACAP). *Proc Natl Acad Sci U S A* 2001;**98**:13355–60.
13. Hashimoto R, Hashimoto H, Shintani N, Chiba S, Hattori S, Okada T, et al. Pituitary adenylate cyclase-activating polypeptide is associated with schizophrenia. *Mol Psychiatry* 2007;**12**:1026–32.
14. Laburthe M, Couvineau A, Tan V. Class II G protein-coupled receptors for VIP and PACAP: structure, models of activation and pharmacology. *Peptides* 2007;**28**:1631–9.
15. Li M, Shuto Y, Somogyvari-Vigh A, Arimura A. Prohormone convertases 1 and 2 process ProPACAP and generate matured, bioactive PACAP38 and PACAP27 in transfected rat pituitary GH4C1 cells. *Neuroendocrinology* 1999;**69**:217–26.
16. Lutz EM, Ronaldson E, Shaw P, Johnson MS, Holland PJ, Mitchell R. Characterization of novel splice variants of the PAC1 receptor in human neuroblastoma cells: consequences for signaling by VIP and PACAP. *Mol Cell Neurosci* 2006;**31**:193–209.
17. Masuo Y, Ohtaki T, Masuda Y, Tsuda M, Fujino M. Binding sites for pituitary adenylate cyclase activating polypeptide (PACAP): comparison with vasoactive intestinal polypeptide (VIP) binding site localization in rat brain sections. *Brain Res* 1992;**575**:113–23.
18. Matsuda K, Maruyama K. Regulation of feeding behavior by pituitary adenylate cyclase-activating polypeptide (PACAP) and vasoactive intestinal polypeptide (VIP) in vertebrates. *Peptides* 2007;**28**:1761–6.
19. Mercer A, Ronnholm H, Holmberg J, Lundh H, Heidrich J, Zachrisson O, et al. PACAP promotes neural stem cell proliferation in adult mouse brain. *J Neurosci Res* 2004;**76**:205–15.
20. Mertens I, Husson SJ, Janssen T, Lindemans M, Schoofs L. PACAP and PDF signaling in the regulation of mammalian and insect circadian rhythms. *Peptides* 2007;**28**:1775–83.
21. Miyata A, Arimura A, Dahl RR, Minamino N, Uehara A, Jiang L, et al. Isolation of a novel 38 residue-hypothalamic polypeptide which stimulates adenylate cyclase in pituitary cells. *Biochem Biophys Res Commun* 1989;**164**:567–74.

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22. Nakamachi T, Farkas J, Watanabe J, Ohtaki H, Dohi K, Arata S, et al. Role of PACAP in neural stem/progenitor cell and astrocyte—from neural development to neural repair. *Curr Pharm Des* 2011;**17**:973–84.
23. Ohtaki H, Nakamachi T, Dohi K, Aizawa Y, Takaki A, Hodoyama K, et al. Pituitary adenylate cyclase-activating polypeptide (PACAP) decreases ischemic neuronal cell death in association with IL-6. *Proc Natl Acad Sci U S A* 2006;**103**:7488–93.
24. Ohtaki H, Nakamachi T, Dohi K, Shioda S. Role of PACAP in ischemic neural death. *J Mol Neurosci* 2008;**36**:16–25.
25. Pinhasov A, Nesher E, Gross M, Turgeman G, Kreinin A, Yadid G. The role of the PACAP signaling system in depression. *Curr Pharm Des* 2011;**17**:990–1001.
26. Reglodi D, Kiss P, Lubics A, Tamas A. Review on the protective effects of PACAP in models of neurodegenerative diseases in vitro and in vivo. *Curr Pharm Des* 2011;**17**:962–72.
27. Reglodi D, Somogyvari-Vigh A, Vigh S, Kozicz T, Arimura A. Delayed systemic administration of PACAP38 is neuroprotective in transient middle cerebral artery occlusion in the rat. *Stroke* 2000;**31**:1411–7.
28. Sherwood NM, Krueckl SL, McRory JE. The origin and function of the pituitary adenylate cyclase-activating polypeptide (PACAP)/glucagon superfamily. *Endocr Rev* 2000;**21**:619–70.
29. Shioda S, Ohtaki H, Nakamachi T, Dohi K, Watanabe J, Nakajo S, et al. Pleiotropic functions of PACAP in the CNS: neuroprotection and neurodevelopment. *Ann N Y Acad Sci* 2006;**1070**:550–60.
30. Somogyvari-Vigh A, Reglodi D. Pituitary adenylate cyclase activating polypeptide: a potential neuroprotective peptide. *Curr Pharm Des* 2004;**10**:2861–89.
31. Ster J, De Bock F, Guerineau NC, Janossy A, Barrere-Lemaire S, Bos JL, et al. Exchange protein activated by cAMP (Epac) mediates cAMP activation of p.38 MAPK and modulation of Ca2+-dependent K+ channels in cerebellar neurons. *Proc Natl Acad Sci U S A* 2007;**104**:2519–24.
32. Vaudry D, Chen Y, Hsu CM, Eiden LE. PC12 cells as a model to study the neurotrophic activities of PACAP. *Ann N Y Acad Sci* 2002;**971**:491–6.
33. Vaudry D, Falluel-Morel A, Bourgault S, Basille M, Burel D, Wurtz O, et al. Pituitary adenylate cyclase-activating polypeptide and its receptors: 20 years after the discovery. *Pharmacol Rev* 2009;**61**:283–357.
34. Vaudry D, Gonzalez BJ, Basille M, Pamantung TF, Fournier A, Vaudry H. PACAP acts as a neurotrophic factor during histogenesis of the rat cerebellar cortex. *Ann N Y Acad Sci* 2000;**921**:293–9.

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Chapter 120

Prolactin-Releasing Peptide

Shuji Hinuma and Norio Iijima

ABSTRACT

Prolactin (PRL)-releasing peptide (PrRP) was discovered as a specific ligand for hGR3 /GPR10 in the course of a search for ligands of orphan G protein-coupled receptors. PrRP has an arginine-amidated phenylalanine in its C-terminus. Peptides with such a structure are referred to as RFamide peptides. The first RFamide peptides to be discovered in mammals were neuropeptides FF and AF. PrRP was thus the second RFamide peptide discovered in mammals. The discovery of PrRP indicated that various RFamide peptides and their genes exist in mammals as a family. Although PrRP was originally reported to stimulate PRL secretion, accumulated evidence demonstrates that PrRP has a variety of regulatory functions including hormone secretion, food intake, stress, blood pressure, and pain. Recent studies indicate that RFamide peptides show crossreactive binding to each others' receptors. The critical functions of PrRP should be considered as part of a network of RFamide peptides/receptors.

DISCOVERY

The discovery of prolactin (PRL)-releasing peptide (PrRP)[8] can be considered in the light of two historical viewpoints. Because PrRP is a member of the RFamide peptide family, one viewpoint would be that of the history of RFamide peptides (i.e., bioactive peptides with arginine and amidated phenylalanine at the C-terminus). The second viewpoint would be that of research into orphan G protein-coupled receptors (GPCRs) (i.e., GPCRs whose ligand is unknown), because PrRP would not have been discovered in the absence of a search for their ligands. The discovery of PrRP could therefore be considered to have happened at a crosspoint of studies on RFamide peptides and orphan GPCRs.

In the 1970s, Price and Greenberg discovered the first RFamide peptide, the tetrapeptide FMRFamide, in the ganglia of the clam, where it functions as a cardioexcitatory peptide. Since then, a number of RFamide peptides have been isolated from invertebrates, and it was expected that FMRFamide-like peptides would exist not only in invertebrates but also in vertebrates. The first report of an RFamide peptide in a vertebrate was the LPLRFamide that was isolated in 1983 from chicken brain by the use of antibodies specific for the C-terminus of FMRFamide.[6] The first discovered mammalian RFamide peptides were neuropeptide FF (NPFF) and neuropeptide AF (NPAF) that were purified from bovine brain extracts. It was later demonstrated that both of these peptides are encoded by a single gene.[27] PrRP, which was reported in 1998, was therefore the second RFamide peptide to be identified in mammals. In addition, it should be noted that the identification of the fish RFamide peptide (C-RFa) made an important contribution to the discovery of mammalian RFamide peptides.[6] To date, a total of five RFamide peptide genes and seven RFamide peptides have been discovered in mammals. Fig. 1 shows the alignment of the seven human RFamide peptides. It is evident from a comparison of their amino acid sequences that these mammalian RFamide peptides share similar primary structures, not only in their C-terminal regions but also in their N-terminal regions.

A breakthrough in the discovery of mammalian RFamide peptides was brought about by the use of a reverse pharmacological approach for the identification of ligands for orphan GPCRs. There are about 800 GPCR genes in the human genome. Half of these genes encode sensory receptors, of which most are olfactory. The other half of these genes are believed to encode receptors that regulate cell functions. There are a number of GPCRs whose ligands are still unknown and these receptors are therefore referred to as orphan GPCRs. When we started our studies of orphan GPCRs in the early 1990s, the exact number of GPCR genes in the human genome was unknown. With the progress of the human genome project, it is now possible to exactly determine the number of GPCR genes in the human genome. Even at present, there remain about 100 orphan GPCRs in the human genome, not including sensory GPCRs. In the mid-1990s, we established a general strategy for the identification of orphan GPCR ligands based on the detection of signal transduction that was induced after stimulation of cells that expressed a targeted orphan GPCR with test samples. Although it is difficult to predict what kind of signal transduction will occur after stimulation of a specific orphan GPCR, we noticed that at least one of three cellular responses was invariably induced by the activation of any regulatory GPCR: (1) an increase in

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00120-2
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Peptide	Amino acid sequence
NPFF	F L F Q P Q R F -NH$_2$
NPAF	A G E G L S S P F W S L A A P Q R F -NH$_2$
PrRP31	S R T H R H S M E I R T P D I N P A W Y A S R G I R P V G R F -NH$_2$
RFRP-1	S L N F E E L K D W G P K N V L K M S T P A V N K M P H S F A N L P L R F -NH$_2$
RFRP-3	A T A N L P L R S G R N M E V S L V R R V P N L P Q R F -NH$_2$
Kisspeptin	G T S L S P P P E S S G S P Q Q P G L S A P H S R Q I P A P Q G A V L V Q R E K D L P N Y N W N S F G L R F -NH$_2$
QRFP/26RFa	< E D E G S E A T G F L P A A G E K T S G P L G N L A E E L N G Y S R K K G G F S F R F -NH$_2$

FIGURE 1 Human RFamide peptides. Amino acid residues with identical sequences in at least two of the species are boxed.

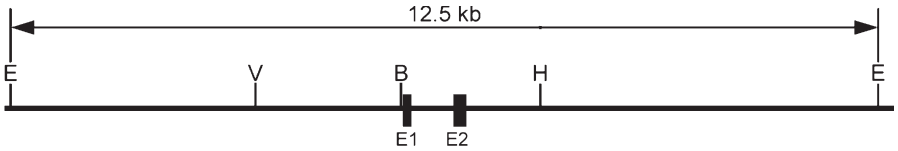

FIGURE 2 Genomic structure of mouse PrRP gene. Exons (E1, E2) are indicated by black boxes. Positions of restriction enzymatic cleavage sites (E, EcoRI; V, VspI; B, BamHI; H, HpaI) and the length of an EcoR I-digested fragment are shown in the figure.

calcium ions; (2) an increase in cAMP; or (3) a decrease in cAMP. Thus, in principle, signal transduction by most orphan GPCRs should be detectable by these three assays. PrRP was discovered during such a search for orphan GPCR ligands. Although we eventually succeeded in determining ligands for various orphan GPCRs, PrRP was the first specific ligand that we identified for an orphan GPCR, hGR3 (now referred to as GPR10). The reasons why we decided to search for ligands of GPR10 was because of the unique distribution of this receptor (GPR10 mRNA is particularly abundant in the pituitary) and because its amino acid sequence shows significant homology with neuropeptide Y receptors. We first constructed GPR10-expressing Chinese hamster ovary (CHO) cells. We then used an arachidonic acid metabolite (AA)-releasing assay to search for a GPR10 ligand because, at that time, convenient methods for the direct detection of Ca^{2+} signaling in multiple samples were unavailable. We purified the obtained ligand from bovine brain extracts using a combination of various chromatographic steps and later named this ligand PrRP based on its ability to induce PRL production by pituitary cells. All of the recently discovered mammalian RFamide peptides, except for NPFF and NPAF, the first of these peptides to be discovered, have been identified in the course of a search for ligands of orphan GPCRs. The discovery of PrRP therefore provided an important clue to explore the existence of other novel mammalian RFamide peptides.

STRUCTURE OF THE PrRP mRNA/GENE

The genomic structure of the mouse PrRP gene is illustrated in Fig. 2.[32] The mouse and human PrRP genes are located on chromosome number 1 and 2, respectively. Both the mouse and the human PrRP genes consist of two exons. Cloning of human, bovine, rat, and mouse PrRP cDNAs showed that mature PrRP is produced from a precursor protein (Fig. 3). Because we purified peptides of two different lengths from bovine hypothalamic tissue extracts, PrRP31 and PrRP20, we predicted that at least two forms of PrRP are produced from this precursor protein. As shown in Fig. 3, the structure of the PrRP precursor protein can be divided into four parts: (1) the N-terminal signal peptide sequence, (2) the mature PrRP protein sequence, (3) an amidation and proteolytic cleavage motif (GRRR), and (4) the C-terminal portion, which is not included in the mature PrRP sequence. Of these four parts, the GRRR motif is completely identical, and the mature PrRP protein sequence is highly conserved between species. The signal peptide sequences are also relatively conserved, whereas the homology of the C-terminal portion that follows the GRRR motif is apparently lower than the homology of other parts of the precursor protein. These results accord very well with what is known regarding molecular evolution, that is, that functionally important amino acid sequences remain highly conserved during the evolution process. Interestingly, the position at which the intron splits the PrRP gene into two exons exactly matches the position of the N-terminus of PrRP20 (noted by an arrowhead in Fig. 3), suggesting that PrRP20 is not merely an artifact resulting from degradation of PrRP31 during the purification procedure.

PROCESSING OF THE PrRP PRECURSOR

As shown in Fig. 3, PrRP precursor proteins derived from human, bovine, rat, and mouse have typical N-terminal signal peptide sequences of 21 or 22 amino acid residues that are important for extracellular secretion. By analyses of

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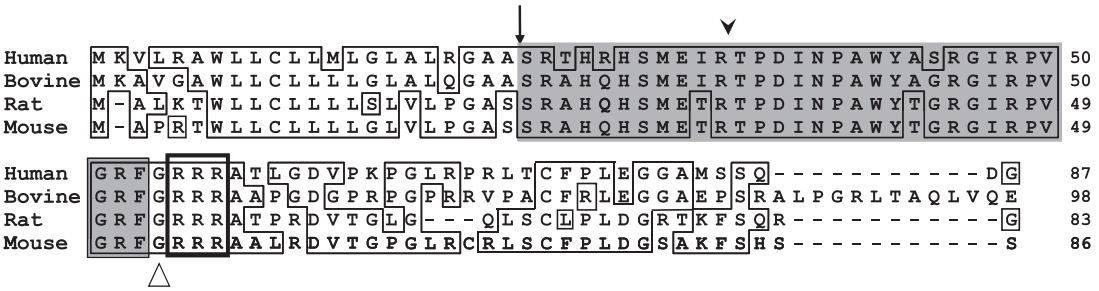

FIGURE 3 Amino acid sequences of human, bovine, rat and mouse precursor proteins containing PrRP. PrRP precursor protein sequences deduced from cDNAs are aligned, and mature PrRP sequences are indicated in gray. The arrow and arrowhead indicate the amino termini of PrRP31 and PrRP20, respectively. The open triangle indicates a glycine residue, which is presumed to react as an amide donor to produce amidated C-termini. Triplet basic amino acid residues constituting the typical motif of a proteolytic cleavage site are boxed with a bold line. Amino acid residues with identical sequences in at least two of the species are boxed.

the N-terminal sequences of active peptides purified from bovine tissue extracts, it has been shown that the N-terminus of one of the mature bovine peptides, PrRP31, starts from S^{23} just after the signal sequence, while that of the other peptide, PrRP20, starts from T^{34}. Because the precursor protein sequences are highly conserved in all species examined, it is expected that mature PrRPs will be produced from these precursor proteins in a similar manner in all species. The process by which mature PrRP is generated from the precursor protein is speculated to be consistent with that of other RFamide peptides. A possible scenario for the production of mature PrRP is illustrated in Fig. 4. First, the signal peptide is removed from the PrRP precursor protein, followed by cleavage of the basic amino acid repeat of R^{55}, R^{56}, and R^{57}. After these basic amino acids have been removed by carboxypeptidase, F^{53} at the carboxy terminus of the mature PrRP is amidated by peptidyl α-amidating monooxygenase (see the chapter on this enzyme in Peptide Biosynthesis/Processing section of this book) through a reaction with G^{54} as an amide donor. Because the amount of PrRP31 obtained from bovine tissue extracts appeared to be greater than that of PrRP20, PrRP31 would be predominantly produced from the precursor protein,[8] though it is unknown at which point of the process to produce mature PrRP, PrRP31 switches to PrRP20.

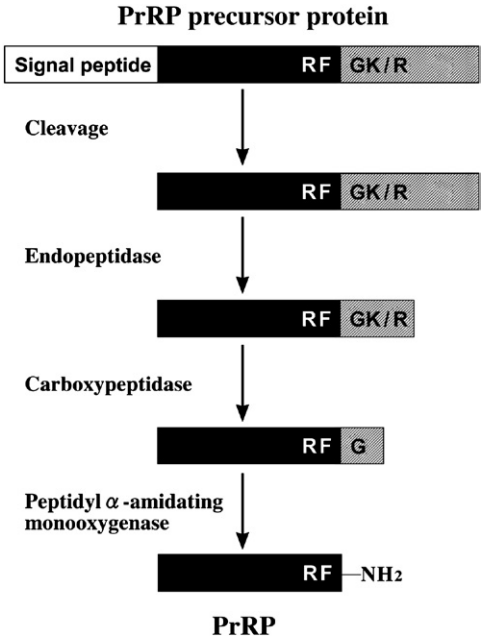

FIGURE 4 Schematic representation of the processing of the PrPR precursor protein leading to mature PrPR.

DISTRIBUTION OF THE mRNA/PEPTIDE

The distribution of immunoreactive PrRP in the brain of rats is shown in Fig. 5. The highest expression of PrRP mRNA in both rats and humans has been detected in the medulla oblongata, whereas moderate levels of mRNA are expressed in the hypothalamus,[26] where the highest concentration of bioactive PrRP has been detected in rats.[20,22] In humans, high levels of immunoreactive PrRP have been reported in both the hypothalamus and the medulla oblongata.[31] In situ hybridization analyses indicate that PrRP mRNA is localized in the neurons of the ventromedial and dorsomedial nuclei of the hypothalamus (VMH and DMH). The strongest PrRP mRNA signals are found in the nucleus of the tractus solitarius (NTS), whereas moderate levels are seen in the lateral reticular nuclei (LRN) of the medulla oblongata.[9,10,28] In rat embryos, PrRP mRNA is seen in the NTS at E18, and in the LRN at E20. On the other hand, PrRP mRNA is first detected by in situ hybridization in the hypothalamus on postnatal day 13. The distribution of PrRP-positive fibers differs considerably between neonatal and adult rat brains. In addition, gonadal steroid hormones regulate PrRP gene expression in the medulla oblongata of rats.[11] Although a high level of PrRP immunoreactivity is detected in the hypothalamus under normal conditions, PrRP-positive nerve fibers are barely detectable in the external layer of the median eminence, which is known

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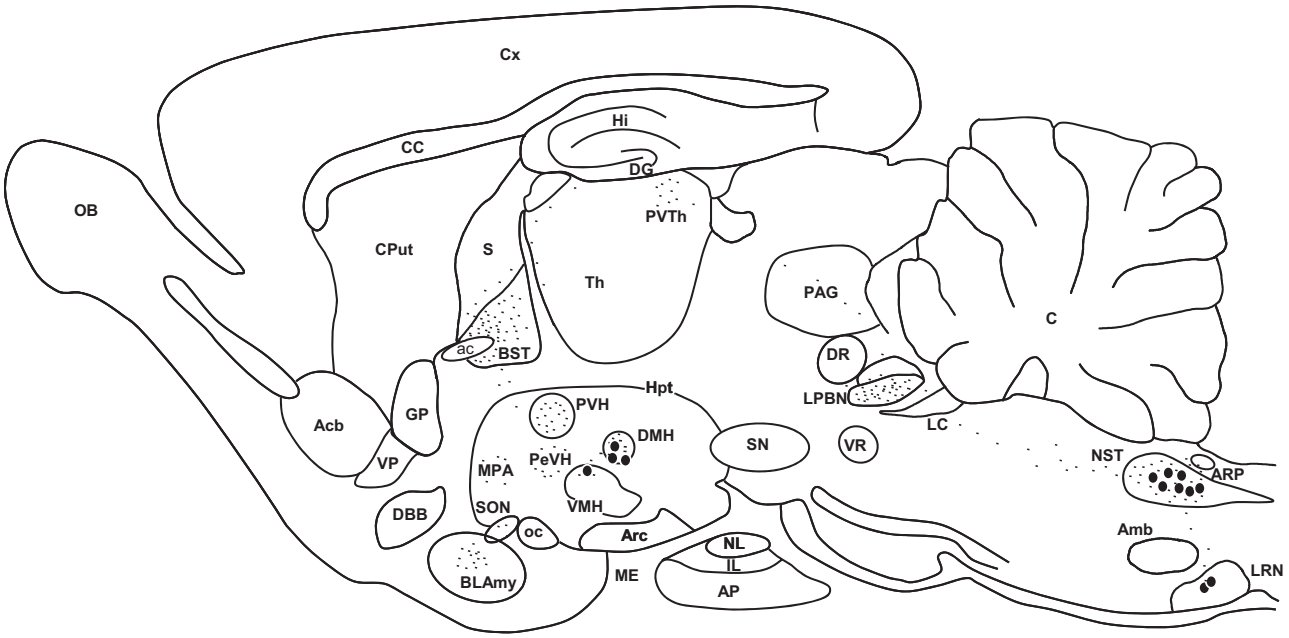

FIGURE 5 Distribution of PrRP-immunoreactive elements in rat brain. Black circles and short lines represent PrRP-immunoreactive cell bodies and fibers, respectively. Acb, nucleus accumbens; Amb, nucleus ambiguus; AP, anterior pituitary; Arc, arcuate nucleus of hypothalamus; ARP, area postrema; BLAmy, basolateral amygdala; BST, bed nucleus of stria terminalis; C, cerebellum; CC, corpus callosum; CPut, caudate putamen; Cx, cerebral cortex; DBB, diagonal band of Broca; DG, dentate gyrus; DMH, dorsomedial nucleus of hypothalamus; DR, dorsal raphe nucleus; GP, globus pallidus; Hi, hippocampus; Hpt, hypothalamus; IL, intermediate lobe of pituitary; LC, locus coeruleus; LRN, lateral reticular nucleus; ME, median eminence; MPA, medial preoptic area; NL, neural lobe of pituitary; NST, nucleus of solitary tract; OB, olfactory bulb; PAG, periaqueductal gray; LPBN, lateral parabrachial nucleus; PeVH, periventricular nucleus of hypothalamus; PVH, paraventricular nucleus of hypothalamus; PVTh, paraventricular nucleus of thalamus; S, septum; SN, substantia nigra; SON, supraoptic nucleus; Th, thalamus; VMH, ventromedial nucleus of hypothalamus; VP, ventral pallidum; VR, ventral raphe nuclei.

to be the release site of the classical hypothalamic hypophysiotropic neurohormones.[9,20] Thus, the pathway of the action of PrRPs on the pituitary, where its receptors are abundantly expressed, remains unclear. Extensive colocalization of PrRP and tyrosine hydroxylase immunoreactive neurons in the A1/A2 areas in the NTS and LRN suggests that PrRP is produced in noradrenergic neurons.[1,34] In peripheral tissues, immunoreactive PrRP has been detected in the adrenal gland.[5,21]

PrRP RECEPTORS AND SIGNALING CASCADES

As mentioned above, PrRP was discovered in the course of a search for specific ligands of the orphan GPCR GPR10 by use of an AA-releasing assay and CHO cells that expressed GPR10.[8] Interaction of a GPCR with an agonistic ligand is considered to induce the following signaling cascade in cells. (1) The binding of agonistic ligand to the GPCR integrated into the cell membrane induces conformational changes in the GPCR. (2) These changes induce activation of receptor-coupling with G proteins, either with Gαq and/or Gαi/o 003F subunits, in the case of GPR10. (3) Activation of G proteins causes subsequent activation of enzymes such as phospholipase A_2 and/or phospholipase C. (4) These activated enzymes catalyze the hydrolysis of membrane phospholipids resulting in the production of AA and/or other metabolites. (5) These events simultaneously cause both Ca^{2+} influx and a decrease in the cAMP content of cells. Based on such assumptions, we succeeded in identifying PrRP as a GPR10 ligand that could induce evident AA release in CHO cells expressing GPR10. Such an approach for the identification of a receptor ligand is referred to as reverse pharmacology. We later confirmed that PrRP induces Ca^{2+} influx and a decrease in cAMP production in CHO cells expressing GPR10. In addition, PrRP stimulates the activation of extracellular signal–regulated protein kinase (ERK), and this PrRP-induced ERK activation was completely blocked by pertussis toxin, which inactivates Gαi/o,[12] indicating that GPR10 couples to both Gαq and Gαi/o. These results indicate that intracellular signaling cascades induced by the interaction of GPR10 and PrRP are almost identical to those induced by other GPCRs. The K_d value of the binding of [^{125}I]-labeled PrRP to GPR10 expressed in CHO cells has been determined to be 26 nM. The binding of PrRP20 and PrRP31 to GPR10 is

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of a similar high affinity. However, both the AA-releasing and receptor-binding activities of PrRP are dramatically decreased in the absence of C-terminal amidation, indicating that the amidated C-terminus is essential for the binding of PrRP and GPR10. It has been reported that the PrRP receptor contains a sequence that closely resembles a PSD-95/Discs-large/ZO-1 (PDZ) domain binding motif in its C-terminal tail and that the PrRP receptor does in fact bind with PDZ domain proteins that are known to interact with α-amino-3-hydroxy-5-methlisoxazole-4-propionic acid receptors.[17] The promoter region of the PrRP receptor gene contains several putative binding sites for transcription factors, including pituitary-specific transcription factor (Pit-1), which is involved in pituitary-specific gene expression, although the 5′-flanking region of the promoter region reportedly shows promoter activity not only in pituitary-derived cells but also in nonpituitary cells.[12,13]

NPFF-2 was originally identified as one of the receptors (NPFF-1 and NFFF-2) for NPFF and NPAF. It has been reported that human PrRP31 has a significantly higher affinity for human NPFF-2 (Ki = 19 nM) than NPFF (Ki = 5.2 nM).[3] Because NPFF-2 can bind to a wide spectrum of RFamide peptides including FMRFamide, its ligand specificity seems to be ambiguous. By contrast, it has also been reported that NPFF does not display any noticeable interaction with GPR10, whereas human PrRP binds to GPR10 with high affinity (Ki = 0.68 nM), indicating that the GPR10 receptor is highly specific for PrRP.[6]

The tissue distribution of the PrRP receptor differs from that of PrRP (Table 1). The unknown hypothalamic receptor-1 (UHR-1), a rat ortholog of human GPR10, was originally isolated from the hypothalamic suprachiasmatic nucleus. UHR-1/GPR10 mRNA is widely distributed throughout the brain, and its highest level of expression is in the anterior lobe of the pituitary.[4] High expression of GPR10 mRNA has also been detected in the normal pituitary and in pituitary adenomas.[35] In situ hybridization analyses have indicated that the highest expression of GPR10 mRNA is in the reticular nucleus of the thalamus and that prominent signals are also detected in the paraventricular nucleus of the hypothalamus (PVH), periventricular nucleus (PeVH), DMH, NTS, and area postrema (AP).[9,18,28] Binding of PrRP to the reticular nucleus of the thalamus has been reported. Although a low level of PrRP binding is also observed in the PeVH, no specific labeling has been detected in any other brain region.[28] In peripheral tissues, GPR10 mRNA has been detected in the adrenal gland, stomach, and femur by reverse transcription-polymerase chain reaction (RT-PCR) analyses.[4] In situ hybridization has revealed that GPR10 mRNA is expressed in the adrenal medulla in rats.[28]

The other PrRP receptor, NPFF-2, is strongly expressed in the central nervous system (CNS), in areas such as the spinal cord, medulla oblongata, and hypothalamus (Table 1).[6] The distribution of NPFF-2 is thus apparently distinct from that of GPR10 and PrRP.

BIOLOGICAL ACTIONS OF PrRP WITHIN THE BRAIN (AND PITUITARY)

PRL-Releasing Activity

We were the first to report that PrRP dose dependently enhances the secretion of PRL from a rat pituitary adenoma-derived cell line, RC-4B/C, and from rat anterior pituitary cells. Since then, accumulated evidence has supported the PRL-releasing activity of PrRP. However, the physiological roles of PrRP are highly controversial. Typical contentious points are as follows: (1) PrRP seems to be less potent in releasing PRL than thyrotropin-releasing hormone (TRH), although the *in vivo* effect of PrRP is strongly affected by the estrous cycle and by gender.[24] (2) PrRP-positive fibers are not apparent in the external layer of the median eminence, where classical hypothalamic hormones acting on the pituitary are detected, under normal conditions. (3) An increase in PrRP mRNA expression in the brain is not detected during lactation in some animals.[16] These results indicate that PrRP clearly differs from known hypothalamic releasing factors that act on the pituitary. In teleost fish, fibers that are immunoreactive with C-RFa (an ortholog of mammalian PrRP) project into the pituitary and terminate close to PRL-producing cells in the rostral pars distalis. Moreover, intraperitoneal injection of C-RFa significantly increases the circulating levels of PRL in fish. C-RFa is therefore considered to be a physiologically relevant candidate stimulator of PRL release in teleost fish. However, recent reports suggest that PrRP plays different roles in PRL release in different species.[14] Therefore, the role of PrRP in PRL secretion remains controversial, even from an evolutionary viewpoint.

Mediation of Stress Responses

PrRP has been proven to affect the hypothalamic–pituitary–adrenal axis by potently stimulating corticotropin-releasing hormone (CRH) neurons in the PVH.[18,21,23] The release of corticosterone and adrenocorticotropic hormone (ACTH) after central administration of PrRP is evoked not only by the modulation of CRH release but also by the release of other ACTH secretagogues such as oxytocin or vasopressin.[23] The highest expression of PrRP mRNA has been detected in the medulla oblongata, while immunoreactive PrRP has been detected in the A1/A2 noradrenergic neurons, which mediate stress signals in the CNS.[1] Water immersion-restraint stress increases c-Fos protein accumulation specifically in PrRP/TH-double immunoreactive cells in A1/A2 neurons.[21] Exposure to acute

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TABLE 1 Summarized Distribution of PrRP and its Receptor mRNA in Rat CNS

Name	PrRP	GPR10	NPFF-2
Piriform cortex			
Cortex–amygdala transition zone			
Ventral pallidum			
Accumbens nucleus		+	
Lateral septal nucleus			
Bed nucleus stria terminalis		+	
Interstitial nucleus of posterior limb of anterior commisure			
Cotex, layer 2		+	
Primary motor cortex			
Secondary motor cortex			
Cingulate cortex			
Amygdaloid nucleus		+	
Preoptic area		++	
Medial preoptic nucleus		+	
Suprachiasmatic nucleus			
Anterior hypothalamic area			
Magnocellular preoptic nucleus			
Paraventricular hypothalamic nucleus		++	
Periventricular hypothalamic nucleus		+++	
Dorsomedial hypothalamic nucleus	++	+++	
Ventromedial hypothalamic nucleus	+	+	
Ventrolateral hypothalamic nucleus		+	
Retrochiasmatic area			
Arcuate nucleus			
Posteror hypothalamic area			
Lateral hypothalamic area			++
Medial mammillary nucleus			+
Supramammillary nucleus			+
Tuberomammillary nucleus			
Subfornical organ			
Reticular thalamic nucleus		+++	++
Laterodorsal thalamic nucleus			++
Anterodorsal thalamic nucleus			

TABLE 1 Summarized Distribution of PrRP and its Receptor mRNA in Rat CNS—*Cont'd*

Name	PrRP	GPR10	NPFF-2
Paraventricular thalamic nucleus			
Centrolateral thalamic nucleus			++
Parafascicular thalamic nucleus			+++
Central medial thalamic nucleus			
Paracentral thalamic nucleus			
Reuniens thalamic nucleus			
Pretectal nucleus			+
Zona incerta			
Intergeniculate leaf			
Habenular nucleus			
Substantia nigra, compacta part			
Superior colliculus			
Marginal zone medial geniculate			
Medial geniculate nucleus			
Nucleus of brachium inferior colliculus			
External cortex of the inferior colliculus			
Periaqueductal gray			
Oculomotor nucleus			
Dorsal raphe nucleus		+	
Median raphe nucleus			
Caudal linear nucleus of raphe			
Interpeduncular nucleus			
Dosal tegmental nucleus			+
Laterodorsal tegmental nucleus			
Parapyramidal nucleus			
Lateral parabrachial nucleus		+	+
Ventral pontine reticular nucleus			++
Locus coeruleus		++	
Central gray			
Area Postrema		+++	
Nucleus of solitary tract	+++	+++	++
Ventrolateral reticular formation	+++	+	
Spinal cord			++

+++ Highest density, ++ moderate density, and + low density by in situ hybridization.

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foot shock also activates substantial numbers of medullary neurons that express PrRP mRNA.[21] In other studies, some PrRP-immunoreactive neurons showed synaptic contact with oxytocin-positive cell bodies in the hypothalamus. Indeed, intracerebroventricular (i.c.v.) administration of PrRP stimulates oxytocin release from the rat pituitary. These results suggest that PrRP-positive noradrenergic neurons that project into the hypothalamus mediate oxytocin release from the pituitary in response to stressful stimuli and demonstrate that PrRP plays an important role in regulating stress responses.

Suppression of Food Intake

PrRP and GPR10 have been detected in the DMH, which is closely involved in the regulation of feeding and energy homeostasis. It was found that PrRP mRNA expression is reduced in both the DMH and NTS during lactation and fasting in rats.[16] I.c.v. administration of PrRP causes a reduction in food intake in free-moving and fasted male rats without affecting water intake. This effect of PrRP is observed when PrRP is injected into the DMH, but not when it is injected into the PVH, indicating that the DMH is important for the inhibitory effect of PrRP on food intake.[29] Analyses of PrRP gene- and PrRP receptor (GPR10) gene-knockout (KO) mice have provided more direct evidence that the PrRP system is involved in the regulation of food intake or energy balance. PrRP KO mice show adult-onset hyperphagia and obesity that are accompanied by impaired glucose tolerance and insulin resistance.[32] Obesity induced by the disruption of PrRP or GPR10 gene seems to be consistent between PrRP and GPR10 KO mice, although the mechanisms responsible for this phenotype are not necessarily identical.[7] In the experiments using PrRP KO mice, there are no significant differences neither in body temperature changes during exposure to a cold environment nor in the cold exposure-induced UCP1 mRNA changes between PrRP-deficient and wild-type mice. Thus, the fact that PrRP KO mice gain more weight than wild-type mice do seems to be a result of their increased food intake rather than reduced energy expenditure. PrRP-positive neurons in the rat brain reportedly possess leptin receptors, and i.c.v. administration of PrRP together with leptin causes additive reductions in nocturnal food intake. These results indicate that PrRP plays important roles in the regulation of food intake.

What is the Physiological Role of PrRP?

As explained above, PrRP has been demonstrated to function in PRL release, as a stress mediator, and in the regulation of food intake. In addition, other functions of PrRP have been reported;[6] (1) induction of the secretion of not only PRL but also of other hormones including growth hormone, somatostatin, luteinizing hormone, and gonadotropin-releasing hormone; (2) induction of an increase in blood pressure and regulation of the cardiovascular system; (3) effects on sleep; and (4) pain control.[15,18] However, although various functions have been reported for PrRP since its discovery, physiological functions exclusive to PrRP have not yet been established. What does this finding mean? We would like to propose the following hypothesis as to how the crucial physiological role of PrRP can be elucidated. PrRP is a member of the RFamide peptide family. To date, seven RFamide peptides, encoded by five distinct genes, have been reported in mammals (Fig. 1). Because RFamide peptides are widely found in both invertebrates and vertebrates, it is speculated that these peptides have evolved through gene duplication. Thus, one or a few ancestral peptides with the same or similar functions have diversified during evolution into the present peptides, which have different functions. The functional differentiation of RFamide peptides continues, even at the present moment. From an evolutionary point of view, it is understandable that some RFamide peptides might have duplicated or redundant functions. Indeed, some peptides share the same receptors. For instance, recent studies indicate that NPFF-2 can bind not only NPFF but also PrRP, RFRPs, kisspeptin, and QRFP /26RFa (Table 2), suggesting that RFamide peptides and their receptors function as a network and that they possibly compensate for each other if one component of the network happens to fail. Indeed, naturally occurring GPR10-deficient rats are not impaired in food intake suppression induced by PrRP administration.[2] In addition, it is shown that PrRP induces high blood pressure and increases the heart rate via NPFF-2 *in vivo* in rats. However, some RFamide peptide/receptor pairs may have specific functions. In the case of kisspeptin and its receptor (GPR54), loss-of-function mutations of the GPR54 gene are known to cause the absence of puberty onset and hypogonadotrophic hypogonadism in humans.

TABLE 2 Cross reaction of Mammalian RFaminde Peptides with Receptors

RFamide peptide[a]	Receptors[a]
NPFF and NPAF[b]	NPFF-1/OT7T022, NPFF-2 /HLWAR77
PrRP	HGR3/GPR10, NPFF-2
RFRP-1 and RFRP-3[b]	NPFF-1, NPFF-2
Kisspeptin	GPR54, NPFF-2
QRFP/26RFa	AQ27/GPR103, NPFF-2

[a]*Representative nomenclature is used here according to the previous report listing RFamide peptides and their receptors.[6] Additional data are obtained from recent articles.[19]*
[b]*The two peptides are derived from a single gene. Alternative name is shown with a slash.*

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Similar phenotypes are produced in kisspeptin-KO and GPR54-KO mice. Kisspeptin and its receptor GPR54 are therefore believed to have differentiated so that they play a selective role in reproduction, which cannot be carried out by other RFamide peptides and their receptors. If this assumption that RFamide peptides and their receptors principally function as a network and complement each other to a certain extent is valid, then a single gene-targeting technique would not always be effective in delineating the physiological functions of the RFamide peptides/receptors. It is expected that analyses of multigene KO animals will be an important approach in the future for resolving the enigma concerning the functions of RFamide peptides and their receptors, especially in the case of PrRP.

PATHOPHYSIOLOGICAL IMPLICATIONS

PrRP receptor (GPR10 or its ortholog) mRNA has been detected in various tumor cell lines including pituitary adenomas, and PrRP stimulation induces the secretion of PRL or other factors from some tumor cells.[30,35] It is probable that the hormone secretion-stimulatory activity of PrRP influences the characteristics of tumors, although there is no direct evidence that PrRP and its receptor are closely related to the growth and phenotypes of tumor cells.

It could be speculated that the regulation of food intake by PrRP and its receptor is related to obesity and/or diabetes. A human genetic study has suggested that polymorphism in GPR10 is associated with high blood pressure, and it was demonstrated, in a strain of rats used as an animal model of diabetes, that a mutated GPR10 gene is responsible for hyperphagia, dyslipidemia, and obesity.[33] In addition, it was found that PrRP gene expression is downregulated in experimental diabetic rats, and that this phenomenon is reversed by insulin or leptin treatment.[25] These reports suggest that PrRP and its receptors are involved in obesity, diabetes, hypertension, and dyslipidemia

REFERENCES

1. Chen C, Dun SL, Dun NJ, Chang JK. Prolactin-releasing peptide-immunoreactivity in A1 and A2 noradrenergic neurons of the rat medulla. *Brain Res* 1999;**822**:276–9.
2. Ellacott KL, Donald EL, Clarkson P, Morten J, Masters D, Brennand J, et al. Characterization of a naturally-occurring polymorphism in the UHR-1 gene encoding the putative rat prolactin-releasing peptide receptor. *Peptides* 2005;**26**:675–81.
3. Engstrom M, Brandt A, Wurster S, Savola JM, Panula P. Prolactin-releasing peptide has high affinity and efficacy at neuropeptide FF2 receptors. *J Pharmacol Exp Ther* 2003;**305**:825–32.
4. Fujii R, Fukusumi S, Hosoya M, Kawamata Y, Habata Y, Hinuma S, et al. Tissue distribution of prolactin-releasing peptide (PrRP) and its receptor. *Regul Pept* 1999;**83**:1–10.
5. Fujiwara K, Matsumoto H, Yada T, Inoue K. Identification of the prolactin-releasing peptide-producing cell in the rat adrenal gland. *Regul Pept* 2005;**126**:97–102.
6. Fukusumi S, Fujii R, Hinuma S. Recent advances in mammalian RFamide peptides: the discovery and functional analyses of PrRP, RFRPs and QRFP. *Peptides* 2006;**27**:1073–86.
7. Gu W, Geddes BJ, Zhang C, Foley KP, Stricker-Krongrad A. The prolactin-releasing peptide receptor (GPR10) regulates body weight homeostasis in mice. *J Mol Neurosci* 2004;**22**:93–103.
8. Hinuma S, Habata Y, Fujii R, Kawamata Y, Hosoya M, Fukusumi S, et al. A prolactin-releasing peptide in the brain. *Nature* 1998;**393**:272–6.
9. Ibata Y, Iijima N, Kataoka Y, Kakihara K, Tanaka M, Hosoya M, et al. Morphological survey of prolactin-releasing peptide and its receptor with special reference to their functional roles in the brain. *Neurosci Res* 2000;**38**:223–30.
10. Iijima N, Kataoka Y, Kakihara K, Bamba H, Tamada Y, Hayashi S, et al. Cytochemical study of prolactin-releasing peptide (PrRP) in the rat brain. *Neuroreport* 1999;**10**:1713–6.
11. Kataoka Y, Iijima N, Yano T, Kakihara K, Hayashi S, Hinuma S, et al. Gonadal regulation of PrRP mRNA expression in the nucleus tractus solitarius and ventral and lateral reticular nuclei of the rat. *Brain Res Mol Brain Res* 2001;**87**:42–7.
12. Kimura A, Ohmichi M, Tasaka K, Kanda Y, Ikegami H, Hayakawa J, et al. Prolactin-releasing peptide activation of the prolactin promoter is differentially mediated by extracellular signal-regulated protein kinase and c-Jun N-terminal protein kinase. *J Biol Chem* 2000;**275**:3667–74.
13. Kishimoto M, Okimura Y, Hinuma S, Fukusumi S, Iguchi G, Fumoto M, et al. Cloning and characterization of the 5′-flanking region of the human prolactin-releasing peptide receptor gene. *Biochem Biophys Res Commun* 2000;**276**:411–6.
14. Kitagawa S, Abe N, Sutoh M, Kasuya E, Sugita S, Aoyama M, et al. Effect of intracerebroventricular injections of prolactin-releasing peptide on prolactin release and stress-related responses in steers. *Anim Sci J* 2011;**82**:314–9.
15. Laurent P, Becker JA, Valverde O, Ledent C, de Kerchove d'Exaerde A, et al. The prolactin-releasing peptide antagonizes the opioid system through its receptor GPR10. *Nat Neurosci* 2005;**8**:1735–41.
16. Lawrence CB, Celsi F, Brennand J, Luckman SM. Alternative role for prolactin-releasing peptide in the regulation of food intake. *Nat Neurosci* 2000;**3**:645–6.
17. Lin SH, Arai AC, Wang Z, Nothacker HP, Civelli O. The carboxyl terminus of the prolactin-releasing peptide receptor interacts with PDZ domain proteins involved in alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptor clustering. *Mol Pharmacol* 2001;**60**:916–23.
18. Lin SH, Leslie FM, Civelli O. Neurochemical properties of the prolactin releasing peptide (PrRP) receptor expressing neurons: evidence for a role of PrRP as a regulator of stress and nociception. *Brain Res* 2002;**952**:15–30.
19. Lyubimov Y, Engstrom M, Wurster S, Savola JM, Korpi ER, Panula P. Human kisspeptins activate neuropeptide FF2 receptor. *Neuroscience* 2010;**170**:117–22.
20. Maruyama M, Matsumoto H, Fujiwara K, Kitada C, Hinuma S, Onda H, et al. Immunocytochemical localization of prolactin-releasing peptide in the rat brain. *Endocrinology* 1999;**140**:2326–33.
21. Maruyama M, Matsumoto H, Fujiwara K, Noguchi J, Kitada C, Fujino M, et al. Prolactin-releasing peptide as a novel stress mediator in the central nervous system. *Endocrinology* 2001;**142**:2032–8.

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22. Matsumoto H, Murakami Y, Horikoshi Y, Noguchi J, Habata Y, Kitada C, et al. Distribution and characterization of immunoreactive prolactin-releasing peptide (PrRP) in rat tissue and plasma. *Biochem Biophys Res Commun* 1999;**257**:264–8.
23. Matsumoto H, Maruyama M, Noguchi J, Horikoshi Y, Fujiwara K, Kitada C, et al. Stimulation of corticotropin releasing hormone-mediated adrenocorticotropin secretion by central administration of prolactin-releasing peptide in rats. *Neurosci Lett* 2000;**285**:234–8.
24. Matsumoto H, Noguchi J, Horikoshi Y, Kawamata Y, Kitada C, Hinuma S, et al. Stimulation of prolactin release by prolactin-releasing peptide in rats. *Biochem Biophys Res Commun* 1999;**259**:321–4.
25. Mera T, Fujihara H, Saito J, Kawasaki M, Hashimoto H, Saito T, et al. Downregulation of prolactin-releasing peptide gene expression in the hypothalamus and brainstem of diabetic rats. *Peptides* 2007;**28**:1596–604.
26. Morales T, Hinuma S, Sawchenko PE. Prolactin-releasing peptide is expressed in afferents to the endocrine hypothalamus, but not in neurosecretory neurones. *J Neuroendocrinol* 2000;**12**:131–40.
27. Perry SJ, Hang EY, Cronk D, Bagust J, Sharma R, Walker RJ, et al. A human gene encoding morphine-modulating peptides related to NPFF and FMRFamide. *FEBS* 1997;**409**:426–30.
28. Roland BL, Sutton SW, Wilson SJ, Luo L, Pyati J, Huvar R, et al. Anatomical distribution of prolactin-releasing peptide and its receptor suggests additional functions in the central nervous system and periphery. *Endocrinology* 1999;**140**:5736–45.
29. Seal LJ, Small CJ, Dhillo WS, Stanley SA, Abbott CR, Ghatei MA, et al. PRL-releasing peptide inhibits food intake in male rats via the dorsomedial hypothalamic nucleus and not the paraventricular hypothalamic nucleus. *Endocrinology* 2001;**142**:4236–43.
30. Takahashi K, Totsune K, Murakami O, Sone M, Noshiro T, Hayashi Y, et al. Expression of prolactin-releasing peptide and its receptor in the human adrenal glands and tumor tissues of adrenocortical tumors, pheochromocytomas and neuroblastomas. *Peptides* 2002;**23**:1135–40.
31. Takahashi K, Yoshinoya A, Arihara Z, Murakami O, Totsune K, Sone M, et al. Regional distribution of immunoreactive prolactin-releasing peptide in the human brain. *Peptides* 2000;**21**:1551–5.
32. Takayanagi Y, Matsumoto H, Nakata M, Mera T, Fukusumi S, Hinuma S, et al. Endogenous prolactin-releasing peptide regulates food intake in rodents. *J Clin Invest* 2008;**118**:4014–24. Erratum in: *J Clin Invest* 2009;**119**:422.
33. Watanabe TK, Suzuki M, Yamasaki Y, Okuno S, Hishigaki H, Ono T, et al. Mutated G-protein-coupled receptor GPR10 is responsible for the hyperphagia/dyslipidaemia/obesity locus of Dmo1 in the OLETF rat. *Clin Exp Pharmacol Physiol* 2005;**32**:355–66.
34. Yano T, Iijima N, Kataoka Y, Hinuma S, Tanaka M, Ibata Y. Developmental expression of prolactin releasing peptide in the rat brain: localization of messenger ribonucleic acid and immunoreactive neurons. *Brain Res Dev Brain Res* 2001;**128**:101–11.
35. Zhang X, Danila DC, Katai M, Swearingen B, Klibanski A. Expression of prolactin-releasing peptide and its receptor messenger ribonucleic acid in normal human pituitary and pituitary adenomas. *J Clin Endocrinol Metab* 1999;**84**:4652–5.

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Chapter 121

Relaxins

Andrew L. Gundlach, Craig M. Smith, Philip J. Ryan, Anna Blasiak, Francisco E. Olucha-Bordonau and Sherie Ma

ABSTRACT

The relaxin family of peptides comprises relaxin-1/2, relaxin-3, and insulin-like peptides (INSL) 3–6. Three of these peptides and their native receptors are present in the brain. Relaxin-1 is expressed by neurons within the anterior olfactory nucleus, piriform/neocortex, hypothalamus, and hippocampus of rat brain, whereas relaxin-3 is highly expressed by discrete populations of neurons in the brainstem of rodents, nonhuman primate, and other species; relaxin-3-positive axons and fibers/terminals are widely distributed throughout the forebrain. By contrast, INSL3 is detected in relatively low levels and only in neurons in some midbrain/brainstem areas. Two types of G protein-coupled receptors—RXFP1 and RXFP2, and RXFP3 and RXFP4—preferentially interact with individual relaxin-family peptides and, apart from RXFP4, are broadly but differentially expressed in the forebrain. Experimental studies implicate relaxin-3/RXFP3 signaling in physiological roles including arousal, stress, and cognition. By contrast, functions of relaxin/RXFP1 and INSL3/RXFP2 signaling within the brain are yet to be clearly identified.

DISCOVERY

The relaxin peptide family consists of seven peptides: relaxin-1/2, relaxin-3, and insulin-like peptide 3-6. Relaxin is a 5 kDa peptide hormone, which was first isolated in 1926 and named for its ability to relax the pubic symphysis. Relaxin was also one of the first genes cloned and contains a highly conserved, characteristic sequence 'RXXXRXX(I/V)' within its B-chain, which was subsequently discovered to be essential for binding to different cognate relaxin receptors. It was this sequence that was used many years later to search the Human Genome and lead to the discovery of the relaxin-3 gene.[2] Similar genomic strategies were used to identify other members of the relaxin peptide family—INSL4-6—all of which have been shown to have distinct biological actions (see Ref. 17 for a review). Insulin-like peptide 3 (INSL3) (also known as relaxin-like factor) is a 57 aa peptide hormone that was discovered in the early 1990s. The *INSL3* gene was subsequently cloned from human, pig, mouse, and rat with high expression in the testis. INSL3 was initially recognized as a powerful regulator of testicular development and function, before more recent studies identified roles in other tissues including kidney and brain (see Ref. 17 for a review).

After years of searching for a presumed tyrosine kinase receptor for relaxin (based on its insulin-like structure), recently four formerly 'orphan' G protein-coupled receptors were identified and characterized as cognate receptors for the relaxin-family peptides: LGR7 (now classified as RXFP1) for relaxin; LGR8 (RXFP2) for INSL3, GPCR135 (RXFP3) for relaxin-3, and GPCR142 (RXFP4) for INSL5 (see Ref. 3 for a review). The leucine rich repeat containing GPCRs, LGR7, and LGR8 were initially identified as receptors for relaxin by Hsu and others;[10] and GPCR135 and GPCR142 were identified by Liu et al. as receptors for relaxin-3.[12] Subsequent intensive characterization using recombinant receptors, chimeric peptides, and/or information from natural and experimentally generated gene deletion mouse models revealed that LGR8 is the native receptor for INSL3 and GPCR142 is the preferred receptor for INSL5 (see Ref. 3 for a review). The relative affinity and cross reactivity of the different relaxin-family peptides (human, rat, and mouse) for the different receptors has been systematically analyzed, and in recent years agonists with far better selectivity for RXFP3 over RXFP1/2 than native relaxin-3[13] and high-affinity RXFP3 antagonists[8,11] have been developed, allowing studies of the respective biological actions of the different ligand-receptor combinations.

STRUCTURE OF THE mRNA/GENE

Relaxin-3

The structure/sequence of the preprorelaxin-3 gene is highly conserved among vertebrates (human, chimpanzee, dog, mouse, and rat). The human relaxin-3 gene (*RLN3*) is located on chromosome 19p13.2, and the rat and mouse genes (*Rln3*) are located on chromosomes 19q11 and 8, respectively (http://www.ncbi.nlm.nih.gov/gene/?term=relaxin 3). The relaxin-3 gene is evolutionarily conserved with 73% homology between

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00121-4
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RLN3 and *Rln3* in the C-peptide domain and 93% homology in the A- and B-domains.[2] There is little homology, however, in the C-peptide domain (<20%) of relaxin-3 and other insulin- and relaxin-family members in human and mouse, which may reflect differences in C-peptide function(s). Phylogenetic analysis indicates that relaxin-3 (also known as insulin-like peptide 7, INSL7) emerged before the divergence of fish and all relaxin-family peptides evolved from an ancestral peptide that was relaxin-3-like. The relaxin-3 gene is under strong purifying selection, indicating a highly conserved and important function.[35]

Relaxin and INSL3

The preprorelaxin gene (*RLN1/2*) is located on human chromosome 9p24.1, and the rat and mouse genes (*Rln1*) are located on chromosomes 1q52 and 19 21.0, respectively (http://www.ncbi.nlm.nih.gov/gene/?term=relaxin). *RLN1* and *RLN2* share high sequence homology, whereas relaxin sequences in different mammalian species vary by up to 42–60%, despite a high similarity in biological activity. The peptide encoded by *RLN2*, relaxin-2, is the predominant stored and circulating form of relaxin in humans and is the functional equivalent of relaxin-1 (or 'relaxin') in rodents, whereas the peptide encoded by *RLN1* is only expressed in humans and great apes in the decidua, placenta, and prostate. *RLN1* and *RLN2* were derived through recent duplication events and are evolutionarily divergent to *RLN3*. The *INSL3*/(*Insl3*) gene is located on human chromosome 19p13.2-p12 and the equivalent rat and mouse genes are located on chromosome 16p14, and chromosome 8 33.0, respectively (http://www.ncbi.nlm.nih.gov/gene/?term=INSL3).

PROCESSING OF THE PEPTIDE PRECURSOR

Relaxin-3

The precursor peptide, preprorelaxin-3, is approximately 23kDa and is organized in a signal sequence-B-C-A domain configuration. The mature peptide is produced by proteolytic cleavage of the C-chain and formation of three disulfide bonds between six highly conserved cysteine residues present on the A- and B-chains (24 and 27 residues, respectively). Solution NMR structure of human relaxin-3 reveals an arrangement in which the A-chain forms two helical segments that are separated by an extended region and lie antiparallel to each other in a U-shape. The B-chain adopts a third helix that lies across the face of the U enclosing a hydrophobic core in an arrangement stabilized by the disulfide array.[2]

Relaxin and INSL3

The precursor peptides, preprorelaxin-1/2, and prepro-INSL3 are processed in a similar way to relaxin-3. There is very little homology between mouse *Rln1* and *Rln3*, other than the key core binding elements required of relaxins in the B-chain, and key structural elements in the A-chain.

DISTRIBUTION OF THE mRNA/PEPTIDE

Relaxin-3

Relaxin-3 mRNA and peptide have been mapped in several species, including mouse, rat, and nonhuman primate. Relaxin-3 mRNA expression is restricted to a relatively small number of neurons in the brainstem, but their extensive projection patterns results in a widespread presence of relaxin-3 in nerve fibers/terminals throughout the CNS, with similar distributions observed amongst all species studied (see Ref. 28 for a review). Because the rat and mouse are the major 'experimental' species, their relaxin-3 expression pattern is described here. A strong correlation exists between areas expressing relaxin-3 mRNA (assessed by in situ hybridization) and areas containing relaxin-3 peptide (assessed by immunohistochemistry) in the two species.[16,27,33] Furthermore, the site of relaxin-3 expression seems to be evolutionarily conserved, as there are analogous populations of neurons in zebrafish (see Ref. 28).

In rodents, relaxin-3 mRNA- and peptide-positive neuronal *cell bodies* are present: (i) in the nucleus incertus pars compacta and dissipata (or nucleus O) of the medial pontine central gray; (ii) in the adjacent pontine raphe nucleus; (iii) scattered along the entire rostrocaudal extent of the ventromedial regions of the periaqueductal gray (PAG), and; (iv) in a region dorsal to the substantia nigra (Fig. 1).[16,27]

Relaxin-3 positive *fibers* are observed in areas immediately adjacent to relaxin-3 soma and in a large number of forebrain and some brainstem areas. Very dense relaxin-3 fiber networks are present in various nodes of the septo-hippocampal pathway, including the median raphe, interpeduncular and supramammillary nuclei, and in the medial septum/nucleus of the diagonal band and hippocampus (ventral>dorsal).[16,27,33] Relaxin-3 fibers are also present in the dorsal raphe nucleus, PAG, tegmentum and tectal areas, in several areas of the hypothalamus, including preoptic and periventricular nuclei and 'lateral hypothalamic area,' and in the extended amygdala (medial/central amygdala, bed nucleus of stria terminalis, and lateral septum) and the amygdalohippocampal area. Medium densities of relaxin-3 fibers are present in the anterior/posterior cingulate and retrosplenial cortices, and midline intralaminar thalamic nuclei, while descending fibers are present in the inferior olive and nucleus of solitary tract.[16,27]

Relaxin-3 neurons are GABAergic (as reflected by the expression of glutamic acid decarboxylase). Based on earlier anatomical studies and data in the Allen Brain Atlas <http://www.brain-map.org/>, relaxin-3 probably coexists with other neuropeptides in nucleus incertus (NI) neurons and

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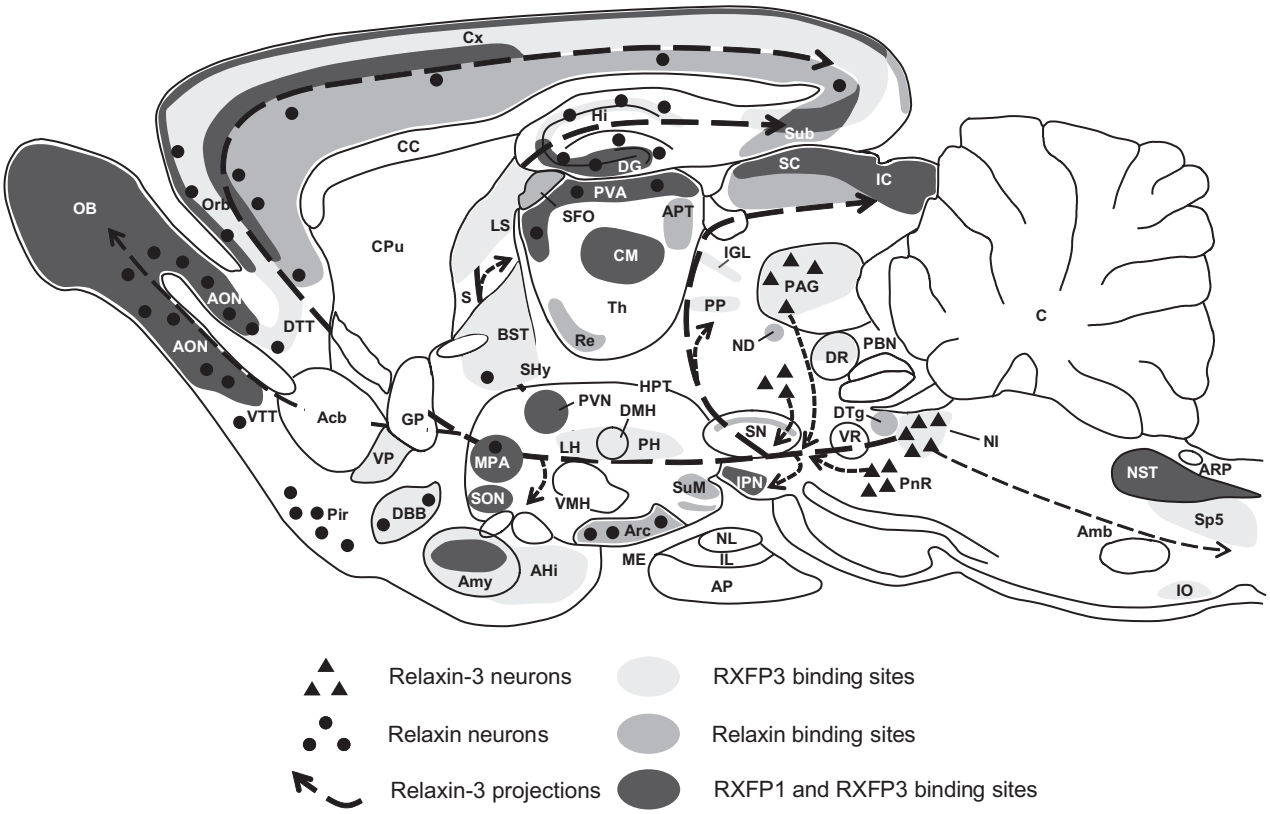

FIGURE 1 Schematic representation of the contrasting distribution of relaxin-3 and relaxin neurons and their receptor distributions in rat brain. In a stylized parasagittal brain section, neurons that express mRNA and immunoreactivity for relaxin-3 (black triangles) and relaxin (black dots) are illustrated, along with the approximate localization of the main relaxin-3 projections (black arrows). The relative distribution of RXFP1 and RXFP3 binding sites and areas in which their regional, but not precise cellular, presence overlaps are also illustrated (different gray shadings).

presumably in the other identified populations. Although a comprehensive and systematic histochemical study has not been conducted, candidates for coexpression with relaxin-3 in NI include cholecystokinin, neurotensin, and neuromedin-B, and a range of other putative markers have been identified, including different receptors, calcium-binding proteins, and transmitter-related enzymes (see Ref. 24 for a review).

Relaxin and INSL3

Relaxin and INSL3 also have a quite restricted expression profile in rat and/or mouse brain. Neurons expressing high levels of relaxin mRNA (as defined as readily detectable by in situ hybridization) are present in the hypothalamic arcuate nucleus and the anterior olfactory nucleus and piriform (or primary olfactory) cortex of rat brain. Relaxin mRNA is also detectable in tenia tecta, orbital cortex, and more generally throughout the cerebral cortex, and in dentate gyrus and CA1-3 fields of hippocampus (Fig. 1) (see Ref. 17 for a review). In contrast to the presence of relaxin-3 in nerve fibers throughout the brain, relaxin immunoreactivity, although detectable in mRNA-expressing neurons and in some fibers within the median eminence, is not observed in fiber networks associated with RXFP1-rich areas and projection target areas for the arcuate nucleus or piriform cortex.[14] This suggests a probable difference in mechanism of action of the relaxin 'peptide hormone,' compared with the 'neuropeptide' relaxin-3, that requires investigation.[17]

Similarly, using oligonucleotide-based in situ hybridization it was not possible to detect neurons expressing INSL3 mRNA in the brain of rats.[25] However, in more recent studies, we have used a characterized INSL3 antiserum to detect INSL3-positive neurons in the midbrain of mice. In a similar fashion to relaxin, INSL3 immunoreactivity is visible within the cytoplasm of the soma, but is not reliably observed in nerve fibers in adjacent or distant areas that contain high levels of RXFP2.[25]

RECEPTORS AND SIGNALING CASCADES

Extensive research using *in vitro* cell-based systems, recombinant and native receptors, and second messenger or associated reporter assays has shown that the different relaxin-family peptide receptors can couple to a variety of signal transduction pathways (see Ref. 7 for a review); however, technical challenges associated with targeting

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these low abundance receptors in complex ex vivo tissue or in intact animals has meant that confirming these findings in endogenous neural systems has proven difficult. INSL5-RXFP4 interactions are more relevant to peptide actions in the gastrointestinal tract, as both INSL5 and RXFP4 are highly expressed in the large intestine. [RXFP4 is a pseudogene in the rat and there is a lack of convincing evidence that INSL5 and RXFP4 are expressed in mouse brain].[31] Nonetheless, independent studies have consistently shown that after activation by relaxin, which involves both the large ectodomain and the transmembrane loops, RXFP1 acts via excitatory G_s-proteins to activate adenylyl cyclase (AC) and cellular cAMP levels and subsequently protein kinases A and C, phosphatidylinositol 3-kinase, and extracellular signaling regulated kinase (ERK1/2), as well as nitric oxide signaling. These events are complex and ligand concentration dependent and are affected by the nature of the intracellular networks available.[7] RXFP2 can couple to G_s-protein and stimulate cellular cAMP levels and has also been reported to couple to inhibitory $G_{i/o}$-proteins and inhibit AC/cAMP in cell lines and native tissues, suggesting a possible influence of the G-protein repertoire of the cell on coupling. RXFP3 has consistently been shown to interact with inhibitory $G_{i/o}$-proteins and reduce cellular cAMP levels activated by forskolin, and relaxin-3 activation of RXFP3 also activates the ERK1/2 pathway. ERK1/2 phosphorylation requires the activation/recruitment of $G_{i/o}$ proteins through receptor internalization and/or compartmentalization into lipid-rich environments.[7] By contrast, Callander et al.[6] reported that RXFP1 does not internalize via conventional β-arrestin-coupled mechanisms, which is consistent with a long, sustained period of activation by relaxin of rat subfornical organ neurons recorded in a brain slice preparation (see Ref. 17 for details). Long-lasting receptor activation/signaling would also favor the maximum effectiveness of low levels of neuropeptide hormone produced in brain or that might enter the brain across the blood-brain barrier from the periphery.

DISTRIBUTION OF mRNA/PROTEIN

The central distribution of the three brain relaxin family receptors has been mapped by in situ hybridization and is described for the rat[15,16,25] (Fig. 1). RXFP1 mRNA is widely distributed in the olfactory bulb, cerebral cortex, thalamus, hippocampus, hypothalamus, midbrain, pons, and medulla, with transcripts most abundant in the basolateral amygdala, subiculum, deep layers of cingulate, somatosensory and motor cortices, and in intralaminar/midline thalamic nuclei. These areas also contain very high densities of [^{33}P]-relaxin binding sites, suggesting a largely somatic localization of RXFP1 and site of action of relaxin.[15]

RXFP2 mRNA is present in a distinct distribution in forebrain, with rich expression in the thalamus. High densities are detected in the parafascicular nucleus, the dorsolateral, ventrolateral and posterior thalamic nuclei, and in the medial habenula. RXFP2 transcripts are also present in frontal and motor cortices. The comparative distribution of RXFP2 protein examined by autoradiography of [^{125}I]-human INSL3 binding sites detected high densities in the thalamus, especially in the parafascicular nucleus, and in the entire striatum – the caudate putamen, islands of Calleja, olfactory tubercle, nucleus accumbens – with lower levels in the distinct layers of cerebral cortex. In contrast to RXFP1, RXFP2 is axonally transported and expressed presynaptically, as well as on neuronal soma, consistent with a different mechanism of action for INSL3-RXFP2 and relaxin-RXFP1 signaling.[25]

The regional pattern of distribution of RXFP3 mRNA (and [^{125}I]-R3/I5 binding sites) largely overlaps with that observed for relaxin-3 positive fibers.[16,30,31] Areas of particular functional interest that contain relaxin-3 fibers *and* RXFP3 mRNA/binding sites include preoptic, periventricular and lateral hypothalamus, paraventricular and supraoptic nuclei; nodes of the septohippocampal pathway—the septum, hippocampus, and median raphe, interpeduncular and supramammillary nuclei; the superior colliculus and PAG; and the extended amygdala, including the bed nucleus of stria terminalis and central/medial amygdala. Some species-based differences in distribution have been identified, where the distribution has been mapped thoroughly in rat and mouse (see Refs. 16, 27 for details).

BIOLOGICAL ACTIONS WITHIN THE BRAIN

Relaxin-3

Relaxin-3 has a proposed role in the control of a range of brain circuits related to behavioral state and arousal, which, in turn, regulate a number of related physiological actions and behaviors, but a full review of all possibilities is beyond the scope of this chapter. Instead, a brief discussion of its proposed role in several key functions will be presented (see Ref. 28 for further details). The neuroanatomical distribution of the nucleus incertus/relaxin-3 system displays a strong similarity to existing or 'characterized' ascending arousal systems. The nucleus incertus lies dorsal and caudal to the median raphe and medial to locus coeruleus and lateral tegmental nucleus, and relaxin-3 positive fibers and RXFP3 are present within areas in which populations of arousal-related neurons are located (although notably, not in locus coeruleus), suggesting relaxin-3/RXFP3 networks act in parallel with the norepinephrine system and in parallel and/or in series with other major arousal systems.

Strong, independent, and consistent pharmacological evidence has shown the ability of exogenous relaxin-3 (and RXFP3-selective agonists) to influence food consumption

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and body weight gain in the rat. There is also some experimental evidence for modulatory actions associated with arousal, circadian rhythm and sleep/wake states, spatial and emotional memory, and responses to stress and affective behavior. Relaxin-3 has not been associated with any particular pathological conditions, but relaxin-3 expression is increased by strong neurogenic stressors[24,28] and relaxin-3 neurons are strongly activated after central administration of corticotropin-releasing hormone (CRH).[33]

Many early 'functional' studies administered human relaxin-3 into the lateral cerebral ventricle or into specific brain areas. However, relaxin-3 can activate RXFP1 and RXFP3 *in vitro* and *in vivo*, and this has increased the value and importance of using agonist peptides selective for RXFP3 over RXFP1 and RXFP2 in studies assessing effects of exogenous peptides *in vivo* that aim to deduce endogenous functions of relaxin-3/RXFP3 signaling.

Feeding and Metabolism

Central (intracerebroventricular, icv) administration of relaxin-3 strongly activates food consumption in satiated adult rats.[19] A similar phagic action has been shown after targeted injections of relaxin-3 into various feeding-related hypothalamic nuclei, including the paraventricular (PVN), and arcuate hypothalamic nuclei.[19] The selective RXFP3 agonist, R3/I5,[13] and the recently developed minimized agonist, RXFP3-A2 agonist[26] also increase food intake after acute icv injection in rats; while prior administration of the RXFP3 antagonists, R3(BΔ23-27)R/I5 or R3B(1-22)R and RXFP3-A3 block R3/I5- and RXFP3-A2-induced feeding, respectively.[11,26] Chronic icv infusion of relaxin-3 (via osmotic minipump) increases food consumption and body weight over a 14-day infusion period, with no marked effect observed on locomotor activity.[9] In a similar study, chronic R3/I5 infusion significantly increases food consumption (~40%) and body weight gain (~10% higher after 14 days). The increased food consumption in the R3/I5 group is reflected in increased epididymal fat, as well as increased plasma insulin and leptin concentrations.[32] Furthermore, when administered in the early part of the light/(inactive) phase, R3/I5 causes a dose-related increase in motor activity over a 2-h period.[32] However, R3(BΔ23-27)R/I5 has no significant effect on locomotor activity, even when given during the dark/active phase, and it does not block food consumption in fasted rats, suggesting that endogenous relaxin-3 does not play a primary role in basal locomotor or feeding behavior. Lastly, in a further chronic study, rats injected centrally with an adeno-associated virus (AAV) capable of producing active RXFP3 agonist secretion into the extracellular space display significantly increased food intake and higher weight gain than do controls over an extended period of >6 weeks (Ganella DE et al., unpublished data).

Despite these consistent pharmacological actions of RXFP3 activation, there is currently no clear evidence that relaxin-3 signaling is essential for the maintenance of 'normal'/basal food consumption and body weight, such as significant alterations in expression produced by fasting, as observed for other key orexigenic peptides such as neuropeptide Y. It has not been determined whether rats injected with relaxin-3 display any preference for fat or carbohydrate intake over other macronutrients, as seen with peptides such as galanin.

Furthermore, in contrast to findings in rats, in normal mice, icv administration of high doses of relaxin-3 or the selective RXFP3 agonist, R3/I5 has no acute stimulatory effect on feeding. Also, despite an early report of metabolic and associated neuroendocrine disruption in a mixed background strain of relaxin-3 null mutation mice, genetically more similar (C57BL/6 backcrossed) relaxin-3 knockout (KO) mice display an identical food consumption and body weight phenotype to wild-type littermates.[29] At present, relaxin-3 overexpressing (OE) mice have not been generated, but it would be interesting to determine whether these display an 'obese' or metabolic phenotype. Exploratory studies of acute and chronic RXFP3 activation or inhibition on feeding and body weight under different dietary conditions or in leptin-deficient obese (*ob/ob*) mice or similar strains might also provide further insights. It will also be important to determine which peptides/transmitters/hormones implicated in feeding control are directly or indirectly altered by RXFP3 activation, particularly under conditions of altered diet and stress such as during the reproductive cycle, and determine the nature of species-specific responses.

In summary, current evidence suggests that although relaxin-3 can modulate feeding, this should not necessarily be thought of as a 'primary' endogenous role of the peptide, and orexigenic effects of exogenous relaxin-3 administration may be best interpreted in terms of the putative overall function of relaxin-3 as an arousal transmitter.

Water Drinking

Several studies have shown rapid increases in water drinking after icv injection of relaxin-3 in rats.[4,19] This effect is, however, similar to that seen after icv injection of human relaxin,[4] and probably results primarily from the activation of RXFP1 in the subfornical organ by high concentrations of exogenous relaxin-3.[16]

Release of Hypothalamic and Pituitary Hormones

Available anatomical results and initial pharmacology studies indicate that relaxin-3 plays a modulatory role in neuroendocrine control—as relaxin-3 positive fibers and

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RXFP3 are present in relevant hypothalamic sites, including magnocellular (and parvocellular) PVN and SON, and in the anterior and posterior periventricular areas. For example, in the hypothalamic–pituitary–gonadal (HPG) axis, icv relaxin-3 increases plasma luteinizing hormone (LH) concentrations, possibly via direct actions on hypothalamic GnRH neurons.[21] Specifically, human relaxin-3 (5 nmol, icv) and intra-PVN (0.5–1.6 nmol) in adult male Wistar rats significantly increase LH plasma levels by 2- to 3-fold 30 min postinjection. This effect is blocked by pretreatment with a peripheral GnRH antagonist. Central administration of human relaxin-2 has no effect on plasma LH levels. In hypothalamic explants, relaxin-3 dose dependently stimulates the release of GnRH by 20–30% above basal values. Relaxin-3, at high doses, stimulates GnRH release from a GT1-7 GnRH hypothalamic neuronal-cell line that express RXFP3. However, these cells also express RXFP1, a possible confound for a simple interpretation of the results. Nonetheless, the probable modulatory effect of relaxin-3 on aspects of reproduction in different species warrants further examination.

McGowan et al.[20] also reported that acute and chronic intra-PVN relaxin-3 injections produces a decrease in plasma thyroid-stimulating hormone (TSH) levels, suggesting a putative role modulating the hypothalamic–pituitary–thyroid axis and energy expenditure.

Central administration of native relaxin-3 also stimulates the hypothalamic–pituitary–adrenal (HPA) axis, increasing CRH expression in the PVN and increasing plasma adrenocorticotropic hormone (ACTH) and corticosterone.[34] Administration of the RXFP3-selective agonist, R3/I5, also increases plasma ACTH and corticosterone levels.[32] Finally, the effect of central relaxin-3 on other hypothalamic peptides and hormone-releasing factors and pituitary hormones has been examined, with reports of decreased plasma growth hormone (GH) levels. Chronic icv delivery of R3/I5 also produces an elevation in plasma testosterone[32] (see Ref. 28 for a review).

Overall, these studies suggest that relaxin-3/RXFP3 signaling may act as an ascending signal to regulate the hypothalamic coordination of appetite and growth, and thyroid and adrenal function, in relation to stress and reproductive status,[20] but further studies are required to better establish the precise mechanisms.

Arousal, Circadian Rhythm, and Sleep

Relaxin-3/RXFP3 networks are present in several brain areas, including the preoptic and lateral hypothalamus and dorsal raphe nucleus that are functionally related to the control of 'arousal' and on this basis the relaxin-3 system has been suggested to act in parallel and/or in series with these other arousal networks.[28] In the lateral hypothalamus, RXFP3 activation may result in altered activity of orexin neurons and in turn, relaxin-3 neurons express receptors for orexin and are activated by this peptide *in vitro* (Blasiak A, unpublished observations). Sleep-active GABA/galanin-containing neurons are located in the subregions of the preoptic area (POA), with a high density in the median preoptic nucleus (MnPN) and the ventrolateral (vl) POA and these areas contain RXFP3.[16,27] Convincing evidence indicates that POA neurons promote sleep onset and maintenance by inhibitory modulation of multiple arousal systems via projections from vlPOA and MnPN to multiple monoaminergic arousal systems in the posterior/lateral hypothalamus and the brainstem and are involved in homeostatic regulation of rapid eye movement (REM) and non-REM sleep. It will be of interest, therefore, to further assess the effect of sleep disturbances on the activity of relaxin-3 neurons in rat and mouse models. Furthermore, relaxin-3/RXFP3 elements are present in the intergeniculate leaflet and suprachiasmatic nucleus (the 'circadian pacemaker'), which receive retinal inputs and integrate photic and nonphotic sensory inputs. In line with this, a preliminary study reported that relaxin-3 mRNA expression varied over the 24-h circadian cycle, with a peak at 2000h (during dark/active phase) and a trough at 0800h (during early light/inactive phase), and RXFP3 activation alters the activity of intergeniculate leaflet neurons *in vitro*.[5] Whether relaxin-3 contributes to the generation of circadian rhythm, or is 'downstream' of these processes, is currently unknown.

In functional studies in rats, icv infusion of R3/I5 increases wakefulness and wakeful behaviors such as locomotor activity/exploration,[32] possibly indicative of increased general arousal. These findings are consistent with the results in two separately C57B/6J backcrossed cohorts of relaxin-3 KO mice, whereby relaxin-3 deficiency leads to increased time spent sleeping during the dark ('active') phase.[28,29]

Motivation and Reward

A primary 'reward pathway' within the brain consists of dopaminergic neurons in the ventral tegmental area that project to the nucleus accumbens, and it is activated when an animal engages in pleasurable behavior such as eating food or mating. It is also involved in the motivational drive to engage in such behavior. Relaxin-3/RXFP3 signaling may impinge directly or indirectly on this dopaminergic pathway and related limbic regions including the amygdala; and preliminary studies in relaxin-3 KO mice suggest that relaxin-3 can modulate motivated behaviors, as KO mice run less far on voluntary running wheels.[29]

Spatial and Emotional Memory

Limited studies of relaxin-3/RXFP3 signaling in learning and memory have been reported. The effect of icv injections

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of RXFP3 agonist or antagonist has not been assessed, but the effect of intraseptal administration of relaxin-3 analogs was explored as part of a wider study to examine the anatomical and functional association of these networks with the septum and the septohippocampal system.[24] Intraseptal injection of the RXFP3 antagonist R3(BΔ23-27)R/I5[11] reduces the performance of adult, male Sprague–Dawley rats in a spatial working memory test (spontaneous alternation task), and this effect is reversed by coinfusion of the RXFP3 agonist, R3/I5.[18] In light of these and related anatomical findings, septal RXFP3 activation is postulated to alter the balance of GABA and acetylcholine release from septal afferents in the hippocampus, but these studies and others pertaining to the direct/indirect actions of RXFP3 activation on hippocampal GABA (inter)neuron and pyramidal neuron physiology and hippocampal-associated learning have not yet been completed. More recently, we also observed a strong inhibitory effect of intraamygdala injection of R3/I5 on fear expression in an auditory fear conditioning model (Ma et al., unpublished data) consistent with the high density of RXFP3 in this region.[16] Further studies are now required for the effect of RXFP3 activation and inhibition on spatial and emotional memory and of the ability of RXFP3 modulation to inhibit or potentiate memory enhancing actions of other drugs in validated preclinical memory tasks.

Relaxin-3 KO mice have been investigated in some learning and memory paradigms—relaxin-3 KO mice display no difference to their wild-type littermates in the Y-maze test—but it will be of interest to assess their performance of more difficult elements of experimental learning and memory tasks available, including Morris water maze (spatial), social transmission of food preference (olfactory), and fear conditioned freezing on the trace-fear conditioning task (emotional). Such studies should better elucidate the precise effects and mechanisms of action of RXFP3 modulation and may reveal the potential of relaxin-3-related drugs as treatment for aged-related memory deficits, perhaps as an adjunct to other treatments.

Stress Responses and Behavioral Activation

Relaxin-3 expression increases markedly after water immersion/restraint stress and repeat forced swim stress,[1,33] and on the basis of this and related findings, relaxin-3 is proposed to play a role in the coordinated stress response, which could include activation of forebrain arousal systems. In this respect, nucleus incertus (NI) relaxin-3 neurons express a high level of CRH-R1 in rat and are activated by CRH;[24] and we have obtained electrophysiological evidence that NI relaxin-3 neurons are activated by oxerins, in line with observations of orexin receptor mRNA in NI (see Ref. 28 for a review).

Relaxin and INSL3

Relaxin is well known for its actions in peripheral systems. Relaxin is produced by the ovary and targets the mammalian reproductive system to ripen the cervix, elongate the pubic symphysis, and inhibit uterine contraction. Additional roles have been proposed in enhancing sperm motility, regulating blood pressure/heart rate, and releasing oxytocin and vasopressin. Relaxin has well-characterized effects on osmotic homeostasis during pregnancy via actions of circulating relaxin on neurons in the circumventricular organs, but its actions within the brain are less well characterized.

Similarly, despite well-described roles for INSL3 in gonadal tissue function in males and females, including development of urogenital tract and female fertility, regulation of growth, and differentiation of gubernaculum and intraabdominal testicular descent, the precise roles of INSL3/RXFP2 signaling in brain are not well characterized. However, available anatomical data on the distribution of relaxin and INSL3 in distinct neuron populations in forebrain, combined with the broad and contrasting distribution of their receptors throughout sensory and motor systems and neuroendocrine networks in rat and mouse brain,[14,15,23,25] suggest that exploring their neurobiological effects in appropriate experimental models will be informative. Central administration of human relaxin inhibits feeding in rats during the early dark phase;[22] but little information is available regarding the regulation of endogenous brain relaxin or INSL3 expression by other peptides/hormones associated with metabolic or reproductive state, including leptin, insulin, and thyroid hormone and estrogen/testosterone, respectively.

Feeding and Metabolism

In initial comparative studies of the effects of relaxin-3 and human relaxin-2 (H2), little or no effect of H2 was reported,[19] but central and peripheral administration of H2 reduces food intake in male, adult Wistar rats.[22] Icv and intra-PVN administration of H2 (180 and 18–540 pmol, respectively) decrease 1-h food intake in the early dark phase by 50–70% and increase grooming and 'head down' behavior, suggesting effects within the PVN and other brain regions.[22]

Water Drinking Behavior and Pregnancy

Relaxin has a well-characterized dipsogenic effect in the brain after iv or icv administration via direct actions on the circumventricular organs (subfornical organ/OVLT), which lie along the rostral border of the third ventricle outside the blood-brain barrier and have neural axonal projections to other regions (MPO, SON, and PVN) that subserve vasopressin secretion and water drinking. Relaxin is thought to activate these brain pathways via angiotensin II signaling. In the

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pregnant rat, injection of a relaxin monoclonal antibody into the brain negates the normal increase in drinking observed during the second half of pregnancy. Exogenous relaxin effects in the rat also vary with time of injection during the light–dark cycle, whereby dose-dependent water drinking caused by relaxin is maximal at night compared with that during the day. This effect, while perhaps predictable in nocturnal animals, may suggest an involvement of relaxin in this and other circadian-based functions (see Ref. 17 for details).

Learning and Emotional Memory

RXFP1 is concentrated in limbic regions including the hippocampus, subiculum, entorhinal cortex, and basolateral amygdala (BLA), known to participate in emotional processing, and learning and memory. The BLA is well characterized with regard to its neural connections and its involvement in modulation of fear memory. Experiments using an aversively motivated, inhibitory avoidance paradigm revealed that infusions of relaxin into the BLA (but not adjacent areas) immediately after a footshock training session significantly impaired 48-h memory retention performance in a dose-related manner.[14] These results indicate relaxin can alter early memory consolidation processes, although further studies are required to investigate the neurochemical mechanisms underlying this effect and whether endogenous relaxin alters BLA function.

Somatosensory and Motor Behavior

Preliminary studies of central administration of different doses of INSL3 (80–320 pmol icv in mice and 10–40 pmol intrastriatal in rats) indicate that INSL3 has complex pharmacological effects on locomotor activity and complex behavior.[17] In light of the presence of low levels of INSL3 in midbrain neurons (in red nucleus and substantia nigra pars reticulata) and the intriguing juxtaposition of these cells to neurons expressing abundant RXFP2 in somatosensory networks,[25] exploring their interaction in appropriate experimental models should help reveal the basis of their neurobiological actions.

PATHOPHYSIOLOGICAL IMPLICATIONS

It is established that transmission systems in the brain can release a cocktail of signaling molecules, including classical transmitters, gases and hormones, and neuropeptides, which are often recruited after severe stress or aversive/pathological events to modulate transmission and promote plasticity and neural repair. Human studies have also shown that dysregulation of neuropeptides is probably of importance for a plethora of neurodegenerative and neuropsychiatric disorders. Although not as extensive as for other similar systems, available anatomical, neurochemical, and pharmacological evidence provides substantial support for the further exploration of relaxin-3-related ligands with good RXFP3 selectivity as treatments for the pathology or associated physiological/behavioral symptoms of stress-related anxiety and depression, forms of addiction, and cognitive impairments associated with such disorders. There is no conclusive evidence linking genetically based alterations in the relaxin-3 system with specific brain pathophysiological disorders, despite some preliminary reports. By contrast, there is the possibility of therapeutically exploiting widespread RXFP3 signaling networks to alleviate the pathophysiology and symptoms of a range of psychiatric conditions. Although speculative in nature, this is considered here for some relevant disorders such as chronic stress and anxiety, mood/sleep disturbances and depression, drug addiction and relapse, and cognitive deficits associated with neurodevelopmental and neurodegenerative diseases.

Relaxin-3

Feeding and Metabolism

Administration of RXFP3 agonists into the brain produces a robust stimulation of food consumption and further studies using rat models of obesity or pathological weight loss are warranted. Studies of ‘life-long’ relaxin-3 deficient mice have provided no strong evidence for the involvement of relaxin-3/RXFP3 signaling in the physiology or pathology of energy balance and related disorders, but conditional relaxin-3- or RXFP3-KO strains or other transgenic lines such as relaxin-3 OE mice may be useful for illustrating any undiscovered role of endogenous relaxin-3 under normal and pathological metabolic conditions. Importantly, chronic secretion of a bioactive relaxin-3 analog into the hypothalamus have produced increased food intake and weight gain over an extended period, indicating that further studies of relaxin-3/RXFP3 signaling related to eating and body weight/obesity disorders may provide new insights.

Stress, Anxiety and Fear

Relaxin-3 modulates the response to stressors, as well as anxiety-like behavior (see Ref. 28 for a review). RXFP3 is expressed in various stress-related brain regions, including the PVN, where it may regulate CRH and vasopressin/oxytocin, which are critical regulators of the HPA or ‘stress’ axis under different physiological conditions. Relaxin-3 expression is increased in nucleus incertus (NI) neurons by neurogenic stressors and NI neurons can be activated by swim stress, social stress, treadmill exercise, and immobilization, and this may act physiologically to promote arousal and alertness. Central relaxin-3 injections increase parvocellular PVN CRH neuron activity, suggesting a positive feedback mechanism,[34] but the precise details of this effect are currently unclear and actions on extra-hypothalamic CRH neurons have not been explored.

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Mood, Reward, Depression, and Addiction

The distribution of relaxin-3 and RXFP3 in key limbic circuits suggests exogenous relaxin-3 analogs and/or endogenous relaxin-3 signaling can modulate arousal, mood, and reward. Given the involvement of the monoamine transmitters, serotonin, and norepinephrine in these modalities, it will be important to determine their interaction with relaxin-3/RXFP3. Relaxin-3 neurons in rat brain express the serotonin-1A receptor ($5HT_{1A}$) and the chronic 'global' depletion of brain serotonin is reported to increase relaxin-3 mRNA in the NI, suggesting a potential inhibitory regulation of relaxin-3 neurons by serotonin-$5HT_{1A}$ transmission.[28] The nature of this interaction and the effect of relaxin-3/RXFP3 signaling on serotonin neurons in the dorsal, median, and pontine raphe nuclei warrants further investigation, as $5HT_{1A}$ receptor function is strongly implicated in depressive-like behaviors.

Preliminary studies with RXFP3-selective peptides suggest relaxin-3/RXFP3 signaling may modulate 'depressive-like' behavior. RXFP-A2[26] injected icv decreases depressive-like behavior (i.e. immobility in the forced swim test), but this needs to be independently verified and shown to be RXFP3 mediated. Mixed background relaxin-3 KO mice display increased 'depressive-like' behavior (i.e. increased immobility in the forced swim test) and further testing of relaxin-3 KO mice in other 'depression' paradigms and an examination of the efficacy of an RXFP3 agonist in validated mouse (and rat) models of depressive symptoms would be valuable. Lastly, relaxin-3 signaling appears to act via RXFP3 as a positive regulator of reward in alcohol-preferring rats, as central RXFP3 agonist administration attenuates alcohol self-administration and inhibits cue-induced reinstatement of drug-seeking behavior (Ryan et al., unpublished data). Thus, further experimental studies in these areas have implications for clinical conditions such as insomnia, depression, and addiction.

Relaxin and INSL3

It is not possible to comment definitively on the involvement of relaxin/RXFP1 and INSL3/RXFP2 signaling in specific disease-based brain pathologies, as there is very little published data available on their neurobiological roles. One can only speculate on such a possibility based purely on the presence of RXFP1 and RXFP2 in different excitatory networks in cortex and subcortical thalamic, hypothalamic, and limbic areas, and the established links between other peptide/hormone systems and the neuropathology of emotional and affective disorders. However, more details about the precise nature of neural actions of these peptides/receptors will facilitate more directed research, and these systems offer some therapeutic potential for disorders associated with sensorimotor deficits and possibly conditions linked to the HPG axis and reproductive behavior and physiology.

ACKNOWLEDGMENTS

The authors wish to acknowledge the contribution of current and former staff and student members of the Gundlach laboratory and our local and international collaborators in generating the experimental data reviewed in this article. Original research in the Gundlach laboratory reviewed in this chapter was supported by NHMRC (Australia); Johnson & Johnson Pharmaceutical Research & Development, LLC, USA; the Howard Florey Biomedical Foundation (USA); the Besen Family and Pratt Foundations; and the Victorian Government's Operational Infrastructure Support Program.

REFERENCES

1. Banerjee A, Shen PJ, Ma S, Bathgate RAD, Gundlach AL. Swim stress excitation of nucleus incertus and rapid induction of relaxin-3 expression via CRF1 activation. *Neuropharmacology* 2010;**58**:145–55.
2. Bathgate RAD, Samuel CS, Burazin TCD, Layfield S, Claasz AA, Reytomas IGT, et al. Human relaxin gene 3 (H3) and the equivalent mouse relaxin (M3) gene: novel members of the relaxin peptide family. *J Biol Chem* 2002;**277**:1148–57.
3. Bathgate RAD, Ivell R, Sanborn BM, Sherwood OD, Summers RJ. International Union of Pharmacology LVII: recommendations for the nomenclature of receptors for relaxin family peptides. *Pharmacol Rev* 2006;**58**:7–31.
4. Bathgate RAD, Lin F, Hanson NF, Otvos LJ, Guidolin A, Giannakis C, et al. Relaxin-3: improved synthesis strategy and demonstration of its high-affinity interaction with the relaxin receptor LGR7 both in vitro and in vivo. *Biochemistry* 2006;**45**:1043–53.
5. Blasiak A, Blasiak T, Hossain MA, Wade JD, Gundlach AL. Relaxin-3 innervation and modulation of the intergeniculate leaflet of the rat thalamus. *Neuropeptides* 2009;**43**:430.
6. Callander GE, Thomas WG, Bathgate RAD. Prolonged RXFP1 and RXFP2 signaling can be explained by poor internalization and a lack of beta-arrestin recruitment. *Am J Physiol Cell Physiol* 2009;**296**:C1058–66.
7. Halls ML, van der Westhuizen ET, Bathgate RAD, Summers RJ. Relaxin family peptide receptors—former orphans reunite with their parent ligands to activate multiple signalling pathways. *Br J Pharmacol* 2007;**150**:677–91.
8. Haugaard-Kedström LM, Shabanpoor F, Hossain MA, Clark RJ, Ryan PJ, Craik DJ, et al. Design, synthesis and characterization of a single-chain peptide antagonist for the relaxin-3 receptor RXFP3. *J Am Chem Soc* 2011;**133**:4965–74.
9. Hida T, Takahashi E, Shikata K, Hirohashi T, Sawai T, Seiki T, et al. Chronic intracerebroventricular administration of relaxin-3 increases body weight in rats. *J Recept Signal Transduct Res* 2006;**26**:147–58.
10. Hsu SY, Nakabayashi K, Nishi S, Kumagai J, Kudo M, Sherwood OD, et al. Activation of orphan receptors by the hormone relaxin. *Science* 2002;**295**:671–4.
11. Kuei C, Sutton S, Bonaventure P, Pudiak C, Shelton J, Zhu J, et al. R3(BΔ23-27)R/I5 chimeric peptide, a selective antagonist for GPCR135 and GPCR142 over relaxin receptor LGR7: in vitro and in vivo characterization. *J Biol Chem* 2007;**282**:25425–35.

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12. Liu C, Eriste E, Sutton S, Chen J, Roland B, Kuei C, et al. Identification of relaxin-3/INSL7 as an endogenous ligand for the orphan G-protein-coupled receptor GPCR135. *J Biol Chem* 2003;**278**:50754–64.
13. Liu C, Chen J, Kuei C, Sutton S, Nepomuceno D, Bonaventure P, et al. Relaxin-3/insulin-like peptide 5 chimeric peptide, a selective ligand for G protein-coupled receptor (GPCR)135 and GPCR142 over leucine-rich repeat-containing G protein-coupled receptor 7. *Mol Pharmacol* 2005;**67**:231–40.
14. Ma S, Roozendaal B, Burazin TCD, Tregear GW, McGaugh JL, Gundlach AL. Relaxin receptor activation in the basolateral amygdala impairs memory consolidation. *Eur J Neurosci* 2005;**22**:2117–22.
15. Ma S, Shen PJ, Burazin TCD, Tregear GW, Gundlach AL. Comparative localization of leucine-rich repeat-containing G protein-coupled receptor-7 (RXFP1) mRNA and [^{33}P]-relaxin binding sites in rat brain: restricted somatic co-expression a clue to relaxin action? *Neuroscience* 2006;**141**:329–44.
16. Ma S, Bonaventure P, Ferraro T, Shen PJ, Burazin TCD, Bathgate RAD, et al. Relaxin-3 in GABA projection neurons of nucleus incertus suggests widespread influence on forebrain circuits via G protein-coupled receptor-135 in the rat. *Neuroscience* 2007;**144**:165–90.
17. Ma S, Gundlach AL. Relaxin-family peptide and receptor systems in brain: insights from recent anatomical and functional studies. *Adv Expl Med Biol* 2007;**612**:119–37.
18. Ma S, Olucha-Bordonau FE, Hossain MA, Lin F, Kuei C, Liu C, et al. Modulation of hippocampal theta oscillations and spatial memory by relaxin-3 neurons of the nucleus incertus. *Learn Mem* 2009;**16**:730–42.
19. McGowan BM, Stanley SA, Smith KL, White NE, Connolly MM, Thompson EL, et al. Central relaxin-3 administration causes hyperphagia in male Wistar rats. *Endocrinology* 2005;**146**:3295–300.
20. McGowan BM, Stanley SA, Smith KL, Minnion JS, Donovan J, Thompson EL, et al. Effects of acute and chronic relaxin-3 on food intake and energy expenditure in rats. *Regul Peptides* 2006;**136**:72–7.
21. McGowan BM, Stanley SA, Donovan J, Thompson EL, Patterson M, Semjonous NM, et al. Relaxin-3 stimulates the hypothalamic–pituitary–gonadal axis. *Am J Physiol Endocrinol Metab* 2008;**295**:E278–86.
22. McGowan BM, Minnion JS, Murphy KG, White NE, Roy D, Stanley SA, et al. Central and peripheral administration of human relaxin-2 to adult male rats inhibits food intake. *Diabetes Obes Metab* 2010;**12**:1090–6.
23. Piccenna L, Shen PJ, Ma S, Burazin TCD, Gossen JA, Mosselman S, et al. Localization of LGR7 gene expression in adult mouse brain using LGR7 knock-out/LacZ knock-in mice: correlation with LGR7 mRNA distribution. *Ann NY Acad Sci* 2005;**1041**:197–204.
24. Ryan PJ, Ma S, Olucha-Bordonau FE, Gundlach AL. Nucleus incertus—an emerging modulatory role in arousal, stress and memory. *Neurosci Biobehav Rev* 2011;**35**:1326–41.
25. Sedaghat K, Shen PJ, Finkelstein DI, Henderson JM, Gundlach AL. Leucine-rich repeat-containing G protein-coupled receptor 8 in the rat brain: enrichment in thalamic neurons and their efferent projections. *Neuroscience* 2008;**156**:319–33.
26. Shabanpoor F, Hossain MA, Ryan PJ, Belgi A, Layfield S, Kocan M, et al. Minimization of relaxin-3 leading to high affinity analogues with increased selectivity for relaxin-family peptide 3 receptor (RXFP3) over RXFP1. *J Med Chem* 2012;**55**:1671–81.
27. Smith CM, Shen PJ, Banerjee A, Bonaventure P, Ma S, Bathgate RAD, et al. Distribution of relaxin-3 and RXFP3 within arousal, stress, affective, and cognitive circuits of mouse brain. *J Comp Neurol* 2010;**518**:4016–45.
28. Smith CM, Ryan PJ, Hosken IT, Ma S, Gundlach AL. Relaxin-3 systems in the brain—the first 10 years. *J Chem Neuroanat* 2011;**42**:262–75.
29. Smith CM, Hosken IT, Sutton SW, Lawrence AJ, Gundlach AL. Relaxin-3 null mutation mice display a circadian hypoactivity phenotype. *Genes Brain Behav* 2012;**11**:94–104.
30. Sutton SW, Bonaventure P, Kuei C, Roland B, Chen J, Nepomuceno D, et al. Distribution of G protein-coupled receptor (GPCR)135 binding sites and receptor mRNA in the rat brain suggests a role for relaxin-3 in neuroendocrine and sensory processing. *Neuroendocrinology* 2004;**80**:298–307.
31. Sutton SW, Bonaventure P, Kuei C, Nepomuceno D, Wu J, Zhu J, et al. G-protein-coupled receptor (GPCR)-142 does not contribute to relaxin-3 binding in the mouse brain: further support that relaxin-3 is the physiological ligand for GPCR135. *Neuroendocrinology* 2006;**82**:139–50.
32. Sutton SW, Shelton J, Smith CM, Williams J, Yun S, Motley T, et al. Metabolic and neuroendocrine responses to RXFP3 modulation in the central nervous system. *Ann NY Acad Sci* 2009;**1160**:242–9.
33. Tanaka M, Iijima N, Miyamoto Y, Fukusumi S, Itoh Y, Ozawa H, et al. Neurons expressing relaxin 3/INSL7 in the nucleus incertus respond to stress. *Eur J Neurosci* 2005;**21**:1659–70.
34. Watanabe Y, Miyamoto Y, Matsuda T, Tanaka M. Relaxin-3/INSL7 regulates the stress-response system in the rat hypothalamus. *J Mol Neurosci* 2010;**277**:4990–7.
35. Wilkinson TN, Speed TP, Tregear GW, Bathgate RAD. Evolution of the relaxin-like peptide family: from neuropeptide to reproduction. *Ann NY Acad Sci* 2005;**1041**:530–3.

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Chapter 122

26RFa

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ABSTRACT

The neuropeptide 26RFa/43RFa (also termed QRFP) is the latest member of the Arg-Phe-NH_2 peptide family discovered in vertebrates. 26RFa/43RFa recognizes the previously orphan G protein-coupled receptor GPR103. In the brain of mammals, 26RFa and GPR103 mRNAs are actively expressed in the dorsolateral and mediobasal hypothalamic areas. Intracerebroventricular injection of 26RFa provokes a dose-dependent increase of food intake. 26RFa also stimulates the gonadotropic axis, inhibits glucose-evoked insulin secretion, increases locomotor activity, and induces analgesic effects. Finally, GPR103-deficient mice suffer from osteopenia indicating that 26RFa plays a physiological role in bone formation.

DISCOVERY

RFamide peptides constitute a superfamily of regulatory peptides that share the Arg-Phe-NH_2 motif at their C-terminus. Numerous RFamide peptides have been identified in various classes of invertebrates (see the Invertebrate Peptides Section in this book), but only 5 groups of RFamide peptides have been characterized in vertebrates: neuropeptides FF/AF (NPFF/NPAF), prolactin-releasing peptide (PrRP, see the chapter on PrRP in this section of the book), gonadotropin-inhibitory hormone (GnIH)/RFamide-related peptide (RFRP) (see the chapter on GnIH in this section), kisspeptin (see the chapter on kisspeptins in this section), and 26RFa/43RFa.[5]

26RFa was initially isolated from the brain of the frog *Rana esculenta* using a nonselective antibody against the RFamide motif.[6] The sequence of 26RFa markedly differs (except for the C-terminal Arg-Phe-NH_2 signature) from all the known vertebrate RFamide peptides, indicating that 26RFa belongs to a new family of RFamide peptides. The amino acid sequence of frog 26RFa was used to characterize the cDNA sequences of the 26RFa precursors in rats and humans.[6] Concomitantly, two groups, using a bioinformatics approach, have identified the 26RFa precursors (that they termed QRFP) in the rat, mouse, bovine, and human genomes and have paired 26RFa with the orphan receptor GPR103.[10,12] Subsequently, the cDNAs encoding the 26RFa precursors have been characterized in goldfish,[17] quail,[25] chicken,[25] and zebra finch[24] indicating that the 26RFa gene is present in virtually all the classes of vertebrates. In the brain of mammals, 26RFa mRNA is primarily expressed in hypothalamic nuclei involved in the control of feeding behavior.[4,6,10,12] Consistent with this observation, intracerebroventricular (icv) injection of 26RFa or 43RFa stimulates food consumption in birds[24,25] and rodents.[6,7,13,14,18,22]

STRUCTURE OF THE 26RFa PRECURSOR

The human 26RFa cDNA encodes a 136-amino acid prepropeptide including a 17-amino acid signal peptide, a 73-amino acid N-terminal flanking peptide and the C-terminally located 43-amino acid peptide 43RFa (Fig. 1).[10,12] The general structure of the 26RFa cDNA has been preserved during evolution although the goldfish precursor sequence (168 residues) is substantially longer than the mammalian ones (120–136 residues).[26] The human 26RFa precursor, but not the precursor of other species, encompasses an additional putative RFamide nonapeptide within the cryptic polypeptide that is delimited by two single Arg residues at its N-(Arg^{79}) and C-terminal (Arg^{90}) extremities (Fig. 1).

PROCESSING OF THE PRECURSOR

The human 26RFa precursor encompasses 5 potential cleavage sites (Arg^{79}, Arg^{90}, Lys^{107}, Arg^{124}-Lys^{125}-Lys^{126}, and Arg^{135}-Arg^{136}) suggesting that processing by prohormone convertases may generate several RFamide peptides (see the chapters on Prohormone convertases in the Peptide Biosynthesis/Processing section of this book). The presence of glycine residues upstream the C-terminal basic cleavage sites (Gly^{89} and Gly^{134}) predicts that two peptides are potentially amidated (Fig. 1). Thus, cleavage at Arg^{90} and Lys^{107} has the potential to generate 43RFa and 26RFa, respectively. Indeed, the mature peptides have now been isolated and sequenced in several representative vertebrate species: in the frog *R. esculenta* and the quail *Coturnix japonica*, the mature

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00122-6
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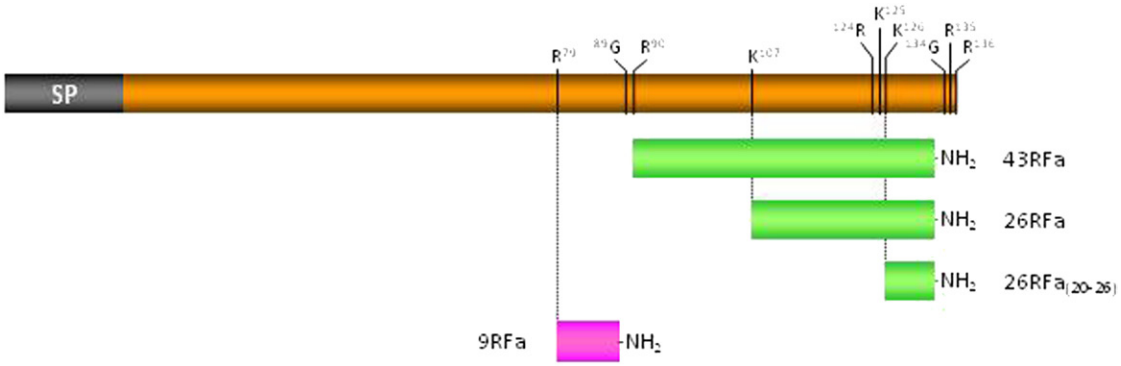

FIGURE 1 Schematic representation of the human prepro26RFa. Potential mono/polybasic cleavage sites that may lead to the generation of RFamide peptides are indicated by vertical bars. G, glycine; K, lysine; R, arginine; SP, signal peptide.

forms of the peptide are 26RFa[6] and its 27 amino acid homolog,[25] respectively, whereas in the rat brain, the mature form is 43RFa.[22] High-performance liquid chromatography analysis combined with radioimmunoassay quantification indicates the existence of both 26RFa- and 43RFa-immunoreactive peptides in the human hypothalamus and spinal cord.[4] The occurrence of a tribasic cleavage site (Arg-Lys-Lys or Arg-Arg-Lys) within the mammalian (except for the duck-billed platypus), avian, reptilian, and amphibian 26RFa sequences (Fig. 2) suggests that 26RFa could be further processed to generate the highly conserved C-terminal heptapeptide $26RFa_{(20–26)}$. Moreover, the presence of two isolated basic residues in the cryptic region of the human precursor (Arg^{79} and Arg^{90}) together with the Arg-Phe-Gly signature at positions 87–89 suggests that the 26RFa precursor could generate an additional RFamide nonapeptide (termed 9RFa) in humans (Fig. 1). However, neither $26RFa_{(20–26)}$ nor 9RFa has been detected in tissue extracts so far.

DISTRIBUTION OF 26RFa mRNA

In rats, 26RFa-expressing neurons are located in the lateral hypothalamic area, the ventromedial hypothalamic nucleus, the lateral aspect of the arcuate nucleus, and the retrochiasmatic area (Fig. 3),[6,10,12] whereas in mice, the expression of 26RFa is restricted to the lateral hypothalamic area and the periventricular nucleus.[22] In humans, 26RFa-containing neurons are present in the ventromedial hypothalamic nucleus and the paraventricular nucleus.[4] The 26RFa gene is also expressed in other extrahypothalamic human brain structures such as the cerebellum, the brainstem, and the dorsal and lateral horns of the spinal cord.[4] In quail, 26RFa mRNA occurs only in the anterior hypothalamic nucleus,[25] whereas in zebra finch, 26RFa transcript is also present in the ventromedial nucleus of the hypothalamus and the lateral hypothalamic area.[24] In teleost fish, the 26RFa gene is expressed in the optic tectum and the hypothalamus.[17]

DISTRIBUTION OF GPR103 mRNA AND SIGNALING CASCADES

26RFa and 43RFa are the ligands of the formerly orphan GPR103, also designated AQ27 or SP9155, a class A G protein-coupled receptor (GPCR).[10,12] Although GPR103 shares substantial sequence similarity with other RFamide receptors, and to a lesser extent with the Y2 neuropeptide Y (NPY) receptor, galanin receptor (Gal-R1), orexin, and cholecystokinin (CCK) receptors,[12,15] it binds selectively 26RFa and 43RFa. In contrast, 26RFa displays a moderate affinity for NPFF-2 and a low affinity for NPFF-1, two other receptors for RFamide peptides.[11] GPR103 exhibits several characteristic features of class A GPCRs including (i) the existence of an Asp residue within the second transmembrane region (TM2) that plays a pivotal role for G protein coupling, (ii) the conserved Glu-Arg doublet sequence at the N-terminal extremity of the second intracellular loop (IL2), (iii) a disulfide bridge between the two Cys residues located in the first and second extracellular loops, and (iv) three conserved residues, that is, Phe, Pro, and Asn, within TM6 and TM7 that are crucial for receptor activation (Fig. 4). GPR103 binds 26RFa and 43RFa with high affinity (IC_{50} = 3.2 and 0.52 nM, respectively).[10,12] In GPR103-transfected CHO cells, 26RFa and 43RFa inhibit cAMP formation with similar efficacy.[10] 26RFa and 43RFa also induce a robust increase in intracellular calcium concentration ($[Ca^{2+}]_i$) in a PTX-independent manner, suggesting that GPR103 is coupled to a $G_{i/0}$ and/or to a G_q protein.[10] The affinity and potency of the C-terminal heptapeptide $26RFa_{(20–26)}$ are several orders of magnitude lower than those of 26RFa indicating that this heptapeptide is not a good ligand for GPR103.[10,16]

Although only one GPR103 gene is found in the human genome, two isoforms of the receptor have been cloned in rodents. These isoforms have been termed GPR103A and GPR103B in mouse[22] and QRFP-r1 and QRFP-r2 in rat.[13] 26RFa binds mouse GPR103A and GPR103B with the same affinity[22] and stimulates inositol triphosphate in rat QRFP-r1 and QRFP-r2 with a similar efficacy.[13]

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Mammals	
Human	QDEGSEATGFLPAAGEKTSGPLGNLAEELNGYSRKKGGFSFRF-NH_2
Chimpanzee*	QDEGSEATGFLPAAGEKTSGPLGNLAEELNGYSRKKGGFSFRF-NH_2
Rhesus macaque*	QDEGSEADDFLPAGGVKASGPLGNLAEELNGYSRKKGGFSFRF-NH_2
Macaque*	QDEGSEADDFLPAGGVKASGPLGNLAEELNGYSRKKGGFSFRF-NH_2
Orangutan*	QDEGSEATGFLPAAGEKASGPLGNLAEELNGYSRKKGGFSFRF-NH_2
Gibbon*	QDEGSEATGFLRAAGEKASGPLGNLAEELNGYSRKKGGFSFRF-NH_2
Marmoset*	QDQGSEATSFLPAEGEKASGPLRNLAEELNGYSRKKGGFSFRF-NH_2
Elephant*	QDDGDEATRFLPADGEKTSGPLGSLAEELTGYSRKKGGFSFRF-NH_2
Horse*	QDDGSEATGFLLADGEKESGPLETLAEELNGYSRKKGGFSFRF-NH_2
Dog*	QDDGSEATGFLPADGEKAHGPLGTLAEELSSYSRKKGGFSFRF-NH_2
Bovine*	QDDGSEATGLLLGEAEKVGGLLGTLAEELNGYSRKKGGFSFRF-NH_2
Sheep*	QDDGSEATGLLLEEAEKVGGPLGTLAEELNGYSRKKGGFSFRF-NH_2
Pig*	QDNGSEAAGFLPGDGEKASGLLGTLAEELHGYSRKKGGFSFRF-NH_2
Panda*	QDDGSEAPSFFPADGEKASGPLGTLAEELSTYSRKKGGFSFRF-NH_2
Rat	QDSGSEATGFLPTDSEKASGPLGTLAEELSSYSRRKGGFSFRF-NH_2
Mouse	QDGSSEAAGFLPADSEKASGPLGTLAEELSSYSRRKGGFSFRF-NH_2
Guinea pig*	QESSETSSFLPAEGSEKTS•PLGNLAEELNGYSRRKGGFSFRF-NH_2
Hamster*	RDRSSQAAGFLPTDAEKASRPLGTLAEELSSYSRRKGGFSFRF-NH_2
Opossum*	QEEDSKATNIYPASEEKQNSLLGSLAEELSGYIRKKGGFSFRF-NH_2
Duck-billed platypus*	QEEESPGAHFVPASGEKRSGLLGNLAEELNGYRRKKGGFSFRF-NH_2
Birds	
Chicken*	GYGQQHSVGPRGRPEGSEKRGGGGTLGDLAEELNGYGRKKGGFAFRF-NH_2
Quail	GGGGTLGDLAEELNGYSRKKGGFAFRF-NH_2
Zebra finch	SGTLGNLAEEINGYNRRKGGFTFRF-NH_2
Reptiles	
Lizard*	ADDNEAASENGEKRAGGLEGLAEELHGYSRRKGGFSFRF-NH_2
Amphibians	
Frog	VGTALGSLAEELNGYNRKKGGFSFRF-NH_2
Fish	
Zebrafish*	PMGTFPDNHIMPLLYPQGGEAEEVGGEKRNEALTSIAGGLQAFNRQKGGFGFRF-NH_2
Goldfish*	PMGTFPDNLIVDVPYPQGGEVEEEGGEKQNEALTSIAGGLQAFNRQKGGFGFRF-NH_2
Fugu*	ILTPLTGGPSVSFRERRQGENGGKKNEALTSIAGELQAVSREKGGFGFRF-NH_2
Stickleback*	LSPFPAGLSLGGVSYQEGGEDGGKRNEALTSIAGGLQAVGREKGGIAFRF-NH_2
Medaka*	LFPGDNSVDSLHHQEEEGEDGWKRNDALTSIAGGLQAVSREKGGFGFRF-NH_2
Cephalochordates	
Amphioxus*	SAMLQQLAQQLKNRPREKGGFTFRF-NH_2

FIGURE 2 Comparison of 26RFa/43RFa sequences from cephalochordate to mammals. Basic amino acids are in bold letters except for the RF-NH_2 motif. To maximize structural similarity, a residue deletion denoted by (•) has been introduced into the guinea pig sequence. *, Peptide sequence deduced from the cDNA sequence.

The distribution of 26RFa binding sites and GPR103 mRNA has been studied by autoradiography and in situ hybridization, respectively. In the rat brain, 26RFa binding sites are widely distributed, the highest densities of recognition sites being found in the granular cell layer of the olfactory bulb, the piriform cortex, the hippocampal formation, the amygdaloid complex, several hypothalamic nuclei, the inferior colliculus, the dorsal raphe, and the accessory abducens, ambiguus, parasolitary, and Roller's nuclei of the medulla oblongata.[3] A very high density of binding sites is also observed in the dorsal horn of the rat spinal cord.[3] QRFP-r1 mRNA is actively expressed in the arcuate, ventromedial, and paraventricular nuclei of the hypothalamus and the amygdala, whereas QRFP-r2 mRNA is particularly abundant in the reuniens and parafascicular nuclei of the thalamus, the medial preoptic area, and the hypoglossal nucleus (Fig. 3).[13] Differential expression of the two receptor isoforms is also observed in the mouse brain: GPR103A mRNA is abundant in the olfactory bulb and the nucleus of the solitary tract, whereas GPR103B mRNA is primarily

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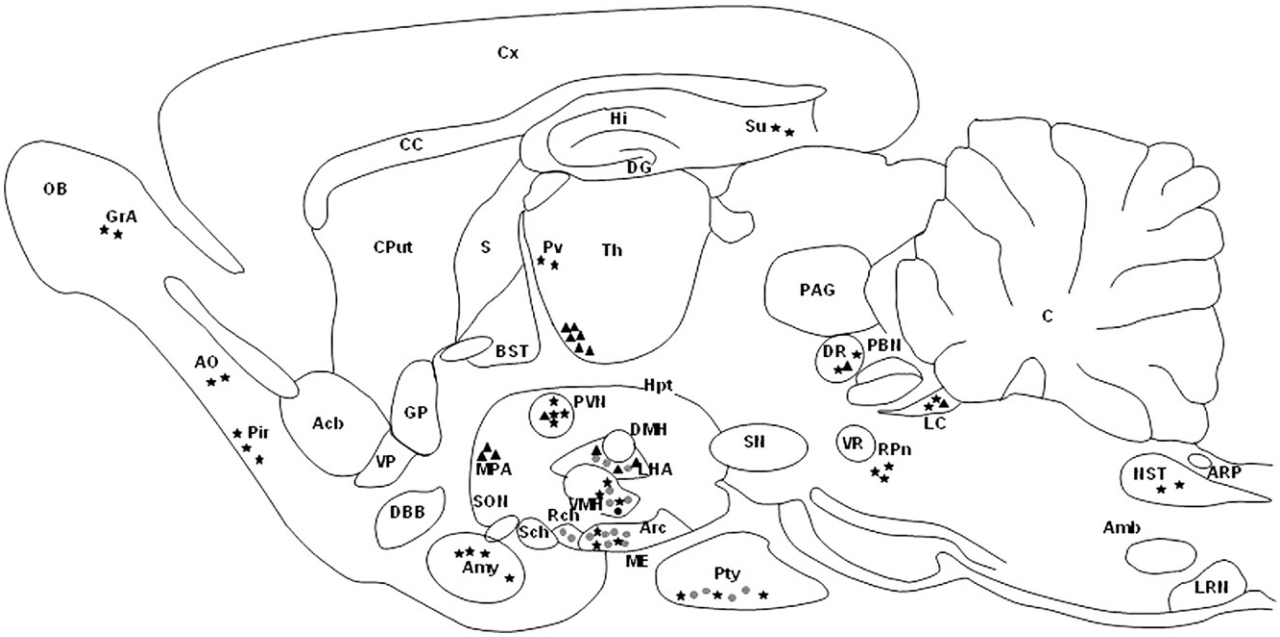

FIGURE 3 Localization of 26RFa and its receptors in the brain. Schematic parasagittal section through the rat brain depicting the distribution of 26RFa-mRNA-, QRFP-r1-mRNA-, and QRFP-r2-mRNA-expressing cells. (●), 26RFa; (★), QRFP-r1; (▲), QRFP-r2; Acb, nucleus accumbens; Amb, nucleus ambiguous; Amy, amygdale; AO, anterior olfactory nucleus; Arc, arcuate nucleus of the hypothalamus; ARP, area postrema; BST, bed nucleus of the stria terminalis; C, cerebellum; CC, corpus callosum; Cput, caudate putamen; Cx, cerebral cortex; DBB, diagonal band of Broca; DG, dentate gyrus; DMH, dorsomedial nucleus of the hypothalamus; DR, dorsal raphe nucleus; GP, globus pallidus; GrA, granular cell layer of the accessory olfactory bulb; Hi, hippocampus; Hpt, hypothalamus; LC, locus coeruleus; LHA, lateral hypothalamic area; LRN, lateral reticular nucleus; ME, median eminence; MPA, medial preoptic area; NST, nucleus of the solitary tract; OB, olfactory bulb; PAG, periaqueductal gray; PBN, parabrachial nucleus; Pir, piriform complex; Pty, pituitary; Pv, paraventricular thalamic nucleus; PVN, paraventricular nucleus of the hypothalamus; Rch, retrochiasmatic nucleus; RPn, raphe pontis nucleus; S, septum; Sch, suprachiasmatic nucleus; SN, substantia nigra; SON, supraoptic nucleus; Su, subiculum; Th, thalamus; VMH, ventromedial nucleus of the hypothalamus; VP, ventral pallidum; VR, ventral raphe nucleus.

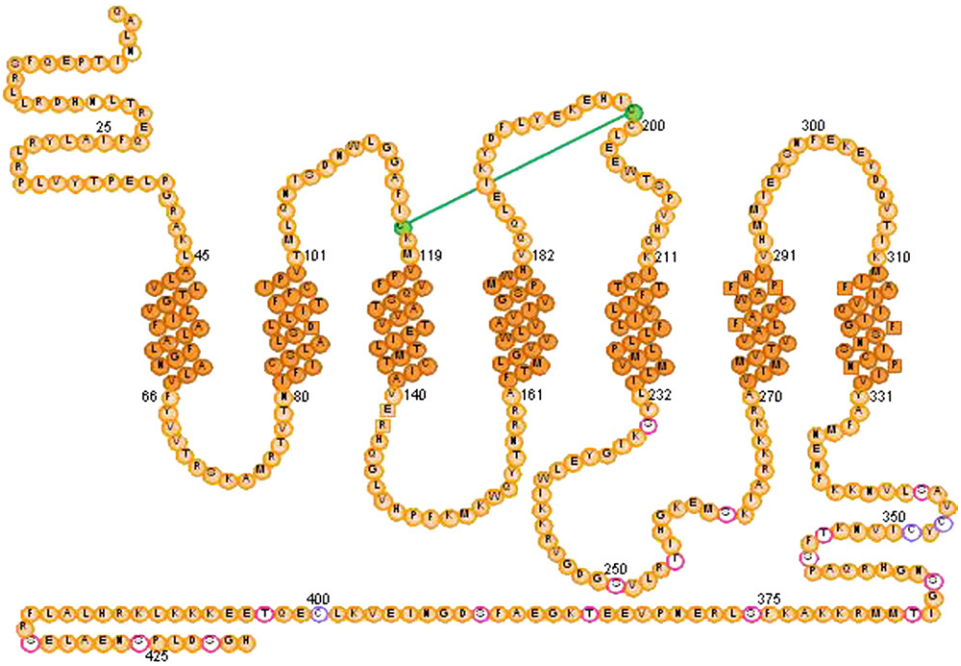

FIGURE 4 Schematic representation of human GPR103. The seven transmembrane domains are represented by dark gray discs and the extra-and intracellular domains by light gray discs. Potential glycosylation, phosphorylation, and palmitoylation sites are shown in white discs. The disulfide bridge between the two cysteine residues located in the first and second extracellular loops is indicated. The Asp residue in TM2 involved in G protein coupling, the conserved Glu-Arg in the second intracellular loop, and the conserved Phe, Pro, and Asn residues in TM6 and TM7 are represented by squares.

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found in the paraventricular hypothalamic nucleus, the magnocellular nucleus of the lateral hypothalamus, the median mammillary nucleus, and the reticular nucleus.[22]

GPR103 is also expressed in several peripheral organs such as the pituitary, the eye, the zona glomerulosa of the adrenal cortex, and the testis in rat,[10] and the eye and bones in mouse.[1] In the human brain, the highest concentrations of GPR103 are observed in the cerebral cortex, hypothalamus, and vestibular nuclei.[12] In human peripheral organs, the GPR103 gene is expressed in the retina, pituitary, heart, kidney, testis, and bone.[1,10,15]

SOLUTION CONFORMATION

The three-dimensional structure of 26RFa has been elucidated by circular dichroism and molecular modeling under NMR constraints in various media.[23] In methanol, 26RFa adopts an α-helical conformation in its central region from Pro^4 to Arg^{17} while the N- and C-terminal extremities are apparently not structured (Fig. 5). The strong conservation, from amphibians to mammals, of the amino acids responsible for the amphipatic characteristic of the α-helix and those of the flexible C-terminal tail suggest that these two regions play a pivotal role in the interaction of 26RFa with GPR103 and/or in receptor activation.

STRUCTURE–ACTIVITY RELATIONSHIPS

Structure–activity relationships of 26RFa have been assessed by measuring the Ca^{2+}-mobilizing activity of human 26RFa-related analogs in *h*GPR103-transfected CHO cells. Peptide truncation studies indicate that the biologically active domain of 26RFa is located in the C-terminal region and that $26RFa_{(20–26)}$ is the shortest analog that retains full efficacy to increase $[Ca^{2+}]_i$ in GPR103-transfected cells.[16] Systematic replacement of each amino acid of $26RFa_{(20–26)}$ by an alanine (Ala scan) or its D-enantiomer (D scan) shows that the last three C-terminal residues are very sensitive to these modifications while position 23 tolerates both substitutions rather well.[16] Most importantly, replacement of Ser^{23} by a norvaline leads to an analog, $[Nva^{23}]26RFa_{(20–26)}$, which is 3-fold more potent than the native heptapeptide.[16]

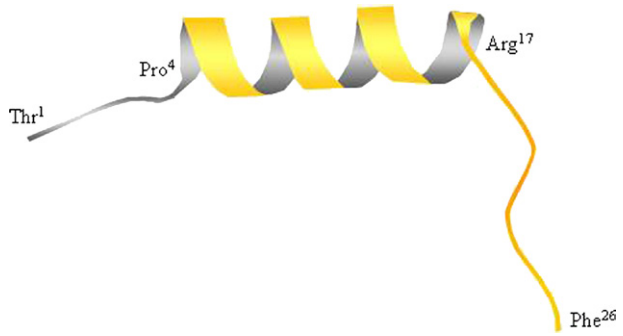

FIGURE 5 Three-dimensional NMR structure of human 26RFa in methanol. 26RFa consists of an α-helix between residues Pro^4 and Arg^{17} flanked by two nonstructured N- and C-terminal segments.

BIOLOGICAL ACTIONS WITHIN THE BRAIN (AND PITUITARY)

The occurrence of 26RFa mRNA, 26RFa immunoreactivity and GPR103 mRNA in hypothalamic nuclei controlling appetite and feeding behavior prompted several groups to investigate the effect of 26RFa and related peptides on food consumption. Acute icv injection of low doses of 26RFa induces a dose-dependent stimulation of food intake in mice.[6,22] 43RFa and the C-terminal fragment $26RFa_{(20–26)}$ both mimic the stimulatory effect of 26RFa on feeding.[7,18] Chronic infusion of 43RFa in mice provokes hyperphagia associated with a significant increase in body weight and fat mass.[18] In rat, icv injection of 26RFa moderately stimulates feeding in normally fed rat without modifying energy balance parameters.[13] 43RFa increases food intake when the animals are fed a high-fat diet but has no effect on rat under regular diet conditions.[20,21] In mouse,[22] as in rat,[14] 26RFa/43RFa-induced food intake is blocked by Y1 receptor antagonists indicating that 26RFa/43RFa exerts its orexigenic effect through activation of NPY neurons.

Reciprocally, energy balance influences 26RFa gene expression. Thus, 26RFa mRNA is upregulated in genetically obese *ob/ob* and *db/db* mice.[22] 26RFa mRNA expression is also enhanced in fasting mice as compared with that of normally fed mice.[22] In rat, 26RFa mRNA level is increased in the ventromedial and arcuate nuclei in animals fed a high-fat diet.[21] Surprisingly, however, 43RFa peptide concentration is higher in rats fed a low-fat diet than in rats under regular diet and is undetectable in rats fed a high-fat diet.[2] In goldfish, food deprivation significantly increases 26RFa and NPY gene expression in the hypothalamus.[17] Finally, in a perfused rat pancreas model, 26RFa inhibits glucose- and arginine-evoked insulin secretion.[8] Altogether, these observations suggest that 26RFa/43RFa plays a physiological role in the regulation of food intake and energy homeostasis.

26RFa and related peptides exert multiple actions on the hypothalamic–pituitary–gonadal axis. Thus, icv injection of 43RFa, 26RFa, or $26RFa_{(20–26)}$ induces an increase in plasma luteinizing hormone (LH) and follicle-stimulating hormone (FSH) concentrations in rat.[19,20] Because high concentrations of GPR103 mRNA and 26RFa binding sites occur in the preoptic area[3] where gonadotropin-releasing hormone (GnRH) neurons are located (see chapter on GnRH in this section of the book), these results suggest that 26RFa may activate the gonadotropic axis through the stimulation of GnRH release. In addition, 43RFa, 26RFa, and $26RFa_{(20–26)}$ stimulate basal and GnRH-induced LH and FSH secretion from rat pituitary explants.[19] This observation, together

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with the presence of GPR103 mRNA in the pituitary,[10,12,19] indicates that 26RFa may act at the pituitary level to stimulate gonadotropin secretion. Finally, GPR103 mRNA is expressed in the testis,[10] suggesting that 26RFa may regulate gonadotropic function through a direct action on the testis. In the goldfish, intraperitoneal injection of 26RFa causes a transient increase in plasma LH concentration, but 26RFa has no effect on LH release from pituitary cells in primary culture,[17] suggesting that, in fish, the peptide may stimulate the gonadotropic axis by acting exclusively at the hypothalamic level.

In the rat, the highest concentration of GPR103 mRNA is found in the adrenal gland.[10] Consistent with this observation, intravenous (iv) injection of 43RFa causes a dose-dependent increase in plasma aldosterone levels.[10] The fact that 43RFa does not modify corticosterone secretion[10] indicates that the peptide acts specifically on zona glomerulosa cells of the adrenal cortex.

The GPR103 gene is actively expressed in the spinal cord of mice.[12] In the spinal cord of rats, high concentrations of GPR103 mRNA and 26RFa binding sites are found in the superficial layers of the dorsal horn.[3] In the human spinal cord, 26RFa mRNA is abundant in the dorsal horn.[4] These observations strongly suggest that 43RFa/26RFa could be involved in nociceptive transmission. In support of this hypothesis, intrathecal administration of 26RFa in rats significantly reduces the frequency of agitation behaviors in the paw formalin injection test and attenuates the level of mechanical allodynia in the paw carrageenan injection test.[28] The analgesic effect of 26RFa in the formalin test is also observed after icv injection of the peptide[27] indicating that 26RFa also modulates nociceptive transmission at a supraspinal level.

Central and peripheral administrations of 26RFa and related peptides induce several other biological responses. In particular, icv injection of 26RFa or 43RFa in mice exerts a dose-dependent stimulatory effect on locomotor activity.[7,22] The action of 26RFa/43RFa on locomotion is not mimicked by the C-terminal heptapeptide $26RFa_{(20–26)}$, suggesting that the effects of 26RFa on food intake and on locomotor activity may be mediated through distinct receptors.[7] Central injection of 43RFa in mice causes a rise in blood pressure and heart rate.[22] Iv injection of 26RFa in anesthetized rats induces a biphasic effect on blood pressure (i.e., hypotension followed by hypertension) associated with tachycardia.[9] The pressure response is inhibited by α- and β-adrenoreceptor antagonists, while the tachycardia response is attenuated by cervical vagotomy and a β-adrenoreceptor antagonist,[9] suggesting that the peripheral cardiovascular effects of 26RFa are mediated via the vagal nerve and involve a (nor)adregernic pathway.

PATHOPHYSIOLOGICAL IMPLICATIONS

Phenotype analysis of GPR103A-deficient mice reveals that homozygous mutant mice are viable and fertile but suffer from severe osteopenia accompanied by a decrease in bone density that is more pronounced in females than in males.[1] In addition, $GPR103A^{-/-}$ mice exhibit pronounced kyphosis similar to patients with osteoporosis.[1] Histological analysis indicates that the decrease in bone density in KO mice results from a reduction in the number of osteoclasts and a deterioration of the osteochondral plate. It thus seems that the activation of GPR103A by 26RFa is involved in bone formation through direct action at the osteochondral plate. Four single-nucleotide polymorphisms (SNPs) have been identified in the osteopenic SAMP6 mouse genomic DNA. In particular, one SNP is found in the promoter region of the 26RFa gene that generates a novel neuron-restrictive silencing factor binding site, a repressor that decreases gene expression in non-neuronal tissues.[29] Accordingly, a significantly lower expression of the 26RFa transcript has been found in lumbar spine, but also in the hypothalamus of SAMP6 mice compared with that of wild-type mice,[29] suggesting that a decrease in 26RFa production may contribute to the osteoporosis phenotype of SAMP6 mice.

ACKNOWLEDGMENTS

Experimental work conducted in the authors' laboratory was supported by INSERM (U413/U982), an INSERM-JSPS exchange program grant to K. Tsutsui and H. Vaudry and the Région Haute-Normandie.

REFERENCES

1. Baribault H, Danao J, Gupte J, Yang L, Sun B, Richards W, et al. The G protein-coupled receptor GPR103 regulates bone formation. *Mol Cell Biol* 2006;**26**:709–17.
2. Beck B, Richy S. Suppression of QRFP 43 in the hypothalamic ventromedial nucleus of Long-Evans rats fed a high-fat diet. *Biochem Biophys Res Commun* 2009;**383**:78–82.
3. Bruzzone F, Lectez B, Alexandre D, Jégou S, Mounien L, Tollemer H, et al. Distribution of 26RFa binding sites and GPR103 mRNA in the central nervous system of the rat. *J Comp Neurol* 2007;**503**:573–91.
4. Bruzzone F, Lectez B, Tollemer H, Leprince J, Dujardin C, Rachidi W, et al. Anatomical distribution and biochemical characterization of the novel RFamide peptide 26RFa in the human hypothalamus and spinal cord. *J Neurochem* 2006;**99**:616–27.
5. Chartrel N, Alonzeau J, Alexandre D, Jeandel L, Alvear-Perez R, Leprince J, et al. The RFamide neuropeptide 26RFa and its role in the control of neuroendocrine functions. *Front Neuroendocrinol* 2011;**32**:387–97.
6. Chartrel N, Dujardin C, Anouar Y, Leprince J, Decker A, Clerens S, et al. Identification of 26RFa, a hypothalamic neuropeptide of the RFamide peptide family with orexigenic activity. *Proc Natl Acad Sci U S A* 2003;**100**:15247–52.

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7. Do Rego JC, Leprince J, Chartrel N, Vaudry H, Costentin J. Behavioral effects of 26RFamide and related peptides. *Peptides* 2006;**27**: 2715–21.
8. Egido EM, Hernández R, Leprince J, Chartrel N, Vaudry H, Marco J, et al. 26RFa, a novel orexigenic neuropeptide, inhibits insulin secretion in the rat pancreas. *Peptides* 2007;**28**:725–30.
9. Fang Q, Liu Q, Jiang TN, Li YL, Yan X, Wang R. Cardiovascular effects of intravenous administered 26RFa, a novel RFamide peptide ligand for GPR103, in anaesthetized rats. *Eur J Pharmacol* 2009;**621**:61–6.
10. Fukusumi S, Yoshida H, Fujii R, Maruyama M, Komatsu H, Habata Y, et al. A new peptidic ligand and its receptor regulating adrenal function in rats. *J Biol Chem* 2003;**278**:46387–495.
11. Gouardères C, Mazarguil H, Mollereau C, Chartrel N, Leprince J, Vaudry H, et al. Functional differences between NPFF1 and NPFF2 receptor coupling: high intrinsic activities of RFamide-related peptides on stimulation of [^{35}S]GTPγS binding. *Neuropharmacology* 2007;**52**:376–86.
12. Jiang Y, Luo L, Gustafson EL, Yadav D, Laverty M, Murgolo N, et al. Identification and characterization of a novel RF-amide peptide ligand for orphan G-protein-coupled receptor SP9155. *J Biol Chem* 2003;**278**:27652–7.
13. Kampe J, Wiedmer P, Pfluger PT, Castaneda TR, Burget L, Mondala H, et al. Effect of central administration of QRFP(26) peptide on energy balance and characterization of a second QRFP receptor in rat. *Brain Res* 2006;**1119**:133–49.
14. Lectez B, Jeandel L, El-Yamani FZ, Arthaud S, Alexandre D, Mardargent A, et al. The orexigenic activity of the hypothalamic neuropeptide 26RFa is mediated by the neuropeptide Y and proopiomelanocortin neurons of the arcuate nucleus. *Endocrinology* 2009;**150**:2342–50.
15. Lee DK, Nguyen T, Lynch KR, Cheng R, Vanti WB, Arkhitko O, et al. Discovery and mapping of ten novel G protein coupled receptor genes. *Gene* 2001;**275**:83–91.
16. Le Marec O, Neveu C, Lefranc B, Dubessy C, Boutin JA, Do Régo JC, et al. Structure–activity relationships of a series of analogues of the RFamide-related peptide 26RFa. *J Med Chem* 2011;**54**:4808–14.
17. Liu Y, Zhang Y, Li S, Huang W, Liu X, Lu D, et al. Molecular cloning and functional characterization of the first non-mammalian 26RFa/QRFP orthologue in goldfish, *Carassius auratus*. *Mol Cell Endocrinol* 2009;**303**:82–90.
18. Moriya R, Sano H, Umeda T, Ito M, Takahashi Y, Matsuda M, et al. RFamide peptide QRFP43 causes obesity with hyperphagia and reduced thermogenesis in mice. *Endocrinology* 2006;**147**:2916–22.
19. Navarro VM, Fernández-Fernández R, Nogueiras R, Vigo E, Tovar S, Chartrel N, et al. Novel role of 26RFa, a hypothalamic RFamide orexigenic peptide, as putative regulator of the gonadotropic axis. *J Physiol* 2006;**573**:237–49.
20. Patel SR, Murphy KG, Thompson EL, Patterson M, Curtis AE, Ghatei MA, et al. Pyroglutamylated RFamide peptide 43 stimulates the hypothalamic–pituitary–gonadal axis via gonadotropin-releasing hormone in rats. *Endocrinology* 2008;**149**:4747–54.
21. Primeaux SD, Blackmon C, Barnes MJ, Braymer D, Bray GA. Central administration of the RFamide peptides, QRFP-26 and QRFP-43, increases high fat food intake in rats. *Peptides* 2008;**29**:1994–2000.
22. Takayasu S, Sakurai T, Iwasaki S, Teranishi H, Yamanaka A, Williams SC, et al. A neuropeptide ligand of the G protein-coupled receptor GPR103 regulates feeding, behavioral arousal, and blood pressure in mice. *Proc Natl Acad Sci U S A* 2006;**103**:7438–43.
23. Thuau R, Guilhaudis L, Ségalas-Milazzo I, Chartrel N, Oulyadi H, Boivin S, et al. Structural studies on 26RFa, a novel human RFamide-related peptide with orexigenic activity. *Peptides* 2005;**26**:779–89.
24. Tobari Y, Iijima N, Tsunekawa K, Osugi T, Haraguchi S, Ubuka T, et al. Identification, localisation and functional implication of 26RFa orthologue peptide in the brain of zebra finch (*Taeniopygia guttata*). *J Neuroendocrinol* 2011;**23**:791–803.
25. Ukena K, Tachibana T, Iwakoshi-Ukena E, Saito Y, Minakata H, Kawaguchi R, et al. Identification, localization, and function of a novel hypothalamic neuropeptide, 26RFa, and its cognate receptor, G protein-coupled receptor-103. *Endocrinology* 2010;**151**:2255–64.
26. Ukena K, Vaudry H, Leprince J, Tsutsui K. Molecular evolution and functional characterization of the orexigenic peptide 26RFa and its receptor in vertebrates. *Cell Tissue Res* 2011;**343**:475–81.
27. Yamamoto T, Miyazaki R, Yamada T. Intracerebroventricular administration of 26RFa produces an analgesic effect in the rat formalin test. *Peptides* 2009;**30**:1683–8.
28. Yamamoto T, Wada T, Miyazaki R. Analgesic effects of intrathecally administered 26RFa, an intrinsic agonist for GPR103, on formalin test and carrageenan test in rats. *Neuroscience* 2008;**157**:214–22.
29. Zhang Q, Qiu P, Arreaza MG, Simon JS, Golovko A, Laverty M, et al. P518/Qrfp sequence polymorphisms in SAMP6 osteopenic mouse. *Genomics* 2007;**90**:629–35.

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Chapter 123

Secretin

Stephanie Y.L. Ng, Carrie Y.Y. Cheng and Billy K.C. Chow

ABSTRACT

Since its initial discovery, secretin is now coined as a classical hormone with widespread distribution. In particular, numerous studies have demonstrated the abundant expression of secretin and its receptor within various brain areas sparking interest of its potential as a brain peptide. With gene knockout animals becoming widely available, recent physiological studies have provided a glimpse of secretin's role in appetite control and osmoregulation, establishing its potential as a brain peptide. This chapter focuses on secretin and its receptor, reviewing our current knowledge in the structural and molecular aspects, central effects and the pathophysiological implications in cognitive behavior.

DISCOVERY

The discovery of secretin in the beginning of the last century introduced the concept of hormone and hence initiated the field of endocrinology. Ivan Petrovich Pavlov showed that the pancreatic secretion was stimulated by the acidity in chyme rather than food, and he believed that the secretion was control by nerves. However, the hypothesis was challenged when researchers showed that the introduction of acid was able to excite pancreatic secretion even after denervation. In 1902, two British physiologists, Ernest Starling and William Bayliss, observed that intravenous injection of a mucosal extract, produced in the duodenum on acid infusion in an anesthetized dog, stimulated pancreatic secretion. This study clearly indicated the presence of an active substance originated from the intestinal mucosa and carried as a blood-borne agent to stimulate pancreatic secretion. This active substance was later named as secretin, and the term "hormone" has been coined ever since, meaning chemical messenger transmitting information from one organ to another via blood circulation.

STRUCTURE OF THE mRNA/GENE

Since its discovery, the secretin genes have been characterized in different species (Fig. 1). Among species, a conservation of gene environment is revealed by chromosomal synteny analysis and a common four-exon structure is also shared (Figs. 2 and 3). In the rat genome, the secretin gene is 813 nucleotides in length. The first exon encodes for the signal peptide and part of the N-terminal peptide; the second exon encodes entirely for the biologically active secretin peptide and processing signal sequences; and the remaining two exons encode for the C-terminal peptide of the prosecretin. Comparison of the secretin gene with genes within the same peptide family reveals similar structural organization suggesting that they are evolved from a common ancestral gene. In particular, a high degree of similarity is shared in the exon(s) encoding the biologically active peptides that are physiologically important, while the C-terminal peptide-encoding exons show a lower level of similarity. In contrast to the active peptides of gastric inhibitory polypeptide (GIP) and growth hormone-releasing hormone (GHRH) that are encoded in two exons, active secretin alongside with other superfamily peptide members (glucagon, glucagon-like peptides (GLPs), vasoactive intestinal peptide (VIP), pituitary adenylate cyclase-activating polypeptide (PACAP), peptide histidine methionine, and PACAP-related peptide (PRP)) are encoded by one exon. Among its superfamily, the SCT gene is unique in encoding for only a single copy of biologically active peptide and lacking a sequestering intron between the transcriptional and translational start sites.

PROCESSING OF THE PRECURSOR

The structure of secretin's precursor has been deduced from a range of mammalian cDNA sequences. In the pig, the precursor consists of a signal peptide, a short N-terminal flanking sequence, a 27 amino acid secretin peptide which is followed by a Gly-Lys-Arg cleavage and amidation sequence

Human	**HSDGTFTSELSRLREGARLQRLLQGLV-**
Pig	**HSDGTFTSELSRLRE**SARLQRLLQGLV-
Rat	**HSDGTFTSELSRLR**QDS**RLQRLLQGLV-**
Mouse	**HSDGMFTSELSRLR**QDS**RLQRLLQGLV-**
R.rugulosa	**H**V**DGMFTSE**F**SRAR**GS**A**AIRKIINSALA
X.laevis	**H**V**DGR**F**TSE**F**SRAR**GS**A**AIRKIINSALA

FIGURE 1 Amino acid sequence alignments of secretin from various species. Residues identical to that of human secretin are boldfaced.

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00123-8
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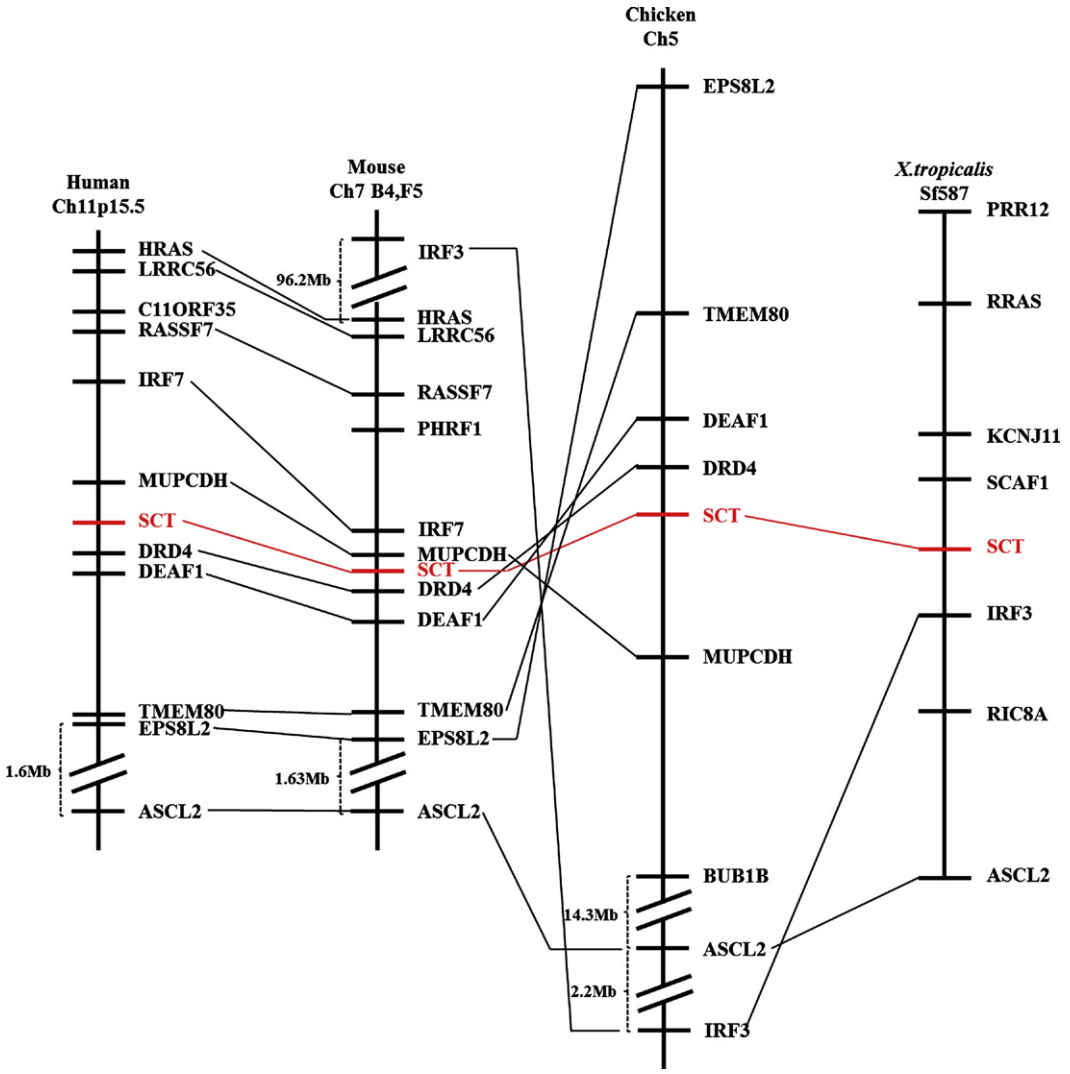

FIGURE 2 Chromosomal locations of secretin in various vertebrate species. Genes adjacent to secretin in various genomes are shown and linked to show their similarities in chromosomal location. The genes are named according to their annotation in the human genome. Secretin genes are highlighted in gray.

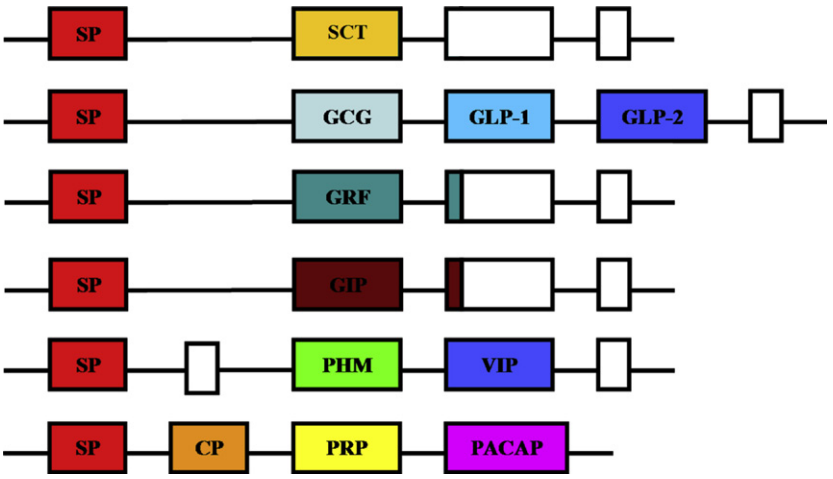

FIGURE 3 The secretin superfamily genes. Boxes denote exons while introns are depicted by lines, which are both not drawn to scale or proportional for ease of gene alignment. The following abbreviations denote: signal peptide, SP; cryptic peptide, CP. See color plate 35.

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and a 72 amino acid C-terminal extension peptide.[33] This was initially believed to be the sole secretin precursor form to exist. However, analysis of porcine intestinal RNAs soon suggested otherwise with findings of at least two scarcer and shorter transcripts whereby in one form a single arginine residue replaces a 32 amino acid sequence preceding the Gly-Lys-Arg motif;[33] and another form which lacks the third exon and only encodes a 44 amino acid C-terminal extension peptide.[33] Differential splicing has then been suggested to be the mechanism responsible for generating these variant transcripts.[33] These transcripts, together with the longer form, have full bioactivity and are the precursor molecules (preprosecretin) from which the 27 amino acid C-terminally amidated secretin (secretin-27) and its Gly (secretin-28), and Gly-Lys-Arg (secretin-30) C-terminally extended intermediates are synthesized from. Two alternative processing pathways are involved such that the different bioactive secretin forms are representatives of intermediate products in the biosynthetic pathway.[4] In the first step of preprosecretin processing, signal peptidase targets the Ala-Arg bond in positions −10 and −9 upstream from secretin's N-terminus to produce an 80 amino acid prosecretin, which then proceeds into one of the two alternative pathways. In one pathway, the signal peptide and N-terminal flanking peptide are cleaved to produce a 71 amino acid intermediate form consisting of secretin extended by the Gly-Lys-Arg amidation region and a C-terminal extension peptide. A trypsin-like peptidase subsequently targets the dibasic site; cleaving C-terminally of the amidation site to produce secretin extended by Gly-Lys-Arg. Secretin-Gly is then obtained by the removal of the latter two residues by carboxypeptidase (see the carboxypeptidase chapter in Peptide Biosynthesis Processing section of this book). Lastly, peptide amide monoamine oxygenase (see the PAM chapter in same section just mentioned) then acts on the intermediate to produce the final product of secretin with a C-terminally amidated valine residue. Alternatively, prosecretin can also be processed first at its C-terminal end before cleavage of its N-terminal flanking peptide.

DISTRIBUTION OF THE mRNA/PEPTIDE

In recent years, our understanding of secretin functioning as a neuropeptide has advanced considerably. Many studies have reported and confirmed the widespread occurrence of secretin protein and transcript in the central nervous system (CNS) by reverse transcriptase-polymerase chain reaction (RT-PCR), Northern blot, in situ hybridization and immunohistochemistry studies. Before 1981, secretin immunoreactivity had been mapped in the CNS of the rat and pig with a highly specific radioimmunoassay. Secretin concentrations are high in the olfactory bulb, thalamus and hypothalamus, moderate in the striatum, septum, hippocampus, midbrain, and cerebellum, and low in the cerebral cortex, medulla oblongata and pons. By using RT-PCR, secretin precursor mRNA was detected in the olfactory bulb, thalamus, hypothalamus, cerebral cortex, hippocampus, midbrain, cerebellum, medulla oblongata, pons, and pituitary. Contrary to the findings of previous studies, the expression level of secretin precursor mRNA is highest in the medulla oblongata, pons and the pituitary at levels comparable with those in duodenum. Secretin has subsequently been found in other brain regions including the brainstem and the sensory ganglia.[19]

Aside from a general view of the expression of secretin in the brain, cellular localization of secretin in some of the brain regions have also been studied in detail. In the cerebellum, secretin immunoreactivity is found in Purkinje cells, basket cells, central cerebellar nuclei, and the superior cerebellar peduncle.[19] On closer examination, secretin is present in the soma, dendrites and axon[19] inside Purkinje cells. In the hypothalamus, secretin is expressed in several hypothalamic nuclei including the paraventricular nucleus (PVN), supraoptic nucleus (SON),[19] and arcuate nucleus (Arc).[7] Secretin is also colocalized with angiotensin type 1 receptors in the PVN.[21] In the cerebral cortex, secretin is found in the hindlimb area of the motor cortex and also pyramidal cells in both external and internal pyramidal layers.[19] In the brainstem, secretin immunoreactivity is present in the mesencephalic nucleus of the trigeminal nerve, the superior olivary nucleus, and scattered cells of the reticular formation.[19]

The distribution of secretin was not only studied in the adult brain but also during embryonic development. Secretin transcripts have been detected in the central amygdala, hippocampus, cerebellum, area postrema, and the nucleus of the solitary tract (NTS) during postnatal day 7 (P7) to P60 in rats. Within most brain regions, secretin transcripts are more abundant in postnatal day 7 and 14 rats when compared with P21, P30, and P60 rats, suggesting that the expression of the secretin gene is developmentally regulated during the first few weeks after birth.[31] The ontogeny of secretin expression has also been studied in the mouse brain. Siu et al. showed that secretin transcripts are expressed as early as at E10.5 in the midbrain and hindbrain regions. In the midbrain region, secretin transcripts and peptides are present in the cephalic mesenschyme at E10.5 and E12.5 and the mesencephalic flexure and tegmentum at E14.5 and E16.5. In the hindbrain region, secretin transcripts and peptides are found in the cerebellar primordium from E10.5 to E16.5 and in the choroid plexus from E12.5 to E16.5.[30] Another research group has also shown the expression of secretin transcripts in discrete areas of the mouse brain during development, including the gigantocellular reticular nucleus, the raphe magnus nucleus, and the lateral paragigantocellular nucleus.[23] These studies complement the previous results on the expression of secretin in the brain of adult mammals and also suggest a functional role of secretin during embryonic development.

Like its ligand, secretin receptor is also widely expressed throughout the CNS. Within the cerebellum, positive signals for secretin receptor knockout (SCTR) are present in Purkinje

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cells and basket cells. Aside from the cerebellum, SCTR is also found in various brain regions. By the use of autoradiography with I^{125}-secretin, it was shown that intense binding is present in the accumbens nucleus, laterodorsal thalamic nucleus and NTS, whereas there is moderate binding in the olfactory bulb, caudate/putamen, lateral septal nucleus, amygdale, hippocampus, hypothalamus, dorsal raphe nucleus, and locus coeruleus, pineal gland, and pituitary (Fig. 4).

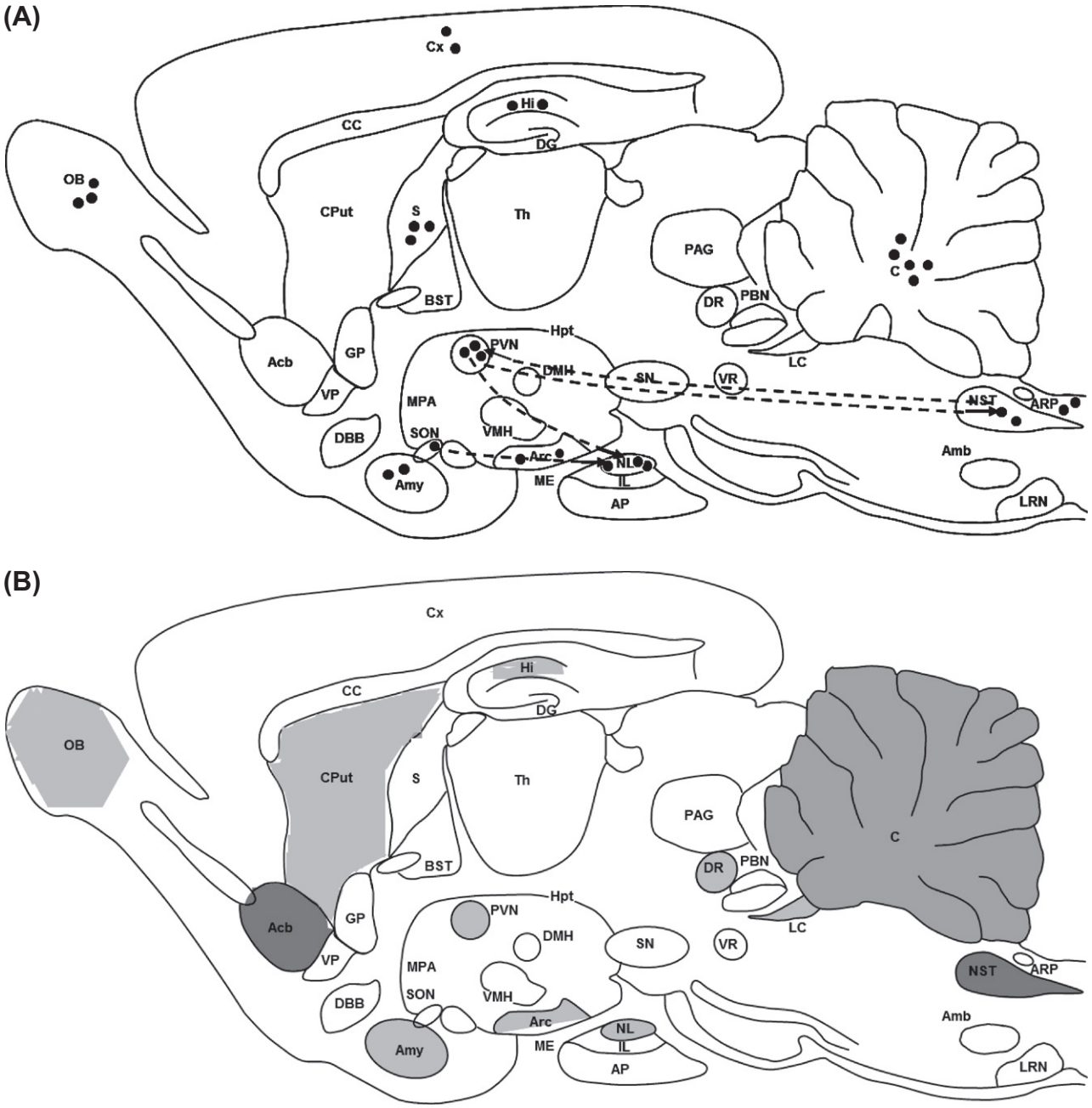

FIGURE 4 Distribution of (A) secretin and (B) secretin receptor in a parasagittal section of the rat brain. Secretin positive perikarya are denoted by black circles, fibers/projections by stippled lines. Regions with positive signals for secretin receptor are denoted by shading ▬ represents regions showing intense binding to I^{125}-secretin; ▭ represents regions showing moderate binding to I^{125}-secretin. Abbreviations: Acb, nucleus accumbens; Amb, nucleus ambiguus; Amy, amygdala; AP, anterior pituitary; Arc, arcuate nucleus of hypothalamus; ARP, area postrema; BST, bed nucleus of stria terminalis; C, cerebellum; CC, corpus callosum; CPut, caudate putamen; Cx, cerebral cortex; DBB, diagonal band of Broca; DG, dentate gyrus; DMH, dorsomedial nucleus of hypothalamus; DR, dorsal raphe nucleus; GP, globus palidus; Hi, hippocampus; Hpt, hypothalamus; IL, intermediate lobe of pituitary; LC, locus coeruleus; LRN, lateral reticular nucleus; ME, median eminence; MPA, medial preoptic area; NL, neural lobe of pituitary; NST, nucleus of solitary tract; OB, olfactory bulb; PAG, periaqueductal gray; PBN, parabrachial nucleus; PVN, paraventricular nucleus of hypothalamus; S, septum; SN, substantia nigra; SON, supraoptic nucleus; Th, thalamus; VMH, ventromedial nucleus of hypothalamus; VP, ventral pallidum; VR, ventral raphe nuclei.

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THE SECRETIN RECEPTOR AND ITS SIGNALING CASCADES

The Structure

By chronological order, the secretin receptor cDNA was first cloned in the rat followed by human, rabbit, and most recently in the lungfish (*Protopterus dolloi*) and frog (*Xenopus laevis*). The secretin receptors cDNAs encode for a 21–28 amino acid residues long N-terminal signal peptide, a single 1320–1392 base pair open reading frame encoding for a 440–464 amino acid mature protein. Among the secretin receptors, 62.6–69.0% sequence identity is shared with most similarity in the ligand binding and seven TM domains, and to a lesser extent in the signal peptide and C-terminal tail domains.

Of the range of secretin receptors, the detailed structure of mammalian forms, particularly of the rat has been most extensively studied. By truncation, site-directed mutagenesis, and chimeric receptor studies on the rat secretin receptor, the large extracellular N-terminal receptor domain has been shown to be a structurally independent, highly folded, disulfide-bonded domain, playing a direct role in recognition with the C-terminal portion of secretin.[12] Included are a series of six conserved cysteine residues ($Cys^{24,44,53,67,85,101}$) and one cysteine (Cys^{11}), which is present only in the most closely related family members. Together, they form three extracellularly located disulfide intradomain bonds that are implicated to be important for both receptor architecture and function.[2] Five putative N-linked glycosylation sites (NXS/T) are also distributed in the N-terminal ectodomain and second exoloop, and are essential in maintaining high affinity binding sites, which ultimately affect receptor function.[28] Secretin receptor structure is further supported by conserved charged amino acids in the extracellular region, namely, Asp^{49}, Arg^{83} in the N-terminus, Asp^{174} and Lys^{194} in the first exoloop, and Arg^{255} in the second exoloop.[10] At least four of the aforementioned amino acids are conserved throughout the secretin receptor superfamily and mutation of any of these residues results in severe reduction in secretin affinity and potency, and the receptor maximal response. Within the third endoloop, two conserved basic motifs, KLR and RLAR/K, have been found crucial in receptor conformation and functionality.[5] Mutagenesis studies reveal KLR to be necessary for efficient G protein coupling while RLAR/K contains the conformational determinant for receptor maturation and stability. Besides structurally supportive components, several regions have also been shown to be associated in ligand binding. These include amino acid residues 1–30, 34–41, and 103–110 distributed throughout the N-terminal domain and complementation by the first and second extracellular loops and the first, second, and sixth transmembrane domains to allow efficient secretin binding and receptor activation.[24]

Receptor Signaling and Regulation

On binding of secretin to its receptor, conformational change is induced to present an α-helical amphiphilic cationic receptor structure that interacts with heterotrimeric G proteins (α, β, and γ). G protein activation subsequently initiates the dissociation of guanosine triphosphate bound to the Gα subunit in exchange for guanosine diphosphate and ultimately triggers a series of downstream signaling cascades. Initially, it was believed that the secretin receptor coupled solely to $G\alpha_s$, stimulating the adenylyl cyclase (AC) pathway to accumulate cAMP. However, it was later discovered that high concentrations of secretin not only resulted in maximal cAMP formation but also transiently increased intracellular calcium (Ca^{2+}) levels in pancreatic acinar cells.[32] As a result, the secretin receptor is now also recognized to mediate independently through $G\alpha_q$ coupling and thereafter activating the inositol triphosphate, intracellular Ca^{2+} and diacylglycerol pathways.

Like some members of the β-adrenergic family of G protein-coupled receptors (GPCRs) where receptor phosphorylation has been identified to be the key, the secretin receptor is also desensitized in a similar fashion in response to agonist stimulation. Alternatively, the secretin receptor has also been suggested to undergo phosphorylation-independent internalization based on findings of restimulation with secretin failing to elicit cAMP response in CHO cells transfected with rat secretin receptors.[16] With later studies using cytosensor microphysiometry on the human secretin receptor, a clearer elucidation of receptor regulation has been postulated, whereby desensitization may be rapidly followed by resensitization.[26]

Receptor Dimerization

GPCRs were previously believed to exist and function as monomeric units; however, mounting evidence now documents the ability for receptors to traffic and signal within higher-order complexes. In fact, dimerization of GPCRs is now a widely accepted constitutive mechanism for both regulation and functional modification. In the past few years, Miller's group has concentrated much effort in exploring the dimerization status of SCTR, making it the most extensively studied class II B GPCR member (see his chapter on secretin in the Gastrointestinal Peptides section of this book). This aspect of receptor association has been investigated predominantly by fluorescence or bioluminescence resonance energy transfer (FRET/BRET) technology, using C-terminally tagged receptors with either *Renilla* luciferase or yellow fluorescent protein. By morphologic FRET microscopy, the secretin receptor has been shown to be capable of agonist-independent self-association (homodimerization), beginning as early as the start of biosynthesis with distribution in organelles (including the endoplasmic

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reticulum, Golgi clusters) of the maturation pathway, and in the plasma membrane.[22] The detailed molecular determinants to secretin receptor homodimerization were later studied with the lipid exposed segment of TM4 (particularly Gly^{243} and Ile^{247}) deemed as an important interface and necessary for efficient stimulation of the AC signaling pathway.[15] Furthermore, cysteine-scanning mutagenesis of 14 TM4 residues provides some information regarding the secretin receptor dimeric state: only three (Gly^{241}, Trp^{242}, and Pro^{245}) of the fourteen cysteine mutated TM4 residue constructs suffered loss in proper trafficking and signaling, and only three (Gly^{243}, Ile^{247}, and Ala^{250}) of the five lipid-facing cysteines exposed to cuprous phenanthroline to induce disulfide bonding were able to stabilize in the high affinity state.[11] In particular, the SCTR does not exist as a higher-order oligomer [13] but is able to heterodimerize with other secretin superfamily members.[14] Clearly, secretin receptor dimerization provides new insights into the molecular components involved in enhancing receptor function.

BIOLOGICAL ACTIONS WITHIN THE BRAIN (AND PITUITARY)

Cerebellum

Among various brain regions, the function of secretin in the brain has been best studied in the cerebellum. In 1981, the presence of secretin in cerebellum was detected by radioimmunoassay, and it was later confirmed by other studies using in situ hybridization and immunohistochemistry. These techniques further detail the cellular location of secretin, showing the expression of secretin in Purkinje neurons of adult humans and rats.[34] Inside Purkinje cells, secretin immunoreactivity is found in both the soma and dendrites.[34] For the secretin receptor, in situ hybridization reveals its expression in the GABAergic interneurons (basket cells) in addition to Purkinje cells.[34] The pattern of expression in the cerebellum suggests that Purkinje cells synthesize secretin which then targets Purkinje cells themselves and also the nearby basket cells. By use of whole-cell patch-clamp recordings from identified Purkinje neurons in acute rat cerebellar slices, it was found that secretin modulates synaptic input to Purkinje cells. Secretin increases the frequency and amplitude of evoked and spontaneous inhibitory postsynaptic currents (IPSCs) as well as the frequency of the miniature IPSCs originating from basket cells.[34]

Taken together, these results indicate an action of secretin in the cerebellum. Depolarization of Purkinje cells triggers the release of secretin from somatodendritic region. Secretin then binds to its receptor located on presynaptic basket cells to facilitate GABA release and thereby stabilize excited Purkinje cells in a negative feedback manner.[34] In addition to direct activation of presynaptic secretin receptors on basket cells, another mechanism of how secretin facilitates the inhibitory neurotransmission in the cerebellum has been suggested. The released secretin acts on an unknown glutamate source causing the release of glutamate which then enhances the release of GABA by activating presynaptic AMPA receptors.[20] Preliminary results also show that secretin may bind to its receptors on the same or nearby Purkinje cells; nevertheless, the function has not yet been identified.

Hippocampus

An earlier study has shown that secretin increases glutamate and GABA levels within the extracellular spaces in the hippocampus.[9] A study using secretin receptor knockout ($SCTR^{-/-}$) mice reveals the importance of secretin and its receptors in hippocampal synaptic plasticity, and social and cognitive behavior.[27] Hippocampal long-term potentiation (LTP) is a use-dependent increase in synaptic efficacy and is widely considered as one of the major cellular mechanisms that underlies memory. Hippocampal LTP was induced by the stimulation of the Schaffer collateral pathway in the CA1 area. In the study, Nishijima et al. showed that loss of secretin receptors causes deficits in synaptic transmission at Schaffer collateral synapses. In addition, loss of secretin receptors also provokes a significant reduction in LTP induction and maintenance, showing the necessity of secretin receptors for normal hippocampal function. The reduced synaptic transmission may correlate with the reduction in the density of dendritic spines displayed in the mutant mice, which is the primary place for connecting excitatory neuronal axons and for synaptic modification. The behavioral consequences brought about by the defect in synaptic plasticity include altered social behaviors and cognitive behaviors.[27]

Amygdala

Fos mapping shows that neurons in the central and medial amygdala are activated by intravenous, intraperitoneal, and intracerebroventricular administrations of secretin, suggesting that secretin may participate in the primary role of the amygdala in emotional learning. Consistent with this notion, it was shown that peripheral administration of secretin dose dependently decreases the magnitude of fear-potentiated startle in rats.[19] In humans, consistent results were also obtained. A functional magnetic resonance imaging (fMRI) study using blood oxygen level dependent (BOLD) changes in response to different facial stimuli showed that secretin treatment causes significant activation of the right amygdala during the viewing of fearful faces,[35] suggesting that secretin alters amygdala responsiveness to affective stimuli. The human and animal studies both suggest the role of secretin in modulating social behavior and recognition.

The amygdala has also been implicated in playing a stimulatory role in pancreatic exocrine secretion via the

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vagus nerve. Electrical stimulation of the medial amygdaloid nucleus significantly increases pancreatic secretion of water and bicarbonate output on secretin or intraduodenal HCl infusion in rats. However, medial amygdaloid stimulation is ineffective in stimulating pancreatic secretion in the control group receiving saline infusion,[19] thus implying that a certain level of plasma secretin is essential for the stimulatory effect of amygdala in pancreatic secretion.

Hypothalamus

The biological role of secretin in body water homeostasis has been studied for many years but not fully substantiated because of inconsistent findings regarding the renal functions of secretin. Recent studies using transgenic mice shed light on the role of secretin in body water homeostasis. *SCTR*$^{-/-}$ mice exhibit polyuria and polydipsia; they drink more water and produce more urine than their wildtype littermates. The underlying mechanism is similar to the way secretin promotes transepithelial solvent flux in cholangiocytes; it induces expression of AQP2 and stimulates trafficking of this water channel from intracellular vesicles to the plasma membrane in renal collecting tubules under hyperosmotic conditions. To regulate water homeostasis, secretin actually works at multiple levels including the hypothalamus and pituitary in addition to the kidneys. Chu et al.[8] showed that chronic hyperosmolality leads to an increase in plasma secretin level. By electrical stimulation of the PVN but not nearby sites in the hypothalamus, secretin is released from the posterior pituitary into the systemic circulation. In hypothalamic and pituitary explants, high concentrations of K^+ in the culture medium leading to K^+-evoked depolarization is able to stimulate secretin release via the activation of L-, N-, and P-type high-voltage-gated calcium channels.[19] Collectively, these results indicate that chronic hyperosmolality could lead to the release of secretin from the posterior pituitary into the systemic circulation by activating magnocellular neurons in the PVN of the hypothalamus. In addition, secretin could upregulate vasopressin expression level in the hypothalamus and stimulate vasopressin release from the hypophysial axis to the circulation.

As a brain-gut peptide, secretin is also involved in appetite control.[7] Central and peripheral secretin injections reduce food intake in wildtype mice but not in *SCTR*$^{-/-}$ mice, implying that secretin exerts its anorectic action via the secretin receptor. Secretin and its receptor are expressed in hypothalamic nuclei including the Arc and PVN that are the primary brain sites engaged in regulating body energy homeostasis. In the Arc, the secretin receptor is expressed in POMC neurons, which are powerful anorexigenic neurons. Consistent with this, secretin injection causes neuronal activation in these two areas, showing the importance of these two hypothalamic nuclei for secretin to control appetite. More importantly, both intraperitoneal and intracerebroventricular secretin are able to modulate transcript levels of several appetite-related genes in these two areas, including activating POMC transcripts in the Arc and melanocortin receptor 4 (MC4R) transcripts in the PVN. Previous injection of the MC4R antagonist, SHU9119, into the PVN is able to significantly reduce secretin-induced anorexia. Taken together, the results show that the anorectic function of secretin is mediated, at least in part, via the hypothalamic melanocortin system.

Lamina Terminalis

As mentioned above, there is a growing body of evidence showing that secretin is important in body fluid homeostasis. It works at multiple levels, including pituitary, hypothalamus, and kidney, to regulate water balance. Very recently, the use of transgenic mice has also revealed the involvement of the secretin/secretin receptor axis in the angiotensin II osmoregulatory pathways in maintaining fluid balance in the body.[21] The lamina terminalis consists of the organum vasculosum of the lamina terminalis (OVLT), the median preoptic nucleus (MnPO), and the subfornical organ (SFO), critical sites for regulating water intake. It has been proposed that angiotensin II stimulates the expression of secretin and its receptors in the SFO and OVLT under hypertonic conditions. The synthesized secretin then induces vasopressin expression and release into the circulation and eventually increases water reabsorption from the kidney. The centrally produced angiotensin II can also stimulate vasopressin and secretin expression and release in the PVN.

Other Brains Areas

In addition to the brain areas discussed above, secretin and its receptors have also been detected in other brain regions including olfactory bulb, septum, striatum, the NTS, and sensory ganglion. Mapping of Fos activation followed by intracerebroventricular injection of secretin reveals that this peptide can influence central networks involved in behavioral, endocrine, and autonomic regulation. Secretin-activated regions include the area postrema, dorsal motor nucleus, medial region of the NTS, lateral tegmentum, locus ceruleus, ventral periaqueductal gray, and paraventricular thalamic nucleus.[19] In the NTS, other than inducing Fos expression, secretin also exerts direct effects on the excitability of the neurons via activation of a nonselective cationic conductance (NSCC), which allows passage of cations (Na^+, K^+, or Ca^{2+}) in varying proportions.[19] The NTS is a pivotal region involved in the integration of cardiovascular, respiratory, gustatory, hepatic, and renal control mechanisms; it receives afferent input from the periphery and sends efferent output to many CNS areas. Consistent with this, previous studies have shown that intracerebroventricular injection of secretin in rats could increase defecation, alter the respiratory rate, and decrease blood pressure.[6]

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Therefore, the NTS might represent an important CNS site where endogenous and/or systemic secretin could act to influence central autonomic regulation and other brain-mediated activities.

PATHOPHYSIOLOGICAL IMPLICATIONS

Autism

Presenting at an early childhood age and having lifelong persistence, autism is a common disorder of neural development characterized by core deficits in social, communicative and behavioral skills. In 1998, the beneficiary effects of secretin in autistic treatment was reported with significant improvements in speech, eye contact, and attention after intravenous secretin treatment trials in three autistic children.[17] This initial success with secretin sparked popularity for its research in the treatment of autism. Despite this, further clinical studies yielded negative results including those with synthetic human secretin and double-blind trails. Although the direct benefits of secretin for autistic treatment remain controversial, experimental results provide support. Autism has been hypothesized to be a hypoglutamatergic disorder, and secretin may be a candidate in autistic treatment with experimental evidence showing its ability when peripherally administered to increase extracellular glutamate and GABA levels in the rat hippocampus.[9] Further, secretin knockout mice show abnormal social and cognitive behavior and impairment in hippocampal synaptic plasticity specifically in the CA1 area,[19] suggesting the involvement of secretin in synaptic function and possible therapeutic value in ameliorating the symptoms of autism. Despite the encouraging results, the most recent information available from controlled trials in autistic children show a lack of impact of secretin in improving various social, communicative and behavioral skill deficits.[18]

Schizophrenia

With the overlap of phenotypic features between autism and schizophrenia, it is not surprising that secretin's potential in alleviating schizophrenia-related disorders has also been explored. This was first shown in a 2004 double-blind study whereby single intravenous injections of secretin in 22 schizophrenia patients was able to significantly improve symptoms more than in those treated with placebo based on the clinical global impression scale.[29] In the same year, the beneficiary effects of secretin was further shown in a patient with chronic undifferentiated schizophrenia and prominent autistic features.[1] Experimentally, the rat has also been used as an animal model for study, and it has been found that secretin is able to dose dependently reverse the phencyclidine-induced impairment in prepulse inhibition, which in schizophrenic patients is in the abnormal range.[25] Secretin administration has also been shown to markedly improve eye-blink conditioning, which is associated with loss of effective associative learning, one of the many symptoms in schizophrenia.[3] With the mounting evidence of secretin improving schizophrenic symptoms, a possible role in enhancing cerebellar function and thus classically conditioned learning has been suggested and deserves further studies.

REFERENCES

1. Alamy SS, Jarskog LF, Sheitman BB, Lieberman JA. Secretin in a patient with treatment-resistant schizophrenia and prominent autistic features. *Schizophr Res* 2004;**66**:183–6.
2. Asmann YW, Dong M, Ganguli S, Hadac EM, Miller LJ. Structural insights into the amino-terminus of the secretin receptor: I. Status of cysteine and cystine residues. *Mol Pharmacol* 2000;**58**:911–9.
3. Bolbecker AR, Hetrick WP, Johannesen JK, O'Donnell BF, Steinmetz JE, Shekhar AS. Secretin effects on cerebellar-dependent motor learning in schizophrenia. *Am J Psychiatry* 2009;**166**:460–6.
4. Bonetto V, Jornvall H, Mutt V, Sillard R. Two alternative processing pathways for a preprohormone: a bioactive form of secretin. *Proc Natl Acad Sci USA* 1995;**92**:11985–9.
5. Chan KY, Pang RT, Chow BK. Functional segregation of the highly conserved basic motifs within the third endoloop of the human secretin receptor. *Endocrinology* 2001;**142**:3926–34.
6. Charlton CG, Miller RL, Crawley JN, Handelmann GE, O'Donohue TL. Secretin modulation of behavioral and physiological functions in the rat. *Peptides* 1983;**4**:739–42.
7. Cheng CY, Chu JY, Chow BK. Central and peripheral administration of secretin inhibits food intake in mice through the activation of the melanocortin system. *Neuropsychopharmacology* 2011;**36**:459–71.
8. Chu JY, Lee LT, Lai CH, Vaudry H, Chan YS, Yung WH, et al. Secretin as a neurohypophysial factor regulating body water homeostasis. *Proc Natl Acad Sci USA* 2009;**106**:15961–6.
9. Clement HW, Pschibul A, Schulz E. Effects of secretin on extracellular GABA and other amino acid concentrations in the rat hippocampus. *Int Rev Neurobiol* 2005;**71**:239–71.
10. Di Paolo E, Vilardaga JP, Petry H, Moguilevsky N, Bollen A, Robberecht P, et al. Role of charged amino acids conserved in the vasoactive intestinal polypeptide/secretin family of receptors on the secretin receptor functionality. *Peptides* 1999;**20**:1187–93.
11. Gao F, Harikumar KG, Dong M, Lam PC, Sexton PM, Christopoulos A, et al. Functional importance of a structurally distinct homodimeric complex of the family B G protein-coupled secretin receptor. *Mol Pharmacol* 2009;**76**:264–74.
12. Gourlet P, Vilardaga JP, De Neef P, Waelbroeck M, Vandermeers A, Robberecht P. The C-terminus ends of secretin and VIP interact with the N-terminal domains of their receptors. *Peptides* 1996;**17**:825–9.
13. Harikumar KG, Happs RM, Miller LJ. Dimerization in the absence of higher-order oligomerization of the G protein-coupled secretin receptor. *Biochim Biophys Acta* 2008;**1778**:2555–63.
14. Harikumar KG, Morfis MM, Sexton PM, Miller LJ. Pattern of intra-family hetero-oligomerization involving the G protein-coupled secretin receptor. *J Mol Neurosci* 2008;**36**:279–85.

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15. Harikumar KG, Pinon DI, Miller LJ. Transmembrane segment IV contributes a functionally important interface for oligomerization of the Class II G protein-coupled secretin receptor. *J Biol Chem* 2007;**282**:30363–72.
16. Holtmann MH, Roettger BF, Pinon DI, Miller LJ. Role of receptor phosphorylation in desensitization and internalization of the secretin receptor. *J Biol Chem* 1996;**271**:23566–71.
17. Horvath K, Stefanatos G, Sokolski KN, Wachtel R, Nabors L, Tildon JT. Improved social and language skills after secretin administration in patients with autistic spectrum disorders. *J Assoc Acad Minor Phys* 1998;**9**:9–15.
18. Krishnaswami S, McPheeters ML, Veenstra-Vanderweele J. A systematic review of secretin for children with autism spectrum disorders. *Pediatrics* 2011;**127**:e1322–5.
19. Lam IP, Siu FK, Chu JY, Chow BK. Multiple actions of secretin in the human body. *Int Rev Cytol* 2008;**265**:159–90.
20. Lee SM, Chen L, Chow BK, Yung WH. Endogenous release and multiple actions of secretin in the rat cerebellum. *Neuroscience* 2005;**134**:377–86.
21. Lee VH, Lee LT, Chu JY, Lam IP, Siu FK, Vaudry H, et al. An indispensable role of secretin in mediating the osmoregulatory functions of angiotensin II. *Faseb J* 2010;**24**:5024–32.
22. Lisenbee CS, Miller LJ. Secretin receptor oligomers form intracellularly during maturation through receptor core domains. *Biochemistry* 2006;**45**:8216–26.
23. Lossi L, Bottarelli L, Candusso ME, Leiter AB, Rindi G, Merighi A. Transient expression of secretin in serotoninergic neurons of mouse brain during development. *Eur J Neurosci* 2004;**20**:3259–69.
24. Miller LJ, Dong M, Harikumar KG. Ligand binding and activation of the secretin receptor, a prototypic family B G protein-coupled receptor. *Br J Pharmacol* 2011.
25. Myers KM, Goulet M, Rusche J, Boismenu R, Davis M. Partial reversal of phencyclidine-induced impairment of prepulse inhibition by secretin. *Biol Psychiatry* 2005;**58**:67–73.
26. Ng SS, Pang RT, Chow BK, Cheng CH. Real-time evaluation of human secretin receptor activity using cytosensor microphysiometry. *J Cell Biochem* 1999;**72**:517–27.
27. Nishijima I, Yamagata T, Spencer CM, Weeber EJ, Alekseyenko O, Sweatt JD, et al. Secretin receptor-deficient mice exhibit impaired synaptic plasticity and social behavior. *Hum Mol Genet* 2006;**15**:3241–50.
28. Pang RT, Ng SS, Cheng CH, Holtmann MH, Miller LJ, Chow BK. Role of N-linked glycosylation on the function and expression of the human secretin receptor. *Endocrinology* 1999;**140**:5102–11.
29. Sheitman BB, Knable MB, Jarskog LF, Chakos M, Boyce LH, Early J, et al. Secretin for refractory schizophrenia. *Schizophr Res* 2004;**66**:177–81.
30. Siu FK, Sham MH, Chow BK. Secretin, a known gastrointestinal peptide, is widely expressed during mouse embryonic development. *Gene Expr Patterns* 2005;**5**:445–51.
31. Tay J, Goulet M, Rusche J, Boismenu R. Age-related and regional differences in secretin and secretin receptor mRNA levels in the rat brain. *Neurosci Lett* 2004;**366**:176–81.
32. Trimble ER, Bruzzone R, Biden TJ, Meehan CJ, Andreu D, Merrifield RB. Secretin stimulates cyclic AMP and inositol trisphosphate production in rat pancreatic acinar tissue by two fully independent mechanisms. *Proc Natl Acad Sci USA* 1987;**84**:3146–50.
33. Yuan Y, Lee LT, Ng SS, Chow BK. Extragastrointestinal functions and transcriptional regulation of secretin and secretin receptors. *Ann N Y Acad Sci* 2011;**1220**:23–33.
34. Yung WH, Leung PS, Ng SS, Zhang J, Chan SC, Chow BK. Secretin facilitates GABA transmission in the cerebellum. *J Neurosci* 2001;**21**:7063–8.
35. Yurgelun-Todd DA, Rogowska J, Gruber SA, Bogorodzki P, Simpson NS, Irvin RW, et al. Increased amygdala fMRI activation after secretin administration. *Exp Clin Psychopharmacol* 2008;**16**:191–8.

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Chapter 124

Somatostatin/Cortistatin

Jacques Epelbaum, Cécile Viollet and Raphaelle Winsky-Sommerer

ABSTRACT

Somatostatin-14 was originally characterized as a hypothalamic neurohormone responsible for the inhibition of pituitary growth hormone secretion. In mammalian brain, two genes encode for the prosomatostatin-derived peptides, somatostatin-14 and somatostatin-28, and procortistatin-related ones, respectively. Each peptide binds with similar affinities to the five cloned sst receptors that belong to the GPCR family and cortistatins bind specifically to MrgX2. Besides the initial neuroendocrine role, somatostatin and cortistatin peptides serve as neuromodulators in the CNS, influencing differently sensory processes, sleep, motor activity, and cognitive functions, as well as playing a role in brain diseases such as affective disorders, epilepsia, and Alzheimer's disease.

DISCOVERY

Somatostatin (SRIF: somatotropin-release inhibiting factor) was discovered in 1972 by Roger Guillemin and his colleagues who were engaged in the quest to purify and characterize the hypothalamic neurohormones involved in the control of pituitary hormone secretion.[29] They were aiming for a growth hormone (GH)-releasing-hormone (GHRH) (see the GHRH chapter in this section of the book) but ended with a 14-amino acid peptide, which actually inhibited GH release from pituitary cells in monolayer culture. Rapidly thereafter, SRIF immunoreactivity was localized in the pancreas and gastrointestinal tract as well as in the brain. Two biologically active forms arise from the C-terminal portion of a single propeptide: SRIF14, originally described in the hypothalamus,[29] and the amino-terminally extended SRIF-28, discovered later in the gut[22] (Fig. 1). SRIF-14 is predominantly produced by neurons and secretory cells in the central and peripheral nervous system, and SRIF-28 in the gastrointestinal tract (see the Somatostatin chapter in Gastrointestinal Peptides section of this book). However, many additional expression sites have been described in other endocrine and exocrine cells, the placenta, the kidney, the retina, and in cells of the immune system.[12]

Cortistatin (CST) was discovered in 1996 by Luis de Lecea[6] who used directional tag PCR subtractive hybridization techniques to fish out genes selectively involved in long-term potentiation (LTP), a phenomenon probably corresponding to cellular memory at the neuronal level. Among several candidates, he sequenced a novel 112-amino acid polypeptide with a striking homology to somatostatin at its distal C-terminal end.[4] The cloning and localization of the gene on human chromosome 1 at 1p36.3–p36.2 increased the complexity of the somatostatin-signaling system. Sharing 11 amino acids with somatostatin, CST is predicted to occur in a 14- (CST-14) or 17-amino acid form (CST-17) in rodents and humans, respectively, and a longer one of 29 amino acids (CST-29) in rats and humans (Fig. 1). In the central nervous system, CST mRNA expression is mainly restricted to the cerebral cortex and the hippocampus. In mouse brain, small levels of preproCST mRNA have been detected in the amygdala and the hypothalamus, an important SRIF expression site.

A novel peptide, neuronostatin (LRQFLQKSLAAAA-NH_2), encoded by the somatostatin gene, has more recently been identified based on bioinformatic analyses of evolutionarily conserved sequences.[26] It is mostly concentrated in spleen, pancreas, and brain, and seems to be involved in cardiovascular, metabolic, and neuronal functions. Some of its actions seem to be mediated through the central melanocortin system.

STRUCTURE OF THE PRECURSOR mRNA/GENES

The preproSRIF gene structure and regulation have been extensively reviewed elsewhere.[30] The SST (human) and Sst (rat and mouse) somatostatin genes are, respectively, located in 3q28, on chromosome 11q23 and 16. The organization of the human, mouse, and rat genes is very similar. A single intron interrupts the two exons encoding preproSRIF. SRIF-14 and SRIF-28, located at the carboxy-terminus of the precursor, are generated by posttranslational enzymatic processing. In brain cells, transcription is regulated positively by many factors acting on *cis*-regulatory elements: bZip proteins acting on a cAMP response element (CRE),

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00124-X
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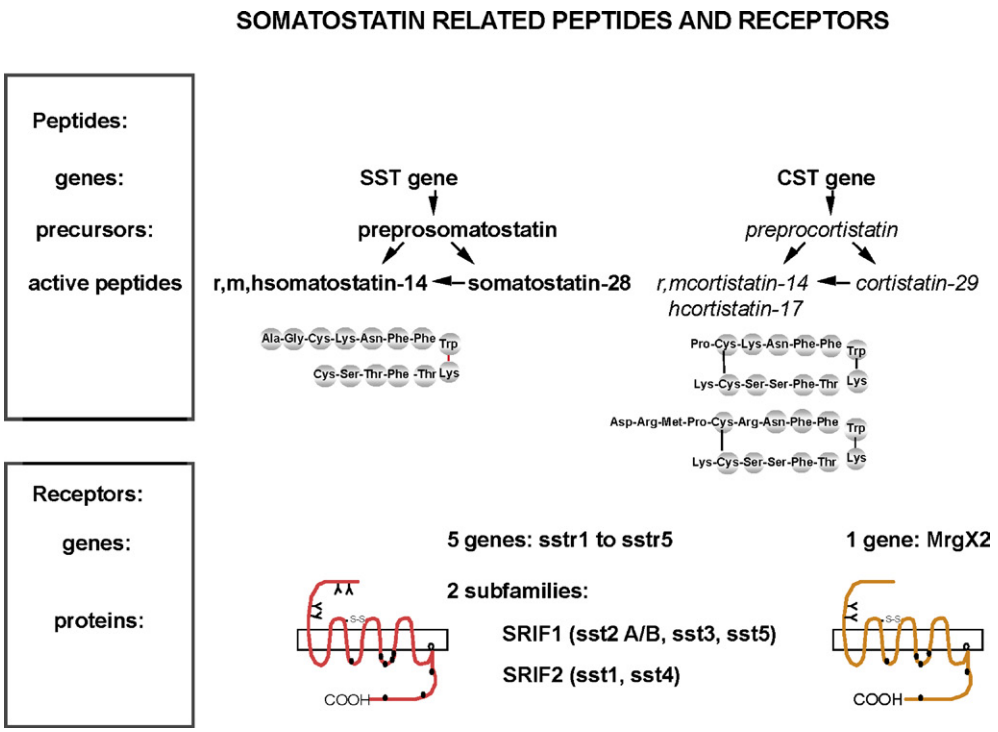

FIGURE 1 Somatostatin-related peptides and receptors. The somatostatin gene gives rise to two bioactive peptides somatostatin-14 and somatostatin-28, the former being totally conserved in mammals and representing the majority of somatostatin immunoreactivity in brain. In contrast, cortistatin gene-derived putative peptides are different in murine and primate species and, according to remaining levels in *Sst*−/− mouse, represent only a minority of somatostatin immunoreactivity in the brain. Somatostatins and putative cortistatins can bind to the six somatostatin receptors which, according to their operational properties, can be subdivided in two families, SRIF1 and SRIF2. Cortistatins can also bind to a formerly orphan receptor, MrgX2, which is highly expressed in dorsal root ganglia.

and homeodomain proteins such as Pdx-1 or bHLH proteins (such as *Sim1* and *arnt2*) on TAAT elements.[20] Transcription is negatively regulated by two proximal silencers, which may be active in cerebrocortical but not hippocampal cells. SST gene regulation seems to be notably different in brain and pancreas since enhancers in the latter (SST-TAAT1, SST-TAAT2 and SST-UE-B) act as silencers in neural cells.

The human CORT and mouse Cort genes are located in 1p36.3–1p36.2 and on chromosome 4, respectively. Very few results are available concerning their regulation.

Although the sequences of SRIF-14 and SRIF-28 are identical in all mammals tested so far, cortistatin sequences vary notably within this lineage, indicating a much more rapid rate of evolution for the latter gene.[4]

DISTRIBUTION OF THE mRNAs AND PEPTIDES

SRIF immunoreactivity is largely distributed in many neurons in mammalian brain, including the human brain (Fig. 2). The highest levels are found in the mediobasal hypothalamus and median eminence, amygdala, preoptic area, accumbens nucleus, cerebral cortex, striatum, olfactory regions, and brainstem. Two main categories of neurons can be identified: those that project to a distance from their soma (long projecting) and short GABAergic interneurons coexpressing the neuropeptide either alone (cortex and hippocampus) or together with neuropeptide Y (striatum and a few cells in the cortex).[32] Preprocortistatin mRNA-expressing neurons are mostly localized in a subset of cortical and hippocampal GABAergic interneurons, which partially colocalize with SRIF-positive neurons. In *Sst*−/− mouse, the levels of immunoreactive material remaining in the brain, which probably correspond to CST, is below the sensitivity of the assay, while CST expression does not change.[18] No study has yet reported the distribution of CST peptides in mammalian brain with selective antibodies, which do not recognize SRIF moieties.

In the rat cortex, a subset of cortistatin-positive neurons contains parvalbumin, whereas SRIF interneurons, which mostly correspond to Martinotti cells, contain calbindin. In the mouse, however, SRIF Martinotti interneurons contains calretinin.[34]

Long-projecting neuronal somatostatinergic systems can be differentiated according to their localizations:

- Hypophysiotropic somatostatin neurons projecting to the median eminence are located within the rostral periventricular nucleus (Pev) and the parvocellular

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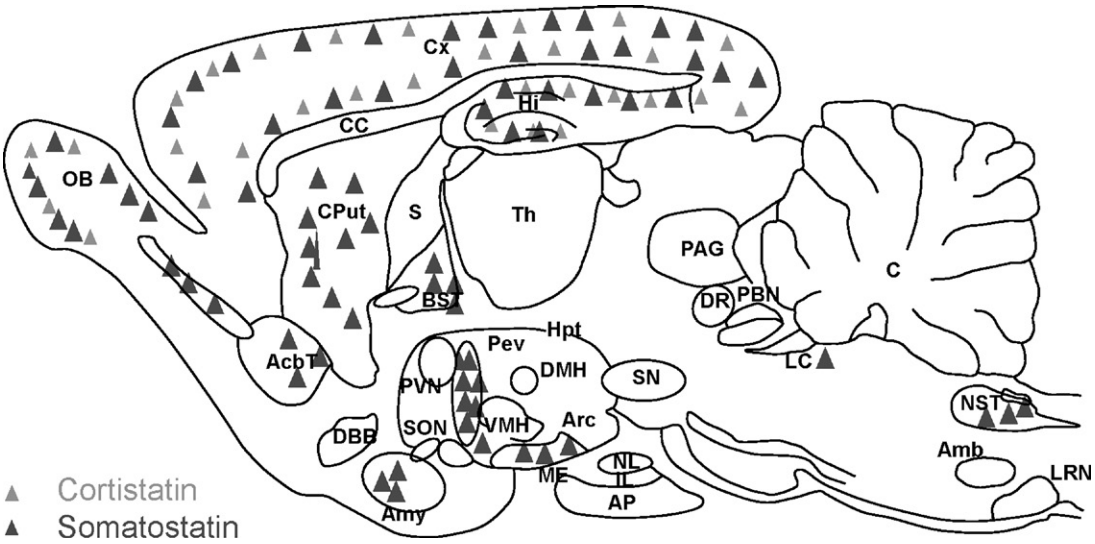

FIGURE 2 Schematic distribution of somatostatin (dark grey) and cortistatin (light grey) neurons in mammalian brain. In adults, somatostatin neurons are essentially distributed in the forebrain (OB, BST, Acb, claustrum, all parts of Cx including HI, entopeduncular nucleus, Amy, and Hpt) and the lower brainstem (central gray, para- and lateral lemniscal nuclei, reticular formation next to the prepositus hypoglossal nucleus, suprageniculate nucleus and NST). Cortistatin neurons are essentially present in the Cx and HI.

paraventricular nucleus. These cells are located close to the third ventricle, in three or four layers that are parallel to the ventricle wall and their axons run laterally and turn caudally through the retrochiasmatic area to enter the median eminence where they make contact with the fenestrated capillary loops of the portal vasculature from which they reach pituitary somatotrophs directly. SRIF cells in the Pev also project to many limbic (olfactory tubercle, septum, habenula, hippocampus) and mesencephalic (substantia nigra and locus coeruleus) regions. Cocaine–amphetamine-regulated transcript (CART) colocalizes in 38% of the somatostatinergic neurons, and CART is the only neuropeptide produced by hypophysiotropic somatostatinergic cells.

- Some hippocampal CA1 somatostatin neurons innervate the subiculum and lateral septal nucleus and also cortical, limbic, and olfactory regions.
- Amygdaloid somatostatin neurons connect the different nuclei of the amygdala with the noticeable exception of central nucleus ones, which innervate many limbic regions through the stria terminalis.
- Solitary tract neurons, through their projections to the ambiguus nucleus, control esophagean motility and, through their ascending projections to magnocellular hypothalamic neurons, cardiovascular processes. These neurons are the only ones in the brain to contain only SRIF-28 (and possibly neuronostatin) but not SRIF-14.
- PreBötzinger complex somatostatin neurons, which are essential for the generation of normal breathing, send widespread projections specifically targeted to other brainstem regions also implicated in the control of breathing.

PROCESSING OF THE PRECURSORS AND DEGRADATION OF THE PEPTIDES

The convertases furin and PACE-4 seem to be involved in the constitutive pathway to generate SRIF-28. In secretory vesicles, proSRIF is a physiological substrate of PC1 and/or PC2, and generation of SRIF-14 in human brain requires this latter convertase. Processing of proSRIF is severely impaired in pancreatic islets of PC2–/– mice pancreatic islets. Further, PC1 and PC2 immunoreactivities are localized in numerous human cortical neurons, and their topographic and cellular distribution broadly coincides with that previously reported for SRIF-containing neurons, that is, mainly in the cortical layers II, III, as well as in V and VI. SRIF peptides are degraded primarily by two endopeptidases: EC.3.4.24.15 (Thimet oligopeptidase) and EC.3.4.24.16 (neprilysin). The former is a soluble enzyme, whereas the latter is membrane associated. Subsequently, exopeptidases, such as aminopeptidases A and M, are able to cleave the N-terminal fragments. SRIF seems also cleared from brain interstitial space by sequestration within microvessels.

No data are available concerning proCST processing and degradation mechanisms in mammalian brain. All these enzymes are discussed in detail in the Peptides Biosynthesis/Processing section of this book.

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RECEPTORS AND SIGNALING CASCADES

SRIF mediates its biological functions by at least six receptor subtypes, termed sst1, sst2A, sst2B, sst3, sst4, and sst5, all of which belong to the family of seven transmembrane domain G-protein-coupled receptors.[20] Truncated human sst5-variants with 4 or 5 transmembrane domains are present in a few normal and some tumoral tissues but they do not seem to be highly expressed in brain.[9] The natural ligands of the different somatostatin receptor subtypes, SRIF-14 and SRIF-28 and CST, bind to all somatostatin receptor subtypes with high affinity. Nonetheless, it has not been shown that CST mediates sst receptor activation *in vivo*. A putative CST receptor has been identified as MrgX2, a member of the Mas-related gene family, which is highly expressed in dorsal root ganglion.[24] Surprisingly, CST also seems to be able to interact with the ghrelin receptor, although this is not the case for SRIF.

The recent generation of specific antibodies against each somatostatin receptor variant allows a precise cellular and subcellular localization of somatostatin receptor subtypes in the central nervous system, which also provides important insights into somatostatin signaling. The development of specific somatostatin analogs and antagonists and the results of experiments investigating genetically modified mice also contribute to a better understanding of the individual somatostatin receptor characteristics.

Many detailed studies[8] have examined, especially in rodents, the pattern of expression of individual sst receptor subtype mRNAs in brain. Sst1–5 receptor transcripts are widely distributed throughout the CNS and the pituitary, displaying distinct but overlapping patterns of expression. However, mRNA levels do not necessarily reflect receptor protein expression. Today, immunolocalization of the sst1–5 proteins in mammalian brain and pituitary has been achieved using selective antibodies (Fig. 3).

Contrasting with the broad distribution of sst1 mRNAs, the sst1 protein seems to be essentially restricted to the rostral part of the periventricular hypothalamic nucleus from the organum vasculosum laminae terminalis extending caudally to the rostral part of the median eminence when using a C-terminally directed antibody. In contrast, a broad distribution of the presumptive deglycosyled form of the sst1 receptor protein in rat brain has been reported, using an antibody directed against the N-terminal sequence. Further experiments are needed to resolve this discrepancy. It remains also to be established whether the presumptive nonglycosylated sst1 form is functional. At any rate, the localization of the sst1 receptors and functional studies in the basal ganglia and retina are consistent with its role as a presynaptic inhibitory autoreceptor.

Using C-terminally directed antibodies, sst2A receptor immunoreactivity seems to be widely distributed, in keeping with the regional distribution of both sst2 mRNA and

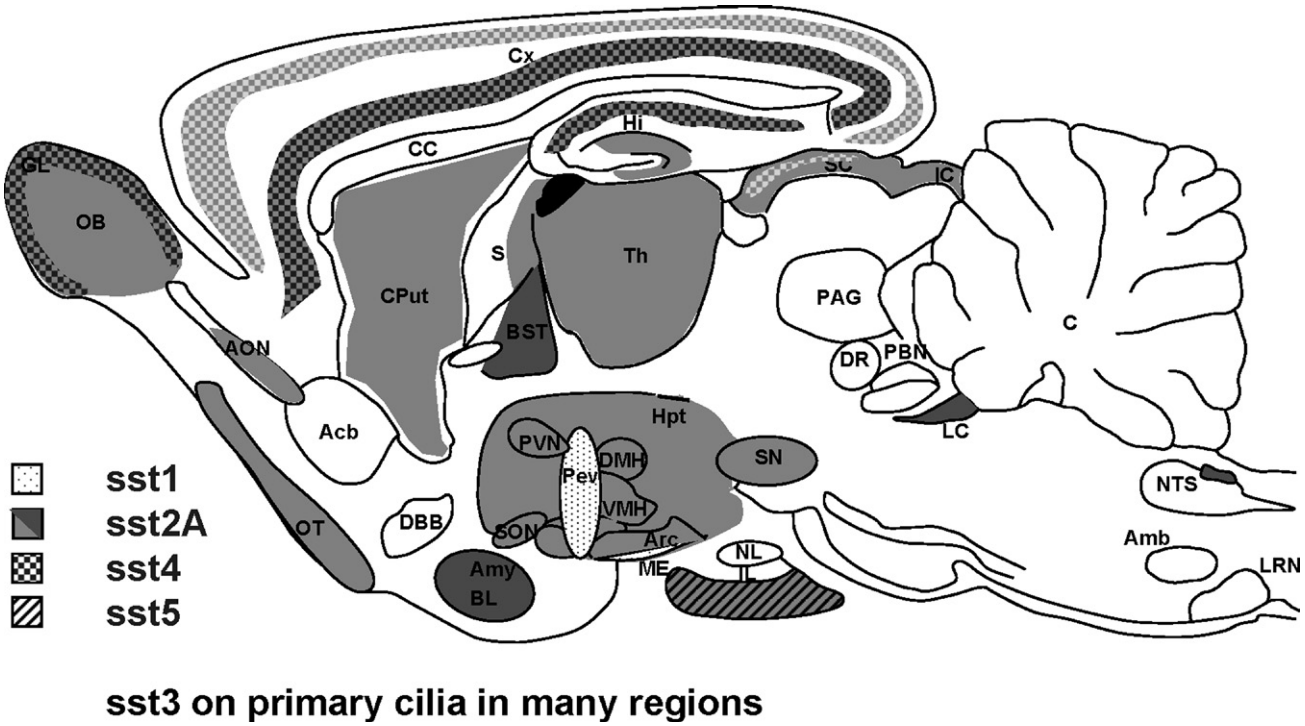

FIGURE 3 Schematic representation of somatostatin receptor immunolocalization in mammalian brain. Color intensity is proportional to the levels of SSTR2A receptor. Same abbreviations as in Figure 2.

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SRIF-1 binding sites. Rostrally, numerous strongly immunoreactive nerve cell bodies are observed throughout the pyramidal layer of the olfactory tubercle, extending into the polymorph layer. More sparse and less intensely labeled nerve cell bodies are also found in layer II of the piriform cortex. Diffuse but intense immunoreactive labeling is present in the endopiriform, anterior olfactory, and lateral olfactory tract nuclei. In the cerebral cortex, the frontal, parietal, temporal, and occipital lobes exhibit prominent perikaryal immunostaining in layers II–III. A few less intensely immunoreactive cell bodies are also evident in layer V. Diffuse but intense sst2A labeling is found in layers V–VI of the cerebral cortex. High densities of immunoreactive perikarya and dendrites are detected in the lateral division of the bed nucleus of the stria terminalis. Low densities of sst2A-positive cells are also found in the vertical limb of the diagonal band of Broca. In the habenula, immunoreactive cell bodies are detected among a dense network of immunoreactive processes in the medial part while the lateral one seems devoid of any positive signal. Moderate densities of immunoreactive nerve cell bodies and proximal dendrites are encountered throughout the ventrolateral and caudal segments of the neostriatum, within the core and the shell divisions of the nucleus accumbens and in the dorsolateral part of the septum. Diffuse but intense immunoreactive labeling is observed in the claustrum.

Within the hippocampus, pyramidal cells including their basal and apical dendrites of CA1–CA2 fields are intensely stained. A few labeled perikarya are also observed in the stratum radiatum. Diffuse but intense immunoreactive signal is present in the stratum lacunosum moleculare and the subiculum and is also apparent, albeit less prominently in the molecular layer of the dentate gyrus. The CA3 subfield remains consistently immunonegative. Both high densities of perikaryal and diffuse labeling are detected in the amygdaloid complex, the former in the central and the latter in the basolateral nucleus. Moderate densities of immunoreactive nerve cell bodies are also encountered in the medial and cortical nuclei. The endopiriform nucleus displays diffuse but intense immunolabeling.

In the hypothalamus, only sparse immunoreactive perikarya are detected except in the tuberomammillary nucleus in which they form a tight cluster of moderately immunoreactive cells. Diffuse labeling is apparent in a few nuclei including the arcuate and paraventricular nuclei. In the midbrain, low densities of labeled cell bodies with long and fine processes are evident in the deep layers of the superior colliculus as well as in periaqueductal gray matter. Additionally, diffuse labeling is found in the dorsal and lateral segment of periaqueductal gray, gray layers of the superior colliculus, pars compacta of the substantia nigra and ventral tegmental area. In the pons, intensely labeled perikarya and processes are detected in the locus coeruleus. Labeled cell bodies are also apparent in the lateral dorsal tegmental area and parabrachial nucleus. More caudally, moderate densities of immunopositive cell bodies are visible in the dorsal motor nucleus of the vagus and in the lateral reticular nucleus. Diffuse labeling is found in the nucleus tractus solitarius and medial vestibular nucleus. The cerebellar cortex and deep cerebellar nuclei are devoid of immunostaining. In the spinal cord, sst2A immunoreactivity is exclusively found in the dorsal horn. A dense network of immunoreactive processes is present in the substantia gelatinosa (Layer II), whereas immunoreactive cells are apparent in the border zone between layers I and II. Only occasional cell bodies are stained in layer VI. Double labeling immunohistochemistry for pituitary hormones and the sst2A receptor reveals an almost complete colocalization of the sst2A receptor with GH expressing cells. Only a small percentage of lactotroph cells (<10%) bear the sst2A receptor, whereas a substantial overlap of the receptor is found with gonadotrophs (LH/FSH) (40–50%), corticotrophs (<60%), and thyrotrophs (<30%).

Sst3 receptor mRNAs are broadly expressed in mammalian central nervous systems. However, SSTR3 is immunolocalized exclusively in neuronal cilia.

Sst4 mRNAs are expressed in a relatively restricted number of rat brain areas, in contrast to the rather broad expression pattern of sst1, sst2, and sst3 receptors. Expression of the sst4 receptor mRNA is the greatest in the pyramidal cells of the CA1 region of the hippocampus, lower in other hippocampal regions, the olfactory bulb, and the cortex and is absent from the striatum, cerebellum, and hypothalamus. SSTR4 immunoreactivity is essentially restricted to the cerebral cortex and CA1 of the hippocampus.

Sst5 receptor immunoreactivity is only sparsely present in neuronal perikarya and proximal dendrites limited to the rostral part of the brain, but the protein is clearly present in the pituitary.

MgrX2 is moderately expressed in susbsets of CA2–4 neurons in the hippocampus, but it is not detected in the cerebral cortex.

SRIF/CST interaction with their cognate receptors in the brain may trigger the recruitment of many intracellular effectors.[20] The five sst receptors can inhibit the adenylyl cyclase–cAMP–protein kinase A pathway through pertussis toxin-sensitive G proteins such as Gαi1–3. Many potassium channels (inward rectifier, noninactivating voltage-sensitive, ATP-sensitive, large conductance Ca^{2+}-activated, M-current) can be opened, either directly or through activation of the phospholipase C-protein kinase C pathway and phospholipase A2–arachidonic acid pathway. By these mechanisms, somatostatin can hyperpolarize cell membranes, therefore reducing $[Ca^{2+}]i$. It may also modulate Ca^{2+} influx either directly through voltage-sensitive Ca^{2+} channels (L-, N- P/Q-types) and Gαo proteins or indirectly

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through the guanylate cyclase-cGMP-dependent-protein kinase pathway. In addition, sst1 receptors can also recruit pertussis toxin-insensitive G proteins such as the Na^+/H^+ exchanger. Stimulated sst receptors can also activate tyrosine (PTP)- and serine/threonine-phosphatases, some of the latter, PP2A and calcineurin (PP2B), being involved in synaptic plasticity processes. Finally, sst receptors seem able to inhibit the mitogen-activated protein kinase pathway, possibly through the activation of PTPs. Such mechanisms may contribute to the monitoring of tumor growth by SRIF. All the previously mentioned signal transduction pathways seem to be effectively modulated by the cloned receptors (see Table 1 for further details). However, it seems that multiple sst receptor subtypes can be expressed in single neurons, the best examples being hypothalamic neurons on which somatostatin can either enhance (through a sst1 pertussis toxin-independent mechanism) or decrease (through a sst2 pertussis toxin-dependent one) AMPA/kainate receptor mediated responses to glutamate, depending on their ability to modulate $[Ca^{2+}]i$.

SRIF receptors can be differentiated by their ability to internalize. With the noticeable exception of sst4, all subtypes internalize in various cellular models after agonist treatment. In the brain, this has only been shown *in vivo* for sst1[27] and sst2[5] receptors. In transfected cell models, SRIF receptors have also been reported to homo- and heterodimerize and interact with PDZ-containing proteins.[20] Such receptor-multidomain protein complexes are likely to intervene in the cell- and tissue-specific modulation of SRIF/CST-mediated signaling processes.

TABLE 1 Characteristics of Human Somatostatin Receptors. Summary of the HSSTRs Characteristics

Subtypes	sst1 (SRIF2A)	sst2 (SRIF1A)	sst3 (SRIF1B)	sst4 (SRIF2B)	sst5 (SRIF1C)
Adenylyl cyclase	–	–	–	–	–
Tyrosine phosphatase	+	+	+	+	+
MAP kinase	+	–	+/–	+	–
K+ channels		+	+	+	+
Ca2+ channels	–	–			
Na+/H+ exchanger	+				
PLP C/IP3 pathway	+	+	+	+	+/–
PLPA2/AA pathway				+	
Gene location	14q13	17q24	22q13.1	20p11.2	16p13.3
Swiss prot.	P30872	P30874	P32745	P31391	P35346
Amino acids	391	369/356	418	388	364
Mol. weight (kDA)	42.7	41.3	45.9	41.9	39.2
Transcript (kb)	4.8	8.5	5.0	4.0	4.0
Glycosylation site	3	4	2	1	3
Phosphorylation site	6	8	5	3	5
Tissue distribution	Brain,	Brain,	Brain,	Brain	Pituitary
	Pituitary	Pituitary	Pituitary	Islets	Islets
	Islets	Islets	Islets	Stomach	Stomach
	Stomach	Stomach	Stomach	Liver	Adrenals
	Liver	Kidneys		Adrenals	
	Kidneys	Adrenals		Lung	
				Placenta	
				Lung	

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INFORMATION ON ACTIVE AND/OR SOLUTION CONFORMATION

SRIF and CST are disulfide bridged peptides, and the receptor pharmacophore lies in the four amino acid sequence: Phe–Trp–Lys–Thr, totally conserved amongst peptides. The lysine and D-tryptophan side chains in cyclic hexapeptide analogs exist in an equatorial orientation relative to the backbone of the peptides. SMS 201-995 [(D)-Phe–c[Cys–Phe–(D)-Trp–Lys–Thr–Cys]–Thr-ol, octreotide] was the first SRIF analog in clinical use for the treatment of acromegaly, and several other endocrine and malignant disorders. It binds predominantly to hsstr2 and hsstr5 and with low affinity to hsstr3, it has no or low affinity to sst1 and sst4, but it also binds to mu-opioid receptors. Octreotide exists in solution in two conformational families, differing mainly by the conformation of the C-terminal tail. The molecule adopts an overall antiparallel ß-sheet conformation, with a type II″ ß-turn centered at the (D)-Trp8–Lys9 region. In one conformational family, the residues after this ß-turn continue the ß-sheet structure. Based on elaborate studies of various somatostatin analogs, it was concluded that this family contains the active conformation(s). In the second conformational family, the residues after the ß-turn adopt a 3–10 helical conformation. A cyclic hexapeptide SRIF substitute, SOM230 c-[(2-amino-ethyl-carbamoyl)-Hyp1–Phg2–DTrp3–Lys4–(4-O-benzyl)-Tyr5–Phe6], pasireotide, has been designed by analogy with octreotide. It binds with high affinity to sst1, sst2, sst3, and sst5 receptors. Strikingly, pasireotide conformers correspond to the two main X-ray structures in the asymmetric unit of SMS 201–995. However, due to a wider range of action and a longer half-life, pasireotide is more potent (longer acting) than octreotide on hormonal inhibition and antiproliferative effects.[15]

BIOLOGICAL ACTIONS WITHIN THE BRAIN AND PITUITARY

The best-characterized action of brain SRIF is the inhibition of pituitary hormone secretion, most notably GH, and also TSH and, in some cases ACTH and TSH.[12] Moreover, SRIF is a widely distributed neuromodulator substance in the brain. Experimental depletion of somatostatin by cysteamine disrupts avoidance learning and decreases locomotor activity, while somatostatin agonists induce the opposite effects. Somatostatin also exerts anxiolytic- and antidepressant-like effects (see Ref. 32 for review, and the Behavior chapter in the last section of this book).

CST shares several functional properties with SRIF such as the depression of neuronal activity. However, some biological functions of CST are unique, for example, the induction of slow-wave sleep and reduction of locomotor activity.[7] Intracerebroventricular (icv) administration of CST-14 induces a twofold increase in deep slow wave sleep (sws) amounts during both light and dark periods; CST mRNA levels depend on the physiological demand for sleep as they oscillate during the light/dark cycle and are induced fourfold on total (but not REM) sleep deprivation, returning to basal levels after 8 hours of rebound sleep. Interestingly, and also in contrast to SRIF, CST antagonizes the effects of acetylcholine at the cortical level (system related to alertness) to promote cortical synchrony and to control sws/wakefulness/sws transitions. These data suggest that CST is a specific sleep factor, which accumulates during wakefulness to generate sleep pressure and then is released during sleep. In contrast to SRIF, CST produces a reduction of locomotor activity. Also unlike SRIF, expression of CST mRNA is not upregulated in response to kainic-acid-induced seizure activity *in vivo*.

Receptor-selective control of several cognitive and sensory processes have been dissected using pharmacological and genetic approaches. In mice, intrahippocampal injection of the sst4 agonist L-803,087 dramatically impairs place memory formation (alike SRIF), whereas sst1–3 agonists do not induce any mnemonic effects. Unlike SRIF, the sst4 agonist also enhances the use of a cue-based memory and acquisition of another striatum-dependent memory task. Hippocampal sst4 thus seems to selectively control the use of cognitive strategies by switching from hippocampus-based multiple associations to simple striatum-based behavioral responses. Selection of striatum-dependent strategies, however, requires a functional cooperation between sst2 and sst4 receptors.[13]

Data on the effect of *sst1–4* and *Sst* gene deletion on brain activity in mice are summarized in Table 2. A change in basal GH release from somatotroph cells has been reported in *sst1−/−* mice, while somatostatin inhibition is reduced in *sst2−/−* mice. Sexual dimorphism in GH pulsatile secretion and ACTH/corticosterone secretion no longer exists in *Sst−/−* mice. Subtle changes in motor and cognitive behavior have been also observed in *sst2−/−* mice. Studies also show that *sst1*, *sst2*, *sst4*, and *sst* gene deletions modify seizure sensibility. *Sst2−/−* mice seem to be less sensitive to ischemia and display disturbed olfactory processing. *Sst3−/−* mice are severely impaired in discriminating novel objects, whereas they retain normal memory for object location. *Sst4−/−* mice display increased sensitivity to seizures. Although the mice have been developed, no report on brain activity is available for *sst5−/−* mice at the present.

PATHOPHYSIOLOGICAL IMPLICATIONS

Long-acting sst2 preferring agonists such as octreotide and lanreotide are used for the clinical management of acromegalic patients, gastroenterological or pancreatic tumors, and other gastrointestinal disorders. Specifically designed

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TABLE 2 Summary of the CNS-Related Phenotypes Observed in the Different Strains Invalidated for the Somatostatin Receptors or Peptide Genes

Neuroendocrine activities	sst1 −/− vs controls	sst2 −/− vs controls	sst3 −/− vs controls	sst4 −/− vs controls	SST −/− vs controls
Pituitary hormones					
GH secretion	−−[16]	=[31,35]			-[18]
ACTH secretion		+[31]			
Behavioral activities					
Motor coordination and learning					
Spontaneous motor activity		=[1,31]			=[18]
Static rod test		=[1,31]			=[18]
Wire hang test		=[1,31]			=[18]
Beam-walking test		−−[1]			
Constant speed rotarod test		=[31]			=[18]
Accelerating rotarod					−−[18]
Open-field exploratory activity					
Open-field test		=[1], −−[31]			=[18]
Anxiety-related behaviors					
Dark-light box test		−−[31]			=[18]
Elevated-plus maze test		+[1], −−[31]			
Forced-swim test		−−[31]			
Reaction to novelty test		−−[31]			
Social interaction					
Resident-intruder test		=[31]			
Sensorimotor responses					
Acoustic startle response					=[18]
Prepulse inhibition					=[18]
Learning and memory					
Radial maze learning		+[10,31]			

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TABLE 2 Summary of the CNS-Related Phenotypes Observed in the Different Strains Invalidated for the Somatostatin Receptors or Peptide Genes—*Cont'd*

Neuroendocrine activities	sst1 −/− vs controls	sst2 −/− vs controls	sst3 −/− vs controls	sst4 −/− vs controls	SST −/− vs controls
Skinner box paradigm		−−[10,31]			
Conditioned fear					=[18]
Object recognition			−−[11]		
Odor recognition		−−[17]			
Seizure susceptibility	+[3] or =[23]	−−[19] or =[23]		+[23]	+[2]
Neurodegeneration after focal ischemia		+[28]			

=, No change; −−, decreased effect/responses; +, improved effect/responses.
Numbers refer to references.

analogs are also used for tumor imaging and in development for radiotherapy. Not surprisingly, the physiopathological implications for neuropsychiatric diseases are less developed. Nevertheless, changes in SRIF and sst receptors have long been known to be associated with dementia, epilepsy, and major affective disorders.[12,13]

Concerning affective disorders, one of the most consistent neuropeptide alterations in depression has been a state-dependent decrease of CSF SRIF. This decrease does not seem to be specific to depression, as it is also observed in other patient populations where cognition is impaired, such as in schizophrenia, Alzheimer's dementia, drug refractory epilepsy, and active multiple sclerosis.[14] The SRIF decrease in affective illness and active multiple sclerosis has been considered state dependent, in that levels normalize with recovery from the acute episode or treatment. In contrast, increased SRIF levels have been reported in psychiatric illness where cognition is accelerated, as in mania, or ruminative, as in obsessive compulsive disorder.

Temporal lobe epilepsy (TLE) is characterized by hippocampal sclerosis together with profound phenotypic changes of different classes of interneurons. Evidence from human studies suggests that hilar SRIF interneurons undergo extensive degeneration in patients with hippocampal sclerosis. Therefore, SRIF receptors may represent potential therapeutic targets for temporal lobe epilepsy. Indeed, SRIF is released under conditions characteristic of seizures; seizures induce changes in the levels of the peptide; SRIF and its analogs affect seizures. However, information on the precise contribution of each SRIF receptor on the SRIF-induced inhibition of epileptiform activity is still limited. Although the sst_2 receptor is the candidate likely to mediate the anticonvulsant effects of SRIF in the rat hippocampus, in the mouse hippocampus, recent observations support a central role of sst4 and/or sst_1 receptors in mediating SRIF inhibition of epileptiform activity.[3,19,23]

Finally, in Alzheimer's disease cortical SRIF deficiency correlates with the dementia score in frontal cortex and with some indices of severity of the illness. Selective assays indicate that somatostatin-related but not cortistatin-related peptides are mainly involved in these deficits.[13] However, overexpression of the human ß-amyloid precursor protein downregulates CST mRNA in PDAPP mice.[33] Pierotti et al. observed that the proportion of proSRIF is significantly lower in the temporal cortex of Alzheimer patients with SRIF-28 proportions significantly elevated.[21] These results suggest that total SRIF deficiency may be related to a reduction in the rate of biosynthesis and/or to a change in the proteolytic processing of the precursor form to yield the bioactive peptides SRIF-28 and SRIF-14 but not through PC2 protein convertases.[13] Monitoring brain SRIF levels may be of interest since the neuropeptide seems to be the only one out of nearly 50 reagents which increases neprylysin[25] activity thereby promoting the degradation of the amyloid ß peptide Aß 42, which is supposed to be a major pathogenic factor in the disease.

REFERENCES

1. Allen JP, Hathway GJ, Clarke NJ, Jowett MI, Topps S, Kendrick KM, et al. Somatostatin receptor 2 knockout/lacZ knockin mice show impaired motor coordination and reveal sites of somatostatin action within the striatum. *Eur J Neurosci* 2003;**17**:1881–95.
2. Buckmaster PS, Otero-Corchon V, Rubinstein M, Low MJ. Heightened seizure severity in somatostatin knockout mice. *Epilepsy Res* 2002;**48**:43–56.
3. Cammalleri M, Cervia D, Langenegger D, Liu Y, Monte MD, Hoyer D, et al. Somatostatin receptors differentially affect spontaneous epileptiform activity in mouse hippocampal slices. *Eur J Neurosci* 2004;**20**:2711–21.

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4. Castaño JP, Ghigo E, Kineman RD, de Lecea L, Malagón MM, Vaudry H. Somatostatin, cortistatin and their receptors in health and disease. *Foreword Mol Cell Endocrinol* 2008;**286**:1–2.
5. Csaba Z, Bernard V, Helboe L, Bluet-Pajot MT, Bloch B, Epelbaum J, et al. In vivo internalization of the somatostatin sst2A receptor in rat brain: evidence for translocation of cell-surface receptors into the endosomal recycling pathway. *Mol Cell Neurosci* 2001;**17**:646–61.
6. de Lecea L, Criado JR, Prospero-Garcia O, Gautvik KM, Schweitzer P, Danielson PE, et al. A cortical neuropeptide with neuronal depressant and sleep-modulating properties. *Nature* 1996;**381**:242–5.
7. de Lecea L. Cortistatin-functions in the central nervous system. *Mol Cell Endocrinol* 2008;**286**:88–95.
8. Dournaud P, Slama A, Beaudet A, Epelbaum J. Somatostatin receptors in peptide receptors. In: Quirion R, Björklund A, Hökfelt T, editors. *Handbook of chemical neuroanatomy*, vol. 16 Part 1. Boston, MA: Elsevier; 2000. p. 1–43.
9. Durán-Prado M, Gahete MD, Martínez-Fuentes AJ, Luque RM, Quintero A, Webb SM, et al. Identification and characterization of two novel truncated but functional isoforms of the somatostatin receptor subtype 5 differentially present in pituitary tumors. *J Clin Endocrinol Metab* 2009;**94**:2634–43.
10. Dutar P, Vaillend C, Viollet C, Billard JM, Potier B, Carlo AS, et al. Spatial learning and synaptic hippocampal plasticity in type 2 somatostatin receptor knock-out mice. *Neuroscience* 2002;**112**:455–66.
11. Einstein EB, Patterson CA, Hon BJ, Regan KA, Reddi J, Melnikoff DE, et al. Somatostatin signaling in neuronal cilia is critical for object recognition memory. *J Neurosci* 2010;**30**:4306–14.
12. Epelbaum J. Somatostatin in the central nervous system: physiology and pathological modifications. *Prog Neurobiol* 1986;**27**:63–100.
13. Epelbaum J, Guillou JL, Gastambide F, Hoyer D, Duron E, Viollet C. Somatostatin, Alzheimer's disease and cognition: an old story coming of age? *Prog Neurobiol* 2009;**89**:153–61.
14. Frye MA, Pazzaglia PJ, George MS, Luckenbaugh DA, Vanderham E, Davis CL, et al. Low CSF somatostatin associated with response to nimodipine in patents with affective illness. *Biol Psychiatry* 2003;**53**:180–3.
15. Interlandi G. Backbone conformations and side chain flexibility of two somatostatin mimics investigated by molecular dynamics simulations. *Proteins* 2009;**75**:659–70.
16. Kreienkamp HJ, Akgun E, Baumeister H, Meyerhof W, Richter D. Somatostatin receptor subtype 1 modulates basal inhibition of growth hormone release in somatotrophs. *FEBS Lett* 1999;**462**:464–6.
17. Lepousez G, Mouret A, Loudes C, Epelbaum J, Viollet C. Somatostatin contributes to in vivo gamma oscillation modulation and odor discrimination in the olfactory bulb. *J Neurosci* 2010;**30**:870–5.
18. Low MJ, Otero-Corchon V, Parlow AF, Ramirez JL, Kumar U, Patel YC, et al. Somatostatin is required for masculinization of growth hormone-regulated hepatic gene expression but not of somatic growth. *J Clin Invest* 2001;**107**:1571–80.
19. Moneta D, Richichi C, Aliprandi M, Dournaud P, Dutar P, Billard JM, et al. Somatostatin receptor subtypes 2 and 4 affect seizure susceptibility and hippocampal excitatory neurotransmission in mice. *Eur J Neurosci* 2002;**16**:843–9.
20. Olias G, Viollet C, Kusserow H, Epelbaum J, Meyerhof W. Regulation and function of somatostatin receptors. *J Neurochem* 2004;**89**:1057–91.
21. Pierotti AR, Harmar AJ, Simpson J, Yates CM. High-molecular-weight forms of somatostatin are reduced in Alzheimer's disease and Down's syndrome. *Neurosci Lett* 1986;**63**:141–6.
22. Pradayrol L, Jornvall H, Mutt V, Ribet A. N-terminally extended somatostatin: the primary structure of somatostatin-28. *FEBS Lett* 1980;**109**:55–8.
23. Qiu C, Zeyda T, Johnson B, Hochgeschwender U, de Lecea L, Tallent MK. Somatostatin receptor subtype 4 couples to the M-current to regulate seizures. *J Neurosci* 2008;**28**:3567–76.
24. Robas N, Mead E, Fidock M. MrgX2 is a high potency cortistatin receptor expressed in dorsal root ganglion. *J Biol Chem* 2003;**278**:44400–4.
25. Saito T, Iwata N, Tsubuki S, Takaki Y, Takano J, Huang SM, et al. Somatostatin regulates brain amyloid beta peptide Abeta(42) through modulation of proteolytic degradation. *Nat Med* 2005;**11**:434–9.
26. Samson WK, Zhang JV, Avsian-Kretchmer O, Cui K, Yosten GL, Klein C, et al. Neuronostatin regulates neuronal, cardiovascular, and metabolic functions. *J Biol Chem* 2008;**283**:31949–59.
27. Stroh T, van Schouwenburg MR, Beaudet A, Tannenbaum GS. Subcellular dynamics of somatostatin receptor subtype 1 in the rat arcuate nucleus: receptor localization and synaptic connectivity vary in parallel with the ultradian rhythm of growth hormone secretion. *J Neurosci* 2009;**29**:8198–205.
28. Stumm RK, Zhou C, Schulz S, Endres M, Kronenberg G, Allen JP, et al. Somatostatin receptor 2 is activated in cortical neurons and contributes to neurodegeneration after focal ischemia. *J Neurosci* 2004;**24**:11404–15.
29. Vale W, Brazeau P, Grant G, Nussey A, Burgus R, Rivier J, et al. Preliminary observations on the mechanism of action of somatostatin, a hypothalamic factor inhibiting the secretion of growth hormone. *C R Acad Sci Hebd Seances Acad Sci D* 1972;**275**:2913–6.
30. Vallejo M. Somatostatin gene structure and regulation. In: Srikant CB, editor. *Somatostatin*. Norwell, MA: Kluwers Academic Publishers; 2004. p. 1–6.
31. Viollet C, Vaillend C, Videau C, Bluet-Pajot MT, Ungerer A, L'Heritier A, et al. Involvement of sst2 somatostatin receptor in locomotor, exploratory activity and emotional reactivity in mice. *Eur J Neurosci* 2000;**12**:3761–70.
32. Viollet C, Lepousez G, Loudes C, Videau C, Simon A, Epelbaum J. Somatostatinergic systems in brain: networks and functions. *Mol Cell Endocrinol* 2008;**286**:75–87.
33. Winsky-Sommerer R, Spier AD, Fabre V, de Lecea L, Criado JR. Overexpression of the human beta-amyloid precursor protein downregulates cortistatin mRNA in PDAPP mice. *Brain Res* 2004;**1023**:157–62.
34. Xu X, Roby KD, Callaway EM. Immunochemical characterization of inhibitory mouse cortical neurons: three chemically distinct classes of inhibitory cells. *J Comp Neurol* 2010;**518**:389–404.
35. Zheng H, Bailey A, Jiang MH, Honda K, Chen HY, Trumbauer ME, et al. Somatostatin receptor subtype 2 knockout mice are refractory to growth hormone-negative feedback on arcuate neurons. *Mol Endocrinol* 1997;**11**:1709–17.

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Chapter 125

Tachykinins

Nigel M. Page

ABSTRACT

The mammalian tachykinins constitute a family of peptides that all share the common C-terminal sequence, FXGLM-NH_2, and that act as neurotransmitters and neuromodulators in the brain. They mediate their biological actions through interactions with three known tachykinin receptors (NK_1, NK_2, and NK_3) that are located widely and in distinct locations in the brain. They are implicated in the control of several autonomic, affective, and higher cerebral functions and in the pathophysiology of some neurogenerative, psychiatric, and reproductive disorders.

DISCOVERY

The history of the tachykinins can be traced back to 1931 with the discovery of substance P (SP) by von Euler and Gaddum. A 'preparation' (and hence the 'P' of SP) was purified from equine brain and intestine that was found to cause vasodilatation and stimulation of gut mobility in rabbits (see the chapter on SP in Gastrointestinal Peptides Section of this book). Numerous attempts to isolate the pure form of SP over the next forty years were unsuccessful. Nonetheless, in 1967, a peptide named sialogen that stimulated salivation was discovered in the bovine hypothalamus, and in the following year, Lembeck and Starke suggested that this peptide might be the same as SP. In 1970, upon purification and comparison of sialogen's biological properties with those of impure preparations of SP, it was realized that these two peptides were indeed the same. This enabled the chemical structure of SP to be determined as that of an amidated undecapeptide (Table 1), allowing its chemical synthesis and the production of a specific radioimmunoassay. Over the next ten years, SP was believed to be the only mammalian tachykinin even though there were indications that this might not be entirely the case. Inference was drawn from the detection of immunoreactive tachykinin-like activity in mammalian tissues other than SP. Additionally, eledoisin and physalaemin, two tachykinins isolated in the early sixties from the Mediterranean octopus and the South American frog, respectively, were found to produce greater pharmacological responses on mammalian tissues than those produced by SP. In 1983, these suppositions gave way to the confirmation of the existence of two further members both isolated from the porcine spinal cord and designated neurokinin A (NKA) and NKB. Two biologically active N-terminally extended forms of NKA—neuropeptide K (NPK) and neuropeptide gamma (NPγ)—were also later discovered in the mid-1980s. More recently (2000 onward), additional species-divergent members have been discovered including hemokinin-1 (HK-1) in the mouse and rat, endokinin-1 (EK-1) in the rabbit, and endokinin A (EKA) and EKB in humans. Furthermore, in 2003/2004, tachykinin gene-related peptides were identified: in rabbit, EK-2, and in humans, EKC and EKD.[21]

STRUCTURE OF THE mRNA/GENE

The mammalian tachykinins are encoded on three different genes, termed preprotachykinin (*TAC*) 1, *TAC*3, and *TAC*4 (Fig. 1). *TAC*1, the first to be cloned in 1983 from bovine brain, was initially believed to be two different genes, one containing SP (*TAC*1) and the second also containing NKA (*TAC*2). In the following year, it was realized that a discrete genomic segment encoded NKA by alternative RNA splicing of the same gene to yield α*TAC*1 and β*TAC*1.[19] The β*TAC*1 splice transcript was determined to be encoded by seven exons with exon 3 and exon 6 containing SP and NKA, respectively. Exon 6 is lacking from α*TAC*1. Existence of a third *TAC*1 precursor was later found in the rat with the elucidation of a sequence corresponding to the precise deletion of exon 4 (γ*TAC*1). In addition, a fourth splice variant δ*TAC*1 lacking both exon 4 and exon 6 was identified. Therefore, in essence, the alternative splicing of exon 6 of *TAC*1 produces either SP alone (α, δ*TAC*1) or along with NKA (β, γ*TAC*1). The differential inclusion or exclusion of exon 4 into β and γ*TAC*1 allows the potential formation of the two extended NKA peptides, either NPK or NPγ, respectively. Marked species-dependent differential expression of the *TAC*1 splice variants has been found, although within a given species no established evidence of tissue-specific alternative splicing has yet been reported. The gene organization for *TAC*3 encoding NKB, like *TAC*1, is composed of seven exons with exon 5 encoding the sequence for NKB. In the bovine mRNA, two

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00125-1
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TABLE 1 Amino Acid Sequences of the Mammalian Tachykinins

Tachykinins	Peptide sequence
SP	RPKPQQFFGLM-NH_2
NKA	HKTDSFVGLM-NH_2
NPK	DADSSIEKQVALLKALYGHGQISHKRHKTDSFVGLM-NH_2
NPγ	DAGHGQISHKRHKTDSFVGLM-NH_2
NKB	DMHDFFVGLM-NH_2
EKA	DGGEEQTLSTEAETWVIVALEEGAGPSIQLQLQEVKTGKASQFFGLM-NH_2
EKB	DGGEEQTLSTEAETWEGAGPSIQLQLQEVKTGKASQFFGLM-NH_2
EK-1	GKASQFFGLM-NH_2
HK-1	SRTRQFYGLM-NH_2

*TAC*3 transcripts have been reported that differ only at the 5′ extremity of their untranslated regions. In humans, three *TAC*3 transcripts (α, β,γ*TAC*3) are found that differ in their C-terminal coding region.[25] Nevertheless, a thorough investigation of *TAC*3 has concluded that it encodes only the single tachykinin. The gene structures of *TAC*1 and *TAC*3 are highly conserved across the mammalian species when compared with the highly species-divergent structure of *TAC*4. Mouse *TAC*4 is encoded by 4 exons, rat by 5, of which exon 2 in both encodes HK-1. In the rabbit, alternative splicing produces α and β*TAC*4, whereas the alternative splicing of exons 3 and 4 in humans produces α, β, γ, and δ*TAC*4. These splicing events allow either a tachykinin (EK-1, EKA, or EKB) to be synthesized alone or in conjunction with one of the tachykinin gene-related peptides (EK-2, EKC, or EKD). The tachykinin gene-related peptides are not predicted in the mouse or rat genome.[21]

PROCESSING OF THE PRECURSOR

The tachykinin peptides are synthesized as large precursor proteins, which undergo posttranslational proteolytic processing to generate the mature peptide products. It is alternative mRNA splicing that leads to the distinct tachykinin precursor proteins. Each precursor has a strongly hydrophobic signal peptide sequence at its N-terminus, responsible for translocating it to the endoplasmic reticulum. Cleavage of this signal sequence in the endoplasmic reticulum forms the procursor, which is further cleaved, normally at dibasic (or monobasic) amino acid residues by prohormone convertases to yield the smaller tachykinin peptides. Each tachykinin is typically flanked upstream (except human EKA and EKB) and downstream by potential dibasic cleavage sites and all possess an adjacent glycine at their C-terminus for amidation. Typically, following prohormone convertase action (see Peptide Biosynthesis/Processing Section later in book), a carboxypeptidase removes the C-terminal dibasic residues and a peptidylglycine α-amidating enzyme converts the exposed glycine into a C-terminal amide. Intermediate products of this process, including SP-glycine and SP-glycine-lysine, can be found throughout the rat brain where the levels of these extended products are the lowest in the amygala and highest in the medulla.[15] Such differences are believed to be an indicator of different rates of tachykinin synthesis coupled to utilization within the different brain regions. However, the most significant differences in tachykinin processing are found at their N-termini. In SP, where the N-terminal dibasic cleavage site is adjacent to a proline residue and because of the resistance of arginine–proline bonds to prohormone converting enzymes, an arginine residue from the dibasic cleavage site is exceptionally maintained. Additionally, in the case of SP, many studies have shown that further processing can occur including the production of SP(1-4) and SP(1-7), which are biologically active SP metabolites. There is also evidence of differential processing at the N-terminal dibasic cleavage site of NKA with such processing responsible for the production of both NPK and NPγ. Indeed, early evidence from the late 1980s noted that although SP cleavage seemed to be easily handled, the fact that multiple peptides were derived from the NKA portion of the *TAC*1 precursor indicated that multiple enzymes might be involved in the cleavage of the different NKA tachykinins. Likewise, the apparent loss of the N-terminal dibasic processing site of EKA and EKB with a monobasic cleavage site could be due to the evolutionary pressure of differential processing.

DISTRIBUTION OF THE mRNA/PEPTIDE

In the brain, distribution of the tachykinins has been most extensively studied in the rat, where widespread and distinct regions containing labeled *TAC*1 and *TAC*3 neurons are found.[20] In the case of rat *TAC*1, these are

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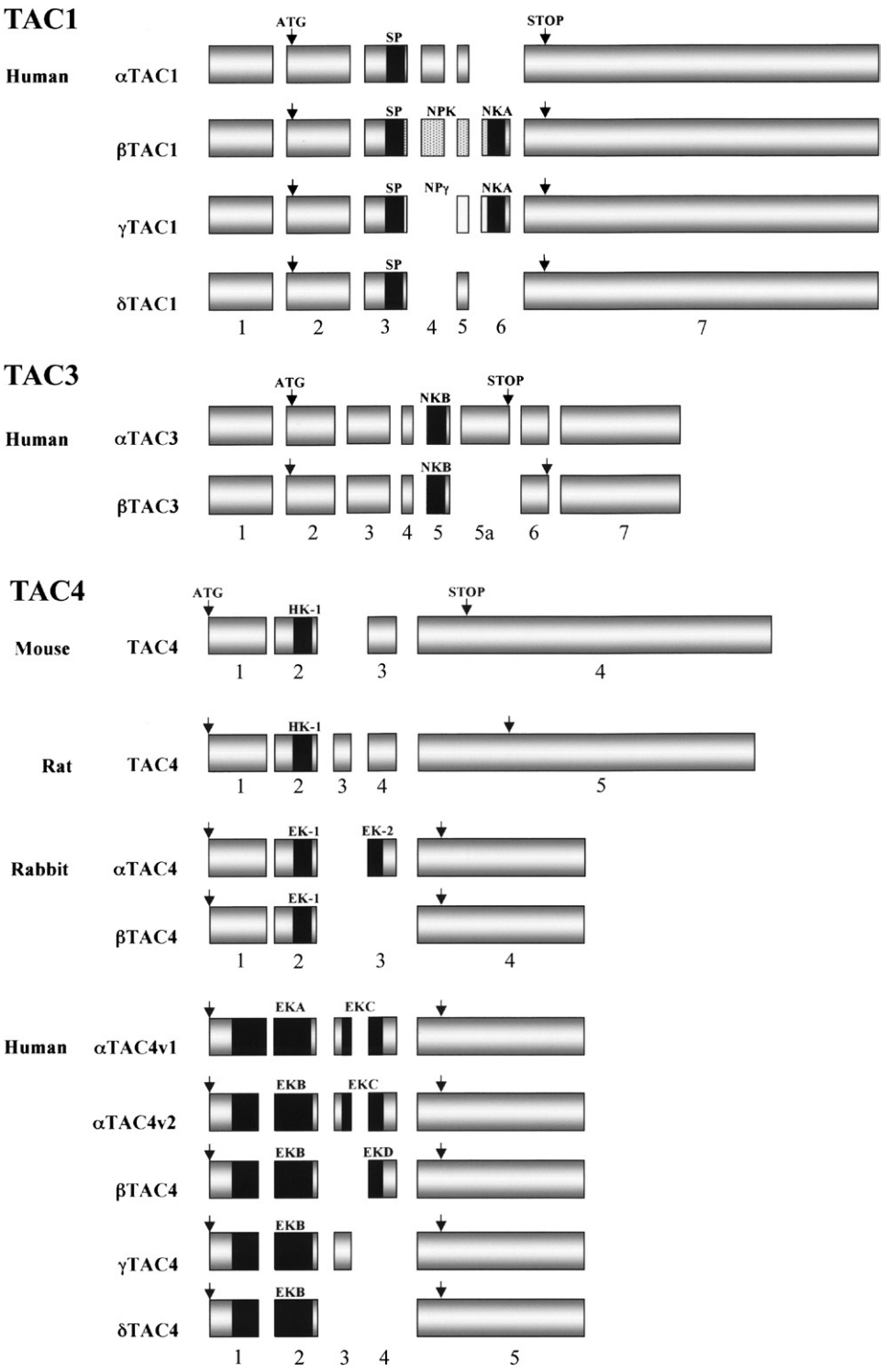

FIGURE 1 Exon organization of the human *TAC*1, human *TAC*3, and mouse, rat, rabbit and human *TAC*4 genes showing the positions of the predicted peptides *(Adapted from Ref. 22)*.

in the anterior olfactory nucleus, olfactory tubercle, the islands of Calleja, the nucleus accumbens, the caudate putamen, amygdala, hypothalamus, the medial habenular nucleus, nuclei of the pontine tegmentum, raphe nuclei, portions of the reticular formation, and the nucleus of the solitary tract.[9] The characterized positions of the SP- and NKA-containing cell groups and fibers in the rat brain are shown in Fig. 2, where it is generally believed that SP is cosynthesized, colocalized, and cosecreted with NKA. Levels of *TAC*1 have also been measured during the

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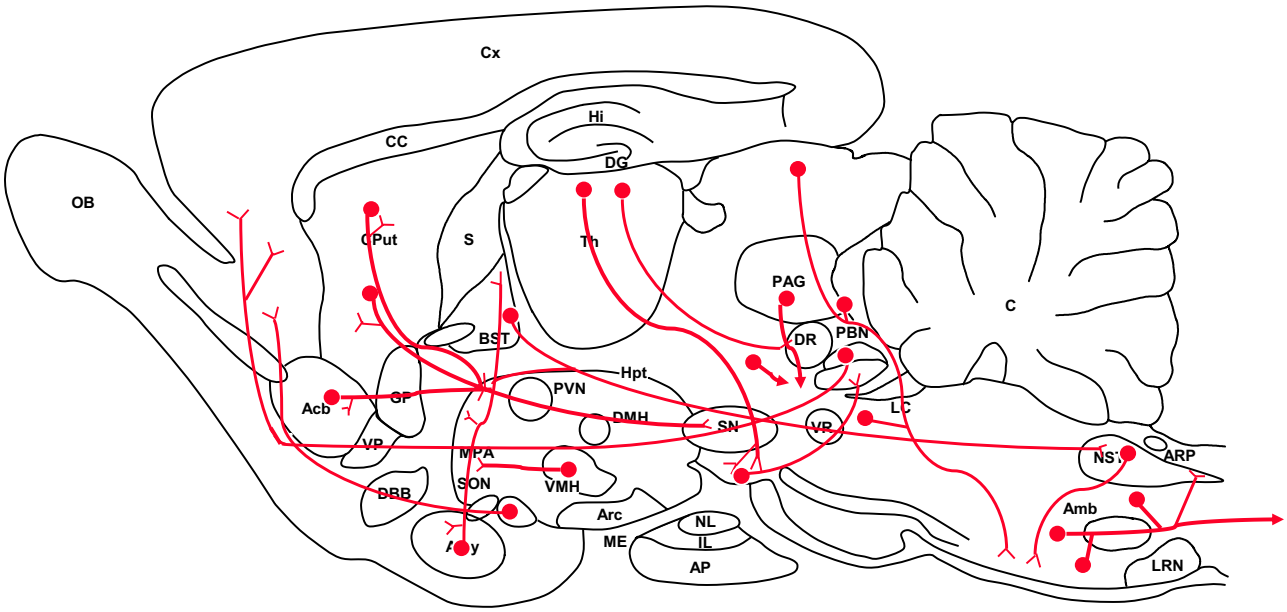

FIGURE 2 Parasagittal section of the rat brain showing substance P and neurokinin A cell groups and fibers.

development of rat brain from embryonic day 15 to adult indicating significant changes in region and time-specific regulation of the *TAC*1 gene, particularly in the cingulate cortex.[1] In the case of rat *TAC*3, neurons are located in the cerebral cortex, the hippocampal formation, the amygdaloid complex, the bed nucleus of the stria terminalis, the ventral pallidum, the habenula, the medial preoptic area, the arcuate nucleus, and the lateral mammillary bodies.[16] In the human brain, there is a distinct and complementary distribution pattern of *TAC*1 and *TAC*3 neurons observed in the hypothalamus and basal forebrain. *TAC*3 is the predominant tachykinin in the rostal hypothalamus, whereas *TAC*1 predominates in the posterior hypothalamus.[3] Numerous *TAC*3 neurons are identified in the magnocellular basal forebrain, the bed nucleus of stria terminalis, and the anterior hypothalamic area with scattered neurons in the infundibular and paraventricular nuclei, paraolfactory gyrus, posterior hypothalamic area, lateral division of the medial mammillary nucleus, and amygdala.[3] Numerous neurons expressing *TAC*1 are identified in the premammillary, supramammillary, and medial mammillary nuclei, the posterior hypothalamic area, and the corpus striatum. Scattered SP neurons are also observed in the preoptic area, the infundibular, intermediate, dorsomedial, and ventromedial nuclei, the infundibular stalk, the amygdala, the bed nucleus of stria terminalis, and the paraolfactory gyrus.[3] The expression levels for *TAC*1 and *TAC*3 have been translated into respective amounts of immunoreactive peptides by immunoassay, which have proved largely confirmatory of the mRNA expression sites. In the different regions of rat brain, the amounts of SP are generally greater than those of NKA, which are in turn greater than those for NKB. *TAC*4 has been found to be predominantly expressed in the periphery. Nevertheless, the analysis of numerous regions of the murine brain (including the brain stem, cerebellum, cortex, caudate, hippocampus, middle brain, hypothalamus, and thalamus) has revealed a similar regional distribution to that for brain *TAC*1, although it is present at consistently (around 100-fold) lower levels, except in the cerebellum that has a 10-fold higher expression level of *TAC*4 than *TAC*1.[5] In terms of some earlier peptide studies of SP in the brain, it is highly possible that these may have also detected HK-1 and EK as some established and commercially available antibodies to SP have been found subsequently to have a 100% crossreactivity with the common C-terminal decapeptide of the HK and EK peptides.[21]

RECEPTORS AND SIGNALING CASCADES

In the early 1980s, after intense pharmacological investigations, the existence of three tachykinin receptors was proposed, termed NK_1, NK_2, and NK_3 (reviewed in Ref. 21). Final confirmation was provided in the late 1980s with the cloning of each subtype from a number of different mammalian species. The receptors all constitute members of the G protein-coupled receptor family and are encoded on five exons. For example, the tachykinin NK_1 receptor contains seven putative hydrophobic α-helical transmembrane domains where the first two extracellular loops are joined by a disulfide bridge. Its extracellular N-terminus contains two predicted asparagine glycosylation sites and its intracellular

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C-terminal tail contains predicted sites of palmitoylation and a series of potential serine and threonine phosphorylation sites. The order of potency of the tachykinin receptors for each tachykinin is as follows: NK_1, SP = HK-1 = EKA/B ≥ NKA > NKB; NK_2, NKA > NKB > SP = EKA/B, and NK_3, NKB > NKA > SP = EKA/B.[24] NPK and NPγ preferentially bind to the NK_2 receptor. However, there is significant opportunity for the crossselectivity of each receptor subtype with each different tachykinin owing to their common 'activating' C-terminal motif, allowing them to act as full agonists at each receptor subtype. Overall, the signal transduction mechanisms of the three tachykinin receptors have been studied in transfected Chinese hamster ovary (CHO) cells. Here, all these receptors markedly activate phospholipase C-β via a pertussis toxin insensitive G protein ($G\alpha_q$ and/or $G\alpha_{11}$), leading to the hydrolysis of membrane-bound inositol phospholipids and elevation of intracellular calcium.[8] Receptor activation can also promote the arachidonic acid cascade; however, the stimulation of adenylate cyclase is less efficient and requires about one order of magnitude higher concentrations of each tachykinin.[18]

The NK_1 receptor has been found distributed in the dorsal striatum, nucleus accumbens, hippocampus, raphe nuclei, medulla oblongata, and cerebral cortex.[20] In the central nervous system, binding sites for NKA were reported despite the finding that NK_2 receptor mRNA transcripts could not be located. Indeed, NK_2 receptors are only expressed in a few regions of the brain such as the nucleus of the medial septum/diagonal band. The NKA binding enigma eventually gave way to the realization that alternative conformations of the NK_1 receptor existed (reviewed in Ref. 23). In CHO cells overexpressing the NK_1 receptor, 'classic' NK_1 receptor binding sites were found coupled to adenylate cyclase whereas 'septide-sensitive' NK_1 receptor sites were found coupled to phospholipase D. At 'classic' NK_1 binding sites, SP was found to be the only endogenous tachykinin to display high affinity, whereas NKA, NPK, NPγ, and NKB, instead exhibited a high affinity for the 'septide-sensitive' NK_1 binding site. However, *in vivo*, 'classic' NK_1 receptor binding sites are associated with phospholipase C, and rarely with adenylate cyclase. Consequently, each NK_1 receptor subtype represents a different receptor conformation that can be coupled to a distinct transduction system with the capacity to activate different functions. Furthermore, the NK_1 receptor also exists as a truncated isoform without its C-terminal tail; this possesses different receptor binding efficiencies and effector system interactions.[7] The full-length NK_1 receptor isoform has been reported to be the most prevalent throughout the human brain, whereas in peripheral tissues, the truncated isoform is the most represented.[2] Nonetheless, more recently, Lai et al.[13] have reported that, in some parts of the brain including the cingulated cortex, nucleus accumbens, and locus coeruleus, mRNA levels of the truncated NK_1 receptor isoform are significantly higher than those of the full-length receptor. In addition to these isoforms and subtypes, it should be noted there are marked species differences in the localization of the central tachykinin receptors, which are also accompanied by significant species-dependent variability in the homologies of the NK_2 and NK_3 receptors when compared with the highly species conserved NK_1 receptor.[23,28]

BIOLOGICAL ACTIONS WITHIN THE BRAIN

There is substantial evidence that, in the brain, tachykinins play very important roles as neurotransmitter, neuromodulatory, and neurotrophic agents. They occur particularly in large quantities in the areas involved in the control of several autonomic and endocrine functions, of affective and emotion responses, and of higher cerebral functions. They are also often colocalized with other neurotransmitters such as dopamine, endomorphin, and serotonin.

The biological actions of tachykinins have been recorded in a number of different brain regions and some of the more defined are summarized here:

1. In the *cerebral cortex*, SP has been found to excite 91% of the spontaneously active neurons tested.[26]
2. In the *striatum*, tachykinins are released by high potassium concentrations. SP has been reported to increase the firing of some striatal neurons and to evoke the release of dopamine, Met-enkephalin, and acetylcholine.
3. In the *globus pallidus*, microinjection of tachykinin antagonists into the entopeduncular nucleus has been shown to reduce muscle tone in genetically spastic rats, suggesting that SP-dependent mechanisms can regulate muscle tone.[29]
4. In the *substantia nigra*, SP is released in a calcium-dependent manner in response to depolarizing stimuli and upon electrophoretic application to produce long-lasting excitation. SP infusion into the substantia nigra produces marked increases in stereotyped behaviors such as grooming, sniffing, and rearing. In addition, SP has also been suggested to have an excitatory regulation on nigrostriatal dopamine release. NKA is also believed to exert potent excitatory action on nigral dopaminergic and nondopaminergic neurons.[12]
5. In the *ventral tegmental area*, a close relationship between dopamine and SP has been suggested. SP infusion into the ventral tegmental area causes a marked increase in locomotion and rearing that can be completely blocked by infusion of dopamine antagonists. An important role for NKA in the ventral tegmental area has also been suggested as it has been found to be 10 times more potent than SP in producing an increase in motor activity.[10]

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6. Dopamine neurons richly innervate the *nucleus accumbens*, where evidence suggests that tachykinins can modulate dopamine release through presynaptic mechanisms. The activation of this pathway is believed to cause an increase in locomotor activity. In addition, SP injection into this nucleus exerts memory-promoting effects.
7. In the limbic system, the *hippocampus* and *amygdala* have been implicated in memory processing, where NKA and NPK administered intracerebroventricularly after footshock avoidance training enhanced memory attention in mice. Localization studies also showed that NPK improved memory when injected directly into the hippocampus and amygdala. The amygdala has also been implicated in the regulation of sexual behavior with higher levels of SP being found in male rather than female rats, and with castration of adult rats reduces amygdala SP staining. It has also been suggested that the medial amygdala is a site of action for a NK_3 mediated mechanism to inhibit salt intake. Weidenhofer et al.[31] have also shown that NK_1 receptors are widely distributed in the amygdala and could be involved in emotion and fear processing. Furthermore, intraseptal activation of all three NK receptors, especially the NK_2 receptor, has been implicated in stress-induced hippocampal acetycholine release.
8. In the *hypothalamo–hypophysial* system, it has been suggested that SP may be secreted directly into the portal circulation to reach the anterior pituitary. In doing so, SP may affect the release of anterior pituitary hormones. Such released SP is believed to inhibit the release of rat growth hormone, whereas SP antagonists increase the concentrations of plasma growth hormone. Also, SP applied to pituitary cells exerts a stimulant effect on prolactin secretion. In the hypothalamus, SP has been shown to act as an orexigenic substance and promotes weight gain via coupling to the NK_1 receptor.[11]
9. In the hypothalamic *infundibular/arcuate nucleus*, NKB and the NK_3 receptor are shown to be the essential elements of the human reproductive axis being coexpressed with kisspeptin, dynorphin, and the estrogen receptor α in a variety of mammalian species.[27] There is evidence in humans that these neurons regulate estrogen negative feedback on gonadotropin-releasing hormone secretion and have been implicated in hypogonadotropic hypogonadism and the menopause.

Furthermore, a number of biological actions of tachykinins in the brain are demonstrated by direct intracerebroventricular injection. These are summarized:

1. *Cardiovascular* responses promoted by SP and NKA typical of a defense reaction that increases blood pressure, heart rate, sympathetic efferent activity, visceral vasoconstriction, and hindlimb vasodilation. These responses are accompanied by behavioral defense reactions including increased locomotion, scratching, skin biting, and grooming.
2. NK_3 agonists that elicit dose-related inhibition of *gastric secretion*, *gastric emptying*, and *colonic propulsion*.
3. The administration of eledoisin that affects the *drinking behavior* evoked by angiotensin II intracerebroventricular injection, which acts by inhibiting water uptake and causing water deprivation and cell dehydration. Injection of eledoisin also causes the release of vasopressin with ensuing antidiuresis and independently displays a potent and lasting inhibitory effect on salt uptake.
4. Changes in *respiration* caused by the dose-dependent administration of SP leading to minute ventilation, due to increased volume stimulated respiration.
5. An increase in *anxiety* with the infusion of NK_1 agonists causing long-lasting and pronounced vocalization in guinea pigs.
6. The administration of SP and some of its N-terminal derived peptides that are shown to control *behavior*. SP(1-7) inhibited not only nociception but also aggressive and grooming behavior, while, like SP, stimulating investigative motor activities.
7. Intrathecal administration of EKA/B is shown to evoke scratching behavior and thermal hyperalgesia through the NK_1 receptor.[32]

PATHOPHYSIOLOGICAL IMPLICATIONS

Tachykinins have been implicated in a number of centrally mediated disease states. These include, among others, the neurodegenerative disorders of Parkinson's and Alzheimer's disease. Parkinson's disease, which is a serious motor disorder, is caused by the degeneration of dopamine neurons in the substantia nigra. However, there are lines of evidence to indicate a role for tachykinins in its pathogenesis.[4] First, decreases of *TAC*1 and SP-immunoreactivity are found in the nigral and striatal regions of animal and post mortem Parkinson's disease patients. Second, tachykinins have been found to exert neuroprotective effects on neurons. In addition, several NK_1 and NK_3 receptors are localized in the dopaminergic neurons of the basal ganglia indicating these neurons are under the physiological regulation of tachykinins. Furthermore, in an animal model of Parkinson's disease, tachykinin receptor agonists were found to modulate motor activity. In the case of Alzheimer's disease, SP function has been related to memory and learning where it is highly expressed in several brain areas from the forebrain to hindbrain.[6] In this regard, the basal forebrain, particularly the nucleus of Meynert, has received attention, as it is these SP-containing nuclei that have been shown to degenerate in the senile dementia of Alzheimer's disease. SP has also been found to interact with the cholinergic ascending system of the nucleus of Meynert and patients with Alzheimer's disease show a marked loss

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of cholinergic neurons from the nucleus of Meynert to the cortex and diminished brain SP immunoreactivity.

SP has also received particular focus in its role in psychiatric disorders. This rationale has been based on the observation that, like clinically used antidepressant/anxiolytic drugs, SP antagonists suppress anxiety-related behaviors and defensive cardiovascular changes in rodents. In addition, mice with selective deletions of the gene encoding the NK_1 receptor or SP itself showed decreased anxiety-related behaviors. More recently, aversive and stressful stimuli have been shown to change SP levels and NK_1 receptor binding with increase SP influx into specific limbic structures such as the amygdala and septum with the magnitude of the response dependent on the severity of the stressor. The mechanism by which SP antagonists bring about their effects is not fully understood, but SP is found in close association with 5-hydroxytryptamine and norepinephrine-containing neurons that are targeted by currently used antidepressant drugs. The NK_1 antagonist MK-869 was shown to display antidepressant activity comparable with that of the selective serotonin reuptake inhibitor paroxetine but with a better side effect profile. However, further research is needed to ascertain the relevance of NK_1 antagonists in depression after the subsequent discontinuation of MK-869 from Phase III trials, after it was shown to be no more effective than placebo. Nevertheless, there still remains an interest in the role of SP in psychiatric illnesses, which also extends to conditions such as panic disorder, obsessive-compulsive disorder, generalized anxiety disorder, and posttraumatic stress disorder. Interestingly, MK-869 has recently received regulatory approval for use as an antiemetogenic therapy in cancer chemotherapy patients along with a combination of $5\text{-}HT_3$ antagonist and dexamethasone. SP is found in neurons of the brain stem and is believed to suppress the centrally controlled vomiting reflex. Because tachykinins are well known for producing marked behavioral changes in animals, they are plausible candidates for playing roles in other pathological behavioral states including schizophrenia and bipolar mood disorders. Changes in brain levels of SP and SP-binding sites have been implicated in schizophrenia. Significant reductions of *TAC*1 mRNA expression are detected in the basal, lateral, and accessory basal amygdaloid nuclei in patients with schizophrenic or bipolar disorders. It has been hypothesized that the involvement of tachykinins in mood disorders and schizophrenia suggests a generalized impairment of the SP system in the amygdala. NK_1 antagonists have also been used in the study of acute migraine where it is believed that SP is a mediator of pain and inflammation through the NK_1 receptor. SP binding to postcapillary NK_1 receptors in the brain vasculature is believed to produce plasma extravasation, platelet aggregation, and white cell endothelial adhesion. These effects could mediate both the inflammatory process and pain reported in migraine. Furthermore, NK_1 antagonists are very effective in animal models of inflammation, thought to be the indicators of activity in acute migraine. SP antagonists have also been implicated as a therapeutic approach for improving the outcome following traumatic brain injury,[30] where SP is released after acute injury leading to not only direct neuronal cell death but also to the development of vasogenic edema. The ability of SP to also augment immune responses of glial cells has important ramifications to the progression of damaging inflammation.[17] Furthermore, in glial cells, SP-NK_1 receptor interaction is involved in glioma development and progression, and NK_1 receptor antagonists may serve as an anticancer therapy.[14]

REFERENCES

1. Brené S, Lindefors N, Friedman WJ, Persson H. Preprotachykinin A mRNA expression in the rat brain during development. *Brain Res Dev Brain Res* 1990;**57**:151–62.
2. Caberlotto L, Hurd YL, Murdock P, Wahlin JP, Melotto S, Corsi M, et al. Neurokinin 1 receptor and relative abundance of the short and long isoforms in the human brain. *Eur J Neurosci* 2003;**17**:1736–46.
3. Chawla MK, Gutierrez GM, Young 3rd WS, McMullen NT, Rance NE. Localization of neurons expressing substance P and neurokinin B gene transcripts in the human hypothalamus and basal forebrain. *J Comp Neurol* 1997;**384**:429–42.
4. Chen LW, Yung KK, Chan YS. Neurokinin peptides and neurokinin receptors as potential therapeutic intervention targets of basal ganglia in the prevention and treatment of Parkinson's disease. *Curr Drug Targets* 2004;**5**:197–206.
5. Duffy RA, Hedrick JA, Randolph G, Morgan CA, Cohen-Williams ME, Vassileva G, et al. Centrally administered hemokinin-1 (HK-1), a neurokinin NK1 receptor agonist, produces substance P-like behavioral effects in mice and gerbils. *Neuropharmacology* 2003;**45**:242–50.
6. Flashner E, Raviv U, Friedler A. The effect of tachykinin neuropeptides on amyloid β aggregation. *Biochem Biophys Res Commun* 2011;**407**:13–7.
7. Fong TM, Anderson SA, Yu H, Huang RR, Strader CD. Differential activation of intracellular effector by two isoforms of human neurokinin-1 receptor. *Mol Pharmacol* 1992;**41**:24–30.
8. Guard S, Watson SP. Tachykinin receptor types: classification and membrane signalling mechanisms. *Neurochem Int* 1991;**18**:149–65.
9. Harlan RE, Garcia MM, Krause JE. Cellular localization of substance P- and neurokinin A-encoding preprotachykinin mRNA in the female rat brain. *J Comp Neurol* 1989;**287**:179–212.
10. Iversen SD, Kelley AE, Stinus L. Dose dependent behavioural stimulation after local infusion of substance P into the ventral tegmental area in the rat [proceedings]. *Br J Pharmacol* 1979;**66**:113P–4P.
11. Karagiannides I, Torres D, Tseng YH, Bowe C, Carvalho E, Espinoza D, et al. Substance P as a novel anti-obesity target. *Gastroenterology* 2008;**134**:747–55.
12. Kelley AE, Iversen SD. Substance P infusion into substantia nigra of the rat: behavioural analysis and involvement of striatal dopamine. *Eur J Pharmacol* 1979;**60**:171–9.
13. Lai JP, Cnaan A, Zhao H, Douglas SD. Detection of full-length and truncated neurokinin-1 receptor mRNA expression in human brain regions. *J Neurosci Methods* 2008;**168**:127–33.
14. Łazarczyk M, Matyja E, Lipkowski A. Substance P and its receptors – a potential target for novel medicines in malignant brain tumour therapies (mini-review). *Folia Neuropathol* 2007;**45**:99–107.

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15. Marchand JE, Shimonaka H, Kream RM. Biochemical characterization and anatomical distribution of a major form of unamidated precursor of substance P in rat brain. *Brain Res* 1991;**567**:290–305.
16. Marksteiner J, Sperk G, Krause JE. Distribution of neurons expressing neurokinin B in the rat brain: immunohistochemistry and in situ hybridization. *J Comp Neurol* 1992;**317**:341–56.
17. Marriott I. The role of tachykinins in central nervous system inflammatory responses. *Front Biosci* 2004;**9**:2153–65.
18. Nakajima Y, Tsuchida K, Negishi M, Ito S, Nakanishi S. Direct linkage of three tachykinin receptors to stimulation of both phosphatidylinositol hydrolysis and cyclic AMP cascades in transfected Chinese hamster ovary cells. *J Biol Chem* 1992;**267**:2437–42.
19. Nawa H, Kotani H, Nakanishi S. Tissue-specific generation of two preprotachykinin mRNAs from one gene by alternative RNA splicing. *Nature* 1985;**312**:729–34.
20. Otsuka M, Yoshioka K. Neurotransmitter functions of mammalian tachykinins. *Physiol Rev* 1993;**73**:229–308.
21. Page NM. Hemokinins and endokinins. *Cell Mol Life Sci* 2004;**61**:1652–63.
22. Page NM. New challenges in the study of the mammalian tachykinins. *Peptides* 2005;**26**:1356–68.
23. Page NM. Characterization of the gene structures, precursor processing and pharmacology of the endokinin peptides. *Vascul Pharmacol* 2006;**45**:200–8.
24. Page NM, Bell NJ, Gardiner SM, Manyonda IT, Brayley KJ, Strange PG, et al. PJ. Characterization of the endokinins: human tachykinins with cardiovascular activity. *Proc Natl Acad Sci U S A* 2003;**100**:6245–50.
25. Page NM, Morrish DW, Weston-Bell NJ. Differential mRNA splicing and precursor processing of neurokinin B in neuroendocrine tissues. *Peptides* 2009;**30**:1508–13.
26. Phillis JW, Limacher JJ. Excitation of cerebral cortical neurons by various polypeptides. *Exp Neurol* 1974;**43**:414–23.
27. Rance NE, Krajewski SJ, Smith MA, Cholanian M, Dacks PA. Neurokinin B and the hypothalamic regulation of reproduction. *Brain Res* 2010;**1364**:116–28.
28. Rigby M, O'Donnell R, Rupniak NM. Species differences in tachykinin receptor distribution: further evidence that the substance P (NK1) receptor predominates in human brain. *J Comp Neurol* 2005;**490**:335–53.
29. Turski L, Klockgether T, Turski WA, Schwartz M, Sontag KH. The entopeduncular nucleus regulates muscle tone in genetically spastic rats: role of substance P and gamma-aminobutyric acid. *Brain Res* 1990;**509**:347–50.
30. Vink R, van den Heuvel C. Substance P antagonists as a therapeutic approach to improving outcome following traumatic brain injury. *Neurotherapeutics* 2010;**7**:74–80.
31. Weidenhofer J, Yip J, Zavitsanou K, Huang XF, Chahl LA, Tooney PA. Immunohistochemical localisation of the NK1 receptor in the human amygdala: preliminary investigation in schizophrenia. *Prog Neuropsychopharmacol Biol Psychiatry* 2006;**30**:1313–21.
32. Yoshioka D, Takebuchi F, Nishimori T, Naono R, Ikeda T, Nakayama T. Intrathecal administration of the common carboxyl-terminal decapeptide in endokinin A and endokinin B evokes scratching behavior and thermal hyperalgesia in the rat. *Neurosci Lett* 2006;**410**:193–7.

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Chapter 126

TRH

A. Eugene Pekary

ABSTRACT

Thyrotropin-releasing hormone (TRH) was the first of the hypothalamic releasing factors to be fully characterized. It consists of the tripeptide pGlu-His-Pro-NH_2 that is derived from a precursor protein with multiple copies of the progenitor sequence Lys-Arg-Gln-His-Pro-Gly-(Lys/Arg)-Arg. PreproTRH is distributed throughout the animal kingdom, occurring in species lacking a pituitary. In addition to its neuroendocrine release from the hypothalamus of mammals, resulting in the secretion of TSH and PRL from the anterior pituitary, it functions as a neurotransmitter, neuromodulator, and neuroprotective agent in the central and peripheral nervous systems. Unlike hypothalamic preproTRH mRNA levels and TRH content that are subject to thyroid hormone negative feedback inhibition, extrahypothalamic brain TRH biosynthesis and release are unresponsive to thyroid hormone status. TRH suppresses glycogen synthase kinase-3β (GSK-3β) expression, a process that may lead to treatments for Alzheimer's disease, depression, bipolar disorder, and diabetes. TRH is also an important regulator of lean body mass.

DISCOVERY OF TRH

The nearly simultaneous reporting of the structure of the first hypothalamic releasing factor, thyrotropin-releasing hormone (TRH), as pGlu-His-Pro-NH_2, by Roger Guillemin and Andrew Schally, was the culmination of a five-decade effort to validate the neuroendocrine hypothesis.[5] This story contains elements of intense rivalry between competing research groups, serendipitous combinations of events, and sheer luck that the substance being isolated contained only three amino acid residues, Glu, His, and Pro. During the synthesis of all possible tripeptides containing these amino acids, Glu-His-Pro was found to have a low, but unmistakable, activity in the dispersed anterior pituitary bioassay being used to detect TSH-releasing activity in chromatographic fractions of hypothalamic extracts. Given that almost all the other hypophysiotropic hormones are much larger than TRH[30] and often require amidation of the C-terminal residue, complete characterization of these other releasing hormones using the technology and funding available at the time would have been nearly impossible. Acceptance of Neuroendocrinology as a legitimate research discipline could have been long delayed but for this extraordinary confluence of chance, rivalry, and scientific skepticism.

STRUCTURE OF THE PRECURSOR mRNA/GENE

Genes for fish, frog, chicken, rat, mouse, and human preproTRH contain three exons and two introns. The first exon codes for the 5′-untranslated region; the second exon contains the signal sequence and most of the N-terminus. The remainder of preproTRH is coded by the third exon (Fig. 1). Two closely related preproTRH genes have been identified in frogs, with sequence differences occurring primarily within the 5′-flanking regions. This may provide additional opportunities for tissue and cell-specific regulation of TRH synthesis in this species, which produces extraordinarily high levels of TRH in its skin.[11] Each preproTRH mRNA contains multiple TRH progenitor coding sequences: 6–8 in teleosts, 7 in frogs, 6 in humans, 5 in rats and mice, and 5 in chicken.[33] Interestingly, one of the 5 TRH progenitor sequences in chicken preproTRH is not flanked by pairs of basic amino acid residues. Peptide sequences connecting some of the TRH progenitors in rat preproTRH have been shown to have biological activity,[20] but these are not strongly conserved among species.[33]

DISTRIBUTION OF PREPROTRH mRNA AND PREPROTRH-DERIVED PEPTIDES

PreproTRH mRNA is expressed at very high levels in mouse hypothalamus and testis.[28] Immunohistochemical staining with antibodies to intervening sequences of rat preproTRH showed staining in neuronal perikarya and processes in all the regions of the brain previously demonstrated to immunostain for TRH, including dense

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00126-3
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innervations of the external zone of the median eminence. Immunocytochemical and in situ hybridization studies localize TRH-producing perikarya in extrahypothalamic brain areas such as the olfactory system, reticular thalamic nucleus, and amygdaloid complex (Fig. 2). Rat preproTRH (160–169) is readily measured in the hippocampus, piriform cortex, amygdala, striatum, and anterior cortex.[20] High levels of TRH immunoreactivity are also found in the posterior pituitary, retina, pineal gland, spinal cord, reproductive, and immune systems.[13,22,30]

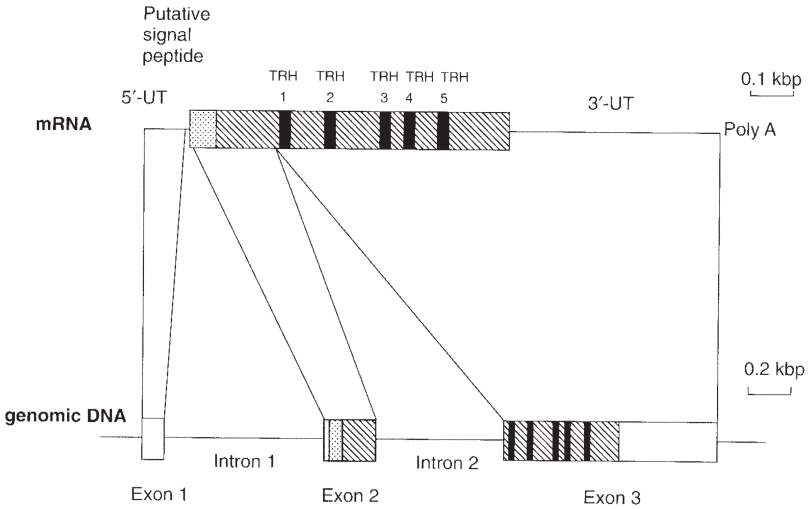

FIGURE 1 Structure of rat preproTRH gene: schematic diagram of the messenger RNA (mRNA) (top line) and genomic DNA (bottom line). The 5′-untranslated region (5′-UT) and 3′-untranslated region (3′-UT) are shown by the solid line (top) and open boxes (bottom). The signal peptide is indicated by a stippled box. The striped boxes indicate connecting and terminal peptides. The solid boxes indicate the TRH progenitor-coding regions. (Ref. 30 with permission from MIT Press).

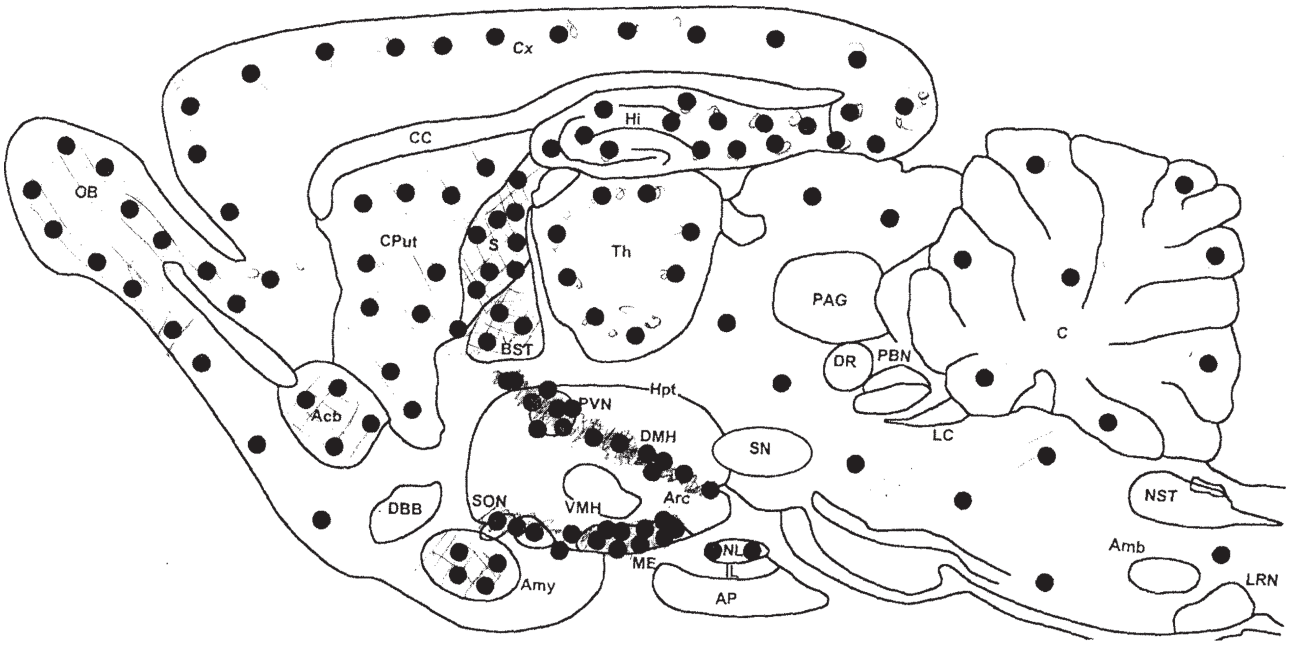

FIGURE 2 PreproTRH mRNA, expressed protein, and derived peptides are widely distributed in rat brain.[7,19,20,26,28,30] However, the extent of processing of the preproTRH precursor protein to form tripeptide TRH, before its extracellular release, is limited in some brain areas but is nearly complete in the hypothalamus.

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PROCESSING OF THE TRH PRECURSOR

Processing of preproTRH begins with the removal of the signal peptide during its passage into the lumen of the rough endoplasmic reticulum.[19] The dibasic amino acid residues flanking the N-terminus and C-terminus of the repeated TRH progenitor sequence Gln-His-Pro-Gly are cleaved by serine proteases, PC1 and PC2, which are constituent membrane proteins of the large dense core vesicles (LDCV) into which preproTRH is packaged after synthesis in the endoplasmic reticulum. The C-terminal basic residues are removed by the zinc-dependent carboxypeptidase H. The remaining C-terminal Gly is cleaved by the copper, ascorbate, and oxygen-requiring peptidylglycine alpha amidating monooxygenase (PAM). The N-terminal Gln is cyclized to pyroglutamate with the aid of an N-glutaminyl cyclase.[19,30]

DISTRIBUTION OF TRH RECEPTORS AND THE TRH DEGRADING ENZYME

Four different receptors for TRH have been reported to occur in a teleost; three in frog; two in rat, mouse; and one in chicken, pig, human, sheep, and cow.[12,18] Three of the teleost TRH receptor genes consist of two exons, whereas the forth consists of 5 exons.[18] The distribution of the rat mRNA for the TRH receptor 1 (TRH-R1) and receptor 2 (TRH-R2) has been studied in detail.[7] TRH-R2 mRNA is widely distributed throughout brain with highest mRNA levels in the thalamus, cerebral and cerebellar cortex, medial habenulae, medial geniculate nucleus, pontine nuclei, and the entire reticular formation. Consistent with the endocrine function of TRH, TRH-R1 predominates in hypothalamic nuclei, the anterior pituitary thyrotrophs, mammotrophs (PRL-secreting cells), and to a lesser extent in somatotrophs (GH-secreting cells).[30] TRH-R1 in brainstem regions and spinal cord motoneurons is involved in autonomic and somatomotor control.[2,5] An overlapping distribution of the TRH degrading enzyme (TRH-DE) mRNA[1] with TRH-R2 mRNA is consistent with the involvement of TRH in higher cognitive functions as well as effects on arousal, locomotor activity, and pain perception.[1,7,8,30]

TRH RECEPTOR SIGNALING CASCADES

TRH-R1 and TRH-R2 are typical G-protein-coupled receptors that bind TRH and N-methyl-TRH with high affinity and specificity.[8] TRH-R3 from frog binds TRH, and other TRH-like peptides weakly, suggesting that it is an orphan receptor (Hinkle and Pekary, unpublished results[16]). TRH-R1 couples to G_q and G_{11},[4] pertussis-toxin-insensitive G proteins that activate phosphoinositide-specific phospholipase C-β (PPI-PLC).[1] PPI-PLC is the enzyme that hydrolyzes phosphatidylinositol 4,5-bisphosphate ($PIPP_2$) to generate two second messengers, inositol 1,4,5-triphosphate and 1,2-diacylglycerol. Inositol trisphosphate binds to the inositol trisphosphate receptor, a calcium channel on the endoplasmic reticulum, resulting in an increase in the cytoplasmic calcium levels.[34] Diacylglycerol, along with calcium, activates protein kinase C (PKC) resulting in the increased phosphorylation of Ser and Thr on a variety of second messenger enzymes.[4] Altered phosphorylation of ion channel, cytoplasmic and plasma membrane proteins, and nuclear transcription factors modify gene expression in the cell nucleus thereby transducing the TRH receptor binding signal.[1]

BIOLOGICAL ACTIONS OF TRH WITHIN THE BRAIN AND PITUITARY

TRH is the principal regulator of the "set point" for thyrotropin (TSH) synthesis and secretion by the anterior pituitary thyrotrophs.[24,32] TSH stimulates the thyroid gland to produce T_4 and T_3, which provide a negative feedback suppression of hypothalamic TRH and pituitary TSH synthesis. Thyroid hormones also accelerate TRH degradation.[19,32] This regulatory network is summarized in Fig. 3. Knock out of the preproTRH gene results in hypothyroidism and diabetes mellitus.[35] Hypothalamic TRH release is altered in many conditions including thyroid disease, cold stress, the onset of sleep, and changes in hormone, monoamine, and cytokine levels resulting from physical or emotional stress or severe illness.[1] Inhibition of "leak" potassium (K^+) channels is a widespread mechanism by which neurotransmitters induce slow excitation of neurons.[31] Transmitter modulation of cellular excitability has been attributed to TASK-1, a two-pore domain K^+ channel, coupled to TRH-R1.[31]

It has long been assumed that TRH has other functions besides the control of TSH given its wide distribution throughout the brain and peripheral tissues, its occurrence in vertebrate and invertebrate species lacking a pituitary, and the unresponsiveness of TRH mRNA and peptide levels of the limbic system to changes in thyroid status.[24] In fish, TRH stimulates growth hormone (GH) and prolactin (PRL) release but does not affect TSH secretion. In amphibians, TRH is a marginal stimulator of TSH release in adult frogs, not in tadpoles, and a major releasing factor for GH and PRL. In birds, TRH induces TSH and GH secretion. In mammals, TRH triggers TSH, GH, and PRL release. In fish and amphibians, TRH also potently stimules α-melanocyte-stimulating hormone release.[3]

TRH has neurotransmitter and neuromodulatory functions.[30,31] It is colocalized and cosecreted with other

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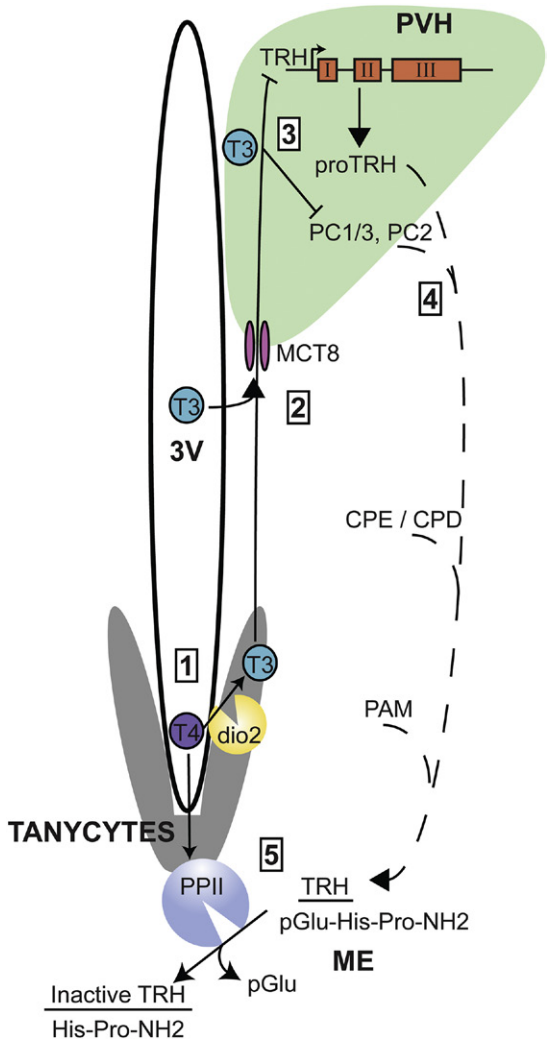

FIGURE 3 Thyroid hormone regulates TRH transcription, posttranslational modification, and degradation through the assistance of tanycytes. 1, tanycytes take up T_4, which is converted to T_3 by type 2 deiodinase (dio2). 2, hypophysiotropic TRH neurons in the paraventricular nucleus of the hypothalamus (PVH) receive T_3 from tanycytes or from the circulation through the monocarboxylate transporter 8 (MCT8). 3, TRH, prohormone convertases 1/3(PC 1/3), and 2 (PC2), are inversely regulated by T3. 4, posttranslational modifications to proTRH by PC1/3 and PC2, carboxypeptidases E and D (CPE and CPD), and peptidyl glycine α-amidating monooxygenase (PAM) occur as it travels down the axon (dashed line). 5, TRH is released in the median eminence (ME) where it can be degraded by tanycyte-bound pyroglutamate aminopeptidase II (PPII), which is positively regulated by thyroid hormone. (Ref. 32 with permission from the Endocrine Society).

neurotransmitters in a variety of different nerve cell types located in both CNS and peripheral tissues.[9,30] CNS regulation of autonomic functions, including arousal, blood flow, heart rate, blood pressure, breathing, thirst, hunger, pain, exocrine and endocrine pancreas functions, gastrointestinal motility, and gastric acid secretion mediated at least in part by TRH release within the CNS.[2,3,5,6,10,14] Interestingly, high levels of TRH also occur within the β-cells of the pancreatic islets[26,35] and Leydig cells of the testis[22] of all mammals so far tested.

PATHOPHYSIOLOGICAL IMPLICATIONS

TRH has an essential neuroprotective function that is partly mediated by its suppression of glycogen synthase kinase-3β (GSK-3β) expression in neurons.[17] This enzyme is responsible for the hyperphsphorylation of tau that results in neurofibrillary tangles that are associated with the neuropathologies of Alzheimer's and other dementias and regulates microtubule assembly in nerve axons and dendrites.[25]

A signal transduction pathway for TRH downregulation of GSK-3β has been proposed.[17] This ability of TRH to suppress GSK-3β has implications not only for Alzheimer's disease but also severe depression, bipolar disorder, diabetes mellitus, stroke, prostate cancer, and aging. GSK-3, in turn, modulates the release of TRH. Antidepressants and the major mood stabilizers for bipolar disorder, lithium and valproate, also inhibit GSK-3β and can reverse the associated neurologic damage with glia and neurons in brain regions associated to mood regulation [see Ref. 25 and references therein]. TRH and TRH-like peptides can be viewed as endogenous antidepressants, anticonvulsants, analeptics, and neuroprotectants.[8,21] Because TRH is rapidly degraded by TRH-DE,[29] development of TRH-DE-resistant TRH analogs,[2,29] use of naturally occurring TRH-like peptides with antidepressant properties,[8,21] or development of nontoxic, selective PAPII inhibitors[29] will be required for practical treatments.

Centrally administered TRH analog has a protective effect on cerulin-induced acute pancreatitis in rats. TRH and TRH receptors are distributed throughout the immune system and play an important role in both normal and pathological responses to bacterial and viral infection.[13]

Genomewide association and replication studies have identified TRHR1 as an important gene for lean body mass.[15] This is consistent with the central role played by the hypothalamic–pituitary–thyroid axis in the regulation of metabolism, muscle development, and maintenance.[15] Chronic ip injection of TRH can mobilize triglycerides, decrease serum leptin, and induce weight loss in obese mice without inducing hyperthyroidism.[27] Leptin and ghrelin, hormones that inhibit and stimulate feeding, respectively, may use TRH and TRH-like peptides in their downstream signaling[23] (Fig. 4).

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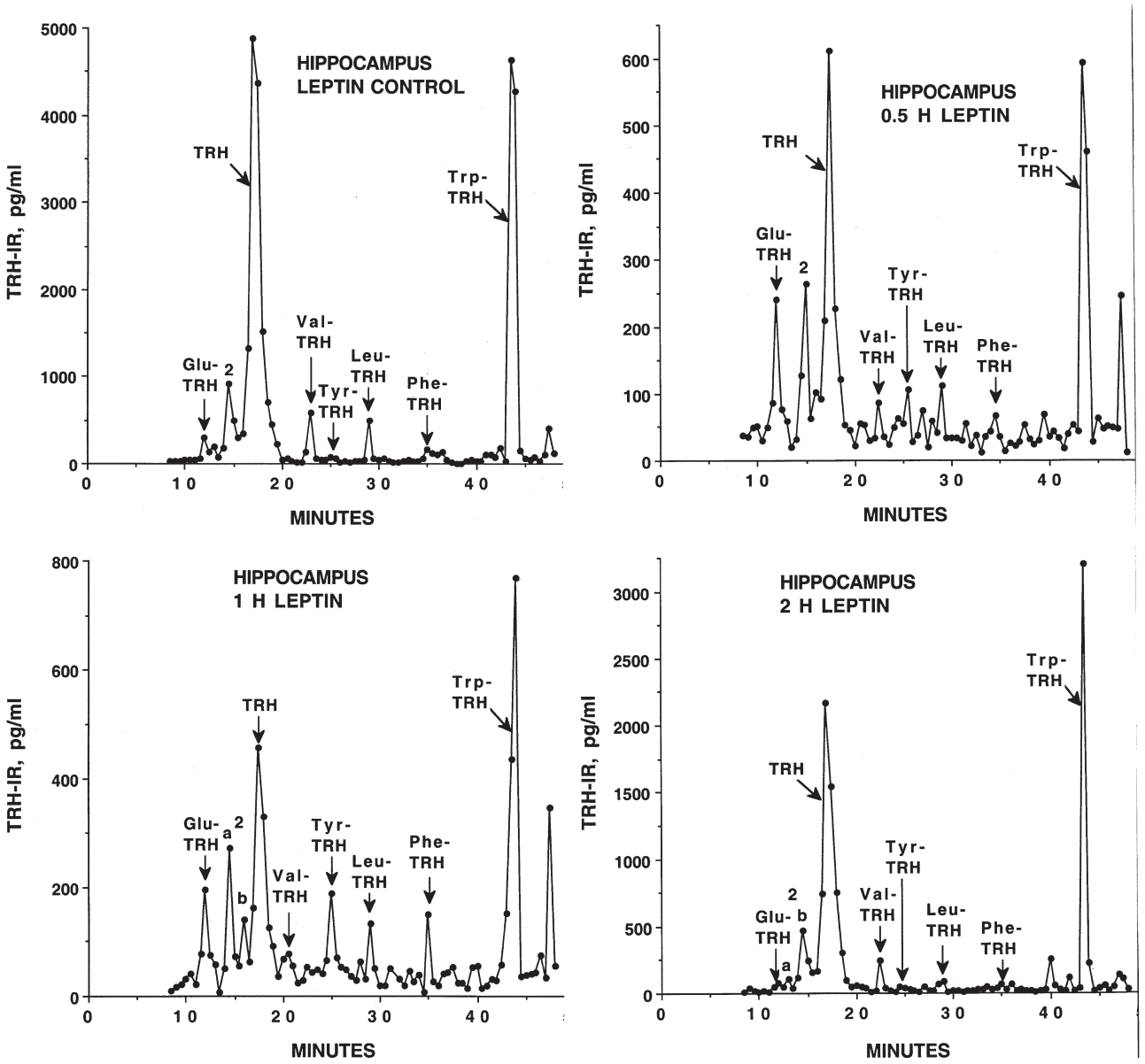

FIGURE 4 Rapid decline in TRH and TRH-like peptide levels (acute release) in hippocampus of male Sprague–Dawley rats following ip injection of 0.1 mg/kg of rat leptin.[23]

REFERENCES

1. Akil H, Campeau S, Cullinan WE, Lechan RM, Toni R, Watson SJ, et al. Neuroendocrine systems I: overview—thyroid and adrenal axes. In: Zigmond MJ, Bloom FE, Landis SC, Roberts JL, Squire LR, editors. *Fundamental neuroscience*. San Diego: Academic Press; 1999. p. 1127–50.
2. Gary KA, Sevarino KA, Yarbrough GG, Prang Jr AJ, Winokur A. The thyrotrophin-releasing hormone (TRH) hypothesis of homeostatic regulation: implications for TRH-based therapeutics. *J Pharmacol Exp Ther* 2003;**305**:410–6.
3. Galas L, Raoult E, Tonon MC, Okada R, Jenks BG, Castano JP, et al. TRH acts as a multifunctional hypophysiotropic factor in vertebrates. *Gen Comp Endocrinol* 2009;**164**:40–50.
4. Gershengorn MC, Osman R. Molecular and cellular biology of thyrotrophin-releasing hormone receptors. *Physiol Rev* 1996;**76**:175–91.
5. Guillemin R. Hypothalamic hormones a.k.a. hypothalamic releasing factors. *J Endocrinol* 2005;**184**:11–28.
6. Hara J, Gerashchenko D, Wisor JP, Sakurai T, Xie X, Kilduff TS. Thyrotropin-releasing hormone increases behavioral arousal through modulation of hypocretin/orexin neurons. *J Neurosci* 2009;**29**:3705–14.

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7. Heuer H, Schafer MK, O'Donnell D, Walker P, Bauer K. Expression of thyrotrophin-releasing hormone receptor 2 (TRH-R2) in the central nervous system of rats. *J Comp Neurol* 2000;**428**:319–36.
8. Hinkle PM, Pekary AE, Senayake S, Sattin A. Role of TRH receptors as possible mediators of analeptic actions of TRH-like peptides. *Brain Res* 2002;**935**:59–64.
9. Hrabovszky E, Liposits Z. Novel aspects of glutamatergic signaling in the neuroendocrine system. *J Neuroendocrinol* 2008;**20**:743–51.
10. Ishibashi H, Nakahata Y, Eto K, Nabekura J. Excitation of locus coeruleus noradrenergic neurons by thyrotropin-releasing hormone. *J Physiol* 2009;**587**:5709–21.
11. Jackson IMD, Reichlin S. Thyrotrophin-releasing hormone: abundance in the skin of the frog, *Rana pipiens*. *Science* 1977;**198**: 414–5.
12. Jiang X, Cai Z, Zhao X, Zhang L, Chen Z, Wang Y, et al. Mapping, cDNA cloning and tissue expression of the porcine thyrotropin-releasing horone receptor gene. *Anim Biotechnol* 2011;**22**:30–6.
13. Kamath J, Yarbrough GG, Prange Jr AJ, Winokur A. The thyrotropin-releasing hormone (TRH)-immune system homeostatic hypothesis. *Pharmacol Ther* 2009;**121**:20–8.
14. Lechan RM, Fekete C. The TRH neuron: a hypothalamic integrator of energy metabolism. *Prog Brain Res* 2006;**153**:209–35.
15. Liu XG, Tan LJ, Lei SF, Liu YJ, Shen H, Wang L, et al. Genome-wide association and replication studies identified *TRHR* as an important gene for lean body mass. *Am J Hum Genet* 2009;**84**:418–23.
16. Lu X, Bidaud I, Ladram A, Gershengorn MC. Pharmacological studies of thyrotrophin-releasing hormone (TRH) receptors from *Xenopus laevis*: is xTRHR3 a TRH receptor? *Endocrinology* 2003;**144**:1842–6.
17. Luo L-G, Stopa EG. Thyrotrophin releasing hormone inhibits tau phosphorylation by dual signaling pathways in hippocampal neurons. *J Alzheimer's Dis* 2004;**6**:527–36.
18. Mekuchi M, Saito Y, Aoki Y, Masuda T, Iigo M. Molecular cloning, gene structure, molecular evolution and expression analyses of thyrotropin-releasing hormone receptors from medaka (*Oryzias latipes*). *Gen Comp Endocrinol* 2011;**170**:374–80.
19. Nillni EA. Regulation of the hypothalamic thyrotropin releasing hormone (TRH) neuron by neuronal and peripheral inputs. *Front Neuroendocrinol* 2010;**31**:134–56.
20. Pekary AE. Is Ps4 (prepro-TRH[160–169]) more than an enhancer for thyrotrophin-releasing hormone? *Thyroid* 1998;**8**:963–8.
21. Pekary AE, Faull KF, Paulson M, Lloyd RL, Sattin A. TRH-like antidepressant peptide, pyroglutamyltyrosylprolineamide, occurs in rat brain. *J Mass Spectr* 2005;**40**:1232–6.
22. Pekary AE, Meyer NV, Vaillant C, Hershman JM. Thyrotrophin-releasing hormone and a homologous peptide in the male rat reproductive system. *Biochem Biophys Res Commun* 1980;**95**:993–1000.
23. Pekary AE, Sattin A, Blood JD. Rapid modulation of TRH and TRH-like peptide release in rat brain and peripheral tissues by leptin. *Brain Res* 2010;**1345**:9–18.
24. Pekary AE, Sattin A, Stevens AS. Rapid modulation of TRH-like peptide levels in rat brain by thyroid hormones. *Peptides* 2006;**27**:1577–88.
25. Pekary AE, Stevens SA, Blood JD, Sattin A. Rapid modulation of TRH and TRH-like peptide release in rat brain, pancreas, and testis by a GSK-3β inhibitor. *Peptides* 2010;**31**:1083–93.
26. Pekary AE, Stevens SA, Sattin A. Valproate and copper accelerate TRH-like peptide synthesis in rat pancreas and reproductive tissues. *Peptides* 2006;**27**:2901–11.
27. Pierpaoli W, Lesnikov VA. Effects of long-term intraperitoneal injection of thyrotropin-releasing hormone (TRH) on aging- and obesity-related changes in body weight, lipid metabolism, and thyroid functions. *Curr Aging Sci* 2011;**4**:25–32.
28. Satoh T, Yamada M, Monden T, Iizuka M, Mori M. Cloning of the mouse hypothalamic preprothyrotrophin-releasing hormone (TRH) cDNA and tissue distribution of its mRNA. *Brain Res Mol Brain Res* 1992;**14**:131–5.
29. Scalabrino GA, Hogan N, O'Boyle KM, Slator GR, Gregg DJ, Fitchett CM, et al. Discovery of a dual action first-in-class peptide that mimics and enhances CNS-mediated actions of thyrotropin-releasing hormone. *Neuropharmacology* 2007;**52**:1472–81.
30. Strand FL. *Neuropeptides. Regulators of physiological processes*. Cambridge: MIT Press; 1999.
31. Talley EM, Sirois JE, Lei Q, Baayliss DA. Review. Two-pore-domain (KCNK) potassium channels: dynamic roles in neuronal function. *Neuroscientist* 2003;**9**:46–56.
32. Vella KR, Hollenberg AN. News and views. The ups and downs of thyrotropin-releasing hormone. *Endocrinology* 2009;**150**:2021–3.
33. Wallis M. Molecular evolution of the thyrotropin-releasing hormone precursor in vertebrates: insights from comparative genomics. *J Neuroendocrinol* 2010;**22**:608–19.
34. Waring P. Redox active calcium ion channels and cell death. *Arch Biochem Biophs* 2005:33–42.
35. Yamada M, Saga Y, Sibusawa N, Hirato J, Murakami M, Iwasaki T, et al. Tertiary hypothyroidism and hyperglycemia in mice with targeted disruption of the thyrotrophin-releasing hormone gene. *Proc Natl Acad Sci USA* 1997;**94**:10861–7.

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Chapter 127

Urotensin II Peptides

Isabelle Lihrmann, Hervé Tostivint, Howard Bern†, and Hubert Vaudry

ABSTRACT

Urotensin II (UII) is a cyclic neuropeptide initially isolated from the urophysis of teleost fish. In the central nervous system (CNS) of tetrapods, UII is almost exclusively produced in motoneurons located in brainstem nuclei and in the ventral horn of the spinal cord. Three paralogs of UII have also been characterized, namely urotensin II-related peptide (URP) both in tetrapods and fish, and URP1 and URP2 in fish. The cyclic region of UII and URPs, which has been fully conserved during evolution, plays a crucial role for the biological activity of these peptides. A single receptor for UII and URP, called the UT receptor, has been identified so far. In agreement with the widespread distribution of UT receptor in the CNS, UII and URP exert a large array of activities in the brain and in peripheral organs.

DISCOVERY

The caudal neurosecretory system of teleost fish incorporates a neurohemal organ called the urophysis, which contains various regulatory factors including neurotransmitters and neuropeptides (Fig. 1). Urotensin II (UII) is a cyclic neuropeptide that has been originally isolated from the goby urophysis on the basis of its ability to induce contractions of the trout hindgut.[2] In all species of fish investigated, the UII sequence encompasses 12 amino acids with a fully conserved cyclic region at position 6–11 that exhibits some structural similarity to somatostatin. It had long been thought that UII was the apanage of the fish urophysis.[2] However, characterization of UII from the brain of the European green frog[7] has revealed that a UII-encoding gene actually exists in certain species of tetrapods and that this gene is not only expressed in the caudal portion of the spinal cord but also in brain nerve cells.[33] Consistent with these notions, the cDNAs encoding the UII precursors have been cloned in various mammalian species,[33] including human.[1,10] Subsequently, a paralog of UII named urotensin II-related peptide (URP) has been characterized in the rat brain and the URP cDNAs have been cloned in mammals, birds, and teleosts fish.[30] Two additional UII paralogs, designed URP1 and URP2, have been recently identified in teleosts.[26] The occurrence of UII, URP and their receptor in the CNS, together with the variety of their behavioral effects, suggest that these peptides act as neurotransmitters and/or neuromodulators.

STRUCTURE

Gene Structure and Evolutionary Aspects

The gene encoding preproUII, also named *uts2*, comprises five exons and maps on human chromosome 1p36. In human, two distinct precursor isoforms with 139 (isoform a) and 124 (isoform b) amino acids have been characterized.[1,10] These two isoforms only differ in their N-terminal extremity and give rise to the same mature UII peptide (Fig. 2). In all other mammalian species studied so far (mouse, rat, pig, and monkey), only isoform b has been identified.[33] In fish, two variants of isoform b are expressed as a result of genome duplication. The *uts2d* gene encoding prepro-URP has five exons and maps on human chromosome 3q23. The human URP precursor consists of 119 amino acids and exhibits very low sequence identity with the UII precursor (<20%). Two additional genes, designed URP1 and URP2, have been characterized in teleost fish, which are not the orthologs of the URP gene.[26] The four distinct UII/URP genes present in vertebrates, namely UII, URP, URP1, and URP2 genes, probably arose during the two rounds of whole genome duplication before the emergence of gnathostomes. However, the URP1 and URP2 genes are thought to have been lost in the tetrapod lineage (Fig. 3). Comparative genomics has further shown that urotensin II and somatostatin gene families likely arose from a common ancestral gene.[31]

Processing of the Precursors

The general organization of the UII and URP precursors is identical, the sequence of each peptide being located at the C-terminal extremity of the protein[1,9,10,26,30,31] (Fig. 2). Somatostatin-14, which possesses structural similarity to UII in the

† This chapter is dedicated to the memory of Professor Howard Bern who first characterized urotensin II (*Proc Natl Acad Sci* 1980;**77**:5021–4).

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00127-5
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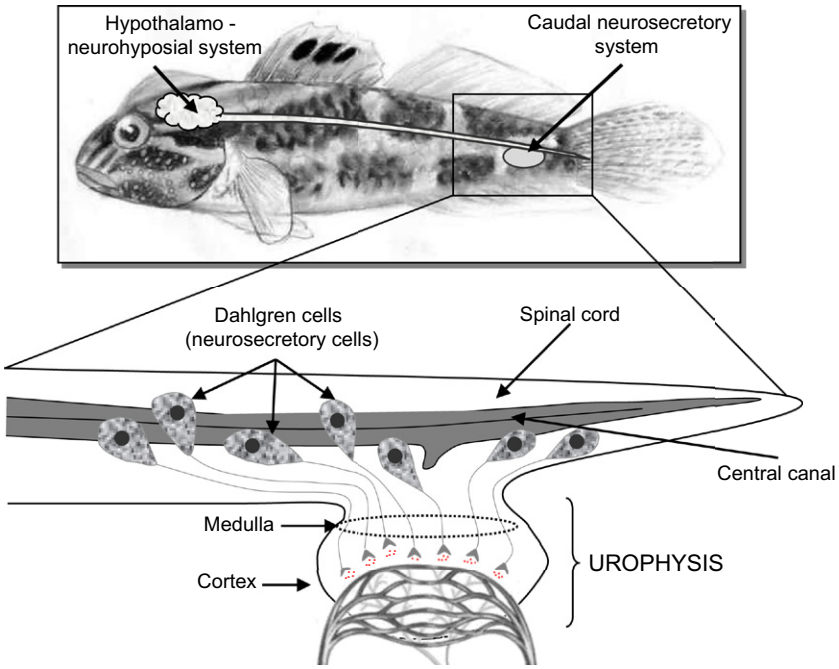

FIGURE 1 The fish caudal neurosecretory system consists in UII-immunoreactive Dahlgren cells located in the caudal portion of the spinal cord in the last (~5 to 6) preterminal vertebrae. Large unmyelinated axons from these cells extend lateroventrally and turn caudally to join the spinal-urophysial tract. UII is released in the vicinity of fenestrated capillaries in the urophysis and ultimately join the renal portal system.

biologically important cyclic region, is also located at the C-terminal end of its precursor (see chapter on Somatostatin in this section of the book). Proteolytic cleavage of proUII and proURP at dibasic (Arg-Lys) or tribasic (Lys-Lys-Arg) sites has the potential to generate mature peptides whose sequences may vary from 8 (tetrapod lineage URP) to 17 (mouse UII) amino acids in length. In tetrapods, up to now, the structure of UII has only been determined in the frog brain[7] and in the human and porcine spinal cord.[5,33] Additional molecular forms have been isolated in the human brainstem[5] which may correspond to large processing products at one of the upstream Arg-Lys dibasic sites. A UII-converting enzyme with furin-like activity has been partially characterized in human epicardial mesothelial cells, vascular endothelial cells, and cardiac fibroblasts by studying the processing of the 25 amino acid C-terminal fragment of proUII.[28]

Peptide Structure

Strong evolutionary pressure has acted to preserve the primary structure of the C-terminal portion of UII and URPs while the N-terminus is highly variable.[22,33] (Fig. 4). Thus, in all species examined, the amino acid sequence of the cyclic region of UII and URPs (Cys-Phe-Trp-Lys-Tyr-Cys) has been fully conserved.[33] In the UII sequence, the cyclic portion is preceded by an acidic residue (Asp or Glu) and is flanked at its C-terminus by a hydrophobic residue (Val or Ile). In URP sequences of tetrapods, the cyclic region is flanked by an Ala residue at its N-terminus and a Val or an Ile residue at its C-terminus. Fish URP1 and URP2 sequences differ from the others by the replacement of the C-terminal Ala by a Val in URP2 and the presence of an N-terminal extension in URP1 and URP2 (Fig. 4).[26]

LOCALIZATION

In the CNS of tetrapods, UII and URP are almost exclusively expressed in motoneurons located in the brainstem and the dorsal horn of the spinal cord.[5,8–10,13,24] In particular, in the rat brain, a high concentration of UII mRNA has been observed in motoneurons located in the trigeminal, abducens, facial, ambiguus and hypoglossal nuclei[9] (Fig. 5). Similarly, intense expression of the UII gene occurs in the ventral horn of the spinal cord in developing rat.[9] UII mRNA is also detectable, albeit at a much lower level, in various human and mouse cerebral structures, including amygdala, hypothalamus, and cerebellum.[9,13] Globally, the profiles of expression of the UII and URP genes in the CNS of mammals are similar.[13,30] except in the human spinal cord where preproURP levels are much lower.[30] In the mouse spinal cord, the UII and URP precursors are expressed within the same motoneuron cell bodies.[24] By contrast, several brain regions in mouse contain mostly URP mRNA. They include the locus coeruleus, the rostroventrolateral medulla, the lateral paragigantocellular nucleus, and the dorsal nucleus of the vagus.[13]

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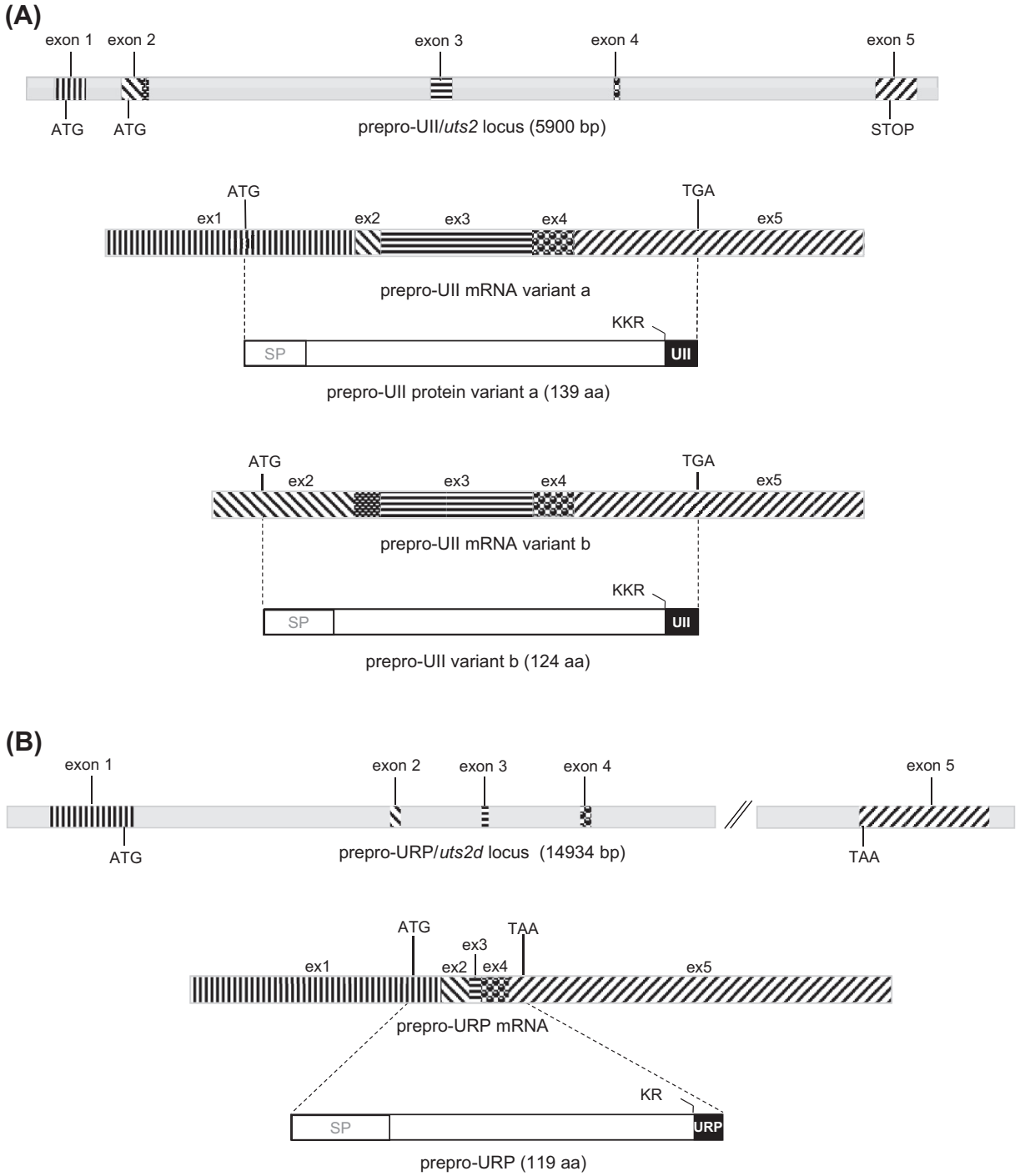

FIGURE 2 Structure of the human preproUII and preproURP genes. **(A)** The preproUII gene comprises five exons and encodes two precursor isoforms (variants a and b) that only differ at their N-terminal extremity. The same mature UII peptide is generated from both isoforms a and b after proteolytic cleavage at a tribasic site (KKR). **(B)** The preproURP gene comprises five exons. The URP sequence is located at the C-terminal extremity of the URP precursor and the mature peptide is generated by proteolytic cleavage at a dibasic site (KR).

In fish, while expression of the UII gene is restricted to the caudal neurosecretory system,[2] the URP1 gene is expressed most abundantly in the medulla oblongata and to a lesser extent in the spinal cord. URP2 mRNA-containing cells are found below the fourth ventricle and at the ventral side of the central canal in the spinal cord. These URP2-expressing cells probably compose the UII-like extra-urophyseal system described earlier.[24] In zebrafish embryos, URP2 gene expression occurs in the floor plate of the neural tube.[26]

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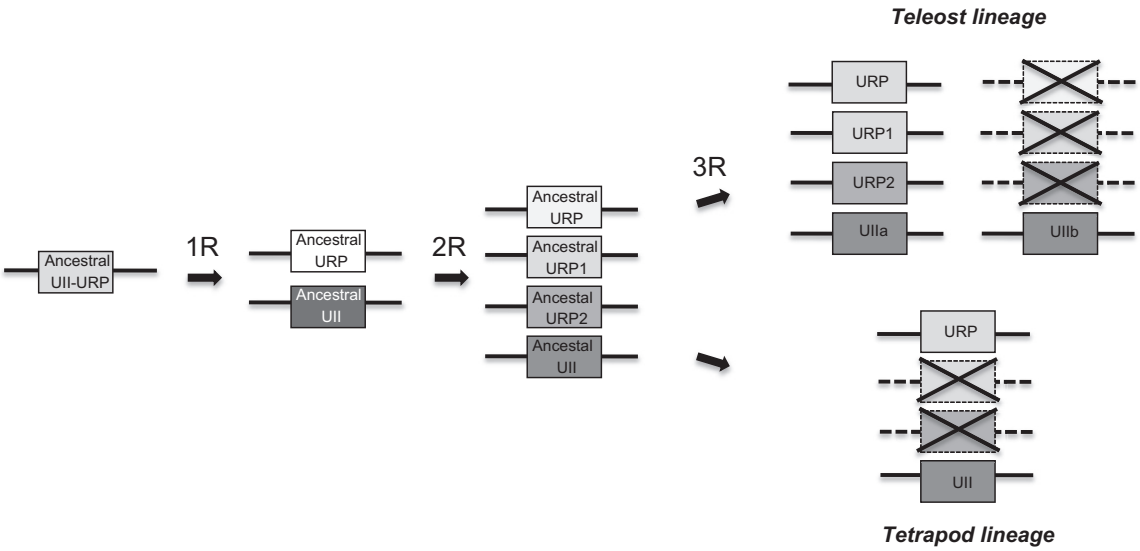

FIGURE 3 A proposed evolutionary scheme for the UII/URP family in vertebrates. R: rounds of whole genome duplications. Crossed-out boxes represent lost genes.

RECEPTORS AND SIGNALING CASCADES

The UT Receptor

The orphan G protein-coupled receptor GPR14, initially characterized by a homology based approach, was subsequently identified by reversed pharmacology as the UII receptor and then renamed UT receptor.[1] This receptor exhibits the highest sequence identity with somatostatin (27%) and opioid (25-26%) receptors. In human, the UT receptor comprises 389 amino acids and is encoded by an intronless gene located on chromosome 17q25.3. The UT receptor belongs to the class A family of GPCRs, characterized by a short N-terminus, an Asp residue in TMD2, a D/ERY motif at the junction of TMD3 and the second intracellular loop, a NPxxY motif in TMD7, and potential Ser/Thr phosphorylation sites in the cytoplasmic tail. UT also exhibits putative N-glycosylation sites in the N-terminal extracellular domain and two Cys residues in the first and second extracellular loops (ECL). Substitution of the Asp residue in TMD2 alters the capacity of receptor transition to an active state, while this modification or mutations in the ERY motif in TMD3 markedly reduce PLC activation.[25] Photolabeling studies with photoprobes have shown that a pair of methionine residues (Met^{184}/Met^{185}) of TMD4 in the rat UT receptor interacts with the Phe residue present in the cyclic region of UII. In addition, the N-terminal domain of UII ligands seems to be in close proximity to a Met residue (Met^{288}) in ECL3 of UT. Within TMDs, an Asp residue in TMD3 could be involved in an ionic interaction with the Lys residue in the cyclic domain of UII while, in TMD6 and TMD7, specific residues may participate in the formation of the binding pocket of the UT receptor.[25]

The UT receptor undergoes internalization via clathrin-coated pits through an arrestin-independent, dynamin-dependent mechanism. A cluster of Ser residues (potential kinase phosphorylation sites) located within the cytoplasmic tail of the UT receptor is crucial for its ability to be internalized efficiently. The existence of caveolin-binding sites in rat UT also suggests a caveolin-dependent endocytotic pathway. In addition to internalization, UT can be regulated by desensitization through an arrestin-dependent mechanism since UT colocalizes with arrestin-1 and -2 in endocytotic vesicles and with arrestin-3 at the plasma membrane. The UT receptor recycles quantitatively within 30 min and the continuous externalization of the UT receptor provides the basis for repetitive and lasting UII-mediated effects.[25]

Signaling Cascades

The biological effects of UII are mediated through activation of a number of transduction pathways. In many cell types and cell lines, including macrophages, endothelial cells, smooth muscle cells, skeletal muscle preparations, cardiomyocytes, and astrocytes, the main transduction mechanism associated with UT is the activation of the $G_{\alpha q/11}$ subtype of heterotrimeric G proteins coupled to phospholipase C (PLC)-IP3-dependent intracellular calcium mobilization.[19,29] However, $G_{\alpha i/0}$ coupling has also been reported in native astrocytes and UT-transfected cell lines.[19,35] UT may also be coupled to $G_{\alpha 12/13}$ since UII activates the small GTPase RhoA and its downsteam effector Rho-kinase, a pathway usually, but not exclusively, associated with $G_{\alpha 12/13}$.[17] One of the most documented downstream signaling pathways of

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Species	AA	Primary structure																		
Human UII	11							Glu	Thr	Pro	Asp	**Cys**	**Phe**	**Trp**	**Lys**	**Tyr**	**Cys**	Val		
Human UII	11							Glu	Thr	Pro	Asp	**Cys**	**Phe**	**Trp**	**Lys**	**Tyr**	**Cys**	Val		
Monkey UII	11							Glu	Thr	Pro	Asp	**Cys**	**Phe**	**Trp**	**Lys**	**Tyr**	**Cys**	Val		
Mouse UII	17	pGlu	His	Lys	Gln	His	Gly	Ala	Ala	Pro	Glu	**Cys**	**Phe**	**Trp**	**Lys**	**Tyr**	**Cys**	Ileu		
Rat UII	14				pGu	His	Gly	Thr	Ala	Pro	Glu	**Cys**	**Phe**	**Trp**	**Lys**	**Tyr**	**Cys**	Ileu		
Porcine UII_A	12						Gly	Pro	Thr	Ser	Glu	**Cys**	**Phe**	**Trp**	**Lys**	**Tyr**	**Cys**	Val		
Porcine UII_B	12						Gly	Pro	Pro	Ser	Glu	**Cys**	**Phe**	**Trp**	**Lys**	**Tyr**	**Cys**	Val		
Chicken UII	12						Gly	Asn	Leu	Ser	Glu	**Cys**	**Phe**	**Trp**	**Lys**	**Tyr**	**Cys**	Val		
Frog UII	13					Ala	Gly	Asn	Leu	Ser	Glu	**Cys**	**Phe**	**Trp**	**Lys**	**Tyr**	**Cys**	Val		
Zebrafish UII_α	12						Gly	Gly	Gly	Ala	Glu	**Cys**	**Phe**	**Trp**	**Lys**	**Tyr**	**Cys**	Val		
Zebrafish UII_β	12						Gly	Ser	Asn	Thr	Glu	**Cys**	**Phe**	**Trp**	**Lys**	**Tyr**	**Cys**	Val		
Goby	12						Ala	Gly	Thr	Ala	Asp	**Cys**	**Phe**	**Trp**	**Lys**	**Tyr**	**Cys**	Val		
Trout	12						Gly	Gly	Asn	Ser	Glu	**Cys**	**Phe**	**Trp**	**Lys**	**Tyr**	**Cys**	Val		
Sucker UII_A	12						Gly	Ser	Gly	Ala	Asp	**Cys**	**Phe**	**Trp**	**Lys**	**Tyr**	**Cys**	Val		
Sucker UII_B	12						Gly	Ser	Asn	Thr	Glu	**Cys**	**Phe**	**Trp**	**Lys**	**Tyr**	**Cys**	Val		
Carp UII_α	12						Gly	Gly	Gly	Ala	Asp	**Cys**	**Phe**	**Trp**	**Lys**	**Tyr**	**Cys**	Val		
Carp $UII_{\beta1}$	12						Gly	Gly	Asn	Thr	Glu	**Cys**	**Phe**	**Trp**	**Lys**	**Tyr**	**Cys**	Val		
Carp $UII_{\beta2}$	12						Gly	Ser	Asn	Thr	Glu	**Cys**	**Phe**	**Trp**	**Lys**	**Tyr**	**Cys**	Val		
Carp UII_γ	12						Gly	Gly	Gly	Ala	Asp	**Cys**	**Phe**	**Trp**	**Lys**	**Tyr**	**Cys**	Ileu		
Flounder UII	12						Ala	Gly	Thr	Thr	Glu	**Cys**	**Phe**	**Trp**	**Lys**	**Tyr**	**Cys**	Val		
Sturgeon UII	12						Gly	Ser	Thr	Ser	Glu	**Cys**	**Phe**	**Trp**	**Lys**	**Tyr**	**Cys**	Val		
Paddlefish UII	12						Gly	Ser	Thr	Ser	Glu	**Cys**	**Phe**	**Trp**	**Lys**	**Tyr**	**Cys**	Val		
Skate UII	12						Asn	Asn	Phe	Ser	Asp	**Cys**	**Phe**	**Trp**	**Lys**	**Tyr**	**Cys**	Val		
Dogfish UII	12						Asn	Asn	Phe	Ser	Asp	**Cys**	**Phe**	**Trp**	**Lys**	**Tyr**	**Cys**	Val		
Lamprey UII	12						Asn	Asn	Phe	Ser	Asp	**Cys**	**Phe**	**Trp**	**Lys**	**Tyr**	**Cys**	Val		
Human URP*	8										Ala	**Cys**	**Phe**	**Trp**	**Lys**	**Tyr**	**Cys**	Val		
Zebrafish URP	10										Asn	**Cys**	**Phe**	**Trp**	**Lys**	**Tyr**	**Cys**	Ileu	Asn	Thr
Zebrafish URP1*	10										Ala	**Cys**	**Phe**	**Trp**	**Lys**	**Tyr**	**Cys**	Val	Thr	Asn
Zebrafish URP2*	10										Val	**Cys**	**Phe**	**Trp**	**Lys**	**Tyr**	**Cys**	Ser	Gln	Asn

FIGURE 4 Alignment of UII and URP sequences identified so far. The number of amino acids (AA) of each peptide is indicated. * indicates that this sequence is conserved in all mammals or all teleosts.

UT, generally associated with cell proliferation, involves the members of the MAPK family (ERK1/2 and to a lesser extent p38) through Gαq/PLC/PKC or Gi/0.[17,27,35] As shown for many GPCRs, a network of interconnected transduction mechanisms occurs in different cell types, which are coupled to diverse cellular responses (proliferation, apoptosis, migration, hypertrophic growth).[17,27] In neural cells, notably motoneurons, UT is coupled to Ca^{2+} influx and neurotransmitter release via both the PKA and the PLC-IP3 pathways[3] while, in astrocytes, activation of UT triggers a Gαq-PLC-InsP3-Ca^{2+} cascade and a Gi/o-PI3 K-PTX-sensitive pathway, the latter being induced by UII, not URP.[19] Alternately, in a number of cell types (cardiomyocytes, renal and smooth muscle cells), UT can activate these pathways indirectly via the transactivation of epidermal growth factor receptor upstream of the MAPK pathway.[17,32]

Distribution of the UT Receptor

In peripheral organs, the highest concentrations of UT receptor mRNA are mainly found in the heart (ventricular myocardium) and vascular tissues (endothelial and smooth muscle).[1,13] UT receptor transcripts are also found in a number of organs including kidney, lung, liver, adipose tissue, skeletal muscle, in the gastrointestinal and genitourinary tracts (stomach, esophagus, small intestine, colon, bladder, ovary, testis, prostate), and endocrine glands (pituitary, thyroid, pancreas, adrenal gland).[1,13] In the CNS, UT receptor mRNA is widely expressed in the cortex, septum, nucleus accubens, striatum, hypothalamus, the pyramidal cell layer and the dentate gyrus of the hippocampus, amygdala, several tegmental nuclei, cerebellum, pons, medulla oblongata, spinal cord and dorsal root ganglia.[1,13,20] UII- and

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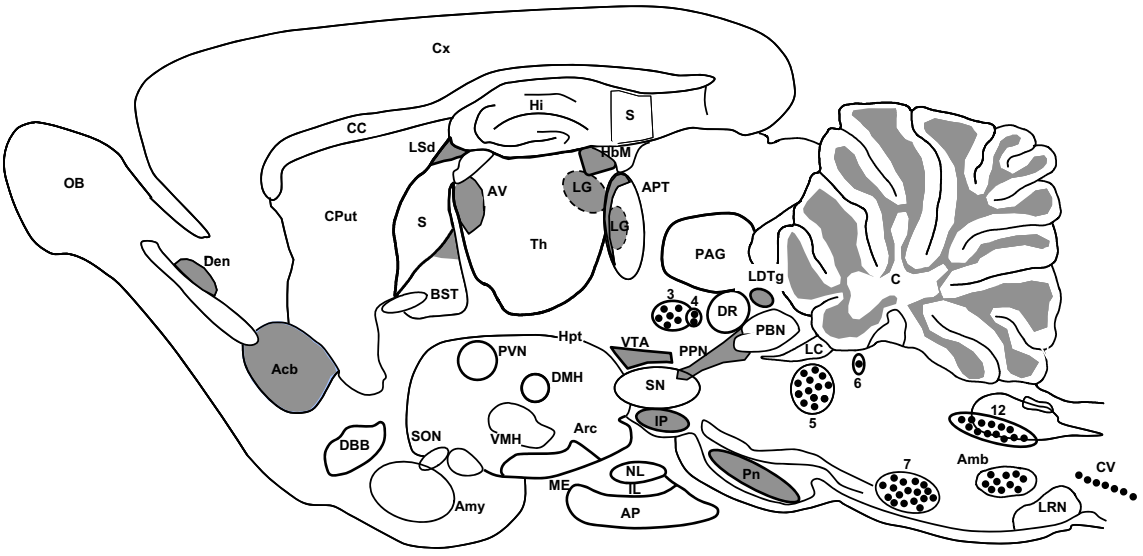

FIGURE 5 Schematic drawing showing the distribution of UII neurons and [^{125}I]UII binding sites in the rat central nervous system.

URP-binding sites totally overlap, but are more restricted than UT mRNA expression pattern.[20] They are found in the lateral septum, accumbens nucleus, dorsal endopiriform nucleus, various amydaloid nuclei, medial preoptic nucleus, anteroventral thalamus, medial habenula, anterior pretectal nucleus, ventral tegmental area, interpeduncular nucleus, parasubiculum, pontine nuclei, some tegmental nuclei, geniculate nuclei and cerebellar cortex.[20]

Structure–Activity Relationships

The cyclic region that is common to UII and URPs (Fig. 4) plays a pivotal role for the biological activity of these peptides on native and recombinant UII receptors.[22,33] $UII_{(4–11)}$ is the minimal sequence required for full biological activity and, in fact, URP which comprises a single Ala upstream of the cycle is as efficient as UII to bind and activate UT.[13,30] Disruption of the disulfide bridge or point-substitution of individual amino acids within the cyclic region by Ala or D-isomers markedly reduces the potency of UII and URP. The triad Phe^6, Lys^8 and Tyr^9 is essential to retain full biological activity. Punctual modifications of these residues generate analogs with agonistic and antagonistic properties.[22,32] The N-terminus, which is reduced to a single Ala in URP, is not necessary for the biological activity, and some substitutions in one of these amino acids increase the stability of the analogs. By contrast, the presence of a hydrophobic residue (Val or Ile) at the C-terminal extremity of UII and URP contributes to the biological potency. Determination of the solution structure of UII and URP by NMR spectroscopy has shown that the cyclic region adopts a highly ordered compact conformation while the N-terminal segment of UII is disordered. In both UII and URP, the side chains of the Phe, Trp and Tyr residues of the cyclic region are oriented on the same side of the peptides suggesting that they may all be involved in docking with the binding pocket of the UII receptor.[22]

BIOLOGICAL ACTIONS WITHIN THE CENTRAL NERVOUS SYSTEM

Central Regulation of Cardiovascular Functions

The genes encoding the UT receptor as well as the UII and URP precursors are all expressed in brainstem nuclei associated with cardiac function and in hypothalamic nuclei such as the paraventricular nucleus,[13,20] which is implicated in the control of corticotropin (ACTH) secretion. As a matter of fact, a number of studies have shown that UII modulates hemodynamics and cardiac function at the CNS level. Studies in conscious sheep and rat have shown that icv UII stimulates the sympatho-adrenal and pituitary-adrenal pathways resulting in epinephrine, but not norepinephrine, release.[34] These effects are accompanied by an increase in blood pressure and heart rate, and similar effects were reported after microinjections of UII into the paraventricular nucleus and the arcuate nucleus in anesthetized rats.[23]

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In fish, at very low doses, UII administered icv increases heart rate without modifying blood pressure, while a higher dose of UII provokes hypertension and takycardia.[21] The chronotropic and inotropic actions of centrally administered UII are blocked by propanolol in sheep and in rat indicating that they are mediated through cardiac β-adrenoreceptors. Furthermore, the observation that the pressor and chronotropic actions of icv-injected UII are attenuated by ganglion blockade indicates that these responses are mediated in part through activation of the sympathetic nervous system.[34] Collectively, these observations show that the inotropic and chronotropic actions of centrally-administered UII in conscious sheep and rat result from stimulation of cardiac β-adrenoreceptors via raised circulating levels of epinephrine and increased cardiac sympathetic nerve activity. Supporting this view, activation of neural activity has been detected in the central amygdala, the paraventricular nucleus, and the nucleus of the solitary tract, after icv UII administration in rat. URP may be directly involved in adrenal sympathetic outflow regulation since its mRNA is highly expressed in neurons that are intermingled with TH-immunoreactive A1/C1 cells in the rostral ventrolateral medulla.[13] Altogether, these observations indicate that central administration of UII/URP causes site- and dose-dependent cardiovascular responses in both fish and mammals.

Neuroendocrine Actions

The regulation of pituitary-sympathoadrenal medullary and pituitary adrenal cortex pathways (see above) are not the unique neurosecretory functions of UII.[34] Indeed, icv administration of UII provokes an increase in plasma prolactin and thyroid-stimulating hormone (TSH) in rat.[16] The stimulatory effect of UII on prolactin, TSH, and ACTH release is probably mediated through activation of thyrotropin-releasing hormone (TRH) and corticotropin-releasing hormone (CRH) neurons that are both located in the paraventricular nucleus[20] (see chapters on TRH and CRH in this section of the book).

Behavioral Responses

Consistent with the widespread distribution of GPR14 mRNA and UII binding sites in the CNS, central administration of UII elicits a number of behavioral effects in rodents.[11] A number of studies have shown that UII is implicated in various mood and sleep processes. Thus, icv injection of UII induces anxiogenic and depressant-like effects in mouse[11] and increases neural activity in rat periaqueductal gray, a midbrain region implicated in anxiety control. In addition, local administration of UII into the pedunculopontine nucleus modulates paradoxical sleep through direct activation of brainstem cholinergic neurons by UT receptors present in the mesopontine tegmental area.[18]

Motor Functions

The expression of the mRNA encoding the UII and URP precursors and the UT receptor in motoneurons of the brainstem and spinal cord indicates that UII/URP may act as autocrine modulators of motoneuron activity.[1,5,8–10,13,20,30] Consistent with this notion, the UT receptor is expressed on a subset of cholinergic nerve terminals in part originating from the recurrent motoneuron axon collaterals in mouse spinal cord.[4] In addition, dissociated rat motoneurons are equipped with functional UT receptors coupled to calcium mobilization.[15] Other results support a role of the UII/URP system as co-transmitter at cholinergic motoneuron terminals, capable of regulating transmitter release and contributing to the excitatory transmission. Thus, UII is present at the neuromuscular junction, in synaptophysin-containing vesicles[13] and retrograde labeling has shown that UII-containing neurons actually innervate muscle.[14] Next, UII stimulates acetylcholine release at the frog neuromuscular junction and in rat and mouse synaptosomes obtained from the spinal cord.[3,4] Finally, UT receptor mRNA is expressed in skeletal muscle and functional UT receptors occur in human skeletal muscle cell lines[12,13] suggesting a post-synaptic regulation of neurotransmitter release. Of note, UII/URP expression may be subject to sexual dimorphic regulation in motoneurons since the androgen receptor is co-localized with UII and URP mRNA in motoneurons, and castration down-regulates the expression of the two genes.[24]

In the brain, icv UII/URP injection increases locomotion in mouse, rat and fish.[11,16,21] High density of UII binding sites has been found in regions of the human and rat brain (cortex, accumbens nucleus, pontine nuclei, cerebellum) which are involved in motor control.[20] Noteworthy, serotoninergic and dopaminergic systems are not altered in these structures following icv UII injection in rat, although they are modulated by other neuropeptides, orexins for instance.

In conclusion, UII exerts an excitatory effect in some cases, since it modulates cardiovascular tone through activation of the sympathetic system and controls REM-on state and acetylcholine release from cholinergic brainstem neurons and motoneurons, respectively. By contrast, UII induces a decrease in neuronal excitability associated to a potentiation of $GABA_A$ receptor-mediated Cl^- current, in neurons of aplysia abdominal ganglion and CA1 area of rat hippocampus.

PHYSIOLOGICAL AND PATHOPHYSIOLOGICAL SIGNIFICANCE

UII was first described as the most potent vasoconstrictor ever discovered, being ~10-fold more potent then endothelin-1,[1] although the magnitude of its effect is highly

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dependent on the species and tissue source of the vessel. In physiological conditions, the UT receptor is present both on smooth muscle and endothelial cells,[1] so that the vascular effect of UII is a balance between vasoconstriction and vasodilation resulting from the release from endothelial cells of nitric oxide, prostaglandins, and endothelium-derived hyperpolarizing factors. The urotensinergic system is thought to participate in the basal vascular tone through the prolonged effect of UII, its pseudo-irreversible binding properties, and the slow dissociation rate of UII at UT.[12] The concentrations of UII in plasma, urine and/or cerebrospinal fluid are increased and the UT receptor is upregulated in many diseases, including essential hypertension, chronic heart failure, atherosclerosis, renal failure, renal dysfunction, portal hypertension/cirrhosis, and diabetes mellitus.[32] Therefore, the urotensinergic system may not contribute to the regulation of organ function in healthy states, but most probably play a significant role in disease states.[27,32]

The upregulation of the urotensinergic system has many physiopathological consequences. In cardiovascular diseases for instance, UII participates to the formation of atherosclerotic plaques and the development of hypertension in several manners: vasoconstriction resulting from endothelial dysfunction and impaired vasodilation, stimulation of acyl-coenzyme A:cholesterol acyltransferase-1 (ACAT-1) activity in macrophages, foam cell infiltration, and smooth muscle cell proliferation.[17,32] In myocardial ischemia and congenital heart failure, the effects of the urotensinergic system on cardiac remodeling and function are still controversial. Patients with significantly elevated plasma UII concentrations display improved outcomes post-myocardial infarction. It has been suggested that UII may play a cardioprotective role in cardiac remodeling, via transactivation of the EGF receptor. By contrast, in animal models of cardiovascular diseases, administration of UT antagonists improves both hemodynamics and cardiac remodeling suggesting the implication of the UT receptor in the development of the disease state.[32] In support of this latter hypothesis, the urotensinergic system has been shown to impair heart function through fibrosis and cardiomyocyte hypertrophy.

The kidney, together with the heart, is one of the major sources of UT, UII, and URP, the expression being highest in the medulla than in the cortex. UT is found in renal tubular cells and collecting ducts, as well as in glomerular arterioles, but the exact roles of UII in tubular reabsorption and secretion of water and electrolytes remain to be clarified. Several studies have reported that UII and URP binding at UT in renal tubules causes a decrease of glomerular filtration rate, urine volume and urinary sodium excretion.[32] The concentrations of UII are increased in biopsies from patients with diabetic nephropathy, in urine of patients with renal tubular disease, and in the plasma of patients with chronic renal failure. The UT antagonist palosuran is effective in diabetic nephropathy, reducing albuminuria in a small number of patients. Furthermore, in patients with moderate-to-severe chronic kidney disease, plasma UII is an inverse predictor of death and fatal cardiovascular events.[32]

UII participates to the metabolic syndrome, via its inhibitory effect on insulin release by pancreatic beta cells and its stimulatory effect on circulating levels of inflammatory cytokines (IL-6), resulting in insulin resistance, atherogenesis and hypertrophy.[27,32] Elevated plasma UII concentrations have been observed in patients with diabetes. Further, association of polymorphisms in the UII gene with type 2 diabetes has been reported in Chinese and Japanese subjects. Hence, UT antagonism may have a beneficial effect on patients with type II diabetes. However, antagonism of the UII system does not influence insulin secretion or sensitivity or daily blood glucose levels in diet-treated patients with type 2 diabetes. By contrast, in a rat model of type 2 diabetes. glycaemia, serum cholesterol, triglycerides, HbA1c, insulin concentrations, renal blood flow, and renal damage are improved.[32]

Although numerous studies have addressed the effects of the urotensinergic system at the periphery, the actions of UII and URP at the central level in pathophysiological states remain poorly documented. UII/URP and UT mRNAs are largely expressed in different brain regions and cell types, including neurons, glial cells and neuromicrovascular endothelial cells.[6,8–10,13,19] In a rat model of cerebral ischemia, icv-injected UII induces a sustained hyperemia through vasodilation and, as a consequence, exacerbates ischemic brain lesion.[6] Injection of UII in the striatum also increases the lesion size in a model of NMDA-induced brain injury.[6] Similar UII concentrations are found in the cerebrospinal fluid and in the serum, indicating that this neuropeptide may be also involved in the maintenance of pial vascular tone. Paradoxically, while numerous *in vitro* results suggest a role of UII and URP in the regulation of neuromuscular function, no results are yet available concerning the possible involvement of the urotensinergic system in neuromuscular disorders.

In hopes of a new therapy for cardiovascular, metabolic and renal diseases, several peptidic and nonpeptide antagonists of the UT receptor have been designed by the combination of biochemical and computational methods.[22,32] However no potent and safe antagonists that could be used in human have been yet developed.

REFERENCES

1. Ames RS, Sarau HM, Chambers JK, Willette RN, Aiyar NV, Romanic AM, et al. Human urotensin-II is a potent vasoconstrictor and agonist for the orphan receptor GPR14. *Nature* 1999;**401**: 282–6.

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2. Bern HA, Pearson D, Larson BA, Nishioka RS. Neurohormones from fish tails: the caudal neurosecretory system. I. "Urophysiology" and the caudal neurosecretory system of fishes. *Recent Prog Horm Res* 1985;**41**:533–52.
3. Brailoiu E, Brailoiu GC, Miyamoto MD, Dun NJ. The vasoactive peptide urotensin II stimulates spontaneous release from frog motor nerve terminals. *Br J Pharmacol* 2003;**138**:1580–8.
4. Bruzzone F, Cervetto C, Mazzotta MC, Bianchini P, Ronzitti E, Leprince J, et al. Urotensin II receptor and acetylcholine release from mouse cervical spinal cord nerve terminals. *Neuroscience* 2010;**170**:67–77.
5. Chartrel N, Leprince J, Dujardin C, Chatenet D, Tollemer H, Baroncini M, et al. Biochemical characterization and immunohistochemical localization of urotensin II in the human brainstem and spinal cord. *J Neurochem* 2004;**91**:110–8.
6. Chuquet J, Lecrux C, Chatenet D, Leprince J, Chazalviel L, Roussel S, et al. Effects of urotensin-II on cerebral blood flow and ischemia in anesthetized rats. *Exp Neurol* 2008;**210**:577–84.
7. Conlon JM, O'Harte F, Smith DD, Tonon MC, Vaudry H. Isolation and primary structure of urotensin II from the brain of a tetrapod, the frog Rana ridibunda. *Biochem Biophys Res Commun* 1992;**107**:578–83.
8. Coulouarn Y, Fernex C, Jegou S, Henderson CE, Vaudry H, Lihrmann I. Specific expression of the urotensin II gene in sacral motoneurons of developing rat spinal cord. *Mech Dev* 2001;**101**:187–90.
9. Coulouarn Y, Jegou S, Tostivint H, Vaudry H, Lihrmann I. Cloning, sequence analysis and tissue distribution of the mouse and rat urotensin II precursors. *FEBS Lett* 1999;**457**:28–32.
10. Coulouarn Y, Lihrmann I, Jegou S, Anouar Y, Tostivint H, Beauvillain JC, et al. Cloning of the cDNA encoding the urotensin II precursor in frog and human reveals intense expression of the urotensin II gene in motoneurons of the spinal cord. *Proc Natl Acad Sci USA* 1998;**95**:15803–8.
11. do Rego JC, Leprince J, Scalbert E, Vaudry H, Costentin J. Behavioral actions of urotensin-II. *Peptides* 2008;**29**:838–44.
12. Douglas SA, Naselsky D, Ao Z, Disa J, Herold CL, Lynch F, et al. Identification and pharmacological characterization of native, functional human urotensin-II receptors in rhabdomyosarcoma cell lines. *Br J Pharmacol* 2004;**142**:921–32.
13. Dubessy C, Cartier D, Lectez B, Bucharles C, Chartrel N, Montero-Hadjadje M, et al. Characterization of urotensin II, distribution of urotensin II, urotensin II-related peptide and UT receptor mRNAs in mouse: evidence of urotensin II at the neuromuscular junction. *J Neurochem* 2008;**107**:361–74.
14. Egginger JG, Camus A, Calas A. Urotensin-II expression in the mouse spinal cord. *J Chem Neuroanat* 2006;**31**:146–54.
15. Filipeanu CM, Brailoiu E, Le Dun S, Dun NJ. Urotensin-II regulates intracellular calcium in dissociated rat spinal cord neurons. *J Neurochem* 2002;**83**:879–84.
16. Gartlon J, Parker F, Harrison DC, Douglas SA, Ashmeade TE, Riley GJ, et al. Central effects of urotensin-II following ICV administration in rats. *Psychopharmacology (Berl)* 2001;**155**:426–33.
17. Guidolin D, Albertin G, Ribatti D. Urotensin-II as an angiogenic factor. Peptides;311:219–1224.
18. Huitron-Resendiz S, Kristensen MP, Sanchez-Alavez M, Clark SD, Grupke SL, Tyler C, et al. Urotensin II modulates rapid eye movement sleep through activation of brainstem cholinergic neurons. *J Neurosci* 2005;**25**:5465–74.
19. Jarry M, Diallo M, Lecointre C, Desrues L, Tokay T, Chatenet D, et al. The vasoactive peptides urotensin II and urotensin II-related peptide regulate astrocyte activity through common and distinct mechanisms: involvement in cell proliferation. *Biochem J* 2010;**428**: 113–24.
20. Jegou S, Cartier D, Dubessy C, Gonzalez BJ, Chatenet D, Tostivint H, et al. Localization of the urotensin II receptor in the rat central nervous system. *J Comp Neurol* 2006;**495**:21–36.
21. Le Mevel JC, Lancien F, Mimassi N, Leprince J, Conlon JM, Vaudry H. Central and peripheral cardiovascular, ventilatory, and motor effects of trout urotensin-II in the trout. *Peptides* 2008;**29**:830–7.
22. Leprince J, Chatenet D, Dubessy C, Fournier A, Pfeiffer B, Scalbert E, et al. Structure-activity relationships of urotensin II and URP. *Peptides* 2008;**29**:658–73.
23. Lu Y, Zou CJ, Huang DW, Tang CS. Cardiovascular effects of urotensin II in different brain areas. *Peptides* 2002;**23**:1631–5.
24. Pelletier G, Lihrmann I, Dubessy C, Luu-The V, Vaudry H, Labrie F. Androgenic down-regulation of urotensin II precursor, urotensin II-related peptide precursor and androgen receptor mRNA in the mouse spinal cord. *Neuroscience* 2005;**132**:689–96.
25. Proulx CD, Holleran BJ, Lavigne P, Escher E, Guillemette G, Leduc R. Biological properties and functional determinants of the urotensin II receptor. *Peptides* 2008;**29**:691–9.
26. Quan FB, Bougerol M, Rigour F, Kenigfest NB, Tostivint H. Characterization of the true ortholog of the urotensin II-related peptide (URP) gene in teleosts. *Gen Comp Endocrinol* 2012;**177**: 205–12.
27. Ross B, McKendy K, Giaid A. Role of urotensin II in health and disease. Am J Physiol Regul Integr Comp Physiol;298:R1156–R1172.
28. Russell FD, Kearns P, Toth I, Molenaar P. Urotensin-II-converting enzyme activity of furin and trypsin in human cells in vitro. *J Pharmacol Exp Ther* 2004;**310**:209–14.
29. Saetrum Opgaard O, Nothacker H, Ehlert FJ, Krause DN. Human urotensin II mediates vasoconstriction via an increase in inositol phosphates. *Eur J Pharmacol* 2000;**406**:265–71.
30. Sugo T, Murakami Y, Shimomura Y, Harada M, Abe M, Ishibashi Y, et al. Identification of urotensin II-related peptide as the urotensin II-immunoreactive molecule in the rat brain. *Biochem Biophys Res Commun* 2003;**310**:860–8.
31. Tostivint H, Joly L, Lihrmann I, Parmentier C, Lebon A, Morisson M, et al. Comparative genomics provides evidence for close evolutionary relationships between the urotensin II and somatostatin gene families. *Proc Natl Acad Sci USA* 2006;**103**:2237–42.
32. Tsoukas P, Kane E, Giaid A. Potential clinical implications of the urotensin II receptor antagonists. *Front Pharmacol* 2011;**2**:1–0.
33. Vaudry H, Do Rego JC, Le Mevel JC, Chatenet D, Tostivint H, Fournier A, et al. Urotensin II from fish to human. *Ann N Y Acad Sci* 2010;**1200**:53–66.
34. Watson AM, May CN. Urotensin II, a novel peptide in central and peripheral cardiovascular control. *Peptides* 2004;**25**:1759–66.
35. Ziltener P, Mueller C, Haenig B, Scherz MW, Nayler O. Urotensin II mediates ERK1/2 phosphorylation and proliferation in GPR14-transfected cell lines. *J Recept Signal Transduct Res* 2002;**22**: 155–68.

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Chapter 128

VIP

Sandrine Passemard, Catalina Abad Rabat, Pierre Gressens and Vincent Lelièvre

ABSTRACT

The vasoactive intestinal neuropeptide VIP is widely expressed throughout the whole body and highly conserved in evolution. Interacting with high affinity receptors, namely, VPAC1 and VPAC2, it plays important roles in regulating peripheral and central functions. This chapter aims to recapitulate some of its most prominent actions in the adult and developing nervous system. In particular, we highlight its regulatory function on cortical development that was recently deciphered at a molecular level, its action in the control of the rhythmic activity of the endogenous brain clocks, and its pivotal role in the expanding field of neuroimmunology. In line with these biological actions, we also point out its striking potentials in pathological conditions including its neuroprotective capability in numerous brain injury models.

INTRODUCTION

Isolated 40 years and 20 years ago, respectively, the related neuropeptides VIP (vasoactive intestinal peptide) and PACAP (pituitary adenylate cyclase activating polypeptide) not only share 68% identity at the amino acid level, common receptors and signaling pathways, but also overlap in patterns of expression and functions. This makes it difficult to comprehensively depict the role of VIP in the brain without referring to PACAP actions. Nevertheless, in this chapter, we attempt to review the most recent results on VIP actions in the brain that highlight previous puzzling findings and also illustrate the potentials of VIP as a diagnostic, preventive or even therapeutic drug in human brain diseases.

STRUCTURE OF THE mRNA/GENE

Fifteen years after its original discovery, the human VIP gene was finally cloned and assigned to chromosome 6q25. Later, orthologs were identified in rats and mice and mapped on chromosome 1p11 and 10, respectively. Just like many other neuropeptides, the VIP gene encodes for a prepropeptide of 170 amino acids.[14] Posttranslational processing finally generates two bioactive peptides encoded on adjacent exons, the 28aa VIP and the 27aa peptide histidine isoleucine and peptide histidine methionine (i.e. PHI/PHM) that solely differ between rodents and humans by their ultimate residue (I vs. M). However, two alternative forms have been observed, namely, peptide histidine valine (PHV42) and PHI-Glu. The 170 amino acid prepro-VIP is initially cut by a signal peptidase in the endoplasmic reticulum to yield the 148 amino acid proVIP. ProVIP is further cleaved by prohormone convertases to VIP-GKR (proVIP125-155) and PHM-GKR (proVIP81-110). These intermediate products are subsequently cleaved by carboxypeptidase-B-like enzymes into VIP-G and PHM-G. The G-forms can then be finalized by PAM enzymes in VIP and PHM, harboring an amidated C-terminus. Conformational analysis of VIP has shown an initial N-terminus sequence made of a β-turn tandem of four amino acid units, followed by two helical segments at residues 7–15 and 19–27 that are connected by an undefined hinge. The three-dimensional structure of VIP confirms the helical segment that is shared between members of the VIP/glucagon family including PACAP27 with minor conformational differences. This may contribute to the selectivity of the peptides for their receptors. Such a hypothesis was confirmed[6] by showing that among the whole VIP sequence the specific domain generated from the α-helix up to the C-terminus interacts with the N-terminal binding domain of the human VPAC1, one of the VIP Receptors.

It is worth noting that the VIP gene has been also identified in numerous lower species, thereby proving its high phylogenetic conversation in sequence and structure.[4] In addition, the PACAP gene structure and processing are very similar: one preproPACAP generating both PRP and PACAP bioactive peptides encoded on similar exons (4 and 5) than PHI/PHM and VIP. The actual hypothesis is that they are derived from a common PACAP-like ancestral gene that duplicated some millions years ago. Other alternative mechanisms including exon skipping or deletion may participate in the full biopic of the VIP/PACAP gene superfamily. There is no evidence of alternative splicing for the mammalian VIP transcript.

Finally phylogenetic studies of the VIP/PACAP gene family have also revealed a very intriguing twist in their

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00128-7
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respective functions (see the receptor sections). Studies of PACAP genes in reptiles reveal that the gila monster expresses a novel peptide family member named exendin. These peptides that exist as four variants of 35–39 amino acids named exendin-1 (helospectin), exendin-2 (helodermin), exendin-3, and exendin-4 are found exclusively in the salivary gland of this venomous lizard native to the southwestern United States and northwestern Mexican deserts. Structurally, exendin-2 is related to human PACAP with 53% sequence identity and exendin-4 to human GLP-1 with a similar identity, although Exendin-2 differentially interacts with the VIP and secretin receptors resulting in an increase in cAMP in rats. However, extensive studies suggest that exendins do not have mammalian homologs.

Expression of the VIP gene is regulated by many factors, including cAMP and PKC. Indeed, promoter studies have revealed that the VIP gene upstream sequences contains three TATAAA boxes, AP2 consensus sites, a 17 bp cAMP-responsive element (CRE) within 100 bases from the start site,[4] and a 180 bp cytokine-responsive area (CyRE) controlled by the ciliary neurotrophic factor (CNTF)/leukemia inhibitory factor (LIF). This element can also bind Smad-containing complexes in response to TGFβ alone or in combination with CNTF in neuroblastoma cells. Estrogen or thyroid hormone can act directly within the pituitary to regulate VIP gene expression in an opposite manner. During embryonic brain development, the neurogenic transcription factor Nurr1 regulates VIP mRNA and protein levels, by transactivating the VIP promoter through Nurr1-responsive cis elements. Loss of heterozygosity in nurr1 reduces VIP transcription and alters the survival of these VIP-producing dopaminergic cells. Dexamethasone studies have also revealed a glucocorticoid response element in the VIP promoter sequence. When a CAT reporter gene is fused to 5.2 kb of the upstream sequence of the human VIP gene, the truncated promoter fails to produce a proper CAT pattern in mutant mice with normal expression in the intestine, but no brain localization. Similarly, a 16.5 kb portion of the mouse VIP promoter fused to beta-galactosidase shows the same partial pattern in adult mice. However, in neonates and embryos, beta-galactosidase staining perfectly matches the original VIP localization in cranial cholinergic and superior cervical ganglia but not in sensory ganglia. These and other results indicate that the multiple transcriptional elements controlling VIP expression are all required for proper *in vivo* patterning.

DISTRIBUTION OF THE mRNA/PEPTIDE

After the initial discovery of VIP, numerous studies have investigated the distribution of this neuropeptide in a large variety of species throughout their whole development.[13] Keeping in mind that VIP can be synthesized and secreted by neurons and also neuroendocrine and immune cells, we focus our review on the central and peripheral nervous system. In the adult brain, VIP is highly expressed in the cerebral cortex, amygdala hippocampus, suprachiasmatic nucleus, and hypothalamus, structures that also express PACAP. In the cerebral cortex, the diversity of cortical GABAergic interneurons is critical to its function. Recent results suggest the existence of three types of regulatory neurons based on the expression of either parvalbumin or somatostatin or again, the ionotropic serotonin 5HT3a receptor known to include the VIPergic neuron subpopulation. In addition, VIP immunoreactivity has been shown in preganglionic sympathetic neurons located in the intermediate zone of the spinal cord and in local nerve fibers in the rat and monkey. These neurons project to parasympathetic ganglia that exhibit VIP-immunoreactive nerve fibers in many vertebrates. In sympathetic ganglia, VIP expression peaks during early development in birds and rodents (from E9 to E14.5) then dramatically decreases later on. VIP immunoreactivity is also observed in large lumbosacral postganglionic neurons and colocalized with catecholamine and CGRP, while it is also codetected with dopamine-β-hydroxylase and NPYin thoracic postganglionic neurons in pig. In mouse embryos, VIP expression detected as early as E10 suggests the possibility of paracrine/autocrine actions during cerebral development to further sustain the placental production of VIP.[27]

RECEPTORS AND SIGNALING CASCADES

Years before the successful cloning of VIP receptors achieved in 1991 from HT29 human adenocarcinoma cell lines, pharmacological studies had already revealed VIP receptors coupling to adenylate cyclase by interacting with GTP binding proteins to stimulate intracellular signaling cascades. In addition, radioactively labeled (iodinated) VIP was generated to develop pharmacological assays that revealed the presence of high affinity binding sites for VIP in a variety of cell types.[13] In parallel, a complete characterization of the pharmacological properties of these VIP binding sites was progressively achieved along with the development of suitable ligands including helodermin and other natural analogs of VIP. In parallel, multiple artificial nonselective "VIP receptor" ligands were created and reported. Most of them were either modified truncated or chimeric VIP peptides, whereas some were proven VIP antagonists. The concept of distinct VIP receptor subtypes was finally accepted to explain how these peptides could show differential affinity with VIP binding sites and exert a wide range potency on cAMP production. Finally, the molecular cloning of three different receptors, the PAC1 receptor that corresponds to PACAP-preferring binding sites and two VIP-preferring receptors namely VPAC1 and

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VPAC2 was achieved. Expression of the cDNA encoding for VPAC1 and VPAC2 in cells confirms their differential pharmacological profiles. In addition, later studies also suspected and/or revealed additional VIP receptors. For instance, type-C natriuretic peptide receptor (NPRC) exhibits high affinities for VIP analogs when overexpressed in Xenopus oocytes.[23] Similarly, PHI/PHM-preferring VIP receptors detected in liver and neuroblastoma cells by biochemical and functional studies were finally cloned in fish.

The VIP-preferring VPAC1 and VPAC2 receptors belongs to the group B GPCR family and are characterized by an N-terminal domain of >120 amino acids that contains a hydrophobic sequence and a cysteine-enriched putative binding domain, a seven transmembrane domain (7TM) region and a long C-terminal segment involved in signal transduction.[13] Numerous structure-function studies have been performed to identify both ligand recognition domain and key motifs necessary for proper signal transduction. Indeed, ligand binding involves both hydrophobic core and N-terminus and extracellular loops of VPACs to initiate physical/conformational changes that permit recruitment of different classes of heterotrimeric G-proteins including the α_s subunit. Directed mutagenesis revealed the role of Tyr239 and Leu240 residues in VPAC1 sequence to circumvent the lack of a G-protein binding E/DRY motif, whereas others proposed that the missing E/DRY motif could be recreated in VPAC1 by three nonadjacent residues Arg174, Glu236, and Tyr239 located in the cytoplasmic end of TM2 and in the distal part of TM3. All in all, studies agreed on the active participation of two specific Tyr residues in TM1, one Lys in TM2 and one Asn in TM3 in VPACs activation.[22] Additional information on VPAC activation was provided by receptor variants. Existence of such variants was proposed as early as 1993 when the group of Gressens revealed the presence of VIP receptors that show no affinity for PACAP to mediated VIP action on mouse brain growth[18] or on neuroprotection against ibotenate-induced lesions.[17] Later, a VPAC2 variant originating from a deletion of exon 11 or 12 was detected in murine thymus and spleen resulting in a weaker coupling to signaling pathways. Finally, in the mononuclear SUPT1 cell line, a larger deletion of 88 aa spanning the TM6-7 region was also detected and associated with a reduced coupling to AC. Moreover, interactions with accessory proteins such as Receptor activity-modifying proteins (RAMPs) can strongly interact with VPAC1 to enhance VIP-induced inositol phosphate hydrolysis without affecting the cAMP pathway.

As already mentioned, the canonical transduction in VPACs requires a coupling with Gαs subunit leading to the activation of adenylate cyclase and subsequent cAMP production. Accumulation of intracellular cAMP may lead to the activation of the protein kinase A that may modulate the ERK (extracellular-signal-regulated kinase) signaling pathway to control cell proliferation and differentiation in cancer cell lines. VIP-induced PKA activation is also responsible for most of the anti-inflammatory activity of VIP by regulating several signaling pathways and transcription factors. VIP receptors are also able to activate PLC in a partly pertussis-toxin-sensitive manner that seems to differ between VPAC1 and VPAC2. Additional couplings may also elicit alternative transduction cascades including phospholipase D and phosphatidylinositol 3-kinase. In the rat pineal gland, VIP stimulates cGMP formation through activation of nitric oxide synthase, nitric oxide (NO) production and activation of cytosolic guanylate cyclase. Ultimately, activation by VIP agonists also initiates the process of receptor desensitization. VPAC1 and VPAC2 are rapidly phosphorylated after agonist exposure. This process starts with phosphorylation of the receptors on Ser/Thr residues in intracellular loops and C-terminus by receptor-activated-dependent kinases (GRKs). GRK-mediated phosphorylation of the receptors initiated a direct interaction with β-arrestins before recruiting the clathrin-dependent endocytic machinery necessary for receptor internalization. VPAC1 and VPAC2 are both rapidly internalized after agonist exposure but differ in their trafficking pattern. After internalization, VPAC1 is not recycled back to the cell surface within a couple of hours, while VPAC2 has already returned.

The receptor distribution has been extensively studied in central and peripheral tissues from numerous species.[30] VPAC1 and VPAC2 transcripts have a completely different and apparently complementary distribution in the rat CNS. Thus, VPAC1-R mRNA is expressed mainly in the cerebral cortex, the hippocampus, and also in deep cerebellar nuclei, thalamus, hypothalamus, and brainstem, whereas VPAC2-R mRNA is detected in the cerebral cortex, hippocampal formation, amygdalar (central) regions, deep cerebellar nuclei, hypothalamus, thalamus and in the pontine nucleus of the brainstem. The distribution of VPAC2-R overlaps with that of VPAC1-R mostly in the hippocampus, playing a key role in adult neurogenesis and memory in a coordinated manner.[35] In the olfactory bulb, VPAC1-R and VPAC2-R mRNAs are differentially distributed, that is, VPAC1-R mRNA is present in the external plexiform layer, whereas VPAC2-R mRNA is expressed in the internal granular layer. In the cerebral cortex, VPAC1-R mRNA is abundant in layers III and V, whereas VPAC2-R mRNA is exclusively localized in layer VI. Both VPAC1-R and VPAC2-R mRNAs are present in astrocytes, whereas VPACs had been identified in neuronal progenitors and preoligodendrocytes undergoing differentiation. In addition, VPACs have also been found in numerous brain tumors, including neuroblastoma and glioma. VPACs have been extensively studied in these pathological tissues and shown to modulate their proliferation and differentiation. They have also been used as prognosis marker or imaging targets for early detection or metastatic development, when overexpressed.[13]

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BIOLOGICAL ACTION WITHIN THE BRAIN (AND PITUITARY) AND PATHOPHYSIOLOGICAL IMPLICATIONS

Based on the wide distribution of VIP and its potential receptors, biological actions of this neuropeptide have been reported in almost all tissues. Indeed, VIP acting as an hormone, a transmitter or a neuromodulator, or again as a tropic factor, exert important roles on metabolism and homeostasis in general that were confirmed by phenotypic analysis of several lines of transgenic mice, including tissue-specific overexpressing animals and complete knockouts.[13] Original transgenic mice expressing the human VIP gene in pancreatic beta cells exhibited a significant reduction in circulation glucose concentration and remained insensitive to glucose intolerance after partial pancreatectomy, suggesting potential values for VIP and related peptides in diabetes treatment. Conversely, the mouse line harboring a chimeric VIP-diptheria toxin transgene produces 20% less cerebral VIP and exhibits cognitive deficits, whereas VPAC2-expressing transgenic mice exhibits impaired rhythmic activity in SCN. However, the first complete VIP-KO mouse line generated in 2003 exhibited a complete loss of both VIP and PHI neuropeptides without compensatory phenomena at ligand (PACAP) or receptor levels. Animals proven viable and fertile suffered from complex behavioral deficits and critical chronobiological symptoms that will be further discussed in the next sections. They also exhibited peripheral deficits in intestinal, immune, and testicular functions.

Three different PAC1-deficient mice have been generated. Numerous *in vivo* studies were performed to evidence the PACAP action on pancreatic and gastric secretion, hippocampus and memory function, light-induced circadian rhythm synchronization, social and psychomotor behavior and also on nociception and sensory system. When animals lack both VIP and PACAP, animal growth and life expectancy are dramatically reduced.[26]

More specifically, VIP may act as an autocrine/paracrine trophic factor on immune and neural cells, leading to important concepts of VIP as a neuromodulator of brain activity in adult and developing brain and also as a neuroimmunomodulator in pathological conditions including brain injury and infection/inflammation situations. These particular actions of VIP are summarized below in greater detail.

VIP Effects on Early Brain Development

VIP regulates growth and embryonic development during the neural tube closure period and the early stages of neurogenesis. Treatment of E9 whole culture mouse embryos with VIP shows a dramatic increase in growth and development over a 4-h period in a dose-dependent manner.[18] PACAP has no effect on embryonic growth during this narrow window of time. VIP receptors are expressed as early as E9 at the floor plate of neural tube, whereas VIP itself is not expressed in embryonic telencephalon before E11–E12.[32] Before E12, VIP comes from T lymphocytes of the maternal decidua where VIP RNA is expressed from E6 to E12 (peak at E8).[29] VIP crosses the placenta, reaches the embryo, and binds to VIP receptors in neural tube or telencephalon.[21] Gressens and coworkers showed that VIP blockade during early neurogenesis using a VIP antagonist (VA), intraperitoneally injected twice a day to the pregnant mice from E9 to E11, leads to microcephaly.[19] They showed how VIP is crucial for cortical development and dissected the VIP signaling pathway in this model.[27] VIP blockade induces a reduction of 20% of cortical thickness, when observed at P5, that affects both upper and lower layers and mimics the phenotype of MCPH patients (autosomal recessive microcephaly, defined by a reduction in brain weight without cortical cytoarchitecture defects causing developmental delay in affected patients). This thinner cortex is the result of a decrease in proliferation of neural progenitors at the ventricular zone at E11.5 by increasing the cell cycle length, accelerating cell cycle exit and promoting early differentiation of cortical progenitors. They showed that VIP blockade disrupts Mcph1 signaling because VA downregulates expression of Mcph1 at both gene and protein levels, as well as its targets, Chk1, a key check point of the cell cycle, and Brca1, in the telencephalon of treated embryos (Fig. 1). *In vitro* silencing of either Mcph1 or Chk1 in neurospheres mimics VA-induced inhibition of cell proliferation, and this inhibition of VIP on neural stem cell progenitors is mediated by VPAC1 receptors coupled with a cAMP/PKA signaling pathway. Taken together, these experiments show that the VIP/Mcph1/Chk1 pathway is the key to normal cortical development and that environmental factors, including maternal ones, such as VIP, can influence neural progenitors proliferation and thereby the size of the brain.

Behavioral consequences of VIP blockade (E9–E11) on neonatal mice are a developmental delay. Pups exhibit retardation in the appearance of 5 (such as eye opening and several motor reflexes) of the 10 developmental milestones, modeling some mental retardation features observed in human MCPH.[33] In adult stages, some other developmental disorders and severe behavioral disorders appear. Adult mice that had been treated by VA between E8 and E10 exhibit anxiety-like behavior and deficits in social behavior such as autism. The developmental delays and social behavior deficits seen in mice treated by VA during embryonic stages are similar to that obtained with $VIP^{-/-}$ mice.[24] But more interestingly, developmental deficit and social behavior disorders are determined by the VIP genotype of the mother, not the genotype of the offspring, because heterozygous offspring of VIP null mother were equally retarded

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in development. These experiments show that deficiency of maternal VIP during neurogenesis contributes greatly to the developmental delay and social behavior deficit in the offspring of VIP-deficient mothers and that expression of VIP by E12 in those offspring with one VIP gene does not ameliorate the developmental deficiency.[24] In conclusion, maternal VIP production influences (1) final brain size by regulating neural progenitor proliferation through Mcph1/Chk1 pathway and (2) the achievement of developmental milestones in offspring and normal social behavior in adult stage.

Immunomodulatory Effects

Immune cells patrol the CNS as a mechanism of defense against infections, and microglia and astrocytes have the ability to produce cytokines in response to insults. However, chronic inflammation of the brain is a key feature in certain autoimmune diseases such as multiple sclerosis (MS), and it has been suggested that aberrant immune responses may perpetuate damage in the brain of Alzheimer's and Parkinson's disease patients as well. Nevertheless, the immune system seems to be a double-edged sword, because in certain circumstances immune cells can secrete neuroprotective molecules that improve axon regeneration and tissue healing. Immunomodulatory therapies that block harmful immune responses and promote beneficial effects of immune cells may be the most promising approaches to ameliorate MS and other neurodegenerative diseases. Extensive evidence reviewed in Refs. 1, 11, 28, and summarized below and in Fig. 2 demonstrates that VIP exhibits such properties.

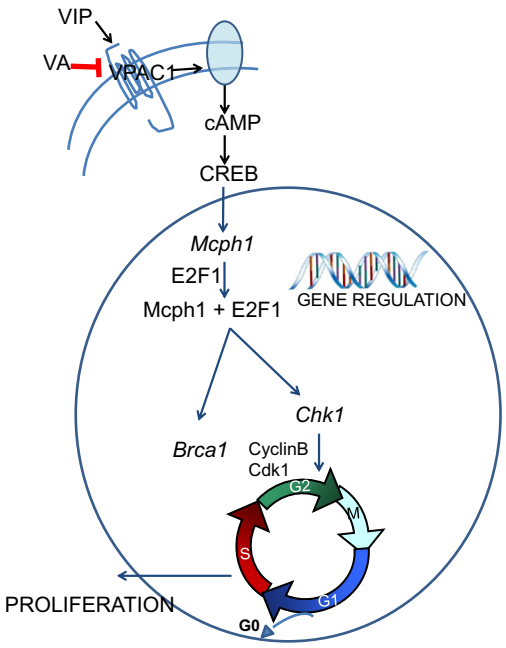

FIGURE 1 Current hypothesis depicting how VIP can induce mouse neural stem cell progenitor proliferation through VPAC1–PKA–Mcph1–Chk1 signaling pathway. VIP proliferative effects are mediated by VPAC1 coupled to the cAMPc–PKA pathway, and this action is blocked by VA. PKA can phosphorylate CREB that potentially binds to one of the 3 cAMP-responsive element sequences on *Mcph1* gene. VIP blockade induces a decrease of *Mcph1* gene expression. *Mcph1* in turn regulates *Chk1* and *Brca1*, two key check points of the cell cycle, through its interaction with E2F1 on the promoter of both genes. VIP blockade then induces a decrease of neural stem cell proliferation through VPAC1–cAMP–PKA–Mcph1–Chk1 signaling pathway. See color plate 36.

The first studies suggesting a link between the neuropeptide VIP and the immune system revealed its presence within the innervation of immune organs and in immune cells. Moreover, VIP receptors are found in the surface of immune cells with a different pattern depending on their activation status: Although in basal conditions macrophages express VPAC1 and PAC1 and lymphocytes express only VPAC1, VPAC2 is expressed after the activation of these cells with stimuli of different nature. VIP modulates different aspects of immune cell physiology, including migration, proliferation, and cytokine secretion. Early studies revealed that VIP exerts strong anti-inflammatory actions *in vitro*. For example, studies using murine peritoneal macrophages show that VIP inhibits the lipopolysaccharide (LPS)-induced production of the proinflammatory cytokines TNFα, IL-6, and IL-12 while stimulating the expression of the anti-inflammatory cytokine IL-10. In addition, VIP may also impede the amplification of the inflammatory response through its inhibitory action on chemokine production, as it reduced the expression of chemokines such as RANTES, KC, MIP-1α, MIP-1β, and MCP-1 in macrophages stimulated with LPS. Moreover, VIP decreased the production of free radicals in macrophages stimulated with LPS at least in part by reducing the expression of inducible nitric oxide synthase (iNOS), and it suppressed cyclooxygenase 2 (COX2) expression in macrophages, microglia, and dendritic cells. Similarly, and relevant to CNS pathogenesis, VIP reduced the LPS-induced production of proinflammatory cytokines, chemokines and other proinflammatory mediators by cultured primary microglia. VIP may inhibit inflammation by acting downstream of toll like receptors (TLRs) and/or other proinflammatory pathways: VIP reduces in naive macrophages the expression of TLR4, the receptor for LPS. Evidence suggests that most of the immunomodulatory effects of VIP are mediated by the inhibition of NF-kB and/or MEKK1/MEK4/JNK pathways, and the induction of cAMP, protein kinase A and CREB phosphorylation.[11] In contrast, this peptide reduces IFNγ signaling in macrophages and microglia by inhibiting Jak1/2-STAT1 phosphorylation.

Most of the anti-inflammatory effects of VIP are believed to be mediated through the VPAC1 receptor, and partially by VPAC2, except IL-6 inhibition, which at least in some contexts depends mainly on the PAC1 receptor. Besides

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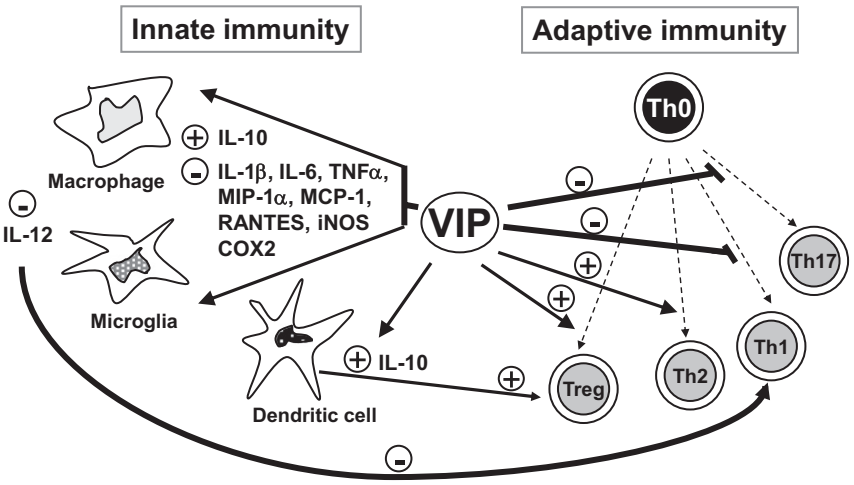

FIGURE 2 VIP modulates the innate and adaptive arms of immunity. VIP exerts its antiinflammatory actions by (1) inhibiting the production of pro-inflammatory cytokines and chemokines from myeloid cells (macrophages and microglia) including IL-1b, IL-6, TNFa, MIP-1a, MCP-1, and RANTES among others, as well as iNOS and COX-2 but stimulating the production of IL-10; (2) inducing a tolerogenic dendritic cell phenotype producing high amounts of IL-10; 3) switching the lymphocyte immune cell response from Th1/Th17 to Th2/Treg, by several mechanisms, including the inhibition of IL-12 production from antigen presenting cells.

its effects on the innate immune system, VIP exhibits important actions on lymphocyte biology and other aspects of adaptive immunity. One of the best known actions of VIP is the ability to potentiate Th2 at the expense of Th1 responses.[11] Although several mechanisms seem to be involved, the modulatory effects of VIP on Th cells are mediated at least partially by its actions on antigen presenting cells (APCs). For example, VIP reduces the production of IL-12, a Th1 promoting cytokine, by macrophages stimulated with LPS. VIP may also selectively modulate Th chemotaxis at the site of antigen presentation by decreasing dendritic cell production of the Th1 chemokine CXCL10, while increasing the Th2 chemokine CCL22. In addition, VIP promotes *in vivo* the generation of memory Th2 cells. Finally, VIP improves Th2 survival at least in part by selectively blocking the expression of granzyme B, FasL, and perforin in these cells. Another mechanism by which VIP may exert its anti-inflammatory action is through the generation of regulatory T cells (Tregs), which has been shown both *in vitro* and *in vivo*.[8] Tregs have been implicated in the resolution phase of inflammation by suppressing effector T cell proliferation and activity. Again, the actions of VIP on dendritic cells may account for their effects on Tregs. Dendritic cells generated from murine bone marrow precursors or human blood monocytes in the presence of VIP were $CD11c^{low}CD45RB^{high}$, secreted high amounts of IL-10 and did not upregulate CD80, CD86, or CD40 after LPS stimulation. These cells were tolerogenic, in the sense that they promoted the production of Tregs *in vitro* and *in vivo*.

The anti-inflammatory effects of VIP have been confirmed *in vivo* in different murine models of disease including LPS-induced endotoxemia, collagen-induced arthritis (CIA), TNBS colitis, and nonobese diabetic (NOD) mice, models for rheumatoid arthritis, Crohn's disease and autoimmune diabetes respectively, reducing the Th1 and inflammatory components that are common to their pathogenesis.[11] Regarding CNS disease, VIP exhibits therapeutic properties in chronic and relapsing-remitting experimental immune encephalomyelitis (EAE) murine models of MS, reducing the clinical symptoms of paralysis, CNS immune cell infiltration and axonal damage while increasing the numbers of oligodendrocytes.[3] MS is believed to be an inflammatory Th1-driven pathology. In the EAE studies, the expression of proinflammatory cytokines in the CNS is significantly reduced in VIP-treated mice while the expression of cytokines generally considered to be anti-inflammatory (IL-10, IL-1Ra, and TGFβ1) is increased after VIP treatment. In addition, the ex vivo analysis of T cell responses suggests that VIP peripheral administration decreases Th1 but favored Th2 responses. The therapeutic action of VIP in EAE is also associated with an induction of Tregs, which may act by blocking the ongoing lymphocytic responses or accelerate the recovery phase of the disease.

Studies in other experimental models of disease highlight a potential therapeutic role for VIP in neuroinflammation. For example, local administration of VIP prevents neurodegeneration induced by intraventricular administration of LPS, reducing microglia activation and TNF-α, IL-1β, and iNOS expression.[10] Likewise, local administration of VIP decreases pathological events occurring after stab wound brain trauma in the periventricular area, including immune cell infiltration, microglia activation, and TNF-α and IL-1β production.[10] VIP administration also decreases microglial activation in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

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(MPTP) model of Parkinson's disease.[9] This peptide also reverses the detrimental effects of IL-1β on ibotenate-induced lesions of the periventricular white matter.[15] Finally, VIP exerts protection against β-amyloid-induced neurodegeneration by inhibiting microglia activation at multiple levels, suggesting a therapeutic potential against Alzheimer's disease.[12]

The direct *in vivo* administration of VIP or receptor analogs is only one option to take advantage of their therapeutic immunomodulatory potential. Initial studies have suggested that the ability of VIP to induce ex vivo dendritic cells with tolerogenic potential and thus capacity to induce Tregs could be successfully exploited for the treatment of autoimmune and other inflammatory diseases.[1] C57BL/6 mice treated with VIP-generated dendritic cells after the onset of EAE show diminished clinical scores compared with control mice. Cells with the ability to migrate to inflammatory sites and immune organs could be also used as carriers of VIP-encoding vectors, allowing the continuous but local production of VIP. Bone marrow derived dendritic cells transduced with a lentiviral vector encoding human VIP (referred to as LentiVIP) result in VIP-secreting dendritic cells, with tolerogenic phenotypic and functional characteristics (secreting little if any of the proinflammatory cytokines but producing high levels of IL-10).[1] When LentiVIP-Dendritic cells pulsed with myelin peptides are administered intravenously to mice with EAE, a reduced severity of the symptoms is observed. Although the mechanisms explaining the *in vivo* effects of these cells remain to be elucidated, it is possible that LentiVIP-dendritic cells exert double beneficial actions first through the secretion of VIP, which may act do inhibit inflammatory cells, and second through their own tolerogenic potential on Treg production.

Neuroprotective Effects

In vitro neuroprotection

In cell culture, VIP stimulates astrocytic mitoses and to protect, at fentomolar concentrations, neurons against oxidative stress, *N*-methyl-D-aspartate (NMDA) receptor-mediated excitotoxicity, HIV-1 gp120 neurotoxicity, 6-hydroxydopamine neurotoxicity, and beta-amyloid neurotoxicity.[2,3,16] VIP protective effects on neurons seem to be mediated by astrocytes as VIP induces the release of trophic factors (such as brain-derived neurotrophic factor—BDNF—and activity-dependent neuroprotective protein—ADNP-), cytokines and chemokines by astrocytes.[2,3,16]

Perinatal brain neuroprotection

Excitotoxicity is a key molecular mechanism of perinatal brain damage, which is associated with cerebral palsy and long-term cognitive deficits. VIP induces a potent neuroprotection against perinatal excitotoxic white matter damage, that is, mimicking lesions observed in preterm neonates.[20] VIP effects are not mimicked by PACAP-27 or PACAP-38. Interestingly, VIP does not prevent the initial appearance of white matter lesion but promotes a secondary repair with axonal regrowth and/or sprouting. This plasticity mechanism involves the following steps: (1) VIP binds to an atypical VPAC2 receptor expressed on astrocytes and activates a PKC pathway; (2) PKC activation promotes astrocytic survival and astrocytic release of soluble factors, including BDNF; (3) released factors such as BDNF activate MAPK and PKC cascades in neurons; (4) MAPK cascade and, possibly PKC, activation leads to axonal repair and/or sprouting.[27] Stable VIP agonists such as stearyl norleucine VIP (a lipophylic VIP agonist that does not activate adenylate cyclase) and RO-25-1553 (a VPAC2 receptor agonist), mimic VIP effects when given systemically and exhibit a large therapeutic window (up to 12 h after the insult).[27]

Adult brain neuroprotection

Neuroprotective effects of stearyl norleucine VIP have also been observed in an animal model of Alzheimer's disease. Mice deficient in apolipoprotein E (ApoE) exhibit decreased cholinergic activity, retardation in the acquisition of developmental milestones, and learning and memory impairments. Daily injections of stearyl norleucine VIP to ApoE-deficient newborn pups increases cholinergic activity and markedly improves the acquisition of behavioral milestones and improves adult cognitive functions.

Neuromodulator Effects

VIP is coreleased with different neurotransmitters to modulate synaptic activity in numerous brain structures. VIP-dependent neuromodulatory activities have been well studied in two central structures, the suprachiasmatic nucleus and the hippocampus.

It is now well established that the suprachiasmatic nuclei (SCN) of the hypothalamus harbors the main mammalian circadian clock.[31] This structure exhibits an endogenous rhythm (pacemaker) synchronized daily and yearly environmental changes through the photic systems. Light controls sleep and locomotor activity monitored by free wheel-running device in rodents. To date, investigations on the neurochemical basis of clock resetting have focused on the influence of SCN afferents. Photic information is conveyed from the retina directly to the SCN by the monosynaptic retinohypothalamic tract (RHT) releasing both glutamate and PACAP. SCN is subdivided into a "shell" and "core." Neurons contain within the shell include AVP- and somatostatin-positive cells, whereas the core neurons produce gastrin-releasing peptide and VIP. This neuroanatomical system perfectly illustrates the synergistic action of VIP and PACAP neuropeptides. In hamsters, the VIP release from the SCN exhibits a daily peak near midday that is completely obliterated under constant darkness. Glutamatergic agonist also stimulates VIP release, whereas selective

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serotonin receptor agonist suppresses it. Abrogation of VIP effects, as observed in VIP null mice or VPAC2 KO, results in the loss of endogenous nocturnal activity rhythm when animals are kept in constant darkness. The rhythmic activity in SCN generates and synchronizes many biological rhythms such as hormones (glucocorticoids, melatonin), neuronal connections, body temperature, metabolites (glucose), and paracrine signals shown to subsequently entrain the peripheral clocks present in numerous brain structures (including hippocampus, prefrontal cortex, amygdala, or VTA) and peripheral tissues. This phenomenon is illustrated by VIP-deficient mice that exhibit weak rhythms in the heart rate and body temperature. In addition, alteration in clock genes or in normal modulation of SCN activity are directly associated with neuropsychiatric disorders in human.[25] For instance, dementia patients, suffering from neurodegenerative disease (Alzheimer's disease, AD) or genetic disorder (Huntington disease, HD) often exhibit sleep disruption and other circadian rhythm disturbances. Therefore, it is not very surprising that PHI concentration in cerebral fluid of patients with AD is reduced or that VPAC receptor expression is altered in the SCN of a mouse model of HD.

A similar situation is observed in the hippocampus. Glutamate, the main excitatory neurotransmitter in the CNS activates hippocampus glutamate receptors that are essential for postsynaptic responses and synaptic plasticity. These synaptic events are crucial for both spatial learning and memory.[7] Long-term potentiation (LTP) and long-term depression (LTD) of the excitatory synapses between CA3 and CA1 hippocampal pyramidal neurons are well-characterized signatures of this plasticity-dependent learning and memory and are almost entirely controlled by AMPA and NMDARs receptors. The activation of VPACs increases evoked NMDA currents via the cyclic AMP/PKA pathway, whereas activation of PAC1-R stimulates a PLC signaling pathway to enhance hippocampal NMDA receptor function.[34] Therefore, it is not surprising to observe memory and learning impairment in PACAP-, PAC1-, and VIP-deficient mice. Further, adult neurogenesis that occurs in hippocampus has been extensively studied in relationship with learning and memory function.[5] Abrogation of neurogenesis in dentate gyrus leads to memory impairment, while stimulation of hippocampal neural stem cell (NSC) proliferation enhances learning and memory in aging animals. Adult VPAC2$^{-/-}$ mice exhibited reduced progeny survival and dentate neurogenesis.[35] Indeed VPAC2 receptor activation shifts the fate of self-renewing NSCs toward nestin-positive progenitors, whereas selective VPAC1 receptor activation differentiates these progenitors into granule cells. This synergistic VIP receptor system on adult hippocampal neurogenesis offers an exciting potential use of pharmacological manipulation of this precursor pool for therapeutic application in the treatment of cognitive deficits.

REFERENCES

1. Abad C, Waschek JA. Immunomodulatory roles of vip and pacap in models of multiple sclerosis. *Curr Pharm Des* **17**:1025–35
2. Brenneman DE. Neuroprotection: a comparative view of vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide. *Peptides* 2007;**28**:1720–6.
3. Brenneman DE, Westbrook GL, Fitzgerald SP, Ennist DL, Elkins KL, Ruff MR, et al. Neuronal cell killing by the envelope protein of hiv and its prevention by vasoactive intestinal peptide. *Nature* 1988;**335**: 639–42.
4. Cardoso JC, Vieira FA, Gomes AS, Power DM. Pacap, vip and their receptors in the metazoa: Insights about the origin and evolution of the ligand-receptor pair. *Peptides* 2007;**28**:1902–19.
5. Castilla-Ortega E, Pedraza C, Estivill-Torrus G, Santin LJ. When is adult hippocampal neurogenesis necessary for learning? Evidence from animal research. *Rev Neurosci* **22**:267–83
6. Ceraudo E, Tan YV, Nicole P, Couvineau A, Laburthe M. The n-terminal parts of vip and antagonist pg97-269 physically interact with different regions of the human vpac1 receptor. *J Mol Neurosci* 2008;**36**:245–8.
7. Citri A, Malenka RC. Synaptic plasticity: multiple forms, functions, and mechanisms. *Neuropsychopharmacology* 2008;**33**:18–41.
8. Delgado M, Chorny A, Gonzalez-Rey E, Ganea D. Vasoactive intestinal peptide generates cd4+cd25+ regulatory T cells in vivo. *J Leukoc Biol* 2005;**78**:1327–38.
9. Delgado M, Ganea D. Neuroprotective effect of vasoactive intestinal peptide (vip) in a mouse model of parkinson's disease by blocking microglial activation. *Faseb J* 2003;**17**:944–6.
10. Delgado M, Ganea D. Vasoactive intestinal peptide prevents activated microglia-induced neurodegeneration under inflammatory conditions: potential therapeutic role in brain trauma. *Faseb J* 2003;**17**:1922–4.
11. Delgado M, Pozo D, Ganea D. The significance of vasoactive intestinal peptide in immunomodulation. *Pharmacol Rev* 2004;**56**:249–90.
12. Delgado M, Varela N, Gonzalez-Rey E. Vasoactive intestinal peptide protects against beta-amyloid-induced neurodegeneration by inhibiting microglia activation at multiple levels. *Glia* 2008;**56**:1091–103.
13. Dickson L, Finlayson K. Vpac and pac receptors: from ligands to function. *Pharmacol Ther* 2009;**121**:294–316.
14. Fahrenkrug J. Vip and pacap. *Results Probl Cell Differ* **50**:221–34
15. Favrais G, Couvineau A, Laburthe M, Gressens P, Lelievre V. Involvement of vip and pacap in neonatal brain lesions generated by a combined excitotoxic/inflammatory challenge. *Peptides* 2007;**28**:1727–37.
16. Gozes I. Vip, from gene to behavior and back: Summarizing my 25 years of research. *J Mol Neurosci* 2008;**36**:115–24.
17. Gressens P, Besse L, Robberecht P, Gozes I, Fridkin M, Evrard P. Neuroprotection of the developing brain by systemic administration of vasoactive intestinal peptide derivatives. *J Pharmacol Exp Ther* 1999; **288**:1207–13.
18. Gressens P, Hill JM, Gozes I, Fridkin M, Brenneman DE. Growth factor function of vasoactive intestinal peptide in whole cultured mouse embryos. *Nature* 1993;**362**:155–8.
19. Gressens P, Hill JM, Paindaveine B, Gozes I, Fridkin M, Brenneman DE. Severe microcephaly induced by blockade of vasoactive intestinal peptide function in the primitive neuroepithelium of the mouse. *J Clin Invest* 1994;**94**:2020–7.
20. Gressens P, Marret S, Hill JM, Brenneman DE, Gozes I, Fridkin M, et al. Vasoactive intestinal peptide prevents excitotoxic cell death in the murine developing brain. *J Clin Invest* 1997;**100**:390–7.

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21. Hill JM, McCune SK, Alvero RJ, Glazner GW, Henins KA, Stanziale SF, et al. Maternal vasoactive intestinal peptide and the regulation of embryonic growth in the rodent. *J Clin Invest* 1996;**97**:202–8.
22. Langer I, Robberecht P. Molecular mechanisms involved in vasoactive intestinal peptide receptor activation and regulation: Current knowledge, similarities to and differences from the a family of G-protein-coupled receptors. *Biochem Soc Trans* 2007;**35**:724–8.
23. Lelievre V, Hu Z, Ioffe Y, Byun JY, Flores A, Seksenyan A, et al. Paradoxical antagonism of pacap receptor signaling by vip in xenopus oocytes via the type-c natriuretic peptide receptor. *Cell Signal* 2006;**18**:2013–21.
24. Lim MA, Stack CM, Cuasay K, Stone MM, McFarlane HG, Waschek JA, et al. Regardless of genotype, offspring of vip-deficient female mice exhibit developmental delays and deficits in social behavior. *Int J Dev Neurosci* 2008;**26**:423–34.
25. Menet JS, Rosbash M. When brain clocks lose track of time: Cause or consequence of neuropsychiatric disorders. *Curr Opin Neurobiol*
26. Niewiadomski P, Coute-Monvoisin AC, Abad C, Ngo D, Menezes A, Waschek JA. Mice deficient in both pituitary adenylyl cyclase-activating polypeptide and vasoactive intestinal peptide survive, but display growth retardation and sex-dependent early death. *J Mol Neurosci* 2008;**36**:200–7.
27. Passemard S, El Ghouzzi V, Nasser H, Verney C, Vodjdani G, Lacaud A, et al. Vip blockade leads to microcephaly in mice via disruption of mcph1-chk1 signaling. *J Clin Invests*
28. Smalley SG, Barrow PA, Foster N. Immunomodulation of innate immune responses by vasoactive intestinal peptide (vip): its therapeutic potential in inflammatory disease. *Clin Exp Immunol* 2009;**157**:225–34.
29. Spong CY, Lee SJ, McCune SK, Gibney G, Abebe DT, Alvero R, et al. Maternal regulation of embryonic growth: the role of vasoactive intestinal peptide. *Endocrinology* 1999;**140**:917–24.
30. Vaudry D, Falluel-Morel A, Bourgault S, Basille M, Burel D, Wurtz O, et al. Pituitary adenylate cyclase-activating polypeptide and its receptors: 20 years after the discovery. *Pharmacol Rev* 2009;**61**: 283–357.
31. Vosko AM, Schroeder A, Loh DH, Colwell CS. Vasoactive intestinal peptide and the mammalian circadian system. *Gen Comp Endocrinol* 2007;**152**:165–75.
32. Waschek JA, Ellison J, Bravo DT, Handley V. Embryonic expression of vasoactive intestinal peptide (vip) and vip receptor genes. *J Neurochem* 1996;**66**:1762–5.
33. Wu JY, Henins KA, Gressens P, Gozes I, Fridkin M, Brenneman DE, et al. Neurobehavioral development of neonatal mice following blockade of vip during the early embryonic period. *Peptides* 1997; **18**:1131–7.
34. Yang K, Trepanier CH, Li H, Beazely MA, Lerner EA, Jackson MF, et al. Vasoactive intestinal peptide acts via multiple signal pathways to regulate hippocampal nmda receptors and synaptic transmission. *Hippocampus* 2009;**19**:779–89.
35. Zaben M, Sheward WJ, Shtaya A, Abbosh C, Harmar AJ, Pringle AK, et al. The neurotransmitter vip expands the pool of symmetrically dividing postnatal dentate gyrus precursors via vpac2 receptors or directs them toward a neuronal fate via vpac1 receptors. *Stem Cells* 2009;**27**:2539–51.

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Chapter 129

VP/OT

John F. Morris

ABSTRACT

Vasopressin (VP) and oxytocin (OT) are the two mammalian representatives of an ancient line of brain peptides central to the investigation of neurosecretion and peptide signaling. Their peripheral actions are well understood; recent research has focused on their central actions and role in behavior. Magnocellular neurons that synthesize VP and OT release them from posterior pituitary axons and from their dendrites; their large size and grouping in the supraoptic and paraventricular (PVN) nuclei have made them robust models for peptidergic neurons in general. Smaller parvocellular neurons in the PVN project to the median eminence, brainstem, and spinal cord. Other small VP and OT neurons are sparsely distributed; their receptors are more widely located. Some VP and OT is produced in peripheral tissues. Systemically, VP is involved in fluid balance and vascular and autonomic control; OT in milk ejection and parturition. Centrally, both the peptides are involved in social behavior including stress, aggression, and pair bonding.

DISCOVERY AND HISTORY[21]

The hormonal activities of vasopressin (VP) and oxytocin (OT) were among the first peptide actions to be discovered. Between 1895 and 1915, Oliver and Schäfer showed vasopressor activity in pituitary extracts, which Howell showed came from its neural lobe (NL); similarly, Schäfer showed antidiuretic activity, and von den Velden showed its NL source. Originally, it was believed that these actions resulted from two hormones, hence the interchangeable use of ADH and VP today. Ott and Scott discovered the mammary effects of OT, Gaines distinguished milk ejection from milk secretion, and Dale showed its uterotonic actions. When these actions were discovered, it was believed that the hormones were synthesized in the NL; it was the studies of Ernst and Berta Scharrer and Wolfgang Bargmann that showed synthesis in large hypothalamic neurons giving rise to the whole concept of neurosecretion into the systemic circulation. The structure of the peptides (Fig. 1a) was elucidated by DuVigneaud in 1953. Acher studying neurophysins and Sachs and Takabatake studying precursor processing showed that OT, VP, and the neurophysins were produced from larger precursors. Molecular understanding dawned in 1984 when Ivell and Richter described the two genes; shortly after, the gene defect giving rise to diabetes insipidus in the Brattleboro rat was identified.

The development of radioactively labeled hormones in the 1950s pioneered the study of receptors for the hormones and the VP receptor subtypes were identified (see Jard in Ref. 35); this led on to the elucidation of the activation cascades initiated by the ligand receptors. The development of electrophysiological techniques showed that the substantial release of both VP and OT relied on bursts of action potentials traveling to the NL but that the pattern of electrical activity differed between VP and OT neurons to the extent that this could be used to identify the two types of cell; VP neurons when activated fire in increasingly long bursts separated by quiescent unresponsive gaps (phasic firing), whereas OT neurons fire in synchronized bursts for parturition before milk ejection (Armstrong et al. in Ref. 3). The use of immunocytochemistry and in situ hybridization has shown that not all VP and OT originate from the magnocellular neurons (MCN) in the SON and PVN and project to the NL. Smaller parvocellular neurons (PCN) in the PVN project to the median eminence to control, in particular, corticotrophs in the anterior pituitary. Other small VP and OT neurons are located in other parts of the CNS and project their actions to specific locations. Combining this with receptor localization shows that there is often a significant mismatch so that many of the CNS receptors apparently respond to peptide that reaches it via diffusion through the CSF and extracellular space.[23] Furthermore, although there is little species variation in the distribution of systemic receptors, the same is not true for the CNS and substantial differences in receptor distribution that relate to behavior have been discovered.

Dendrites of MCN secrete VP or OT independently of the axons (Morris and Ludwig in Ref. 18) to act locally and in an autocrine manner on the MCN.[23,24] Because all peptidergic neurons have peptides in their dendrites, dendritic release is presumably the norm. A host of molecular biology techniques are now used to investigate the system. Germ line and increasingly specific and inducible knockouts and

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00129-9
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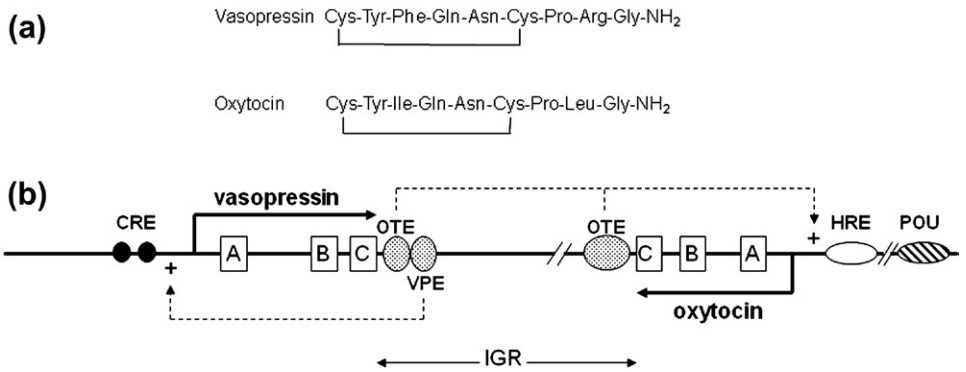

FIGURE 1 (a) Amino acid structure of arginine vasopressin (Mr 1084.38) and oxytocin (Mr 1007.35). In pigs and some marsupials, the Arg in vasopressin is replaced by lysine; the Arg (or Lys) is critical for vasopressin action. Oxytocin has the same structure in all mammals; the Ile is critical for oxytocin action. In birds, reptiles, amphibian, and lungfishes, vasotocin and mesotocin are the VP and OT equivalents; in bony fish, vasotocin and isotocin. (b) VP and OT genes and their regulatory domains (mouse). Enhancers of OT and VP (OTE, VPE) are in a 178-bp segment next to OT exon 3. Cyclic AMP response elements (CRE) are present in the VP promoter; the OT promoter contains a composite hormone response element (HRE) (between −70 and −195) and a POU homeodomain (between −1270 and −1535).

knock-ins have been created for both the peptides and their receptors. Genetic analysis of a large number of humans is gradually revealing not only the wide variety of mutations that occur but also the role of microsatellites and epigenetic silencing in both normal control and disease.

STRUCTURE AND REGULATION OF THE PRECURSOR mRNAs/GENES (FIG. 1)[4,5,21]

The human VP and OT genes (Fig. 1) are on the short arm of chromosome 20 (20p13) (in rats on chromosome 2; mice, chromosome 2; http://www.ncbi.nlm.nih.gov/), tail to tail and transcribed in opposite directions, reflecting evolutionary duplication. The intergene sequence contains a LINE sequence and enhancers for both the OT genes. The close linkage of the genes allows regulatory communication between the two transcription units, but the full extent of controlling sequences remains unclear.

Each gene consists of three exons (~2 kb). Exon A encodes the signal, hormone, a Gly-Lys-Arg cleavage sequence and nine N-terminal amino acids of the hormone-specific neurophysin (NP); exon B encodes the midpart of NP (aa 10–76), which is highly conserved, reflecting its role as a chaperone and hormone binder within large dense-cored vesicles (LDVC). Exon C encodes the remainder of the NP, a single Arg and, for VP only, a 39 aa glycopeptide (GP) the function of which is still unknown. The precursor proteins form homodimers in the rER. Occasional cells containing hybrid VP/OTNP and OT/VPNP mRNAs and proteins have been found and these proteins derived from mutant genes accumulate in the rRER and are not packaged (see Section 7)

Transcription of the genes in MCN is controlled by the activity of the neurons in ways that are still unclear. In both VP and OT neurons, gene transcription and precursor synthesis generally increase with c-fos induction (marker of activation) presumably via the AP-1 site (actions via c-jun and ATF on TPA and cAMP response elements have been less studied). Steroids affect both genes: glucocorticoids act via an inducible GR to stimulate VP synthesis; estrogens stimulate synthesis of OT. Osmotic stimulation doubles the poly(A) tail length on the VP and OT mRNAs to affect their stability. Epigenetic silencing of the genes via methylation is now being studied as an important control; testosterone maintains expression of VP in the BNST in relation to aggressive behavior.[1]

PROCESSING OF THE PRECURSORS (FIG. 2)[4,21]

Transcription of the VP and OT genes yields heteronuclear RNA, which is then spliced to form mRNA, that leaves the nucleus and attaches to free ribosomes. VP mRNA has a long half life (1.9 days). Translation of the signal peptide causes attachment to the rRER, and preprovasopressin and preprooxytocin are translated and translocated into the rER lumen where the signal is cleaved, S–S bonds and folding are established, and proximal glycosylation of the GP moiety of provasopressin occurs, to produce the prohormones. These then pass to the Golgi where terminal glycosylation of cleavage of the GP occurs, and the mature prohormone together with processing enzymes is packaged by the transGolgi into LDCV (the neurophysin moiety is essential for this). As the LDCV are transported into the axons and dendrites, the prohormone is cleaved and the C-terminal glycine of the hormone is amidated. The acidic pH in the LDCV is optimal for the cleavage enzymes and for binding the hormones to their neurophysins. VP neurons express prohormone convertases (PC)

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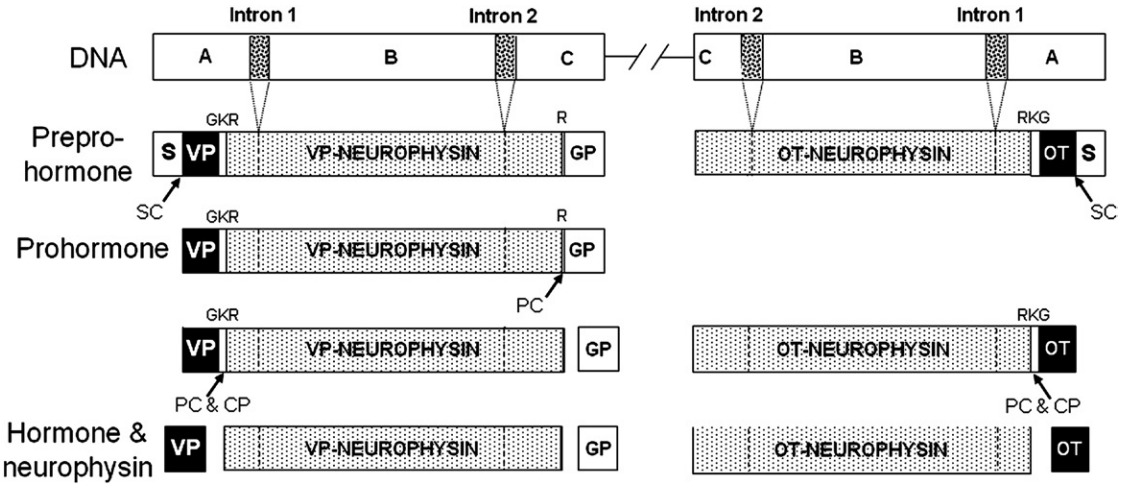

FIGURE 2 Processing of vasopressin (left) and oxytocin (right). The signal-cleaving peptidase (SC) removes the signal peptide from the preprohormone. Prohormone convertases first remove the CP in provasopressin, then cleave between hormone and neurophysin within the large dense-cored vesicles.

1 and 3, but PC5 predominates in OT neurons. The PCs cleave the bond between hormone and neurophysin, carboxypeptidase H/E then trims the remaining C-terminal to reveal glycine, which is amidated by peptidylglycine α-amidating monoxygenase (see Peptide Biosynthesis/Processing section of the book). Both transcription and processing are upregulated in activated neurons.

DISTRIBUTION OF VP AND OT PRODUCING CELLS; SITES OF RELEASE (FIGS. 3 AND 4)[4,5,10,22,25]

Magnocellular neurons:[10,17,24] The SON consists almost entirely of MCN; VP neurons are mostly situated ventrally/caudally, OT neurons dorsally/rostrally. In the PVN OT, neurons are situated more rostrally, VP neurons more caudally. Other scattered MCN are found in the preoptic area, lateral hypothalamic and perifornical areas, in the subcommissural nucleus and nucleus circularis. Most MCN axons project to the NL, but have sparse collateral branches in the hypothalamus; the dendrites of both SON and PVN MCN are located close to the CSF; those from the SON in a plexus on the ventral brain surface, those from the PVN beneath the wall of the third ventricle. Although MCN were believed to produce exclusively VP or OT, most MCN produce small amounts of the other peptides even in unstimulated animals; in lactation, 17% produce large amounts of both peptides. It is now clear that MCN, like many if not all peptide-secreting neurons, also produce and copackage small amounts of many other neuropeptides (including: apelin, CCK, CRH, enkephalin, dynorphin, galanin, nesfatin, PHI, salusin β, secretin, TRH [other chapters in this volume]). The copackaged peptides exert local paracrine and autocrine actions, some of which are known.

In addition to release from their axons, MCN releases OT and VP from their dendrites and somata by calcium-dependent exocytosis. The peptides then act on local and autoreceptors and diffuse via the extracellular fluid and CSF to distant receptors—"volume transmission."[24]

Parvocellular neuroendocrine neurons: In the PVN, parvocellular neurons (PCN) predominate in the medial/caudal part. In medial PVN, CRH neurons coexpress VP when stimulated by stress/adrenalectomy. These CRH/VP neurons project to the median eminence to control pituitary corticotrophs.[28,32]

Other small VP neurons are widely distributed. A population in the shell of the suprachiasmatic nucleus (SChN) rhythmically displays clock gene expression, which is reduced in aging; they project dorsally to the PVN, DMH, POA, and caudally to the retrochiasmatic area and VMH. Other VP neurons are located in the BNST and medial amygdala (involved in aggression/fear); expression is sexually dimorphic (more VP in males); these project to the lateral septum, lateral habenula, locus coeruleus and hypothalamus. In the POA, males have less VP than females do. VP in periglomerular neurons in the olfactory bulb has been linked to social recognition.[30]

Parvocellular OT neurons in the posterior PVN project to the brainstem and spinal cord where OT is involved in erection. Other OT neurons are located in the MBH, median POA, periventricular area, and BNST. In general, OT projections are more widespread than those for VP, being found in the hypothalamus, limbic system, nucleus accumbens, central gray, with large amounts in the olfactory system and insular cortex.

In addition to these CNS sources, VP mRNA has been detected in NL pituicytes, and OT mRNA and peptide in ovarian follicles, corpora lutea, endometrium, testicular interstitial cells, and other peripheral tissues involved in reproductive functions.

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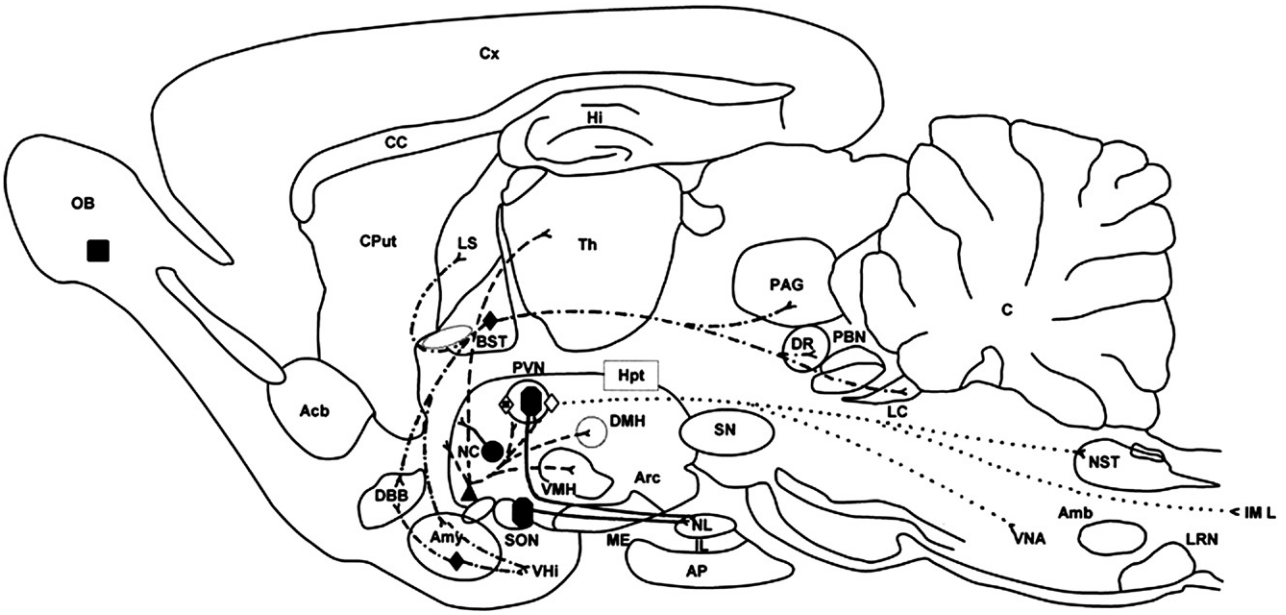

FIGURE 3 Distribution of VP neurons and fibers. Magnocellular neurons (⬮; —). Parvocellular neurons: (a) projecting to median eminence (⟐; fibers not shown); (b) circadian rhythm neurons (▲; - - -); (c) sex dimorphic neurons (♦; -•-•-); (d) autonomic group (◇; ••••); (e) periglomerular olfactory group (■; act largely locally). Abbreviations in Figures 3 and 4 not in the main text: A aminergic neurons in ventral medulla; C cingulated cortex; F frontal cortex; IML, intermediolateral column autonomic neurons; LS, lateral septum; NC, nucleus circularis; POA, preoptic area; PV, periventricular group; VH, ventral hippocampus.

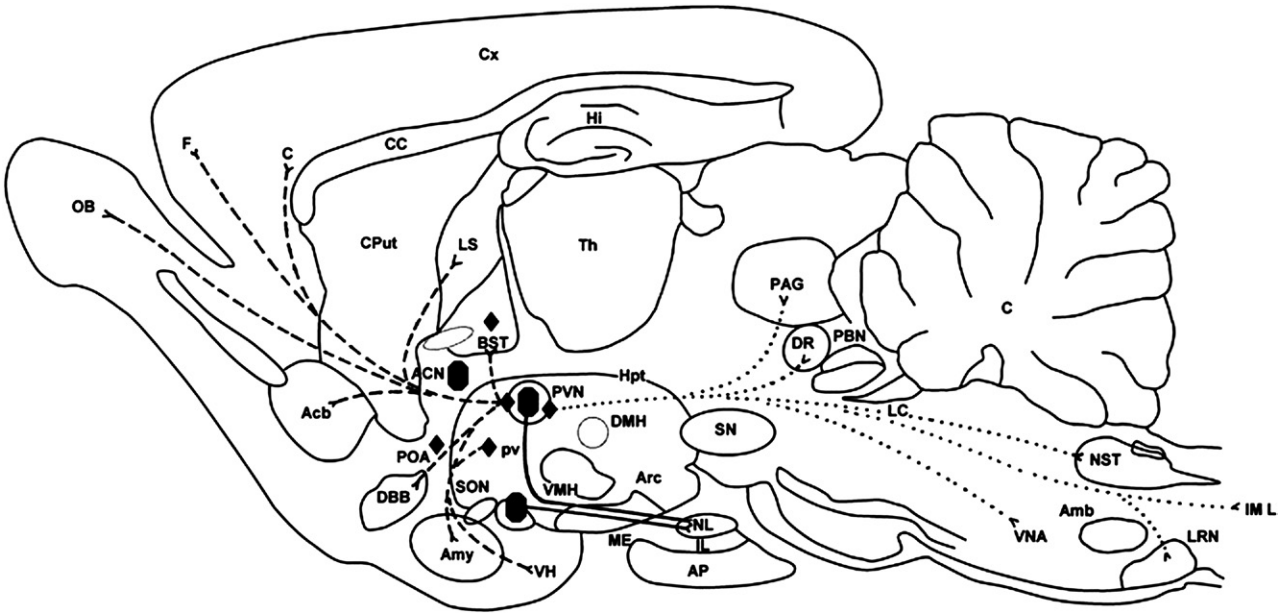

FIGURE 4 Distribution of OT neurons and fibers. Magnocellular neurons (⬮; —). Parvocellular neurons: projecting to forebrain (♦; - - - -); projecting to brainstem and cord (♦; ••••). For abbreviations, see Fig. 3.

RECEPTORS FOR VP AND OT; SIGNALING CASCADES (FIG. 5)[21]

Receptors for VP and OT are all members of the G protein–coupled 7-transmembrane family. As with the hormones, there is a high degree of homology of the receptors and their cis and trans control elements across species[8,11] and about one-quarter of the receptor amino acids are invariant across all VP and OT receptors. Three types of VP receptor (VR) have been identified (AVPR1a, AVPR1b (V3), and AVPR2), but only a single OT receptor (OXTR). The VP receptors are relatively specific for VP ($K_i \cong 1$ nM)

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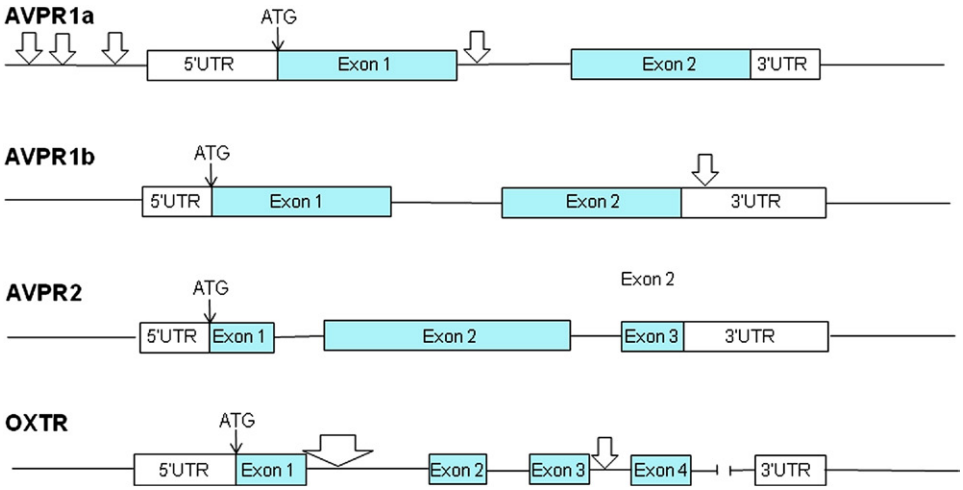

FIGURE 5 Diagrammatic structure (not to scale) of genes for the receptors for VP (AVPR1a, AVPR1b, AVPR2) and OT (OXTR). Block arrows mark the regions where microsatellites in AVPR1 and SNPs in the OXTR have been linked to autism in some studies. The block arrow in AVPR1b marks, a haplotype linked to depression (van West in Ref. 27). For the location of the numerous mutations in AVPR2 linked to nephrogenic DI, see Ref. 2.

compared with OT ($K_i > 64$ nM) whereas the OT receptor is relatively nonspecific (K_i 1 nM for OT, 16 nM for VP). The human AVPR1a is located on chromosome 12 (q14–15), AVPR1b on 1(q32), AVPR2 on the X(q28); they are, respectively, 418, 424, and 371 aa in length. The OXTR is on chromosome 3(p25.3). The genes are unusual among GPCRs in that there is an intron between transmembrane segments 6 and 7 (Fig. 5).[16]

The AVPR1a acts via $G_{q/11}$ to regulate various phospholipases (C, D, A2). It is expressed in liver, vascular smooth muscle, in most peripheral tissues that express VR, and is probably the most common VR in brain, including expression on VP MCN.[15] AVPR1b also acts via $G_{q/11}$ to stimulate PLCs; it was first identified in pituitary corticotrophs but is now known to occur in many brain regions including the VP MCNs, and in kidney, heart, lung, spleen, thymus, uterus, and breast[27] (Jard in Ref. 35). AVPR2 preferentially couples to $G\alpha_s$, stimulating adenylyl cyclase and the cAMP cascade. It is expressed only in the kidney.[2] The OXTR is also coupled via G_q to PLC-β. It is expressed in the myometrium, breast, and many other reproductive structures, including many parts of the brain with reproductive functions.[11,16] OXTR are abundant in parts of the olfactory system (anterior olfactory nucleus and tubercle (dependent on sex-steroids)), BNST, limbic system (ventral hippocampus, amygdala), neostriatum, and spinal cord (dorsal horn).[31]

There is some evidence that VPR expression can be regulated by cortisol, testosterone, and epigenetically, but OXTRs are strongly regulated by estradiol (E_2) (though they do not disappear entirely after gonadectomy) and adrenal steroids.[5] In nonexpressing tissues, OTR is constitutively repressed by hypermethylation.[7] Regulatory elements that control tissue-specific expression are present in the 5′ sequences but not yet fully elucidated.[16] On ligand binding the receptors are rapidly (5–10 min) internalized; AVPR1 but not OXTR or AVPR2 are recycled to the membrane. Homo and heterodimerization of the receptors occurs and control the amount of receptor on the cell surface. A possible direct progesterone effect on OXTR is controversial; the brain is known to produce progesterone derivatives but whether these affect OTR is not known.

BIOLOGICAL ACTIONS[21]

Systemic effects of VP and OT are well known, but evidence for actions of neurophysins and GP is lacking. Systemic VP is the main controller of body water and osmolality via AVPR2 and aquaporin 2 in the distal nephron; it is a more minor player in vascular regulation via AVPR1.[13] Pituitary portal vein VP acts in the adenohypophysis via AVPR1b to facilitate ACTH secretion. VP also acts in the liver, pancreas, adrenals, myometrium, kidney mesangium and medullary vasculature, endothelial cells, and platelets;[10] systemic OT stimulates milk ejection and myometrial contraction at term (when OXTR increases 200-fold); in fetal membranes, OT stimulates prostaglandin production near term. In the pituitary, OT influences the stress axis and, toward the end of gestation, stimulates prolactin release via E2-induced OXTR and is required for the development of the neurovascular interface.[14] In the ovary, OT stimulates follicular steroidogenesis and is luteotropic.[22] In the testis, Leydig cell–produced OT stimulates tubule movement; it also affects the prostate and epididymis where there is little evidence for OXTR. OT is produced and acts in the heart[13] and acts in the pancreas, thymus, adipocytes (where it has insulin-like actions), and adrenal.

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In the CNS, one would expect a good correlation between OT or VP receptors and axonal terminals containing the peptides, but this is often not the case. The peptides act both by axonal secretion at their synaptic targets and also by "volume transmission" after release by axons and dendrites.[23] In the SON and PVN VP inhibits VP neurons, but OT facilitates OT neuronal activity (separation of the respective MCN and bundling of OT dendrites at parturition/lactation apparently compensates for the non-selectivity of OXTR).

The distribution of OXTR and AVPR1a vary markedly.[1,11,27,31] VP and OT and their receptors are located in centers involved in osmotic and cardiovascular regulation (OVLT), median preoptic nucleus, area postrema, dorsal motor nucleus of the vagus. In the SChN and its targets, VP is involved in rhythm generation via AVPR1a and 1b. Both AVP and OT are released by noxious stimuli; in the limbic system, VP reinforces aversion-related learning and memory, whereas OT attenuates it by reducing NMDA receptor activity.

A major emphasis of research on the peptides and their receptors currently focuses on reproductive and related social behaviors such as recognition memory, pair bonding/grooming, care of offspring, maternal and male competitor aggression,[5,8,10,16,19,22,25,27,31] Although many key observations were made in animals (voles, rats, sheep) it is now becoming clear that similar effects occur in humans. Large amounts of OT are released in parturition and lactation and at orgasm. In both males and females, OT is secreted centrally in stress, activates stress-associated grooming, and has anxiolytic properties that reduce stress-activated behaviors, which might impair maternal care and pair bonding. In females, OT influences receptive (lordosis) and seems to be critical for the initiation and development of maternal behavior; in males OT causes erection.

PHYSIOLOGICAL AND PATHOLOGICAL IMPLICATIONS (SEE CHAPTERS IN CARDIOVASCULAR AND RENAL PEPTIDES SECTION OF THE BOOK)

Vasopressin. The obvious pathological conditions related to VP and its receptors are diabetes insipidus (DI) and the syndrome of inappropriate ADH secretion (SIADH) leading to hyponatremia. DI results from a lack of VP action in the kidney. Disruption of the hypothalamic–neurohypophysial system causes "central" (neurohypophyseal) DI. Causes include accidental stalk shearing, tumors, and mutations of the VP gene. In Brattleboro rats, a single base deletion in exon B causes a frameshift that prevents the mRNA being efficiently translated, and no VP is packed in LDCV. Human central DI differs and may be partial or complete. Mutations (>60 are reported) in the human VP gene are autosomal dominant or X-linked recessive; most affect the NP part of the molecule, to cause protein misfolding, dimerization, and aggregation in the rER; rare mutations involve the signal- and hormone-coding regions. Most result in progressive destruction of VP magnocellular neurons.[2]

Mutations in the AVPR2 gene (X-linked, semirecessive) or aquaporin 2 gene (autosomal dominant) cause nephrogenic (renal) DI. More than 200 mutations are known and affect all aspects of receptor function: the most common cause misfolding and retention in the rER; others cause truncation, misfolding that affects interaction with VP or with the $G\alpha_s$.[2] (In nephrogenic *di/di* mice, overactive phosphodiesterase prevents AVPR2 action, but this has not been reported in humans.)

In SIADH, plasma VP is inappropriately high for plasma osmolality. It can be caused by ectopic expression of the VP gene by carcinoma of the breast or lung, by a variety of CNS disorders with no obvious link, and by serotonin reuptake inhibitors, particularly in the elderly. Marked changes in the VP and OT neurons of the PVN and SON also occur in Alzheimer's disease.[17]

In many species, changes in central VP adjust behavior in stressful situations.[9] Overexpression can lead to hyperanxiety, whereas a deficit can reduce anxiety. Polymorphisms in the central receptors seem to reflect anxiety status.[10]

In mice, knockout of OT prevents milk ejection but not parturition. No deletions of the human OT gene have been reported. Studies across vertebrate species suggest that the distribution of central VP and OT receptors is strongly correlated with social behavior, and that deficits in bonding and social interaction which occur in many situations but particularly in autism, may reflect deficits in the function of these receptors. Variation in rat maternal care is heritable and appears to result from estrogen–OT interactions and epigenetic effects on hypothalamic estrogen receptors.[7] OXTR knockout mice do show maternal behavior deficits and autism-like behaviors.[26] Two species of voles, one promiscuous, one monogamous, show marked variation in the distribution of central OT and VP receptors. They show no difference in OT or VP neurons or in the essential structure of the receptors,[5,16] although increasing OT in the nucleus accumbens enhances partner bonding.[19] VPR1a receptor distribution may be controlled by polymorphisms in a complex 5′ DNA microsatellite.[8] Despite the lack of human data on the distribution of VP and OT receptors, there is now growing, if controversial evidence for effects of the peptides on human behavior, and for mutations and polymorphisms in their receptors in autism.[6,8,12,16,25,26,29,32] Variations in the human AVPR1a gene locus are associated with diverse social behavior including partner bonding[33] and the same locus is implicated in autism.[8] Intranasal OT can enhance "trust" between gaming competitors[20] and reduces social anxiety disorder.[16] Reduced plasma OT has been reported in autism, but this and attempts to link autism to specific genetic/epigenetic

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effects on the receptors remain controversial. That said, there is encouraging evidence that intranasal OT can ameliorate some symptoms of autism.[6,12,16,29,34]

Space considerations preclude consideration of VP and OT agonists and antagonists, but both peptide and nonpeptide varieties are widely used both in research and treatment.

REFERENCES

1. Auger CJ, Coss D, Auger AP, Forbes-Lorman RM. Epigenetic control of vasopressin expression is maintained by steroid hormones in the adult male rat brain. *Proc Natl Acad Sci USA* 2011;**108**:4242–7.
2. Babey M, Kopp P, Robertson GL. Familial forms of diabetes insipidus: clinical and molecular characteristics. *Nat Rev Endocrinol* 2011;**7**:701–14.
3. Brown C, Oiso Y, Ueta Y (eds). Vasopressin and oxytocin: focus in the post-genomic era. *J Neuroendocrinol* 2010;**22**:317–482.
4. Burbach JPH, Luckman SM, Murphy D, Gainer H. Gene regulation in the magnocellular hypothalamo-neurohypophysial system. *Phys Rev* 2001;**81**:1197–267.
5. Caldwell HK, Lee H-J, Macbeth AH, Young III WS. Vasopressin: behavioral roles of an "original" neuropeptide. *Prog Neurobiol* 2008;**84**:1–24.
6. Campbell DB, Datta D, Jones STJ, Lee EB, Sutcliffe JSM, Dammock EAD, et al. Association of oxytocin receptor *(OXTR)* gene variants with multiple phenotype domains of autism spectrum disorder. *J Neurodevelop Disord* 2011;**3**:101–12.
7. Champagne FA. Epigenetic mechanisms and the transgenerational effects of maternal care. *Front Neuroendocrinol* 2008;**29**:386–97.
8. Donaldson ZR, Young LJ. Oxytocin, vasopressin, and the neurogenetics of sociality. *Science* 2008;**322**:900–4.
9. Erhardt A, Müller MB, Rödel A, Welt T, Ohl F, Holshoer F, et al. Consequences of chronic social stress on behaviour and vasopressin gene expression in the PVN of DBA/2OlaHsd mice—influence of treatment with the CRHR1-antagonist R121919/NBI 30775. *J Psychopharm* 2009;**23**:31–9.
10. Frank E, Landgraf R. The vasopressin system—from antidiuresis to psychopathology. *Eur J Pharmacol* 2008;**583**:226–42.
11. Gimpl G, Fahrenholz F. The oxytocin receptor system: structure, function and regulation. *Phys Rev* 2001;**81**:629–84.
12. Gregory SG, Connelly JJ, Towers AJ, Johnson J, Biscocho D, Markunas CA, et al. Genomic and epigenetic evidence for oxytocin receptor deficiency in autism. *BMC Med* 2009;**7**:62–75.
13. Gutkowska J, Jankowski M. Vasopressin/oxytocin, this volume.
14. Gutnick A, Blechman J, Kaslin J, Herwig L, Belting H-G, Affolter M, et al. The hypothalamic neuropeptide oxytocin is required for formation of the neurovascular interface of the pituitary. *Dev Cell* 2011;**21**:642–54.
15. Hurbin A, Orcel H, Alonso G, Moos F, Rabié A. The vasopressin receptors colocalize with vasopressin in the magnocellular neurons of the rat supraoptic nucleus and are modulated by water balance. *Endocrinology* 2002;**143**:456–66.
16. Insel TR. The challenge of translation in social neuroscience: a review of oxytocin vasopressin and affiliative behavior. *Neuron* 2010;**65**:768–79.
17. Ishunina TA, Swaab DF. Neurohypophyseal peptides in aging and Alzheimer's disease. *Ageing Res Rev* 2002;**1**:537–58.
18. Kawata M, Leng G. Neurohypophysial hormones. *J Neuroendocrinol* 2004;**16**:293–408.
19. Keebaugh AC, Young LJ. Increasing oxytocin receptor expression in the nucleus accumbens of pre-pubertal female prairie voles enhances alloparental responsiveness and partner preference formation as adults. *Horm Behav* 2011;**60**:498–504.
20. Kosfeld M, Heinrichs M, Zak PJ, Fischbacher U, Fehr E. Oxytocin increases trust in humans. *Nature* 2005;**435**:673–6.
21. Laycock JF. *Perspectives on vasopressin*. Imperial College Press; 2010.
22. Lee H-J, Macbeth AH, Pagani JH, Young III WS. Oxytocin: the great facilitator of life. *Prog Neurobiol* 2009;**88**:127–51.
23. Leng G, Ludwig M. Information processing in the hypothalamus: peptides and analogue computation. *J Neuroendocrinol* 2006;**18**: 379–92.
24. Ludwig M, Leng G. Dendritic peptide release and peptide-dependent behaviours. *Nature Rev Neurosci* 2006;**7**:126–36.
25. MacDonald K, MacDonald TM. The peptide that binds: a systematic review of oxytocin and its prosocial effects in humans. *Harv Rev Psychiatry* 2010;**18**:1–21.
26. Pobbe RLH, Pearson BL, Defensor EB, Bolivar VJ, Young III WS, Lee H-J, et al. Oxytocin receptor knockout mice display deficits in the expression of autism-related behaviors. *Horm Behav* 2011. http://dx.doi.org/10.1016/j.yhbeh.2011.10.010 (on line).
27. Roper JA, O'Carroll A-M, Young III WS, Lolait SJ. The vasopressin Avpr1b receptor: molecular and pharmacological studies. *Stress* 2011;**14**:98–115.
28. Suzuki H, Kawasaki M, Ohnishi H, Nakamura T, Ueta Y. Regulatory mechanism of the arginine vasopressin-enhanced green fluorescent protein fusion gene expression in acute and chronic stress. *Peptides* 2009;**30**:1763–70.
29. Tansey KE, Brookes KJ, Hill MJ, Cochrane LE, Gill M, Skuse D, et al. Oxytocin receptor (OXTR) does not play a major role in the aetiology of autism: genetic and molecular studies. *Neurosci Lett* 2010;**474**:163–7.
30. Tobin VA, Hashimoto H, Wacker DW, Takayanagi Y, Langnaese K, Caquineau C, et al. An intrinsic vasopressin system in the olfactory bulb is involved in social recognition. *Nature* 2010;**464**:413–7.
31. Viero C, Shibuya I, Kitamura N, Verkhratsky A, Fujihara H, Katoh A, et al. Oxytocin: crossing the bridge between basic science and pharmacology. *CNS Neurosci Ther* 2010;**16**:e138–56.
32. Ueta Y, Dayanithi G, Fujihara H. Hypothalamic vasopressin response to stress and various physiological stimuli: visualization in transgenic animal models. *Horm Behav* 2011;**59**:221–6.
33. Walum H, Westberg L, Henningsson S, Neiderhiser JM, Reiss D, Iqi W, et al. Genetic variation in the vasopressin receptor 1a gene (AVPR1A) associates with pair-bonding behavior in humans. *Proc Natl Acad Sci U S A* 2008;**105**:14153–6.
34. Wu S, Jia M, Ruan Y, Liu J, Guo Y, Shuang M, et al. Positive association of the oxytocin receptor gene (OXTR) with autism in the Chinese Han population. *Biol Psychiatry* 2005;**58**:74–7.
35. Zingg HH, Bourque CW, Bichet DG. Vasopressin and oxytocin: molecular cellular, and clinical advances. *Adv Exp Biol Med* 1998; **449**:1–483.

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Chapter 130

Adiponectin

Frederik J. Steyn and Chen Chen

ABSTRACT

Adiponectin was isolated, and its molecular structure was identified nearly two decades ago. Although after initial observations it was hoped to characterize adiponectin as a key intermediate that signals from adiposity, recent developments clearly illustrate that the actions of adiponectin extend well beyond that of a simple indicator of adiposity. Significant progress has been made regarding the identification and characterization of adiponectin intermediates, including adiponectin receptors and binding proteins; more work is still needed to clarify the complex role of adiponectin under normal healthy and diseased conditions. This chapter provides a brief overview of the better-demonstrated aspects of adiponectin and established mechanisms underlying well-known functions of adiponectin.

DISCOVERY

Lodish and colleagues first described Adiponectin as a 30-kDA protein similar to complement factor C1q and consequently named it Acrp30 (adipocyte complement–related protein of 30 kDa).[25] Within months of this initial publication, a number of similar observations were reported.[10,18,21] These equally contributed to the identification of this adipokine, and as a result, adiponectin came to be known as Acrp30, AdipoQ, apM, or GBP28. Although the naming of adiponectin may have varied between these early discoveries, all initial observations found that the expression of adiponectin is highly specific to adipose tissue. As such, adiponectin was heralded as a key adipokine that may participate in energy homeostasis and in particular the regulation of adiposity.

THE ADIPONECTIN GENE AND PROTEIN STRUCTURE

The adiponectin gene is located on chromosome 3q27. This gene encodes a 244-amino acid protein composed of an amino-terminal signal peptide, a collagen-like domain at the N-terminus, and a globular domain at the C-terminus (Fig. 1). The globular domain is homologous to proteins such as collagens VIII and X and complement factor C1q[25] and facilitates binding to the adiponectin receptor. The N-terminal of adiponectin is structurally unique, and this collagen-like domain allows for the formation of three distinct multimer complexes of adiponectin. Hydroxylation and glycosylation of the lysine residues within the collagenous domain of adiponectin gives rise to trimers. Adiponectin trimers can associate into hexamers and high-molecular-weight (HMW) adiponectin[30] (Fig. 1). These multimers are the predominant form of circulating adiponectin and typical measures of plasma adiponectin range from 2 to 20 μg/ml[1]; however, some observations record adiponectin concentrations of up to 30 μg/ml.[7] In serum, a second version of adiponectin exists as a proteolytic fraction corresponding to the globular domain known as globular adiponectin (gAdiponectin).[8] It is believed that proteolytic cleavage of adiponectin multimers to give rise to gAdiponectin is modulated by leukocyte elastase secreted from activated monocytes and/or neutrophils.[29] Compared with full-length adiponectin multimers, gAdiponectin circulates in very small quantities (<1% total adiponectin). Although still under investigation, many observations confirm that the different multimeric forms of adiponectin may target tissues differentially.[13] For example, HMW adiponectin may be more active in the liver, whereas trimers and hexamers may act on a wider range of tissues. Tissue-specific interaction may occur in response to differential expression of adiponectin receptor subtypes, as well as local interactions with binding proteins and downstream modulators of adiponectin action.

PRODUCTION OF ADIPONECTIN

The production and release of adiponectin into circulation are not well understood. Although predominantly isolated from adipose tissue,[10,18,25] observations confirm the expression of adiponectin in many tissues including the pituitary gland, liver, diencephalon, skeletal muscle, ovary, spleen, and the kidney.[17] In human tissue, adiponectin is further expressed in bone marrow cells, osteoblasts, fetal cells, myocytes, cardiomyocytes, and epithelial cells of the salivary glands.[22] The functional significance of adiponectin expression outside of adipose tissue still remains unknown, whereas isolated

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00130-5
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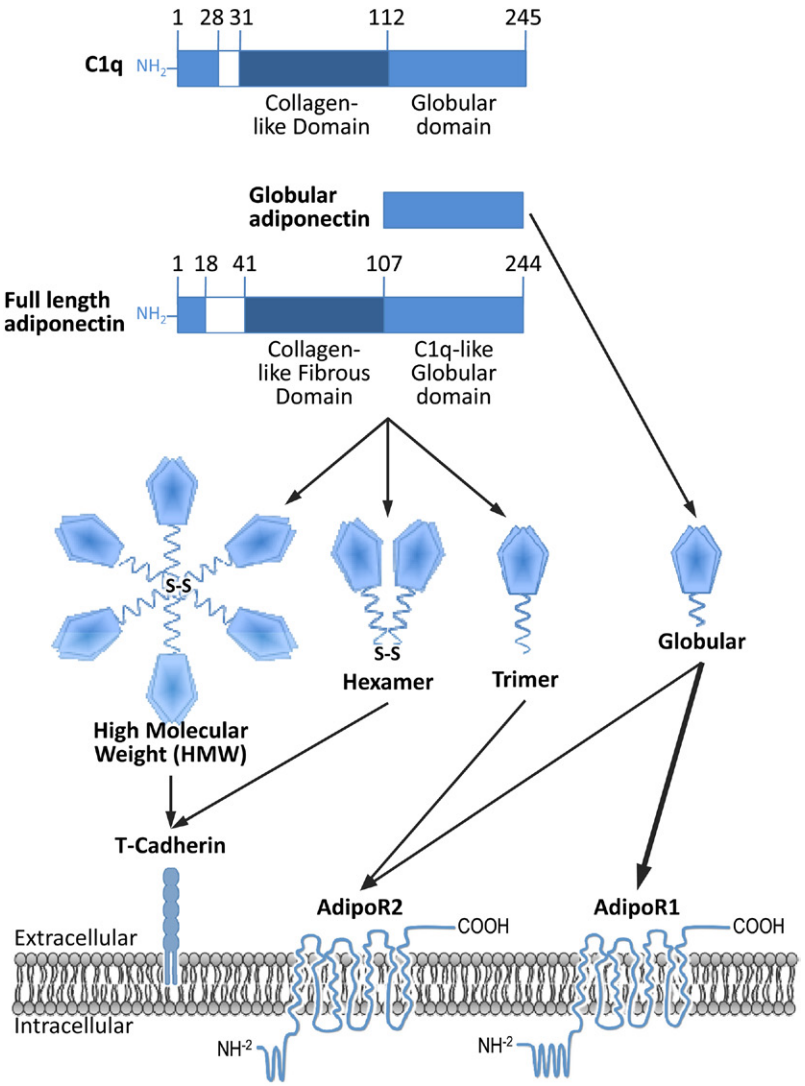

FIGURE 1 Adiponectin is a 244-amino acid protein homologous to complement factor C1q. In the circulation, adiponectin exists in globular form and as trimers, hexamers, and high-molecular-weight (HMW) adiponectin. The actions of adiponectin are mediated via binding to one of three receptors, Adiponectin receptor subtype 1 (AdipoR1), adiponectin receptor subtype 2 (AdipoR2), and T-cadherin. Globular and trimetric adiponectin interacts with AdipoR1 and AdipoR2, whereas HMW and adiponectin hexamers are believed to predominantly interact with T-cadherin.

expression in distinct cell populations suggests a possible autocrine/paracrine role.

Circulating concentrations of adiponectin may vary significantly between individuals. This reflects a large number of potential factors that may influence adiponectin production, release, and metabolism. Adiponectin concentrations in females tend to be elevated compared with that of males. Similarly, adiponectin concentrations may vary depending on nutritional and disease states.[6] Generally, unlike other adipocyte-derived hormones such as leptin, the amount of adiponectin in circulation is inversely correlated with adiposity and conditions associated with insulin resistance including cardiovascular diseases, hypertension, and metabolic syndrome.[13]

ADIPONECTIN RECEPTORS

Adiponectin signals through three distinct receptors. Of these, adiponectin receptor 1 (AdipoR1) and adiponectin receptor 2 (AdipoR2) are seven-transmembrane domain receptors. These receptors are structurally and functionally distinct from G protein-coupled receptors (GPCRs) in that the locations of their external carboxy terminus and intracellular amino terminus are reversed.[32] AdipoR1 and R2 belong to the progestin and AdipoQ receptor (PAQR) family of receptors.[28] Currently, the PAQR family consists of 11 proteins grouped into three classes based on sequence similarities. Class I is widely expressed in eukaryotes and includes AdipoR1 (PAQR1) and AdipoR2 (PAQR2). Class

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II proteins include nonclassical receptors for progestin including mPRα (PARQ7), while members for Class III receptors (PAQR10 &11) are currently orphan receptors.[28]

Structurally, AdipoR1 and AdipoR2 share 67% protein sequence homology. The N-terminal amino acid sequence for AdipoR1 and AdipoR2 is specific for each receptor, meaning that the remainder of these proteins are highly homologous (80% homology).[13] Structural homology between species (humans and mice) is highly conserved (95% for AdipoR1 and 97% for AdipoR2). In addition to AdipoR1 and AdipoR2, adiponectin may also signal via T-cadherin.[11] This putative adiponectin receptor is a unique cadherin molecule that lacks transmembrane and cytoplasmic domains and is bound to the cell surface via a glycosylphosphatidylinositol anchor.[28] Although T-cadherin may lack an intracellular domain, and thus may not directly induce intracellular signaling, it may still mediate intracellular signaling by competing with AdipoR1 and R2 receptors. Consequently, some debate exists to suggest that T-cadherin may not necessarily exist as a functional adiponectin receptor but rather that T-cadherin acts as a functional adiponectin binding protein.[3]

Binding affinity for adiponectin trimers and gAdiponectin vary significantly between adiponectin receptors. AdipoR1 has a high binding affinity for gAdiponectin, whereas AdipoR2 will bind full-length and gAdiponectin without significant preference.[32] T-cadherin may bind hexameric or HMW forms of adiponectin with very low or no affinity for globular or trimeric forms of adiponectin (Fig. 1).

AdipoR1 and AdipoR2 are universally expressed; however, abundance of expression varies significantly among tissues. AdipoR1 is expressed predominantly in skeletal muscle, whereas AdipoR2 is expressed within skeletal muscle and liver. As with AdipoR1 and AdipoR2, T-cadherin is ubiquitously expressed; however, an abundance of expression is observed in the cardiovascular system with high levels of expression predominating in the heart, aortic, carotid, and iliac arteries and the arteries of the kidney.[14] Distribution of AdipoR1 and R2 expression within various tissues is summarized in Table 1.

ADIPONECTIN SIGNALING

Details regarding the activation of specific signaling cascades by adiponectin are still emerging. Knockout studies reveal that although both AdipoR1 and AdipoR2 are involved in energy metabolism, opposing effects are observed after deletion of either receptor. Deletion of AdipoR1 results in an obese phenotype associated with glucose intolerance and decreased energy expenditure. By contrast, deletion of AdipoR2 results in a lean phenotype associated with resistance to dietary induced obesity and increased energy expenditure.[2] Given the largely universal expression of adiponectin receptors, differential functions may therefore arise from distinct activation of intracellular signaling pathways. Indeed, observations suggest that these receptors act via distinct signaling cascades wherein AdipoR1 signals predominantly via AMP-kinase (AMPK) phosphorylation, whereas AdipoR2 actions are predominantly mediated via PPARα activation.[4]

APPL1, an Adaptor protein containing a Pleckstrin homology domain, a Phosphotyrosine domain, and a Leucine zipper motif, was recently identified as an adiponectin receptor binding protein. Current observations principally focus on potential binding interactions between AdipoR1 and APPL1. The N-terminal amino acids (4–142) of AdipoR1 interact with APPL1 to form an AdipoR1/APPL1 complex.[19] Formation of this complex is enhanced after adiponectin treatment, whereas APPL1 is abundantly expressed in mouse skeletal muscle, c2c12 myotubes and to some degree in hepatocytes.[19] It is therefore probable that the action of adiponectin in modulating glucose sensing within the muscle may occur via this adaptor/binding protein. Interestingly, APPL2 (an isoform of APPL1 that shares 54% protein homology) binds to adiponectin receptors.[20] Unlike APPL1, however, APPL2 seems to inhibit AdipoR1 dependent signaling and may form heterodimers with APPL1. Consequently, APPL2 may inhibit or downregulate AdipoR1 mediated APPL1 signaling by competing with APPL1 binding to the AdipoR and by preventing APPL1 binding through the formation of APPL1/APPL2 complexes. Interactions between adiponectin receptors and binding proteins contribute significantly toward adiponectin signaling and future clarification of these interactions will provide significant insights regarding adiponectin actions.

ADIPONECTIN AND ENERGY HOMEOSTASIS

Adipose tissue, liver, and muscle act in concert to regulate energy homeostasis. Given the predominant expression of adiponectin in adipose tissue and the abundant expression of adiponectin receptors in liver and muscle tissue, it is not surprising that the actions of adiponectin within these tissues, and regarding energy homeostasis are well studied. Here, adiponectin may serve as a key adipokine that modulated muscle and liver metabolism relative to adiposity.

Within the liver, adiponectin modulates insulin sensitivity and fatty acid flux by decreasing gluconeogenesis and free fatty acid flux into the liver, and by increasing fatty acid oxidation. In addition to these metabolic effects, adiponectin has antifibrotic actions in the liver and downregulates the expression of aldehyde oxidase, transforming growth factor and connective tissue growth factor. Furthermore, adiponectin has anti-inflammatory actions within the liver and suppresses proinflammatory cytokines

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TABLE 1 Distribution of Adiponectin Receptors (AdipoR1, AdipoR2 and T-Cadherin) in Human, Rodent and Pig Tissue. Adiponectin R1 is Predominantly Expressed in Skeletal Muscle while AdipoR2 is Abundantly Expressed in Liver Samples. T-cadherin Expression is Absent from Liver Samples while Some or No Expression is Apparent within the Spinal Cord

		AdipoR1	AdipoR2	T-Cadherin
Integumentary system	Skin	✓*	✓*	✓
	Subcutaneous adipose	✓*	✓*	✓
	Visceral adipose	✓	✓	✓
Muscular system	Skeletal muscle	✓ ✓*	✓*	✓*
Nervous system	Brain	✓*	✓*	✓*
	Cerebral cortex			✓*
	Medulla			✓*
	Thalamus			✓*
	Midbrain			✓*
	Hypothalamus			✓*
	Spinal cord			✓* (low)
Circulatory system	Heart	✓*	✓*	✓*
	Blood vessels	✓*	✓*	✓*
Endocrine system	Adrenal gland	✓*	✓*	
	Pituitary gland	✓	✓	
	Pancreatic β-cells	✓	✓	
	White adipose tissue	✓	✓	
	Brown adipose tissue	✓	✓	
Digestive system	Stomach	✓*	✓*	
	Small intestine	✓*	✓*	
	Large intestine/Colon	✓*	✓*	
	Liver	✓*	✓ ✓*	✗
	Pancreas	✓*	✓*	
Immune system	Thymus	✓*	✓*	
	Lymph node	✓*	✓*	
	Bone marrow	✓*	✓*	
Respiratory system	Lungs	✓*	✓*	
Urinary system	Bladder	✓*	✓*	
	Kidney	✓*	✓*	
Reproductive system	Testes	✓*	✓*	
	Prostate	✓*	✓*	
	Breast	✓*	✓*	

Signifies confirmation of expression in human tissue.
Data collated from [3,12,13,15,26,27].

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while stimulating anti-inflammatory cytokines (Reviewed in Ref. 3). Given these metabolic, antifibrotic and anti-inflammatory actions of adiponectin, a reduction in circulating concentrations of adiponectin may contribute to the development of nonalcoholic fatty liver (NAL) and nonalcoholic steatohepatitis (NAS). Although some contention remains, current opinion is that a reduction in circulating levels of adiponectin is associated with the development of nonalcoholic fatty liver disease (NAFLD). One should be careful when interpreting these observations, however, as a meta-analysis of clinical observations reveals that reduced adiponectin may contribute to the progression of NAL to NAS; however, whether an association with reduced circulating adiponectin and the development of NAFLD still remains unclear.[23] Interestingly, adiponectin prevents the development of NAFLD in ob/ob mice.[31] This effect is partly explained by improved hepatic fatty acid oxidation and a reduction in fatty acid synthesis. Therefore, although it remains unclear whether hypoadiponectinemia may contribute to the development of NAFLD, effective treatment strategies to recover liver function may include treatment with adiponectin.

As with the liver, adiponectin may target muscle tissue to regulate energy metabolism. Acting through AMPK, p38 MAPK, and PPARα, adiponectin improves lipid metabolism in muscle resulting in increased glucose use and fatty acid oxidation. In this way, adiponectin may directly contribute to glucose metabolism and improved insulin sensitivity.[3] Although many observations still focus on the potential effects of circulating adiponectin, other observations, including the work of Liu et al. clearly illustrate the functional significance of locally produced adiponectin in improving glucose uptake by muscle.[16] Liu et al. found that an increase in adiponectin message and protein within L6 muscle cells after treatment with rosiglitazone (an antidiabetic drug) is associated with an increase in insulin-induced glucose uptake.[16] Similarly, treatment with rosiglitazone in dietary induced obese mice not only corrects the expression for muscle adiponectin message and protein (HMW) but this also improves whole-body insulin sensitivity. These observations illustrate the metabolic potential of locally produced adiponectin.

The expression of adiponectin receptors within the skeletal muscle is inversely associated with insulin. Consequently, an alteration in insulin levels associated with fasting/feeding or hypoinsulinemia or hyperinsulinemia will greatly influence adiponectin sensitivity. For example, undernourishment during early development results in enhanced insulin sensitivity and fatty acid oxidation. This is associated with an increase in circulating adiponectin and the expression of AdipoR1 in skeletal muscle.[24] Although Prior and colleagues hypothesized that this increase in adiponectin and AdipoR expression in muscle may play a protective role to ensure balanced energy homeostasis after early and prolonged calorie restriction, further clarification of the role in adiponectin in improving insulin sensitivity within muscle is warranted.

ADIPONECTIN AND OBESITY

The paradoxical decrease in circulating levels of adiponectin with increased adiposity was first observed by Arita et al. in 1999.[1] They found that adiponectin is abundantly present in the plasma of nonobese subjects (ranging from 1.9 to 17.0 mg/ml). However, circulating concentrations of adiponectin decrease significantly (to <4 mg/ml on average) in obese subjects. Moreover, Arita et al. reported a negative correlation between plasma adiponectin levels and BMI in both men and women.[1] Follow-up studies reconfirmed these initial observations, and extended measures to suggest that adiponectin concentrations are determined by intraabdominal fat mass, and that additional independent effects depend on age and sex.[5] The reduction in adiponectin associated with obesity recovers after weight loss, and although this may suggest that excess adiposity directly contributes to the suppression of adiponectin, specific factors that directly contribute to decreased adiponectin in obesity remain unclear. Proposed mechanisms include a reduction in insulin-stimulated adiponectin secretion (as a consequence of insulin insensitivity), and direct suppression by TNF-α and IL-6.

It remains unclear whether suppressed levels of adiponectin are a cause or consequence of obesity. Furthermore, the negative consequences regarding altered adiponectin secretion in obesity remain unclear. Evidence implicating adiponectin in insulin-sensitizing and adiponectin's anti-inflammatory and antiatherogenic properties suggest that reduced adiponectin levels after weight gain may contribute to the development of many pathologies associated with the metabolic syndrome. Indeed, elevated concentrations of circulating adiponectin are inversely correlated with insulin resistance, the development of type-2-diabetes, dyslipidemia, and cardiovascular disease. Measures of circulating adiponectin, and in particular HMW adiponectin, may provide a suitable alternative to assess patients with metabolic syndrome-related disorders.[9]

CONCLUSIONS

Since its discovery nearly two decades ago, significant progress has been made regarding the characterization and physiological role of adiponectin. Continued efforts to elucidate signaling mechanisms in many tissues, including the liver, muscle, and adipose tissue will further clarify the physiological role of this adipokine. Although current observations suggest that altered adiponectin secretion is a

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key factor that contributes to the onset of pathologies associated with excessive weight gain and obesity (including the development of insulin insensitivity), significant work is still needed to determine whether altered secretion of adiponectin is simply a by-product of obesity, or whether this directly contributes to obesity-associated pathologies. Given the abundant expression of adiponectin receptors throughout the body, additional observations investigating the impact of metabolic disorders on adiponectin secretion and actions may identify additional roles for adiponectin in contributing to normal physiology and the impact of altered adiponectin secretion on developing pathology. Consequently, the physiological role adiponectin in healthy physiology will become better defined. These observations are necessary if we are to understand the physiological functions of adiponectin as an antiatherogenic, anti-inflammatory, and antidiabetic adipokine, and the role of adiponectin in contributing to the development or treatment of cancer and NAFLD.

REFERENCES

1. Arita Y, Kihara S, Ouchi N, Takahashi M, Maeda K, Miyagawa J, et al. Paradoxical decrease of an adipose-specific protein, adiponectin, in obesity. *Biochem Biophys Res Commun* 1999;**257**:79–83.
2. Bjursell M, Ahnmark A, Bohlooly YM, William-Olsson L, Rhedin M, Peng XR, et al. Opposing effects of adiponectin receptors 1 and 2 on energy metabolism. *Diabetes* 2007;**56**:583–93.
3. Brochu-Gaudreau K, Rehfeldt C, Blouin R, Bordignon V, Murphy BD, Palin MF. Adiponectin action from head to toe. *Endocrine* 2010;**37**:11–32.
4. Buechler C, Wanninger J, Neumeier M, Adiponectin receptor binding proteins–recent advances in elucidating adiponectin signalling pathways. *FEBS Lett* 2010;**584**:4280–86.
5. Cnop M, Havel PJ, Utzschneider KM, Carr DB, Sinha MK, Boyko EJ, et al. Relationship of adiponectin to body fat distribution, insulin sensitivity and plasma lipoproteins: evidence for independent roles of age and sex. *Diabetologia* 2003;**46**:459–69.
6. Combs TP, Berg AH, Rajala MW, Klebanov S, Iyengar P, Jimenez-Chillaron JC, et al. Sexual differentiation, pregnancy, calorie restriction, and aging affect the adipocyte-specific secretory protein adiponectin. *Diabetes* 2003;**52**:268–76.
7. Corbetta S, Bulfamante G, Cortelazzi D, Barresi V, Cetin I, Mantovani G, et al. Adiponectin expression in human fetal tissues during mid- and late gestation. *J Clin Endocrinol Metab* 2005;**90**:2397–402.
8. Fruebis J, Tsao TS, Javorschi S, Ebbets-Reed D, Erickson MR, Yen FT, et al. Proteolytic cleavage product of 30-kDa adipocyte complement-related protein increases fatty acid oxidation in muscle and causes weight loss in mice. *Proc Natl Acad Sci U S A* 2001;**98**:2005–10.
9. Hirose H, Yammato Y, Seino-Yoshihara Y, Kawabe H, Saito I, Serum high-molecular-weight adiponectin as a marker for the evaluation and care of subjects with metabolic syndrome and related disorders.. *J Atheroscler Thromb* 2010;**17**:1201–11.
10. Hu E, Liang P, Spiegelman BM. AdipoQ is a novel adipose-specific gene dysregulated in obesity. *J Biol Chem* 1996;**271**:10697–703.
11. Hug C, Wang J, Ahmad NS, Bogan JS, Tsao TS, Lodish HF. T-cadherin is a receptor for hexameric and high-molecular-weight forms of Acrp30/adiponectin. *Proc Natl Acad Sci U S A* 2004;**101**:10308–13.
12. Ivanov DB, Philippova MP, Tkachuk VA. Structure and functions of classical cadherins. *Biochemistry (Mosc)* 2001;**66**:1174–86.
13. Kadowaki T, Yamauchi T. Adiponectin and adiponectin receptors. *Endocr Rev* 2005;**26**:439–51.
14. Kudriashova E, Bashtrykov PP, Ivanov DB, Antropova Iu G, Il'inskaia OP, Tararak EM, et al. Expression of T-cadherin in the rat carotid artery wall after balloon injury and in different rat organs. *Tsitologiia* 2002;**44**:285–95.
15. Liu BH, Wang PH, Wang YC, Cheng WM, Mersmann HJ, Ding ST. Fasting regulates the expression of adiponectin receptors in young growing pigs. *J Anim Sci* 2008;**86**:3377–84.
16. Liu Y, Chewchuk S, Lavigne C, Brule S, Pilon G, Houde V, et al. Functional significance of skeletal muscle adiponectin production, changes in animal models of obesity and diabetes, and regulation by rosiglitazone treatment. *Am J Physiol Endocrinol Metab* 2009;**297**:E657–64.
17. Maddineni S, Metzger S, Ocon O, Hendricks 3rd G, Ramachandran R. Adiponectin gene is expressed in multiple tissues in the chicken: food deprivation influences adiponectin messenger ribonucleic acid expression. *Endocrinology* 2005;**146**:4250–6.
18. Maeda K, Okubo K, Shimomura I, Funahashi T, Matsuzawa Y, Matsubara K. cDNA cloning and expression of a novel adipose specific collagen-like factor, apM1 (adiPose most abundant gene transcript 1). *Biochem Biophys Res Commun* 1996;**221**:286–9.
19. Mao X, Kikani CK, Riojas RA, Langlais P, Wang L, Ramos FJ, et al. APPL1 binds to adiponectin receptors and mediates adiponectin signalling and function. *Nat Cell Biol* 2006;**8**:516–23.
20. Miaczynska M, Christoforidis S, Giner A, Shevchenko A, Uttenweiler-Joseph S, Habermann B, et al. APPL proteins link Rab5 to nuclear signal transduction via an endosomal compartment. *Cell* 2004;**116**:445–56.
21. Nakano Y, Tobe T, Choi-Miura NH, Mazda T, Tomita M. Isolation and characterization of GBP28, a novel gelatin-binding protein purified from human plasma. *J Biochem* 1996;**120**:803–12.
22. Nishida M, Funahashi T, Shimomura I. Pathophysiological significance of adiponectin. *Med Mol Morphol* 2007;**40**:55–67.
23. Polyzos SA, Toulis KA, Goulis DG, Zavos C, Kountouras J. Serum total adiponectin in nonalcoholic fatty liver disease: a systematic review and meta-analysis. *Metabolism* 2011;**60**:313–26.
24. Prior LJ, Velkoska E, Watts R, Cameron-Smith D, Morris MJ. Undernutrition during suckling in rats elevates plasma adiponectin and its receptor in skeletal muscle regardless of diet composition: a protective effect? *Int J Obes (Lond)* 2008;**32**:1585–94.
25. Scherer PE, Williams S, Fogliano M, Baldini G, Lodish HF. A novel serum protein similar to C1q, produced exclusively in adipocytes. *J Biol Chem* 1995;**270**:26746–9.
26. Steyn FJ, Boehme F, Vargas E, Wang K, Parkington HC, Rao JR, et al. Adiponectin regulate growth hormone secretion via adiponectin receptor mediated Ca(2+) signalling in rat somatotrophs in vitro. *J Neuroendocrinol* 2009;**21**:698–704.
27. Takeuchi T, Misaki A, Liang SB, Tachibana A, Hayashi N, Sonobe H, et al. Expression of T-cadherin (CDH13, H-Cadherin) in human brain and its characteristics as a negative growth regulator of epidermal growth factor in neuroblastoma cells. *J Neurochem* 2000;**74**:1489–97.
28. Tang YT, Hu T, Arterburn M, Boyle B, Bright JM, Emtage PC, et al. PAQR proteins: a novel membrane receptor family defined by an ancient 7-transmembrane pass motif. *J Mol Evol* 2005;**61**:372–80.

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29. Waki H, Yamauchi T, Kamon J, Kita S, Ito Y, Hada Y, et al. Generation of globular fragment of adiponectin by leukocyte elastase secreted by monocytic cell line THP-1. *Endocrinology* 2005;**146**:790–6.
30. Wang Y, Lam KS, Chan L, Chan KW, Lam JB, Lam MC, et al. Post-translational modifications of the four conserved lysine residues within the collagenous domain of adiponectin are required for the formation of its high molecular weight oligomeric complex. *J Biol Chem* 2006;**281**:16391–400.
31. Xu A, Wang Y, Keshaw H, Xu LY, Lam KS, Cooper GJ. The fat-derived hormone adiponectin alleviates alcoholic and nonalcoholic fatty liver diseases in mice. *J Clin Invest* 2003;**112**:91–100.
32. Yamauchi T, Kamon J, Ito Y, Tsuchida A, Yokomizo T, Kita S, et al. Cloning of adiponectin receptors that mediate antidiabetic metabolic effects. *Nature* 2003;**423**:762–9.

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Chapter 131

Adrenomedullin Peptides

Josune García-Sanmartín, Ignacio M. Larráyoz, Laura Ochoa-Callejero and Alfredo Martínez

ABSTRACT

Adrenomedullin (AM) is a 52-amino acid regulatory peptide that is expressed in many organs of the body, including the endocrine glands. Pituitary, adrenals, pancreas, gonads, and some other endocrine organs produce AM and its related peptides (PAMP and AM-2) and have the receptors and/or binding sites for these molecules, suggesting the existence of autocrine and paracrine loops within these organs. This family of peptides influences the secretion of several hormones such as ACTH, aldosterone, corticosterone, catecholamines, insulin, progesterone, and testosterone. The gland architecture is also relevant in understanding local regulation: where is the peptide expressed, what is the direction of blood flow inside the gland, and where are the receptors and binding proteins located with respect to the peptide secretion site. New tools are being developed that may be very helpful in the near future to better understand the local regulation of endocrine physiology through AM and related peptides.

INTRODUCTION

Most peptide hormones are secreted by particular cells and travel through the organism till they find their specific membrane receptors to elicit physiological actions. Depending on how far the receptors are from the secretory cell, classically, we talk about endocrine, paracrine, or autocrine modes of action. Adrenomedullin (AM) and proadrenomedullin N-terminal 20-peptide (PAMP) are not an exception to this rule, and numerous examples can be found where these peptides act at different distances from their point of origin.[12] Nevertheless, at some point in the history of AM, several authors have expressed their doubts on the true endocrine nature of AM. These opinions were based on the extremely low levels of the peptide that were found in the blood stream (between 2 and 20 pmol/liter in humans), which were not enough to activate the AM receptors (discussed elsewhere in this book) with dissociation constants of about 2–3 nM. It was the discovery of a tremendously abundant binding protein in the plasma that recovered the status of AM as a potential endocrine factor. This binding protein, identified as complement factor H,[25] circulates in the blood at extremely high concentrations (~500 μg/ml), has at least 2 different binding sites for AM, and prevents bound AM from being quantified in radioimmunoassays. Therefore, all the studies in which AM levels have been quantified in the past represent gross underestimations of the actual AM values. Unfortunately, a reliable method for quantifying total AM is not yet available. Moreover, the most physiologically relevant pool of AM could be the one that is bound to factor H rather than the free peptide, because the binding protein protects the peptide from protease degradation but does not interfere with the binding of the peptide to its receptor.[25]

But, unlike most peptides, AM and PAMP also have intracellular properties. These peptide hormones bind to the cytoskeleton (Fig. 1), either to the tubulin dimer itself (PAMP) or to microtubule-associated proteins (AM). In the case of PAMP, the binding to the microtubules results in a delayed polymerization of tubulin, thus producing a more plastic cytoskeleton. When the gene that codes for both AM and PAMP is downregulated through siRNA or gene targeting, the cytoskeleton acquires a hyperpolymerized state, and the cells show defects on cell cycle regulation and motility capabilities.[27] Interestingly, this association with the cytoskeleton seems to occur predominantly in nonendocrine cells, such as neurons or epithelial cells. By contrast, in classical endocrine cells, AM and PAMP are located in the secretory granules.[21] Hopefully, after the recent publication of the three-dimensional structures of PAMP and AM,[24] it will be possible to investigate the interactions of the peptides with the components of the cytoskeleton.

Another protein, named AM-2 or intermedin, displays a high homology to AM but is coded by a different gene. Although most of its actions seem to be homologous to those of AM, there are areas where differences have been described.[32]

Regardless, all authors agree on the great potential of AM-related peptides in local regulation. In this chapter, the physiological regulation of the main endocrine organs by these peptides is reviewed.

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00131-7
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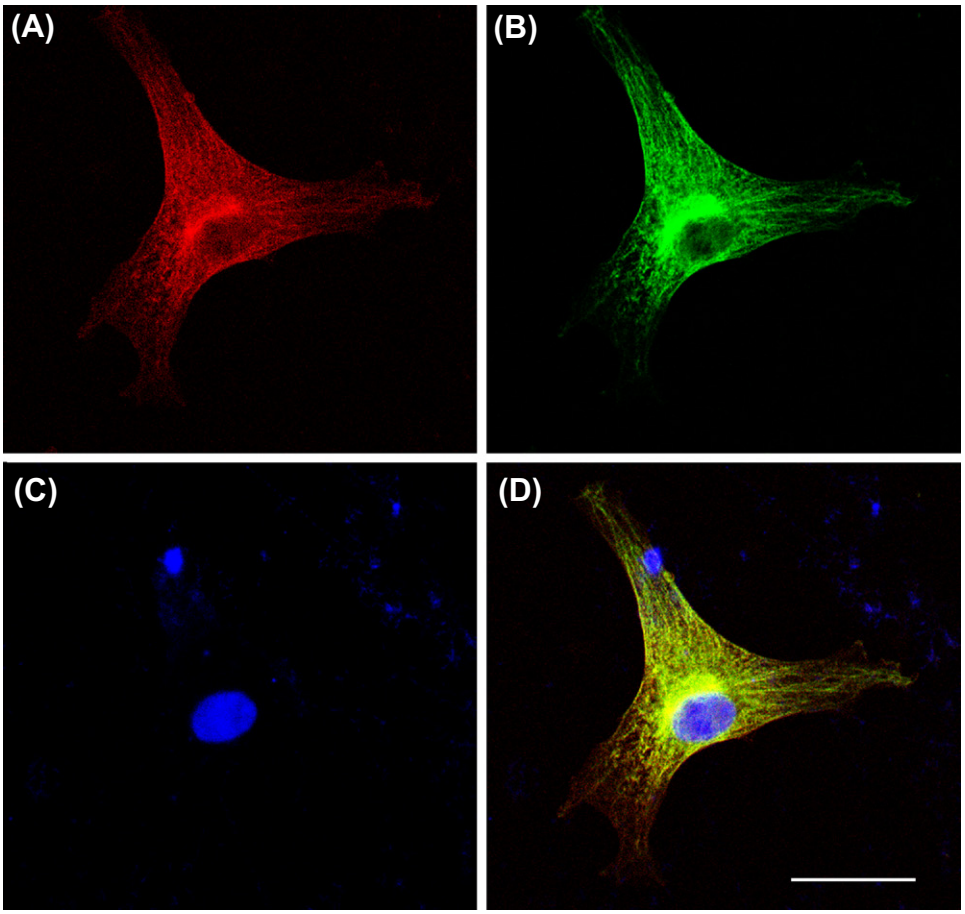

FIGURE 1 Confocal images of Cos7 cells in culture stained for AM (A), tubulin (B), DAPI (C), and the combination of the 3 colors (D). Note that AM decorates all the microtubules of the cell. Bar = 20 μm. See color plate 37.

PITUITARY

AM, PAMP, and AM-2 are expressed in all components of the hypothalamic–pituitary–adrenal (HPA) axis.[32] Immunohistochemical studies have shown a diffuse staining in all cells of the anterior pituitary for both AM and AM-2, whereas PAMP was restricted to the FSH-expressing gonadotrophs. This lack of overlapping colocalization between AM and PAMP, two peptides produced by the same prohormone, has been observed in several locations and may be explained by an alternative splicing mechanism. Immunohistochemical results were confirmed by RIA quantification and gene expression studies, which showed higher levels of AM in the pituitary than in the brain.

Several studies have looked into the functional roles of AM-related peptides in pituitary physiology. The addition of AM to dispersed cells of the anterior pituitary results in a dose-dependent decrease in the secretion of ACTH and an increase on prolactin and GH.[9,29] AM-2 presents a different pattern than AM on the regulation of pituitary hormone secretion. On the one hand, AM-2 increases prolactin secretion as does AM but, on the other hand, it increases ACTH and decreases GH in clear contrast with AM.[32] For the effects of PAMP, we find two studies with opposite results; the first one found that PAMP reduced ACTH secretion,[29] whereas the second report found an increase of this hormone after injection of PAMP in the rat.[14] Perhaps this discrepancy may be explained due to the different approaches of both teams; the first group was working on dispersed pituitary cells in culture, whereas the second was using the complete animal, so all the regulatory elements, external to the pituitary, were in place.

No formal studies on AM receptors in the pituitary are available, although binding sites have been reported. Interestingly, however, female mice lacking one allele for the AM receptor component RAMP2 exhibit enlarged pituitaries and hyperprolactinemia,[8] suggesting important roles for this receptor in pituitary physiology.

The HPA axis is involved in the regulation of the stress response, and AM-related peptides seem to be part of this system, as well. Several situations of experimental and clinical stress induce an increase of the expression of these peptides and, conversely, the deletion of the AM gene resulted in an excessive stress response,[4] suggesting that AM is part of a defensive mechanism against stress.

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ADRENALS

AM was initially isolated from a pheochromocytoma, a tumor of the adrenal medulla. The peptide and its mRNA have been found in both compartments of the adrenals, with higher expression in the medulla. In the cortex, AM is highly expressed by the zona glomerulosa and, at lower levels, by the zona fasciculata and reticularis (Fig. 2). A similar distribution has been reported for AM-2.[23] These peptides are costored with catecholamines in the secretory granules of chromaffin cells and are cosecreted with them on activation of these cells.

Binding sites for AM and PAMP have been found throughout the adrenals, and the components of the AM receptors share a common distribution with that of the ligands, suggesting the existence of autocrine and/or paracrine mechanisms in the regulation of the effects mediated by such peptides. Two types of functional effects mediated by peptides of the AM family have been studied in the adrenals: regulation of the secretion of adrenal hormones and growth effects during experimental regeneration of the gland.

The main hormone secreted by the zona glomerulosa is aldosterone, and there are contradictory reports on whether AM and PAMP induce or inhibit aldosterone secretion in the adrenal cortex. In support of an inhibitory function, AM has been shown to decrease the induction of aldosterone secretion elicited by angiotensin II, KCl, and ACTH.[28] PAMP has also been described as an inhibitor of aldosterone secretion, being more potent than AM. This view is also supported by more recent studies using antisense approaches.[26] On the other hand, there are several reports of a stimulating role for both AM and PAMP.[7] This controversy was difficult to reconcile until we realized that different methods were used in these studies. When isolated cells are used, they contain the receptors, but they lack all other environmental cues such as local paracrine effects, which are present in perfusion studies or in *in vivo* models. For instance, in dispersed cell preparations, if there is a mixture of cortical and medullary cells, AM can induce catecholamine release from the chromaffin cells which, in turn, can enhance aldosterone secretion from cells belonging to the zona glomerulosa. All these studies coincide in pointing to AM and PAMP as important autocrine and/or paracrine players in the regulation of aldosterone.

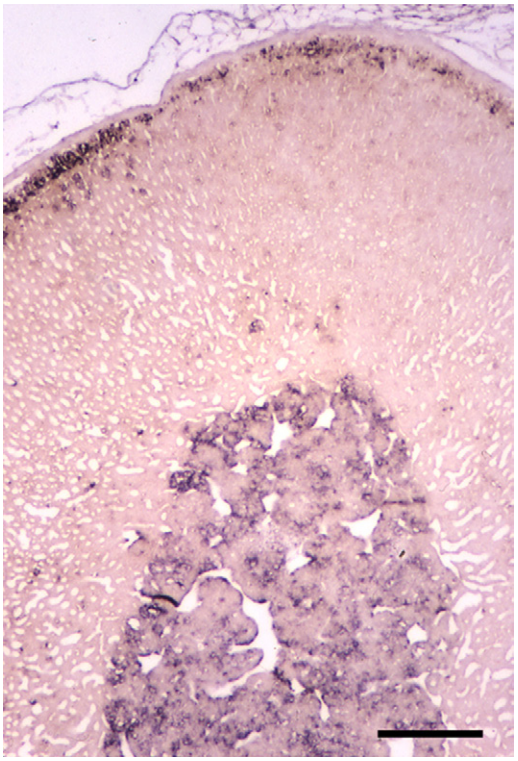

FIGURE 2 Distribution of proadrenomedullin mRNA in a section of rat adrenal gland by in situ hybridization. Proadrenomedullin expression is highest in the medulla and in the zona glomerulosa of the cortex. Bar = 150 μm. See color plate 38.

In the adrenal medulla, AM increases catecholamine release by stimulating Na^+/Ca^{2+} exchange in the membrane of the chromaffin cells whereas PAMP has the reverse effect.[22] Because both AM and PAMP are cosecreted with catecholamines from the chromaffin cells, PAMP would exert an autocrine feedback mechanism on chromaffin cell secretion, whereas AM would tend to multiply the initial secretory stimulus. Nevertheless, AM has been shown to reduce tyrosine hydroxylase activity,[35] so, in the long run, AM may be also part of the negative feedback loop. No studies are as yet available for the effects of AM-2 on the secretion regulation of adrenal hormones.

Enucleation of the adrenal gland provides a good model to study organ regeneration and cell proliferation *in vivo*. During regeneration of the medulla, it has been shown that the levels of AM and its receptor components, especially RAMP2, experiment a marked increase. In this context, AM increases proliferation and reduces apoptosis,[26] in a similar manner to its functions in tumor cells (which are discussed in the Cancer/Anticancer section of this book).

ENDOCRINE PANCREAS

AM has been found in the endocrine pancreas of all vertebrates, from fish all the way to humans. In lower vertebrates, AM colocalizes with all pancreatic hormones in a complex pattern, but in birds and mammals, the cells expressing high levels of AM are exclusively the F cells, which also produce pancreatic polypeptide in the periphery of the islets of Langerhans.[21] This phylogenetic variation is recapitulated during ontogenesis. In early embryos, when the pancreatic bud is being formed, AM expression is already present and colocalizes with all other pancreatic hormones. As the organ matures, AM colocalization pattern is progressively restricted until it reaches its final location just in the F cells.[18]

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The AM receptor and binding protein, complement factor H, are expressed by the β-cells that synthesize insulin in the center of the islet.[20] This architecture and the fact that blood flow in the islet goes from the periphery to the center suggests a clear anatomical model for local regulation of pancreatic physiology through AM. Indeed, AM has been shown to reduce insulin secretion when applied to isolated islets. Even more impressive is the observation that the application of a monoclonal antibody against AM to isolated islets increased insulin secretion fivefold, demonstrating that AM exerts a tonic inhibition on insulin secretion under normal conditions.[21] This was later confirmed on isolated β-cells in culture.[30] This physiological effect of AM was also shown *in vivo*. Injection of AM in rats increased circulating glucose levels whereas the monoclonal antibody reduced these levels.[19]

As expected from the close relationship that exists between β-cell function and metabolic diseases such as diabetes, obesity, or metabolic syndrome, important variations of AM levels have been reported in such patients. The levels of circulating AM are always higher in patients than in normal controls, and patients with symptoms of advanced disease such as retinopathy, microvascular, or renal complications present even higher levels than do patients without these complications.[1] Based on these observations, some authors have proposed that increasing AM levels are just a consequence of endothelial degeneration, something that occurs late in the natural history of diabetes and other metabolic conditions. Nevertheless, analysis of two populations of patients with a very recent onset of diabetes revealed the existence of two subsets of patients, depending on their AM levels. All diabetic patients had a higher concentration of AM than normal controls did, but some of the diabetic patients exhibited an exceedingly high concentration of AM. In these patients, preexisting excessive AM may have triggered the onset of the disease.[19]

Type-2 diabetes is characterized by a secretory defect at the islet of Langerhans and also by desensitization of peripheral tissues to insulin action. AM seems to play a role at both ends. On the one hand, an excess of AM reduces insulin secretion at the islet level. On the other hand, it has been shown that mice haploinsufficient for AM have increased insulin resistance when compared with that of normal controls.[31]

AM is also involved in adipogenesis. AM delays adipocyte differentiation and insulin reduces AM expression in these cells. Furthermore, in obese patients, AM plasma levels are negatively correlated with insulin levels and always elevated when compared with that of lean controls,[6] suggesting that AM may play an important role in the relationship between diabetes, obesity, and other metabolic conditions.

No information is available on the effects of either PAMP or AM-2 on pancreatic physiology.

DIFFUSE ENDOCRINE SYSTEM OF THE GUT

The diffuse endocrine system consists of a variety of individual cells with secretory endocrine morphology that appear scattered among other epithelial cells throughout the digestive tract. These cells act as chemo and mechanoreceptors of the conditions occurring in the gut and release their secretory peptides to neighboring cells to regulate digestive physiology (Fig. 3). Both AM and PAMP have been found in the secretory granules of a subset of these cells, colocalizing with serotonin and gastrin. The number of PAMP-positive cells in the gut, including the stomach and the small and large intestines, seems to be higher than the number of AM-immunoreactive cells.

The levels of AM increase in pathological situations such as gastric ulcers, microbial infection, colitis or even after chronic starvation,[15] suggesting that AM may constitute in the gut, as it does in other organs, a protective system that maintains homeostasis.

Studying the function of the diffuse endocrine system has always been complex given the dispersion of the cells that constitute the system and the paracrine mechanism of action. This is particularly complicated when we realize that there is another source of AM and PAMP in the gut, namely the parasympathetic nervous system which innervates the smooth muscle layers, the blood vessels, and even the epithelium. Nevertheless, several functions have been ascribed to AM and PAMP that relate to digestive physiology. In most cases, these actions can be better understood from a local perspective rather than involving a bona fide endocrine mechanism. For instance, AM increases somatostatin secretion in the stomach and, probably as a direct consequence, decreases histamine and acid secretion. Both AM and PAMP influence the intestinal absorption of sugars. In addition, AM stabilizes epithelial barrier function in the gut,[34] thus contributing to the well-known protective effect of AM

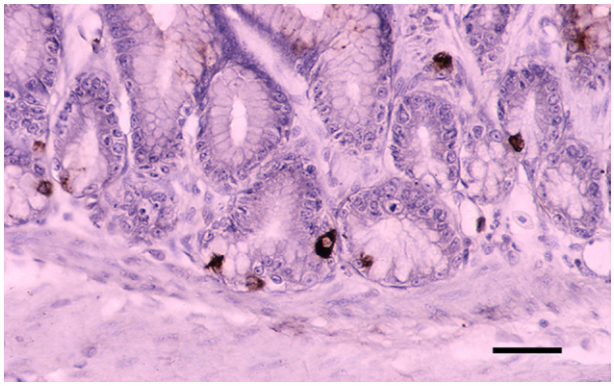

FIGURE 3 Paraffin section of the stomach of the lizard *Anolis sagrei*, stained for PAMP. Note the numerous PAMP-positive cells belonging to the diffuse endocrine system located in the deep portion of the gastric glands. Hematoxylin was used to provide nuclear counterstaining (blue). Bar = 25 μm. See color plate 39.

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against stomach ulcers, colitis, and other inflammatory conditions of the gut. Interestingly, some of these effects may be mediated by the impact of AM on the regulation of the hypoxia inducible factor (HIF) system.[13]

OTHER ENDOCRINE ORGANS

AM and its receptor components have been found in the ovary, mainly in the granulosa and thecal layers of the follicles and in the corpora lutea. In the follicles, treatment with AM increases estradiol production but does not affect follicular growth or ovulation rate. In the corpora lutea, AM affects progesterone production in a pregnancy-stage dependent manner, being inhibitory at early and late pregnancy but stimulatory at midpregnancy. Therefore, AM may contribute to the high levels of progesterone that are produced at mid gestation by the corpora lutea.[11] Plasma AM levels change during the human menstrual cycle in a profile similar to the one displayed by luteinizing hormone. AM concentrations inside the follicular fluid correlate negatively with the success of *in vitro* fertilization techniques.[17]

The testis is another organ where AM and its receptors are produced, and they play an important autocrine/paracrine role in the physiology of the organ. AM and the receptor components can be mainly located in Leydig and Sertoli cells. The levels of AM in the seminal plasma are 8- to 9-fold higher than in circulating plasma and correlate with sperm motility.[16] AM has a clear autocrine effect on Leydig cell steroidogenesis by reducing testosterone production.[3] In the Sertoli cells, AM increases inhibin B production, thus having a direct effect on regulating spermatogenesis.[2] In addition, AM levels in the rat testis increase with age.

AM is also present in the thyroid, in both the follicular epithelium and the C-cells, colocalizing with calcitonin (Fig. 4). No specific action has been reported for AM in this gland, but the peptide fragment PAMP(12–20) decreases thyroid-stimulating hormone (TSH) levels and epithelium/colloid ratio in the rat thyroid gland.[5] AM and PAMP levels are increased in animal models and in patients with hyperthyroidism,[33] but the implications of these peptides in the pathophysiology of this disease are not yet clear. AM is also elevated in patients with hyperparathyroidism, where it is mainly produced by the chief cells, although the peptide is not expressed at all by the normal gland.[10]

CONCLUSIONS

AM and PAMP are expressed by many endocrine organs and constitute important regulators of local physiology in these places. To study local functions of these peptides, it may be helpful to use genetically modified mouse models. It is well known that the systemic elimination of either the gene coding for AM/PAMP or for the different components of the AM receptor result in embryo lethality,[8] hence the need for conditional constructs. "Floxed" models are already available for AM,[4] but they have not been specifically targeted to endocrine organs yet. Hopefully, in the near future, these conditional models may help in unraveling the local contributions of AM-related peptides with more detail.

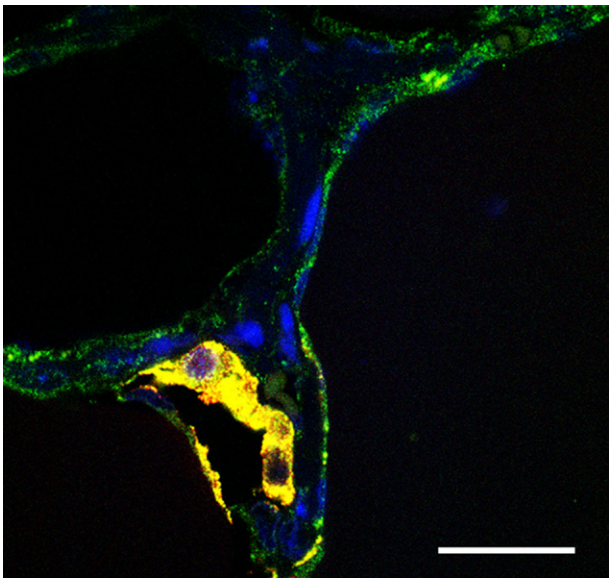

FIGURE 4 Confocal microscopy image of a monkey thyroid gland stained for AM (green), calcitonin (red), and DAPI (blue). Colocalization of both immunoreactivities in the C-cells is shown by the yellow hue. Bar = 30 μm. See color plate 40.

ACKNOWLEDGMENTS

The authors are supported by grants from Spain's Ministry of Science and Innovation (SAF2009-13240-C02-01) and Ministry of Health (DIN-021).

REFERENCES

1. Caliumi C, Balducci S, Petramala L, Cotesta D, Zinnamosca L, Cianci R, et al. Plasma levels of adrenomedullin, a vasoactive peptide, in type 2 diabetic patients with and without retinopathy. *Minerva Endocrinol* 2007;**32**:73–8.
2. Chan YF, Tang F, O WS. Adrenomedullin in the rat testis. II: its production, actions on inhibin secretion, regulation by follicle-stimulating hormone, and its interaction with endothelin 1 in the sertoli cell. *Biol Reprod* 2008;**78**:780–5.
3. Chan YF, O WS, Tang F. Adrenomedullin in the rat testis. I: its production, actions on testosterone secretion, regulation by human chorionic gonadotropin, and its interaction with endothelin 1 in the leydig cell. *Biol Reprod* 2008;**78**:773–9.
4. Fernandez AP, Serrano J, Tessarollo L, Cuttitta F, Martinez A. Lack of adrenomedullin in the mouse brain results in behavioral changes, anxiety, and lower survival under stress conditions. *Proc Natl Acad Sci U S A* 2008;**105**:12581–6.
5. Ginda WJ, Nowak KW, Malendowicz LK. Decrease of TSH levels and epithelium/colloid ratio in rat thyroid glands following administration of proadrenomedullin N-terminal peptide (12–20). *Horm Metab Res* 2000;**32**:10–4.

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6. Harmancey R, Senard JM, Rouet P, Pathak A, Smih F. Adrenomedullin inhibits adipogenesis under transcriptional control of insulin. *Diabetes* 2007;**56**:553–63.
7. Hinson JP, Kapas S. The role of endothelial cell products in the regulation of adrenocortical function: actions of endothelin, nitric oxide, adrenomedullin and PAMP. *Horm Metab Res* 1998;**30**:334–40.
8. Kadmiel M, Fritz-Six K, Pacharne S, Richards GO, Li M, Skerry TM, et al. Research resource: haploinsufficiency of receptor activity-modifying protein-2 (RAMP2) causes reduced fertility, hyperprolactinemia, skeletal abnormalities, and endocrine dysfunction in mice. *Mol Endocrinol* 2011;**25**:1244–53.
9. Kimata-Hayashi N, Takano K, Yasufuku-Takano J, Teramoto A, Fujita T. Mechanism of adrenomedullin-induced prolactin release from human prolactin-releasing adenoma cells. *Endocr J* 2005;**52**:769–73.
10. Letizia C, Ricci F, De TG, Cianci R, Alo P, Celi M, et al. Adrenomedullin immunoreactivity tissue distribution in parathyroids of the patients with primary hyperparathyroidism. *Horm Metab Res* 2004;**36**:480–4.
11. Li L, O WS, Tang F. Adrenomedullin in rat follicles and corpora lutea: expression, functions and interaction with endothelin-1. *Reprod Biol Endocrinol* 2011;**9**:111.
12. Lopez J, Martinez A. Cell and molecular biology of the multifunctional peptide, adrenomedullin. *Int Rev Cytol* 2002;**221**:1–92.
13. MacManus CF, Campbell EL, Keely S, Burgess A, Kominsky DJ, Colgan SP. Anti-inflammatory actions of adrenomedullin through fine tuning of HIF stabilization. *FASEB J* 2011;**25**:1856–64.
14. Malendowicz LK, Macchi C, Nowak M, Nussdorfer GG, Majchrzak M. Effects of proadrenomedullin N-terminal 20 peptide on the rat pituitary-adrenocortical axis under basal and stressful conditions. *Int J Mol Med* 2006;**18**:285–7.
15. Man SY, Hwang IS, Li YY, O WS, Sheng HP, Tang F. Differential regulation of adrenomedullin gene expression in the fundic and pyloric regions of the rat stomach during acute and chronic starvation. *Neuropeptides* 2007;**41**:177–87.
16. Marinoni E, Di IR, Villaccio B, Vellucci O, Di NT, Sessa M, et al. Adrenomedullin in human male reproductive system. *Eur J Obstet Gynecol Reprod Biol* 2005;**122**:195–8.
17. Marinoni E, Feliciani E, Muzzonigro F, Letizia C, Tranquilli A, Tranquilli D, et al. Intrafollicular concentration of adrenomedullin is associated with IVF outcome. *Gynecol Endocrinol* 2010;**26**: 435–9.
18. Martinez A, Cuttitta F, Teitelman G. Expression pattern for adrenomedullin during pancreatic development in the rat reveals a common precursor with other endocrine cell types. *Cell Tissue Res* 1998;**293**:95–100.
19. Martinez A, Elsasser TH, Bhathena SJ, Pio R, Buchanan TA, Macri CJ, et al. Is adrenomedullin a causal agent in some cases of type 2 diabetes? *Peptides* 1999;**20**:1471–8.
20. Martinez A, Kapas S, Miller MJ, Ward Y, Cuttitta F. Coexpression of receptors for adrenomedullin, calcitonin gene-related peptide, and amylin in pancreatic beta-cells. *Endocrinology* 2000;**141**:406–11.
21. Martinez A, Weaver C, Lopez J, Bhathena SJ, Elsasser TH, Miller MJ, et al. Regulation of insulin secretion and blood glucose metabolism by adrenomedullin. *Endocrinology* 1996;**137**:2626–32.
22. Masada K, Nagayama T, Hosokawa A, Yoshida M, Suzuki-Kusaba M, Hisa H, et al. Effects of adrenomedullin and PAMP on adrenal catecholamine release in dogs. *Am J Physiol* 1999;**276**:R1118–24.
23. Morimoto R, Satoh F, Murakami O, Hirose T, Totsune K, Imai Y, et al. Expression of adrenomedullin 2/intermedin in human adrenal tumors and attached non-neoplastic adrenal tissues. *J Endocrinol* 2008;**198**:175–83.
24. Perez-Castells J, Martin-Santamaria S, Nieto L, Ramos A, Martinez A, de Pascual-Teresa B, et al. Structure of micelle-bound adrenomedullin, a first step towards the analysis of its interactions with receptors and small molecules. *Biopolymers* 2012;**97**:45–53.
25. Pio R, Elsasser TH, Martinez A, Cuttitta F. Identification, characterization, and physiological actions of factor H as an adrenomedullin binding protein present in human plasma. *Microsc Res Tech* 2002;**57**:23–7.
26. Rossi GP, Conconi MT, Malendowicz LK, Nussdorfer GG. Role of the endogenous adrenomedullin system in regulating the secretion and growth of rat adrenal cortex. *Hypertens Res* 2003;**26**(Suppl.):S85–92.
27. Sackett DL, Ozbun L, Zudaire E, Wessner L, Chirgwin JM, Cuttitta F, et al. Intracellular proadrenomedullin-derived peptides decorate the microtubules and contribute to cytoskeleton function. *Endocrinology* 2008;**149**:2888–98.
28. Salemi R, McDougall JG, Hardy KJ, Wintour EM. Effect of adrenomedullin infusion on basal and stimulated aldosterone secretion in conscious sheep with cervical adrenal autotransplants. *J Endocrinol* 2000;**166**:389–99.
29. Samson WK, Murphy TC, Resch ZT. Proadrenomedullin N-terminal 20 peptide inhibits adrenocorticotropin secretion from cultured pituitary cells, possibly via activation of a potassium channel. *Endocrine* 1998;**9**:269–72.
30. Sekine N, Takano K, Kimata-Hayashi N, Kadowaki T, Fujita T. Adrenomedullin inhibits insulin exocytosis via pertussis toxin-sensitive G protein-coupled mechanism. *Am J Physiol Endocrinol Metab* 2006;**291**:E9–14.
31. Shimosawa T, Ogihara T, Matsui H, Asano T, Ando K, Fujita T. Deficiency of adrenomedullin induces insulin resistance by increasing oxidative stress. *Hypertension* 2003;**41**:1080–5.
32. Takahashi K, Morimoto R, Hirose T, Satoh F, Totsune K. Adrenomedullin 2/intermedin in the hypothalamo–pituitary–adrenal axis. *J Mol Neurosci* 2011;**43**:182–92.
33. Taniyama M, Kitamura K, Ban Y, Sugita E, Ito K, Katagiri T. Elevation of circulating proadrenomedullin-N terminal 20-peptide in thyrotoxicosis. *Clin Endocrinol* 1997;**46**:271–4.
34. Temmesfeld-Wollbruck B, Brell B, zu Dohna C, Dorenberg M, Hocke AC, Martens H, et al. Adrenomedullin reduces intestinal epithelial permeability in vivo and in vitro. *Am J Physiol Gastrointest Liver Physiol* 2009;**297**:G43–51.
35. Yuksel S, Yurekli M. The effect of adrenomedullin (ADM) on tyrosine hydroxylase (TH) enzyme activity and blood pressure in cold exposed rats. *Endocr J* 2003;**50**:553–9.

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Chapter 132

Ghrelin

Marcos C. Carreira, Ana B. Crujeiras and Felipe F. Casanueva

ABSTRACT

Ghrelin, the endogenous ligand of the growth hormone (GH) secretagog (GHS) receptor (GHS-R), is a 28 amino acid peptide originally isolated from the stomach with GH-releasing and appetite-inducing activities. During the years after its discovery, ghrelin and its receptor have also been detected in other organs, including hypothalamus, pituitary, thyroid, parathyroid, pancreas, adrenal glands, placenta, male and female gonads, and almost every other normal human tissue studied. In addition, it is present in neuroendocrine, pituitary, pancreatic, and thyroid tumors. Thus, ghrelin is involved in many processes, and its endocrine, paracrine, and autocrine effects play an important role in physiological and pathophysiological functions.

INTRODUCTION

Ghrelin is a ubiquitously expressed hormone, which since it was discovered has been proposed as a much-needed therapeutic option for several diseases. This is because of its endocrine, cardiovascular, immunological, reproductive, and behavioral effects that have been described based on the expression of ghrelin and its receptor in a wide variety of tissues.[6,23,32]

This hormone was originally discovered as a strong GH-releasing activity mediated by the activation of GH secretagog receptor type 1a (GHS-R1a). The human GHS-R1a is a G protein-coupled receptor of seven transmembrane domains and 366 amino acids, which transduce information provided by ghrelin.[23] This receptor is mainly expressed in central neuroendocrine tissues. However, a widespread pattern of GHS-R1a expression is observed in a variety of peripheral tissues including the thyroid gland, pancreas, spleen, myocardium, adrenal gland, testis, ovaries, and stomach.[20] The peripheral distribution suggests that ghrelin is involved in functions in addition to the regulation of GH release and energy homeostasis.[20] Moreover, according to the role of ghrelin in regulating proliferation in some tumors, GHSR-1a is expressed in a wide variety of cancers including neuroendocrine cancers, breast carcinomas, prostate cancer cell lines, neoplastic thyroid tissue, and ovarian tumors.[6,23,32]

After the binding of ghrelin, GHS-R1a acts through the G α11 subunit of G protein to activate PLC resulting in the hydrolysis of membrane-bound phosphatidylinositol biphosphate (PIP2) to generate inositol triphosphate (IP3) and diacylglycerol (DAG). Intracellular calcium concentration increases result from the release from IP3-responsive cytoplasmatic storage pools and inflow of Ca^{2+} through the activation of L-type calcium channels (Fig. 1). It has been proposed that GHRH interacts directly with GHS-R1a and modifies the ghrelin-associated intracellular signaling pathway. This interaction may represents a new form of regulation, which could plays a role in the physiology of GH regulation and appetite control.[5]

Ghrelin is derived from the ghrelin gene, which produces three peptides by a posttranslational mechanism (Fig. 2). The ghrelin mature molecule, a 28 amino acid residue peptide, is obtained from the proteolytic cleavage of preproghrelin and exists in two major molecular forms, acylated ghrelin and unacylated ghrelin. The acylation, catalyzed by the ghrelin O-acyltransferase (GOAT)[19,34] occurs on the third residue (Ser) and is essential for its functional activity through GHS-R1a.[19] Ghrelin seems to be the sole substrate for GOAT. Expression of GOAT is limited to the major ghrelin-secreting tissues as gastrointestinal tract, intestine, testis, and pancreas.[19,34]

Unacylated ghrelin (UAG) was initially believed to be nonfunctional but has subsequently been proven to exert biological activities, some of which are similar to ghrelin and others divergent. Both ghrelin and unacylated ghrelin exert functions in cells and tissues that do not express the GHS-R1a receptor involving the existence of an unidentified receptor.[23,32]

The third ghrelin gene product is obestatin (OB), a 23 amino acid amidated peptide derived from the preproghrelin gene.[35] See the obestatin chapter in the Gastrointestinal Peptides section of this book. It was originally believed that obestatin binds to the G protein-associated receptor GPR 39 and postulated as a physiological opponent of ghrelin inhibiting food intake.[35] However, currently, the effects of this peptide on food intake remain unclear. In addition, obestatin is able to produce several biological functions

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00132-9
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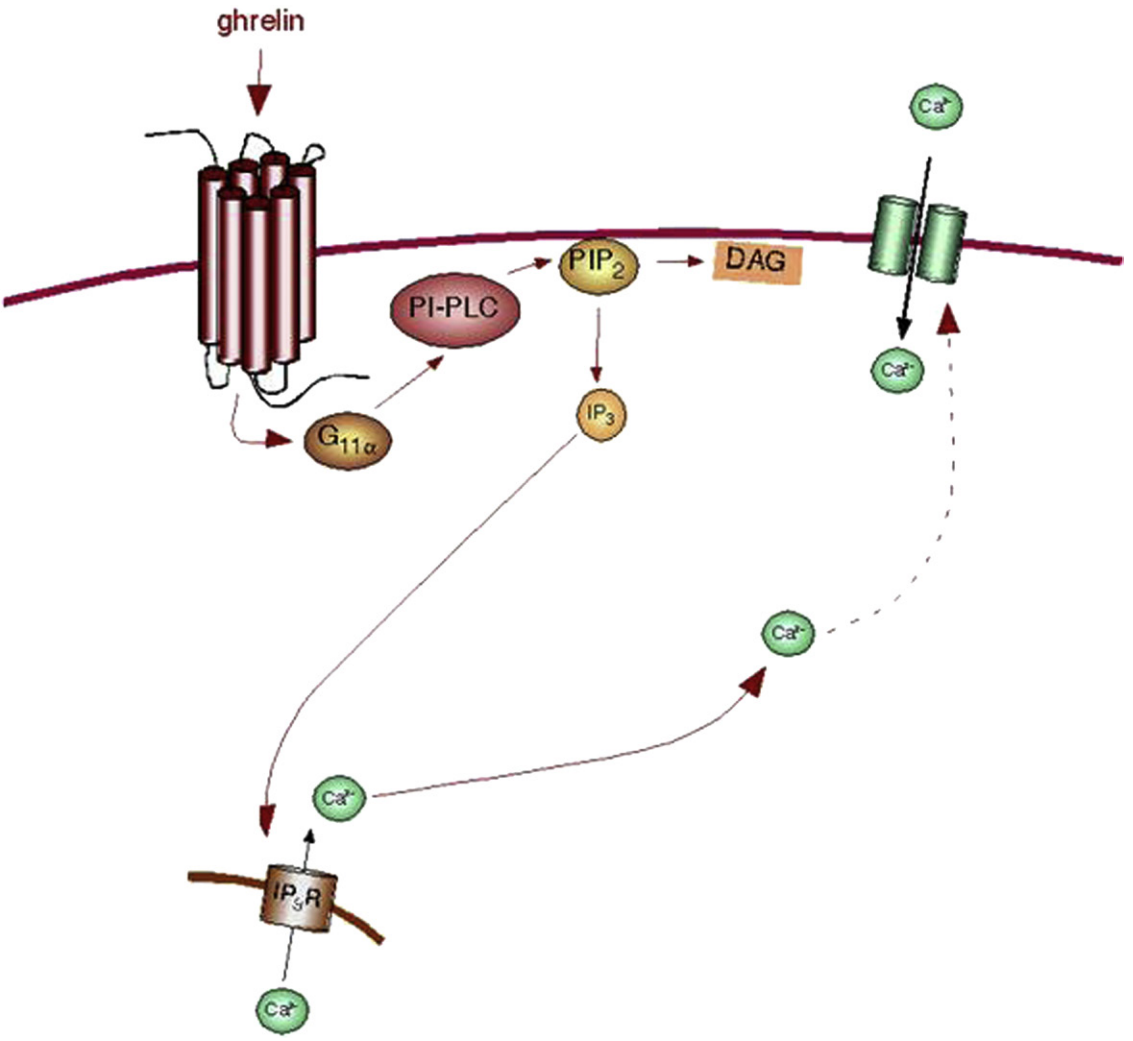

FIGURE 1 Proposed model of the signal transduction pathway activated by the GHS-R1a receptor to elicit intracellular calcium peak.

at the central level such as improving memory, anxiolytic effects, and activation of cortical neurons, and it also has peripheral effects. At the cellular level, obestatin stimulates GH release from rat tumor somatotroph cells[25] and regulates adipocyte metabolism and adipogenesis.[18]

Ghrelin is predominantly produced and secreted by the stomach and in lower amounts in bowel, kidney, pancreas, lung, immune system, placenta, tested, thyroid, hypothalamus, and pitutitary.[23,32]

It was primarily postulated that the main function of ghrelin is to regulate appetite and energy homeostasis by an endocrine or a paracrine signal directly at the stomach level and to activate specific neuronal circuits situated in the hypothalamus although the activation of the afferent vagal nervous system has been proposed.[23,32] However, the coexpression of ghrelin and the functional ghrelin receptor shows that ghrelin has direct effects on several organs and is involved in many more processes than was initially postulated. In this context, this chapter reports the direct effect of ghrelin, and some effects of desacyl ghrelin and obestatin on different endocrine organs at physiological and pathophysiological levels.

GHRELIN SYSTEM IN THE PITUITARY

In addition to the probable hypothalamic effects of ghrelin, this peptide is synthesized locally within the pituitary gland where ghrelin immunostaining is colocalized to prolactin, GH, and TSH secreting cells but not to ACTH or gonadotroph-secreting cells. Thus, within the pituitary gland, ghrelin may influence the release of GH in an autocrine or paracrine manner. GH secretion is pulsatile and, classically, it was believed to be controlled by GHRH and somatostatin, but several studies suggested that other factors play a

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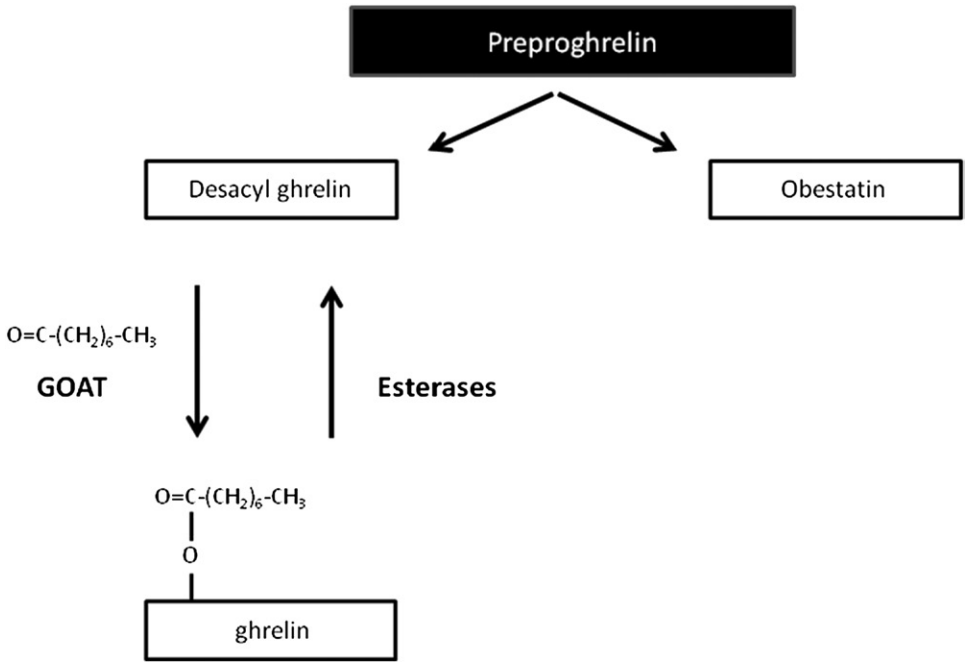

FIGURE 2 Production of the three ghrelin gene products.

role in the regulation of GH release from the pituitary. In accordance with the identification of ghrelin as the endogenous ligand for the GH hormone secretagogue receptor, the initial studies with exogenous synthetic GH secretagogs and ghrelin have shown a strong and dose-dependent GH releasing effect, but the physiological role of ghrelin in the regulation of GH release is still uncertain.

It has been proposed that ghrelin releases GH by a dual mechanism involving the hypothalamus and the pituitary. The effect of ghrelin *in vivo* is stronger than the *in vitro* effect, and it is dependent on the presence of GHRH. In addition, ghrelin shows an additive or a synergistic effect on GHRH stimulated GH release and prevents the cyclic refractoriness to GHRH. The ghrelin effect is markedly reduced, but not totally abolished, in animals with a lesion of the pituitary stalk, suggesting that the most important action of ghrelin to release GH occurs at the hypothalamic level.[6,23,32]

At the hypothalamic level, ghrelin acts via GHRH-secreting neurons because immunization against GHRH or treatment with GHRH antagonists reduces its stimulatory effect on GH secretion. *In vivo* studies have shown an increase release of GHRH in portal blood of the pituitary after synthetic secretagogue administration. In the lit/lit mouse, which has nonfunctional pituitary GHRH receptors, GHS has no effect on GH release. In humans with GHRH receptor mutations, a very small but significant GH response is observed.[23,32] Peripheral or central administration of ghrelin stimulates hypothalamic GHS-R1a and the pituitary receptor directly to release GH. In contrast, some results indicate that peripheral ghrelin stimulates GH release and feeding via a vagus nerve-mediated abdominal action.[8]

The expression of ghrelin in the pituitary in male and female rats is higher during ontogeny and decreases during development in females, whereas in males, there is no variation during development. This pattern of expression is contrary to that observed in the stomach where ghrelin expression occurs in the fetus and increases after birth in an age-dependent manner.[23,32] Ghrelin is expressed in somatotrophs, lactotrophs, and thyrotrophs. Given that GHS-R1a is also expressed in the pituitary, the possibility exists that locally produced ghrelin in the pituitary may have paracrine or autocrine effects on pituitary function. In fact, under experimental conditions in which hypothalamic GHRH expression is increased (GH or glucocorticoid deficiency, hypothyroidism), pituitary ghrelin levels are also increased.[23,32] Consistent with this correlation, pituitary ghrelin expression levels are decreased in conditions in which hypothalamic GHRH expression is decreased, indicating a physiological role of GHRH in stimulating pituitary ghrelin expression.[23,32] Another source of ghrelin is the hypothalamus, although it is not clear that ghrelin-produced neurons project to GHRH and somatostatin neurons. In addition, GHS-R antisense transgenic animals have much less GHS-R expression but maintain typical pulsatile GH secretion and IGF-1 levels.[23,32] Moreover, a direct interaction occurs between the GHRH-R and the GHS-R in the pituitary somatotroph.[7]

Based on its strong and reproducible GH-releasing effect, particularly when combined with GHRH, ghrelin

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can be used as a diagnostic tool in suspected GH deficiency. Ghrelin when administrated together with GHRH exerts a synergistic action on GH secretion. This combination is the most potent GH releaser.[6,23,32] Clinical studies have demonstrated that ghrelin–GHRH administration may be considered a test of GH reserve in humans because the GH response elicited is not altered by gender, age, or adiposity. This test is able to discriminate between healthy subjects and patients with GH deficiency. In this condition, the somatotroph response to ghrelin is reduced, whereas the lactotroph and corticotroph response is fully preserved.[6,23,32] These results suggest that ghrelin may represent a reliable test to evaluate the maximal secretory capacity although they do not support the idea that impaired ghrelin response is the major cause of GH deficiency.

Moreover, it has been proposed that ghrelin rejuvenates the GH/IGF-1 axis with possible clinical applications for the treatment of aging. Chronic administration of ghrelin is able to increase the low GH levels associated with aging.[23,32]

In addition to its regulatory role on GH secretion, *in vivo* ghrelin induces a significant secretion of prolactin and ACTH/cortisol without significantly altering the release of LH, FSH, and TSH.[23,32]

The mechanisms of adrenocorticotropin regulation by ghrelin may include hypothalamic involvement through the release of CRH, arginine vasopressin, and NPY and/or gamma-aminobutyric acid (GABA).[23,32]

It is possible that ghrelin exerts other effects on somatotroph cell function independently of GH release. Ghrelin influences the expression of the transcription factor Pit-1 through a sequence that contains two cAMP response element.[6,23,32]

Both ghrelin and GHS-R1a receptor, as well as the isoform GHS-R1b obtained by alternative splicing, are expressed in normal human pituitaries and adenomas.[6,23,32] Ghrelin mRNA is expressed at relatively low levels in corticotroph adenomas and relatively high levels in somatotroph adenomas in comparison with normal pituitaries as this expression pattern correlates with the expression of Pit-1.[23,32] GHS-R is expressed in somatotrophinomas, prolactinomas, thyrotropinomas, and some corticotroph adenomas.[24] The presence of ghrelin and GHS-R in pituitary adenomas indicates that adenoma-produced ghrelin may contribute to the development of adenoma cells through an autocrine/paracrine mechanism.

GHRELIN SYSTEM AND THYROID GLAND

Hormones of the hypothalamus-pituitary-thyroid (HPT) axis are involved in energy homeostasis and body weight as well as in the maintaining of body temperature. This axis stimulates the metabolic rate of tissues such as the heart, bone, liver, adipose tissue, and muscle, increasing thermogenesis as well as energy expenditure. As is well known, hyperthyroidism is associated with weight loss and increased body temperature, whereas hypothyroidism is associated with weight gain and lower temperature.

Ghrelin is present in human thyroid tissue. Fetal, but not adult thyrocytes, present significant amounts of ghrelin suggesting that follicular cells are an additional source of ghrelin during thyroid development.[6,23,32]

Ghrelin binding sites have however been detected in the human thyroid, and ghrelin enhances *in vitro* TSH-mediated proliferation of rat thyrocytes and diminished cell proliferation in human thyroid carcinoma cells. It has been suggested that ghrelin affects the HPT axis in humans by direct action over the thyroid by increasing the levels of free thyroxine and decreasing TSH concentrations in plasma.[6,23,32]

The intracerebroventricular administration of ghrelin reduces the size of pituitary TSH-producing cells and TSH plasma concentrations, resulting in less active thyroid follicles and reduced plasma thyroxine (T4) concentrations. Hyperthyroidism is also associated with decreased ghrelin levels and becomes normalized by antithyroid treatment. In addition, ghrelin levels in hypothyroidism are similar to those of healthy subjects, although in animal models ghrelin is increased.

It has been suggested that ghrelin plays an important role in the generation and development of thyroid tumors. It is expressed in malignant epithelial carcinomas, but not in benign cases, suggesting the involvement of ghrelin in the development of thyroid epithelial carcinomas. Both follicular and papillary carcinomas show intense levels of ghrelin expression.[6,23,32] The high proportion of adenomas containing high levels of ghrelin suggests that neoplastic transformation involves the expression or reexpression of ghrelin. No apparent differences in ghrelin expression were observed in thyroid tumors despite tumor grade and differentiation, indicating that ghrelin does not play an important role in tumor progression. Interestingly, high concentrations of ghrelin has antiproliferative properties in thyroid carcinoma cell lines, suggesting that an autocrine mechanism is involved in control of the growth of follicular tumors.[6,23,32]

GHRELIN SYSTEM IN THE PARATHYROID GLAND

Rat and human parathyroid glands present an intense and diffuse cytoplasmic distribution of immunoreactivity for ghrelin.[24] Given the antiproliferative actions of ghrelin in the thyroid and different thyroid cell lines, it is possible that ghrelin could also be involved in the inhibition of the proliferation of parathyroid in both normal and tumor cells. However, specific binding of the radiolabeled synthetic secretagogue hexarelin to membranes from the parathyroid gland is negligible.[27] The parathyroid adenomas are diffusely positive for ghrelin.[24] Ghrelin expression in the

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parathyroid gland indicates the existence of an additional source of ghrelin in this gland involved in autocrine regulation of parathyroid cell function.

GHRELIN SYSTEM IN THE TESTIS AND OVARY

Evidence suggests that ghrelin and its functional receptor are expressed in mammalian testis and ovaries, where ghrelin may have specific regulatory actions. Thus, ghrelin may participate in the regulation of gonadotropin secretion, and it may influence the timing of puberty.[12] In addition, it has been shown that ghrelin has direct gonadal effects since the expression of ghrelin and the functional ghrelin receptor have been reported in the reproductive system including follicular, luteal, surface epithelial, and interstitial hilus cells in the ovary, as well as in leydig and sertoli cells in the testis.[2,12]

Physiological Functions of Ghrelin in Female Gonads

In addition to the recognized regulatory hormones, several reports have described that the control of the ovary is also exerted by ghrelin. This assumption was shown by the expression of ghrelin and its functional receptor in animal models and the human ovary.[12]

Ghrelin is found in ovarian hilus interstitial cells as well as young and mature *corpora lutea*, whereas ghrelin is not detected in ovarian follicles at any developmental stage.[14] Assessment of relative mRNA levels of ghrelin through the different stages of the estrous cycle has shown that ghrelin mRNA levels vary significantly depending on the phase of the cycle. The lowest expression levels occur in the luteal phase and the highest when the *corporea lutea* enter the functional phase, while remaining lower during *corporea lutea* formation and regression.[4] Further substantiation is provided by immunohistochemical analyses, which show intense and specific ghrelin immunoreactivity in the cytoplasm of steroidogenic luteal cells.[4]

Similarly, ovarian expression of GHS-R1a protein shows a wider pattern of tissue distribution, with detectable specific signals in oocytes as well as somatic follicular cells, luteal cells from young, mature, old, and regressing *corporea lutea*, interstitial hilus cells, and fallopian tube epithelium.[15] Therefore, the presence of both components (ligand and receptor) of the ghrelin-signaling system within the ovary opens up the possibility of a potential regulatory role of ghrelin in ovarian function.[12] In this regard, the recent assessment of the biological actions of ghrelin in the human ovary by an *in vitro* model showed that ghrelin is able to significantly inhibit, in a dose-dependent manner, estradiol (E2) and progesterone (P4) production by granulosa-lutein cells by acting through GHS-R1a.[33] Such a mechanism could contribute to the suppression of the reproductive axis in situations of negative energy balance, such as starvation, which is characterized by low blood gonadotropins and E2 levels and high ghrelin concentrations. However, ghrelin does not inhibit, but rather promotes, the function of chicken, rabbit, and porcine ovarian cells in cultures. In these cells, ghrelin directly promotes ovarian cell proliferation (measured by an increase in the proliferation-related peptides PCNA, cyclin B1, and MAPK/ERK1,2), inhibits their apoptosis (measured by a decrease in the apoptosis-related substances bax, p53, and caspase 3), and stimulates the release of ovarian derived hormones (progesterone, testosterone, estradiol, IGF-1, prostaglandin, and others).[29] Therefore, the role of ghrelin in controlling ovarian secretory activity requires further study.

Despite the proliferative actions of ghrelin on the ovarian, analysis of metaplastic lesions and tumors of the ovary shows that the pattern of expression and cellular distribution of the GHS-R1a is similar to that of normal and neoplastic ovaries, but malignant tumors that derive from the ovarian surface epithelium show weak or no GHS-R1a immunoreactivity.[24]

Physiological Functions of Ghrelin in Male Gonads

The testis is a complex endocrine organ in which different cell types interplay to ensure male fertility under the control of an array of extragonadal and intragonadal hormones and growth factors. Current results suggest that ghrelin participates in such a regulatory network.[30]

Within the testis, expression of ghrelin has been observed in mature Leydig cells of both rats and humans. In addition, expression of the functional ghrelin receptor, the GHS-R type 1a, is seen in Sertoli and Leydig cells. In contrast, expression of GHS-R1a in the seminiferous tubules strongly suggests that the seminiferous epithelium may be a target for ghrelin action that directly regulates seminiferous tubule functions.[30]

These results pertaining to the rat testis as a model *in vitro* show that ghrelin is able to significantly inhibit, in a dose-dependent manner, human choriogonadotropin (hCG)-and cAMP-stimulated testosterone secretion.[12] This inhibitory effect on testosterone secretion is associated with a significant decrease in hCG-stimulated expression of genes related to the steroidogenic route, suggesting that ghrelin may participate in the autolimitation of testicular secretion of testosterone in responses to gonodotropic stimulation.[30] In addition, in *in vivo* models, ghrelin is able to inhibit the proliferative rate of immature leydig cells during puberty by inhibiting the expression of the gene encoding stem cell factor, a Sertoli cell product involved in the germ cell development and survival factor for the spermatogenic

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cells.[3] Reinforcing these effects, shown by acute intratesticular administration of ghrelin, the chronic treatment of ghrelin induces a significant decrease in the number of the different spermatogenic cell types except for spermatogonia, a decrease in the thickness of the germinal epithelium of the seminiferous tubules, as well as an intracellularly reduction in the size and number of mitochondrial while increasing vacuolated areas within the matrix of the cells.[22]

Overall, these results confirm that ghrelin has antiproliferative effects on different testicular cell types and is a negative modulator of male reproductive system in situations of negative energy balance, such as starvation, where limited resources are allocated to major physiological processes.[12,22]

At the pathophysiogical level, ghrelin expression is conserved in Leydig cell tumors and is linked to the degree of cell differentiation.[13,24] Therefore, this peptide is suggested as a novel regulator of testicular development and operates as marker of Leydig cell differentiation, both during normal development and in tumor transformation.[13,24]

GHRELIN SYSTEM IN THE PANCREATIC ISLETS

Pancreatic islets consist of four major endocrine cell types that produce the hormones necessary for maintaining appropriate levels of circulating glucose. The α-cells synthesize glucagon, β-cells synthesize insulin, Δ-cells synthesize somatostatin, and pancreatic polypeptide (PP) cells synthesize PP. A few years ago, a fifth mammalian islet cell type, the ghrelin-producing epsilon cell, was described. Besides these novel islet cells, multiple experimental systems have also shown ghrelin-immunoreactivity in α-cells and β-cells.[10] In regards to the ghrelin receptors in pancreatic islets, messenger RNAs (mRNAs) encoding GHS-R are expressed in the pancreas of rats and humans as well as in β-cell lines, and double immunohistochemistry shows GHS-R-like immunoreactivity mainly colocalized extensively with glucagon-pancreatic islets. Moreover, rodent studies show that the expression of pancreatic ghrelin changes dramatically during fetal development and that ε-cells are the major source of ghrelin during fetal life rather than the fetal stomach.[16]

Besides the expression pattern of ghrelin in the pancreatic islets mentioned above, radioimmunoassays comparing the concentration of ghrelin in the pancreatic vein with that in the pancreatic artery rats show ghrelin release from pancreatic islets.[10] However, the regulation of ghrelin release from the pancreas as compared with that from the stomach remains to be clarified.[10]

Little is known about the role of ghrelin within the pancreas, but it seems that ghrelin could play a paracrine/autocrine role in the regulation of β-cell survival and function. In this context, ghrelin has recently been reported to promote proliferation and cell growth and also to inhibit apoptosis of pancreatic β-cell and human islets. This suggests that one function of ghrelin in the developing pancreas could be to promote cell growth and maturation locally.[16] Study of the effect of ghrelin on pancreatomized rats showed an increase in the regenerative capacity of the remaining pancreas after pancreatectomy.[21]

Ghrelin also exerts inhibitory effects on the expression and production of inflammatory cytokines and attenuates the development of acute pancreatitis in rats by blocking the inflammatory signal transduction pathways.[11] Moreover, pharmacological, immunological, and genetic blockade of ghrelin or ghrelin action in pancreatic islets markedly enhances glucose-induced insulin release, suggesting an insulinostatic function of islet-derived ghrelin.[10] *In vitro*, ghrelin inhibits glucose-stimulated insulin secretion in a dose-dependent manner from culture pancreas, isolated pancreatic islets, and immortalized β-cells lines, suggesting that it acts directly on β-cells to achieve this effect. In this regard, the infusion of acyl-ghrelin, the bioactive endogenous ligand of the GHSR-1a, reduces glucose-stimulated insulin secretion and glucose disappearance in healthy humans after an intravenous glucose tolerance test (IVGTT), suggesting that endogenous ghrelin has a role in physiologic insulin secretion, and that ghrelin antagonists could improve β-cells function.[31] However, a significant inverse correlation of ε-cell mass with body mass index (BMI), consistent with reports of an inverse correlation between plasma ghrelin and BMI, leads to speculation that the loss of ε-cells mass associated with increase in BMI could produce increased insulin secretion and also an enhanced susceptibility to apoptosis and inflammation damage in pancreatic islets.[1] Moreover, a preventive action of ghrelin was reported on the development of diabetes in streptozotocin-treated newborn rats via enhanced regeneration of β-cells[17] and the ghrelin-induced increase in the regeneration of pancreatic exocrine and endocrine cells after pancreatectomy, together with high proliferative rate of islet cells, resulted in decreased plasma glucose concentration as compared with the group treated with a ghrelin antagonist.[21]

In regard to the role of ghrelin on α-cell function, increased glucagon secretion by ghrelin has been seen *in vitro*, but the effect of ghrelin on its release is less impressive *in vivo*, with levels unchanged or mildly increased after ghrelin administration. In fact, no relevant change in glucagon level is seen with pharmacologic doses of ghrelin during fasting or IVGTT in healthy humans.[31]

Acylated ghrelin, unacylated ghrelin, and obestatin may prevent pancreatic β-cell death and improve β-cell function after treatment with cytokines, which are the major causes of β-cell

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destruction in diabetes. UAG and OB improve glucose metabolism and preserve islet cell mass in streptozotocin-treated newborn rats, suggesting therapeutic potential in medical conditions associated with impaired beta-cell function.[17]

Coexpression of ghrelin and its receptor have also been seen in pancreatic adenocarcinoma and endocrine pancreatic tumors. This hormone increases proliferation of a pancreatic adenocarcinoma expressing the ghrelin receptor. However, few patients with pancreatic tumors have high concentrations of circulating ghrelin, and no correlation has been found between the expression of ghrelin and its receptor and malignant features. Therefore, the clinical influence of ghrelin production from pancreatic tumors remains unknown.[24]

Combining all the results, pancreatic secreted ghrelin seems to play a role in early islet biology and in β-cell survival and function. Further studies are necessary to delineate mechanisms by which endogenous ghrelin may affect islet function, maturation during fetal life, and pancreas reorganization in adulthood.

GHRELIN SYSTEM IN THE ADRENAL GLAND

The adrenal gland has two distinct structures, the adrenal cortex and the medulla, both of which produce hormones. The human adrenal cortex consists of the *zona glomerulosa* (ZG), which produces aldosterone, the *zona fasciculata* (ZF) responsible for cortisol production and secretion, and the *zona reticularis*, which is essential for adrenal androgen production. The adrenal medulla is the core of the adrenal gland and is surrounded by the adrenal cortex. The chromaffin cells of the medulla, named for their characteristic brown staining with chromic acid salts, are the body's main source of the circulating catecholamines, epinephrine and norepinephrine.

The function of adrenal gland is regulated by the hypothalamus–pituitary–adrenal axis, in which ghrelin plays an important role by the stimulating effect on CRH, ACTH, and cortisol secretion. Besides the indirect effect of ghrelin on adrenal gland, this peptide seems to have a direct effect on adrenal gland. The presence of ghrelin binding sites and the expression of preproghrelin and ghrelin receptors GHS-R1a and GHS-R1b at the mRNA and protein levels in both the cortex and medulla have been shown.[28] However, the direct effect of ghrelin on the secretory activity of this gland is under discussion, while a potent effect of the peptide on raising proliferative activity and decreasing apoptotic deletion rate of adrenocortical cells is established. In cultured human ZG cells, ghrelin does not significantly affect either basal or agonist-stimulated aldosterone secretion but exerts proliferative and antiapoptotic effects via MAPK.[9] As in humans, ghrelin does not affect corticosteroidogenesis in rat adrenocortical cells or adrenal slices.[9] In contrast, ghrelin can potently stimulate corticosterone secretion in cultured cells.[28] This study was performed in freshly isolated and cultured rat adrenocortical cells where the prolonged exposure to ghrelin resulted in a potent effect, comparable to ACTH, stimulating the effect of ghrelin on corticosterone secretion. This steroidogenic, ACTH-like effect of ghrelin on cultured cells was accompanied by a notable increase in the expression of P450scc, a cholesterol side chain cleavage (CYP11A1), the gene-coding synthesis of a key regulatory protein involved in corticosteroidogenesis.[28] The action of ghrelin on adrenocortical cells seems to have been performed with the acylated ghrelin form as shown by the presence of GOAT mRNAs. The concomitant expression of preproghrelin and GOAT genes in adrenal ZG suggests that newly synthesized ghrelin peptide may be rapidly octanoylated into an active form in glomerulosa cells.[28]

With respect to the role and localization of ghrelin in the medulla, the ghrelin precursor, proghrelin and mature ghrelin expression have been found in the adrenal medulla, as in the stomach, showing that ghrelin is synthesized by the adrenal medulla in both humans and rats.[26] Therefore, ghrelin synthesized in the adrenal medulla could be involved in the autocrine/paracrine regulation of chromaffin cell secretion. Indeed, ghrelin stimulates dopamine secretion from rat pheochromocytoma PC 12 cells.[26] However, further studies are needed to clarify the direct role of ghrelin on the regulation of catecholamines release.

GPR39, the putative obestatin receptor (GPR39v1 and GPR39v2) whose mRNAs levels are similar in adrenocortical zones and adrenal medulla, is present in all compartments of the rat adrenal gland and cultured adrenocortical cells.[28] However, the role of obestatin in the regulation of adrenocortical cell growth and function remains unknown.

CONCLUDING REMARKS

Ghrelin is secreted from the stomach into the circulation, but it is also synthesized in many endocrine and nonendocrine tissues suggesting a possible dual role as autocrine/paracrine and/or endocrine factor (Fig. 3). Moreover, ghrelin provides information from the periphery and from within the brain's interconnecting hormonal axis and produces an integrative response on the endocrine control of the organism. In addition, the characteristic acyl modification is necessary for some functions but not for others. The production of obestatin from a common precursor messenger RNA with a complex gene structure and transcription regulation as well as the probable existence of new nondescribed receptors increase the challenge of understanding

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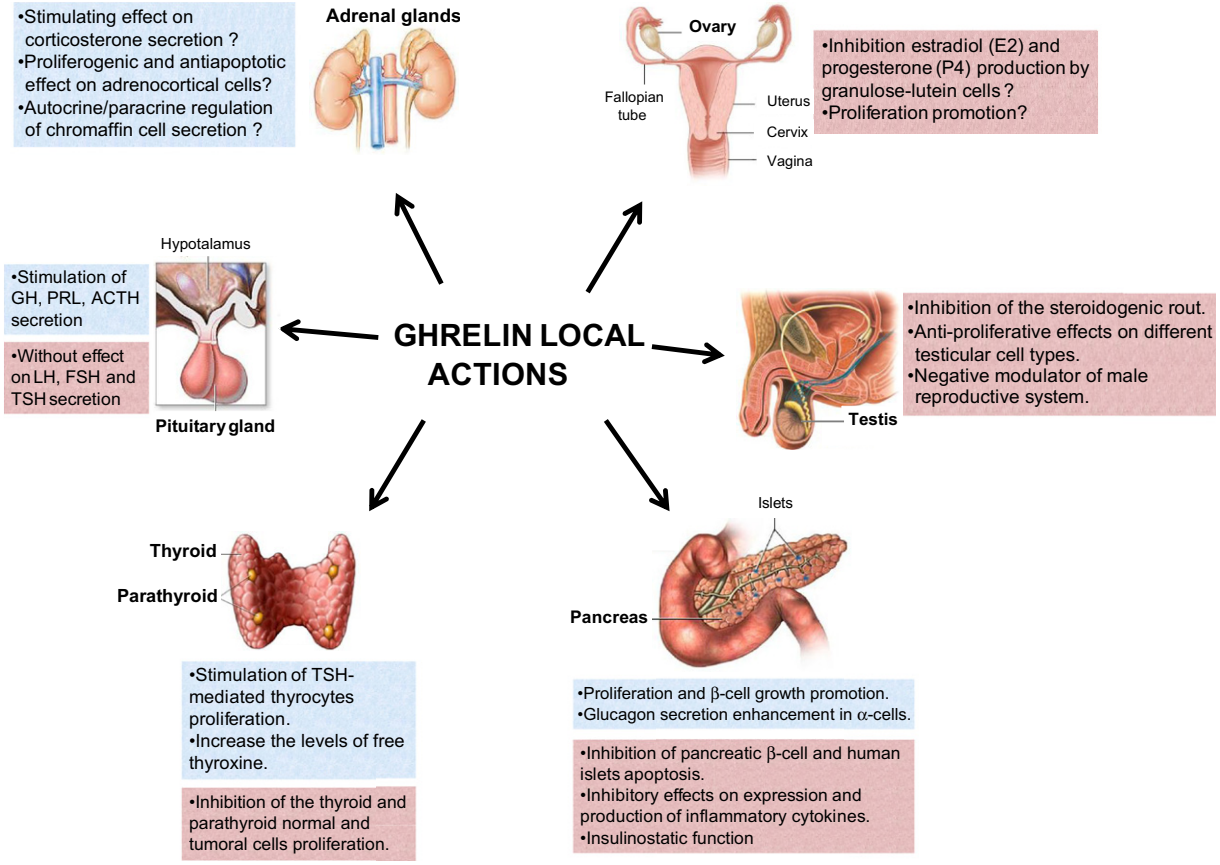

FIGURE 3 Postulated autocrine/paracrine actions of ghrelin within the endocrine glands. A question mark (?) indicates that there is controversial evidence or that the effect was not studied.

the exact role of ghrelin in the physiology and pathophysiology of endocrine glands.

REFERENCES

1. Andralojc KM, Mercalli A, Nowak KW, Albarello L, Calcagno R, Luzi L, et al. Ghrelin-producing epsilon cells in the developing and adult human pancreas. *Diabetologia* 2009;**52**:486–93.
2. Barreiro ML, Tena-Sempere M. Ghrelin and reproduction: a novel signal linking energy status and fertility? *Mol Cell Endocrinol* 2004;**226**:1–9.
3. Barreiro ML, Gaytan F, Castellano JM, Suominen JS, Roa J, Gaytan M, et al. Ghrelin inhibits the proliferative activity of immature Leydig cells in vivo and regulates stem cell factor messenger ribonucleic acid expression in rat testis. *Endocrinology* 2004;**145**:4825–34.
4. Caminos JE, Tena-Sempere M, Gaytan F, Sanchez-Criado JE, Barreiro ML, Nogueiras R, et al. Expression of ghrelin in the cyclic and pregnant rat ovary. *Endocrinology* 2003;**144**:1594–602.
5. Casanueva FF, Camina JP, Carreira MC, Pazos Y, Varga JL, Schally AV. Growth hormone-releasing hormone as an agonist of the ghrelin receptor GHS-R1a. *Proc Natl Acad Sci U S A* 2008;**105**:20452–7.
6. Casanueva FF, Dieguez C. Ghrelin: the link connecting growth with metabolism and energy homeostasis. *Rev Endocr Metab Disord* 2002;**3**:325–38.
7. Cunha SR, Mayo KE. Ghrelin and growth hormone (GH) secretagogues potentiate GH-releasing hormone (GHRH)-induced cyclic adenosine 3′,5′-monophosphate production in cells expressing transfected GHRH and GH secretagogue receptors. *Endocrinology* 2002;**143**:4570–82.
8. Date Y, Murakami N, Toshinai K, Matsukura S, Niijima A, Matsuo H, et al. The role of the gastric afferent vagal nerve in ghrelin-induced feeding and growth hormone secretion in rats. *Gastroenterology* 2002;**123**:1120–8.
9. Delhanty PJ, van Koetsveld PM, Gauna C, van de Zande B, Vitale G, Hofland LJ, et al. Ghrelin and its unacylated isoform stimulate the growth of adrenocortical tumor cells via an anti-apoptotic pathway. *Am J Physiol Endocrinol Metab* 2007;**293**:E302–9.
10. Dezaki K, Sone H, Yada T. Ghrelin is a physiological regulator of insulin release in pancreatic islets and glucose homeostasis. *Pharmacol Ther* 2008;**118**:239–49.
11. Zhou X, Xue C. Ghrelin inhibits the development of acute pancreatitis and nuclear factor kappaB activation in pancreas and liver. *Pancreas* 2009;**38**:752–7.
12. Garcia MC, Lopez M, Alvarez CV, Casanueva F, Tena-Sempere M, Dieguez C. Role of ghrelin in reproduction. *Reproduction* 2007;**133**:531–40.
13. Gaytan F, Barreiro ML, Caminos JE, Chopin LK, Herington AC, Morales C, et al. Expression of ghrelin and its functional receptor, the type 1a growth hormone secretagogue receptor, in normal human testis and testicular tumors. *J Clin Endocrinol Metab* 2004;**89**:400–9.

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14. Gaytan F, Barreiro ML, Chopin LK, Herington AC, Morales C, Pinilla L, et al. Immunolocalization of ghrelin and its functional receptor, the type 1a growth hormone secretagogue receptor, in the cyclic human ovary. *J Clin Endocrinol Metab* 2003;**88**:879–87.
15. Gaytan F, Morales C, Barreiro ML, Jeffery P, Chopin LK, Herington AC, et al. Expression of growth hormone secretagogue receptor type 1a, the functional ghrelin receptor, in human ovarian surface epithelium, mullerian duct derivatives, and ovarian tumors. *J Clin Endocrinol Metab* 2005;**90**:1798–804.
16. Granata R, Settanni F, Biancone L, Trovato L, Nano R, Bertuzzi F, et al. Acylated and unacylated ghrelin promote proliferation and inhibit apoptosis of pancreatic beta-cells and human islets: involvement of 3′,5′-cyclic adenosine monophosphate/protein kinase A, extracellular signal-regulated kinase 1/2, and phosphatidyl inositol 3-Kinase/Akt signaling. *Endocrinology* 2007;**148**:512–29.
17. Granata R, Volante M, Settanni F, Gauna C, Ghe C, Annunziata M, et al. Unacylated ghrelin and obestatin increase islet cell mass and prevent diabetes in streptozotocin-treated newborn rats. *J Mol Endocrinol* 2010;**45**:9–17.
18. Gurriaran-Rodriguez U, Al-Massadi O, Roca-Rivada A, Crujeiras AB, Gallego R, Pardo M, et al. Obestatin as a regulator of adipocyte metabolism and adipogenesis. *J Cell Mol Med* 2010;**210**:R1–7.
19. Gutierrez JA, Solenberg PJ, Perkins DR, Willency JA, Knierman MD, Jin Z, et al. Ghrelin octanoylation mediated by an orphan lipid transferase. *Proc Natl Acad Sci U S A* 2008;**105**:6320–5.
20. Howard AD, Feighner SD, Cully DF, Arena JP, Liberator PA, Rosenblum CI, et al. A receptor in pituitary and hypothalamus that functions in growth hormone release. *Science* 1996;**273**:974–7.
21. Kerem M, Salman B, Ozsoy S, Pasaoglu H, Bedirli A, Haziroglu R, et al. Exogenous ghrelin enhances endocrine and exocrine regeneration in pancreatectomized rats. *J Gastrointest Surg* 2009;**13**:775–83.
22. Kheradmand A, Roshangar L, Taati M. The role of ghrelin on the morphometry and intracellular changes in the rat testis. *Tissue Cell* 2009;**41**:105–11.
23. Korbonits M, Goldstone AP, Gueorguiev M, Grossman AB. Ghrelin—a hormone with multiple functions. *Front Neuroendocrinol* 2004;**25**:27–68.
24. Leontiou CA, Franchi G, Korbonits M. Ghrelin in neuroendocrine organs and tumours. *Pituitary* 2007;**10**:213–25.
25. Pazos Y, Alvarez CJ, Camina JP, Al-Massadi O, Seoane LM, Casanueva FF. Role of obestatin on growth hormone secretion: an in vitro approach. *Biochem Biophys Res Commun* 2009;**390**:1377–81.
26. Raghay K, Garcia-Caballero T, Bravo S, Alvarez CV, Gonzalez R, Dieguez C, et al. Ghrelin localization in the medulla of rat and human adrenal gland and in pheochromocytomas. *Histol Histopathol* 2008;**23**:57–65.
27. Raghay K, Garcia-Caballero T, Nogueiras R, Morel G, Beiras A, Dieguez C, et al. Ghrelin localization in rat and human thyroid and parathyroid glands and tumours. *Histochem Cell Biol* 2006;**125**:239–46.
28. Rucinski M, Ziolkowska A, Tyczewska M, Malendowicz LK. Expression of prepro-ghrelin and related receptor genes in the rat adrenal gland and evidences that ghrelin exerts a potent stimulating effect on corticosterone secretion by cultured rat adrenocortical cells. *Peptides* 2009;**30**:1448–55.
29. Sirotkin AV, Meszarosova M, Grossmann R, Benco A, Valenzuela F. Effect of inhibitor and activator of ghrelin receptor (GHS-R1a) on porcine ovarian granulosa cell functions. *Gen Comp Endocrinol* 2010.
30. Tena-Sempere M. Ghrelin: novel regulator of gonadal function. *J Endocrinol Invest* 2005;**28**:26–9.
31. Tong J, Prigeon RL, Davis HW, Bidlingmaier M, Kahn SE, Cummings DE, et al. Ghrelin suppresses glucose-stimulated insulin secretion and deteriorates glucose tolerance in healthy humans. *Diabetes* 2010;**59**:2145–51.
32. van der Lely AJ, Tschop M, Heiman ML, Ghigo E. Biological, physiological, pathophysiological, and pharmacological aspects of ghrelin. *Endocr Rev* 2004;**25**:426–57.
33. Viani I, Vottero A, Tassi F, Cremonini G, Sartori C, Bernasconi S, et al. Ghrelin inhibits steroid biosynthesis by cultured granulosa-lutein cells. *J Clin Endocrinol Metab* 2008;**93**:1476–81.
34. Yang J, Brown MS, Liang G, Grishin NV, Goldstein JL. Identification of the acyltransferase that octanoylates ghrelin, an appetite-stimulating peptide hormone. *Cell* 2008;**132**:387–96.
35. Zhang JV, Ren PG, Avsian-Kretchmer O, Luo CW, Rauch R, Klein C, et al. Obestatin, a peptide encoded by the ghrelin gene, opposes ghrelin's effects on food intake. *Science* 2005;**310**:996–9.

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Chapter 133

Leptin

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ABSTRACT

Adipocytes are the major source of leptin production, and plasma leptin concentrations directly correlate with the amount of total body fat. Leptin fulfills a variety of pivotal physiological functions as a signaling molecule between peripheral organs and the central nervous system, regulating numerous endocrine functions relevant for the maintenance of energy expenditure, appetite regulation, body weight control, and the function of endocrine organs. This review focuses on the description of the expression of leptin and leptin receptors and delineation of the major endocrine activities of leptin in regulating various functions of the endocrine pancreas, hypothalamus–pituitary–thyroid axis, reproductive organs, and the hypothalamus–pituitary–adrenal gland axis. In addition, well-known, less understood novel aspects, as well as controversies regarding the roles of leptin and its receptors in the pathophysiology of metabolic disorders are summarized and discussed.

INTRODUCTION

Leptin (Greek *leptos* meaning thin) was discovered in 1994 at the Rockefeller University by Jeffrey M. Friedman and coworkers during the study of leptin-deficient *ob/ob* mice. Leptin, also termed ob protein, is a hormone consisting of 167 amino acids (16 kDa) encoded by the *ob* gene. Strong leptin expression was reported in the adipocytes of white adipose tissue. In addition, leptin is also produced in much lower quantities by brown adipose tissue, placenta (syncytiotrophoblasts), ovaries, skeletal muscle, the lower part of fundic glands of the stomach, mammary epithelial cells, bone marrow, pituitary, and liver. Plasma concentrations of leptin are directly proportional to the total amount of fat in the body, suggesting that leptin plays a role in the pathophysiology of obesity.[22]

Six splice variants of leptin receptors (Ob-Ra–Ob-Rf, or LepRa–LepRf) are known, which are encoded by a single gene, Ob-R or LEPR. The "short forms" of the leptin receptor are widely expressed in many tissues, whereas the "long form" (OB-Rb, containing a longer cytoplasmic domain) is highly expressed in the CNS. Ob-Rb is a type 1 cytokine receptor, which stimulates the JAK/STAT3 pathway and PI3K,[22] possibly explaining the regulation of inflammatory processes by leptin.

Because endocrine glands highly express leptin and its receptors, this chapter focuses on the distribution and actions of leptin on the tissues of peripheral endocrine system (Fig. 1).

REGULATION OF PANCREATIC B-CELL FUNCTIONS BY LEPTIN

Leptin regulates whole body metabolism and controls the energy homeostasis. In this context, regulation of various functions of the endocrine pancreas by leptin is of important physiological relevance. Leptin receptors are expressed in pancreatic islets. In physiological concentrations, leptin decreases insulin secretion from B-cells in healthy lean and leptin-deficient *ob/ob* mice.[9,18] By contrast, leptin fails to affect the secretion of insulin from islets derived from *db/db* mice.[9] *db/db* mice fail to express functional leptin receptors, which explains the incapability of leptin to influence insulin secretion from pancreatic islets isolated from these animals.[9]

Leptin inhibits glucose-stimulated insulin secretion via cAMP/PKA and phospholipase C/protein kinase C pathways. By contrast, at low glucose levels, leptin fails to influence insulin secretion.[28] These *in vitro* observations are in agreement with the results of *in vivo* studies showing that leptin decreases plasma insulin levels only in fed, but not in fasted mice.[18]

The mechanism regulating glucose-stimulated insulin release encompasses depolarization of B-cells, as a consequence of closure of ATP-sensitive potassium channels. Hyperpolarization of cell membranes resulting from the opening of these channels by the leptin-dependent decrease in intracellular Ca^{2+} is one of the additional possible mechanisms of leptin action.[17] Furthermore, leptin decreases insulin biosynthesis by reducing proinsulin mRNA expression in rodent B-cells.[30] The suppression of insulin biosynthesis is presumably independent on the K_{ATP} channels activation because diazoxide, the K_{ATP} channel opener, has no effect on leptin-dependent suppression of insulin biosynthesis.[30]

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00133-0
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(A)

	Leptin	Ob-Rb
PANCREAS	-	+
THYROID	-	+
OVARY	+	+
PLACENTA	+	+
TESTIS	-	+
ADRENAL CORTEX	-	+
ADRENAL MEDULLA	-	+

(B)

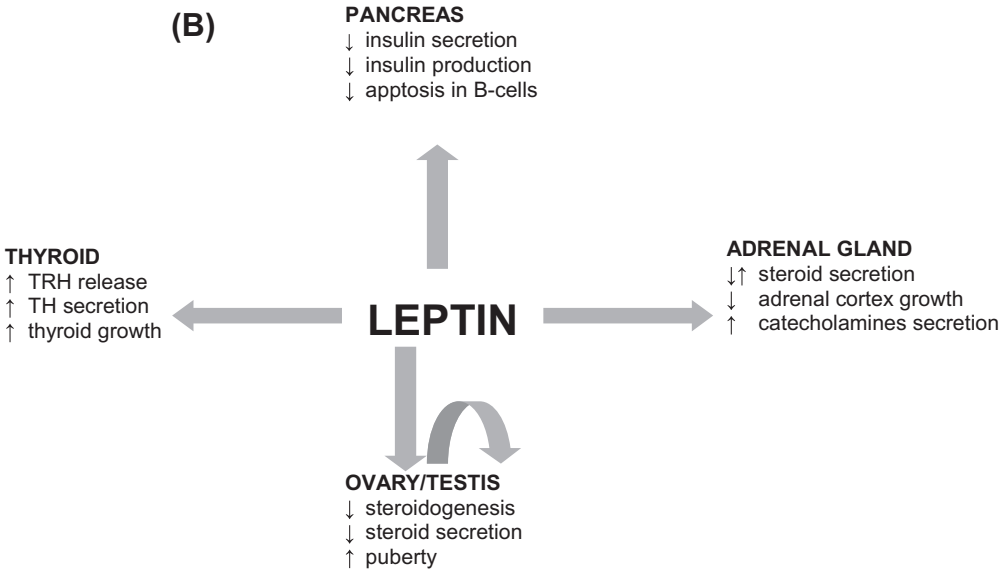

FIGURE 1 (A) Expression of leptin and its active form of the receptor (Ob-Rb) in endocrine glands; (B) effects of leptin in endocrine system (TSH, thyroid-stimulating hormone; TH, thyroid hormones).

Several studies shown that leptin displays insulinotrophic activity, by prolonging B-cell survival and protecting B-cells from apoptotic cell death. Zucker diabetic fatty rats have a full length but dysfunctional leptin receptor resulting from a mutation. In comparison with Zucker fatty or lean rats, Zucker fatty rats with type 2 diabetes mellitus have markedly lower B-cell mass, which results primarily from the increased rate of B-cell death.[27] Increased triglyceride accumulation in B-cells in type 1 and type 2 diabetes mellitus accelerates B-cell death. Leptin can decrease lipid content in B-cells by accelerating the intracellular fatty acid oxidation.[31] Noteworthy, leptin decreases insulin-stimulated glucose transport, lipogenesis and protein synthesis in isolated rat adipocytes.[24] One of the possible mechanisms by which leptin affects the anabolic activities of insulin is the attenuation of insulin binding, as reported in isolated hepatocytes.[26] Thus, leptin influences B-cell functions by controlling the secretion, and biosynthesis of insulin. In addition, leptin modulates the anabolic activities of insulin. These activities of leptin are relevant in the context of regulation of glucose homeostasis.

ROLE OF LEPTIN IN REGULATING THYROID FUNCTIONS

The hypothalamus–pituitary–thyroid (HPT) axis plays a significant role in controlling energy expenditure and whole body metabolism. Therefore, the modulation of HPT axis by peripheral signals of energy deposition has a pivotal relevance for the maintenance of metabolic homeostasis.

Leptin stimulates thyroid functions by increasing thyrotropin-releasing hormone (TRH) secretion.[19] Leptin also increases the growth of the thyroid gland tissue and stimulates the secretion of thyroid hormones (TH).[19,25] In the hypothalamus, leptin induces STAT 3 phosphorylation of Ob-Rb via Y1138 in nuclei of TRH neurons.[15] This observation suggests that STAT 3 is critical in mediating the effects of leptin on preproTRH gene expression. Indeed, stimulation of preproTRH gene expression by leptin via the STAT 3 response element, which is located in the preproTRH promoter, was confirmed by the use of heterologous expression system (transfected 293T cells).[13] In addition, STAT 3 mediates the transcriptional activity of leptin *in vivo*.[12] Moreover, systemic leptin administration into fasting animals not only

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increases preproTRH mRNA in the PVN but also fully restores thyroid hormone levels and TRH to normal or even higher levels.[19]

Animal studies have shown that prolonged fasting is accompanied by the fall of circulating leptin, and that fasting lowers circulating T3 and T4 levels at low or normal TSH levels are restored to normal after leptin injections.[19] Leptin administration in hyperthyroid rats is followed by an increase in serum TSH concentrations.

Rat thyroid gland expresses the long form of leptin receptor (Ob-Rb) mRNA. Furthermore, Ob-Rb-like immunoreactivity is found in membranes of the thyroid epithelial cells and in C-cells of the gland.[25] These findings suggest that leptin can directly act on the thyroid gland.

Acute leptin administration into animals does not affect thyroid ^{125}I uptake; however, serum TSH, T3, and T4 concentrations are increased. In contrast, no changes in serum TSH and TSH secretion *in vitro* were observed in leptin-treated hypothyroid rats.[6] Furthermore, chronic continuous leptin infusion in rats lowers plasma concentrations of TSH, while those of thyroid hormones (free T3 and total T4) are raised.[25] Contrary to the *in vivo* results, *in vitro* studies on rat FRTL-5 cells show that leptin reduces iodine uptake.[16]

Apart from influencing the HPT-thyroid axis, leptin modulates the monodeiodinases in various tissues, depending on the energetic status.[12] Thus, leptin controls energy expenditure at the level of TH secretion and TH synthesis by acting on the HPT-TH axis and by controlling the conversion of TH in numerous extrathyroidal tissues.

LEPTIN AND THE REPRODUCTIVE SYSTEM

Leptin Expression in Reproductive System

Leptin is expressed in the reproductive tissues in rodents and humans, including ovary, placenta, and also in the fetus. On the cellular level, leptin is expressed in murine trophoblastic cells and in human trophoblastic placental cell lines JAR and JEG-3. *In situ* hybridization and immunohistochemistry reveal leptin-immunoreactivity in murine fetus including cartilage and bone, brain, heart, liver, and hair follicles.[22] Within the ovaries leptin is expressed in steroid-producing cells including granulosa, thecal, and luteal cells as well as in interstitial cells of rodents, and in both healthy women and patients with polycystic ovary syndrome (PCOS).[22]

Ob-Rs Expression in Reproductive Tissues

Six Ob-Rs isoforms are known, which are differentially expressed in numerous mammalian peripherial tissues, including heart, liver kidneys, lungs, small intestine, placenta, pancreas, adrenal gland, ovaries, testes, and adipose tissue. Within the ovarian follicles, functional Ob-Rb isoform is detected in steroidogenic granulosa (GC) and theca cells of many examined mammalian species.[22] Moreover, after ovulation, luteal cells occur, which also express leptin receptor.

The expression of the long Ob-Rb and short Ob-Ra isoforms vary throughout the rat estrous cycle. Low expression of both isoforms reported in proestrus and diestrus II rather than in estrus and diestrus I could result from changes of plasma concentrations of steroids and leptin as well as from responses to the changing environment of the ovary.[7] *In situ* hybridization reveals leptin receptor expression in spermatic and Leydig cells in murine testis.[4] In mouse testis germ cells, the Ob-R immunoreactivity shows stage- and age-dependent variability, suggesting that the leptin system may play a role in regulating the proliferation and differentiation of germ cells.[8]

Leptin Function in Ovaries and Testis

The majority of studies show that leptin inhibits ovarian steroidogenesis. *In vitro*, leptin directly suppresses IGF-1-, TGF-beta-, and glucocorticoid–stimulated secretion of steroids from GC.[3] Downregulation of Ob-Rb expression in ovarian cells with the consecutive restriction of its binding sites during the estrous cycle may represent a compensatory mechanism to attenuate the inhibition of steroidogenesis by leptin.

In contrast to studies in isolated GC or TC cells, Gregoraszczuk et al. showed differential effects of leptin on estradiol secretion in whole follicles of different sizes obtained through the porcine estrus cycle.[11] Leptin fails to alter estradiol secretion from small- and moderate-sized follicles. However, in cultured large, preovulatory follicles leptin inhibits basal, GH- and IGF-1 stimulated estradiol secretion, and increases progesterone secretion. These results suggest that leptin may promote the luteinization process.

Numerous reports indicate that leptin participates in controlling the growth and maturation of ovarian follicles and acts as a proliferative factor.[1] In healthy women and patients with PCOS leptin levels in follicular fluids serve as a predictor of successful pregnancy.[21] Leptin can cross the murine blood–testis barrier[2] and has the ability to modulate the paracrine network that controls testicular steroidogenesis.[4,34] In primary cultured rat Leydig cells and the Leydig tumor cell line (MLTC-1), leptin inhibits LH-[4] and hCG-stimulated[34] testosterone secretion.

Leptin Controls Fertility and Puberty

Genetically obese ob/ob mice of both, male and female sexes, are infertile. The infertility is reversible upon administration of exogenous leptin,[5] which restores ovulation,

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pregnancy, parturition, and lactation. Leptin induces an early puberty in healthy mice, indicating that leptin acts as a signal initiating the onset of puberty.

Role of Leptin in Pregnancy

During pregnancy, hyperleptinemia occurring in the second and third trimesters is not associated with physiological changes of food intake or energy expenditure.[23]

Because leptin is expressed in placenta and in many fetal tissues, it was hypothesized that leptin may act as a growth factor and serve as a messenger controlling energy status between mother and a fetus. In the placenta, leptin probably acts as an auto- and/or paracrine manner because syncytiotrophoblasts express leptin receptor.[29]

REGULATION OF ADRENAL GLANDS BY LEPTIN

The interactions of peptides regulating food intake, and especially leptin with the hypothalamus–pituitary–adrenal (HPA) axis are of great physiological relevance because glucocorticoids hormones are known to be involved in the control of energy homeostasis and adipogenesis. In addition, a strong correlation between serum concentrations of cortisol and leptin is well documented. Several reports clearly show that leptin modulates the functions of both the HPA axis and the systemic symphatic/adrenomedullary (SS/AM) system.[20] This part focuses on reviewing the interaction of leptin with the adrenal gland.

Localization of Leptin in Adrenal Glands

In contrast to the remaining members of HPA axis (hypothalamus and pituitary) ob mRNA is not expressed in adrenal glands, neither in adrenocortical nor medullary cells. Therefore, paracrine or autocrine mechanisms of leptin's action on the adrenal glands are unlikely.[20] Remarkably, adrenal glands are naturally surrounded by adipose tissue and presumably exposed to high local concentrations of leptin. In addition, some evidence suggests that leptin is expressed in intra-adrenal mast cells.[20]

Localization Ob-R Protein in Adrenal Glands

Leptin receptors (Ob-Rs) are also present in the adrenal glands of humans, rats, pigs, and cows, suggesting a direct regulation of cortex and medullary adrenal tissues functions by leptin.[20] Real-time PCR studies show the expression of Ob-Ra, Ob-Rb, Ob-Rc, Ob-Re, and Ob-Rf in both adrenal cortex and medulla.[20] Except for Ob-Re, the level of expression of all ObR isoforms is significantly higher in the medulla.[20]

Role of Leptin in Regulating Adrenal Cortex Functions

Growing evidence from *in vivo* studies collectively shows that leptin inhibits the HPA axis activity in humans, monkeys, sheep, mice, and rats (for detailed review see Ref. 20). It should be noted that the net effects of leptin reported in *in vivo* studies represent the sum of complex direct or indirect interactions of leptin with central and peripheral organs. In light of the rather scarce data available to date, the mechanisms of a direct interaction of leptin with the receptors of adrenocorticals cells remain not well understood. The initial studies performed by Malendowicz et al.[20] reveal that leptin upregulates basal but not ACTH-stimulated steroid secretion in dispersed rat adrenocorticals cell cultures. On the contrary, Bornstein and colleagues reported that leptin inhibits both basal and ACTH-stimulated steroid release from cultured human and bovine primary adrenocorticals cells, and the NCI-H295 cell line.[10] In addition to the regulation of steroid secretion, leptin has antiproliferative activity by suppressing the growth of the adrenal cortex in the rats.[20] More recently, the mechanism of leptin-dependent inhibition of ACTH controlled adrenal steroidogenesis was proposed.[14] The mechanism encompasses modulation of ACTH-regulated JAK2-PI3K/Akt-PDE3-cAMP pathways with the consecutive downregulation of the expression of steroidogenic genes.[14]

Role of Leptin in Regulating the Functions of the Adrenal Medulla

There are few studies available that assess the functional interaction between leptin and adrenal catecholamines. Most of the studies were performed in cultured cells, showing that leptin stimulates the synthesis and secretion of catecholamines.[33] The mechanism of action encompasses the activation of the voltage-gated Ca^{2+} L- and N-type channels.[32]

Role of Leptin in Pathophysiological Conditions

Leptin has been reported to dampen the HPA-axis responses (ACTH and/or corticosteroids) to various stressors with the exception of cold stress.[20] However, mechanisms underlying this regulation remain unclear.

Several studies (extensively reviewed in Ref. 20) suggested that leptin may play a role in the pathophysiology of adrenal tumorigenesis by affecting the growth, proliferation, and secretion processes. However, the results of the studies are ambiguous.

In obesity, the ability of leptin to decrease food intake is impaired. Obese individuals frequently develop central leptin resistance. It has been proposed that the attenuated

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leptin sensitivity in the adrenal medulla could contribute to the higher catecholamine production previously observed after neonatal hyperleptinemia. Later, it was shown that neonatal hyperleptinemia leads to medullary adrenal hypertrophy and high catecholamine production.[35] These effects do not seem to result from a direct action of leptin on the medulla because the leptin signaling pathways are down-regulated in this tissue, and leptin treatment in adulthood has limited effects.[35]

CONCLUSIONS

The discovery of leptin about 2 decades ago allowed a better understanding of the regulation of crucial physiological processes including food intake, body weight control, and maintenance of energy homeostasis. Because leptin was one of the first adipokines described in fat cells, the discovery led to a better understanding of the adipose tissue compartment serving not only as a simple storage of excessive energy as lipids, but also playing a considerable role as "endocrine active tissue" that influences numerous physiological functions in the peripheral and central nervous system. Because of its well-documented impact on the endocrine pancreatic B-cells, leptin can control glucose homeostasis and play a role in the pathophysiology of type 2 diabetes mellitus, at least in animal models of this disease. Resulting from its interaction with organs such as the pituitary, regulation of the HPA axis activity, and modulation of the fate of pituitary hormones in peripheral tissues, leptin plays a considerable role in regulating energy expenditure and obesity. Importantly, leptin serves as a "messenger molecule" between peripheral organs (most importantly fat cells) and the central nervous system, which is of crucial relevance as a feedback mechanism for the control and maintenance of metabolic homeostasis. Lastly, leptin regulates the function of ovaries and testes and plays an important role in controlling fertility and puberty. Although the wide range of pleiotropic endocrine activities of leptin is well known, the role of this adipokine in the context of neoplastic (see the Leptin chapter in the Cancer/Anticancer section of this book) and hormonal disorders, with the resulting concomitant potential introduction into therapy remains to be established.

REFERENCES

1. Almog B, Gold R, Tajima K, Dantes A, Salim K, Rubinstein M, et al. Leptin attenuates follicular apoptosis and accelerates the onset of puberty in immature rats. *Mol Cell Endocrinol* 2001;**183**:179–91.
2. Banks WA, McLay RN, Kastin AJ, Sarmiento U, Scully S. Passage of leptin across the blood–testis barrier. *Am J Physiol* 1999;**276**: E1099–104.
3. Barkan D, Jia H, Dantes A, Vardimon L, Amsterdam A, Rubinstein M. Leptin modulates the glucocorticoid-induced ovarian steroidogenesis. *Endocrinology* 1999;**140**:1731–8.
4. Caprio M, Isidori AM, Carta AR, Moretti C, Dufau ML, Fabbri A. Expression of functional leptin receptors in rodent Leydig cells. *Endocrinology* 1999;**140**:4939–47.
5. Chehab FF, Lim ME, Lu R. Correction of the sterility defect in homozygous obese female mice by treatment with the human recombinant leptin. *Nat Genet* 1996;**12**:318–20.
6. de Oliveira E, Teixeira Silva Fagundes A, Teixeira Bonomo I, Curty FH, Fonseca Passos MC, de Moura EG, et al. Acute and chronic leptin effect upon in vivo and in vitro rat thyroid iodide uptake. *Life Sci* 2007;**81**:1241–6.
7. Duggal PS, Weitsman SR, Magoffin DA, Norman RJ. Expression of the long (OB-RB) and short (OB-RA) forms of the leptin receptor throughout the oestrous cycle in the mature rat ovary. *Reproduction* 2002;**123**:899–905.
8. El-Hefnawy T, Ioffe S, Dym M. Expression of the leptin receptor during germ cell development in the mouse testis. *Endocrinology* 2000;**141**:2624–30.
9. Emilsson V, Liu YL, Cawthorne MA, Morton NM, Davenport M. Expression of the functional leptin receptor mRNA in pancreatic islets and direct inhibitory action of leptin on insulin secretion. *Diabetes* 1997;**46**:313–6.
10. Glasow A, Bornstein SR. Leptin and the adrenal gland. *Eur J Clin Invest* 2000;**30**(Suppl. 3):39–45.
11. Gregoraszczuk EL, Ptak A, Wojtowicz AK, Gorska T, Nowak KW. Estrus cycle-dependent action of leptin on basal and GH or IGF-I stimulated steroid secretion by whole porcine follicles. *Endocr Regul* 2004;**38**:15–21.
12. Guo F, Bakal K, Minokoshi Y, Hollenberg AN. Leptin signaling targets the thyrotropin-releasing hormone gene promoter in vivo. *Endocrinology* 2004;**145**:2221–7.
13. Harris M, Aschkenasi C, Elias CF, Chandrankunnel A, Nillni EA, Bjoorbaek C, et al. Transcriptional regulation of the thyrotropin-releasing hormone gene by leptin and melanocortin signaling. *J Clin Invest* 2001;**107**:111–20.
14. Hsu HT, Chang YC, Chiu YN, Liu CL, Chang KJ, Guo IC. Leptin interferes with adrenocorticotropin/3',5'-cyclic adenosine monophosphate (cAMP) signaling, possibly through a Janus kinase 2-phosphatidylinositol 3-kinase/Akt-phosphodiesterase 3-cAMP pathway, to down-regulate cholesterol side-chain cleavage cytochrome P450 enzyme in human adrenocortical NCI-H295 cell line. *J Clin Endocrinol Metab* 2006;**91**:2761–9.
15. Huo L, Munzberg H, Nillni EA, Bjorbaek C. Role of signal transducer and activator of transcription 3 in regulation of hypothalamic trh gene expression by leptin. *Endocrinology* 2004;**145**: 2516–23.
16. Isozaki O, Tsushima T, Nozoe Y, Miyakawa M, Takano K. Leptin regulation of the thyroids: negative regulation on thyroid hormone levels in euthyroid subjects and inhibitory effects on iodide uptake and Na+/I- symporter mRNA expression in rat FRTL-5 cells. *Endocr J* 2004;**51**:415–23.
17. Kieffer TJ, Heller RS, Leech CA, Holz GG, Habener JF. Leptin suppression of insulin secretion by the activation of ATP-sensitive K+ channels in pancreatic beta-cells. *Diabetes* 1997;**46**: 1087–93.
18. Kulkarni RN, Wang ZL, Wang RM, Hurley JD, Smith DM, Ghatei MA, et al. Leptin rapidly suppresses insulin release from insulinoma cells, rat and human islets and, in vivo, in mice. *J Clin Invest* 1997;**100**:2729–36.

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19. Legradi G, Emerson CH, Ahima RS, Flier JS, Lechan RM. Leptin prevents fasting-induced suppression of prothyrotropin-releasing hormone messenger ribonucleic acid in neurons of the hypothalamic paraventricular nucleus. *Endocrinology* 1997;**138**:2569–76.
20. Malendowicz LK, Rucinski M, Belloni AS, Ziolkowska A, Nussdorfer GG. Leptin and the regulation of the hypothalamic–pituitary–adrenal axis. *Int Rev Cytol* 2007;**263**:63–102.
21. Mantzoros CS, Cramer DW, Liberman RF, Barbieri RL. Predictive value of serum and follicular fluid leptin concentrations during assisted reproductive cycles in normal women and in women with the polycystic ovarian syndrome. *Hum Reprod* 2000;**15**:539–44.
22. Margetic S, Gazzola C, Pegg GG, Hill RA. Leptin: a review of its peripheral actions and interactions. *Int J Obes Relat Metab Disord* 2002;**26**:1407–33.
23. Masuzaki H, Ogawa Y, Sagawa N, Hosoda K, Matsumoto T, Mise H, et al. Nonadipose tissue production of leptin: leptin as a novel placenta-derived hormone in humans. *Nat Med* 1997;**3**:1029–33.
24. Muller G, Ertl J, Gerl M, Preibisch G. Leptin impairs metabolic actions of insulin in isolated rat adipocytes. *J Biol Chem* 1997;**272**:10585–93.
25. Nowak KW, Kaczmarek P, Mackowiak P, Ziolkowska A, Albertin G, Ginda WJ, et al. Rat thyroid gland expresses the long form of leptin receptors, and leptin stimulates the function of the gland in euthyroid non-fasted animals. *Int J Mol Med* 2002;**9**:31–4.
26. Nowak KW, Mackowiak P, Nogowski L, Szkudelski T, Malendowicz LK. Acute leptin action on insulin blood level and liver insulin receptor in the rat. *Life Sci* 1998;**63**:1347–52.
27. Pick A, Clark J, Kubstrup C, Levisetti M, Pugh W, Bonner-Weir S, et al. Role of apoptosis in failure of beta-cell mass compensation for insulin resistance and beta-cell defects in the male Zucker diabetic fatty rat. *Diabetes* 1998;**47**:358–64.
28. Poitout V, Rouault C, Guerre-Millo M, Briaud I, Reach G. Inhibition of insulin secretion by leptin in normal rodent islets of Langerhans. *Endocrinology* 1998;**139**:822–6.
29. Senaris R, Garcia-Caballero T, Casabiell X, Gallego R, Castro R, Considine RV, et al. Synthesis of leptin in human placenta. *Endocrinology* 1997;**138**:4501–4.
30. Seufert J, Kieffer TJ, Habener JF. Leptin inhibits insulin gene transcription and reverses hyperinsulinemia in leptin-deficient ob/ob mice. *Proc Natl Acad Sci USA* 1999;**96**:674–9.
31. Shimabukuro M, Koyama K, Chen G, Wang MY, Trieu F, Lee Y, et al. Direct antidiabetic effect of leptin through triglyceride depletion of tissues. *Proc Natl Acad Sci USA* 1997;**94**:4637–41.
32. Takekoshi K, Ishii K, Kawakami Y, Isobe K, Nanmoku T, Nakai T. Ca(2+) mobilization, tyrosine hydroxylase activity, and signaling mechanisms in cultured porcine adrenal medullary chromaffin cells: effects of leptin. *Endocrinology* 2001;**142**:290–8.
33. Takekoshi K, Motooka M, Isobe K, Nomura F, Manmoku T, Ishii K, et al. Leptin directly stimulates catecholamine secretion and synthesis in cultured porcine adrenal medullary chromaffin cells. *Biochem Biophys Res Commun* 1999;**261**:426–31.
34. Tena-Sempere M, Pinilla L, Gonzalez LC, Dieguez C, Casanueva FF, Aguilar E. Leptin inhibits testosterone secretion from adult rat testis in vitro. *J Endocrinol* 1999;**161**:211–8.
35. Trevenzoli IH, Pinheiro CR, Conceicao EP, Oliveira E, Passos MC, Lisboa PC, et al. Programming of rat adrenal medulla by neonatal hyperleptinemia: adrenal morphology, catecholamine secretion, and leptin signaling pathway. *Am J Physiol Endocrinol Metab* 2010;**298**:E941–9.

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Chapter 134

ANP

Jolanta Gutkowska

ABSTRACT

Atrial natriuretic peptide (ANP) was the first member of the natriuretic peptide (NP) family isolated from heart tissue, and since its discovery, other members of this family, including BNP, CNP, urodilatin, and DNP were isolated from the brain, kidney cells, and snake venom. The physiological actions of NPs are mediated by two guanylyl cylase (GC) receptors, known as A- and B-type, and a third receptor called "clearance" C receptor, whose main role was initially believed to internalize and degrade NPs. However, recent results suggest that the C receptor may mediate physiological actions of NPs. Activation of GC receptors leads to the increase in cGMP and subsequent intracellular events. NPs were originally associated with cardiovascular homeostasis, but their presence and the distribution of their cognate receptors in a various organs suggest a much broader range of functions. Indeed, NPs have antiproliferative, antifibrotic, anti-inflammatory, insulin-like functions, and are involved in the regulation of bone growth. NPs are essential for the growth and maturation of ovarian follicles and act as regulators of various functions at the level of several glands.

DISCOVERY

The existence in the heart of a hormone regulating blood volume was predicted long ago before its discovery. Pioneering studies by Gauer and Henry[13] advanced the concept that volume regulation is an integral part of an overall cardiovascular system. The search for a natriuretic hormone was stimulated by the experiments of de Wardener et al.[7] who observed a potent natriuretic effect in renal artery-clamped dogs, which could not be explained by a change in aldosterone levels or in glomerular filtration rate, which remained constant under these experimental conditions. With the advent of electron microscopy, discovery and characterization of specific atrial granules, presumably of a secretory nature, gave new direction to the search for a natriuretic hormone. Atrial granularity was shown to depend on both the hydromineral status of the body and intact adrenal function. The relevance of these observations was confirmed by de Bold et al.[5] who performed landmark experiments and reported that, in anesthetized rats, injection of rat atrial tissue extract caused rapid but short duration diuresis and natriuresis.

The discovery of the cardiac hormone, ANP, was considered a major breakthrough in the search for natriuretic hormones.

STRUCTURE OF THE PRECURSOR mRNA GENE

ANP was originally purified from the rat heart, and cDNA clones encoding the ANP precursor were isolated from atrial cDNA libraries. In the adult heart, ANP gene is expressed predominantly in the atria where ANP mRNA represents 1–3% of total mRNA.

ANP is the first member of the NP family, which consists of four other peptides: BNP and CNP, both isolated from the porcine brain.[31] The BNP and CNP genes seem to each synthesize only one peptide hormone, BNP-32 and CNP-22, from their respective biosynthetic precursors, i.e., (prepro) $BNP_{1–108}$ and (prepro) $CNP_{1–103}$. The fourth peptide of this family, a 32-amino acid peptide known as urodilatin is a product of alternative processing of proANP in kidney cells and found in urine. The recently discovered dendroaspis-type natriuretic peptide (DNP) is a 38-amino acid peptide isolated from the venom of the *Dendroaspis angusticeps* snake, which has a structural and functional similarity to other NPs. Like ANP and BNP, DNP activates the GC-A receptor resulting in natriuretic and diuretic effects. A DNP-like peptide has been reported to be present in human plasma and heart and is elevated in human congestive heart failure.

All NPs have structural homology in the 17-amino acid ring formed by a disulfide bond between cysteine residues at positions 105 and 121 in humans. The biological activity of the peptides depends on the integrity of the ring structure (Fig. 1). The ANP structure is highly conserved during evolution, and there is a difference of only one amino acid between human and rat ANP. Methionine in human ANP replaces isoleucine in rats in position 110.[23] The gene encoding proANP consists of three coding exon sequences separated by two intron sequences (Fig. 2).

All NPs are synthesized by different genes and stored in the form of prohormones: ANP (126 amino acids), BNP

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00134-2
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(108 amino acids), CNP (126 amino acids) in secretory granules. However, the DNP prohormone gene structure is still unknown in humans. The ANP gene is present not only in the animal kingdom but also in plants. The pro-ANP gene is located on the distal short arm of chromosome 1 in band 1P36 in humans and on chromosome 4 in mice.

The availability of ANP-specific antibodies allowed the development of immunocytochemical and radioimmunoassay analyses for ANP. Further developments in molecular biology led to the mapping of ANP-expressing tissue. With these tools, ANP has also been detected in several extraatrial tissues,[16] including brain, lungs, endocrine, and exocrine glands where it exerts physiological paracrine or autocrine effects. ANP is highly expressed in various regions of the brain, particularly in the rostral diencephalon, and the ability of NPs to interact with the secretion of hypothalamic neuroendocrine systems regulating, and various hypothalamic releasing and inhibiting hormones has been established.[27] Furthermore, ANP is synthesized and secreted by rat adrenal chromaffin cells and is detected in peripheral ganglia, superior cervical nodosa, and in the cell bodies of superior celiac mesenteric ganglia.[16]

ANP prohormone is present in the blood vessels, namely, in inferior and superior vena cavae, extrapulmonary and intrapulmonary veins and in the smaller vasculature of the lungs, from which ANP is released as a 28-amino acid peptide. The digestive system and gastrointestinal tract contain NPs with functions well established, as mentioned later in this chapter. Linkage between ANP and the immune system has been supported by the demonstration of ANP in thymus and lymphoid tissues.[33] Other tissues, such as the thyroid, male and female reproductive organs, and liver contain NPs, and in some of them, a physiological role of NPs has been postulated.

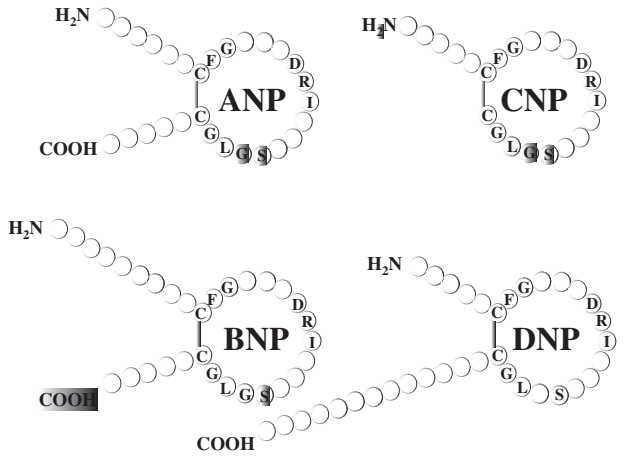

FIGURE 1 Primary structure of the NP family. Each peptide consists of a highly conserved 17 aa ring created by a disulfide bond which is required for receptor binding. Marked residues in the circles denote aa that are conserved between peptides.

PROCESSING

Since the discovery of ANP, enormous progress has been made in identifying the structural and functional properties of this peptide. The human ANP precursor peptide gene encodes a 151-amino acid preprohormone, a molecule that contains a 25-amino acid signal peptide sequence. The signal peptide is cleaved during the transport of the peptide across the endoplasmic membrane reticulum producing a prohormone $ANP_{(1–126)}$. The final proteolytic cleavage of $ANP_{(1–126)}$ to the major, biologically active peptide $ANP_{(99–126)}$ takes place during or just after the release of the peptide by exocytosis in secretory vesicles, which are transferred to the plasma membrane after increases in levels of cytoplasmic calcium. Several groups have purified biologically active ANP peptides of different lengths from rat atrial homogenates. These other "related" peptides are most probably artefacts of proteolytic digestion during purification. Several enzymes have been shown to convert $proANP_{(1–126)}$ to $ANP_{(99–126)}$, such as carboxypeptidases, kallikrein, trypsin, thrombin, corine, and others, but those of physiological relevance have not yet been established.[23]

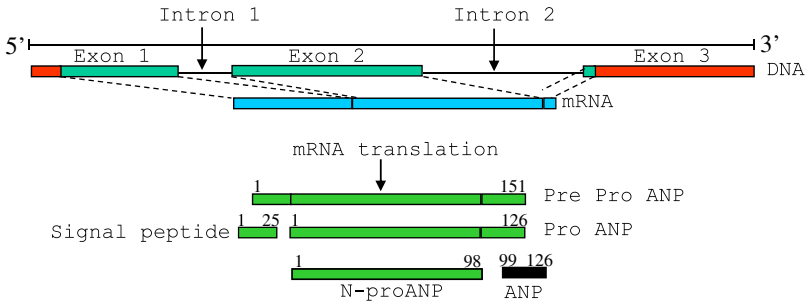

FIGURE 2 Structure of the ANP gene. ANP is synthesized as a prepropeptide from mRNA originating from three exons of ANP gene. Prepropeptide and its derivative ANP propeptide are stored in the cells. Proteolytic cleavage of the propeptide sequences results in the formation of mature (low molecular mass) $ANP_{99–126}$ and N-terminal $N\text{-}proANP_{1–98}$.

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RELEASE OF NPs

ANP release is controlled by many neural and hormonal factors, but the principal stimulus controlling ANP synthesis and release is atrial and ventricular wall stretch, resulting from volume expansion. Angiotensin II, endothelin, glucocorticoids, norepinephrine, oxytocin, arginine–vasopressin, morphine, and other factors, acting via hemodynamic stress or by direct effects on cardiac cells, have been shown to stimulate ANP release.[23]

RECEPTORS AND SIGNALING CASCADE

Molecular cloning techniques revealed the existence of three NP receptors, two, membrane GCs with tyrosine-like kinase domain GC-A and GC-B and C or "clearance" receptors, which exhibit distinct structural features and are widely distributed and display different affinities to the members of the NP family. ANP and BNP bind with higher affinity to GC-A and GC-B, whereas CNP binds to GC-B. All NPs bind with a relatively similar affinity to a third, very abundant C receptor whose role as originally believed was to internalize and degrade NPs (Fig. 3). However, recent results revealed that the GC-C receptor is a G protein-coupled receptor with an intracellular domain coupled to adenylyl cyclase inhibition through an inhibitory guanine nucleotide regulatory protein and/or to phospholipase C.[2] Therefore, the C receptor may mediate the biological actions of NPs by the inhibition of cAMP or stimulation of phosphoinositide hydrolysis. In the intestine and kidney, the activation of GC-C by intestinal peptide uroguanylin induces diuretic and natriuretic activity, providing an endocrine link between the intestines and kidney to modulate renal function in response to oral salt load.[17] The GC receptors belong to a family of seven isoforms of transmembrane enzymes, which convert guanosine triphosphate into cyclic 3′.5′ guanosine monophosphate (cGMP). The activation of intracellular cGMP subsequently modulates the activity of regulatory proteins such as cGMP-regulated phosphodiesterases, ion channels, and cGMP-dependent protein kinases types I and II. These proteins, which are differentially expressed in various cell types, modify the cellular functions of cGMP. There is considerable homology between guanylyl cyclase-containing receptors and between the species for particular receptors.

BIOLOGICAL ACTIVITIES OF NPs

Although ANP was found to be a potent diuretic and natriuretic substance, ANP and other peptides of this family are now recognized as hormones with a wide spectrum of central and peripheral, autocrine/paracrine actions, including cell

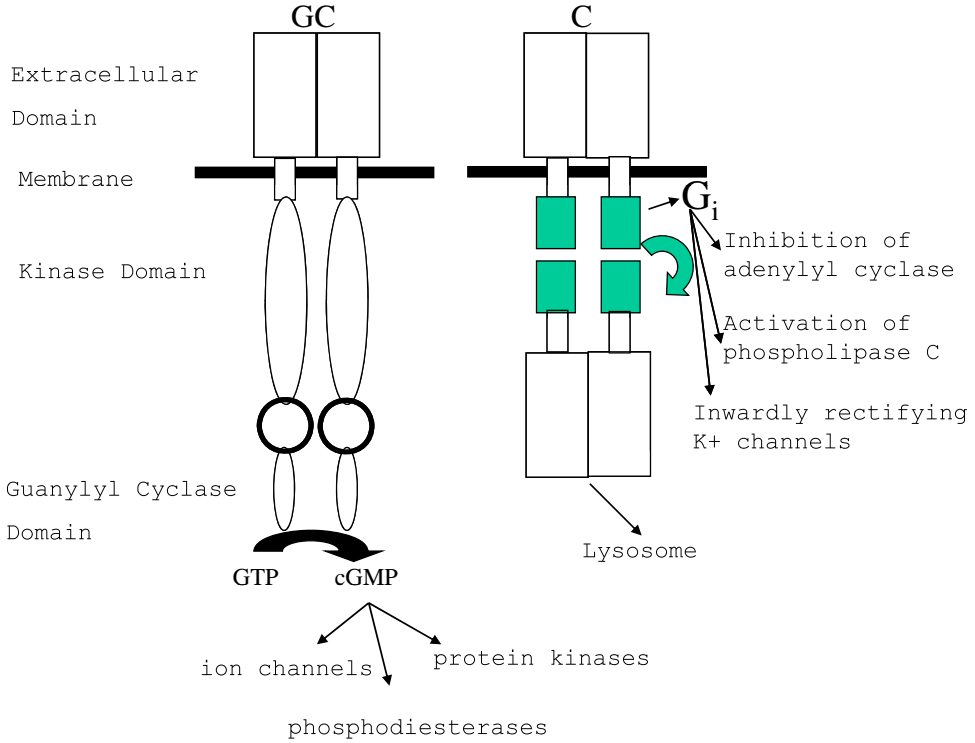

FIGURE 3 Natriuretic peptide receptor subtypes and their signaling pathways. GC-A, GC-B are particulate GC receptor producing cGMP. Receptor-C internalizes bound NPs and subjects them to lysosomal degradation. Receptor-C can also lead to G protein-coupled inhibition of adenylyl cyclase, activation of phospholipase C and G protein-gated inwardly rectifying K+ channels.

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proliferation and differentiation in various tissues, regulation of reproductive function, and bone formation.

Heart

Several studies have established that ANP, by activating the GC-A/cGMP pathway, regulates blood pressure and the cardiorenal axis through its ability to antagonize the renin–angiotensin–aldosterone system and sympathetic nervous systems, or through its direct vasodilatory and natriuretic responses, modulating endothelial permeability and fluid shift from vascular beds into the lymphatic system.

NPs exert several and important autocrine and paracrine actions within the heart and coronary circulation. All NPs have effects on cardiac contractility, considered as being negatively inotropic, although conflicting results have been reported. NPs are involved in the regulation of coronary blood flow, cardiomyocyte growth, inhibition of cardiac fibroblast proliferation, and extracellular matrix secretion. Robust cardioprotective and anti-ischemic effects as well as a vasorelaxant influence in the coronary circulation have been documented.[17] Most of these diverse physiological actions within the heart are mediated by cGMP.[4]

The generation of animal models with deletion or overexpression of genes has given a new insight into the molecular physiology and diverse biological functions of NPs and their receptors. The first GC-A and ANP knockout mice showed that this hormonal system is not only essential for the maintenance of blood pressure and volume homeostasis but also has local growth-mediated functions. Mice lacking the GC-A receptor present marked cardiac hypertrophy and fibrosis, disproportionate to their increase in blood pressure, and chronic antihypertensive treatment of these animals normalizes blood pressure without a marked effect on cardiac hypertrophy.[20] The targeted deletion of the BNP gene has indicated that BNP may act as a local antifibrotic factor and may be involved in the mechanism that inhibits the development of cardiac hypertrophy and fibrosis.[17] Further studies with deletion of CNP, BNP, or GC-B receptor showed that these natriuretic peptides are in fact unlikely regulators of volume and sodium excretion, but exert autocrine and paracrine effects mediated by cGMP on cellular proliferation and differentiation in the different tissues, in particular vasculature, bones, and fibroblasts.[17]

Endocrine and Exocrine Pancreas

All NPs and their receptors are expressed in the pancreas and gastrointestinal tract.[26] Rat pancreatic islet α and β cells, and insulinoma cells (INS-1E) express GC-A receptor, whereas C receptors are only expressed in pancreatic α cells.

The close link between ANP and diabetes mellitus has been shown.[18] Acute hyperglycemia in the type 1 diabetic patient is associated with increased ANP mRNA and plasma ANP levels.[18] Although the short-term effect of ANP on insulin secretion is controversial, recent studies have shown that long-term exposure of isolated islets to ANP inhibits glucose-stimulated insulin secretion and ATP generation.[35] Furthermore, ANP activation of GC-A receptor in pancreatic β cells and in the glucose-responsive insulinoma (INS-1E) cell line, results in growth involving SER-THR kinase AKT/PKB, FoxO1a pathways, and cyclin D2-mediated by activation of phosphatydylinositol 3`kinase (PI3K). Insulin secretion in response to other secretagogues is also markedly inhibited by ANP. ANP significantly increased the level of islet-activated hormone-sensitive lipase and the expression of uncoupling protein-2 and peroxisome proliferator-activated receptors γ and α. Interestingly, although islet GC-A receptor expression was reduced by 60% after 7 days in culture with ANP, cGMP levels remained similar to control islet levels.[35] Finally, the GC-A knockout mice showed glucose-stimulated islet insulin secretion, supporting the concept of inhibition of insulin secretion by ANP.[22]

Gastrointestinal Tract

All three NP receptors have also been shown in isolated acini from rat pancreas,[25] and ANP is present in acinar and centroacinar cells and in nerve fibers in the pancreas of porcine and other species. Both CNP and ANP stimulate amylase release secretion from isolated pig pancreatic segments. Because of the close association between peptide localization in nerve fibers with pancreatic secretory cells, it has been suggested that nerve fibers release ANP, which subsequently stimulates protein and amylase secretion.[1] ANP exerts a direct stimulatory action on the pancreatic secretion of acinar fluid and protein, through C receptors coupled to the phosphoinositide pathway,[26] independent of nitric oxide or parasympathetic activity. ANP has an inhibitory effect on glucagon secretion by a mechanism that involves cGMP inhibition of calcium uptake, likely via C receptors.

Several findings support the participation of ANP in the modulation of gastrointestinal physiology and digestive exocrine glands. All NPs and their receptors are expressed in the human liver.[34] ANP and CNP administered centrally or peripherally decrease spontaneous and sodium dechlorocholate-evoked bile secretion and modify the bile composition by a direct effect, independent of the autonomic nervous system.[26] Some results suggest that the inhibitory response of bile secretion to ANP and CNP occurs by a calcium-dependent mechanism.

ANP has been localized in enterochromaffin cells in gastric antrum and GC-A and C receptors are present in antral mucosa, and ANP has been shown to be involved in the regulation of gastric acid secretion. Intracerebroventricularly administered ANP increases gastric secretion in rat

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through the vagal-dependent mechanism. However, intravenous injection of ANP has biphasic effect on gastric secretion. Although a low dose enhances vagal-stimulated gastric secretion, high dose decreases it, indicating also paracrine/autocrine action and supporting the involvement of the peptidergic pathway.[24]

The effect of ANP and all NP receptors have been described in exocrine glands related to water and salt homeostasis including the submaxillary and parotid glands. ANP has been localized in both acinar and parotid duct cells. Several studies have shown that ANP plays a regulatory role on gland function such as enhancement of the salivary amylase secretion and on electrolyte composition of the saliva. Although ANP has no effect on salivation per se, it enhances salivation stimulated by various sialogogic agonists. The ANP effect on amylase secretion is mediated by the increase in intracellular calcium level linked to phosphoinositide metabolism within acinar and the ductal cells.[24]

Taken together, these observations clearly indicate an important neuromodulatory role of NPs in the control of digestive functions and may contribute to the development of new strategies for the diagnosis, treatment, and prevention of digestive diseases.

Pituitary Gland

NPs are specifically involved in the regulation of the hypothalamic–pituitary–adrenal axis. ANP immunoreactivity was detected in both anterior and posterior lobes of the pituitary gland. However, ANP synthesis occurs only in the anterior pituitary by detection of prohormone. ANP is a hypothalamic neurohormone that regulates blood pressure and volume by direct effects on blood vessels and kidney and indirectly by inhibiting the secretion of arginine vasopressin (AVP), renin, and aldosterone. In various species, ANP inhibits AVP and oxytocin release in response to osmotic stimuli or after hemorrhage and reduces plasma AVP in euhydration and dehydration.[15]

Recent evidence indicates that CNP has emerged as an important neuroendocrine regulator.[11] Although the highest tissue concentration of CNP is found in anterior pituitary, comparative studies of gene expression in mouse pituitary localized the CNP mRNA specifically in gonadotrophs. Furthermore, gonadotroph-derived cells αT3-1 secrete the mature form of CNP. The accumulation of cGMP in primary cell culture of pituitary gland and in the pituitary cell lines with characteristics of gonadotrophs (αT3-1), lactotrophs (GH3), corticotrophs (AtT-20), and folliculo-stellate cells (TtT-GF) is higher after administration of CNP than ANP or BNP. This indicates that the cGMP in the pituitary gland is predominantly stimulated by the CNP-specific, GC-B receptor.[11]

ANP probably plays an important inhibitory part in the neural control of adrenocorticotrophic hormone (ACTH) release.[10] ANP is a potent inhibitor of basal ACTH secretion, and secretion induced by hemorrhage, central angiotensin II administration, or stress. The latter may be mediated, at least partly, by suppression of AVP release from the neural lobe. One important aspect of the hypothalamic and pituitary action of NPs is their ability to interrupt the mechanism controlling the release of CRH, the hypothalamic releasing hormone involved in the peripheral stress hormone secretion. ANP, BNP, and CNP inhibit CRH-evoked ACTH secretion in mice hemipituitary preparations, with CNP being the most effective.[14] NPs also significantly reduce pituitary pro-opiomelanocortin mRNA expression. ANP can act directly on the pituitary to stimulate luteinizing hormone and follicle-stimulating hormone release, and to inhibit prolactin release, although the effects of ANP on release of these hormones are yet to be confirmed. ANP does not influence the release of thyrotropin and prolactin from dispersed anterior pituitary cells; nevertheless, central administration of high doses of ANP can cause a significant inhibition of prolactin, suggesting that it has no direct influence on lactotroph cells in inhibiting prolactin secretion but may activate the hypothalamic–dopaminergic system.[19] On the other hand, endogenous ANP plays a pathophysiological role in augmenting prolactin release during stress, either by inhibiting the release of prolactin-inhibiting factors or, alternatively, by enhancing the release of prolactin-releasing factors.[12] Intracerebroventricular injection of ANP increases plasma growth hormone levels in both conscious and urethane-anesthetized rats.

Adrenal Glands

Human and rat adrenal medulla is a site of synthesis of NPs and their three receptors. Adrenal chromaffin cells are endocrine cells embryologically derived from neural crest and biochemical analysis of bovine chromaffin cell extracts revealed the presence of mature form of ANP and its precursor indicating local synthesis. Several lines of evidence have shown the presence of NP receptors in adrenal chromaffin cells of various species, suggesting that these peptides secreted from adrenal medulla regulate chromaffin function in an autocrine manner. In the adrenal medulla, NPs participate in the inhibitory control of release and increase in catecholamine uptake. In tissue slices obtained from humans with pheochromocytoma, a tumor derived from the adrenal medulla, the basal secretion of cathecholamines is inhibited by ANP, suggesting that NPs could play a role in the pathology of adrenocortical neoplasms and pheochromocytomas.[21]

NPs are known to inhibit basal and angiotension-II-stimulated aldosterone secretion through an increase of

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intracellular cGMP and inhibition of T-type calcium channels.[28] NPs also inhibit the growth of cells in zona glomerulosa. The role of ANP in the regulation of aldosterone, which plays a central part in the control of blood pressure, has been suggested in the development of hypertension in offspring from food-restricted mothers. An elevated plasma aldosterone level is associated with decreased plasma ANP and an increased density of adrenal C receptors, which could explain, at least in part, the observed increase in arterial blood pressure.[28] The adrenomedullary synthesis of ANP is increased in patients with primary aldosteronism, and ANP mRNA is upregulated in the adrenal medulla and cortex of DOCA-salt treated rats, confirming the role of ANP in regulating both aldosterone release and blood pressure.

Adipose Tissue

Stress-related chronic stimulation of hypothalamic–pituitary–adrenal axis and increased glucocorticoid exposure are important pathophysiological factors in the development of obesity. It has been recently hypothesized that NPs, which mediate endocrine and behavioral responses to stress, could also be involved in the pathophysiology of obesity. Several lines of research have shown that obese patients have lower plasma levels of NPs than lean patients do, even though obesity is associated with an increased risk of cardiovascular disease such as heart failure and hypertension, which are paradoxically accompanied by increased NPs. Therefore, it could be hypothesized that reduced peripheral NPs could lead to reduced lipolysis and excessive weight gain, which would contribute to the development of obesity.[3]

Until recently, ß-adrenergic agonists were considered as main hormones controlling lipolysis in human adipocytes, but results now indicate that part of lipid mobilization could result from the direct effect of NPs both in situ in human adipose tissue and *in vitro* in human fat cells.[30] ANP and BNP stimulate lipolysis with the greater potency than CNP in human isolated fat cells, and this effect is accompanied by cGMP accumulation. The activation of cGMP-dependent protein kinase may be a key mechanism of action of NPs in human adipocytes. Understanding the potential mechanism of the association between adiposity and NP status could allow the development of new therapeutic strategies.

Thyroid and Parathyroid Glands

The entire NP system is present in the thyroid gland, and some results point to a functional role of NPs in the regulation of thyroid hormone secretion. Plasma concentrations of ANP are decreased in patients with hypothyroidism, whereas in hyperthyroidism, a two-fold increase in circulating plasma ANP is observed in comparison with normal healthy subjects. Thyroxine treatment of hypothyroid patients normalizes ANP circulating levels. ANP infusion in humans decreases plasma triiodothyronine and T_4 levels, resulting in a reciprocal increase in TSH secretion, indicating that the ANP action occurs directly in the thyroid not via the pituitary or hypothalamus; otherwise, TSH levels would decrease in the circulation if the inhibitory effects occurred at hypothalamic or pituitary sites.[32]

In terms of thyroglobulin production, ANP suppresses production in cultured human thyroid cells, and this is correlated with a reduction of cAMP, indicating that the effect is mediated by C receptors. The role of GC receptors is supported by another study where ANP and CNP altered the morphology of human thyroid cells by increasing cells of the retracted phenotype.[29] The upregulation of the ANP gene expression by thyroid hormones together with the observation that T_4 increases plasma ANP in patients with hypothyroidism indicates the clinical relevance of NPs in the thyroid gland.

The functional interaction between parathyroid hormone (PTH) and NPs have been observed in modulating cyclic nucleotides and cell proliferation in chondrocytes and osteoblasts, indicating a possible role of ANP on calcium and phosphate metabolism The direct action of ANP on the release of endothelin from cultured rat parathyroid cells has been shown, indicating the presence of NP receptors in the cells. The identical circadian rhythms of NPs and PTH together with the evidence that PTH may release ANP let to conclusion that some of the effects of PTH may be mediated by NPs. Some evidence suggested the possible interactions of ANP in the PTH (1–84) regulation of blood pressure and volume homeostasis in healthy subjects. Volume depletion by bolus injection of ANP was associated with the decrease of PTH (1–84) in healthy subjects but not in patients with liver cirrhosis or nephrotic syndrome; however, the clinical relevance is yet to be shown.[6]

Reproductive Organs

Although all NPs are highly expressed in male and female reproductive organs, and stimulate the release of gonadotropin factors from the pituitary gland, there is strong evidence that CNP acts as a local paracrine or autocrine regulator in female reproduction, pregnancy, and fetal development. CNP also plays a distinct role in male reproduction.[19]

In rodent ovaries and uteri, both CNP and GC-B show estrus cycle-dependent gene expression, with maximal expression at proestrus.[8] The modulation of follicular atresia in follicles at preantral to antral transition has been postulated. During pregnancy, the synthesis of CNP shows an organ-specific expression in maternal reproductive tissues with the highest concentration occurring in the placenta. The highest expression of CNP mRNA in the placenta would indicate a defined biological function of this peptide in gestation, possibly to antagonize overactive vasoconstrictive systems. Higher CNP levels have been found in the fetal

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rather than in the maternal circulation, suggesting the need for and ability of the fetus to synthesize de novo CNP and ANP. Pathological pregnancies, such as intrauterine growth retardation or preeclampsia, present differential regulation of CNP synthesis. When compared with normal pregnancy, CNP decreases in the placenta with an increase in the myometrium, indicating CNP organ-specific function in these pathologies. Because maternal plasma levels do not correlate with those alterations, it can be suggested that the CNP/GC-B/cGMP system acts locally in an autocrine/paracrine fashion exhibiting organ-specific effects. Interestingly, the offspring of CNP-deficient mice show dwarfism resulting from impaired endochondral ossification. Neonatal mice show growth retardation and high mortality, although surviving animals are fertile. Transgenic mice overexpressing CNP also show bone malformation suggesting that the main function of CNP in fetus is the regulation of bone growth by the chondrocyte proliferation and cartilage matrix production.

NPs stimulate testosterone production by Leydig cells. In humans, acute α-hANP injection significantly increases testosterone concentrations in the spermatic vein but not in peripheral blood. The role of NPs in the early phase of fetal testicular steroidogenesis has been postulated before the onset of pituitary luteinizing hormone secretion.[9] Furthermore, the paracrine action, particularly of CNP, includes relaxation of seminiferous tubules necessary to regulate sperm transport and testicular blood supply.

Support for ANP function in the maintenance of serum testosterone and testicular steroidogenesis is provided by a study in Leydig cells isolated from GC-A –/– and transgenic mice. ANP had no effect on testosterone production in GC-A –/– mice. However, Leydig cells isolated from animals having extra copies of GC-A gene showed increased steroidogenesis.

CNP and GC-B receptors are expressed in the testis[19] and in porcine seminal plasma. CNP regulates testicular function, such as testosterone release, modulation of spermatozoa mobility, and testicular cell germ development. The possible involvement of CNP in penile erection has also been hypothesized, because CNP activates GC-B receptors and increases production of cGMP localized on the cavernosal membrane. The specific relevance of cGMP system for the reproductive functions has recently been shown by the successful use of sildenafil, an inhibitor of cGMP-specific phosphodiesterase type-5, for the treatment of erectile dysfunction. Because NPs are involved in multiple local functions in testis, it may be speculated that NPs via cGMP- mediated processes might influence the *potentia coeundi* within the penis and *potential generandi* at different levels within the testis.[19]

PATHOPHYSIOLOGICAL IMPLICATIONS

In patients with congestive heart failure, plasma concentrations of ANP and BNP and their N-terminal fragments of prohormones are elevated because the cardiac hormonal system is activated by volume overload and pressure. NPs have a beneficial, compensatory action in heart failure by inducing vasodilatation, natriuresis, and inhibition of the sympathetic nervous and renin–angiotensin–aldosterone systems. Furthermore, with their well-documented autocrine/paracrine effects within the heart, such as regulation of coronary blood flow, proliferative responses contributing to vascular remodeling, and cardioprotection, NP therapy could greatly benefit the patient with cardiovascular disease. These diverse actions may have a significant counterregulatory role to the various neuroendocrine mechanisms activated in cardiovascular diseases.

Although the effects of NPs have been reported in various glands in several experiments, the intracellular signal transduction pathways remain to be identified in the organ in which the effects have been observed. The elucidation of these regulatory mechanisms would allow us to understand the importance of autocrine/paracrine regulation by the NPs in physiology and in pathophysiological states. During the past decades, great progress has been seen in our understanding of cGMP regulation of many endocrine functions. However, the question still remains whether the most important role in cGMP generation occurs by the action of natriuretic peptides or nitric oxide. Ultimately, an understanding of autocrine/paracrine actions of NPs may lead to their therapeutic application in various pathologies.

REFERENCES

1. Adeghate E, Ember Z, Donath T, Pallot DJ, Singh J. Immunohistochemical identification and effects of atrial natriuretic peptide, pancreastatin, leucine–enkephalin, and galanin in the porcine pancreas. *Peptides* 1996;**17**:503–9.
2. Anand-Srivastava MB. Natriuretic peptide receptor-C signaling and regulation. *Peptides* 2005;**26**:1044–59.
3. Beleigoli AM, Diniz MF, Ribeiro AL. Natriuretic peptides: linking heart and adipose tissue in obesity and related conditions——a systematic review. *Obes Rev* 2009;**10**:617–26.
4. D'Souza SP, Davis M, Baxter GF. Autocrine and paracrine actions of natriuretic peptides in the heart. *Pharmacol Ther* 2004;**101**:113–29.
5. de Bold AJ, Borenstein HB, Veress AT, Sonnenberg H. A rapid and potent natriuretic response to intravenous injection of atrial myocardial extract in rats. *Life Sci* 1981;**28**:89–94.
6. De Feo ML, Bartolini O, Orlando C, Maggi M, Serio M, Pines M, et al. Natriuretic peptide receptors regulate endothelin synthesis and release from parathyroid cells. *Proc Natl Acad Sci U S A* 1991;**88**:6496–500.
7. De Wardener HE, Mills IH, Clapham WF, Hayter CJ. Studies on the efferent mechanism of the sodium diuresis which follows the administration of intravenous saline in the dog. *Clin Sci* 1961;**21**:249–58.
8. Dos Reis AM, Fujio N, Dam TV, Mukaddam-Daher S, Jankowski M, Tremblay J, et al. Characterization and distribution of natriuretic peptide receptors in the rat uterus. *Endocrinology* 1995;**136**:4247–53.
9. El-Gehani F, Tena-Sempere M, Ruskoaho H, Huhtaniemi I. Natriuretic peptides stimulate steroidogenesis in the fetal rat testis. *Biol Reprod* 2001;**65**:595–600.

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10. Fink G, Dow RC, Casley D, Johnston CI, Lim AT, Copolov DL, et al. Atrial natriuretic peptide is a physiological inhibitor of ACTH release: evidence from immunoneutralization in vivo. *J Endocrinol* 1991;**131**:R9–12.
11. Fowkes RC, McArdle CA. C-type natriuretic peptide: an important neuroendocrine regulator? *Trends Endocrinol Metab* 2000;**11**:333–8.
12. Franci CR, Anselmo-Franci JA, McCann SM. The role of endogenous atrial natriuretic peptide in resting and stress-induced release of corticotropin, prolactin, growth hormone, and thyroid-stimulating hormone. *Proc Natl Acad Sci U S A* 1992;**89**:11391–5.
13. Gauer OH, Henry JP. Circulatory basis of fluid volume control. *Physiol Rev* 1963;**43**:423–81.
14. Guild SB, Cramb G. Characterisation of the effects of natriuretic peptides upon ACTH secretion from the mouse pituitary. *Mol Cell Endocrinol* 1999;**152**:11–9.
15. Gutkowska J, Antunes-Rodrigues J, McCann SM. Atrial natriuretic peptide in brain and pituitary gland. *Physiol Rev* 1997;**77**:465–515.
16. Gutkowska J, Nemer M. Structure, expression, and function of atrial natriuretic factor in extraatrial tissues. *Endocr Rev* 1989;**10**:519–36.
17. Kuhn M. Molecular physiology of natriuretic peptide signalling. *Basic Res Cardiol* 2004;**99**:76–82.
18. McKenna K, Smith D, Tormey W, Thompson CJ. Acute hyperglycaemia causes elevation in plasma atrial natriuretic peptide concentrations in type 1 diabetes mellitus. *Diabet Med* 2000;**17**:512–7.
19. Middendorff R, Davidoff MS, Behrends S, Mewe M, Miethens A, Muller D. Multiple roles of the messenger molecule cGMP in testicular function. *Andrologia* 2000;**32**:55–9.
20. Oliver PM, Fox JE, Kim R, Rockman HA, Kim HS, Reddick RL, et al. Hypertension, cardiac hypertrophy, and sudden death in mice lacking natriuretic peptide receptor A. *Proc Natl Acad Sci U S A* 1997;**94**:14730–5.
21. Porzionato A, Macchi V, Rucinski M, Malendowicz LK, De Caro R. Natriuretic peptides in the regulation of the hypothalamic–pituitary–adrenal axis. *Int Rev Cell Mol Biol* 2010;**280**:1–39.
22. Ropero AB, Soriano S, Tuduri E, Marroqui L, Tellez N, Gassner B, et al. The atrial natriuretic peptide and guanylyl cyclase-A system modulates pancreatic beta-cell function. *Endocrinology* 2010;**151**: 3665–74.
23. Ruskoaho H. Atrial natriuretic peptide: synthesis, release, and metabolism. *Pharmacol Rev* 1992;**44**:479–602.
24. Sabbatini ME. Natriuretic peptides as regulatory mediators of secretory activity in the digestive system. *Regul Pept* 2009;**154**:5–15.
25. Sabbatini ME, Rodriguez M, di Carlo MB, Davio CA, Vatta MS, Bianciotti LG. C-type natriuretic peptide enhances amylase release through NPR-C receptors in the exocrine pancreas. *Am J Physiol Gastrointest Liver Physiol* 2007;**293**:G987–94.
26. Sabbatini ME, Villagra A, Davio CA, Vatta MS, Fernandez BE, Bianciotti LG. Atrial natriuretic factor stimulates exocrine pancreatic secretion in the rat through NPR-C receptors. *Am J Physiol Gastrointest Liver Physiol* 2003;**285**:G929–37.
27. Samson WK. Here the heart may give a useful lesson to the head. *Endocrinology* 1996;**137**:3629–30.
28. Schiebinger RJ, Kem DC, Brown RD. Effect of atrial natriuretic peptide on ACTH, dibutyryl cAMP, angiotensin II and potassium-stimulated aldosterone secretion by rat adrenal glomerulosa cells. *Life Sci* 1988;**42**:919–26.
29. Sellitti DF, Lagranha C, Perrella G, Curcio F, Doi SQ. Atrial natriuretic factor and C-type natriuretic peptide induce retraction of human thyrocytes in monolayer culture via guanylyl cyclase receptors. *J Endocrinol* 2002;**173**:169–76.
30. Sengenes C, Berlan M, De Glisezinski I, Lafontan M, Galitzky J. Natriuretic peptides: a new lipolytic pathway in human adipocytes. *FASEB J* 2000;**14**:1345–51.
31. Sudoh T, Minamino N, Kangawa K, Matsuo H. C-type natriuretic peptide (CNP): a new member of natriuretic peptide family identified in porcine brain. *Biochem Biophys Res Commun* 1990;**168**:863–70.
32. Vesely DL, San Miguel GI, Hassan I, Gower Jr WR, Schocken DD. Atrial natriuretic hormone, vessel dilator, long-acting natriuretic hormone, and kaliuretic hormone decrease the circulating concentrations of total and tree T4 and free T3 with reciprocal increase in TSH. *J Clin Endocrinol Metab* 2001;**86**:5438–42.
33. Vollmar AM, Lang RE, Hanze J, Schulz R. A possible linkage of atrial natriuretic peptide to the immune system. *Am J Hypertens* 1990;**3**:408–11.
34. Vollmar AM, Paumgartner G, Gerbes AL. Differential gene expression of the three natriuretic peptides and natriuretic peptide receptor subtypes in human liver. *Gut* 1997;**40**:145–50.
35. You H, Laychock SG. Long-term treatment with atrial natriuretic peptide inhibits ATP production and insulin secretion in rat pancreatic islets. *Am J Physiol Endocrinol Metab* 2011;**300**:E435–44.

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Chapter 135

Neuromedins U/S

Ludwik K. Malendowicz, Marcin Rucinski and Agnieszka Ziolkowska

ABSTRACT

Neuromedin U (NMU) and neuromedin S (NMS) are structurally related and highly conserved neuropeptides that act via two receptors designated NMUR1 and NMUR2. NMUR1 is expressed predominantly in the periphery, whereas NMUR2 is expressed in the central nervous system. NMU and NMS share a C-terminal pentapeptide and bind to the same receptors. Therefore, it is believed that both the peptides have similar putative physiological actions. A growing body of evidence suggests that NMU and NMS function in the regulation of smooth muscle contractions, central and peripheral energy homeostasis, ion transport, circadian rhythms, stress responses, vasoconstriction, nociception, and bone remodeling. This system has also been implicated in the pathogenesis of septic shock, mast cell-mediated inflammation, and cancer. This chapter focuses on the expression and function of NMU and NMS in the endocrine system. Progress in molecular biology techniques provided new information on this subject, allowing a better understanding of the role played by both the neuromedins in the endocrine system.

INTRODUCTION

In 2000, neuromedin U (NMU), a multifunctional neuropeptide originally isolated from porcine spinal cord, was identified as an endogenous ligand for the orphan receptors FM-3/GPR66 and FM-4/TGR1 Subsequently, these receptors were named NMUR1 and NMUR2, respectively. NMUR1 is expressed predominantly in the periphery while NMUR2 is highly expressed in specific regions of the central nervous system.[1,8,24] In 2005, another endogenous ligand for NMUR1 and NMUR2 receptors was identified in the rat. This ligand is highly expressed in the suprachiasmatic nucleus of the hypothalamus, and therefore, it was designated as neuromedin S (NMS).[25] In various species, numerous NMU molecular forms have been identified and their amino acid sequences extend from 8 (NMU8) to 38 (NMU38) aa.[24] NMS, however, is a 36-amino-acid neuropeptide, which shares the amidated C-terminal pentapeptide with NMU. Interestingly, NMS analogs—NMS17 and NMS33—have also been identified. Despite these similarities, the human NMU and NMS genes have been mapped to different chromosomes. Thus, NMS is not a splice variant of NMU. NMUR2 has a greater affinity for NMS than NMU and in the rat NMS mRNA has a more limited distribution than NMU.[25] Experimental results suggest that NMS exerts more potent activity than NMU in the central nervous system but both peptides exert similar activity in peripheral tissues and *in vitro*.

Controversy exists over the presence of endogenous NMU and NMS in the general circulation. The manufacturers' manuals for various kits contain data on the neuropeptide concentration in plasma (human NMU – 0.1–5 ng/mL; NMS – 0.2–0.9 ng/mL) and comparable figures are shown in clinical studies.[18] However, experimental results suggest that neither endogenous NMU nor NMS are circulating, at least in the rat. Further, the t1/2 of NMU is shorter than 5 min. These findings suggest that NMU and NMS may function in an endocrine manner, but they also may exert paracrine or autocrine effects on target cells.

Both NMU and NMS contract smooth muscle, increase blood pressure, stimulate pain, modify intestinal ion transport, and regulate feeding and circadian rhythms. In recent years, the interest in neuromedins U and S significantly increased because of the discovery of their involvement in the regulation of energy homeostasis.[10,12,13,19,26,27] Further, both neuromedins are involved in the regulation of neuroendocrine axes and may modulate the growth and secretory activity of endocrine glands.[1,22,24]

This chapter focuses on the expression and function of NMU and NMS in the endocrine system. Progress in molecular biology techniques provided new information on this subject, allowing better understanding of the role played by both the neuromedins in the endocrine system.

Elements of the NMU/NMS and NMUR1 and NMUR2 system are present at both mRNA and protein levels in the hypothalamus and all classic endocrine glands. Examples of results for NMU/NMS and NMUR1 and NMUR2 mRNA expression in the rat are shown in Figures 1 and 2.

HYPOTHALAMUS

The earliest studies indicated a high level of NMU-like immunoreactivity in the rat, mouse, and human hypothalamus.[4,11] Modern molecular biology techniques have enabled

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00135-4
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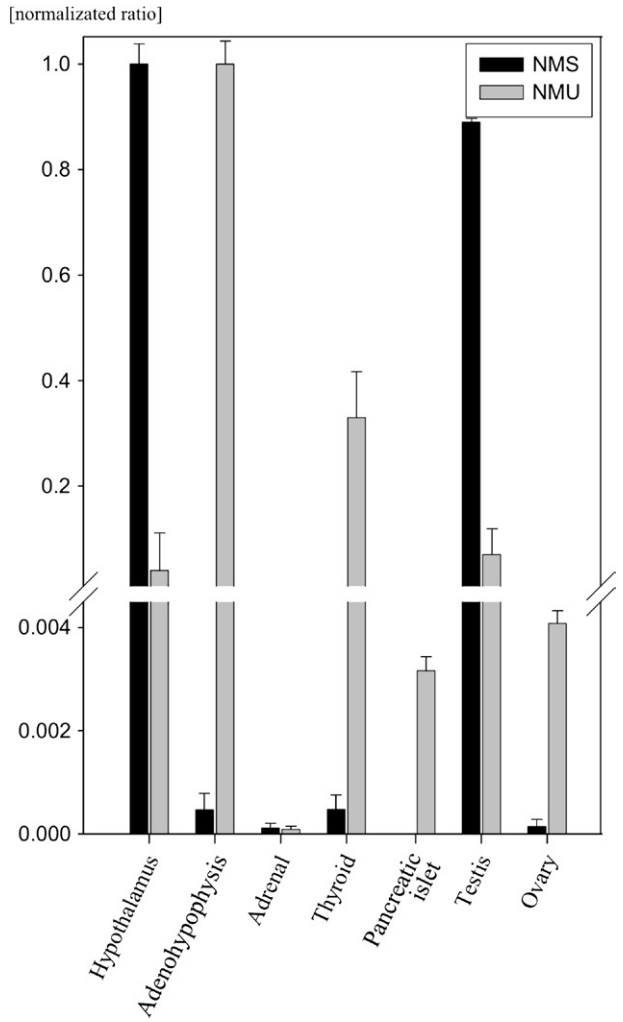

FIGURE 1 Results of Q-PCR analysis of neuromedin U (NMU) and neuromedin S (NMS) genes expression in the hypothalamus and endocrine glands of the adult rat. Data present relative expression in relation to HPRT gene expression.

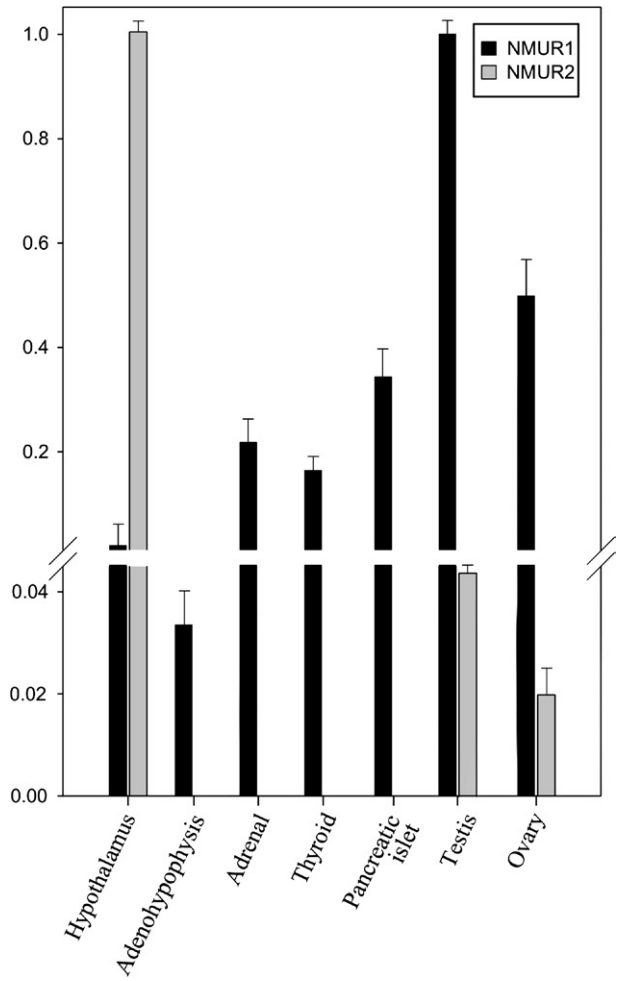

FIGURE 2 Results of Q-PCR analysis of neuromedin U (NMU) receptor genes expression (NMUR1 and NMUR2) in the hypothalamus and endocrine glands of the adult rat. Data present relative expression in relation to HPRT gene expression.

a more precise localization of NMU to hypothalamic nuclei and cell types. Generally, in the anterior and middle hypothalamus, which are the regions involved in neuroendocrine regulation, NMU is localized in nerve fibers while an intense NMS-like immunoreactivity is present in cell bodies of the suprachiasmatic nucleus.[1,24,25] In the rat hypothalamus, expression of the NMS gene is nearly 3-fold higher than that of the NMU gene.[29] These regions of the hypothalamus are also provided with highly specific NMU-binding sites, which have been identified as NMUR2. Among others, NMUR2 mRNA is expressed within the paraventricular nucleus (NPV) and the wall of the third ventricle (ependymocytes). The presence of NMUR2 receptors in these regions of the brain has also been confirmed by receptor autoradiography.[1,24]

The above-described localization of elements of the NMU/NMS and NMUR2 system in the hypothalamus forms a basis of regulation by NMU and NMS of neuroendocrine function. It is well documented that NMU administered icv enhances expression of c-Fos immunoreactivity or its mRNA in PVN, supraoptic nucleus, arcuate nucleus, and lateral hypothalamic area. Further, in the rat NMU administered icv, subcutaneously or into the NPV increases blood ACTH and glucocorticoid concentrations via the stimulation of CRH release.[15,22,32] Also, NMS administered icv stimulates corticosterone secretion in the rat, and similar changes were observed in steers administered icv with NMU or NMS.[14] These findings suggest that both NMU and NMS activate a main part of the hypothalamic–pituitary–adrenal (HPA) axis.

Several reports suggest the involvement of NMU and NMS in the hypothalamic regulation of gonadotropin release; however, the available results are controversial. In the rat icv, NMU administration inhibited LH and FSH release or had no effect.[8,31] Increased release of LH, however, was observed

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only after icv, but not after intravenous administration of NMS. NMS (from 1 to 1000 nM) directly stimulated GnRH release from the pig hypothalamic explants. It has also been suggested that NMU administration to the rat slows the onset of puberty.[33]

Both NMUR2 and oxytocin mRNA are coexpressed in the rat NPV. Therefore, it is not unexpected that icv NMU administration stimulates arginine vasopressin and oxytocin release.[32] Of interest is that after icv administration output evoked by NMS is more pronounced than that induced by the equimolar dose of NMU. Rapid NMS-induced oxytocin release may indicate that NMS may mimic the suckling-induced oxytocin release.

The above-described NMU and NMS-induced alterations in functioning of the central part of the neuroendocrine axes suggest that both neuromedins, acting by the activation of different controlling systems of the brain, may indirectly regulate secretion of pituitary hormones. However, evidence is available that they may also exert a direct effect on hypothalamic structures controlling these axes. A direct stimulating effect of NMU on CRH and AVP release from rat medial basal hypothalamic explants *in vivo* was shown by Wren et al.[32] Further, by means of whole cell patch-clamp recordings, Qui et al.[28] observed that NMU selectively depolarized the subpopulation of PVN parvocellular neurons (which are known to secrete, among other peptides, CRH) via enhancement of the hyperpolarization-activated inward current.

PITUITARY GLAND

Soon after the discovery of NMU, RIA and immunohistochemistry showed the presence of NMU-immunoreactive substances in the intermediate and anterior pituitary gland lobes of various species, among them mouse, rat, and human.[4,5,23,30] In the mouse, rat, and human adenohypophysis, NMU immunoreactivity is found in corticotrophs.[2,30] Electron microscopy studies revealed NMU-like immunoreactivity in some thyrotrophs and most corticotrophs of the rat pituitary gland. NMU is colocalized with galanin and ACTH in the same secretory granules.[22]

NMU mRNA is highly expressed in the rat adenohypophysis, while expression of NMUR1 is relatively low and that of NMUR2 practically absent. In contrast to NMU, NMS mRNA expression in adenohypophysis is very low and in rat expression of NMU gene is almost 1000-fold higher when compared with that of the NMS gene.[29] In the pituitary of the pig, on the other hand, relatively high expression of NMS and NMR2 mRNA was reported.[34]

The physiological role of NMU in pituitary gland is unclear. At the protein level, anterior pituitary expression of this neuropeptide is highly regulated by thyroid hormones.[5]

Only scanty results are available on the direct effect of NMU or NMS on the secretory activity of the adenohypophysis. A direct suppressive effect of NMU on LH and FSH release by rat primary pituitary cell cultures was reported.[7] To clarify the role of NMU in the regulation of gonadotropin secretion, examination of gonadal function in NMU-KO mice revealed an early onset of vaginal opening. NMU-KO mice maintain normal concentrations of NMS and therefore the authors suggested that "it is unlikely that NMS is also involved in the regulation of LH and FSH releases from pituitary." In this context, rather unexpected results had been published on the effects of NMS on the release of LH and FSH from isolated pig anterior pituitary cells.[33] In these studies, NMS dose dependently increased LH release from pituitary cells, while none of the NMS doses changed FSH release.

THYROID GLAND

The NMU content of the whole thyroid gland of the rat is relatively low and thyrotoxicosis induced by exogenous T4 fails to alter it while antithyroid drug treatment lowers it.[3] Immunostaining localized NMU to a minor (~5%) population of parafollicular C-cells, while no staining was observed in nerve fibers.

In rat thyroid gland, there is expression of NMU and NMUR1 mRNA although expression of NMUR2 is very low.[8,29] In the rat thyroid gland, expression of NMU gene is almost 1000-fold higher when compared with the expression of NMS gene. Expression of NMS and, unexpectedly, NMUR2 has been found in the pig thyroid.[34] The functional relevance of NMU and NMS in the thyroid gland remains completely unknown. In this regard, only Gartlon et al.[8] reported that in the rat icv, NMU administration did not affect plasma TSH levels.

PANCREATIC ISLETS

Earlier results on the expression of NMU in the pancreas were obtained from the samples of the entire pancreas. However, in isolated rat pancreatic islets, NMU but not NMS mRNA and NMUR1 but not NMUR2 mRNA have been identified.[16,17,29] Further, in pancreatic islets, NMUR1 protein was also identified by Western blotting and immunohistochemistry showed NMUR1-like substances in peripheral cells of the islets.

A functional study revealed that NMU in concentrations from 1 to 100 nmol/L dose dependently reduces insulin output by isolated rat pancreatic islets.[17] Moreover, NMU also lowers insulin secretion from in situ perfused rat pancreas. The insulinostatic effect of NMU on insulin release is accompanied by increased somatostatin (SST) secretion. As is known, SST is a potent inhibitor of insulin secretion and results of subsequent experiments suggest that SST may mediate the NMU-dependent inhibition of insulin secretion. It should be emphasized that the direct, NMU-evoked and SST-mediated reduction of insulin secretion is in accord with the anorexigenic action of NMU.

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ADRENAL GLAND

Involvement of NMU in the regulation of adrenal cortex growth and function was recognized soon after the identification of this neuropeptide.[22] In view of these findings, the NMU/NMS system in the adrenal has also been studied recently by means of molecular biology techniques. Very low expression of NMU gene in the rat adrenal has been reported by several groups. The human adrenal gland contains both NMUR1 and NMUR2 receptors. Detailed studies on the rat adrenal gland reveals very low expression of NMU and NMS genes at the mRNA level.[29] In immature rat adrenal, NMUR1 is expressed at both mRNA and protein levels, while the signal from NMUR2 is absent. NMUR1 mRNA is present in all adrenocortical zones and in the medulla.[35] Moreover, the presence of NMUR2 mRNA in isolated zona glomerulosa and fasciculata/reticularis cells eliminates the possibility that the expression results from the presence in the assayed specimens of non-parenchymal components of the gland. In contrast to rats, expression of NMS, and (again unexpectedly) NMUR2, has been described in the pig adrenal.[34]

Earlier results on the interrelationship between NMU and HPA axis have been reviewed.[22] Apart from the above-mentioned central CRH-mediated action of NMU on the HPA axis, NMU also directly affects adrenocortical function. As shown by our group, NMU8 has no effect on basal and ACTH-stimulated corticosterone secretion by isolated inner zone adrenocortical cells. This neuropeptide, however, stimulates corticosterone output by adrenal slices, an effect shown only at an NMU concentration of 10^{-8} M. Detailed analysis of the NMU8 action on the rat adrenal slices reveals that at all concentrations tested (10^{-8}–10^{-6} M) NMU8 increases basal pregnenolone and total post-pregnenolone steroid output by gland slices containing both cortex and medulla.[20,21] However, NMU8 is able to stimulate corticosterone secretion only at the lowest concentration. At a higher concentration, the NMU8-stimulating effect is masked by the strong enhancement of 18-hydroxylation. This effect of NMU8 is not observed in adrenocortical autotransplants, which are deprived of chromaffin cells and which display a normal secretory response to ACTH. These results suggest that at the adrenal level NMU8 affects the steroid secretion indirectly by acting on the medullary chromaffin cells, which in turn may paracrinally stimulate the adrenal cortex. It has been suggested that the medullary mediator of NMU8 action on the adrenal cortex may specifically activate 18-hydroxylation and aldosterone synthase activity. Recent results also show a direct stimulating effect of NMU on proliferative activity of immature rat inner adrenocortical cells in primary culture.[35]

As mentioned in the "Hypothalamus" paragraph, growing evidence indicates that the NMU/NMS system plays an integral role in mediating the stress response.[9] In view of the available results, it seems legitimate to suggest that NMU/NMS integration of the stress response is mediated at both a central level (CRH pathway) and at the level of the adrenal cortex.

TESTIS

Rat testes contain NMU mRNA, a finding that is in accordance with earlier results on the presence of NMU-like immunoreactive substances in this tissue.[4,24] Further, in testes expression of NMS was reported by Mori et al.[25] In the rat testis, expression of the NMS gene is almost three-fold higher than that of NMU.[29] There is expression of NMUR1 and NMUR2 in human and rat testis. Unfortunately, no results are available on the cytological localization of the genes in the gonads.

The role of NMU/NMS in the testes remains puzzling. To our knowledge, no results are available either on the presence of particular elements of the MU/NMS system in the spermatogenic or endocrine compartments of the testis or on its function.

OVARY

The presence of NMU-like immunoreactive substances in rat ovaries was reported by Domin et al.,[5] and NMU mRNA was also found. Moreover, in rat ovary, NMS mRNA has been identified.[29] Initially, in the rat ovary, only NMUR2 mRNA was found but subsequent studies revealed the expression of both NMUR1 and NMUR2 genes. In addition to the rat, there is NMS and NMUR2 mRNA in the pig ovary.[33,34] In this species, expression levels of NMS and NMUR2 genes are dependent on the stage of the estrous cycle. Gene expression is the highest in diestrus and lowest in proestrus.

As in the case of testis, no results are available on the presence of particular elements of NMU/NMS system in ovarian compartments. However, NMS at a concentration of 10, but not 1, 100, or 1000 nM was found to stimulate estradiol output from cultured ovarian granulosa cells of the puberal pig.[33] Moreover, in ovarian tumor cell lines, NMU gene is overexpressed when compared with ovarian surface epithelial cell lines.[6]

CONCLUSIONS

The physiologic role of the NMU/NMS system is not fully recognized yet. The available experimental results indicate that neuromedin U and S may exert both endocrine and/or paracrine/autocrine effects on target cells.

Discovery of the anorexigenic action of NMU/NMS triggered great interest in this field and there are new findings on their physiological role. Well documented is the role of NMU/NMS in central and peripheral energy homeostasis regulation and, in this regard, CRH stimulation and direct

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insulinostatic effects of both neuropeptides. Further, a direct effect of these neuropeptides on the secretory activity of the rat adrenal cortex may also contribute to their anorexigenic action.

Growing evidence indicates that the NMU/NMS system plays an integral role in mediating the stress response, not only through the CRH pathway. This response may also be mediated by neuromedins' action on vasopressin release and via their direct action on the adrenal gland.

Recent results also suggest involvement of NMU/NMS in the regulation of the reproductive system. Scarce experimental results suggest that NMU/NMS indirectly and directly stimulate GnRH release at the hypothalamic level. Moreover, NMU/NMS directly affects FSH secretion from the adenohypophysis and estradiol output from ovarian granulosa cells. Further, NMU affects puberty onset in the mouse.

It has also been suggested that NMU exerts a tumorigenic action that may be involved in cancers such as that of the pancreas. However, results on NMU overexpression are only available for ovarian and pancreatic cancer ductal cells. In this context, the role of NMU expression in pituitary corticotrophs of the normal human gland and ACTH secreting tumors remains to be elucidated.

REFERENCES

1. Brighton PJ, Szekeres PG, Willars GB. Neuromedin U and its receptors: structure, function, and physiological roles. *Pharmacol Rev* 2004;**56**:231–48.
2. Cimini V. Neuropeptides in anterior pituitary development. *Int J Dev Neurosci* 2003;**21**:291–301.
3. Domin J, Al-Madani AM, Desperbasques M, Bishop AE, Polak JM, Bloom SR. Neuromedin U-like immunoreactivity in the thyroid gland of the rat. *Cell Tissue Res* 1990;**260**:131–5.
4. Domin J, Ghatei MA, Chochan P, Bloom SR. Characterization of neuromedin U-like immunoreactivity in rat, porcine, guinea pig and human tissue extracts using a specific radioimmunoassay. *Biochem Biophys Res Commun* 1986;**140**:1127–34.
5. Domin J, Steel JH, Adolphus N, Burrin JM, Leonhardt U, Polak JM, et al. The anterior pituitary content of neuromedin U-like immunoreactivity is altered by thyreotropin-releasing hormone and thyroid status in the rat. *J Endocrinol* 1989;**122**:471–6.
6. Euer NI, Kaul S, Deissler H, Möbus VJ, Zeilunger R, Weidle UH. Identification of L1CAM, Jagged2 and Neuromedin U as ovarian cancer-associated antigens. *Oncol Rep* 2005;**13**:375–87.
7. Fukue Y, Sato T, Teranishi H, Hanada R, Takahashi T, Nakashima Y, et al. Regulation of gonadotropin secretion and puberty onset by neuromedin U. *FEBS Lett* 2006;**580**:3485–8.
8. Gartlon J, Szekeres P, Pullen M, Sarau HM, Aiyar N, Shabon U, et al. Localisation of NMU1R and NMU2R in human and rat central nervous system and effects of neuromedin-U following central administration in rats. *Psychopharmacology (Berl)* 2004;**177**:1–4.
9. Hanada R, Nakazato M, Murakami N, Sakihara S, Yoshimatsu H, Toshinai K, et al. A role for neuromedin U in stress response. *Biochem Biophys Res Commun* 2001;**289**:225–8.
10. Hanada R, Teranishi H, Pearson JT, Kurokawa M, Hosoda H, Fukushima N, et al. Neuromedin U has a novel anorexigenic effect independent of the leptin signaling pathway. *Nat Med* 2004;**10**:1067–73.
11. Honzawa M, Sudok T, Minamino N, Tokyama M, Matsuo H. Topographic localization of neuromedin U-like structures in the rat brain: an immunohistochemical study. *Neuroscience* 1987;**23**:1103–22.
12. Ida T, Mori K, Miyazato M, Egi Y, Abe S, Nakahara K, et al. Neuromedin S is a novel anorexigenic hormone. *Endocrinology* 2005;**146**:4217–23.
13. Ivanov TR, Lawrence CB, Stanley PJ, Luckman SM. Evaluation of neuromedin U actions in energy homeostasis and pituitary function. *Endocrinology* 2002;**143**:3813–21.
14. Jászberényi M, Bagosi Z, Thurzó B, Földesi I, Telegdy B. Endocrine and behavioral effects of neuromedin S. *Horm Behav* 2007;**52**:631–9.
15. Jethwa PH, Smith KL, Small CJ, Abbott CR, Darch SJ, Murphy KG, et al. Neuromedin U partially mediates leptin-induced hypothalamo-pituitary adrenal (HPA) stimulation and has a physiological role in the regulation of the HPA axis in the rat. *Endocrinology* 2006;**147**:2886–92.
16. Kaczmarek P, Malendowicz LK, Fabis M, Ziolkowska A, Pruszynska-Osmalek E, Sassem M, et al. Does somatostatin confer insulinostatic effects of neuromedin U in the rat pancreas? *Pancreas* 2009;**38**:208–12.
17. Kaczmarek P, Malendowicz LK, Pruszynska-Osmalek E, Wojciechowicz T, Szczepankiewicz D, Szkudelski T, et al. Neuromedin U receptor 1 expression in the rat endocrine pancreas and evidence suggesting neuromedin U suppressive effect on insulin secretion from isolated pancreatic islets. *Int J Mol Med* 2006;**18**:951–5.
18. Ketterer K, Kong B, Frank D, Giese NA, Bauer A, Hoheisel J, et al. Neuromedin U is overexpressed in pancreatic cancer and increases invasiveness via the hepatocyte growth factor c-Met pathway. *Cancer Lett* 2009;**277**:72–81.
19. Kowalski TJ, Spar BD, Markowitz L, Maguire M, Golovko A, Yang S, et al. Transgenic overexpression of neuromedin U promotes leanness and hypophagia in mice. *J Endocrinol* 2005;**185**:151–64.
20. Malendowicz LK, Andreis PG, Markowska A, Nowak M, Warchol JB, Neri G, et al. Effects of neuromedin U-8 on the secretory activity of the rat adrenal cortex: evidence for an indirect action requiring the presence of the zona medullaris. *Res Exp Med* 1994;**194**:69–79.
21. Malendowicz LK, Nussdorfer GG, Markowska A, Tortorella C, Nowak M, Warchol JB. Effects of neuromedin U (NMU)-8 on the rat hypothalamo–pituitary–adrenal axis. Evidence of a direct effect of NMU-8 on the rat adrenal gland. *Neuropeptides* 1994;**26**:47–53.
22. Malendowicz LK. Role of neuromedins in the regulation of adrenocortical function. *Horm Metab Res* 1998;**30**:374–83.
23. Minamino N, Kangawa K, Matsuo H. Neuromedin U-8 and U-25: novel uterus stimulating and hypertensive peptides identified in porcine spinal cord. *Biochem Biophys Res Commun* 1985;**130**:1078–85.
24. Mitchell JD, Maguire JJ, Davenport AP. Emerging pharmacology and physiology of neuromedin U and the structurally related peptide neuromedin S. *Br J Pharmacol* 2009;**158**:87–103.
25. Mori K, Miyazato M, Kangawa K. Neuromedin S: discovery and functions. *Results Probl Cell Differ* 2008;**46**:201–12.
26. Nakahara K, Katayama T, Maruyama K, Ida T, Mori K, Miyazato M, et al. Comparison of feeding suppression by the anorexigenic hormones neuromedin U and neuromedin S in rats. *J Endocrinol* 2010;**207**:185–93.
27. Nakazato M, Hanada R, Murakami N, Date Y, Mondal MS, Kojima M, et al. Central effects of neuromedin U in the regulation of energy homeostasis. *Biochem Biophys Res Commun* 2000;**277**:191–4.

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28. Qiu DL, Chu CP, Shirasaka T, Nabekura T, Kunitake T, Kato K, et al. Neuromedin U depolarizes rat hypothalamic paraventricular nucleus neurons in vitro by enhancing IH channel activity. *J Neurophysiol* 2003;**90**:843–50.
29. Rucinski M, Ziolkowska A, Neri G, Trejter M, Zemleduch T, Tyczewska M, et al. Expression of neuromedins S and U and their receptors in the hypothalamus and endocrine glands of the rat. *Int J Mol Med* 2007;**20**:255–9.
30. Steel JH, Van Noorden S, Ballesta J, Gibson SJ, Ghatei MA, Burrin J, et al. Localization of 7B2, neuromedin B, and neuromedin U in specific cell types of rat, mouse, and human pituitary, in rat hypothalamus, and in 30 human pituitary and extrapituitary tumors. *Endocrinology* 1988;**122**:270–82.
31. Vigo E, Roa J, López M, Castellano JM, Fernandez-Fernandez R, Navarro VM, et al. Neuromedin s as novel putative regulator of luteinizing hormone secretion. *Endocrinology* 2007;**148**:813–23.
32. Wren AM, Small CJ, Abbott CR, Jethwa PH, Kennedy AR, Murphy KG, et al. Hypothalamic actions of neuromedin U. *Endocrinology* 2002;**143**:4227–34.
33. Yang G, Su J, Li X, Yao Y, Lei Z, Yang X, et al. Expression of NMS and NMU2R in the pig reproductive axis during the estrus cycle and the effect of NMS on the reproductive axis in vitro. *Peptides* 2009;**30**:2206–12.
34. Yang G, Su J, Yao Y, Lei Z, Zhang G, Liu Y, et al. Distribution of neuromedin S and its receptor NMU2R in pigs. *Res Vet Sci* 2011.
35. Ziolkowska A, Macchi C, Trejter M, Rucinski M, Nowak M, Nussdorfer GG, et al. Effects of neuromedin-U on immature rat adrenocortical cells: in vitro and in vivo studies. *Int J Mol Med* 2008;**21**:303–7.

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Chapter 136

Neuropeptide B/W

Fumiko Takenoya, Haruaki Kageyama, Ki Sung Kang and Seiji Shioda

ABSTRACT

Neuropeptides B and W (NPB/NPW) have been identified as endogenous ligands of the G protein-coupled receptors NPBWR1 and NPBWR2, which belong to the orphan G protein-coupled receptor (GPCR) family. NPB/NPW is expressed widely in brain including the hypothalamus, pituitary, and adrenal gland, suggesting that NPB/NPW may have a role in the modulation of neuroendocrine functions. Moreover, intracerebroventricular infusion of NPW suppresses food intake and body weight and increases both heat production and body temperature, suggesting that NPW functions as an endogenous catabolic signaling molecule. Moreover, the expression of NPB/NPW and NPBWR1 mRNA is detected in the periaqueductal gray and amygdala where opioid receptors exist, suggesting that NPB/NPW may be related to pain function. Thus, NPB and NPW play very important physiological functions. In this chapter, we describe the neuroendocrine function of NPB/W and its role in regulating feeding behavior, energy homeostasis, and inflammatory pain.

INTRODUCTION

GPCRs compose the largest family of cell-surface mediators of signal transduction. Therefore, the continued discovery of genes encoding GPCRs has provided an extensive reserve of potential therapeutic targets. Both NPBWR1 and NPBWR2 belong to the GPCR family, the members of which are characterized by their seven transmembrane domains. These two GPCRs were identified by cloning opioid–somatostatin-like receptor genes from human genomic DNA.[21] NPBWR1 is expressed in the brain and peripheral organs of both human beings and rodents but is not expressed in rodents. NPBWR1 mRNA is expressed in the hypothalamus such as the arcuate nucleus (ARC), ventromedial hypothalamus (VMH), paraventricular nucleus (PVN), and dorsomedial nuclei, which are believed to be involved in the regulation of feeding and energy balance. NPBWR1 is most abundantly expressed in the central amygdaloid nucleus (CeA) and bed nucleus of the stria terminalis (BST), suggesting that NPBWR1 may be involved in the regulation of stress, emotion, fear, and anxiety.[13,27] NPBWR1 and NPBWR2 mRNAs are also localized in the pituitary and adrenal glands, suggesting that the peptides may influence the neuroendocrine response to stress via the hypothalamic–pituitary–adrenal (HPA) axis.[16] Moreover, NPBWR1 mRNA is expressed in the spinal cord and thus may be involved in pain regulation.

Between 2002 and 2003, neuropeptide B (NPB) and neuropeptide W (NPW) were identified by several research groups as the ligands for two orphan GPCRs, NPBWR1 and NPBWR2.[2,7,26,30] Both NPB and NPW consist of two forms, 23 and 29, 23 and 30 amino acids in length, respectively. Subsequently, several immunohistochemical studies have shown that NPBs/NPWs are expressed widely in brain including the PVN, midbrain, and in tissues such as the pituitary and adrenal, suggesting that NPBs/NPWs have roles in modulating neuroendocrine functions.[6,13,24,26,28–30]

NEUROENDOCRINE FUNCTION OF NPB/NPW

Immunohistochemical studies have reported that NPBWR1 is expressed in the PVN, supraoptic nucleus (SO), ARC, suprachiasmatic nucleus (SCh), pituitary gland, and adrenal medulla in the human, mouse, and rat.[2,14,21,30] Moreover, NPW23-immunoreactive cells are detected in the PVN, mainly in the parvocellular subdivisions, SO, accessory neurosecretory nuclei, dorsal and lateral hypothalamic areas, perifornical region, ARC, and anterior and posterior pituitary. These observations may indicate that NPBs/NPWs interact with neuroendocrine systems that control hormone release from the anterior pituitary gland. In addition, restraint stress induces c-Fos expression in several brain regions, including hypothalamic and amygdaloid nuclei,[3] where NPW-like immunoreactive (-LI) neurons are abundantly distributed. Moreover, c-Fos expression in the PVN is significantly increased by intracerebroventricular (icv) infusions of NPW in the mouse. This suggests that NPW seems to respond to stress and to release pituitary hormones directly through NPWBR1 on the HPA axis.[20,26] However, NPB and NPW are not reported to influence the release of other anterior pituitary hormones. These neuroendocrine

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00136-6
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effects of NPB/NPW are not mediated directly through NPWBR1 but may be mediated indirectly through control of the release of the hypothalamic hormone corticotropin-releasing factor (CRH).[2,23]

NPB/NPW is colocalized with urocortin, a stress-related neuropeptide of the CRH family, in the Edinger–Westphal nucleus (EW). Tanaka et al.[30] have reported, with icv infusion of NPB, the anorexic effect of NPB is enhanced by pretreatment with CRH, which also has anorexic effects. Infusion of these two peptides almost completely suppresses food intake. These results suggest that NPB markedly enhances the anorexic effect of CRH. In addition, NPB-mediated release of corticosterone can be attributed to the release of CRH, as passive immunization using anti-CRH serum results in a significant attenuation of NPB release of corticosterone.[23] In addition, others have reported that NPB increases prolactin secretion but decreases growth hormone.[23]

Infusion of NPW23 stimulates prolactin release in the rat.[26] An *in vitro* study has reported that NPW23, in log molar concentrations ranging from 1.0 pM to 100 nM, significantly alters prolactin, growth hormone, and ACTH release from dispersed anterior pituitary cells harvested from male rats.[1] In *in vivo* studies, 1.0 nM NPW23 stimulates an approximately 2-fold increase in plasma prolactin levels, which attain significance at 60 min.[1] After icv infusion of 3.0 nM NPW23, plasma prolactin concentrations increase to levels 5- to 7-fold higher than those of controls. In addition, icv infusion of NPW23 (1.0 nM) results in a significant, 2-fold elevation in plasma corticosterone concentrations at 10 min after injection.[1] Increase of both prolactin and corticosterone plasma concentrations suggest a role for NPW in the hypothalamic response to stress. However, plasma growth hormone concentrations are inhibited by icv. infusion of NPW23. These findings suggest that NPW is the endogenous ligand for both GPR7 and GPR8 and acts as a mediator of the central control of neuroendocrine function.[1,26] Moreover, Taylor et al.[3] have reported that icv infusion of NPW activates the HPA axis, as shown by increased plasma corticosterone concentrations in conscious rats without stimulating the release of oxytocin (OXT) or vasopressin (AVP) in the peripheral circulation or altering blood pressure or heart rate. Additionally, icv infusion of a CRH antagonist does not significantly reduce corticosterone concentrations. However, pretreatment with a CRH antagonist significantly reduces the capacity of centrally administered NPW to increase corticosterone concentrations.[31] These observations suggest an action of NPW in the parvocellular subdivision of the PVN. Moreover, Taylor et al.,[31] using electrophysiological studies with whole cell patch recording of hypothalamic slice preparations, have shown that NPW depolarizes and increases the spike frequency of the majority of the identified putative neuroendocrine PVN neurons. Furthermore, the response of PVN neurons to NPW in the presence of TTX confirms the postsynaptic actions of NPW on these neuroendocrine cells.[31] Therefore, it is suggested that endogenous NPW may play a physiologically relevant role in the neuroendocrine response to stress in the brain. In addition, Nimi et al.[20] have examined the effect of immobilization stress and cold exposure stress on NPW neurons in the rat hypothalamus. They have found that a double immunostaining for both NPW and c-Fos is significantly increased in response to these stresses. Immobilization stress increases parasympathetic outflow, and cold stress is primarily considered to increase sympathetic outflow, suggesting that NPW may participate in the regulation of both the sympathetic and parasympathetic branches of the autonomic nervous system.

In the PVN, AVP neurons are known to regulate endocrine and autonomic stress responses into hormonal outputs.[32] Kawasaki et al.[11] showed that icv NPW30 significantly increased plasma concentrations of AVP and OXT 5 15 min later. The icv infusion of NPW significantly increases Fos-positive neurons in the SO and parvocellular and magnocellular divisions of the PVN. Moreover, dual detection of Fos-LI and OXT-/AVP-LI in the SO and PVN shows that both AVP-LI cells and OXT-LI cells exhibit nuclear Fos-LI in the SO and PVN after icv infusion of NPW30.[11] These results suggest that in the SO and PVN, NPW may stimulate the secretion of AVP and OXT in magnocellular neurosecretory neurons.

By using a cell patch clamp recording on the somatostatin neurons in the hypothalamic ARC, Price et al.[22] showed that NPW administration in the ARC results in the depolarization of most of the somatostatin neurons, suggesting a direct effect of NPW on the excitability of somatostatin neurons in the ARC. Therefore, the influence of NPW to suppress growth hormone release is caused by the activation of somatostatin neurons in the ARC. We summarize the neuroendocrine function of NPB/W in Fig. 1.

ENDOCRINE FUNCTION OF NPB/W

Hochol et al.[8] have shown that NPW increases ACTH-stimulated aldosterone secretion but does not affect either basal or ACTH-stimulated corticosterone production by dispersed zona fasciculata/reticularis cells. Moreover, bolus intraperitoneal injections of NPB or NPW enhance the plasma concentrations of parathyroid hormone, corticosterone, and testosterone as determined by radioimmunoassay. Furthermore, NPB increases the blood concentration of thyroxine, and NPW raises ACTH and estradiol levels in the rat, suggesting that NPB and NPW play a role in the autocrine–paracrine functional regulation of the endocrine system.[9] In addition, there is an ACTH-independent extraadrenal effect, because NPB and NPW stimulate adrenal glucocorticoid secretion *in vivo* but not mineralocorticoid secretion. In addition, NPW stimulates aldosterone secretion *in vitro* by enhancing the release of medullary catecholamines and subsequently activating beta-adrenoreceptors.[9]

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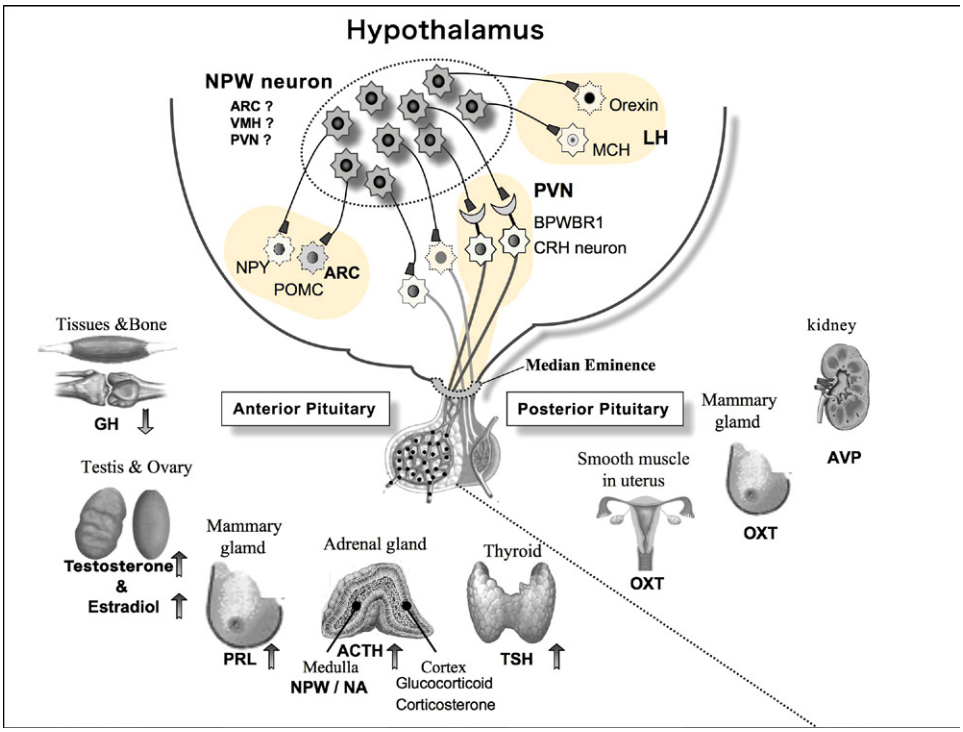

FIGURE 1 Schematic illustrating the HPA axis under conditions of neuroendocrine systems via NPW neuron. The arrows indicate stimulatory or inhibitory effects. GH: growth hormone; PRL: prolactin; ACTH: adrenocorticotropic hormone; TSH: thyroid-stimulating hormone; OXT: oxytocin; AVP" arginine vasopressin; NPY: neuropeptide Y; POMC: proopiomelanocortin; MCH: melanin-concentrating hormone; ARC: arcuate nucleus; VMH: ventromedial hypothalamic nucleus; LH: lateral hypothalamus; PVN: paraventricular hypothalamic nucleus.

RT-PCR analysis of peripheral tissues has identified NPB mRNA gene expression in the human kidney, uterus, ovary, testis, and placenta. High levels of NPB mRNA have been detected by Northern blot analysis in the rat stomach, spinal cord, testis, liver, and kidney.[1,30] Moreover, we have studied NPW expression in the rat adrenal gland, Total RNA was prepared from the hypothalamus, pituitary, and adrenal. RT-PCR analysis revealed the expression of NPW mRNA in these tissues, whereas in situ hybridization histochemistry showed the presence of NPW mRNA in the adrenal medulla. Immunohistochemical examination of tissue sections of the adrenal gland showed that NPW-LI is expressed only in the medulla but not in the cortex.[25] Moreover, we have shown that NPW-LI is colocalized with norepinephrine, but not epinephrine, in the adrenal chromaffin cells. NPW may play an important role in the regulation of the endocrine system response to stress, not only via the activation of the HPA axis but also via the paracrine pathway from the medulla to the cortex in the adrenal gland.[25]

In addition, NPW-containing cells have been identified by both light and electron microscopy in peripheral tissues such as the gastric antral glands in the rat, mouse, and human being. A double-immunolabeling study showed that NPW is present in the antral gastrin (G)-containing cells. NPW-positive cells accounted for 90% of chromagranin A-LI and 85% of gastrin-LI endocrine cells in rat gastric glands.[19] These results suggest an additional role for NPW in the regulation of gastric function. In the ontogenetic study of the rat stomach, NPW gene expression was initially detected at embryonic day 14 (E14) and gradually increased during development until postnatal day 1 (P1). Thereafter, the NPW mRNA level increased again from the weaning period (P21) through until adulthood. NPW-LI cells in the P1 antral stomach gradually increase during maturation throughout development.[19]

Furthermore, Mondal et al.[18] have reported that the levels of NPW in stomach antral cells significantly decrease after 15-h fasting but increase after refeeding. From these observations, feeding regulation by NPW may be affected through peripheral tissues including the stomach. NPW has also been shown to increase insulin release from isolated pancreatic islet cells in the presence of high concentrations of glucose. Fura-2 microfluorometry experiments with rat single β-cells show a concentration-dependent increase of NPW in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) at high glucose levels, which is abolished under external Ca^{2+}-free conditions and in the presence of an L-type Ca^{2+} channel blocker. Double immunohistochemical analysis shows that NPW-LI is present in pancreatic islets and is colocalized with insulin-LI in B cells, but not glucagon-LI A cells or somatostatin-LI D cells.[5] These results suggest that NPW serves as a local modulator of glucose-induced insulin release in rat islet cells.

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REGULATION OF FEEDING AND ENERGY METABOLISM BY NPW

With reference to the hypothalamic nuclei, it is well known that the LH is a feeding regulation center, the VMH is a center for satiety, and the ARC is an integrated center for feeding regulation and energy homeostasis. NPBW1 mRNA is detected in the VMH, the dorsal medial hypothalamic nucleus (DMH), the PVH, and the ARC, which regulate feeding behavior and metabolic disorders. Moreover, several studies have shown the presence of NPW mRNA and NPW neurons in the hypothalamus, in particular the hypothalamic nuclei related to feeding regulation. NPBWR1 knockout mice are hyperphagic and show decreased energy expenditure, suggesting that NPW may act as a modulator of feeding and could modulate feeding behavior. Initial studies indicated that icv infusion of NPW in male rats increases food intake for the first 2 h in the light phase.[26] Similarly, Levine et al.[15] reported an orexigenic action of NPW by showing that administration of NPW into the PVN and LH results in hyperphagia. From these results, NPW was initially considered to be an acute orexigenic peptide.[26]

Our immunohistochemical studies show the colocalization of NPW with other feeding-regulating peptides such as orexigenic and anorexigenic peptides in the rat hypothalamus. NPW-containing nerve fibers and orexin- or melanin-concentrating hormone (MCH)-containing neuronal cell bodies and nerve fibers have been identified in the LH in rat.[28] Moreover, Levine et al.,[15] using a dual immunostaining method, showed that c-Fos-LI is upregulated in orexin-containing neurons in the perifornical region of the LH after icv infusion of NPW. Orexin is recognized as a peptide that promotes food intake and plays a role in the control of sleep and wakefulness. These findings strongly suggest that the effect of NPW on food intake and wakefulness may be mediated via the activation of the orexin pathway.

By contrast, an orexigenic action of NPW in the dark phase in response to continuous icv infusion was reported by Mondal et al.,[17] who showed that this treatment decreases food intake in the rat for 48 h. In addition, the icv infusion of anti-NPW IgG stimulates food intake, suggesting that NPW plays a role as a food intake inhibitory peptide.[17] The action of NPW differs in its effect on feeding action depending on whether animals are maintained in a light phase or dark phase. NPBWR1 is localized in the SCh,[14,27] and we have observed NPW-IL fibers in this area,[29] suggesting that NPW may modulate circadian rhythms.

We have identified NPW-LI cell bodies in the VMH, which is known as a center for satiety. Leptin acts on neurons in the VMH, where its receptors are abundantly expressed, and reduces food intake. Moreover, Date et al.,[4] reported that NPW-LI neurons and leptin receptors are colocalized in the VMH, and that NPW expression is significantly upregulated in ob/ob (leptin-deficient) and db/db (leptin receptor–deficient) mice. Therefore, NPW may play important roles in feeding and energy metabolism, functioning as a substitute for leptin. Moreover, NPW reduces food intake via the melanocortin-4-receptor signaling pathway. Thus, NPW may activate POMC-containing neurons and inhibit NPY-containing neurons to control feeding regulation in the ARC (Fig. 2).[4]

Mondal et al.[17] have also reported that they did not detect enhanced locomotor activity when NPW is administered to rats, but they observed increased O_2 consumption and CO_2 production while showing an increase in body temperature compared with the administration of vehicle alone. This effect, independent of locomotor activity, may result from protein and triacylglycerol catabolism induced by corticosterone. Interestingly, Mondal et al.[17] have found that the levels of NPW isolated from rat stomach antral cells are decreased in fasted animals and increased in animals that had been refed. By contrast, female NPBWR1-/- mice do not display hyperphagic activity compared with wild-type mice.[10] Dun et al.[6] have reported that differences between male and female rats exist with respect to the distribution

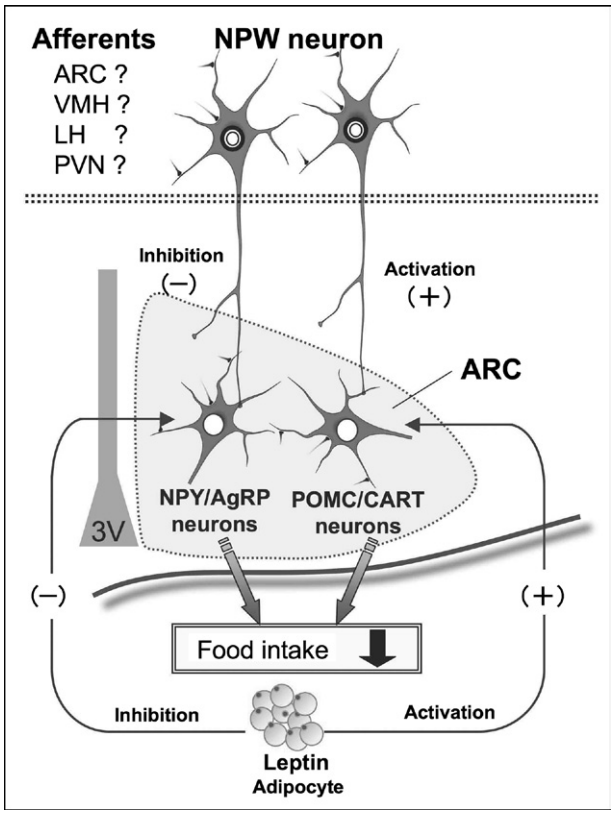

FIGURE 2 Schematic illustration based on the findings of morphological and physiological studies of appetite regulation in the hypothalamus by NPW neurons and feeding-related peptides. The plus or minus indicates stimulatory (+) or inhibitory (−) effects, respectively. ARC: arcuate nucleus; VMH: ventromedial hypothalamic nucleus; LH: lateral hypothalamus; PVN: paraventricular hypothalamic nucleus; 3V: third ventricle.

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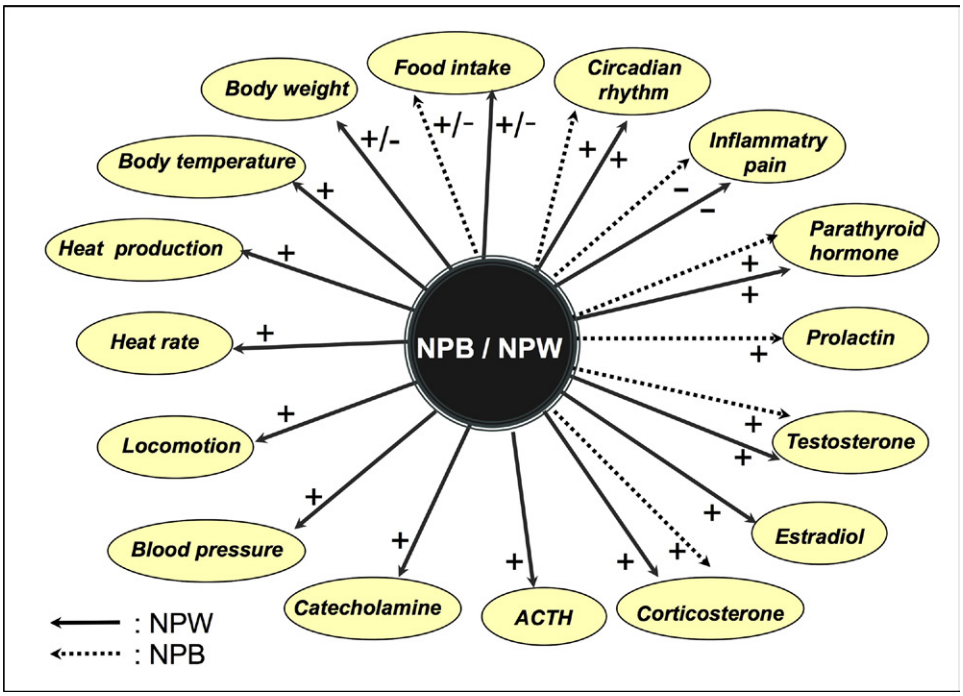

FIGURE 3 Multiple pharmacological effects of NPB/NPW. The plus or minus indicates stimulatory (+) or inhibitory (−). A solid line shows the action of NPW, and a dashed line shows the action of NPB.

of NPW. Further studies to elucidate sex differences of the action of NPW are required.

We have hypothesized that NPW may have several actions related to feeding regulation, including not only the regulation of food intake but also salivary secretion, chewing, and tongue movements. Consistent with the observation that the LH, amygdala, and BST are implicated in the regulation of feeding and taste, NPW is also expressed in these areas. In particular, nerve fibers in the LH project to premotoneurons in the NST and LPB. These premotor neurons are well known to innervate the salivary glands and tongue. On the other hand, Tanaka et al.[30] have reported that icv infusion of NPB causes acute effects on feeding behavior in mice; however, the long-term effect significantly reduces feeding behavior. Moreover, in the light phase, injection of NPB has no significant effect on feeding action.[30] By contrast, in the dark phase, icv infusion of NPB (3 nM) increases feeding activity for approximately 2 h. Moreover, a high dose (10 nM) of NPB reduces food intake. In addition, coadministration of NPB and CRH shows a completely inhibitory effect compared with that seen when NPB or CRH are administered separately. Urocortin, a CRH family member, is well known to be observed in the EW nucleus where NPB/NPW is localized. Moreover, we have confirmed NPW mRNA expression in the PVH by RT-PCR analysis.[29] Infusion of NPW icv upregulates c-Fos expression in the PVH region; this c-Fos expression is colocalized with CRH-containing neurons and its expression is decreased by spontaneous locomotor activity, which is considered to be a form of stress relief; spontaneous exercise results in the resumption of the anorexigenic effect of NPW (unpublished data). Therefore, NPB/W may be implicated both in feeding behavior and regulation of the stress response (Fig. 3).

ANTIHYPERALGESIC EFFECT OF NPB/W

NPBWR1 and NPBWR2 belong to the opioid and somatostatin receptor families, though GPR7 receptor binds nonselective opioid ligands with low affinity. In brain, the expression of NPB/NPW and GPR7 mRNA is located in the periaqueductal gray (PAG) and amygdala, which also contain opioid receptors, suggesting that NPB and NPW may be involved in nociception. Tanaka et al.,[30] for example, have used the paw flick test (heat-induced pain) in rat to investigate the participation of NPB in nociception. They found that NPB does not affect the behavioral response against an acute thermal stimulus. However, when the formalin test (chemically induced pain) is used, icv infusion of NPB significantly reduces the duration of licking. Moreover, inflammation induced by carrageenan significantly shortens latency in paw flicking. Later, Yamamoto et al. reported that intrathecal (i.t.) infusion of NPW or NPB decreases the number of agitation behaviors in response to formalin injection into the paw and attenuates the level of mechanical allodynia in rat, but not the level of thermal hyperalgesia induced by paw carrageenan injection in a

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dose-dependent manner.[33] In addition, antihyperalgesic activity was not affected by the opioid receptor antagonist naloxone, suggesting that the analgesic and anti-allodynia pathways are independent of the activation by opioid peptides.[33] On the other hand, i.t. infusion of NPW23 or NPB does not affect the outcomes of the hot-plate test or mechanical nociceptive test.[33] These results suggest that the effects of NPB result in part from differences of function involving supraspinal regulation of pain. Yamamoto et al.[33] have also observed that NPW participates in pain regulation, but only NPW23 or NPB significantly reduces the expression of c-Fos in the spinal dorsal horn (L4-5) induced by formalin injection into the paw. These results suggest that spinally applied NPW23 and NPB suppress the input of nociceptive information to the spinal dorsal horn, and they produce an analgesic effect in response to inflammatory pain.[33] Kelly et al.,[12] found a significant hyperalgesia to an acute inflammatory stimulus in NPB −/− mice induced by intraperitoneal application of acetic acid.

In addition, a low level of GPR7 receptor expression is observed in normal human and rat nerves and in primary rat Schwann cell cultures. In patients exhibiting inflammatory/immune-mediated neuropathies, increased GPR7 receptor expression is observed in myelin-forming Schwann cells in a manner similar to that seen in an immune-inflammatory animal model. These results suggest that the regulated expression of GPR7 receptor in myelin-forming Schwann cells in inflammatory/immune-mediated neuropathies modulates the effect of pain transmission.[34]

ACKNOWLEDGMENTS

This study was supported in part by the Ministry of Education, Culture, Sports, Science and Technology of Japan via a Grant-in-Aid for Scientific Research (C) (to F. Takenoya (23500863) and H. Kageyama (21590222)). Support was also received from the Ministry of Education, Culture, Sports, Science and Technology of Japan via a Grant-in-Aid for Exploratory Research 21659059 (to S. Shioda) and by a High-Technology Research Center project grant from the Ministry of Education, Culture, Sports, Science and Technology of Japan (to SS).

REFERENCES

1. Baker JR, Cardinal K, Bober C, Taylor MM, Samson WK. Neuropeptide W acts in brain to control prolactin, corticosterone, and growth hormone release. *Endocrinology* 2003;**144**:2816–21.
2. Brezillon S, Lannoy V, Franssen JD, Le Poul E, Dupriez V, Lucchetti J, et al. Identification of natural ligands for the orphan G protein-coupled receptors GPR7 and GPR8. *J Biol Chem* 2003;**278**:776–83.
3. Chen X, Herbert J. Regional changes in c-fos expression in the basal forebrain and brainstem during adaptation to repeated stress: correlations with cardiovascular, hypothermic and endocrine responses. *Neuroscience* 1995;**64**:675–85.
4. Date Y, Mondal MS, Kageyama H, Ghamari-Langroudi M, Takenoya F, Yamaguchi H, et al. Neuropeptide W: an anorectic peptide regulated by leptin and metabolic state. *Endocrinology* 2010;**151**:2200–10.
5. Dezaki K, Kageyama H, Seki M, Shioda S, Yada T. Neuropeptide W in the rat pancreas: potentiation of glucose-induced insulin release and Ca^{2+} influx through L-type Ca^{2+} channels in beta-cells and localization in islets. *Regul Pept* 2008;**145**:153–8.
6. Dun SL, Brailoiu GC, Yang J, Chang JK, Dun NJ. Neuropeptide W-immunoreactivity in the hypothalamus and pituitary of the rat. *Neurosci Lett* 2003;**349**:71–4.
7. Fujii R, Yoshida H, Fukusumi S, Habata Y, Hosoya M, Kawamata Y, et al. Identification of a neuropeptide modified with bromine as an endogenous ligand for GPR7. *J Biol Chem* 2002;**277**:34010–6.
8. Hochol A, Albertin G, Nussdorfer GG, Spinazzi R, Ziolkowska A, Rucinski M, et al. Effects of neuropeptides B and W on the secretion and growth of rat adrenocortical cells. *Int J Mol Med* 2004;**14**:843–7.
9. Hochol A, Tortorella C, Ricinski M, Ziolkowska A, Nussdorfer GG, Malendowicz LK. Effects of neuropeptides B and W on the rat pituitary–adrenocortical axis: in vivo and in vitro studies. *Int J Mol Med* 2007;**19**:207–11.
10. Ishii M, Fei H, Friedman JM. Targeted disruption of GPR7, the endogenous receptor for neuropeptides B and W, leads to metabolic defects and adult-onset obesity. *Proc Natl Acad Sci U S A* 2003;**100**:10540–5.
11. Kawasaki M, Onaka T, Nakazato M, Saito J, Mera T, Hashimoto H, et al. Centrally administered neuropeptide W-30 activates magnocellular neurosecretory cells in the supraoptic and paraventricular nuclei with neurosecretion in rats. *J Endocrinol* 2006;**190**:213–23.
12. Kelly MA, Beuckmann CT, Williams SC, Sinton CM, Motoike T, Richardson JA, et al. Neuropeptide B-deficient mice demonstrate hyperalgesia in response to inflammatory pain. *Proc Natl Acad Sci U S A* 2005;**102**:9942–7.
13. Kitamura Y, Tanaka H, Motoike T, Ishii M, Williams SC, Yanagisawa M, et al. Distribution of neuropeptide W immunoreactivity and mRNA in adult rat brain. *Brain Res* 2006;**1093**:123–34.
14. Lee DK, Nguyen T, Porter CA, Cheng R, George SR, O'Dowd BF. Two related G protein-coupled receptors: the distribution of GPR7 in rat brain and the absence of GPR8 in rodents. *Brain Res Mol Brain Res* 1999;**71**:96–103.
15. Levine AS, Winsky-Sommerer R, Huitron-Resendiz S, Grace MK, de Lecea L. Injection of neuropeptide W into paraventricular nucleus of hypothalamus increases food intake. *Am J Physiol Regul Integr Comp Physiol* 2005;**288**:R1727–32.
16. Mazzocchi G, Rebuffat P, Ziolkowska A, Rossi GP, Malendowicz LK, Nussdorfer GG. G protein receptors 7 and 8 are expressed in human adrenocortical cells, and their endogenous ligands neuropeptides B and W enhance cortisol secretion by activating adenylate cyclase and phospholipase C-dependent signaling cascades. *J Clin Endocrinol Metab* 2005;**90**:3466–71.
17. Mondal MS, Yamaguchi H, Date Y, Shimbara T, Toshinai K, Shimomura Y, et al. A role for neuropeptide W in the regulation of feeding behavior. *Endocrinology* 2003;**144**:4729–33.
18. Mondal MS, Yamaguchi H, Date Y, Toshinai K, Kawagoe T, Tsuruta T, et al. Neuropeptide W is present in antral G cells of rat, mouse, and human stomach. *J Endocrinol* 2006;**188**:49–57.
19. Mondal MS, Yamaguchi H, Date Y, Tsuruta T, Shimbara T, Toshinai K, et al. Ontogeny of a new enteric peptide, neuropeptide W (NPW), in the developing rat stomach. *Regul Pept* 2008;**145**:141–6.
20. Niimi M, Murao K. Neuropeptide W as a stress mediator in the hypothalamus. *Endocrine* 2005;**27**:51–4.

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21. O'Dowd BF, Scheideler MA, Nguyen T, Cheng R, Rasmussen JS, Marchese A, et al. The cloning and chromosomal mapping of two novel human opioid–somatostatin-like receptor genes, GPR7 and GPR8, expressed in discrete areas of the brain. *Genomics* 1995;**28**:84–91.
22. Price CJ, Samson WK, Ferguson AV. Neuropeptide W influences the excitability of neurons in the rat hypothalamic arcuate nucleus. *Neuroendocrinology* 2008;**88**:88–94.
23. Samson WK, Baker JR, Samson CK, Samson HW, Taylor MM. Central neuropeptide B administration activates stress hormone secretion and stimulates feeding in male rats. *J Neuroendocrinol* 2004;**16**:842–9.
24. Schulz S, Stumm R, Hollt V. Immunofluorescent identification of neuropeptide B-containing nerve fibers and terminals in the rat hypothalamus. *Neurosci Lett* 2007;**411**:67–71.
25. Seki M, Kageyama H, Takenoya F, Hirayama M, Kintaka Y, Inoue S, et al. Neuropeptide W is expressed in the noradrenalin-containing cells in the rat adrenal medulla. *Regul Pept* 2008;**145**:147–52.
26. Shimomura Y, Harada M, Goto M, Sugo T, Matsumoto Y, Abe M, et al. Identification of neuropeptide W as the endogenous ligand for orphan G protein-coupled receptors GPR7 and GPR8. *J Biol Chem* 2002;**277**:35826–32.
27. Singh G, Maguire JJ, Kuc RE, Fidock M, Davenport AP. Identification and cellular localisation of NPW1 (GPR7) receptors for the novel neuropeptide W-23 by [125I]-NPW radioligand binding and immunocytochemistry. *Brain Res* 2004;**1017**:222–6.
28. Takenoya F, Kitamura S, Kageyama H, Nonaka N, Seki M, Itabashi K, et al. Neuronal interactions between neuropeptide W- and orexin- or melanin-concentrating hormone-containing neurons in the rat hypothalamus. *Regul Pept* 2008;**145**:159–64.
29. Takenoya FYM, Kageyama H, Shiba K, Endo K, Nonaka N, Date Y, et al. Distribution of neuropeptide W in the rat brain. *Neuropeptides* 2010;**44**:99–106.
30. Tanaka H, Yoshida T, Miyamoto N, Motoike T, Kurosu H, Shibata K, et al. Characterization of a family of endogenous neuropeptide ligands for the G protein-coupled receptors GPR7 and GPR8. *Proc Natl Acad Sci U S A* 2003;**100**:6251–6.
31. Taylor MM, Yuill EA, Baker JR, Ferri CC, Ferguson AV, Samson WK. Actions of neuropeptide W in paraventricular hypothalamus: implications for the control of stress hormone secretion. *Am J Physiol Regul Integr Comp Physiol* 2005;**288**:R270–5.
32. Ulrich-Lai YM, Herman JP. Neural regulation of endocrine and autonomic stress responses. *Nat Rev Neurosci* 2009;**10**:397–409.
33. Yamamoto T, Saito O, Shono K, Tanabe S. Anti-hyperalgesic effects of intrathecally administered neuropeptide W-23, and neuropeptide B, in tests of inflammatory pain in rats. *Brain Res* 2005;**1045**:97–106.
34. Zaratin PF, Quattrini A, Previtali SC, Comi G, Hervieu G, Scheideler MA. Schwann cell overexpression of the GPR7 receptor in inflammatory and painful neuropathies. *Mol Cell Neurosci* 2005;**28**:55–63.

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Chapter 137

Orexins

Naima Ahmed, Harman S. Mattu and Harpal S. Randeva

ABSTRACT

Orexins are named after the ancient Greek word *Orexis* meaning hunger or appetite. This was in reference to what was initially believed to be the primary role of Orexins, that is, stimulating feeding. Orexins A and B (hypocretins 1 and 2), the excitatory neuropeptides first discovered in the lateral hypothalamus (LH), regulate feeding, wakefulness, sleep, and energy homeostasis. Previous studies have demonstrated that orexins are produced exclusively in the LH. Recent studies have found orexin peptides and their receptors (OX1R and OX2R) in a wide range of tissues including the intestines, pancreas, adrenal gland, reproductive tract, and adipose tissue. The central orexinergic pathways that originate in the medulla can stimulate the hypothalamic–pituitary–adrenal axis and thus affect sympathetic tone. In the intestines, the receptors are modulated by feeding status and therefore affect insulin secretion and intestinal motility. Therefore, orexins play an important role not only in the CNS but also in the periphery.

OVERVIEW OF THE STRUCTURE OF OREXINS AND ITS RECEPTORS

Orexin, also referred to as hypocretin (Hcrt), was initially discovered in 1998 by two separate independent research groups with the help of orphan receptor technologies and substractive cDNA cloning. The latter involved the analysis of the expression patterns of substracted hypothalamus-enriched cDNAs, which unveiled the original 569-nucleotide clone encoding a 130-amino acid protein residue (prepro-orexin). This putative secretory protein had an apparent signal sequence. Potential maturation and cleavage of this peptide suggested the possibility of two homologous peptides being generated, which shared a weak sequence homology with secretin. These peptides were later named hypocretin-1 and hypocretin-2 because of their hypothalamic localization.[13] The other group, led by Sakurai and colleagues, screened mRNA extracts of rat brain to induce intracellular Ca^{2+} transients in a battery of cells expressing orphan G protein-coupled receptors. Because of their orexigenic effects, the two peptides were named orexin A and orexin B. Orexin B is 28 amino acids in size and was identical to hypocretin 2. Similarly, orexin A, which is 33 amino acids in size, was found to be identical to hypocretin 1.[17]

Apart from the hypothalamus, this peptide is also expressed in various other endocrine gland tissues: pituitary, thyroid, parathyroid, adrenals, testes, ovaries, and the pancreatic islets. The prepro-orexin of mammalians including pigs, dogs, rats, mice, and humans consists of 130–131 amino acids. The human prepro-orexin gene is encoded on chromosome 17. It has two exons, one of which has the full coding region of the finalized peptide. The prepro-orexin gene found in mice consists of a promoter 3.2 kb in size, sufficient enough to direct expression of the genes located downstream of the lateral hypothalamus.[12]

Both orexins A and B can stimulate orexin receptors (OX1R and OX2R). In addition, according to the findings of Sakurai and colleagues, the OX1R shows an affinity that is ten times higher for orexin A than for orexin B, whereas OX2R does not seem to show any preference between the two peptides. The actual cell signaling pathways that become activated remain quite obscure. However, the receptors stimulate Gi, Gs, and Gq proteins.[16] Moreover, an increase in Ca^{2+} is a prevalent response observed in almost all cell lines that have been heterologously expressed.[17]

OX1R seems to act by coupling with adenylyl cyclase regulation via multiple mechanisms.[7] One study involved the production of a recombinant that expressed OX1R in Chinese hamster ovary cells to observe the regulatory pathway of this receptor with respect to adenylyl cyclase. OX1R receptors stimulate adenylyl cyclase through a low potency Gs coupling and a high potency phospholipase C/PKC coupling.[7] After reversing the inhibition by using pertussis toxin, observations suggested the involvement of Gi/o proteins. In addition, orexin B is almost as effective as orexin A in increasing the cAMP levels compared with the increase in Ca^{2+} and phospholipase C activation where orexin A is ten times more potent. Moreover, OX2R seems to be coupled to Gq/11 in addition to the Gi protein when expressed in cell lines. Food deprivation shows a differential effect on the coupling that occurs between orexin receptors and different G proteins.

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00137-8
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ADIPOSE TISSUE

Both receptors of orexin play a role in regulating various physiological mechanisms including energy metabolism and body weight. Furthermore, because adipose tissue also plays a role in body weight regulation, the focus was whether components of orexin are also expressed in the adipose tissue. We observed the effect of orexins A and B in human adipose tissue by incubating adipose tissue explants with orexins A and B. This shows an increased expression of PPAR-γ-2, which is known to regulate adipose differentiation.[3] In addition, orexins A and B decrease hormone-sensitive lipase enzyme expression in the omental adipose tissue compartment. Orexin A reduces the release of glycerol indicating that orexin presumably has a role in regulating fat tissue metabolism through the inhibition of lipolysis.[3] Another study involving the treatment of 3T3-L1 cells with low doses of orexin A also shows induction of cell proliferation, whereas orexin B inhibits this action.

GASTROINTESTINAL TRACT

A study conducted in 2003 showed the presence of a subset of neurons in the gastrointestinal tract that has an orexin-like immunoreactivity and is able to express functional receptors that can bind orexin peptides.[10] These also happen to express receptors for leptin. The same study also revealed the role of orexin in increasing GI motility via secretomotor neurons in the submucosal plexus derived from a guinea pig. This combined with the observation that the orexins are present in the lateral hypothalamus and that the lateral hypothalamus has an important role in the control of gut-sensitive neurons in the dorsal vagal complex indicate that orexins might be involved in the central control of the gastrointestinal tract.

Fasting increases the levels of the phosphorylated cAMP response binding protein (pCREB) in orexin immunoreactive neurons.[10] Another study was the first to show the importance of the role of orexin in glucose metabolism and energy homeostasis after using orexin knockout mice.[22] This study showed that orexin deficiency causes age-linked development of impaired glucose tolerance and insulin resistance in both nonobese male mice and mildly obese female mice that are on a normal diet. In the stomach, secretion of gastric acid in response to orexin A occurs in conscious rats, indicating that orexin A might act on the brain to activate acid secretion through the regulation of the vagal system. Moreover, intraperitoneal administration of orexin in rats is ineffective; vagotomy or atropine diminishes the action of central orexin. Nevertheless, some species do express endogenous orexin in their stomach.

HYPOTHALAMIC–PITUITARY–ADRENAL AXIS

Orexin peptides regulate the adrenal glands either directly or indirectly by acting via the HPA axis. A study involving intravenous injection of either peptide has been found to increase the concentration of adrenocorticotropic hormone (ACTH), which is partially modulated by the action of the corticotropic releasing hormone (CRH). Orexins and their receptors were initially found to be expressed in rat and human brains as discussed earlier, and there have been numerous reviews on the presence of mRNA of orexin peptides and receptors in the hypothalamus. However, the orexin peptides are not expressed in the human pituitary gland as no mRNA or protein has been found. Studies conducted in other mammalians have yielded similar results.[8] Interestingly, immunocytochemistry and PCR studies on rat pituitary glands show the expression and distribution of both receptor types for orexin peptides. This has also been observed in both sheep and human studies.[8]

Blanco et al. conducted a study in 2001 focusing on the expression of orexin receptors in the human pituitary, which also showed (in agreement with previous studies in animals) that both the orexin receptors are expressed in the human pituitary gland.[9] In addition, their immunostaining showed that the OX1R is distributed in larger numbers compared with OX2R. Through various other techniques, they also found that the cells that expressed OX1R are somatotrophs, whereas the cells expressing OX2R are largely corticotrophs. Furthermore, they discovered that OX2R is only expressed in the basophil cells of the pars intermedia in the human pituitary.

ADRENAL GLANDS

The initial RT-PCR and immunocytochemistry studies conducted on orexin receptor expression in the rat adrenal gland showed the presence of OX1R and OX2R in the adrenal medulla. Later studies also show the presence of these receptors in the rat adrenal cortex and cortical cells (using RT-PCR, western blotting, and quantitative real time PCR).[19]

In vivo studies (rat) observing the effects of orexin on the adrenal gland show an elevation of plasma concentrations of both ACTH and corticosterone within two hours of a single systemic administration of orexin A, whereas orexin B do not have any stimulatory effect.[19] These findings suggest that orexin A is able to cross the blood–brain barrier relatively quickly, whereas orexin B may be quickly broken down in the circulation, and this confirms direct quantification in 1999 by the highly sensitive multiple time-regression analysis method that orexin A, but not orexin B, rapidly enters brain tissue from blood. Other studies have

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also shown a direct function of orexins in the adrenal cortex without relying on the HPA axis through a prolonged subcutaneous administration of orexin A and orexin B in rats, leading to increased corticosterone,[2] but not an increase in the ACTH plasma levels. Orexins A and B increase norepinephrine concentrations. High doses of orexin A also increased plasma epinephrine indicating that orexins stimulate the sympathetic outflow and the adrenal medulla.

With regard to cell signaling and coupling of orexin receptors in the adrenal cortex, there have been varying results thus far. The effect of orexin A on the release of glucocorticoid in both rat and human adrenocortical cells are inhibited by adenylyl cyclase or protein kinase A (PKA) inhibitors indicating that the coupling of the involved receptor subtype with Gs increased cAMP production within an hour in the adrenocortical cells. Moreover, local orexin receptors are capable of coupling with Gq, Gi, and Go, which was dependent on the feeding state of the rats.[23] The second messenger studies supported the dose-dependent increase of cAMP and the increase in inositol triphosphate (IP3) after stimulation with orexin A. Furthermore, the human adrenal membranes showed an increase in the labeling of Gq, Gs, and Gi but not Go after the stimulation with orexin A.[2]

Concerning the functional role of orexin in the adrenal glands, one study observed the role of orexins on steroidogenesis in the human adrenocortical NCI H295R cell line.[23] Their findings showed an increase in cortisol secretion and a strong elevation in intracellular Ca^{2+} after the stimulation of the cell line with orexin. Additionally, through the use of quantitative PCR, they studied the steroidogenic enzymes after the stimulation of orexin A. Their findings indicate that mRNA levels of CYP11B1 and CYP11B2 are elevated from the early stages of stimulation with orexin A, whereas HSD3B2 and CYP21 mRNA levels are elevated after a prolonged stimulation. CYP11A and CYP17 levels do not respond to stimulation by orexins. PKC and ERK1/2 are also involved, and there are also high levels of OX2R mRNA compared with those of OX1R in the human adrenals, indicating that OX2R is present in the human and rat adrenal cortices.[23]

Wenzel et al. also confirmed (after comparing the effect of angiotensin II or forskolin on the CYP11B and CYP11B1 expression) that orexin A is low and that the effect of orexin A on the mRNA of HSD3B2 is similar to that of angiotensin II. This study has successfully shown the role of orexin in the differential regulation of the enzymes involved in steroidogenesis and more so in increasing the synthesis of cortisol in the human adrenocortical cell line. Furthermore, the study shows the effect of orexin A on the autoregulatory function of OX2R where the orexins modulate the expression of HSD3B2, which involves PKA and Ca^{2+} signaling pathways and the ERK1/2 kinase activation.[23]

HYPOTHALAMIC–PITUITARY–THYROID AXIS

The paraventricular nucleus (PVN) is one of many sites in the brain, which receive input signals from orexin. The nucleus contains neurons that synthesize the thyrotropin-releasing hormone (TRH). During fasting, the thyroid hormone secretion is reduced and the expression of mRNA prepro-orexin is increased. Therefore, a study led by Kok and colleagues speculated that an orexin neural circuit could regulate the activity of the hypothalamic TRH neurons and hence affect the Hypothalamic–Pituitary–Thyroid (HPT) axis.[11] There are no complete studies on the putative effects of orexins on the HPT axis in animals. An intracerebroventricular or an intravenous administration of orexin A in rats results in decreased concentrations of circulating thyroid-stimulating hormone (TSH). However, numerous other studies have shown that orexin A does not have a significant effect on the pituitary–thyroid axis. However, central administration of orexin A has been shown to increase circulating levels of TSH. Even though the topography of the orexin and thyrotrope neural circuits indicate that orexin input determines the TRH neuroactivity, the characteristics of the signal have been obscure as orexins A and B might have differential effects resulting from their unique chemical structure.

TESTES AND OVARIES

Orexin is involved as one of pleotropic regulators of different biological systems including the reproductive organs. This results from the observations seen in the peripheral expression and functions of orexin and orexin type-1 receptor mRNAs in the testis.[1] The biological mechanisms of orexin and its expression patterns are yet to be studied in male gonads. However, the immunoreactivity of OXA and OXB has been seen. One study took the initiative to analyze orexin A and B immunoreactivity and showed its presence in the rat testis. Moreover, the expression of prepro-orexin mRNA has thus far been found to be expressed in the penis and epididymis but not in the testes. Conversely, in humans, both OX1R and OX2R mRNA are expressed in the testis, the seminal vesicle, penis, and epididymis.[1] Nevertheless, there is no evidence for major reproductive defects in orexin knockout rat models, indicating that orexin might not have a vital role in the control of testicular function. However, regardless of the proven role of orexin in the regulation of food intake from previous studies, the orexin knockout mice that were used are also devoid of the major metabolic phenotype because they exhibit a modest trend to decrease food intake and mild tendency toward obesity.

Orexin receptors OX1R and OX2R are both expressed in the rat ovaries, whereas prepro-orexin was not found

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to be expressed in this area and differs according to the estrus cycle.[18] This same group also discovered that during proestrus, the expression of orexin receptors increases in the afternoons and evenings. However, this does not occur during other phases of the estrus cycle. After blocking the action or the release of gonadotropin-releasing hormone, Silveyra and colleagues also found that it inhibits the upregulation of expression in the pituitary and the ovaries. However, a similar effect is not observed in the hypothalamus. In addition, an injection of OX1 and OX2 antagonists inhibits the actions of luteinizing hormone and follicle-stimulating hormone as well as ovulation, resulting in the ovaries appearing hyperemic. These studies collectively show that the hormonal environment during the female cycle in the ovaries controls orexin expression. Hence, blocking the signaling pathway in orexin receptors reduces the release of gonadotropin.

PANCREATIC ISLETS

Both orexins A and B are expressed in the pancreas, and the expression varies in different species. For example, Kirchgessner and Liu discovered in 1999 that the rat and guinea-pig pancreas showed receptor OX1R and OX2R immunoreactivity in the β-cell pancreatic islets, nerve bundles, which are involved in blood vessels, and in nerve fibers located in the pancreatic ganglia.[10] Additionally, orexin A is colocalized with insulin in addition to colocalization with somatostatin and glucagon positive cells in humans. However, in rats, orexin A immunoreactivity is seen in β cells and pancreatic polypeptide-containing cells, but this is not observed in human α cell nor in δ cell islets.[4] Another study involving isolated rat islets showed the release of orexin A that is dependent on low glucose concentration. The pathway to this mechanism remains elusive, but the presence of orexin in the pancreas indicates that orexin may be involved in the regulation of pancreatic hormone secretion and the modulation of glucose.[22] The hypothalamic neurons, which are able to sense glucose, have an essential role in activating the adaptive metabolic and behavioral responses. The glucose-excited neurons and the glucose-inhibited neurons are responsible for sensing glucose in the hypothalamus. As the name suggests, the glucose-inhibited neurons decrease the firing rate, whereas the glucose-excited neurons increase it because the glucose levels outside the cells increase within the physiological range in the celebrospinal fluid.[22]

The suprachiasmatic nuclei (SCN) and the PVN are where the circadian clock outputs are initiated in the hypothalamus. The sympathetic and parasympathetic activities are also regulated here. The SCN modulates the daily rhythm in the plasma glucose concentration and is not dependent on feeding activities.[22]

The circadian clock of SCN can be reset via arousal promoting signals from orexin neurons. Reciprocally, the activation of orexin neurons is tightly controlled by the circadian rhythm. After restricting the feeding of orexin knockout mice, the activity and core body temperature rhythms remain, indicating that orexin may not be required for the signal transduction of the food-entrainable oscillator, presumed to be situated in the dorsomedial hypothalamus.[22] Hyperglycemia linked to peripheral insulin resistance suppresses the expression of orexin. Moreover, as this could have otherwise maintained the hypothalamic insulin sensitivity, it leads to a cycle being formed between the peripheral and hypothalamic insulin resistance via the suppression of orexin levels.[22]

ENERGY EXPENDITURE

When comparing the energy expenditure between orexins A and B, the very first study conducted on the correlation between energy expenditure and orexins revealed that not only did orexin increase energy expenditure, but it also showed that orexin B did not increase the metabolic rate as much as orexin A did.[20] The same study also microfused orexin into the third ventricle, resulting in stimulation of the metabolic rate. This stimulation appeared to be higher during the dark cycle when compared with that of the light cycle in mice, indicating that circadian differences of orexin A increase/influence the metabolic rate. Another study found (by using Fos immunoreactivity, which is generally known for providing information on the sites of action of various stimuli) that there is not only an increase in fos immunoreactivity in the orexin neurons but that this increase also coincides with the increase in orexin A-induced metabolic rate in the dark cycle compared with that of the light cycle.[20] ATP and substrate oxidation is required for muscle contraction and other physical activities. Thus, the effects of orexin on physical activities such as locomotion, grooming, and rearing all require muscle contraction, which subsequently results in a calorific cost leading to energy expenditure. To confirm these ideas, one study showed that intra-PVH orexin A increases physical activity and energy expenditure in a dose dependent manner.[20]

Appetite and Food Intake

The lateral hypothalamus is known for regulating metabolism and food intake. This was discovered by studies involving the effect of lesions in the lateral hypothalamus, which leads to reduced food intake and maintenance of the newly achieved reduced weight in obese rats.[14] This fact combined with the expression of mRNA of prepro-orexin in this area of the brain led to studies focusing on the effects of orexin on feeding behavior. Sakurai and colleagues administered orexins centrally, which led to the

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stimulation of food intake, indicating that orexins play a role in central regulation of feeding.[17] They followed by observing whether this regulation of food intake is modulated by nutritional state and compared the expression of mRNA prepro-orexins in the hypothalamus of both fed and fasting rats. There is an upregulation of mRNA prepro-orexin expression in the fasting rats. This increase in expression is 2.4 times higher compared with that of the nonfasting rats. Moreover, the elevated expression of orexin precursor mRNA surpasses that of the increase observed in NPY mRNA.[16] Another study also found that enhanced orexin receptor-2 signaling prevents diet-induced obesity and improves leptin sensitivity.[5]

PHYSIOLOGICAL IMPLICATIONS

Insulin resistance stemming from impaired glucose metabolism plays a major role in the pathogenesis of type 2 diabetes and occurs during the aging process. In 2008, Tsuneki et al. studied the involvement of orexin on glucose metabolism by using orexin knockout mice.[21] They discovered that orexin has a vital role in maintaining normal insulin sensitivity with advancing age. The male mice with orexin knocked out show an impaired insulin sensitivity and hyperinsulinemia at nine months of age. However, this characteristic is not seen in male mice aged 2–6 months. Curiously, glucose tolerance is not affected in orexin knockout middle-aged males. A plausible reason could be that the insulin resistance is compensated by hyperinsulinemia to maintain glucose tolerance. The orexin knockout female mice also show impaired glucose tolerance and hyperinsulinemia at the same ages as their male counterparts. Interestingly, only the female orexin knockout mice show late-onset mild obesity suggesting that male orexin knockout mice developed age-related insulin resistance without obesity as opposed to insulin resistance combined with other factors related to obesity, which might act synergistically because of impaired glucose tolerance in female orexin knockout mice.[21] The findings collectively suggest that orexin is essential in maintaining hypothalamic insulin signaling. This signaling is probably responsible for preventing the onset of peripheral insulin resistance, which tends to increase with age. This may explain why type 2 diabetes incidence increases in normal weight patients with orexin deficiency.

The orexin/ataxia-3 mice show a late-onset of obesity despite being on a 30% lower food intake during the dark cycle. This pattern of obesity is observed in both male and female mouse models. This finding combined with the observation that orexin is known to increase energy expenditure, provides a plausible explanation as to why these mice gain weight regardless of their diet. Therefore, study of spontaneous motor activity with lower energy expenditure may be attributed to variations therein. Their findings are consistent with the lower energy expenditure in transgenic mice as their spontaneous activity is lower than the wild-type mice. This observation was noted only during the dark period, with no difference observed during the light period.[6] This is an indication of the essential role of orexin-containing neurons in controlling energy homeostasis and wakefulness as well as regulating a susceptible genetic background. Thus, this study and other studies have collectively shown the orexin/ataxin-3 mice model to be an important tool not only for studying the pathophysiology and treatment of narcolepsy but also other neuroendocrinological conditions.

Okumura and Nozu have also proposed that lack of orexin signaling within the brain may possibly trigger the progressive development of functional gastrointestinal disorders (FGID). They also suggested that the increase of orexin signaling by pharmacological manipulation may provide a treatment for FGID.[15]

Peripheral orexin A reduces the rate of gastric emptying in humans. Orexin A significantly insulin plasma concentrations and reduces plasma leptin concentrations. However, orexin A does not have any effect on the plasma concentrations of either glucagon or glucose. Also, appetite does not seem to be affected.[20] Orexin A and orexin receptor-like immunoreactivity are present in the human gut. The physiological implications and mechanisms related to the rate of gastric emptying (which is reduced by orexin A) might reflect a predominant inhibitory tone on gastric emptying while fasting because the gastric acid accumulates to prepare for the next digestive process. Additionally, orexin A plasma concentrations are elevated during fasting, and therefore, the probable pharmacological elevation of orexin A plasma concentrations after eating might reflect the artificial condition that would otherwise have a major effect on appetite ratings. Nevertheless, as these were not affected, it suggests that the peripheral orexin A constitutes its own pool with no interaction with the central orexin A pathways.[20]

CONCLUSIONS

Orexins have various roles and influence many functions including autonomic regulation, endocrine function, food intake, appetite, wakefulness, and sleep. Orexins and their receptors are present in a wide range of organs outside the CNS. These molecules are involved in various physiological mechanisms. Based on the current findings, there is strong evidence supporting their functional roles in the periphery. Moreover, many studies have shown that disturbances in the expression or the levels of orexin peptides can result in pathological disorders such as late-onset obesity, impaired insulin sensitivity, hyperinsulinemia, and disturbances in gut function. Nevertheless, the precise mechanisms through which orexins act are yet to be fully understood.

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REFERENCES

1. Barreiro ML, Pineda R, Gaytan F, Archanco M, Burrell MA, Castellano JM, et al. Pattern of orexin expression and direct biological actions of orexin-A in rat testis. *Endocrinology* 2005;**146**:5164–75.
2. Blanco M, Lopez M, Gargia-Caballero T, Gallego R, Vazquez-Boquete A, Morel G, et al. Cellular localization of orexin receptors in human pituitary. *J Clin Endocrinol Metab* 2001;**86**:1616–9.
3. Digby JE, Chen J, Tang JY, Lehnert H, Matthews RN, Randeva HS. Orexin receptor expression in human adipose tissue: effects of orexin-A and orexin-B. *J Endocrinol* 2006;**191**:129–36.
4. Ehrström M, Gustafsson T, Finn A, Kirchgessner A, Grybäck P, Jacobsson H, et al. Inhibitory effect of exogenous orexin a on gastric emptying, plasma leptin, and the distribution of orexin and orexin receptors in the gut and pancreas in man. *Clin Endocrinol Metab* 2005;**90**:2370–7.
5. Funato H, Tsai AL, Willie JT, Kisanuki Y, Williams C, Sakurai T, et al. Enhanced orexin receptor-2 signaling prevents diet-induced obesity and improves leptin sensitivity. *Cell Metab* 2009;**9**:64–76.
6. Hara J, Beuckmann CT, Nambu T, Willie JT, Chemelli RM, Sinton CM, et al. Genetic ablation of orexin neurons in mice results in narcolepsy, hypophagia, and obesity. *Neuron* 2001;**30**:345–54.
7. Holmqvist T, Johansson L, Östman M, Ammoun S, Åkerman K, Kukkonen JP. OX1 orexin receptors couple to adenylyl cyclase regulation via multiple mechanisms. *J Biol Chem* 2005;**280**:6570–9.
8. Kagerer SM, Jöhren O. Interactions of orexins/hypocretins with adrenocortical functions. *Acta Physiol* 2010;**198**:361–71.
9. Karteris E, Machado RJ, Chen J, Zervou S, Hillhouse EW, Randeva HS. Food deprivation differentially modulates orexin receptor expression and signaling in rat hypothalamus and adrenal cortex. *Am J Physiol Endocrinol Metab* 2005;**288**:E1089–100.
10. Kirchgessner AL. Orexins in the brain-gut axis. *Endocr Rev* 2002;**23**:1–5.
11. Kok SW, Roelfsema F, Overeem S, Lammers GJ, Frölich M, Meinders AE, et al. Altered setting of the pituitary-thyroid ensemble in hypocretin-deficient narcoleptic men. *Am J Physiol Endocrinol Metab* 2004;**288**:E892–9.
12. Kukkonen JP, Holmqvist T, Ammoun S, Åkerman K. Functions of the orexinergic/hypocretinergic system. *Am J Physiol Cell Physiol* 2002;**283**:C1567–91.
13. Lecea L, Kilduff TS, Peyron C, Gao XB, Foye PE, Danielson PE, et al. The hypocretins: hypothalamus-specific peptides with neuroexcitatory activity. *Proc Natl Acad Sci U S A* 1998;**95**:322–7.
14. Milam KM, Stern JS, Storlien LH, Keesey RE. Effect of lateral hypothalamic lesions on regulation of body weight and adiposity in rats. *Am J Physiol Regul Integr Comp Physiol* 1980;**239**:R337–43.
15. Okumura T, Nozu TJ. Role of brain orexin in the pathophysiology of functional gastrointestinal disorders. *Gastroenterol Hepatol* 2011;**3**:61–6.
16. Randeva HS, Karteris E, Grammatopolous D, Hillhouse EW. Expression of orexin-A and functional orexin type 2 receptors in the human adult adrenals: implications for adrenal function and energy homeostasis. *J Clin Endocrinol Metab* 2001;**86**:4808–13.
17. Sakurai T, Amemiya A, Ishii M, Matsuzaki I, Chemelli RM, Tanaka H, et al. Orexins and orexin receptors: a family of hypothalamic neuropeptides and G protein-coupled receptors that regulate feeding behavior. *Cell* 1998;**92**:573–85.
18. Silveyra P, Lux-Lantos V, Libertun C. Both orexin receptors are expressed in rat ovaries and ovulation fluctuate with the estrous cycle: effects of orexin receptor antagonists on gonadotropins and ovulation. *Am J Physiol Endocrinol Metab* 2007;**293**:E977–85.
19. Spinazzi R, Andreis PG, Rossi GP, Nussdorfer GG. Orexins in the regulation of the hypothalamic–pituitary–adrenal axis. *Pharmacol Rev* 2006;**58**:46–57.
20. Teske JA, Billington CJ, Kotz CM. Hypocretin/orexin and energy expenditure. *Acta Physiol* 2010;**198**:303–12.
21. Tsuneki H, Anzawa Y, Soeda Y, Tokai E, Wada T, Kimura M, et al. Age-related insulin resistance in hypothalamus and peripheral tissues of orexin knockout mice. *Diabetologia* 2008;**51**:657–67.
22. Tsuneki H, Wada T, Sasaoka T. Role of orexin in the regulation of glucose homeostasis. *Acta Physiol* 2010;**198**:335–48.
23. Wenzel J, Grabinski N, Knopp CA, Dendorfer A, Ramanjaneya M, Randeva HS, et al. Hypocretin/orexin increases the expression of steroidogenic enzymes in human adrenocortical NCI H295R cells. *Am J Physiol Regul Integr Comp Physiol* 2009;**297**:R1601–9.

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Chapter 138

PACAP

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ABSTRACT

Pituitary adenylate cyclase-activating polypeptide (PACAP), a peptide of the vasoactive intestinal polypeptide (VIP)-glucagon superfamily, was initially characterized by virtue of its ability to stimulate cAMP formation in cultured rat anterior pituitary cells. Three PACAP receptors have been cloned so far: a PACAP-selective receptor termed PAC1 and two PACAP/VIP-mutual receptors termed VPAC1 and VPAC2. PACAP and its receptors are widely expressed in the brain and in peripheral organs, notably in the hypothalamus and in endocrine glands. Indeed, there is now clear evidence that PACAP exerts neuroendocrine, paracrine, and/or autocrine control of the activity of the pituitary, thyroid, testis, ovary, adrenal medulla, adrenal cortex, and endocrine pancreas. These observations suggest that selective PACAP agonists and antagonists could have therapeutic value for the treatment of various endocrine disorders.

INTRODUCTION

Pituitary adenylate cyclase-activating polypeptide (PACAP) was first isolated from ovine hypothalamic extracts on the basis of its ability to stimulate cAMP formation in cultured rat anterior pituitary cells.[22] Characterization of the peptide revealed that it consists of 38 amino acids and is C-terminally α-amidated. PACAP38 exhibits an internal cleavage site (Gly^{28}-Lys^{29}-Arg^{30}) and can thus generate a 27-amino acid, α-amidated peptide (PACAP27), which is present, in most tissues, at a much lower concentration than PACAP38.[32] PACAP27 exhibits 68% sequence identity with vasoactive intestinal polypeptide (VIP), identifying PACAP as a member of the VIP–glucagon–secretin superfamily. The sequence of PACAP has been remarkably well conserved during evolution from protochordate to mammals, suggesting that the peptide is involved in the regulation of vital biological functions.[32] Knockout of the PACAP gene in zebrafish and mice has shown that PACAP is important for development, reproduction, metabolism, and feeding.[24] Characterization of the PACAP precursor cDNA has revealed the existence of a PACAP-related peptide flanking PACAP on its N-terminal side, whose activity remains unknown. Two types of PACAP binding sites have been characterized: type I binding sites exhibit a high affinity for PACAP and a much lower affinity for VIP, whereas type II binding sites have similar affinity for PACAP and VIP. Molecular cloning of PACAP receptors has shown the existence of three distinct receptor subtypes, the PACAP-specific receptor PAC1, which is coupled to several transduction systems, and the two PACAP/VIP-mutual receptors VPAC1 and VPAC2, which are primarily coupled to adenylyl cyclase. PACAP and its receptors are widely distributed in the brain (see the Chapter on PACAP in the Brain Peptides section of this book) and peripheral organs, notably in endocrine glands including the pituitary, thyroid, gonads, adrenals, and pancreas (Table 1).

EFFECTS ON THE PITUITARY GLAND

Various hypothalamic nuclei, including the paraventricular and arcuate nuclei, contain PACAP-producing neurons that project toward the external zone of the median eminence,[21] and high concentrations of PACAP have been measured in the hypophyseal portal blood, supporting the view that PACAP may act as a hypophysiotrophic neuropeptide. Besides, the occurrence of PACAP has also been detected in a subpopulation of gonadotroph cells[21] indicating that the peptide is expressed within the adenohypophysis and may act as a paracrine and/or autocrine regulator. Indeed, the ability of PACAP to stimulate cAMP formation in anterior pituitary cells provides clear evidence that the peptide controls the activity of the adenohypophysis.[22] The diversity of actions of PACAP on the pituitary has been detailed in previous reviews.[26] Among the different hypophysiotrophic neuropeptides identified so far, the situation of PACAP is rather unique in that functional PACAP receptors are present in all endocrine cell types, and in folliculo-stellate cells of the adenohypophysis. PACAP stimulates the release of growth

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00138-X
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TABLE 1 Localization and Relative Abundance of PACAP and Its Binding Sites in Various Endocrine Tissues

	PACAP		PACAP Binding Sites	
Structures	**Cell**	**Fibers**	**PAC1**	**VPAC1 & VPAC2**
Anterior pituitary	-/++		++/+++	++
FSH, GH, PRL and ACTH cells	++			
TSH, LH, FSH cells	+			
Neurohypophysis		++		
Thyroid				+
Parathyroid		+		
Testis		+		
Spermatocytes	++		++	
Leydig cells	-		+	++
Epithelial cells from epidymydal tubules	+		+	
Ovary		+		++
Granulosa and cumulus cells	++			
Adrenal gland				
Cortex	-	+	-	+
Medulla	-/+	-/+	++	-/+
Chromaffin cells	-/+	-/+	++	-/+
Subcapsular region		+		
Endocrine pancreas	++	++		++

The symbols provide a semi-quantitative evaluation of the level of expression: +++, high density; ++, moderate density; +, low density; -, no hybridization or immunohistochemical signal detected.

hormone, adrenocorticotropic hormone, luteinizing hormone (LH), follicle-stimulating hormone (FSH), and prolactin.[26,32] PACAP is even more potent than "classical" hypothalamic hormones such as gonadotropin-releasing hormone at increasing NOS1 levels in cultured rat anterior pituitary cells,[7] indicating that PACAP exerts important biological functions in these cells. PACAP also enhances the secretion of α-melanocyte-stimulating hormone from cultured rat pars intermedia melanotroph cells. The hypotrophic action of PACAP has been documented in representative species of submammalian vertebrates including fish, amphibians, and birds, indicating that this function has been conserved during evolution. The effects of PACAP on pituitary cells are mediated through activation of adenylyl cyclase, cGMP, phospholipase C (PLC) and/or mobilization of cytosolic calcium (Table 2). PACAP is also released by neuroendocrine tumors and may be involved in their growth regulation. A detailed description of the effects of PACAP on each cell type can be found elsewhere.[32]

EFFECTS ON THE THYROID GLAND

In humans, VPAC1 is the predominant type of PACAP receptors expressed in the thyroid, whereas in mice, thyroid follicular cells primarily express VPAC2.[13] In the human and porcine thyroid, PACAP stimulates cAMP production, which is probably involved in the activation of thyroxin secretion.[4] Further, in PACAP knockout animals, the thyroid axis is altered, leading to a reduction of TRH in the hypothalamus and of deiodinase in brown fat and brain, which could explain the reduction in fat mass in these animal.[1]

EFFECTS OF PACAP ON THE ENDOCRINE TESTIS AND OVARY

The presence of PACAP and its receptors in the testis and ovary strongly suggests that the peptide may operate as a local regulator of gonadal activity.[13,17] The rat testis contains the highest level of PACAP in any peripheral organs.[2]

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TABLE 2 Effects of PACAP on Pituitary Cells

Cell type	Second-Messenger Coupling	Hormone Release and/or mRNA Expression
Gonadotrope cells	↑ cAMP, ↑ IP turnover, ↑ $[Ca^{2+}]_i$, ↑ NOS 1	↑/0 LH release,↑/0 FSH release ↑ LH mRNA, 0 FSH mRNA
Somatotrope cells	↑ cAMP, ↑ $[Ca^{2+}]_i$	↑/0 GH release
Lactotrope cells	↑ $[Ca^{2+}]_i$	↑/↓/0 PRL release, ↑/0 PRL mRNA
Corticotrope cells	↑ $[Ca^{2+}]_i$	↑/0 ACTH release
Thyrotrope cells	↑ $[Ca^{2+}]_i$	0 TSH release
Folliculo-stellate cells	↑ cAMP, ↑ $[Ca^{2+}]_i$	↑ IL-6 release
Fibroblasts	↑ cAMP	
Melanotrope cells	↑ cAMP	↑ α-MSH release,↑ BDNF release

↑, stimulatory effect; ↓, inhibitory effect; 0, no effect; ACTH, adrenocorticotrophic hormone; FSH, follicle-stimulating hormone; GH, growth hormone; IL-6, interleukin-6; LH, luteinizing hormone; PRL; prolactin; TSH, thyroid-stimulating hormone.

The concentration of PACAP in the testis is significantly reduced after hypophysectomy and is restored by FSH administration, indicating that testicular expression of the PACAP gene is under the control of pituitary gonadotropins. *In vitro*, PACAP induces a dose-dependent stimulation of testosterone secretion from isolated rat Leydig cells and regulates protein synthesis in spermatocytes and spermatids.[32] In Leydig cells, PACAP acting through PAC1, stimulates both adenylyl cyclase and PLC. The effect of PACAP on Leydig cells may also be mediated *via* another signaling pathway coupled to sodium channels through a pertussis-toxin-sensitive G protein. The effects of PACAP on protein synthesis in spermatocytes and spermatids are both mimicked by dbcAMP. In cultured Sertoli cells, PACAP increases cAMP concentration and stimulates estradiol and inhibin secretion. In the epididymal epithelium, PACAP increases chloride secretion, which is important for sperm activation and storage. The occurrence of PACAP-immunoreactive material in epididymal tubules indicates that PACAP is locally synthesized and thus may act as a paracrine regulator of sperm maturation. The epididymal epithelium-derived PACAP may also stimulate epididymal spermatozoa that have lost PACAP synthesis ability but still possess PACAP binding sites. In human, PACAP dose dependently relaxes norepinephrine- and electrically contracted isolated preparations of corpus cavernosum, suggesting that the peptide may be involved in the induction and maintenance of penile erection. Consistent with this notion, a stearic acid VIP conjugate increases the copulatory activity and penile reflex in testosterone-treated, castrated rats. These results suggest that some PACAP resistant analogs could be useful for the treatment of impotence.

In the rat ovary, most granulosa and cumulus cells from large preovulatory follicles contain both PACAP mRNA and PACAP peptide.[10] Human chorionic gonadotropin (hCG) stimulates the expression of PACAP mRNA and progesterone receptor mRNA.[16] The peak of expression of progesterone receptor mRNA occurs 3 hours after hCG treatment and the peak of PACAP mRNA only after 6 hours, suggesting that progesterone receptor activation is required for PACAP gene expression.[16] Indeed, the progesterone receptor antagonist ZK98299 blocks the effect of hCG on PACAP gene expression.[16] The hCG-evoked stimulation of PACAP gene transcription is abolished by cycloheximide, indicating the requirement of protein synthesis for PACAP mRNA expression.[16] Exposure of cultured granulosa cells to PACAP causes a dose-dependent increase in progesterone production.[9] Reciprocally, immunoneutralization of endogenous PACAP reduces progesterone formation and impairs subsequent luteinization, suggesting that PACAP plays an important role in LH-induced progesterone secretion during the periovulatory period[9] which could, at least in part, explain the reduced fertility observed in PACAP-deficient mice. Incubation of immature rat preovulatory follicles with PACAP or VIP induces a dose-dependent inhibition of follicle apoptosis and reduces FSH-stimulated follicle growth. In luteinized granulosa cells, PACAP seems to be more potent than LH in stimulating cAMP accumulation. In the human female genital tract, PACAP is located in fibers innervating blood vessels and smooth muscle cells of the internal cervical os, suggesting that the peptide could regulate local blood flow and lubrication of the vagina. High concentrations of PACAP are also found throughout the human uteroplacental unit. *In vitro*, PACAP induces relaxation of nonvascular smooth muscle strips from the Fallopian tube and myometrium and stem villous and intramyometrial arteries, indicating that PACAP may regulate the vascular tone in the human female

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reproductive tract. In placental cells, PACAP enhances cAMP formation, hCG, and interleukin-6 production.

EFFECTS OF PACAP ON THE ADRENAL GLAND

PACAP and its receptors are actively expressed in the adrenal medulla.[32] PACAP exerts a stimulatory action on catecholamine secretion from chromaffin cells,[32] induces the release of brain natriuretic peptide and enkephalins, two regulatory peptides, which are cosequestered with catecholamines in chromaffin granules,[3] and causes a robust increase in VIP mRNA expression in bovine chromaffin cells through a cAMP-dependent, PKA-independent pathway.[11] *In vivo* studies show that PACAP and VIP stimulate catecholamine release in anesthetized dogs through the activation of dihydropyridine-sensitive L-type calcium channels.[8] PACAP-induced catecholamine secretion is significantly enhanced by hypoglycemia, suggesting that PACAP may play a beneficial role in glucose counterregulatory mechanisms in the adrenal medulla during hypoglycemia.[34] The effect of PACAP on catecholamine secretion is mediated through PAC1 and associated with an increase in adenylyl cyclase activity[19] and calcium influx.[23] Incubation of adrenomedullary cells in calcium-free medium or blockage of voltage-operated calcium channels suppresses the PACAP-evoked stimulation of catecholamine secretion, indicating that the effect of PACAP on chromaffin cells is mediated through calcium influx. Concurrently, PACAP increases calcium mobilization from ryanodine/caffeine-sensitive calcium stores. Treatment of chromaffin cells with PACAP activates the expression of tyrosine hydroxylase, dopamine β-hydroxylase, and phenylethanolamine *N*-methyltransferase,[32] and the stimulatory effect of PACAP on tyrosine hydroxylase activity is mediated through the activation of the adenylyl cyclase/PKA signaling cascade.[20] Divergent results have been reported regarding the possible effect of PACAP on multiplication of adrenochromaffin cells: PACAP seems to stimulate proliferation of rat chromaffin cells in primary culture and to inhibit the mitogenic action of nerve growth factor on chromaffin cells. High levels of PACAP and its receptors are expressed in most pheochromocytomas where the peptide could act in an autocrine manner to regulate the secretory activity or the differentiation of tumor cells.[27]

The pheochromocytoma PC12 cell line has been widely used as a model to investigate the neuroendocrine and neurotrophic effects of PACAP. In PC12 cells, PACAP acting through PAC1 inhibits cell proliferation, stimulates neurite outgrowth, and prevents apoptosis.[32] The effect of PACAP on neuritogenesis is mediated through a cAMP/ERK-dependent, PKA-independent mechanism.[25] In PC12 cells, PACAP also stimulates tyrosine hydroxylase and chromogranin A gene expression, and activates the transcription of the transfected neuropeptide Y and proenkephalin A genes.[5] The effect of PACAP on global gene expression has also been investigated in PC12 cells using microarray approaches.[31] Many of the genes or proteins regulated by PACAP are associated with neuritogenesis (ornithine decarboxylase, annexin A2, or EGR1), survival (stathmin), cell growth (growth arrest specific 1 or cyclin B2), cell morphology remodeling (actin, tubulin), vesicle trafficking (synaptotagmin IV), or cell adhesion (Mcma, attractin).[6,25,31]

Intravenous administration of PACAP causes elevation of plasma cortisol levels in dog and calf.[15] PACAP stimulates corticosterone and aldosterone secretion from human, rodent, chicken, and frog adrenal slices.[32] Recent studies highlight the critical role of PACAP along the hypothalamic–pituitary–adrenal axis to mediate a sustained hormone secretion in response to emotional stressors.[30] Exposure of human adrenal slices to the β-adrenoreceptor blocker l-alprenolol totally suppresses the steroidogenic effect of PACAP. Similarly, the action of PACAP on dehydroepiandrosterone and cortisol secretion by the fetal human adrenal gland is suppressed by the β-adrenoreceptor antagonist propranolol. Altogether, these observations indicate that, in most mammalian species, the effect of PACAP on corticosteroid secretion can be ascribed to the stimulatory action of the peptide on catecholamine secretion. In contrast, PACAP stimulates corticosteroid release from dispersed bovine and frog adrenocortical cells. That PACAP enhances cAMP and inositol phosphate formation in bovine glomerulosa cells and calcium mobilization in individual frog adrenocortical cells provides additional evidence for a direct stimulatory effect of the peptide on steroidogenesis in these two species.

EFFECTS OF PACAP ON PANCREATIC ISLETS

In the pancreas, PACAP-immunoreactive fibers innervate both the exocrine acini and the islets of Langerhans, as well as the small arteries of the connective tissue (Table 1). Electrical stimulation of the vagus nerve causes the release of PACAP from the isolated perfused pig pancreas, suggesting that PACAP may control exocrine and/or endocrine pancreatic secretions. PACAP seems to be much more potent than VIP or other regulatory peptides in stimulating pancreatic hormone secretion. *In vivo* administration of PACAP stimulates insulin secretion in mice, calf, dog, and human.[32] Mice with a targeted deletion of the PACAP gene have a more profound insulin-induced hypoglycemia than wild-type animals.[12] The stimulatory effect of PACAP on insulin release has also been documented on perfused rat and pig pancreas and on cultured islet cells. On these cells, PACAP and VIP activate VPAC2 to increase the number of pancreatic β-cells.[28] The role of PACAP in the control of β-cell proliferation has been confirmed in mice overexpressing PACAP in the pancreas under the control of a human

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insulin promoter.[35] Pancreatic β-cells express cell-surface ectopeptidases capable of degrading PACAP.[14] The amplitude and kinetics of the PACAP-evoked stimulation of insulin release depends on glucose concentration in the incubation medium. PACAP induces a biphasic effect on insulin secretion, that is, a rapid and transient stimulation (acute phase) followed by a rebound of the secretory response (plateau phase).[29] The plateau phase may result from the ability of PACAP to regulate insulin gene expression. The phosphatidylinositol 3-kinase inhibitor wortmannin inhibits the plateau phase but not the acute phase of PACAP-evoked insulin release.[29] Exposure of pancreatic β-cells to PACAP causes calcium influx through L-type calcium channels[33] and the stimulatory effect of PACAP on insulin secretion is abolished by nitrendipine,[18] indicating that activation of voltage-sensitive L-type calcium channels is involved in the insulinotropic effect of PACAP. Paradoxically, the combination of glucose, PACAP and carbachol stimulates insulin release while being unable to elevate intracellular calcium.[18] Incubation of isolated rat islets with specific PACAP antisera inhibits the ability of glucose to stimulate insulin release,[33] suggesting that endogenous PACAP acts as a physiological regulator of pancreatic β-cell activity. PACAP is also a potent stimulator of glucagon secretion. Intravenous injection of PACAP increases plasma glucagon concentration in mouse, dog and human.[32] Likewise, in the perfused rat pancreas, PACAP enhances glucagon secretion. The stimulatory effect of PACAP on insulin and glucagon release is completely abolished by somatostatin. In contrast, the endozepine octadecaneuropeptide (a potent inhibitor of insulin release) has no effect on PACAP-evoked insulin secretion.

REFERENCES

1. Adams BA, Gray SL, Isaac ER, Bianco AC, Vidal-Puig AJ, Sherwood NM. Feeding and metabolism in mice lacking pituitary adenylate cyclase-activating polypeptide. *Endocrinology* 2008;**149**:1571–80.
2. Arimura A, Somogyvari-Vigh A, Miyata A, Mizuno K, Coy DH, Kitada C. Tissue distribution of PACAP as determined by RIA: highly abundant in the rat brain and testes. *Endocrinology* 1991;**129**:2787–9.
3. Babinski K, Bodart V, Roy M, De Lean A, Ong H. Pituitary adenylate-cyclase activating polypeptide (PACAP) evokes long-lasting secretion and de novo biosynthesis of bovine adrenal medullary neuropeptides. *Neuropeptides* 1996;**30**:572–82.
4. Chen W, Inui T, Hachiya T, Ochi Y, Nakajima Y, Kajita Y. Stimulatory action of pituitary adenylate cyclase-activating polypeptide (PACAP) on thyroid gland. *Biochem Biophys Res Commun* 1993;**194**:923–9.
5. Colbert RA, Balbi D, Johnson A, Bailey JA, Allen JM. Vasoactive intestinal peptide stimulates neuropeptide Y gene expression and causes neurite extension in PC12 cells through independent mechanisms. *J Neurosci* 1994;**14**:7141–7.
6. Dejda A, Chan P, Seaborn T, Coquet L, Jouenne T, Fournier A, et al. Involvement of stathmin 1 in the neurotrophic effects of PACAP in PC12 cells. *J Neurochem* 2010;**114**:1498–510.
7. Garrel G, Lozach A, Bachir LK, Laverriere JN, Counis R. Pituitary adenylate cyclase-activating polypeptide stimulates nitric-oxide synthase type I expression and potentiates the cGMP response to gonadotropin-releasing hormone of rat pituitary gonadotrophs. *J Biol Chem* 2002;**277**:46391–401.
8. Geng G, Gaspo R, Trabelsi F, Yamaguchi N. Role of L-type Ca2+ channel in PACAP-induced adrenal catecholamine release in vivo. *Am J Physiol* 1997;**273**:R1339–45.
9. Gras S, Hannibal J, Fahrenkrug J. Pituitary adenylate cyclase-activating polypeptide is an auto/paracrine stimulator of acute progesterone accumulation and subsequent luteinization in cultured periovulatory granulosa/lutein cells. *Endocrinology* 1999;**140**:2199–205.
10. Gras S, Hannibal J, Georg B, Fahrenkrug J. Transient periovulatory expression of pituitary adenylate cyclase activating peptide in rat ovarian cells. *Endocrinology* 1996;**137**:4779–85.
11. Hamelink C, Lee HW, Chen Y, Grimaldi M, Eiden LE. Coincident elevation of cAMP and calcium influx by PACAP-27 synergistically regulates vasoactive intestinal polypeptide gene transcription through a novel PKA-independent signaling pathway. *J Neurosci* 2002;**22**:5310–20.
12. Hamelink C, Tjurmina O, Damadzic R, Young WS, Weihe E, Lee HW, et al. Pituitary adenylate cyclase-activating polypeptide is a sympathoadrenal neurotransmitter involved in catecholamine regulation and glucohomeostasis. *Proc Natl Acad Sci U S A* 2002;**99**:461–6.
13. Harmar AJ, Sheward WJ, Morrison CF, Waser B, Gugger M, Reubi JC. Distribution of the VPAC2 receptor in peripheral tissues of the mouse. *Endocrinology* 2004;**145**:1203–10.
14. Hupe-Sodmann K, Goke R, Goke B, Thole HH, Zimmermann B, Voigt K, et al. Endoproteolysis of glucagon-like peptide (GLP)-1 (7-36) amide by ectopeptidases in RINm5F cells. *Peptides* 1997;**18**:625–32.
15. Kawai K, Yokota C, Ohashi S, Isobe K, Suzuki S, Nakai T, et al. Pituitary adenylate cyclase-activating polypeptide: effects on pancreatic-adrenal hormone secretion and glucose-lipid metabolism in normal conscious dogs. *Metabolism* 1994;**43**:739–44.
16. Ko C, In YH, Park-Sarge OK. Role of progesterone receptor activation in pituitary adenylate cyclase activating polypeptide gene expression in rat ovary. *Endocrinology* 1999;**140**:5185–94.
17. Koh PO, Noh HS, Kim YS, Cheon EW, Kim HJ, Kang SS, et al. Cellular localization of pituitary adenylate cyclase-activating polypeptide in the rat testis. *Mol Cells* 2003;**15**:271–6.
18. Komatsu M, Schermerhorn T, Straub SG, Sharp GW. Pituitary adenylate cyclase-activating peptide, carbachol, and glucose stimulate insulin release in the absence of an increase in intracellular Ca2+. *Mol Pharmacol* 1996;**50**:1047–54.
19. Lamouche S, Yamaguchi N. Role of PAC(1) receptor in adrenal catecholamine secretion induced by PACAP and VIP in vivo. *Am J Physiol Regul Integr Comp Physiol* 2001;**280**:R510–8.
20. Marley PD, Cheung CY, Thomson KA, Murphy R. Activation of tyrosine hydroxylase by pituitary adenylate cyclase-activating polypeptide (PACAP-27) in bovine adrenal chromaffin cells. *J Auton Nerv Syst* 1996;**60**:141–6.
21. Mikkelsen JD, Hannibal J, Fahrenkrug J, Larsen PJ, Olcese J, McArdle C. Pituitary adenylate cyclase activating peptide-38 (PACAP-38), PACAP-27, and PACAP related peptide (PRP) in the rat median eminence and pituitary. *J Neuroendocrinol* 1995;**7**:47–55.
22. Miyata A, Arimura A, Dahl RR, Minamino N, Uehara A, Jiang L, et al. Isolation of a novel 38 residue-hypothalamic polypeptide which stimulates adenylate cyclase in pituitary cells. *Biochem Biophys Res Commun* 1989;**164**:567–74.

This book belongs to Central Library BADJI Mokhtar-ANNABA UNIVERSITY Copyright Elsevier 2024

23. Morita K, Sakakibara A, Kitayama S, Kumagai K, Tanne K, Dohi T. Pituitary adenylate cyclase-activating polypeptide induces a sustained increase in intracellular free Ca(2+) concentration and catechol amine release by activating Ca(2+) influx via receptor-stimulated Ca(2+) entry, independent of store-operated Ca(2+) channels, and voltage-dependent Ca(2+) channels in bovine adrenal medullary chromaffin cells. *J Pharmacol Exp Ther* 2002;**302**:972–82.
24. Otto C, Hein L, Brede M, Jahns R, Engelhardt S, Grone HJ, et al. Pulmonary hypertension and right heart failure in pituitary adenylate cyclase-activating polypeptide type I receptor-deficient mice. *Circulation* 2004;**110**:3245–51.
25. Ravni A, Vaudry D, Gerdin MJ, Eiden MV, Falluel-Morel A, Gonzalez BJ, et al. A cAMP-dependent, protein kinase A-independent signaling pathway mediating neuritogenesis through Egr1 in PC12 cells. *Mol Pharmacol* 2008;**73**:1688–708.
26. Rawlings SR, Hezareh M. Pituitary adenylate cyclase-activating polypeptide (PACAP) and PACAP/vasoactive intestinal polypeptide receptors: actions on the anterior pituitary gland. *Endocr Rev* 1996;**17**:4–29.
27. Reubi JC, Laderach U, Waser B, Gebbers JO, Robberecht P, Laissue JA. Vasoactive intestinal peptide/pituitary adenylate cyclase-activating peptide receptor subtypes in human tumors and their tissues of origin. *Cancer Res* 2000;**60**:3105–12.
28. Sato A, Wang PC, Ohgawara H. Effect of stimulators such as GLP-1, PACAP, and nicotinamide on glucose-stimulated insulin secretion from porcine pancreatic endocrine cells in long-term culture. *Pancreas* 2002;**25**:86–93.
29. Straub SG, Sharp GW. A wortmannin-sensitive signal transduction pathway is involved in the stimulation of insulin release by vasoactive intestinal polypeptide and pituitary adenylate cyclase-activating polypeptide. *J Biol Chem* 1996;**271**:1660–8.
30. Stroth N, Liu Y, Aguilera G, Eiden LE. Pituitary adenylate cyclase-activating polypeptide controls stimulus-transcription coupling in the hypothalamic–pituitary–adrenal axis to mediate sustained hormone secretion during stress. *J Neuroendocrinol* 2011;**23**:944–55.
31. Vaudry D, Chen Y, Ravni A, Hamelink C, Elkahloun AG, Eiden LE. Analysis of the PC12 cell transcriptome after differentiation with pituitary adenylate cyclase-activating polypeptide (PACAP). *J Neurochem* 2002;**83**:1272–84.
32. Vaudry D, Falluel-Morel A, Bourgault S, Basille M, Burel D, Wurtz O, et al. Pituitary adenylate cyclase-activating polypeptide and its receptors: 20 years after the discovery. *Pharmacol Rev* 2009;**61**:283–357.
33. Yada T, Sakurada M, Nakata M, Yaekura K, Kikuchi M. PACAP as low as 10(-13) M raises cytosolic Ca2+ activity in pancreatic B-cells by augmenting Ca2+ influx through L-type Ca2+ channels to trigger insulin release. *Adv Exp Med Biol* 1997;**426**:165–71.
34. Yamaguchi N, Lamouche S. Enhanced reactivity of the adrenal medulla in response to pituitary adenylate cyclase activating polypeptide1-27 (PACAP) during insulin-induced hypoglycemia in anesthetized dogs. *Can J Physiol Pharmacol* 1999;**77**:819–26.
35. Yamamoto K, Hashimoto H, Tomimoto S, Shintani N, Miyazaki J, Tashiro F, et al. Overexpression of PACAP in transgenic mouse pancreatic beta-cells enhances insulin secretion and ameliorates streptozotocin-induced diabetes. *Diabetes* 2003;**52**:1155–62.

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Chapter 139

Adiponectin/Resistin

Rexford S. Ahima

ABSTRACT

Adiponectin is synthesized and secreted by adipocytes in rodents and humans, and it enhances insulin sensitivity and fat oxidation. Resistin, however, is produced by adipocytes in rodents and macrophages in humans, and it induces insulin resistance in liver, muscle, and fat. Adiponectin and resistin are both capable of controlling feeding and energy balance. This chapter provides an overview of the central and peripheral effects of adiponectin and resistin on energy homeostasis.

ADIPONECTIN AND ENERGY HOMEOSTASIS

Adiponectin is synthesized almost exclusively by adipocytes, and it circulates as multimeric protein complexes and regulates metabolism in peripheral tissues.[32] Adiponectin lowers serum glucose through the suppression of hepatic glucose production.[33,34] Epidemiological studies have shown that adiponectin is inversely correlated to insulin resistance and other components of the metabolic syndrome.[6,14,32] In rodents, adiponectin deficiency leads to glucose intolerance, insulin resistance, dyslipidemia, and increased susceptibility to vascular injury and atherosclerosis.[17,33,34] Peripheral administration of adiponectin enhances insulin sensitivity and fatty acid oxidation and protects against vascular injury.[17,33,34] The effects of adiponectin on glucose and lipid metabolism are attributed to adiponectin receptors, AdipoR1 and R2, which signal through AMP-activated kinase (AMPK) and peroxisome proliferator-activated receptor α (PPARα) in liver and muscle.[14,32] Adiponectin also acts through IRS-2, STAT3, NFκB, and IL-6 to regulate glucose homeostasis and inflammation.[18,32]

There is a wide distribution of adiponectin receptors in the brains of rodents and humans.[5,10,12] AdipoR1 is widely expressed in rat brain, whereas AdipoR2 expression is limited to the hypothalamus, cortex, and hippocampus.[5,12] Within the arcuate and paraventricular hypothalamic nuclei, AdipoR1 and AdipoR2 are expressed by neurons as well as astrocytes.[5] AdipoR1 and AdipoR2 are colocalized in proopiomelanocortin (POMC) and neuropeptide Y (NPY) neurons in the arcuate nucleus.[5] Intracerebroventricular (icv) injection of adiponectin induces AMP-activated protein kinase (AMPK) phosphorylation in the rat hypothalamus.[5,12] Other studies have shown potent electrophysiological actions of adiponectin in the rat hypothalamus and brainstem.[4,7,8]

Despite the presence of adiponectin receptors in the brain, it is controversial whether adiponectin is transported across the blood–brain barrier.[19,29] Nonetheless, adiponectin is detectable in cerebrospinal fluid (CSF) in rodents and humans,[10,12,13,24] and CSF adiponectin concentration can be increased after intravenous administration of adiponectin in mice.[12,24] Adiponectin concentrations in human CSF is 0.1% of serum concentration and mainly consists of trimeric and low-molecular-mass hexameric complexes.[13] Men have a higher CSF:serum ratio of adiponectin than women have, but it is unknown whether this sex difference affects metabolism.[13]

We first reported that administration of adiponectin in the brain has potent effects on energy expenditure, and glucose and lipid metabolism in mice.[24] Administration of adiponectin icv potently decreases body weight by stimulating energy expenditure.[24] Lepob/ob mice display greater sensitivity to icv adiponectin compared with that of intraperitoneal adiponectin treatment.[24] Adiponectin stimulates thermogenesis and reduces body weight and fat, and serum glucose and lipid levels.[24] Adiponectin increases Fos immunoreactivity in the arcuate and paraventricular nuclei.[24] In contrast to wildtype and Lepob/ob mice, agouti ($A^{y/a}$) mice do not respond to icv adiponectin treatment, suggesting that melanocortin signaling plays a critical role in the CNS action of adiponectin.[24] These results in mice were later corroborated in rats.[21] Chronic icv infusion of adiponectin enhances the signaling of insulin and leptin in the hypothalamus of normal and diabetic rats, and also decreases epididymal fat mass by increasing energy expenditure and fat oxidation.[21] Unlike peripheral adiponectin treatment that enhances insulin action in liver via activation of AMPK, the effects of icv adiponectin on insulin signaling, energy expenditure and fat oxidation are not related to AMPK activation.[21,32]

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00139-1
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Masaki et al. compared the CNS and peripheral actions of adiponectin in $A^{y/a}$ mice.[15] They showed that chronic intraperitoneal adiponectin administration reduces body weight and fat, accompanied by increased expression of UCP1 in brown adipose tissue (BAT), UCP2 in white adipose tissue (WAT), and UCP3 mRNA in skeletal muscle.[15] Acute peripheral administration of adiponectin increases sympathetic nervous activity in BAT, rectal temperature, and Fos immunoreactivity in the hypothalamus.[15] In agreement with an earlier report,[24] icv injection of adiponectin has no effect on energy homeostasis in $A^{y/a}$ mice.[15]

Adiponectin icv has opposite effects on food intake in rats.[3,12] Coope et al. reported that icv adiponectin inhibits food intake, and this effect is accompanied by activation of IRS1/2, ERK, Akt, FOXO1, JAK2, and STAT3 in the hypothalamus.[3] AdipoR1 and AdipoR2 are both localized in the arcuate and lateral hypothalamic nuclei; however, inhibition of AdipoR1, and not AdipoR2, prevents the anorexigenic action of icv adiponectin.[3] By contrast, Kubota et al. showed that adiponectin stimulates food intake and decreases energy expenditure.[12] These effects of adiponectin on energy homeostasis require AdipoR1 in the arcuate nucleus and are attenuated by blockade of AMPK activity.[12] Adiponectin knockout mice have reduced AMPK phosphorylation in the arcuate nucleus, associated with reduced food intake and increased energy expenditure, exhibiting resistance to diet-induced obesity.[12] Adiponectin levels in the blood and CSF are elevated during fasting and reduced after refeeding, suggesting that adiponectin serves as a signal from adipose tissue to the brain during energy deficiency.[12]

Ablation of AdipoR1 and AdipoR2 in mice results in different metabolic phenotyes.[2] AdipoR1 knockout mice show increased fat accumulation, associated reductions in spontaneous locomotor activity and energy expenditure, and impaired glucose tolerance.[2] By contrast, AdipoR2 knockout mice are resistant to obesity, and show improved glucose tolerance, and increased spontaneous locomotor activity and energy expenditure.[2] AdipoR1 and AdipoR2 are localized in warm sensitive neurons of the hypothalamic preoptic area (POA).[9] Microinjection of adiponectin in the POA induces hyperthermia, increases oxygen consumption and decreases the respiratory exchange ratio (RER), indicating oxidation of fat.[9] The hyperthermic effect of adiponectin treatment is blunted, while oxygen consumption and fat oxidation are unchanged in AdipoR1 knockout mice. In contrast, the hyperthermic effect of adiponectin treatment is blunted in AdipoR2 knockout mice, but POA adiponectin is able to stimulate fat oxidation.[9] These findings show that adiponectin modulates core body temperature, energy expenditure and fat metabolism via distinct neuronal circuits in the brain.[2,9]

Although the evidence for adiponectin's effects on energy balance is strong, further research is needed to address the controversies surrounding the central versus peripheral responses to different protein forms of adiponectin. The icv route of administration of adiponectin is artificial and may exert different actions in the brain from the true physiological roles of adiponectin. The mechanisms by which adiponectin reaches the CSF from the circulation is crucial. It is still unclear whether a blood-to-brain transport system for adiponectin exists in rodents, humans, and other species. Moreover, the precise signaling pathways in the brain and peripheral organs that mediate the effects of adiponectin on food intake and energy expenditure need to be clarified.

RODENT VERSUS HUMAN RESISTIN

Resistin is a 12.5 kDa cysteine-rich polypeptide that is secreted by rodent adipocytes and suppressed by antidiabetic thiazolidinedione drugs.[27] Resistin belongs to a family of secreted proteins known as resistin-like molecules (RELMs) or found in inflammatory zone (FIZZ) proteins.[27] In rodents, adipose resistin expression is suppressed in obesity while circulating resistin levels are increased.[25] Administration of exogenous resistin or transgenic overexpression of resistin (Retn) induces insulin resistance, whereas blocking resistin activity or ablation of *Retn* improves insulin sensitivity.[1,26,30] Resistin also induces hepatic inflammation and insulin resistance when administered icv in rodents.[16,28]

Although resistin exerts direct actions on adipose, liver, muscle, vascular endothelium, cardiomyocytes, and inflammatory cells, the nature of the resistin receptor and downstream signaling pathways that mediate the actions of resistin are uncertain.[27] Resistin binds to the endotoxin receptor Toll-like receptor 4 (TLR4) and an isoform of decorin. Potential downstream signaling molecules of resistin include AMPK in mouse and rat liver and muscle, and cytokine signaling-3 (SOCS-3) in mouse adipose tissue.[27]

Unlike rodent resistin, human resistin is predominantly expressed in macrophages.[27] The lack of human resistin expression in adipocytes may result from the loss of a genomic binding site for PPARγ that normally controls Retn expression in mouse adipocytes. The expression and secretion of resistin by human macrophages is increased in response to inflammation.[27] Although initial reports on the role of resistin in humans were conflicting, large epidemiologic studies have shown significant correlations of resistin levels with insulin resistance, type 2 diabetes and inflammation.[27] To address the biology of human resistin, the Lazar laboratory generated mice that lack adipocyte-derived mouse resistin but produce human resistin in macrophages.[22] When fed a high-fat diet, the humanized resistin mice rapidly develope white adipose tissue (WAT) inflammation, leading to increased lipolysis and serum free fatty acid levels. Subsequently, diacylglycerol accumulated in muscle, leading to activation of protein kinase C (PKC)-q activity and insulin resistance in muscle and WAT.[30] An

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"even more humanized resistin mice" lacking murine resistin but transgenic for a bacterial artificial chromosome containing human resistin (BAC-Retn) has provided new insights into the link between resistin and inflammation.[20] As in humans, lipopolysaccharide (LPS) treatment increased resistin expression and serum resistin levels in BAC-Retn mice.[20] Hypoglycemia induced by acute LPS treatment is attenuated in BAC-Retn mice. Moreover, BAC-Retn mice developed hepatic insulin resistance in response to chronic LPS treatment.[20] These mouse models will be valuable for exploring the connection between resistin and inflammation and the development of insulin resistance and type 2 diabetes.

RESISTIN AND ENERGY HOMEOSTASIS

Several lines of evidence in rodents suggest that resistin is involved in energy homeostasis. Resistin protein levels are reduced during fasting and increased in response to refeeding.[25] Lepob/ob mice lacking resistin develop severe obesity because of a reduction in energy expenditure.[23] In rats, resistin decreases food intake, and this anorexigenic effect is associated with decreased expression of agouti-related peptide and neuropeptide Y, and increased expression of cocaine and amphetamine-regulated transcript within the arcuate nucleus of the hypothalamus.[31] Resistin also increases the phosphorylation of AMPK and its downstream target acetyl-coenzyme A carboxylase (ACC), and decreases the expression of fatty acid synthase in the ventromedial nucleus of the hypothalamus.[31] Chronic icv resistin infusion decreases body weight and fat, and suppresses the expression of lipogenic enzymes in liver and adipose tissue.[31]

Kosari et al. studied the effects of icv resistin on sympathetic nervous activity (SNA) in rats.[11] Acute icv resistin treatment increases lumbar SNA but decreased BAT SNA. However, mean arterial pressure and heart rate, were not affected by resistin. Resistin treatment decreases BAT temperature and core body temperature.[11]

CONCLUSIONS

Adiponectin and resistin levels are altered in obesity and metabolic syndrome. Adiponectin has potent actions in the brain and modulates feeding and energy expenditure via AdipoR1 and AdipoR2. Electrophysiologic and genetic manipulation of AdipoR1 and AdipoR2 reveal distinct actions of adiponectin in the hypothalamus and other regions of the brain. By contrast, there is little known about the role of resistin in energy homeostasis. Although resistin has been detected in human CSF,[10] the blood–brain transport mechanisms are unknown. The resistin receptors and their signaling pathways in the brain, and how these specifically regulate feeding and energy expenditure are also unknown. A better understanding of the effects of adiponectin and resistin on energy balance could provide novel insights on the pathogenesis and treatment of obesity, diabetes, and related disorders.

ACKNOWLEDGMENTS

Supported by P01-DK-049210/DK/NIDDK NIH HHS/United States.

REFERENCES

1. Banerjee RR, Rangwala SM, Shapiro JS, Rich AS, Rhoades B, Qi Y, et al. Regulation of fasted blood glucose by resistin. *Science* 2004;**303**:1195–8.
2. Bjursell M, Ahnmark A, Bohlooly YM, William-Olsson L, Rhedin M, Peng XR, et al. Opposing effects of adiponectin receptors 1 and 2 on energy metabolism. *Diabetes* 2007;**56**:583–93.
3. Coope A, Milanski M, Araujo EP, Tambascia M, Saad MJ, Geloneze B, et al. AdipoR1 mediates the anorexigenic and insulin/leptin-like actions of adiponectin in the hypothalamus. *FEBS Lett* 2008;**582**:1471–6.
4. Fry M, Smith PM, Hoyda TD, Duncan M, Ahima RS, Sharkey KA, et al. Area postrema neurons are modulated by the adipocyte hormone adiponectin. *J Neurosci* 2006;**26**:9695–702.
5. Guillod-Maximin E, Roy AF, Vacher CM, Aubourg A, Bailleux V, Lorsignol A, et al. Adiponectin receptors are expressed in hypothalamus and colocalized with proopiomelanocortin and neuropeptide Y in rodent arcuate neurons. *J Endocrinol* 2009;**200**:93–105.
6. Hara K, Horikoshi M, Yamauchi T, Yago H, Miyazaki O, Ebinuma H, et al. Measurement of the high-molecular weight form of adiponectin in plasma is useful for the prediction of insulin resistance and metabolic syndrome. *Diabetes Care* 2006;**29**:1357–62.
7. Hoyda TD, Ferguson AV. Adiponectin modulates excitability of rat paraventricular nucleus neurons by differential modulation of potassium currents. *Endocrinology* 2010;**151**:3154–62.
8. Hoyda TD, Samson WK, Ferguson AV. Adiponectin depolarizes parvocellular paraventricular nucleus neurons controlling neuroendocrine and autonomic function. *Endocrinology* 2009;**150**:832–40.
9. Klein I, Sanchez-Alavez M, Tabarean I, Schaefer J, Holmberg KH, Klaus J, et al. AdipoR1 and 2 are expressed on warm sensitive neurons of the hypothalamic preoptic area and contribute to central hyperthermic effects of adiponectin. *Brain Res* 2011;**1423**:1–9.
10. Kos K, Harte AL, da Silva NF, Tonchev A, Chaldakov G, James S, et al. Adiponectin and resistin in human cerebrospinal fluid and expression of adiponectin receptors in the human hypothalamus. *J Clin Endocrinol Metab* 2007;**92**:1129–36.
11. Kosari S, Rathner JA, Chen F, Badoer E. Centrally administered resistin enhances sympathetic nerve activity to the hindlimb but attenuates the activity to brown adipose tissue. *Endocrinology* 2011;**152**:2626–33.
12. Kubota N, Yano W, Kubota T, Yamauchi T, Itoh S, Kumagai H, et al. Adiponectin stimulates AMP-activated protein kinase in the hypothalamus and increases food intake. *Cell Metab* 2007;**6**:55–68.
13. Kusminski CM, McTernan PG, Schraw T, Kos K, O'Hare JP, Ahima R, et al. Adiponectin complexes in human cerebrospinal fluid: distinct complex distribution from serum. *Diabetologia* 2007;**50**:634–42.

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14. Kusminski CM, Scherer PE. The road from discovery to clinic: adiponectin as a biomarker of metabolic status. *Clin Pharmacol Ther* 2009;**86**:592–5.
15. Masaki T, Chiba S, Yasuda T, Tsubone T, Kakuma T, Shimomura I, et al. Peripheral, but not central, administration of adiponectin reduces visceral adiposity and upregulates the expression of uncoupling protein in agouti yellow (Ay/a) obese mice. *Diabetes* 2003;**52**:2266–73.
16. Muse ED, Lam TK, Scherer PE, Rossetti L. Hypothalamic resistin induces hepatic insulin resistance. *J Clin Invest* 2007;**117**:1670–8.
17. Ouchi N, Kihara S, Funahashi T, Matsuzawa Y, Walsh K. Obesity, adiponectin and vascular inflammatory disease. *Curr Opin Lipidol* 2003;**14**:561–6.
18. Ouchi N, Parker JL, Lugus JJ, Walsh K. Adipokines in inflammation and metabolic disease. *Nat Rev Immunol* 2011;**11**:85–97.
19. Pan W, Tu H, Kastin AJ. Differential BBB interactions of three ingestive peptides: obestatin, ghrelin, and adiponectin. *Peptides* 2006;**27**:911–6.
20. Park HK, Qatanani M, Briggs ER, Ahima RS, Lazar MA. Inflammatory induction of human resistin causes insulin resistance in endotoxemic mice. *Diabetes* 2011;**60**:775–83.
21. Park S, Kim DS, Kwon DY, Yang HJ. Long-term central infusion of adiponectin improves energy and glucose homeostasis by decreasing fat storage and suppressing hepatic gluconeogenesis without changing food intake. *J Neuroendocrinol* 2011;**23**:687–98.
22. Qatanani M, Szwergold NR, Greaves DR, Ahima RS, Lazar MA. Macrophage-derived human resistin exacerbates adipose tissue inflammation and insulin resistance in mice. *J Clin Invest* 2009;**119**:531–9.
23. Qi Y, Nie Z, Lee YS, Singhal NS, Scherer PE, Lazar MA, et al. Loss of resistin improves glucose homeostasis in leptin deficiency. *Diabetes* 2006;**55**:3083–90.
24. Qi Y, Takahashi N, Hileman SM, Patel HR, Berg AH, Pajvani UB, et al. Adiponectin acts in the brain to decrease body weight. *Nat Med* 2004;**10**:524–9.
25. Rajala MW, Qi Y, Patel HR, Takahashi N, Banerjee R, Pajvani UB, et al. Regulation of resistin expression and circulating levels in obesity, diabetes, and fasting. *Diabetes* 2004;**53**:1671–9.
26. Rangwala SM, Rich AS, Rhoades B, Shapiro JS, Obici S, Rossetti L, et al. Abnormal glucose homeostasis due to chronic hyperresistinemia. *Diabetes* 2004;**53**:1937–41.
27. Schwartz DR, Lazar MA. Human resistin: found in translation from mouse to man. *Trends Endocrinol Metab* 2011;**22**:259–65.
28. Singhal NS, Lazar MA, Ahima RS. Central resistin induces hepatic insulin resistance via neuropeptide Y. *J Neurosci* 2007;**27**:12924–32.
29. Spranger J, Verma S, Gohring I, Bobbert T, Seifert J, Sindler AL, et al. Adiponectin does not cross the blood–brain barrier but modifies cytokine expression of brain endothelial cells. *Diabetes* 2006;**55**:141–7.
30. Steppan CM, Bailey ST, Bhat S, Brown EJ, Banerjee RR, Wright CM, et al. The hormone resistin links obesity to diabetes. *Nature* 2001;**409**:307–12.
31. Vazquez MJ, Gonzalez CR, Varela L, Lage R, Tovar S, Sangiao-Alvarellos S, et al. Central resistin regulates hypothalamic and peripheral lipid metabolism in a nutritional-dependent fashion. *Endocrinology* 2008;**149**:4534–43.
32. Yamauchi T, Kadowaki T. Physiological and pathophysiological roles of adiponectin and adiponectin receptors in the integrated regulation of metabolic and cardiovascular diseases. *Int J Obes (Lond)* 2008;**32**(Suppl. 7):S13–8.
33. Yamauchi T, Kamon J, Minokoshi Y, Ito Y, Waki H, Uchida S, et al. Adiponectin stimulates glucose utilization and fatty-acid oxidation by activating AMP-activated protein kinase. *Nat Med* 2002;**8**:1288–95.
34. Yamauchi T, Nio Y, Maki T, Kobayashi M, Takazawa T, Iwabu M, et al. Targeted disruption of AdipoR1 and AdipoR2 causes abrogation of adiponectin binding and metabolic actions. *Nat Med* 2007; **13**:332–9.

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Chapter 140

Amylin/CGRP

Thomas A. Lutz

ABSTRACT

This chapter focuses on the anorectic action of the pancreatic hormone amylin and the structurally related peptide calcitonin-gene-related peptide (CGRP). Both peptides when given peripherally or centrally produce potent anorectic effects and delay gastric emptying. The anorectic action of CGRP seems to parallel amylin's effect on eating in many aspects; however, in this chapter, more emphasis will be placed on the effects of amylin on eating because amylin's effects may be of physiological relevance, but this is less clear in the case of CGRP. Other effects of CGRP are mentioned in other chapters of this book.

INTRODUCTION

The acute and chronic eating-inhibitory effects of amylin seem to be one important factor in amylin's overall role to control the influx of nutrients into the circulation. Apart from this effect, amylin's action to reduce gastric acid secretion, to limit the rate of gastric emptying, and to diminish pancreatic glucagon and digestive enzyme secretion are other factors serving the same purpose.[12,29] Therefore, by regulating nutrient appearance and the postprandial glucose concentration, amylin seems to be a necessary and complementary factor to insulin in the control of nutrient flux.

Amylin, or islet amyloid polypeptide (IAPP), was first isolated from pancreatic amyloid deposits, which typically occur in human type 2 diabetes mellitus, in feline diabetes mellitus, and in insulinomas in animals and humans. Amylin, however, is also a physiological product of pancreatic B-cells from where it is cosecreted with insulin. It is generally assumed that the pancreatic B-cells constitute the main source for circulating and postprandially released amylin and that the increase in amylin levels in response to eating is the physiological basis for amylin's satiating effect. Accordingly, pancreatectomized cats have very low baseline amylin (and insulin) levels and virtually no postprandial increase in blood amylin levels (unpublished); unfortunately, no measures of eating or meal pattern were determined in these studies.

Eating results in a marked increase in the plasma amylin concentration. This meal-induced increase is rapid and correlates with the size of the pertinent meal. Blood levels of CGRP show only a modest and late increase in response to eating. Therefore, a physiological role for CGRP in satiation seems questionable, at least referring to a hormonal action of circulating CGRP. The source for this increase in circulating CGRP may be by spillover from neuronal cells.

RECEPTORS FOR AMYLIN AND CGRP ACTION

Amylin and CGRP binding sites belong to the family of G-protein-coupled receptors. The functional amylin receptor involves the calcitonin receptor (CT-R) as a core receptor, whose amylin specificity and affinity results from the coexpression of one of several receptor activity modifying proteins (RAMPs).[9,13,24] The CT-R without coexpression of RAMPs represents the classical CT receptor, whereas a typical amylin receptor arises from the interaction of RAMP1 or RAMP3 with the CT-R. A similar principle leads to functional receptors for CGRP; they rely on the coexpression of the calcitonin receptor-like receptor (CL-R) with appropriate RAMPs.

Amylin binding sites are widely distributed in the central nervous system and also occur in high densities in brain areas involved in the control of eating. The area postrema (AP) which seems to mediate amylin's and CGRP's effects on eating, contains all components of functional amylin receptors, as shown by the presence of RAMP1 and RAMP3 mRNA and positive immunostaining for RAMP1, RAMP3, and the CT-R.

Detailed studies of the presence of CL-R protein as part of functional CGRP receptors in the brain and especially in brain areas involved in the control of eating are not available. However, *in situ* hybridization studies have shown that CL-R mRNA is present in the rat brain. Other than in the AP, the physiological relevance of amylin and CGRP binding sites for the anorectic actions of amylin and CGRP remains unknown.

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00140-8
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CHARACTERIZATION OF THE EFFECTS OF AMYLIN AND CGRP ON EATING AS SATIATING HORMONE

Amylin shares the typical characteristics of satiating hormones,[12] which are involved in the control of meal size. Amylin is rapidly released during food ingestion, and it dose dependently reduces meal size. Amylin has a rapid onset and brief duration of action, and amylin antagonism increases eating by a specific meal size effect.[14,20]

In contrast to some other satiating hormones such as cholecystokinin (CCK), amylin is also active under conditions of chronic delivery; amylin reduces eating by decreasing average meal size, but it may also reduce meal number by increasing the duration of the intermeal interval. Similarly, amylin increases the latency to eat under certain experimental conditions. Nonetheless, the best characterized function of amylin is its effect to reduce meal size.

Qualitatively, CGRP reduces eating by affecting the same meal pattern parameters as amylin, that is, CGRP reduces meal size after acute and chronic administration and affects the intermeal interval during chronic infusion. Because of the lack of 100% specific antagonists against amylin or CGRP receptors and the lack of studies with specific receptor deficient animals, it remains unclear whether CGRP's effects on eating result from an interaction at amylin receptors or from a specific, amylin independent effect. However, based on its release pattern with an increase of plasma CGRP levels only occurring at least 1 h after meal onset, it seems unlikely that blood-borne CGRP constitutes a physiological satiating agent.[7]

Meal pattern analysis after the injection of amylin or CGRP reveals another potential difference between amylin and CGRP action because CGRP reduces the eating rate in rats, at least at higher doses. This suggests that some aversive or illness-producing effect may at least partly underlie the anorectic action of CGRP, indicating that its action on feeding is not specific. This contrasts with amylin which does not reduce feeding by producing a conditioned taste aversion or by an unspecific effect (e.g., via a reduction in drinking).[12]

The lowest dose of exogenous amylin that produces a significant reduction in feeding yields plasma amylin levels that were about two times higher than the concentrations measured postprandially. Strictly speaking, this means that physiological amylin concentrations that occur at the end of spontaneous meals have not yet been shown to reduce feeding. However, different kinetics of the blood amylin concentration after exogenous amylin delivery rather than endogenous secretion may play a role. Further, under natural feeding conditions many gastrointestinal hormones that individually induce satiation are released at the same time and may interact in a way that may be difficult to mimic in an experimental setting. Overall, we believe that amylin is a physiological control of meal size and that this effect is based on circulating amylin; importantly, several studies have clearly shown that peripherally or centrally delivered amylin antagonists produce an effect opposite to that of amylin, that is, an increase in meal size.[14]

AMYLIN AS AN ADIPOSITY SIGNAL

The role of amylin as a satiating hormone seems to depend on phasic changes in amylin levels, that is, the meal-induced increase in plasma amylin levels. In addition, amylin may also exert a tonic effect on eating, that is, it may play a role in the long-term control of eating or body weight.[10,27]

First, basal levels of amylin also seem to depend on the prevailing body weight and body adiposity, in both rats and humans. High-fat fed, obese rats have higher baseline amylin levels than age matched lean controls: of note; this does not distinguish between a body weight and a diet effect. We also investigated whether changes in body adiposity in single individuals are reflected in changing amylin levels and whether such changes in adiposity and amylin in fact follow the same temporal pattern; rats were rendered overweight by chronic intragastric overfeeding leading to a 30% excess body weight above normal weight rats. To our surprise, basal amylin levels did not change significantly during or after termination of overfeeding. Whether amylin levels may only increase after extended periods of overweight or whether the type of diet used during overfeeding may play a role is currently unknown. It seems clear that further studies are required to define the exact contribution of time, diet, extent of overweight and other potential factors on the obesity related increase in plasma amylin levels.

Second, chronic amylin infusion in rats lowers eating and body weight gain by reducing body adiposity while chronic peripheral or central infusion of amylin antagonists increases body weight with a major effect on body fat mass while sparing lean body mass.[20,23] We showed that chronic third ventricular amylin infusions reduce body weight gain in rats regardless of previous weight manipulations. This means that amylin infused rats, whether temporarily food restricted, overfed or left unmanipulated, eventually reached similar body weight which was consistently lower than in saline infused controls.[27] Hence, the effect of a chronic modulation of amylin signaling is similar to that of leptin or insulin. The relative contribution of amylin versus leptin or insulin to the control of body weight is difficult to judge but it is clear that the lack of leptin in *ob/ob* mice results in a more dramatic obese phenotype than the lack of amylin in amylin-deficient mice.[8,12] However, because obesity-associated leptin resistance prevents leptin from being effective when the effect apparently would be needed most, additional amylin may be a very effective

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alternative and amylin may play an important modulatory role. Further, even animals with defective leptin and insulin signaling (e.g., the Zucker *fa/fa* rat) keep their body weight relatively stable, even though this occurs at a higher level. Hence, amylin may play some role as an adiposity feedback signal similar to leptin and insulin, at least when the leptin and insulin feedback signaling systems are deficient.

EATING IN KNOCKOUT ANIMALS[16]

Amylin knockout animals have been developed to study the role of amylin in nutrient and bone metabolism.[8] The specific phenotypes of amylin-deficient mice that are not related to amylin's role in the control of eating include a lower bone mass resulting from a higher number of osteoclasts and reduced late phase nociception in the paw formalin test; the latter implies that endogenous amylin increases the perception of chemical pain.

In the same animals, adult body weight does not differ between knockout and wildtype animals in some studies, but amylin knockou*t* mice show a slightly higher rate of body weight gain compared with that of corresponding wildtype controls; the knockout mice grow significantly faster up to about 4 months of age. Eating is slightly though not significantly higher than in control animals.[12]

Interestingly, amylin knockout mice also differ from control animals in their interaction with other factors co ntrolling eating, indicating that endogenous amylin may have a facilitating role for other controls of eating (e.g., CCK;[15] or leptin). Together, these studies suggest a role for endogenous amylin in the overall control of energy balance, most probably by an effect on eating and in interaction with other satiating and adiposity signals.[12]

Eating in amylin receptor knockout animals has not been determined so far. The effect of haplodeletion of amylin and its receptor protein (calcitonin receptor; CT-R) on bone metabolism has been determined in crossbred heterozygous amylin$^{+/-}$ and heterozygous CT-R$^{+/-}$ mice, but neither eating nor body weight were reported.[16]

EATING IN ANIMALS OVEREXPRESSING AMYLIN

To my knowledge, no detailed studies are available on the effect of amylin[16] overexpression on eating or body weight. Rats that were transgenic for the overexpression of human amylin gained weight comparably until 5 months of age but then had a slightly lower body weight than wildtype controls.[4] The authors argued that lower body weight may have been a consequence of energy loss because of the development of glycosuria and diabetes in the transgenic rats, but no detailed data on eating were provided. Hence, reduced eating which in principle would be consistent with the effects of exogenous amylin on eating and body weight cannot be excluded. The rather small difference between transgenic and control animals may have resulted from receptor desensitization or the redundant system controlling body weight. Because the major difference in body weight between amylin knockout and wildtype mice occurs during a certain period of life, the same may also hold true for animals overexpressing amylin. Another article with mice that are transgenic for the overexpression of human amylin also did not report data on eating and body weight.[28]

EATING IN ANIMALS DEFICIENT OF CGRP OR OVEREXPRESSING CGRP

Although no detailed results are available related to the effect of CGRP[16] overexpression on eating or body weight, a later article describes the effect of genetic CGRP deficiency (CGRP knockout) on eating and body weight in mice. CGRP ko mice fed different diets ate slightly but significantly more than control mice of the same diet group; total eating and eating corrected for body weight gain were both increased significantly. This effect on eating in principle is consistent with the effect of exogenous CGRP to reduce energy intake. Surprisingly however, and despite overeating, the ko mice seem to be protected from the development of diet-induced obesity because both their body weight and body adiposity are lower than in wildtype animals. The latter effect may be explained by the increase in core body temperature and energy expenditure in knockout versus wildtype mice.[28]

ENERGY EXPENDITURE AS AFFECTED BY AMYLIN AND CGRP

Many studies have shown that acute (central) and chronic amylin administration increase energy expenditure as assessed by indirect calorimetry (reviewed in Ref. 12; see also Ref. 27). The effect under chronic conditions may in part be secondary to the reduction in adiposity by amylin and hence the relative increase in metabolically more active lean body mass. Acute peripheral injection of an anorectic dose of amylin fails to increase energy expenditure in rats, whereas the long acting amylin agonist salmon calcitonin (sCT) increases it; we therefore believe that this difference may result from the different biological half-life of amylin versus sCT. The central neural targets which may mediate the effect of exogenous amylin on energy expenditure and the underlying mechanisms are not clear.

Given the similar eating-inhibitory effects of amylin and CGRP, the report that CGRP knockout mice have increased rather than decreased energy expenditure is surprising. Whether CGRP replacement would have corrected for this phenotype was not tested.[28]

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ENERGY METABOLISM IN ANIMALS WITH INCREASED AMYLIN OR CGRP SIGNALING PROPENSITY

Another study has provided an interesting insight into the potential mechanisms of enhanced amylin or CGRP receptor signaling.[30] This study used transgenic mice overexpressing the human RAMP1 gene specifically in the central nervous system; because RAMP1 is a chaperone to the amylin (CT-R) and CGRP (CL-R) receptors, any effect observed in these mice could result from enhanced signaling of amylin, of CGRP or both. Total eating is temporarily reduced in the transgenic mice and eating related to body weight gain is increased. Interestingly, energy expenditure is significantly higher in the transgenic mice, probably explaining the lower body weight and adiposity. Because the effect of exogenous central amylin but not CGRP on body weight gain is markedly enhanced in the transgenic mice, the phenotype of the RAMP1 transgenic mice may mainly result from enhanced brain amylin signaling via the CT-R/RAMP1 receptor complex. This may also explain the apparent paradox between lower body adiposity in these mice, and the effect of the knockout of CGRP in mice that results in a protection from diet-induced obesity.[28]

REGULATION OF AMYLIN AND CGRP SYNTHESIS AND SECRETION IN LEAN AND OBESE INDIVIDUALS

Because of their cosynthesis and colocalization in secretory vesicles, insulin and amylin are normally cosecreted on activation with the appropriate stimuli;[2,3] this typically occurs at a fixed ratio of about 1:10–1:100. Interestingly, divergent secretion of insulin and amylin is possible, but it remains unclear how differential release of insulin and amylin is regulated. Human obesity, various rat models of obesity, diabetes mellitus, pancreatic cancer, and pharmacological intervention (e.g., dexamethasone) may lead to relative overexpression and over-secretion of amylin versus insulin. Clear evidence for a preferential transcription of amylin or increased stability of amylin mRNA under these conditions has not been found so far. It has however been hypothesized that differential regulation of amylin and insulin mRNA production may involve the cAMP and protein kinase A (PKA) pathways and that different transcription factors for insulin and amylin may be involved.

We investigated whether obesity or maintenance on a high-fat diet (HF diet) affect meal induced amylin release in rats. HF fed rats, whether classified as diet-induced obese (DIO) or diet-resistant (DR) exhibit an earlier meal-induced rise in amylin compared with lean chow-fed controls. Further, DR rats have higher amylin:insulin ratios before and after a meal. In principle, these findings are consistent with the demonstration that elevated fatty acids, which can result from the consumption of a diet high in fat, induce enhanced mRNA expression and release of amylin, but not insulin. One important overall conclusion from these experiments in respect to amylin's role in the control of eating and in obesity is that amylin secretion does not seem to be blunted in obesity; it therefore seems unlikely that insufficient release of amylin contributes to overeating in obesity or in individuals on HF diets.

Obese individuals have been reported to have higher CGRP levels than lean controls,[7] but the physiological relevance of this finding is unclear. A direct link between baseline CGRP levels and the degree of adiposity seems unlikely because CGRP does not decrease in response to body weight loss. Plasma concentrations of CGRP increase in response to eating mainly by spillover from sensory nerve endings rather than controlled release from endocrine cells. This is only seen after a high fat, but not after a high carbohydrate meal, and the late increase (>1 h) argues against an effect of CGRP on individual meals. Specific studies directly comparing the meal-induced changes in CGRP secretion and blood concentrations in lean versus obese individuals or in individuals on specific diets have not been reported.

CENTRAL PATHWAYS MEDIATING THE ANORECTIC EFFECTS OF AMYLIN AND CGRP

Experimental evidence supports the idea that the anorectic effect of peripheral amylin is mediated by direct humoral action on the area postrema (AP) in the hindbrain (reviewed in Refs. 12, 17). Amylin binding sites have been described in various CNS locations involved in the control of eating, but behavioral tests with peripheral or local AP infusions, immunohistological, and electrophysiological experiments all indicate that the AP seems to be necessary as primary site of action for peripheral amylin to reduce eating.

Blood-borne amylin has easy access to AP neurons because of the lack of a functional blood–brain barrier in this area; several studies from different laboratories have shown that peripheral amylin's anorectic action is abolished in rats with lesions in the AP. Endogenous blood-borne amylin also seems to act via the AP because an infusion of the amylin antagonist AC 187 into the AP increases eating because of an increase in meal size; further, AC 187 infused into the AP also partly blocks the anorectic effect induced by peripheral amylin injection, providing the best *in vivo* evidence for the importance of the AP in mediating peripheral amylin's anorectic action.

Electrophysiological and immunohistochemical studies indicate that AP neurons are directly and dose dependently stimulated by amylin and that exogenous and endogenous

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amylin produce a strong expression of the immediate early gene product c-Fos protein as a marker of neuronal activation in AP neurons. Both responses are effectively blocked by AC 187.

PHENOTYPE OF AMYLIN RESPONSIVE NEURONS IN THE AP

Expression of c-Fos protein was used to determine the phenotype of amylin responsive neurons in the AP.[19] We found that about 50% of amylin-activated AP neurons are noradrenergic because they expressed dopamine-beta-hydroxylase (DBH), the enzyme catalyzing noradrenaline (NA) synthesis. These neurons seem to play a specific functional role in amylin's effect on eating because even a partial (~50%) lesion of the NA-containing neurons in the AP is sufficient to block the acute eating effect of peripheral amylin. Further, amylin did not induce a significant c-Fos response in AP neurons in NA-lesioned rats.

INTRACELLULAR SIGNALING PATHWAYS IN AP NEURONS ACTIVATED BY AMYLIN

Despite the widespread use as activation marker, we believe that the amylin-induced c-Fos response in the AP has no functional implications because the kinetics of c-Fos expression and the onset of amylin's anorectic effect are inconsistent.[12,17] We have provided evidence for a functional role of phosphorylation of the extracellular signal regulated kinase (pERK1/2) cascade in amylin's effect; amylin-induced ERK1/2 phosphorylation, and (partial) blockade of this effect-attenuated amylin's feeding response. How this relates functionally to amylin-induced cGMP formation in AP neurons remains to be investigated.

PROJECTIONS OF AP NEURONS IMPLICATED IN AMYLIN'S AND CGRP'S EFFECTS AND HYPOTHALAMIC INVOLVEMENT IN THE ANORECTIC ACTION OF PERIPHERAL AMYLIN.[18,21]

Amylin elicits a strong c-Fos response in AP projection areas, namely, the nucleus of the solitary tract (NTS), the lateral parabrachial nucleus (lPBN), the central nucleus of the amygdala (CeNA), and the bed nucleus of the stria terminalis (BNST). The AP, NTS, and the lPBN form a necessary part of the CNS pathway conveying amylin's anorectic signal to higher brain structures; their activation seems to be secondary to an action of amylin on AP neurons.

Retrograde and anterograde tracer studies confirm reciprocal projections between these amylin-activated areas. We identified dense projections from the amylin-activated neurons in the lPBN and sparse projections from the NTS to the dorsal lateral hypothalamus (dLHA) where amylin reduces fasting induced c-Fos expression; hence, the lPBN may thus mediate the amylin-induced inhibition of the dLHA. This inhibition may be linked to the downregulation of the expression of orexin and melanin-concentrating hormone in the dLHA. Dense efferents are also observed from the lPBN to other hypothalamic areas, namely, to the ventromedial (VMH), dorsomedial, paraventricular (PVN), and arcuate nuclei (ARC). Their functional role is not entirely clear, but see paragraphs on amylin/leptin interactions and the role of the ventromedial hypothalamus.

SITE OF CGRP ACTION

The site of action for CGRP's anorectic action has been investigated in less detail than that of amylin. Similar to amylin, the anorectic action of peripheral CGRP is abolished in rats with an AP lesion. Immunohistochemical and electrophysiological studies have not been performed to the same extent as for amylin, so that it is difficult to integrate these findings into the whole brain network for the physiological control of eating.

FOREBRAIN EFFECTS OF AMYLIN

The role of forebrain amylin[12] receptors is still unclear. It seems probable that these receptors mediate the anorectic action of amylin infused into the third brain ventricle; this effect is very potent and long-lasting, and third ventricular infusion of AC 187 increases eating in rats. The latter is associated with an increase in body weight and the weight of the retroperitoneal fat pads. Nonetheless, the functional relevance of these findings under physiological conditions is not clear, and at present, there is no evidence to suggest that forebrain amylin receptors are involved in the regulation of eating by peripheral amylin.

CNS production of amylin has not been shown unequivocally, except in an interesting study pointing to a potential role of hypothalamic amylin in the modulation of maternal behavior.[6] The physiological relevance of these findings is currently under investigation.

FOREBRAIN EFFECTS OF CGRP

Injection of CGRP into the PVN which contains CGRP binding sites[5] produces a strong anorectic effect, and it increases the concentration of various anorectic neuropeptides in the hypothalamus. However, the physiological relevance of these findings is unclear because very high doses of CGRP had been used, exceeding the EC_{50} for amylin to elicit an anorectic effect after administration into the third

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brain ventricle by a factor of >200. Further, the PVN content of CGRP was elevated in response to fasting, which is difficult to reconcile with the idea of CGRP acting as an anorectic hormone or neuropeptide. Hence, the role of CGRP as a brain-intrinsic neuropeptide in the control of feeding remains to be investigated.

INTERACTION BETWEEN AMYLIN AND ESTRADIOL

Only few studies have tested the specific anorectic effect of amylin in females.[11,25] Similar to CCK and other satiating hormones, single acute injections of amylin are more effective in ovariectomized rats with physiological estradiol replacement than in rats without replacement. Further, the amylin antagonist AC 187 increases eating more in estradiol replaced ovariectomized animals, pointing to the physiological relevance of the modulating effect of estradiol on amylin action. Surprisingly, the effect is opposite under chronic conditions; in other words, the action of chronic amylin on eating and body weight is stronger in ovariectomized rats lacking estradiol than in control rats or in ovariectomized rats receiving estradiol replacement. Chronic amylin also at least partly restores the metabolic rate that was decreased as a consequence of ovariectomy. Hence, the interaction between amylin and estradiol seems to be complex and these apparently opposing phenomena require intensified study.

SHORT-TERM INTERACTION WITH OTHER HORMONES CONTROLLING INGESTIVE BEHAVIOR

Subthreshold doses of peripheral insulin and amylin reduce eating in rats when coadministered, and central insulin enhances the action of peripheral amylin.[12,22] Further, amylin and CCK also seem to interact in their effect on eating. CCK and amylin synergistically reduce eating; similar synergistic effects have been reported with PYY and for the combination of sCT with the GLP-1 agonist exendin-4 in nonhuman primates.[1] Further, part of the satiating action of CCK seems to be mediated by amylin, because amylin antagonists attenuated CCK's anorectic actions in rats and because the anorectic effect of CCK is almost abolished in amylin knockout mice; the latter effect is restored by amylin. In other words, endogenous amylin seems to modulate the anorectic effect of CCK.

INTERACTION BETWEEN AMYLIN AND LEPTIN

Amylin and leptin[22,26] seem to interact acutely and chronically. Acute central leptin increases the eating-inhibitory effect of peripheral amylin. Further, two-week peripheral infusions of amylin and leptin were performed in leptin resistant DIO rats. Amylin alone reduces eating and leads to a decrease in body weight. Interestingly, rats pair-fed to the amylin group and that received leptin do not lose more weight than amylin-treated rats. However, combined application of exogenous leptin and amylin decreases eating and body weight more than amylin alone, and body fat is lowest after leptin/amylin. Hence, amylin strongly enhances the sensitivity of obese rats to the catabolic effect of leptin, including an increase in energy expenditure. Body fat loss is more in the amylin/leptin-treated rats than in the pair-fed controls; this is consistent with a lower respiratory quotient, indicating preferential fat oxidation. One important point to consider is that the interactions of amylin and leptin on eating and adiposity have mainly been shown under pharmacological, not under physiological conditions.

ROLE OF THE VMH IN THE LEPTIN AND AMYLIN INTERACTION

The interaction[26] between amylin and leptin seems to result from a direct effect of amylin on central leptin signaling, most probably in the VMH and potentially the ARC. Amylin enhances leptin signaling as gauged by increased immunoreactivity of pSTAT3 specifically in the VMH; this effect may be linked to an amylin-induced upregulation of leptin receptor expression and hence direct enhancement of leptin action in VMH neurons. Further, amylin-deficient mice have reduced leptin receptor expression in the mediobasal hypothalamus.

INTERACTION BETWEEN AMYLIN AND METABOLITES

Higher doses of amylin are necessary to inhibit sham feeding than real feeding, which may be because of the lack of cooperative effects between amylin and metabolites (e.g., elevated glucose) under these conditions.[12] Similar to amylin's effect on gastric emptying, amylin's eating-inhibitory effect seems to be reduced in rats with induced hypoglycemia. This is in line with coactivation of AP neurons by physiological concentrations of amylin and glucose. The co-responsiveness of AP neurons to amylin and glucose is similar to that for glucose and CCK; AP neurons may therefore integrate metabolic and hormonal signals that control eating.

PHYSIOLOGICAL AND PATHOPHYSIOLOGICAL IMPLICATIONS

Relatively little is known about the possible role of amylin's effect on feeding under pathophysiological conditions. It has been hypothesized that amylin may contribute to cancer

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anorexia in certain pancreatic neoplasia, which may be associated with chronically supraphysiological plasma amylin levels.

An altered ratio between amylin and insulin has been supposed to play a role in eating disorders and hence to contribute to abnormal feeding behavior in diabetes mellitus. In other words, the lack of amylin in type 1 diabetes and later stages of type 2 diabetes could result in increased eating or body weight. The clinical experience with the amylin analog pramlintide in the treatment of type 1 and 2 diabetes is consistent with this idea. Individuals treated with pramlintide plus insulin lost body weight while patients treated with insulin alone gained weight. Subsequent trials showed that pramlintide decreases eating and body weight in obese or diabetic patients. Because obesity and chronic hyperamylinemia, which is typically associated with obesity, do not seem to reduce amylin effectiveness, amylin may be a promising and potent approach to treat obese patients. Similar to the results in rats, this effect can be potentiated when amylin is combined with leptin.

Similar results are not available for CGRP. Hence, it is unknown whether CGRP or CGRP analogs produce similar or even more potent effects than amylin in a clinical setting. Given the current availability of data, amylin seems to be a more promising and safer approach than CGRP.

SUMMARY

Both amylin and the structurally related neuropeptide CGRP reduce eating. Amylin may be a physiological control of meal size, but this is less clear for CGRP. The effects of peripheral amylin and CGRP on eating are centrally mediated by the AP in the hindbrain as the most probable primary target area. Chronic exposure to amylin decreases eating and body weight gain. Next to a satiating action, amylin may also constitute an adiposity feedback signal. The anorectic action of amylin is pharmacologically exploited because amylin analogs are under clinical consideration for their effect to reduce eating and body weight in humans; this effect can be potentiated with leptin co-therapy.

REFERENCES

1. Bello NT, Kemm MH, Ofeldt EM, Moran TH. Dose combinations of exendin-4 and salmon calcitonin produce additive and synergistic reductions in food intake in nonhuman primates. *Am J Physiol Regul Integr Comp Physiol* 2010;**299**:R945–52.
2. Boyle CN, Lutz TA. Amylinergic control of food intake in lean and obese rodents. *Physiol Behav* 2011;**105**:129–37.
3. Boyle CN, Rossier MM, Lutz TA. Influence of high-fat feeding, diet-induced obesity, and hyperamylinemia on the sensitivity to acute amylin. *Physiol Behav* 2011;**104**:20–8.
4. Butler AE, Jang J, Gurlo T, Carty MD, Soeller WC, Butler PC. Diabetes due to a progressive defect in beta-cell mass in rats transgenic for human islet amyloid polypeptide (HIP Rat): a new model for type 2 diabetes. *Diabetes* 2004;**53**:1509–16.
5. Dhillo WS, Small CJ, Jethwa PH, Russell SH, Gardiner JV, Bewick GA, et al. Paraventricular nucleus administration of calcitonin gene-related peptide inhibits food intake and stimulates the hypothalamo–pituitary–adrenal axis. *Endocrinology* 2003;**144**:1420–5.
6. Dobolyi A. Central amylin expression and its induction in rat dams. *J Neurochem* 2009;**111**:1490–500.
7. Geary N. Effects of glucagon, insulin, amylin and CGRP on feeding. *Neuropeptides* 1999;**33**:400–5.
8. Gebre-Medhin S, Mulder H, Pekny M, Westermark G, Tornell J, Westermark P, et al. Increased insulin secretion and glucose tolerance in mice lacking islet amyloid polypeptide (amylin). *Biochem Biophys Res Commun* 1998;**250**:271–7.
9. Hay DL, Christopoulos G, Christopoulos A, Poyner DR, Sexton PM. Pharmacological discrimination of calcitonin receptor: receptor activity-modifying protein complexes. *Mol Pharmacol* 2005;**67**:1655–65.
10. Isaksson B, Wang F, Permert J, Olsson M, Fruin B, Herrington MK, et al. Chronically administered islet amyloid polypeptide in rats serves as an adiposity inhibitor and regulates energy homeostasis. *Pancreatology* 2005;**5**:29–36.
11. Lutz TA. Amylin may offer (more) help to treat postmenopausal obesity. *Endocrinology* 2011;**152**:1–3.
12. Lutz TA. The role of amylin in the control of energy homeostasis. *Am J Physiol Regul Integr Comp Physiol* 2010;**298**:R1475–84.
13. McLatchie LM, Fraser NJ, Main MJ, Wise A, Brown J, Thompson N, et al. RAMPs regulate the transport and ligand specificity of the calcitonin-receptor-like receptor. *Nature* 1998;**393**:333–9.
14. Mollet A, Gilg S, Riediger T, Lutz TA. Infusion of the amylin antagonist AC 187 into the area postrema increases food intake in rats. *Physiol Behav* 2004;**81**:149–55.
15. Mollet A, Meier S, Grabler V, Gilg S, Scharrer E, Lutz TA. Endogenous amylin contributes to the anorectic effects of cholecystokinin and bombesin. *Peptides* 2003;**24**:91–8.
16. Muff R, Born W, Lutz TA, Fischer JA. Biological importance of the peptides of the calcitonin family as revealed by disruption and transfer of corresponding genes. *Peptides* 2004;**25**:2027–38.
17. Potes CS, Lutz TA. Brainstem mechanisms of amylin-induced anorexia. *Physiol Behav* 2010;**100**:511–8.
18. Potes CS, Lutz TA, Riediger T. Identification of central projections from amylin-activated neurons to the lateral hypothalamus. *Brain Res* 2010;**1334**:31–44.
19. Potes CS, Turek VF, Cole RL, Vu C, Roland BL, Roth JD, et al. Noradrenergic neurons of the area postrema mediate amylin's hypophagic action. *Am J Physiol Regul Integr Comp Physiol* 2010;**299**:R623–31.
20. Reidelberger RD, Haver AC, Arnelo U, Smith DD, Schaffert CS, Permert J. Amylin receptor blockade stimulates food intake in rats. *Am J Physiol Regul Integr Comp Physiol* 2004;**287**:R568–74.
21. Riediger T, Zuend D, Becskei C, Lutz TA. The anorectic hormone amylin contributes to feeding-related changes of neuronal activity in key structures of the gut-brain axis. *Am J Physiol Regul Integr Comp Physiol* 2004;**286**:R114–22.
22. Roth JD, Trevaskis JL, Turek VF, Parkes DG. "Weighing in" on synergy: preclinical research on neurohormonal anti-obesity combinations. *Brain Res* 2010;**1350**:86–94.
23. Rushing PA, Hagan MM, Seeley RJ, Lutz TA, D'Alessio DA, Air EL, et al. Inhibition of central amylin signaling increases food intake and body adiposity in rats. *Endocrinology* 2001;**142**(5035).

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24. Sexton PM, Paxinos G, Kenney MA, Wookey PJ, Beaumont K. In vitro autoradiographic localization of amylin binding sites in rat brain. *Neuroscience* 1994;**62**:553–67.
25. Trevaskis JL, Turek VF, Wittmer C, Griffin PS, Wilson JK, Reynolds JM, et al. Enhanced amylin-mediated body weight loss in estradiol-deficient diet-induced obese rats. *Endocrinology* 2010;**151**:5657–68.
26. Turek VF, Trevaskis JL, Levin BE, Dunn-Meynell AA, Irani B, Gu G, et al. Mechanisms of amylin/leptin synergy in rodent models. *Endocrinology* 2010;**151**:143–52.
27. Wielinga PY, Löwenstein C, Muff S, Munz M, Woods SC, Lutz TA. Central amylin acts as an adiposity signal to control body weight and energy expenditure. *Physiol Behav* 2010;**101**:45–52.
28. Wong WP, Scott DW, Chuang CL, Zhang S, Liu H, Ferreira A, et al. Spontaneous diabetes in hemizygous human amylin transgenic mice that developed neither islet amyloid nor peripheral insulin resistance. *Diabetes* 2008;**57**:2737–44.
29. Young A, Denaro M. Roles of amylin in diabetes and in regulation of nutrient load. *Nutrition* 1998;**14**:524–7.
30. Zhang Z, Liu X, Morgan DA, Kuburas A, Thedens DR, Russo AF, et al. Neuronal receptor activity modifying protein-1 promotes energy expenditure in mice. *Diabetes* 2011;**60**:1063–71.

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Chapter 141

Apolipoprotein (apo) A-IV/apoE

Patrick Tso and Min Liu

ABSTRACT

Although circulating apolipoprotein A-IV (apo A-IV) is mainly produced from the intestine, and apolipoprotein E (apoE) from the liver, both proteins are also synthesized in brain areas involved in the energy homeostasis. Both the proteins have been proposed to physiologically control food intake, and this inhibitory effect is mainly centrally mediated. Intracerebroventricular (icv) administration of apo A-IV or apoE significantly reduces food intake without causing malaise, whereas icv infusion of their antibodies stimulates feeding, implying that these endogenous apolipoproteins tonically inhibit food intake. Both genetically obese (*ob*/*ob*) mice and rats with high-fat-diet-induced obesity have significantly reduced hypothalamic apo A-IV and apoE mRNA levels compared with that of their lean controls. Compelling evidence has thus shown that apo A-IV and apoE exert their potent catabolic influence to regulate energy homeostasis through integration with other central signals.

INTRODUCTION

Apolipoprotein A-IV (apo A-IV), discovered in the late 1970s,[31] is a glycoprotein found in many species; the human form has a molecular weight of 46 kDa. The jejunum is the major site of apo A-IV synthesis, and its synthesis and secretion are associated with chylomicron formation. Once chylomicrons enter the circulation, apo A-IV rapidly dissociates from chylomicron remnants, with the result that the majority of apo A-IV in the circulation exists as free protein, whereas the remainder is associated with circulating high-density lipoproteins. Several studies have provided evidence that apo A-IV plays a role in cholesterol and lipoprotein metabolism, protection against lipoprotein oxidation, and atherosclerosis.[3,4,23] Compelling evidence also supports a primary role for apo A-IV in the control of ingestive behavior.[7,8,16] Although the reduced gastric motility and acid secretion induced by apo A-IV partially contribute to its anorectic effect,[22] compelling evidence suggests that the inhibitory action on food intake is mainly centrally mediated.[17,27,30,32]

Apolipoprotein E (apo E), first described in 1975, is a 299-amino-acid protein with a 34-kDa molecular weight.[33] ApoE is synthesized in several areas of the body, but the liver accounts for the major portion of circulating apoE. When synthesized by the liver as part of very low-density lipoproteins (VLDLs), apoE functions in the transport of triglycerides to the tissues. ApoE is also found in high-density lipoproteins (HDLs) and functions in cholesterol homeostasis. In the CNS, apoE is produced by diverse cell types, including astrocytes and neurons. In the cerebrospinal fluid (CSF), apoE is the most abundant component of HDL-like lipoproteins, which are bound and internalized via receptor-mediated endocytosis by members of the low-density lipoprotein receptor (LDLR) family.[1] Previous studies have shown that apoE plays additional roles in the brain, regulating both neuronal and astrocytic functions, including preventing excitotoxicity, promoting neuron survival and sprouting, protecting neurons against oxidative stress, and regulating innate and adaptive immune responses.[11,14] More recent results indicate that brain apo E is also an important regulator of food intake and body weight in rats.[19,29]

Effects on Food Intake and Body Weight

The role of Apo A-IV in regulating food intake was first proposed by Fujimoto et al.[7] Because chylomicron transport and apo A-IV output in mesenteric lymph increase significantly after a lipid meal, Fujimoto et al. infused mesenteric lymph collected from lymph-fistula rats after lipid administration intravenously (iv) in 24-h fasted rats and observed that those animals eat significantly less, compared with saline controls. By contrast, lymph collected before lipid infusion and lymph collected from a rat fed lipid plus Pluronic L-81 (a potent inhibitor of chylomicron formation) has no such anorectic effect.[7] After specific immunoprecipitation of apo A-IV by a monospecific antibody, chylomicrons and apo A-IV-rich lymph lose their anorectic effect. In 24-h fasted rats, iv infusion of purified apo A-IV, the same amount of apo A-IV as the level in chylous lymph 6–8 h after a lipid meal, suppresses food intake. Fujimoto et al. therefore proposed that apo A-IV is a circulating satiation

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00141-X
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signal released by the small intestine in response to fat feeding and probably helps mediate the anorectic effect of fat consumption.[7]

Feeding behavior is influenced by many circulating factors, and chemosensitive monitoring systems for these factors exist both in the CNS and in peripheral organs.[35] To investigate whether the anorectic effect of apo A-IV after lipid feeding is mediated via the CNS, Fujimoto et al. infused different doses of apo A-IV icv and found that apo A-IV inhibits food intake in a dose-dependent manner.[8] Administration of goat antirat apo A-IV serum into the third ventricle of rats results in increased food intake, indicating that apo A-IV may act centrally to control food intake.

It has been reported that targeted absence of apoE yields significantly elevated food intake and body weight in mice fed a diabetogenic diet.[24] Based on that, Shen et al. hypothesized that apoE participates in regulating energy homeostasis. They administered different doses apoE into the third cerebral ventricle of rats, and found that it produces a significant and dose-dependent inhibition of food intake in both ad libitum and 24-h fasted conditions, and the reduction of food intake is not associated with illness.[29] To further determine whether the CNS is the critical site for the anorectic action of apoE, food intake of rats administered iv apoE at doses that are effective centrally does not differ from those receiving vehicle at any time point, implying that icv apoE acts within the CNS to suppress food intake rather than leaking into the blood and acting at peripheral apoE receptors.

To determine whether endogenous apoE signaling influences food intake, Shen et al. administered apoE antiserum icv to *ad libitum* fed rats in the middle of the light phase and observed significantly increased food intake, but icv infusion of preabsorbed apoE serum fails to elicit a feeding response. Further, the anorectic action induced by icv apoE is attenuated by icv-infused apoE antibody.[29] Because food intake is a major determinant of energy balance, Liu et al. hypothesized that chronic icv infusion of apoE would lead to a reduction of body weight. To determine this, rats received a continuous infusion of apoE or vehicle via osmotic minipump, and their food intake, body weight were monitored daily for 10 days. In parallel with the reduction of food intake, there is a significant decrease in body weight in rats by Day 4 of treatment with apoE, compared with that of vehicle controls (Fig. 1). The body weight of pair-fed rats is comparable with that of apoE-treated rats during the entire period of observation, implying that the effect of apoE on body weight is mainly the result of a reduction of food intake.

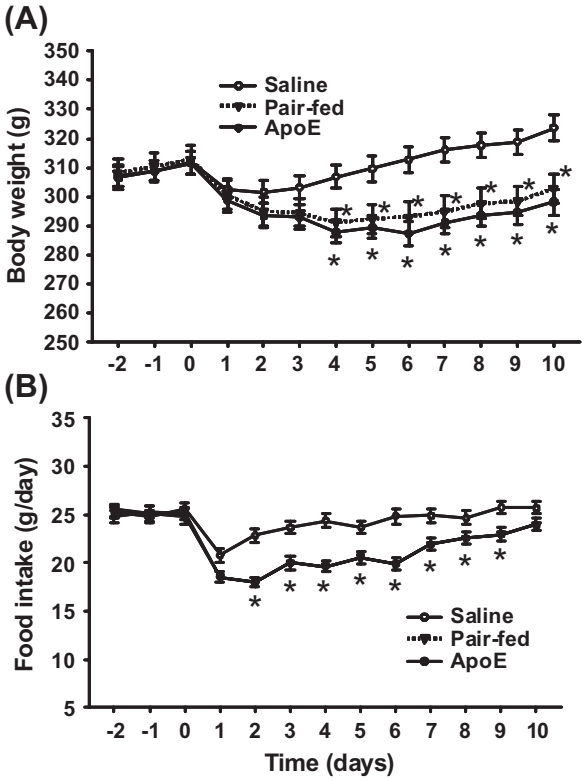

FIGURE 1 Changes in body weight (A) and food intake (B) during chronic central apoE infusion. ApoE (4 μg/day) or saline was infused into the third cerebral ventricle via Alzet osmotic minipump (0.5 μl/h). Data represent the mean ± SEM; $n = 7–8$, $*P < 0.05$ vs. saline controls.

Distribution in Brain Areas Involved in Energy Homeostasis

Liu et al. have provided direct evidence that apo A-IV mRNA and protein are present in the rat hypothalamus.[15] Using immunohistochemical techniques, Shen et al. further characterized the distribution of apo A-IV in brain areas involved in energy homeostasis.[27] Dense apo A-IV staining is detected in the arcuate (ARC) and ventromedial hypothalamic (VMH) nuclei with less staining in cells in the paraventricular (PVN) and dorsomedial (DMN) nuclei. In the brainstem, apo A-IV staining is found in the nucleus of the solitary tract (NTS). Double-staining immunohistochemistry revealed the coexistence of apo A-IV with neuronal nuclei (a neuronal marker), but less with glial fibrillary acidic protein (GFAP, a glial marker), in ARC, suggesting that apo A-IV is largely present in neurons. To ascertain whether the apo A-IV detected in the brain is derived from the circulation, ^{125}I-labeled recombinant rat apo A-IV was injected iv into mice. No increase of radioactive apo A-IV was found in the brain, consistent with a lack of uptake of coinjected ^{99m}Tc-labeled albumin, indicating that circulating apo A-IV is unable to cross the blood–brain barrier.[27]

ApoE is also in the hypothalamus.[28] Shen et al. used immunofluorescence labeling and observed a prominent presence of apoE protein in the ARC and PVN, consistent with its role in the regulation of energy homeostasis. To determine the apoE-producing cell type, Shen et al. investigated the colocalization of apoE with specific cell type

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markers in the hypothalamus and observed that 87% of the apoE-positive cells are astrocytes, 6% are neurons, 3% are microglial cells, 2% are oligodendrocytes, and 2% are other brain cells.[28]

REGULATION OF APO A-IV AND APOE PRODUCTION

Fasting and Refeeding

Circulating and central apo A-IV levels are influenced by nutritional status.[15] Food-deprived animals have a pronounced decrease in apo A-IV gene expression in the hypothalamus, with a concomitant decrease in the jejunum. Refeeding fasted rats with low-fat chow for 4 h evokes a significant increase of apo A-IV mRNA in jejunum but not in the hypothalamus. However, lipid refeeding to the fasted animals restores apo A-IV mRNA levels in both the hypothalamus and the jejunum. Currently, it is unclear how lipid feeding regulates apo A-IV expression, that is, whether it is a direct or indirect effect. Nonetheless, hypothalamic and intestinal apo A-IV respond similarly to fasting followed by lipid feeding.[15]

Similar responses were observed with rat hypothalamic apoE.[29] Shen et al. measured hypothalamic apoE mRNA levels in rats that were ad libitum fed, 36-h fasted, and 32-h fasted followed by 4-h chow refeeding. The fasted rats had significantly reduced hypothalamic apoE mRNA levels compared with ad libitum fed controls. ApoE mRNA expression was reversed to levels of ad libitum fed rats after 4 h of refeeding.

Chronic High-Fat (HF) Feeding

Weinberg et al. first reported that human plasma apo A-IV levels adapt in response to prolonged consumption of fat.[34] Humans who chronically consume an HF diet initially develop significantly elevated plasma apo A-IV levels that disappear over time. The investigators therefore concluded that intestinal apo A-IV production is autoregulated in response to diets with HF or high-energy density. Kalogeris et al. also examined the effect of daily fat supplementation on intestinal gene expression and protein synthesis and apo A-IV plasma levels.[13] Male rats were administered bolus intragastric injections of either saline or Intralipid for 0, 1, 2, 4, 8, or 16 days. They found plasma apo A-IV to initially increase 40% in rats fed the fat emulsion compared with that in the saline-injected controls. Daily consumption of fat produces a plasma apo A-IV response that rapidly and progressively diminishes, such that, by Day 4, plasma apo A-IV is the same in both the fat- and saline-fed groups. Jejunal mucosal apo A-IV synthesis and mRNA levels also have a time-dependent reduction in response to fat feeding. Both rodent and human results suggest that intestinal apo A-IV synthesis and secretion become less responsive to fat after its chronic consumption.

To further determine whether diet-induced obesity alters apo A-IV gene expression in the intestine and hypothalamus, Liu et al. fed rats a HFD, LFD, or standard chow (CHOW) for 2, 4, 6, 8, or 10 weeks.[18] Rats fed the HFD have significantly greater body weights than rats given the LF and CHOW diets. Intestinal and plasma apo A-IV levels are comparable across dietary groups and time. LF and CHOW rats have comparable hypothalamic apo A-IV mRNA across the course of the experiment. However, HFD rats have a slow and progressive diminution in hypothalamic apo A-IV mRNA over time that becomes significantly lower than that of LFD or CHOW rats by 10 weeks. Intragastric infusion of lipid emulsion to overnight-fasted animals significantly stimulates hypothalamic apo A-IV mRNA in LF and CHOW rats but has no effect in HF rats. These results show that chronic consumption of a HFD significantly reduces apo A-IV mRNA levels and the response of apo A-IV gene expression to dietary lipids in the hypothalamus.[18] This raises the possibility that dysregulation of hypothalamic apo A-IV could contribute to diet-induced obesity.

Similar to the response of apo A-IV gene expression to chronic HFD feeding (10 weeks), diet-induced obesity also alters apoE gene expression in the hypothalamus. Compared with LFD rats, HFD rats have significantly reduced hypothalamic apoE gene expression. Hypothalamic apoE levels of the LFD and pair-fed groups were not statistically different.[29]

Diurnal Rhythm

Fukagawa et al. showed that serum apo A-IV concentration has a circadian rhythm concomitant with the feeding pattern.[9] In 24-h fasted rats, serum apo A-IV maintains a circadian rhythm, being high during the dark. With mesenteric lymph diversion, serum apo A-IV concentration diminishes and the circadian rhythm is abolished. The lymph flow and lymph apo A-IV all exhibit the same circadian rhythm in fasting, with the levels higher in the dark. These circadian rhythms are abolished after bile diversion, indicating that bile is an important determinant of the circadian rhythm of lymph flow and lymph apo A-IV output.

To further understand the roles of central apo A-IV in the management of daily food intake, Liu et al. examined the diurnal patterns of hypothalamic apo A-IV gene and protein expression in freely feeding and food-restricted (food provided 4 h daily) rats.[17] In freely feeding rats, the hypothalamic apo A-IV mRNA and protein levels fluctuate, with highest levels during the light phase, peaking 3 h after lights on, and with a nadir 3 h after lights off. The daily patterns of the fluctuation, however, are altered in food-restricted rats, which have a marked decrease in hypothalamic apo A-IV mRNA and protein levels during the 4-h feeding period of the light phase. Although corticosterone (CORT) secretion temporally coincides with the decreasing phase of apo A-IV

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in the hypothalamus, depletion of CORT by adrenalectomy significantly decreases, rather than increases, hypothalamic apo A-IV mRNA and protein levels.[17] These results indicate that the diurnal expression of hypothalamic apo A-IV is regulated by factors other than the circulating CORT, for example, the reduced food intake and body weight in adrenalectomized animals. The fact that hypothalamic apo A-IV level and food intake are inversely related during the normal diurnal cycle as well as in the period of restricted feeding suggests that hypothalamic apo A-IV is involved in the regulation of daily food intake.

Different from apo A-IV, hypothalamic apoE mRNA and protein levels fluctuate with peaks occurring during the dark phase and nadirs occurring during the light in freely fed rats.[26] This pattern is altered in food-restricted (4 h food daily) rats, which have a marked increase in hypothalamic apoE mRNA and protein levels during the 4-h feeding period in the light phase. Depletion of CORT by adrenalectomy (ADX) does not significantly influence hypothalamic apoE levels during either period, implying that the circadian pattern of hypothalamic apoE is regulated by factors other than circulating CORT.[26] That hypothalamic apoE and food intake are positively associated during the normal circadian cycle as well as in the period of restricted feeding suggests that hypothalamic apoE is food entrained and probably involved in the physiological regulation of daily food intake.

Leptin

Leptin is a polypeptide synthesized and secreted by adipocytes (see the leptin chapters in Endocrine, Cancer, Ingestive, and Gastrointestinal Peptides sections of the book). Its administration decreases food intake and increases energy expenditure. Doi et al. showed that increased lipid-induced levels of intestinal apo A-IV are significantly attenuated by iv leptin. Apo A-IV immunostaining is reduced by an average of 50% in the jejunum and 35% in the ileum in animals infused iv with leptin.[2] These findings are consistent with those of Morton et al. who observed that leptin reduces apo A-IV mRNA levels 90 min after ingestion of a pure fat meal.[21]

Shen et al. reported that leptin-deficient obese (*ob/ob*) mice have significantly reduced hypothalamic apo A-IV mRNA levels.[30] Intragastric infusion of a lipid emulsion significantly stimulates hypothalamic apo A-IV gene expression in lean controls but not in *ob/ob* mice. Daily ip administration of leptin for 5 days significantly increases hypothalamic apo A-IV mRNA levels of *ob/ob* mice relative to pair-fed controls. In addition, centrally administered leptin raises the reduced apo A-IV gene expression induced by fasting.[30] Using immunohistochemistry, Shen et al. showed that apo A-IV is present in leptin-sensitive phosphorylated signal transducer and activator of transcription-3 (pSTAT3)-positive cells of the ARC. Knockdown of STAT3 expression by small interfering RNA (siRNA) significantly attenuates the stimulatory effect of leptin on apo A-IV protein expression in cultured primary hypothalamic neurons, implying that the hypothalamic apo A-IV is regulated by leptin, and at least partially via the STAT3 signaling pathway. Administration of a subthreshold dose of leptin (1 μg icv) potentiates apo A-IV-induced (subthreshold dose, 0.5 μg) reduction of feeding, indicating the existence of a functional synergistic interaction between leptin and apo A-IV, leading to suppression of food intake.

Consistent with the response of hypothalamic apo A-IV to leptin, Shen et al. showed that apoE mRNA levels in the hypothalamus are also significantly reduced in both genetically obese *ob/ob* mice and HFD-induced obese (leptin resistant) rats, raising the possibility that deficient leptin signaling might be related to the change in apoE gene expression.[29] Leptin or vehicle ip to *ob/ob* or *db/db* mice for 5 days significantly increases hypothalamic apoE mRNA levels in *ob/ob*, but not in pair-fed *ob/ob* or *db/db*, mice.[28] These results indicate that leptin upregulates hypothalamic apoE gene expression and requires an intact leptin receptor, and that this effect is not related to the changes in food intake and body weight. The reduced apoE gene expression caused by fasting, which also results in relatively lower leptin level, is restored by icv leptin. In addition, leptin is significantly less efficacious in apoE KO mice, that is, these animals consume more food and lose less weight after leptin treatment compared with that of wild-type controls.[28] These observations imply that apoE signaling, at least partially, mediates the inhibitory effects of leptin on feeding.

Estrogen

Estrogens have potent suppressive effects on food intake and body weight in many species, including humans. Compelling evidence suggests that the anorectic action of estrogen is through an indirect mechanism by enhancing the strength of other physiological signals that reduce meal size. To determine whether estradiol, the primary form of estrogen, modulates the anorectic effect of apo A-IV, intra-4th-ventricular administration of low doses of apo A-IV was found to reduce food intake to a greater extent in ovariectomized (OVX) rats cyclically treated with estradiol than in vehicle-treated OVX controls, implying that cyclic estradiol replacement increases the satiating potency of apo A-IV. OVX significantly increases food intake and body weight but decreases apo A-IV gene expression in the NTS. All these alterations are reversed by cyclic estradiol treatment. The finding of colocalization of apo A-IV with estrogen receptor-alpha in the NTS suggests that estradiol might act locally in the NTS to upregulate apo A-IV gene expression. Further, OVX apo A-IV knockout mice have a smaller feeding response to estradiol, eating significantly more food and gaining more body weight than OVX wild-type controls

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during the period of cyclic estradiol replacement. These results indicate that an increased signaling of endogenous apo A-IV may partially mediate estradiol-induced inhibitory effect on feeding.

PERIPHERAL AND NEURAL NETWORKS AND SIGNALING PATHWAYS AFFECTED

Apo A-IV and CCK

Apo A-IV and CCK act both peripherally and centrally to reduce food intake by decreasing meal size. Lo et al. examined the interaction of apo A-IV and CCK-8 in the control of food intake in rats.[20] Ip apo A-IV at 100 μg/kg reduces food intake significantly for 1 h, as do doses of CCK-8 at or >0.125 μg/kg. Doses of apo A-IV (50 μg/kg) or CCK (0.06 μg/kg) alone have no effect on food intake. However, when these subthreshold doses are administered together, the combination produced a significant inhibition of food intake relative to controls, and the duration of the effect is longer than that caused by the administration of either apo A-IV or CCK alone. The satiation effect produced by CCK-8 plus apo A-IV is attenuated by lorglumide, a CCK1 receptor antagonist.[20] Lo et al. therefore concluded that the coadministration of subthreshold doses of the two peptides is highly satiating and works via CCK1 receptor.

Central Melanocortin System

The hypothalamic melanocortin system is important in the homeostatic regulation of body weight. α-Melanocyte-stimulating hormone (α-MSH) derived from proopiomelanocortin (POMC) neurons in the ARC is the primary agonist for melanocortin type 3 and 4 receptors (MC3/4-R) in the brain (see the chapters in the Brain and Ingestive Peptides sections of the book). α-MSH is believed to exert a tonic inhibitory influence over feeding. Agouti-related protein (AgRP) is a peptide produced in non-POMC cells in the ARC and antagonizes MC3/4-R. Consistent with this, AgRP elicits hyperphagia when it is centrally administered.[5] The i3vt administration of metallothionein-II (MT-II), a synthetic MC3/4-R agonist, potently reduces feeding,[6] whereas i3vt administration of SHU9119, a synthetic MC3/4-R antagonist, blocks the anorectic effect of MT-II and elicits feeding when administered alone at higher doses.[12]

Shen et al. showed that apo A-IV is colocalized with POMC in the ARC, and that apo A-IV administration stimulates ARC POMC gene expression, suggesting that the brain apo A-IV system suppresses food intake by stimulating the ARC POMC system.[27] Gotoh et al. showed that icv administration of a subthreshold dose of apo A-IV potentiates icv MT-II-suppression of 30-min feeding in rats.[10] A subthreshold dose of SHU9119 completely attenuates the anorectic effect of icv apo A-IV. Icv apo A-IV also significantly elevates the expression of c-Fos in PVN neurons but not in the ARC or median eminence.[10] These results support a synergistic interaction between apo A-IV and melanocortins that reduces food intake by acting downstream of the ARC.

Shen et al. reported that apoE interacts with the central melanocortin system to control food intake.[29] A subthreshold dose of SHU9119 was administered concomitantly with apoE in rats. Although icv SHU9119 alone does not produce a reliable effect on food intake, apoE significantly reduces food intake 4 h after injection. However, when the two injections are combined, the SHU9119 completely attenuates the anorectic effect of apoE, implying that an interaction occurs between apoE and the melanocortin system with regard to feeding regulation by the brain.[29]

Signaling Pathways

Phosphatidylinositol 3-kinases (PI3Ks) are heterodimeric complexes composed of regulatory and catalytic subunits that recruit lipids as second messengers and control a broad spectrum of cellular functions including survival, growth and metabolism. The PI3 K signaling pathway is important in the regulation of food intake and energy balance by hypothalamic neurons.

Using primary cultured hypothalamic neurons, Shen et al. showed that apoE treatment elicits rapid activation of the PI3 K/Akt signaling cascade.[25] Specifically, apoE induces the phosphorylation of Akt, peaking at 30 min, and the increased phosphorylation of Akt is significantly attenuated after pretreatment with LY294002, an inhibitor of the PI3 K signaling pathway. To determine whether activation of PI3 K by apoE is required for the ability of apoE to reduce food intake, LY294002 was infused into the third-cerebral ventricle before injection of an anorectic dose of apoE. Consistent with previous reports, apoE exerts significant reduction of food intake in the 4-h fasted rats, compared with that of saline. Pretreatment with LY294002 significantly attenuates the potency of exogenous apoE to induce satiation, whereas the same dose of PI3 K inhibitor by itself causes only a slight nonsignificant decrease of food intake. These results indicate that the activation of the PI3 K/Akt pathway is necessary for the acute effects of apoE on food intake.

Receptors

Liu et al. showed that low-density lipoprotein receptor-related protein 1 (LRP1) regulates leptin signaling and energy homeostasis.[19] Conditional deletion of the *Lrp1* gene in the brain results in an obese phenotype characterized by increased food intake, decreased energy consumption, and decreased leptin signaling. LRP1 directly binds to leptin and the leptin receptor complex and is required for leptin receptor phosphorylation

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and Stat3 activation. Liu et al. further found that deletion of the *Lrp1* gene specifically in the hypothalamus by Cre lentivirus injection is sufficient to trigger accelerated weight gain.[19] These results indicate that LRP1, which is critical for lipid metabolism, also regulates food intake and energy homeostasis in the adult CNS.[19]

To summarize, the control of food intake and energy expenditure is extremely complex and involves many different messengers. Different lines of investigation suggest that disturbances in eating behavior are associated with abnormal hypothalamic neurotransmission and neuropeptide concentrations. Apo A-IV and apoE have been proposed as satiation factors. Both genetically obese (*ob/ob*) mice and rats with HFD-induced obesity have significantly reduced hypothalamic apo A-IV and apoE mRNA levels, suggesting that decreased apo A-IV/apoE may contribute to hyperphagia in these obese animals. Compelling evidence shows that apo A-IV and apoE exert their potent catabolic influence to regulate energy homeostasis through integration with other central signals. A better understanding of the detailed mechanisms underlying apo A-IV and apoE's anorectic actions is needed to develop new therapeutic approaches to obesity.

REFERENCES

1. Beffert U, Stolt PC, Herz J. Functions of lipoprotein receptors in neurons. *J Lipid Res* 2004;**45**:403–9.
2. Doi T, Liu M, Seeley RJ, Woods SC, Tso P. Effect of leptin on intestinal apolipoprotein AIV in response to lipid feeding. *Am J Physiol Regul Integr Comp Physiol* 2001;**281**:R753–9.
3. Duverger N, Tremp G, Caillaud JM, Emmanuel F, Castro G, Fruchart JC, et al. Protection against atherogenesis in mice mediated by human apolipoprotein A-IV. *Science* 1996;**273**:966–8.
4. Dvorin E, Gorder NL, Benson DM, Gotto Jr AM. Apolipoprotein A-IV. A determinant for binding and uptake of high density lipoproteins by rat hepatocytes. *J Biol Chem* 1986;**261**:15,714–8.
5. Ebihara K, Ogawa Y, Katsuura G, Numata Y, Masuzaki H, Satoh N, et al. Involvement of agouti-related protein, an endogenous antagonist of hypothalamic melanocortin receptor, in leptin action. *Diabetes* 1999;**48**:2028–33.
6. Fan W, Boston BA, Kesterson RA, Hruby VJ, Cone RD. Role of melanocortinergic neurons in feeding and the agouti obesity syndrome. *Nature* 1997;**385**:165–8.
7. Fujimoto K, Cardelli JA, Tso P. Increased apolipoprotein A-IV in rat mesenteric lymph after lipid meal acts as a physiological signal for satiation. *Am J Physiol* 1992;**262**:G1002–6.
8. Fujimoto K, Fukagawa K, Sakata T, Tso P. Suppression of food intake by apolipoprotein A-IV is mediated through the central nervous system in rats. *J Clin Invest* 1993;**91**:1830–3.
9. Fukagawa K, Gou HM, Wolf R, Tso P. Circadian rhythm of serum and lymph apolipoprotein AIV in ad libitum- fed and fasted rats. *Am J Physiol* 1994;**267**:R1385–90.
10. Gotoh K, Liu M, Benoit SC, Clegg DJ, Davidson WS, D'Alessio D, et al. Apolipoprotein A-IV interacts synergistically with melanocortins to reduce food intake. *Am J Physiol Regul Integr Comp Physiol* 2006;**290**:R202–7.
11. Gutman CR, Strittmatter WJ, Weisgraber KH, Matthew WD. Apolipoprotein E binds to and potentiates the biological activity of ciliary neurotrophic factor. *J Neurosci* 1997;**17**:6114–21.
12. Hagan MM, Benoit SC, Rushing PA, Pritchard LM, Woods SC, Seeley RJ. Immediate and prolonged patterns of Agouti-related peptide-(83–132)-induced c-Fos activation in hypothalamic and extrahypothalamic sites. *Endocrinology* 2001 Mar;**142**(3):1050–6.
13. Kalogeris TJ, Painter RG. Adaptation of intestinal production of apolipoprotein A-IV during chronic feeding of lipid. *Am J Physiol Regul Integr Comp Physiol* 2001;**280**:R1155–61.
14. Lee Y, Aono M, Laskowitz D, Warner DS, Pearlstein RD. Apolipoprotein E protects against oxidative stress in mixed neuronal-glial cell cultures by reducing glutamate toxicity. *Neurochem Int* 2004;**44**:107–18.
15. Liu M, Doi T, Shen L, Woods SC, Seeley RJ, Zheng S, et al. Intestinal satiety protein apolipoprotein AIV is synthesized and regulated in rat hypothalamus. *Am J Physiol Regul Integr Comp Physiol* 2001;**280**:R1382–7.
16. Liu M, Maiorano N, Shen L, Pearson K, Tajima D, Zhang DM, et al. Expression of biologically active rat apolipoprotein AIV in *Escherichia coli*. *Physiol Behav* 2003;**78**:149–55.
17. Liu M, Shen L, Liu Y, Tajima D, Sakai R, Woods SC, et al. Diurnal rhythm of apolipoprotein a-iv in rat hypothalamus and its relation to food intake and corticosterone. *Endocrinology* 2004;**145**:3232–8.
18. Liu M, Shen L, Liu Y, Woods SC, Seeley RJ, D'Alessio D, et al. Obesity induced by a high-fat diet downregulates apolipoprotein A-IV gene expression in rat hypothalamus. *Am J Physiol Endocrinol Metab* 2004;**287**:E366–70.
19. Liu Q, Zhang J, Zerbinatti C, Zhan Y, Kolber BJ, Herz J, et al. Lipoprotein receptor LRP1 regulates leptin signaling and energy homeostasis in the adult central nervous system. *PLoS Biol* 2011;**9**:e1000575.
20. Lo CM, Zhang DM, Pearson K, Ma L, Sun W, Sakai RR, et al. Interaction of apolipoprotein AIV with cholecystokinin on the control of food intake. *Am J Physiol Regul Integr Comp Physiol* 2007;**293**:R1490–4.
21. Morton NM, Emilsson V, Liu YL, Cawthorne MA. Leptin action in intestinal cells. *J Biol Chem* 1998;**273**:26194–201.
22. Okumura T, Fukagawa K, Tso P, Taylor IL, Pappas TN. Mechanism of action of intracisternal apolipoprotein A-IV in inhibiting gastric acid secretion in rats. *Gastroenterology* 1995;**109**:1583–8.
23. Qin X, Swertfeger DK, Zheng S, Hui DY, Tso P. Apolipoprotein AIV: a potent endogenous inhibitor of lipid oxidation. *Am J Physiol* 1998;**274**:H1836–40.
24. Schreyer SA, Vick C, Lystig TC, Mystkowski P, LeBoeuf RC. LDL receptor but not apolipoprotein E deficiency increases diet-induced obesity and diabetes in mice. *Am J Physiol Endocrinol Metab* 2002;**282**:E207–14.
25. Shen L, Wang DQH, Tso P, Jandacek RJ, Woods SC, Liu M. Apolipoprotein E reduces food intake via PI3K/Akt signaling pathway in the hypothalamus. *Physiol Behav* 2011;**105**:124–8.
26. Shen L, Carey K, Wang DQ, Woods SC, Liu M. Food-entrained rhythmic expression of apolipoprotein E expression in the hypothalamus of rats. *Brain Res* 2009;**1273**:66–71.
27. Shen L, Pearson KJ, Xiong Y, Lo CM, Tso P, Woods SC, et al. Characterization of apolipoprotein A-IV in brain areas involved in energy homeostasis. *Physiol Behav* 2008;**95**:161–7.
28. Shen L, Tso P, Wang DQ, Woods SC, Davidson WS, Sakai R, et al. Up-regulation of apolipoprotein E by leptin in the hypothalamus of mice and rats. *Physiol Behav* 2009;**98**:223–8.

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29. Shen L, Tso P, Woods SC, Clegg DJ, Barber KL, Carey K, et al. Brain apolipoprotein E: an important regulator of food intake in rats. *Diabetes* 2008;**57**:2092–8.
30. Shen L, Tso P, Woods SC, Sakai RR, Davidson WS, Liu M. Hypothalamic apolipoprotein A-IV is regulated by leptin. *Endocrinology* 2007;**148**:2681–9.
31. Swaney JB, Braithwaite F, Eder HA. Characterization of the apolipoproteins of rat plasma lipoproteins. *Biochemistry* 1977;**16**:271–8.
32. Tso P, Liu M. Apolipoprotein A-IV, food intake, and obesity. *Physiol Behav* 2004;**83**:631–43.
33. Utermann G. Isolation and partial characterization of an arginine-rich apolipoprotein from human plasma very-low-density lipoproteins: apolipoprotein E. *Hoppe Seylers Z Physiol Chem* 1975;**356**:1113–21.
34. Weinberg RB, Dantzker C, Patton CS. Sensitivity of serum apolipoprotein A-IV levels to changes in dietary fat content. *Gastroenterology* 1990;**98**:17–24.
35. Woods SC. The control of food intake: behavioral versus molecular perspectives. *Cell Metab* 2009;**9**:489–98.

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Chapter 142

Bombesin

Ellen E. Ladenheim

ABSTRACT

Bombesin (BN), a tetradecapeptide originally isolated from anuran skin, belongs to a large family of structurally related amphibian and mammalian peptides. Since their discovery, BN and two mammalian BN-like peptides, gastrin-releasing peptide (GRP), and neuromedin B (NMB) have been shown to exhibit diverse behavioral and physiological actions in a number of species. Receptor subtypes for GRP and NMB and a third receptor subtype, BRS-3, have been identified. BN-like peptides have been shown to suppress food intake after peripheral or central administration and have been proposed as putative satiety signals. Specific agonists and antagonists have been instrumental in determining the individual contributions of BN receptor subtypes to food intake and energy homeostasis. Studies in knockout mice have provided an additional means to evaluate their physiological function. Recent studies have shown that BN-like peptides and receptors participate in long-term effects on food intake and body weight.

BACKGROUND

Bombesin (BN) is a tetradecapeptide isolated in 1970 by Erspamer et al. from the skin of the European frog *Bombina bombina*. It is part of a large family of amphibian and mammalian peptides that are biologically active in mammals (see Bombesin-like Peptides in Amphibian/Skin Peptides section of this book). Since their discovery, BN-like peptides have been shown to affect many gastrointestinal (GI) processes including gastrin release, gastric acid secretion, pancreatic enzyme secretion, gallbladder contraction, and intestinal motor activity. Aside from their well-known effects on gastrointestinal function, BN-like peptides also participate in a number of important neuroregulatory processes such as thermoregulation, glucose homeostasis, circadian rhythms, and food intake.[10]

Although BN itself is not present in mammals, several peptides that are structurally homologous to BN have been detected in mammalian viscera and central nervous system (CNS). These peptides include several forms of gastrin-releasing peptide ($GRP_{1–27}$, $GRP_{1–29}$, and $GRP_{18–27}$, also known as neuromedin C) and several forms of neuromedin B ($NMB_{1–32}$, $NMB_{2–32}$, and $NMB_{23–32}$). Bombesin and GRP share an identical carboxyl terminal heptapeptide sequence, the minimal fragment necessary for their biological activity. NMB contains a portion of these amino acid residues but more closely resembles the amphibian peptides, ranatensin, and litorin.[24]

The distribution of BN/GRP-like immunoreactivity in the gastrointestinal tract is mainly confined to nerve cell bodies in the myenteric ganglia and in nerve fibers of these ganglia. Similarly, NMB-like immunoreactivity is found throughout nerve fibers in the gastrointestinal tract, as well as the pancreas, spinal cord, and pituitary gland. In the CNS, immunohistochemical mapping of GRP and NMB, as well as mRNA studies, have shown distinct and discrete distribution of the two peptides in rat brain. GRP mRNA is highly expressed in the isocortex, hippocampal formation, anterior olfactory nucleus, and several hypothalamic nuclei. NMB mRNA expression is most prominent in the olfactory bulb, dentate gyrus, and dorsal root ganglion.[7]

Because of structural similarities between BN and GRP it was initially believed that GRP was the mammalian counterpart to BN and thus all BN binding sites in the periphery and brain represented GRP receptors. Early work evaluating the effect of BN-like peptides on smooth muscle contraction found that they displayed varying degrees of potency suggesting the existence of multiple receptor subtypes. Subsequent studies by von Schrenck et al.[10] identified and characterized two pharmacologically distinct BN receptor subtypes in pancreatic acinar cells and esophageal muscularis mucosa. BN binding sites in the pancreas displayed a high affinity for GRP and BN and a low affinity for NMB, whereas esophageal BN binding sites had a high affinity for NMB and BN and a low affinity for GRP. Because of these pharmacological characteristics esophageal BN receptors were termed "NMB-preferring" or BB1 and pancreatic BN receptors were termed "GRP-preferring" or BB2. BN has a high and equal affinity for NMB-R and GRP-R, and thus, its activity may be attributed to binding to either or both receptor subtypes. These receptors were later cloned, and an additional mammalian BN receptor subtype, BN receptor subtype-3 (BRS-3 or BB3) was identified through homology screening and found to

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00142-1
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share approximately 50% structural identity with GRP-R and NMB-R. Unlike GRP-R and NMB-R, BRS-3 has a low affinity for all naturally occurring BN-like peptides. To date, no endogenous ligand for BRS-3 has been identified and it is termed an "orphan" receptor.[10]

Receptors for BN-like peptides have been identified in a variety of tissues in the periphery and central nervous system. Using an approach similar to that used to characterize peripheral BN receptor subtypes, initial studies evaluated the relative ability of BN, GRP, and NMB to inhibit binding of radioactively labeled BN in rat brain. These studies revealed two distinct binding patterns consistent with those reported in peripheral tissues. Subsequent autoradiographic mapping studies determined that the predominant BN receptor subtype in the rat brain is NMB-R with the highest densities in the anterior olfactory nucleus, nucleus accumbens, and thalamus. The distribution of GRP-R is more limited with the highest densities in several hypothalamic nuclei, basal ganglia and central nucleus of the amygdala.[16] The results of the binding experiments were in general agreement with *in situ* hybridization studies examining expression patterns of GRP-R and NMB-R mRNA. Localization of BRS-3 mRNA was initially reported to be much more limited with the highest expression levels in the arcuate (Arc), dorsomedial (DMH), ventromedial (VMH), and paraventricular (PVN) nuclei of the hypothalamus. A later study using an antibody directed toward BRS-3 found a much wider distribution in the CNS with particularly strong immunoreactivity in the cerebral cortex, hippocampal formation, hypothalamus, and thalamus.[7]

In the periphery, BN binding sites in the stomach were similarly differentiated where the majority of binding was GRP-R. In gastric regions, BN binding sites were localized to the circular muscle layer with the highest density in the gastric fundus. In primates, the highest levels of NMB-R mRNA were detected in the testis and stomach, whereas in rodent peripheral tissue, significant NMB-R expression was found in esophagus, intestine, testis, and uterus.[7] Immunohistochemical evaluation of gastrointestinal BRS-3 distribution demonstrated BRS-3-like immunoreactivity at all levels of the gastrointestinal tract primarily in neurons of the myenteric and submucosal ganglia and in fibers in the longitudinal and circular muscle layers.[29]

In general, the distinct distributions of BN-like peptides and receptors support the view that they function as separate regulatory agents in the periphery and CNS.

PERIPHERAL EFFECTS OF BN-LIKE PEPTIDES ON FOOD INTAKE

A role for BN as a putative satiety signal stems from experiments showing that exogenous administration of BN dose dependently suppresses food intake in a variety of experimental paradigms, and in a number of animal species, including humans. The inhibition of food intake produced by peripherally administered BN is followed by a normal postprandial sequence of behaviors that are characteristic of meal ingestion. The reduction of food intake by BN is not accompanied by behavioral signs of toxicity, such as sickness or malaise, nor are there nonspecific effects on water intake. The decrease in meal size produced by BN is consistent with a behavioral profile that indicates an increase in post-ingestive feedback signaling. Studies in human subjects have demonstrated that intravenous administration of BN reduced intake of a test meal without producing significant side effects.[6]

Although an increase in meal-related plasma BN-like immunoreactivity has been reported, the general consensus is that BN's actions on food intake are mediated through neurocrine or paracrine mechanisms. Supporting this view are studies demonstrating an increase in BN-like immunoreactivity in the antral region of the stomach in response to meal intake, coupled with the localization of BN-like immunoreactivity in gut neurons.[23]

BN has an equal and high affinity for both GRP and NMB receptors. The discovery that BN interacted equally with GRP and NMB receptors presented the possibility that BN was mimicking the actions of GRP and NMB but that both GRP and NMB produced their effects on food intake at distinct receptor populations. A comparison of feeding suppression produced by peripheral administration of BN, $GRP_{1–27}$, $GRP_{18–27}$ and NMB in rats showed that, at intermediate doses, the rank order of potency is $BN > GRP_{1–27} = GRP_{18–27} > NMB$. However, at the lowest and highest doses, BN, $GRP_{1–27}$, and $GRP_{18–27}$ are equivalent. The biphasic nature of the dose response curve for $GRP_{1–27}$ and $GRP_{18–27}$ suggested that these peptides may be interacting with GRP-R at low doses and with both GRP-R and NMB-R at high doses. NMB is consistently less potent than BN in reducing food intake, suggesting that it is interacting only with NMB-R. A more recent study examining the satiety effects of $GRP_{1–29}$ in rats (the predominant form in this species) found equivalent potency to $GRP_{1–27}$. It is believed that the increased potency of BN to reduce food intake, compared with its mammalian counterparts, partially results from a relative resistance to enzymatic degradation. Supporting this idea are studies showing that acetylation of $GRP_{18–27}$, which improves resistance to degradation, increased its ability to reduce feeding, making it equivalent to that of BN.[21]

The notion that the effects of BN on food intake may result from the combined action at both receptor subtypes is also supported by studies showing that peripheral administration of equimolar doses of GRP and NMB reduces food intake and elicits a microstructural licking pattern equivalent to that of BN.[31] Moreover, when a dose of NMB that produces a maximal suppression of food intake is given in combination with a submaximal dose of GRP, the reduction in food intake is significantly enhanced beyond suppression

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produced by either peptide administered alone.[21] Additional studies show that a specific NMB-R receptor antagonist blocks feeding inhibition by peripherally administered NMB, but not by GRP, suggesting that NMB and GRP reduce food intake by acting at distinct receptor populations.[20] Although several studies found that peripherally administered GRP-R antagonists prevent the suppression of food intake by peripherally administered BN, attempts to increase food intake using GRP-R antagonists, or to block feeding suppression produced by intragastric loads, have been inconsistent.[4,23] The reason for this is unresolved; however, it is possible that the antagonists have limited potency or bioavailability and are unable to access sites that are activated by endogenously released BN-like peptides.

CENTRAL EFFECTS OF BN-LIKE PEPTIDES ON FOOD INTAKE

The presence of BN-like immunoreactivity and BN binding sites in brain regions implicated in controlling food intake suggested that central BN-like peptides may also play a role in satiety. Several early studies showed that BN administered into the lateral cerebral ventricles (LV) in rats produces a potent suppression of food intake.[12] However, the reduction in food intake is accompanied by a decrease in water intake and an increase in grooming and locomotion, suggesting a nonspecific effect of BN on feeding that is dissimilar to the actions of peripherally administered BN.

Subsequently, studies were undertaken to examine the specific brain sites where BN may produce its anorexic effects. Early studies using site specific injections of high doses of BN into various hypothalamic nuclei including the PVN, DMH, VMH, and lateral hypothalamus (LH) significantly reduced food intake, as did injections into extrahypothalamic areas such as the amygdala and periaqueductal gray. However, in all instances, feeding suppression was accompanied by increased grooming activity suggesting that the reduction in food intake may be secondary to competing behaviors.[13] Other experiments using smaller BN doses found that direct injections into the LH and amygdala specifically reduce food intake without producing competing behavioral effects.[6,33]

Initial studies focused primarily on forebrain mediation of BN satiety; however the presence of binding sites for BN-like peptides and BN-like immunoreactive nerve terminals and cell bodies in hindbrain regions known to participate in ingestive control (e.g., nucleus of the solitary tract (NTS)) suggest that this region may be responsive to BN's feeding effects. Several investigators found that injections of BN into the fourth cerebral ventricle (4V), in close proximity to the NTS, are highly effective in reducing food intake in rats[4] The doses required to suppress feeding after 4V administration are up to 100 times lower than those that elicit reduced food intake after LV administration. Further, this action is specific for food and does not produce a conditioned taste aversion or elicit competing behaviors such as grooming and locomotor activity that are associated with injection at other brain sites. Studies evaluating the effect of direct microinjections of BN into the NTS found this brain region to be particularly sensitive for feeding inhibition by BN.[23]

Although several studies have examined the feeding effects of centrally administered BN receptor agonists, a systematic comparison across peptides has not been conducted. It is assumed that the order of potency is BN>GRP>NMB based on studies examining each peptide individually or comparing one peptide against BN using a limited range of doses. Although the majority of BN binding sites in the vicinity of the 4V are NMB-R, studies using specific GRP-R antagonists have identified the contribution of hindbrain GRP-R to the feeding inhibitory actions of BN. Two reports showed that pretreatment with selective GRP-R antagonists facilitates feeding in sated rats, supporting a role for endogenously released GRP in reducing food intake.[5,23] Injections of $GRP_{1–27}$ and $GRP_{18–27}$ into the central amygdala have also been reported to transiently decrease food intake without producing changes in body temperature or stereotypic behaviors.[3]

Relatively little attention has been given to the role of central NMB-R in food intake. Agonist studies have not been particularly informative because central NMB exhibits very poor potency to inhibit feeding. However a role for hindbrain NMB-R in ingestive control was determined in studies using a specific NMB-R antagonist.[19] As shown with GRP-R antagonists, 4V administration of an NMB-R antagonist alone also increases food intake and blocks the effects of centrally administered BN. In addition, 4V administration of GRP-R and NMB-R antisense oligonucleotides prevents the suppression of food intake by centrally administered BN, supporting a role for both receptor subtypes in mediating BN's central feeding effects.[23]

NEURAL PATHWAYS MEDIATING THE EFFECTS OF BN-LIKE PEPTIDES

Several studies have been conducted to determine the site of action and neural substrates underlying the satiety actions of peripheral and central BN-like peptides. Early work evaluating the effect of adrenalectomy, hypophysectomy, vagotomy, and lesions of the ventromedial and dorsomedial hypothalamus reported that none of these surgical manipulations were effective in preventing BN's ability to suppress food intake.[6] By contrast, Stuckey et al. examined the effect of individual or combined lesions of the afferent innervation of the GI tract and found that, while vagotomy alone fails to reduce BN satiety, combined bilateral subdiaphragmatic vagotomy, spinal cord transaction (T6), and dorsal rhizotomy (T1–T6) significantly attenuate the ability of peripheral

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BN to reduce food intake.[6] A subsequent study found that rhizotomy and spinal cord section, in the absence of vagotomy, also attenuate BN's feeding effects. More recently, it was shown that vagotomy combined with splanchnic afferent and efferent denervation of the gut lessen the effects of GRP1–29 on meal size in rats.[35]

To more clearly define the pathways through which BN-like peptides exert their satiety actions, studies were undertaken to evaluate the effects of capsaicin on the suppression of food intake produced by BN. Capsaicin, a derivative of red pepper, is a neurotoxin that selectively damages a subset of primary sensory fibers that include both vagal and spinal neurons. Adult rats treated systemically with capsaicin exhibit a significant attenuation in the feeding inhibitory response to peripheral BN administration.[18] A subsequent report showed that neonatal capsaicin treatment, which produces a more complete deafferentation, completely abolishes peripheral BN's feeding effects in adult rats without affecting the inhibition of food intake produced by third ventricular (3V) administration.[23] Because of the distinct roles of BN-like peptides to reduce food intake, additional studies were conducted in adult rats treated neonatally with capsaicin to examine whether different neural substrates underlie the feeding suppression by BN, GRP, and NMB.[17] The suppression of food intake by peripheral administration of BN and NMB is abolished or attenuated by capsaicin treatment, whereas the effects of GRP remained intact except at the highest dose. These results suggest that BN-like peptides use distinct neural pathways to reduce feeding.

The presence of gastric BN binding sites and changes in gastrointestinal BN-like immunoreactivity in response to meal intake led to the proposal that BN may produce its effects on food intake through a gastric mechanism, although evidence to support this has been equivocal. In support of a gastric site of action for BN, it was shown that local celiac artery infusions of BN are much more effective in suppressing food intake than either intraperitoneal or superior mesenteric artery infusions.[11] However, another experiment that compared the feeding effects of various doses of BN with the gastric inhibitory effects of BN, concluded that BN's satiety actions are dissociated from its effects on gastric emptying, as is the potency of BN to reduce food intake compared with its relative affinity for gastric receptors.[9] These results suggest that BN's effects on food intake are not mediated by a gastric site or via a gastric inhibitory mechanism. Although BN-like peptides have been shown to excite mechanosensitive gastric branch vagal afferents, electrophysiological studies have determined that this is an indirect action secondary to its stimulation of gastric motor effects, consistent with the lack of BN binding sites in the vagus nerve.

The neuronal pathways through which peripheral and central BN-like peptides exert their effects on food intake have been also been examined by evaluating patterns of c-fos immunoreactivity, a marker of neuronal activation. Peripheral injection of BN induces c-fos in the area postrema (AP), medial NTS, parvocellular PVN, and to a lesser extent in central nucleus of amygdala, parabrachial nucleus, supraoptic, and magnocellular PVN. A comparison between peripheral and central (4V) BN administration found that both routes of administration show strong fos-LI in the PVN. Fos-like immunoreactivity was also detected in the medial NTS, central nucleus of amygdala, and bed nucleus of the stria terminalis, though the regional distribution differs depending on the route of administration. Differences were also found in the AP where peripheral, but not central, administration induced c-fos activation.[1,30]

A more recent study compared the effects systemic administration of different forms of GRP on c-fos activation in the gastrointestinal tract and hindbrain. Myenteric neurons in the stomach and neurons in the area postrema are activated by $GRP_{18–27}$, $GRP_{1–27}$, and $GRP_{1–29}$. In the duodenum, both $GRP_{1–27}$ and $GRP_{1–29}$ increase c-fos activation in the myenteric plexus, whereas only $GRP_{1–29}$ increases fos-LI in the submucosal plexus.[34]

The necessity of central BN-like peptide receptors to the peripheral effects of BN-like peptides has also been examined. Merali et al. (1988) first reported that 3V infusion of BN antisera, or a GRP-R antagonist, blocks the suppression of food intake by peripheral BN administration. Other studies have shown that ablation of the AP/NTS complex or blockade of hindbrain GRP receptors with 4V GRP-R antagonists attenuate the ability of peripherally administered BN or GRP to suppress feeding.[23]

Converging behavioral and anatomical evidence indicates that the NTS is a critical region for the feeding effects of BN. This region receives sensory input from the gastrointestinal tract via vagal and spinal neurons. Within the NTS, BN-ir is located in the medial subnucleus, an area that is associated with nerve terminals that synapse on cell bodies or dendrites of vagal motoneurons. Compelling evidence for the importance of the caudal hindbrain to BN's feeding effects is provided in experiments showing that either peripheral or 4V injections of BN reliably suppress sucrose intake in chronic decerebrate rats, a surgical preparation that severs communication between the hindbrain and forebrain. These results support the idea that local neural circuitry residing in the caudal hindbrain, independent of forebrain systems, is sufficient to mediate the effects of BN-like peptides on ingestive behavior.[4]

Although the necessity of central neural substrates, particularly in the NTS, play a critical role in BN's effects on food intake there are still questions remaining concerning how the signal is transmitted. Whether peripheral administration of BN-like peptides causes a central release of GRP (or NMB) or whether they gain direct access to critical hindbrain receptors has not been fully determined.

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CONTRIBUTION OF BN RECEPTOR SUBTYPES TO FOOD INTAKE USING GENETICALLY MODIFIED MICE

The development of mice with targeted deletions for each BN receptor subtype has provided a complementary means to evaluate the individual roles of BN receptor subtypes to ingestive control and other physiological effects of BN-like peptides.[26] The first of these to be developed are mice lacking the GRP-R. In studies to evaluate the overall contribution of GRP-R to food intake, GRP-R KO mice fail to suppress intake after exogenously administered of either BN or GRP, suggesting that GRP-R alone mediates the satiety effect of BN-like peptides in mice. An analysis of 24-h meal patterns reveals that GRP-R KO mice exhibit an increase in meal size consistent with a deficit in satiety signaling, but 24-h food consumption is not different from control mice because of a reduction in the total number of meals consumed. A long-term analysis of body weight found that body weights between GRP-R KO mice and WT controls are comparable in early life although a small but significant elevation in body weight occurs in aged GRP-R KO mice. Additional studies show GRP-R deficient mice display increased night-time locomotor activity, loss of GRP-induced hypothermia, and increased social investigatory behaviors. Metabolic disturbances, including decreased secretion of glucagon-like peptide 1 and abnormalities in insulin release and glucose tolerance, are also present in GRP-R KO mice.

Unlike GRP-R KO mice, previous studies in mice lacking NMB-R (NMB-R KO) found no differences in food intake and body weight between NMB-R KO and WT control mice. However, a subsequent study showed that although female NMB-R KO mice fed a chow diet show no differences in body weight, when presented with a high fat diet they are partially protected against body weight gain and adipose accumulation and exhibit normal glucose tolerance compared with that of WT littermates.[28]

Recent attention has been directed toward the role of BRS-3 in food intake and energy homeostasis. Progress in determining the physiological function of BRS-3 was hindered by the lack of an identifiable natural ligand or a suitable synthetic ligand. To assess the function of BRS-3, Ohki-Hamazaki and colleagues generated mice with a targeted deletion of BRS-3 (BRS-3 KO) and found that they develop obesity accompanied by hypertension, impairment of glucose tolerance, and insulin resistance. In addition, BRS-3 KO mice exhibit reduced metabolic rate, increased feeding efficiency and hyperphagia, and showed alterations in glucose transport.[26]

Additional behavioral and physiological studies were conducted to identify factors contributing to increased food consumption and body weight in BRS-3 KO mice.[15] A primary deficit leading to the obese phenotype of BRS-3 deficient mice is an uncompensated increase in meal size resulting in an overall increase in total food intake. Although the increase in body weight could be overcome by pair-feeding to the caloric intake of WT controls, they still exhibit significantly elevated leptin concentrations and body fat distribution, confirming a metabolic dysregulation in BRS-3 KO mice. Despite the finding that BRS-3 KO mice increase their meal size, they exhibit a normal response to peripherally administered peptides (e.g., cholecystokinin and BN) that participate in meal-related controls of food intake. A study evaluating the effects of centrally administered peptides reported that mice lacking BRS-3 are leptin resistant, hyperresponsive to the orexigenic effects of exogenously administered melanin concentrating hormone (MCH), and have elevated levels of MCH mRNA in the lateral hypothalamus.

Studies have also suggested that hyper-responsiveness to taste stimuli may contribute to overeating in BRS-3 deficient mice particularly when exposed to highly palatable foods.[26] BRS-3 KO mice exhibit a stronger taste preference for saccharin solution and a stronger aversion to quinine solution compared with that of WT littermates. Using a conditioned taste aversion paradigm, BRS-3 KO mice display a stronger aversion to both saccharin and sodium chloride solutions after intraperitoneal lithium chloride administration. A preliminary study reported exaggerated hyperphagia and weight gain in BRS-3 KO mice maintained on a high fat diet, a characteristic that is consistent with other mouse models of obesity.

BN-LIKE PEPTIDES/RECEPTORS AND ENERGY HOMEOSTASIS

Although a major focus for determining a role for GRP and NMB in ingestive control has been to evaluate their function as short-term or meal-related satiety signals there is also evidence to suggest that they interact with energy regulatory pathways and may contribute to long-term energy balance.

Previous studies show that peptides that play a critical role in energy homeostasis work in concert with gastrointestinal signaling peptides to reduce food intake. Leptin, the protein product of the ob gene, is synthesized and released by adipocytes and circulates at levels proportional to body fat. Although many of the hypothalamic signaling pathways involved in leptin's actions have been identified, the specific mechanisms mediating their effects on food intake are not clear. Several studies show that the effect of leptin on food intake is characterized by a reduction in meal size without affecting meal frequency, a behavioral profile shared by satiety peptides. A study evaluating the interaction of leptin with BN found that when a subthreshold dose of leptin administered into the 3V is combined with a peripheral injection of BN, there is a significant potentiation in the reduction of food intake. Consistent with the behavioral results, c-fos activation in the NTS is increased

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by combined dosages of leptin and BN beyond the individual response to either peptide. These results support the idea that the effects of leptin on food intake may be mediated, in part, by modulating meal-related satiety signals such as BN.[25]

In the brain, the preponderance of GRP and GRP receptors are found in several hypothalamic nuclei that play important roles in energy homeostasis. GRP mRNA is localized in the hypothalamic PVN in a region that receives input from neurons in the Arc. Previous studies have shown that direct bilateral PVN infusion of BN (presumably acting on GRP-R) results in a decrease in food intake and a robust increase in blood glucose, free fatty acids, and corticosterone,[8] suggesting that BN binding sites in the PVN may participate in regulating circulating metabolic fuels and the hypothalamic–pituitary–adrenal (HPA) axis. Other studies show that food ingestion evokes a rapid elevation of GRP levels in various hypothalamic sites, including the PVN and Arc.[23] A follow-up study determined how these changes correlate with release during different phases of ingestion and found that during food consumption there is a substantial decrease in the release of BN-like peptides in the PVN which is then followed by an increase in release during the postprandial phase. These results suggest that the decline in release during the prandial phase is conducive to meal initiation, whereas the elevation in release provides a signal for meal termination.

A more recent study found that challenges that represent different nutritional states alter expression of GRP mRNA expression in the PVN.[14] Food deprivation significantly decreases GRP mRNA expression in the PVN, whereas activation of the melanocortin system with the melanocortin 4 receptor (MC4-R) agonist MTII increases levels of PVN GRP mRNA and prevents the reduction in GRP mRNA induced by fasting. Further, this population of neurons respond to MTII injection, as seen by c-fos activation, and co-expresses MC4-R. These results show that food deprivation and stimulation of the melanocortin system produce opposing changes in GRP gene expression in the PVN, suggesting that GRP-containing neurons in the PVN may be part of the hypothalamic signaling pathway controlling food intake and energy balance.

Electrophysiological studies have evaluated the effects of GRP and NPY on identified Arc NPY cells.[32] The results indicate that nanomolar amounts of both the peptides produce strong direct excitatory effects on NPY neurons, surpassing the actions of other energy regulatory peptides such as ghrelin and orexin. Similar excitation is also seen in Arc POMC neurons. Because these two sets of neuronal populations are generally activated in opposition to one another it is surmised that BN-like peptides may function to initiate broad activation, rather than excitation or inhibition of specific cellular populations in the Arc.

Several studies have also identified NMB as an important contributor to energy balance regulation. NMB and NMB-R are significant regulators of the HPA axis through modulation of thyrotropin secretion.[27] Hoggard et al. have reported that NMB mRNA is present in human and rodent adipose tissue and that the expression levels can be changed through alterations in energy balance and leptin signaling.

Neuromedin B has also been proposed as a strong candidate for gene linking eating behaviors to the susceptibility to obesity in humans.[2] The Quebec Family Study, a large prospective study designed to identify the genetics of obesity and related diseases, found that a missense mutation within exon 2 of the NMB gene was significantly associated with eating behaviors and obesity, resulting in twice as much body fat gain over a six-year period compared with those without the mutation. Subjects that are homozygous for the mutation are twice as likely to exhibit high levels of disinhibition and susceptibility to hunger and to exhibit positive associations for sweet craving.

As mentioned above, the physiological role for BRS-3 has been examined primarily by evaluating the consequences of BRS-3 deletion in genetically engineered mice. A recent advance in studying the role of BRS-3 in energy homeostasis has been the development of specific agonist and antagonist ligands for BRS-3.[22] Experiments using a specific BRS-3 antagonist, show that chronic LV infusion results in increased food intake, body weight, and adiposity. Conversely, acute BRS-3 agonist administration reduces food intake and increases fasting metabolic rate. Chronic delivery is effective in lowering food intake and fat mass in diet-induced obese mice.

Additional studies examined the interaction of BRS-3 activation with other central neural pathways that are important in energy homeostasis.[22] Results obtained in a variety of KO mice (i.e., Npy(−/−), Mc4r(−/−), Cnr1(−/−) and Lepr(db/db)) indicate that BRS-3 agonists are still capable of reducing food intake in these mice and thus activation of BRS-3 seems to be independent of these pathways. BRS-3 also plays a role in body temperature regulation, but this seems to be secondary to its effects on energy metabolism. In general, results from experiments in BRS-3 KO mice and with BRS-3 agonist and antagonist compounds have led to the conclusion that BRS-3 plays a more significant role in long-term or metabolic controls of energy balance than either GRP-R or NMB-R.

ACKNOWLEDGMENT

The author was supported by Grant DK-019302 from the National Institute of Diabetes, Digestive and Kidney Diseases.

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REFERENCES

1. Bonaz B, De Giorgio R, Tache Y. Peripheral bombesin induces c-fos protein in the rat brain. *Brain Res* 1993;**600**:353–7.
2. Bouchard L, Drapeau V, Provencher V, Lemieux S, Chagnon Y, Rice T, et al. Neuromedin beta: a strong candidate gene linking eating behaviors and susceptibility to obesity. *Am J Clin Nutr* 2004;**80**:1478–86.
3. Fekete EM, Bagi EE, Toth K, Lenard L. Neuromedin C microinjected into the amygdala inhibits feeding. *Brain Res Bull* 2007;**71**:386–92.
4. Flynn FW. Bombesin-like peptides in the regulation of ingestive behavior. *Ann N Y Acad Sci* 1994;**739**:120–34.
5. Flynn FW. Fourth ventricular injection of selective bombesin receptor antagonists facilitates feeding in rats. *Am J Physiol* 1993;**264**:R218–21.
6. Gibbs J, Smith GP. The actions of bombesin-like peptides on food intake. *Ann N Y Acad Sci* 1988;**547**:210–6.
7. Gonzalez N, Moody TW, Igarashi H, Ito T, Jensen RT. Bombesin-related peptides and their receptors: recent advances in their role in physiology and disease states. *Curr Opin Endocrinol Diabetes Obes* 2008;**15**:58–64.
8. Gunion MW, Tache Y. Bombesin microinfusion into the paraventricular nucleus suppresses gastric acid secretion in the rat. *Brain Res* 1987;**422**:118–28.
9. Hostetler AM, McHugh PR, Moran TH. Bombesin affects feeding independent of a gastric mechanism or site of action. *Am J Physiol* 1989;**257**:R1219–24.
10. Jensen RT, Battey JF, Spindel ER, Benya RV. International Union of Pharmacology. LXVIII. Mammalian bombesin receptors: nomenclature, distribution, pharmacology, signaling, and functions in normal and disease states. *Pharmacol Rev* 2008;**60**:1–42.
11. Kirkham TC, Gibbs J, Smith GP. Satiating effect of bombesin is mediated by receptors perfused by celiac artery. *Am J Physiol* 1991;**261**:R614–8.
12. Kulkosky PJ, Gibbs J, Smith GP. Feeding suppression and grooming repeatedly elicited by intraventricular bombesin. *Brain Res* 1982;**242**:194–6.
13. Kyrkouli SE, Stanley BG, Leibowitz SF. Bombesin-induced anorexia: sites of action in the rat brain. *Peptides* 1987;**8**:237–41.
14. Ladenheim EE, Behles RR, Bi S, Moran TH. Gastrin-releasing peptide messenger ribonucleic acid expression in the hypothalamic paraventricular nucleus is altered by melanocortin receptor stimulation and food deprivation. *Endocrinology* 2009;**150**:672–8.
15. Ladenheim EE, Hamilton NL, Behles RR, Bi S, Hampton LL, Battey JF, et al. Factors contributing to obesity in bombesin receptor subtype-3-deficient mice. *Endocrinology* 2008;**149**:971–8.
16. Ladenheim EE, Jensen RT, Mantey SA, Moran TH. Distinct distributions of two bombesin receptor subtypes in the rat central nervous system. *Brain Res* 1992;**593**:168–78.
17. Ladenheim EE, Knipp S. Capsaicin treatment differentially affects feeding suppression by bombesin-like peptides. *Physiol Behav* 2007;**91**:36–41.
18. Ladenheim EE, Ritter RC. Capsaicin attenuates bombesin-induced suppression of food intake. *Am J Physiol* 1991;**260**:R263–6.
19. Ladenheim EE, Taylor JE, Coy DH, Carrigan TS, Wohn A, Moran TH. Caudal hindbrain neuromedin B-preferring receptors participate in the control of food intake. *Am J Physiol* 1997;**272**:R433–7.
20. Ladenheim EE, Taylor JE, Coy DH, Moran TH. Blockade of feeding inhibition by neuromedin B using a selective receptor antagonist. *Eur J Pharmacol* 1994;**271**:R7–9.
21. Ladenheim EE, Wirth KE, Moran TH. Receptor subtype mediation of feeding suppression by bombesin-like peptides. *Pharmacol Biochem Behav* 1996;**54**:705–11.
22. Majumdar ID, Weber HC. Biology and pharmacology of bombesin receptor subtype-3. *Curr Opin Endocrinol Diabetes Obes* 2012;**19**:3–7.
23. Merali Z, McIntosh J, Anisman H. Role of bombesin-related peptides in the control of food intake. *Neuropeptides* 1999;**33**:376–86.
24. Minamino N, Kangawa K, Matsuo H. Neuromedin B and neuromedin Ca. Two mammalian bombesin-like peptides identified in porcine spinal cord and brain. *Ann N Y Acad Sci* 1988;**547**:373–90.
25. Moran TH, Aja S, Ladenheim EE. Leptin modulation of peripheral controls of meal size. *Physiol Behav* 2006;**89**:511–6.
26. Ohki-Hamazaki H, Iwabuchi M, Maekawa F. Development and function of bombesin-like peptides and their receptors. *Int J Dev Biol* 2005;**49**:293–300.
27. Oliveira KJ, Cabanelas A, Veiga MA, Paula GS, Ortiga-Carvalho TM, Wada E, et al. Impaired serum thyrotropin response to hypothyroidism in mice with disruption of neuromedin B receptor. *Regul Pept* 2008;**146**:213–7.
28. Paula GS, Souza LL, Cabanelas A, Bloise FF, Mello-Coelho V, Wada E, et al. Female mice target deleted for the neuromedin B receptor have partial resistance to diet-induced obesity. *J Physiol* 2010;**588**:1635–45.
29. Porcher C, Juhem A, Peinnequin A, Bonaz B. Bombesin receptor subtype-3 is expressed by the enteric nervous system and by interstitial cells of Cajal in the rat gastrointestinal tract. *Cell Tissue Res* 2005;**320**:21–31.
30. Rowland NE, Crews EC, Gentry RM. Comparison of Fos induced in rat brain by GLP-1 and amylin. *Regul Pept* 1997;**71**:171–4.
31. Stratford TR, Gibbs J, Smith GP. Simultaneous administration of neuromedin B-10 and gastrin-releasing peptide(1–27) reproduces the satiating and microstructural effects of bombesin. *Peptides* 1996;**17**:107–10.
32. van den Pol AN, Yao Y, Fu LY, Foo K, Huang H, Coppari R, et al. Neuromedin B and gastrin-releasing peptide excite arcuate nucleus neuropeptide Y neurons in a novel transgenic mouse expressing strong Renilla green fluorescent protein in NPY neurons. *J Neurosci* 2009;**29**:4622–39.
33. Vigh J, Lenard L, Fekete E, Hernadi I. Bombesin injection into the central amygdala influences feeding behavior in the rat. *Peptides* 1999;**20**:437–44.
34. Washington MC, Sayegh AI. Gastrin releasing peptides increase Fos-like immunoreactivity in the enteric nervous system and the dorsal vagal complex. *Peptides* 2011;**32**:1600–5.
35. Wright SA, Washington MC, Garcia C, Sayegh AI. Gastrin releasing peptide-29 requires vagal and splanchnic neurons to evoke satiation and satiety. *Peptides* 2012;**33**:125–31.

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Chapter 143

CART

Graham J. Dockray and Guillaume de Lartigue

ABSTRACT

Peptides derived from cocaine and amphetamine-regulated transcript (CARTp) are expressed in central and peripheral neurons associated with ingestive behavior. CART is coexpressed with proopiomelanocortin (POMC) in arcuate nucleus (ARC) neurons that are linked to satiety. Expression is decreased by food withdrawal and stimulated by leptin; intracerebroventricular (icv) administration of CARTp inhibits food intake. Mice null for CART have a tendency toward mild obesity and are prone to diet-induced obesity. However, microinjection of CARTp into some hypothalamic nuclei stimulates food intake and the functional significance of this observation remains unknown. In vagal afferent neurons, CARTp is expressed in a cholecystokinin (CCK)- and leptin-dependent manner and seems to act as an autocrine enhancer of the satiety effect of CCK. Mutations of the *CART* gene are linked to human obesity syndromes. Collectively, the data indicate roles for CARTp at multiple levels in controlling ingestion through mechanisms that are still poorly understood.

INTRODUCTION

Cocaine and amphetamine-regulated transcript (CART) was discovered by differential mRNA display of the striatum from rats treated with cocaine or amphetamine.[12] Soon after, peptide sequences predicted to be derived from CART were identified, CART expression was shown to vary in the arcuate nucleus (ARC) with food deprivation, to be regulated by leptin, and a predicted peptide (CARTp) was shown to inhibit food intake after intracerebroventricular (icv) injection.[20] The main active forms of CARTp correspond to the 55–102 and 62–102 sequences of one of the splice variants first characterized in the rat (the numbering differs for peptides derived from an alternative rat transcript, and from the human transcript). In addition to roles in the control of food intake, there is also growing evidence for a role of CARTp in reward mechanisms and in endocrine functions.[25] We focus here on the evidence for the role of CARTp in controlling ingestive behavior.

EFFECTS ON FEEDING BEHAVIOR

A variety of experimental approaches indicate that exogenous CARTp inhibits food intake. Many different laboratories have reported that exogenous CARTp inhibits food intake when administered by icv injection; set against this, however, a few studies using microinjection of hypothalamic nuclei have reported increased food intake. Intracerebroventricular injection of CARTp 55–102 was first shown to reduce food intake in 24 h fasted rats.[20] Similarly, icv injection of the shorter CARTp fragment 62–102, but not of 55–59, decreases food intake.[21] Inhibition of food intake with icv administration is dose (~0.1–1 nmol) and time (20 min–8 h) dependent. Chronic icv infusion of CARTp 55–102 also inhibits feeding and results in 15% body weight loss in lean rats after 3–5 days.[22] Sustained expression of CART after icv administration of an adeno-associated virus (AAV) CART construct is also associated with reduced food intake and decreased body weight gain in rats on a high-fat diet.[24] Importantly, there is direct evidence supporting a role for CARTp as an endogenous satiety factor from the results of experiments with icv injection of CARTp antibodies, which show increased food intake in rats.[20,21] Moreover, microinjections of CARTp 55–102 into the nucleus accumbens (NAcc) shell decrease food intake,[32] whereas knocking down CART expression by microinjection of short interfering (si)RNAs into the NAcc increases food intake in mice.[15]

In contrast to the evidence for satiety effects of CARTp, direct injection of CARTp into several hypothalamic nuclei has been reported to stimulate food intake in fasted rats. Thus, administration into the ARC, ventromedial nucleus (VMN), paraventricular nucleus (PVN), lateral hypothalamic area (LH), anterior hypothalamic area (AHA), dorsomedial nucleus (DMH), and supraoptic nucleus (SON) all increase food intake.[1] There are differences between nuclei in the time course of the response and sensitivity to CARTp; for example, there is an orexigenic response to 0.04 nmol CARTp 55–102 in the VMN and ARC but only at 0.2 nmol after 4 h in the PVN, LHA, AHA, DMH, and SON.[1] The orexigenic response has a delayed onset (1–2 h for VMN, ARC, and DMN; 2–4 h for the other regions) compared with the relatively rapid onset of the anorexic response to icv injection. Orexigenic responses have also been reported in studies overexpressing CART through delivery of an AAV construct; there is increased food intake and increased weight gain, which are accentuated on a high-fat diet after

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00143-3
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injection of the construct into the rat PVN.[27] Moreover, overexpression of CARTp in the ARC results in increased food consumption and body weight gain in rats[17]. The reasons for the different responses to icv compared with that of local injection remain unclear and further work is needed to determine the precise sites and mechanisms of action of CARTp in different experimental conditions.

Locomotor changes with CARTp administration have been reported in several studies. For example, icv administration is associated with movement related tremor, body flattening, and a change in licking microstructure.[3,20] It seems possible, therefore, that at least some of the effects of CARTp on ingestive behavior, particularly after icv administration, are secondary to behavioral changes influencing locomotion. In addition, it should be noted that icv administration of CARTp 55–102 (1 μg, 0.19 nmol) has been associated with conditioned taste aversion.[2] For these reasons, it is appropriate to be cautious in interpreting the physiological relevance of responses to icv administration.

STUDIES FROM GENETICALLY MODIFIED ORGANISMS

Mice null for CART exhibit a relatively mild phenotype, which is nevertheless consistent with a role in inhibiting food intake. Asnicar et al. reported that CART$^{-/-}$ mice on a C57Bl/6 background exhibit modest increases in food intake and increased adiposity on a high-fat diet, on a normal diet; however, body weight in these mice is similar to wildtype up to 17 weeks.[4] Using a different strain of CART$^{-/-}$ mice, Wierup et al. found increased body weight at 40 weeks on a normal diet, but no difference in food intake, indicating a modest phenotype with late onset.[31]

FUNCTIONAL RESPONSES IN DIFFERENT METABOLIC STATES

Reports of the effects of CARTp on food intake come from studies in both freely feeding animals and previously fasted animals. In specific circumstances, the effects of CARTp have been shown to be dependent on nutritional status. For example, microinjections of CARTp 55–102 into the NAcc shell had a greater inhibitory effect on food intake in freely fed compared with that of 48-h fasted rats.[32] Direct comparison has been made of the effects of CARTp administration icv for 6 days in control rats on normal chow compared with that of obese rats fed a high-fat diet, and the results suggest similar inhibition of food intake.[26]

There is good evidence that the expression of CART in the hypothalamus is strongly influenced by nutritional status. Thus, Kristensen et al. first reported decreased CART mRNA in the ARC of 24- and 48-h fasted rats,[20] and there is also decreased CARTp measured by radioimmunoassay in the hypothalamus of 24-h fasted rats.[29] The abundance of CART mRNA is strongly dependent on leptin stimulation. In Zucker *fa/fa* rats (in which there is mutation of the leptin receptor), there is a profound decrease in CART mRNA in the ARC, and a lesser decrease in the DMH; similarly, in *ob/ob* mice (in which the *leptin* gene is mutated), there are also profound decreases in CART mRNA in the ARC, and modest decreases in the DMH.[20] Because neurons expressing CART also express the leptin receptor (ObR) and because leptin stimulates these neurons (see below), the results suggest that CART abundance in this system is strongly dependent on circulating leptin status.

The expression of CART in peripheral neurons is also dependent on nutrient status. In vagal afferent neurons where CART is coexpressed with the cholecystokinin (CCK)-1 receptor,[6] there is a sharply decreased expression in fasted animals, which is promptly reversed by the administration of exogenous CCK8s, or by refeeding via a mechanism dependent on endogenous CCK.[8] In addition, fasting decreases CARTp concentrations in blood and abolishes its diurnal rhythm.[28] Multiple organs probably contribute to circulating CARTp including the adrenal gland, and a role for glucocorticoids in regulating circulating CART concentrations has been suggested. In general, peripheral administration of CARTp (intraperitoneal, ip) alone has a weak effect on feeding behavior. The response is, however, dependent on previous nutrient availability. Thus, after fasting rats for 15 h, ip CARTp has a small effect in increasing food intake, whereas after 2.5 h, fasting there is a small decrease in food intake. Importantly, however, ip CARTp prolongs and potentiates the satiating effects of ip CCK8s after the short, but not the long, duration of fasting.[9]

NEURAL NETWORKS INVOLVED

The notion that CARTp in hypothalamic neurons is implicated in control of food intake has been grounded in the early observations that (a) CART is expressed in neurons that are strongly associated with control of ingestion, (b) these neurons are stimulated by leptin, (c) CART expression in them changes in response to leptin and food deprivation, (d) there are changes in food intake on administration of exogenous peptide, and (e) there are changes in food intake on inhibiting endogenous CARTp by either siRNA knockdown or antibody administration. However, CARTp in other systems may also play a role in regulating ingestive behavior, including, for example, NAcc, hind brain sites and vagal afferent neurons. Because fourth ventricular injection of CARTp 55–102 reduces the short-term intake of a liquid meal,[2,34] and because plugging the cerebral aqueduct between the third and fourth ventricles blocks the anorectic effect of third ventricular injection of CARTp,[3] it has been proposed that icv CARTp reduces food intake from

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hind brain sites albeit with questions about the physiological relevance of responses by this route of administration.[2] Even so, the role of CART acting within the brain stem is interesting in the context of observations of the expression and actions CARTp in vagal afferent neurons that terminate the nucleus tractus solitarius (NTS, see below).

Hypothalamus

In the ARC, CART is expressed in neurons that also express proopiomelanocortin (POMC).[13,14] There is a well-defined role for the POMC-derived peptide, α-melanocyte stimulating hormone (αMSH), in inhibiting food intake via actions on melanocortin-(MC)3 and MC4 receptors expressed by target neurons. The POMC/CART-expressing neurons are distinct from those expressing neuropeptide Y (NPY) and agouti-related peptide (AgRP) in the ARC, both of which are associated with stimulation of food intake. There is, however, a dense network of NPY-immunoreactive terminals surrounding CARTp-positive nerve cell bodies.[5] The CART-expressing neurons of the ARC also express ObR, and leptin stimulates expression of c-fos and suppressor of cytokine signaling-3 (SOCS-3) in these neurons, as well as stimulating CART expression, supporting the idea that these cells are a target for direct actions of leptin in the hypothalamus.[13,14]

In the parvicellular PVN, there are strong in situ hybridization signals for CART,[12] and a high density of cells by immunohistochemistry.[19] These neurons seem to coexpress CARTp and thyrotrophin releasing hormone[5,14] and intravenous leptin administration induces c-fos expression in them.[13] Varicosities containing NPY-immunoreactivity form dense pericellular baskets around the CARTp-immunoreactive PVN cell bodies suggesting a functional link between NPY and CARTp in this system.[21] Interestingly, Cota et al. have reported that CARTp in PVN neurons is colocalized with cannabinoid, CB1, receptors, which are linked to orexigenic responses.[7] The results suggest, therefore, that both anorexigenic factors, such as leptin, and orexigenic stimuli, such as NPY and endocannabinoids, may interact to control CART expression, or release, in the PVN.

In both the LHA and DMH, CARTp colocalizes extensively with the orexigenic peptide melanin-concentrating hormone (MCH).[5,14] Intravenous leptin activates many of these neurons, as determined by the expression of c-fos and SOCS-3, and as they also express ObR, it has been suggested that there is direct leptin stimulation of them.[13,14] The CARTp-expressing neurons of the DMH are surrounded by a population of NPY-immunoreactive terminals that may originate from the ARC and consistent with the suggestion that CARTp is downstream of NPY.[5] The CART-expressing neurons of the LHA and DMH neurons also express CB1 receptors, and in $CB1^{-/-}$ mice, there was decreased CART mRNA abundance,[7] which may be relevant in the context of orexigenic responses to CART on local injection into these nuclei and the colocalization of CART with MCH.

Limbic System

In both the shell and the core of the rat NAcc, there is a high density of CARTp-immunoreactivity fibers.[18] Retrograde tracing suggests that many of the CARTp-immunoreactive fibers reflect projections from the ARC as well as ventral striatum and perifornical region.[32] There are, however, some CARTp-immunoreactive cell bodies in the NAcc, and CART mRNA in the NAcc shell was shown to be suppressed after fasting, suggesting that CARTp may be involved in feeding-induced reward and reinforcement pathways.[32] As noted above, microinjection of CARTp into the NAcc decreases food intake in both fed rats and fasted mice,[15,32] while knocking down CART by the use of siRNAs increases food intake in fed mice.[15] Both 5-HT_4 receptors and CB1 receptors regulate CART expression in the NAcc. Stimulation of 5-HT_4 receptors in the NAcc increases CART abundance and decreases food intake. Conversely, reducing CARTp in food-deprived mice by administration of siRNA abolishes the anorectic effects of a 5-HT_4 receptor agonist,[15] suggesting that CARTp is a mediator of 5-HT_4 receptor-induced inhibition of food intake.

Hind Brain and Vagal Afferent Neurons

There is a high density of CARTp-expressing nerve fibers in the commissural and medial parts of the NTS and also the lateral part of the area postrema.[18,35] There are some CARTp-immunoreactive perikarya in the area postrema, but importantly, there are abundant CART-expressing neurons in the nodose ganglia, and the central projections of these neurons contribute to the CARTp-immunoreactive fibers in the NTS.[6,18,35] Although there are projections to the dorsal vagal complex from hypothalamic nuclei including PVN, LHA, and ARC, retrograde tracing experiments suggest the CARTp-expressing neurons in these regions do not make a major contribution to the CARTp-positive fibers in the dorsal vagal complex.[35]

Administration of CARTp, into the fourth ventricle in doses that inhibit food intake (80 pmol) stimulates c-fos expression in NTS neurons;[35] moreover, it is well established that there is an induction of c-fos in NTS neurons after vagal afferent stimulation by CCK or food intake. Interestingly, however, it has been reported that microinjection of CARTp into the NTS has little or no effect on food intake.[35]

Approximately 50% of vagal afferent cell bodies in the nodose ganglia express CART[6] and CARTp,[8,9] and the

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same neurons also express CCK1 receptors.[6] In retrograde tracing studies, CARTp is present in a higher proportion of nodose neurons projecting to the duodenum compared with the stomach (although it should be noted that the total number of labeled neurons projecting to the stomach is higher than to the duodenum).[35] The CARTp synthesized in cell bodies of vagal afferent neurons is transported to both central (NTS) and peripheral sites.[6,9] These neurons also express the orexigenic peptide MCH. However, the expression of MCH and CART is differentially regulated depending on nutritional status.[8] Thus, in rats fed ad libitum, CARTp expression is high while MCH expression is low in nodose ganglion neurons, while after 24 h fasting, CART expression is reduced and MCH expression is upregulated. This switch in expression is regulated by CCK. Injection of CCK8s in fasted animals is sufficient to upregulate CARTp expression (and decrease MCH expression), while a CCK1 receptor antagonist inhibits feeding-induced CARTp expression. In cultured vagal afferent neurons, CCK8s also induces synthesis and release of CARTp. These effects are potentiated by leptin[10] and inhibited by ghrelin.[8,9]

RECEPTORS AND SIGNALING PATHWAYS

The molecular mechanism of action of CART remains uncertain. Notably, the putative CARTp receptor has not, so far, been characterized although binding studies on cell lines, and on dissociated hypothalamic neurons have been described.[16,25] There is some evidence for actions at a G protein-coupled receptor acting via $G_{i/o}$ although this conclusion must remain provisional until molecular characterization of the receptor. Potential intracellular mediators of CARTp effects include the transcription factor c-fos, the expression of which has been widely used as a marker of cells responding to CARTp.[30] Many of the latter include neuronal systems implicated in control of ingestion. But in addition, other transcriptional mediators of CARTp include the immediate early gene (early growth response-1) EGR-1 and phosphoCREB that are activated in hypothalamic, brain stem, or vagal afferent neurons. In the vagal neurons, there is evidence that EGR-1 and phosphoCREB mediate the effects of CARTp in regulating its own expression and that of Y2 receptors.[9]

INTERACTIONS WITH OTHER SYSTEMS

Many different interactions between CARTp and other systems have been suggested that have implications for the control of energy balance. These include interactions with endocrine control mechanisms, with reward systems and components of the limbic system, and with autonomic mechanisms controlling digestion and energy expenditure. There may also be interactions with other peripheral systems including the pancreatic hormones.[25]

Cholecystokinin and Leptin

In addition to the evidence for leptin control of CART expression already discussed in the context of hypothalamic neurons, there is also evidence of strongly cooperative interactions between CCK and leptin in determining CART expression in vagal afferent neurons. The action of CCK in stimulating CART expression is mediated by the activation of EGR-1, which is achieved by stimulating its translocation from cytoplasm to nucleus; CCK does not seem to increase EGR-1 expression. Leptin strongly potentiates CCK-stimulated nuclear translocation of EGR-1, but it has no effect on its own. Leptin does, however, strongly stimulate EGR-1 expression. Thus, leptin is able to set the level of EGR-1 abundance but not its activity, whereas CCK determines activation but not abundance, and together, they determine CART expression.[10] These actions are also a target for orexigenic peptides such as ghrelin, which inhibits both leptin stimulation of EGR-1 expression and CCK stimulation of EGR-1 nuclear translocation.[10] Acutely, CCK stimulates CARTp release from cultured vagal afferent neurons, and there is a small effect of leptin in increasing this response. Interestingly, inhibition of CART expression in cultured vagal afferent neurons reduces some of the responses to CCK. For example, CCK stimulates both CART and Y2 receptor expression, and both seem to be partially dependent on CARTp secretion. However, CCK also inhibits the expression of CB1 receptors and MCH in vagal afferent neurons, and CART is not implicated in these responses.[9] Thus, CARTp seems to act as an autocrine enhancer of some, but not all, CCK effects on vagal afferent neurons which may underlie its effects on peripheral administration in potentiating CCK inhibition of food intake. The interaction with CCK may also account for the observation that administration of CARTp alone into the NTS has little effect on consumption of a non-CCK releasing meal (sucrose).[34,35]

Endocannabinoids, NPY, and MCH

Both morphological and functional evidence indicates interactions between CARTp and orexigenic factors including NPY, MCH and endocannabinoids. Further work is still required to elucidate these interactions particularly in hypothalamic nuclei implicated in ingestive behavior. Based on present evidence, it seems that the CB1 antagonist rimonabant inhibits food intake in wildtype mice but not in $CART^{-/-}$ mice; moreover, in mice, null for fatty acid amide hydrolase (FAAH), which metabolizes endocannabinoids, there is reduced CARTp in ARC and DMH and treatment

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of these mice with rimonabant increases CARTp.[23] These results are compatible with the view that expression and release of CART are inhibited by CB1 receptor stimulation, and that suppression of CART is a component of the orexigenic effect of endocannabinoids.

PATHOPHYSIOLOGICAL IMPLICATIONS

There is evidence that mutations in the *CART* gene are associated with obesity. A missense mutation that results in substitution of Leu at position 34 with Phe was reported to cosegregate with obesity over three generations in one family and was not found in a control population. The mutation is adjacent to a pair of lysine residues, which constitute a site of precursor cleavage raising the possibility of impaired posttranslational processing.[11] In keeping with this, the mutation was also associated with decreased circulating concentrations of CARTp, but increased concentrations of partially processed precursor peptide. In the AtT20 cell line, the mutant precursor was missorted and poorly processed consistent with the idea that this mutation leads to deficiency in CARTp via failure to generate the active peptide.[33]

PHYSIOLOGICAL OVERVIEW

Nearly 15 years after the first demonstration that CARTp inhibited food intake, there remain many unanswered questions. The results of studies in mice null for CART and in humans with *CART* gene mutations support the idea of a role in inhibition of food intake. Moreover, the experimental evidence extends these findings by indicating that CARTp may be a satiety factor at multiple levels including peripherally at vagal afferent neurons, and centrally within the brain stem, hypothalamus, and components of the limibic system. In recent years, some of the cellular mechanisms that govern the relationships between CARTp and other well-established satiety signals including CCK and leptin have started to emerge, particularly with respect to vagal afferent neurons. Set against this, however, there remains the problem that putative CARTp receptors are uncharacterized, and so it is difficult to examine cellular mechanisms of action in any detail, or for that matter to examine the consequences of receptor gene deletion or to develop antagonists. The issue is important given the work of some groups raising questions about the physiological relevance of actions of CARTp (notably when given icv) in inhibiting food intake, together with the work of others suggesting orexigenic actions. Moreover, CARTp has possible roles in other systems that might impact directly or indirectly on energy intake and expenditure, including those associated with pain, anxiety, reward, endocrine regulation, cardiovascular and gastrointestinal systems.[25] Further progress using existing laboratory methods should help to elucidate these inter-relationships and resolve seemingly contradictory findings. Nevertheless, the system will inevitably remain incompletely understood until it is possible to explore fully the relevant receptor mechanisms.

REFERENCES

1. Abbott CR, Rossi M, Wren AM, Murphy KG, Kennedy AR, Stanley SA, et al. Evidence of an orexigenic role for cocaine- and amphetamine-regulated transcript after administration into discrete hypothalamic nuclei. *Endocrinology* 2001;**142**:3457–63.
2. Aja S, Robinson BM, Mills KJ, Ladenheim EE, Moran TH. Fourth ventricular CART reduces food and water intake and produces a conditioned taste aversion in rats. *Behav Neurosci* 2002;**116**:918–21.
3. Aja S, Sahandy S, Ladenheim EE, Schwartz GJ, Moran TH. Intracerebroventricular CART peptide reduces food intake and alters motor behavior at a hindbrain site. *Am J Physiol Regul Integr Comp Physiol* 2001;**281**:R1862–7.
4. Asnicar MA, Smith DP, Yang DD, Heiman ML, Fox N, Chen YF, et al. Absence of cocaine- and amphetamine-regulated transcript results in obesity in mice fed a high caloric diet. *Endocrinology* 2001;**142**:4394–400.
5. Broberger C. Hypothalamic cocaine- and amphetamine-regulated transcript (CART) neurons: histochemical relationship to thyrotropin-releasing hormone, melanin-concentrating hormone, orexin/hypocretin and neuropeptide Y. *Brain Res* 1999;**848**:101–13.
6. Broberger C, Holmberg K, Kuhar MJ, Hokfelt T. Cocaine- and amphetamine-regulated transcript in the rat vagus nerve: a putative mediator of cholecystokinin-induced satiety. *Proc Natl Acad Sci USA* 1999;**96**:13506–11.
7. Cota D, Marsicano G, Tschop M, Grubler Y, Flachskamm C, Schubert M, et al. The endogenous cannabinoid system affects energy balance via central orexigenic drive and peripheral lipogenesis. *J Clin Invest* 2003;**112**:423–31.
8. de Lartigue G, Dimaline R, Varro A, Dockray GJ. Cocaine- and amphetamine-regulated transcript: stimulation of expression in rat vagal afferent neurons by cholecystokinin and suppression by ghrelin. *J Neurosci* 2007;**27**:2876–82.
9. de Lartigue G, Dimaline R, Varro A, Raybould H, de la Serre CB, Dockray GJ. Cocaine and amphetamine regulated transcript mediates the actions of cholecystokinin on rat vagal afferent neurons. *Gastroenterology* 2010;**138**:1479–90.
10. de Lartigue G, Lur G, Dimaline R, Varro A, Raybould H, Dockray GJ. EGR1 is a target for cooperative interactions between cholecystokinin and leptin, and inhibition by ghrelin, in vagal afferent neurons. *Endocrinology* 2010;**151**:3589–99.
11. Del Giudice EM, Santoro N, Cirillo G, D'Urso L, Di TR, Perrone L. Mutational screening of the CART gene in obese children: identifying a mutation (Leu34Phe) associated with reduced resting energy expenditure and cosegregating with obesity phenotype in a large family. *Diabetes* 2001;**50**:2157–60.
12. Douglass J, McKinzie AA, Couceyro P. PCR differential display identifies a rat brain mRNA that is transcriptionally regulated by cocaine and amphetamine. *J Neurosci* 1995;**15**:2471–81.
13. Elias CF, Lee C, Kelly J, Aschkenasi C, Ahima RS, Couceyro PR, et al. Leptin activates hypothalamic CART neurons projecting to the spinal cord. *Neuron* 1998;**21**:1375–85.

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14. Elias CF, Lee CE, Kelly JF, Ahima RS, Kuhar M, Saper CB, et al. Characterization of CART neurons in the rat and human hypothalamus. *J Comp Neurol* 2001;**432**:1–9.
15. Jean A, Conductier G, Manrique C, Bouras C, Berta P, Hen R, et al. Anorexia induced by activation of serotonin 5-HT4 receptors is mediated by increases in CART in the nucleus accumbens. *Proc Natl Acad Sci USA* 2007;**104**:16335–40.
16. Keller PA, Compan V, Bockaert J, Giacobino JP, Charnay Y, Bouras C, et al. Characterization and localization of cocaine- and amphetamine-regulated transcript (CART) binding sites. *Peptides* 2006;**27**:1328–34.
17. Kong WM, Stanley S, Gardiner J, Abbott C, Murphy K, Seth A, et al. A role for arcuate cocaine and amphetamine-regulated transcript in hyperphagia, thermogenesis, and cold adaptation. *FASEB J* 2003;**17**:1688–90.
18. Koylu EO, Couceyro PR, Lambert PD, Kuhar MJ. Cocaine- and amphetamine-regulated transcript peptide immunohistochemical localization in the rat brain. *J Comp Neurol* 1998;**391**:115–32.
19. Koylu EO, Couceyro PR, Lambert PD, Ling NC, DeSouza EB, Kuhar MJ. Immunohistochemical localization of novel CART peptides in rat hypothalamus, pituitary and adrenal gland. *J Neuroendocrinol* 1997;**9**:823–33.
20. Kristensen P, Judge ME, Thim L, Ribel U, Christjansen KN, Wulff BS, et al. Hypothalamic CART is a new anorectic peptide regulated by leptin. *Nature* 1998;**393**:72–6.
21. Lambert PD, Couceyro PR, McGirr KM, Dall Vechia SE, Smith Y, Kuhar MJ. CART peptides in the central control of feeding and interactions with neuropeptide Y. *Synapse* 1998;**29**:293–8.
22. Larsen PJ, Vrang N, Petersen PC, Kristensen P. Chronic intracerebroventricular administration of recombinant CART(42–89) peptide inhibits and causes weight loss in lean and obese Zucker (fa/fa) rats. *Obes Res* 2000;**8**:590–6.
23. Osei-Hyiaman D, Depetrillo M, Harvey-White J, Bannon AW, Cravatt BF, Kuhar MJ, et al. Cocaine- and amphetamine-related transcript is involved in the orexigenic effect of endogenous anandamide. *Neuroendocrinology* 2005;**81**:273–82.
24. Qing K, Chen Y. Central CART gene delivery by recombinant AAV vector attenuates body weight gain in diet-induced-obese rats. *Regul Pept* 2007;**140**:21–6.
25. Rogge G, Jones D, Hubert GW, Lin Y, Kuhar MJ. CART peptides: regulators of body weight, reward and other functions. *Nat Rev Neurosci* 2008;**9**:747–58.
26. Rohner-Jeanrenaud F, Craft LS, Bridwell J, Suter TM, Tinsley FC, Smiley DL, et al. Chronic central infusion of cocaine- and amphetamine-regulated transcript (CART 55–102): effects on body weight homeostasis in lean and high-fat-fed obese rats. *Int J Obes Relat Metab Disord* 2002;**26**:143–9.
27. Smith KL, Gardiner JV, Ward HL, Kong WM, Murphy KG, Martin NM, et al. Overexpression of CART in the PVN increases food intake and weight gain in rats. *Obesity (Silver Spring)* 2008;**16**:2239–44.
28. Vicentic A. CART peptide diurnal variations in blood and brain. *Peptides* 2006;**27**:1942–8.
29. Vicentic A, Lakatos A, Hunter R, Philpot K, Dominguez G, Kuhar MJ. CART peptide diurnal rhythm in brain and effect of fasting. *Brain Res* 2005;**1032**:111–5.
30. Vrang N, Tang-Christensen M, Larsen PJ, Kristensen P. Recombinant CART peptide induces c-fos expression in central areas involved in control of feeding behaviour. *Brain Res* 1999;**818**:499–509.
31. Wierup N, Richards WG, Bannon AW, Kuhar MJ, Ahren B, Sundler F. CART knock out mice have impaired insulin secretion and glucose intolerance, altered beta cell morphology and increased body weight. *Regul Pept* 2005;**129**:203–11.
32. Yang SC, Shieh KR, Li HY. Cocaine- and amphetamine-regulated transcript in the nucleus accumbens participates in the regulation of feeding behavior in rats. *Neuroscience* 2005;**133**:841–51.
33. Yanik T, Dominguez G, Kuhar MJ, Del Giudice EM, Loh YP. The Leu34Phe ProCART mutation leads to cocaine- and amphetamine-regulated transcript (CART) deficiency: a possible cause for obesity in humans. *Endocrinology* 2006;**147**:39–43.
34. Zheng H, Patterson C, Berthoud HR. Fourth ventricular injection of CART peptide inhibits short-term sucrose intake in rats. *Brain Res* 2001;**896**:153–6.
35. Zheng H, Patterson LM, Berthoud HR. CART in the dorsal vagal complex: sources of immunoreactivity and effects on fos expression and food intake. *Brain Res* 2002;**957**:298–310.

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Chapter 144

CCK

Claire Barbier de La Serre and Timothy H. Moran

ABSTRACT

Peripheral administration of the brain/gut peptide cholecystokinin (CCK) inhibits food intake. The actions of peripheral CCK in feeding are consistent with a role in satiety. CCK is rapidly released during a meal, exogenous peptide administration terminates an individual meal without long-term effects on food intake, and CCK antagonists or genetic manipulations that alter CCK-1R receptor signaling result in increases in meal size. A necessary role for CCK-induced vagal afferent activation in CCK satiety has been shown. CCK not only activates vagal afferents providing a feedback signal to the dorsal hindbrain but it also plays a major role in altering gene expression of other orexigenic and anorexigenic peptides and receptors in vagal afferent cell bodies in the nodose ganglion.

INTRODUCTION

In their classic 1973 article, Gibbs, Young, and Smith showed the ability of exogenous administration of the brain-gut peptide cholecystokinin (CCK) to inhibit food intake in rats.[15] The pattern of behavioral changes produced by CCK was consistent with the production of satiety. CCK reduced meal size and meal duration and resulted in an earlier appearance of a behavioral sequence of satiety similar to that seen after ingestion of a normal size meal. From these results, Gibbs et al. suggested that exogenously administered CCK was mimicking a physiological hormonal action of the endogenous peptide.[15] These initial reports have stimulated almost 40 years of work in a variety of laboratories aimed at showing a physiological role for CCK in the control of food intake and at understanding the mechanisms underlying the satiety actions of both exogenously administered and endogenously released CCK. The results of these investigations have shown an important role for CCK as a within meal negative feedback satiety signal. The following chapter will detail the current state of our understanding of the role of peripheral CCK in satiety and the mechanisms through which these actions are mediated.

CCK AS A GUT PEPTIDE

CCK is released from I enteroendocrine cells found in the upper small intestine (duodenum and jejunum). Digestion products of lipids and proteins are the most potent elicitors of CCK secretion with carbohydrates inducing a much smaller response.[9] Different receptors, such as GPR93 or PEPT-1 for proteins and GPR120 for fatty acids, are involved in nutrient sensing and nutrient-induced CCK release. Although these receptors mobilize different intracellular signaling pathways, they have a common outcome, an increase in intracellular Ca^{2+} and CCK release.[25] CCK plays a variety of roles in coordinating digestive processes. CCK delays gastric emptying, stimulates gall bladder contraction and pancreatic secretion, and reduces food intake, all of which optimize digestive capacities.

FEEDING INHIBITORY ACTIONS OF PERIPHERAL CCK

Exogenous peripheral administration of CCK results in a dose-related suppression of short-term food intake in a variety of species and under a variety of experimental situations. CCK effectively reduces food intake in birds, rodents, pigs, sheep, nonhuman primates, and humans. CCK affects both solid and liquid food intake and is effective in spontaneous food intake as well as feeding that is stimulated by access to a palatable food source or in response to deprivation. CCK inhibits both real and sham feeding, in which ingested food is swallowed but drains from the stomach through a gastric fistula, demonstrating that CCK is a sufficient stimulus for reducing food intake even in the absence of other postingestive feedback.[16]

CCK primarily affects food intake by reducing meal size. Repeated meal-contingent CCK administration that occurs at meal onset results in a reliable reduction in meal size without an overall change in food intake.[32] That is, in response to the repeated CCK-induced reductions in meal size, rats compensate for the missed calories through increasing their meal frequency. By contrast, when CCK administration is timed to meal termination, there is no effect on the duration of the intermeal interval and no overall changes in the amounts consumed.[33]

Such meal termination actions of CCK are consistent with the dynamics of CCK in circulation. Exogenously

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00144-5
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administered CCK is rapidly degraded with estimates of half-life in the circulation in the range of a few minutes. In response to a meal, plasma CCK levels increase rapidly, peaking within a few minutes of meal initiation, and then fall to less elevated levels that are sustained over a longer time period.[19] This rapid release and short duration in circulation suggest a role in the termination of a single meal without actions on the reoccurrence of eating at the next meal.

The satiating action of CCK is characterized by an inhibition of food intake without abnormal or competing behaviors. CCK elicits the normal behavioral sequence of satiety.[1] Rats treated with CCK stop eating sooner but go through the normal sequence of post-prandial behaviors that characterize response to a spontaneous meal—meal termination is accompanied by a period of grooming and exploration followed by resting or sleep. Doses of CCK that inhibit food intake in food deprived rats do not affect water intake in water deprived rats, showing that the actions of CCK are not secondary to an abnormal alteration in oral motor patterns.[15] CCK inhibits food intake at doses that do not produces a conditioned taste aversion and, in fact, at the low end of the effective dose range for food intake, CCK produces a conditioned preference.[26] Results from human experiments in which the subjective sensations of subjects receiving CCK are assessed show significant suppression in food intake in the absence of significant reports of side effects.[18] Such results support the view that the feeding inhibitory actions of exogenous CCK are specific to satiety.

FEEDING INHIBITORY ACTIONS OF ENDOGENOUS CCK

The availability of CCK receptor antagonists provided the ability to critically assess the physiological significance of endogenous CCK in the control of meal size. Assessments of the CCK receptor subtype mediating the actions of exogenous CCK in food intake point to a critical role for CCK-1R receptors. Sulfated CCK-8 inhibits food intake, whereas nonsulfated CCK-8 or shorter CCK fragments do not. As receptor subtype specific CCK antagonists became available a similar pattern of results was found. CCK antagonists with specificity for the CCK-1R receptor subtype increase food intake in a variety of species and across a range of testing paradigms while antagonists with CCK-2R specificity do not.[20] Overall, the results show that the increases in food intake stimulated by CCK-1R antagonists are dose related, and the majority of the effect is secondary to increases in the size of meals.

The importance of CCK acting through CCK-1R for feeding control has also been seen in studies in Otsuka Long Evans Tokushima Fatty (OLETF) rats lacking the CCK-1R and in CCK 1-R receptor knockout mice. OLETF rats arose from a spontaneous mutation in a colony of outbred Long Evans rats that resulted in obesity and diabetes. In examining overall pancreatic function in OLETF rats, it was discovered that they lacked an amylase response to exogenous CCK, leading to the demonstration that OLETF rats have a 6-kb deletion in the gene for CCK-1R that spans the promotor region and the first and second exons resulting in the absence of CCK-1R in these rats.[13] Functional studies in OLETF rats have revealed that they do not reduce food intake in response to CCK administration and examination of their meal pattern showed that they have a satiety deficit.[22] OLETF rats consume meals that are about twice the normal size. In response to this increase in meal size, meal number is decreased but not sufficiently to normalize overall food intake, resulting in about a 40% hyperphagia (Fig. 1).

In contrast to the OLETF rats, CCK-1R knockout mice consume normal amounts of food intake over 24 h and do not become obese. However, CCK-1R receptor knockout mice consume larger meals than that of wildtype mice of the same background strain. The increase is relatively smaller than that seen in OLETF rat and the mice do compensate for the increase such that total food intake is not significantly elevated. Thus, although these two models result in different overall metabolic phenotypes (suggesting that the OLETF rat has further deficits that those involving peripheral CCK satiety signaling), they do both support the view that CCK signaling is important for the normal controls of meal size.

SITES OF ACTION FOR PERIPHERAL CCK IN SATIETY

Peripherally administered CCK induces a variety of changes in gastrointestinal motility and secretion, and some of these actions are direct local effects of the peptide and some depend on the activation of vagal afferent fibers. A role for CCK-induced vagal activation in CCK satiety has been shown in a variety of experimental settings. Total subdiaphragmatic vagotomy completely blocks the ability of CCK to inhibit food intake and vagal deafferentation significantly attenuates the feeding inhibitory effects of CCK.[21,30] The nature of this attenuation is to eliminate the ability of low doses of CCK to affect food intake while reducing the efficacy of higher doses. Surgical removal of the pyloric sphincter also significantly attenuates CCK satiety. CCK-1Rs are expressed in nodose ganglion neurons and transported both peripherally and central to vagal terminal fields.[23] CCK-1Rs are also expressed in circular muscle fibers in the pyloric sphincter.[31] CCK activates vagal afferent fibers and contracts the pyloric sphincter, an action that contributes to CCK's ability to inhibit gastric emptying and secondary to that effect, reduce food intake.[24]

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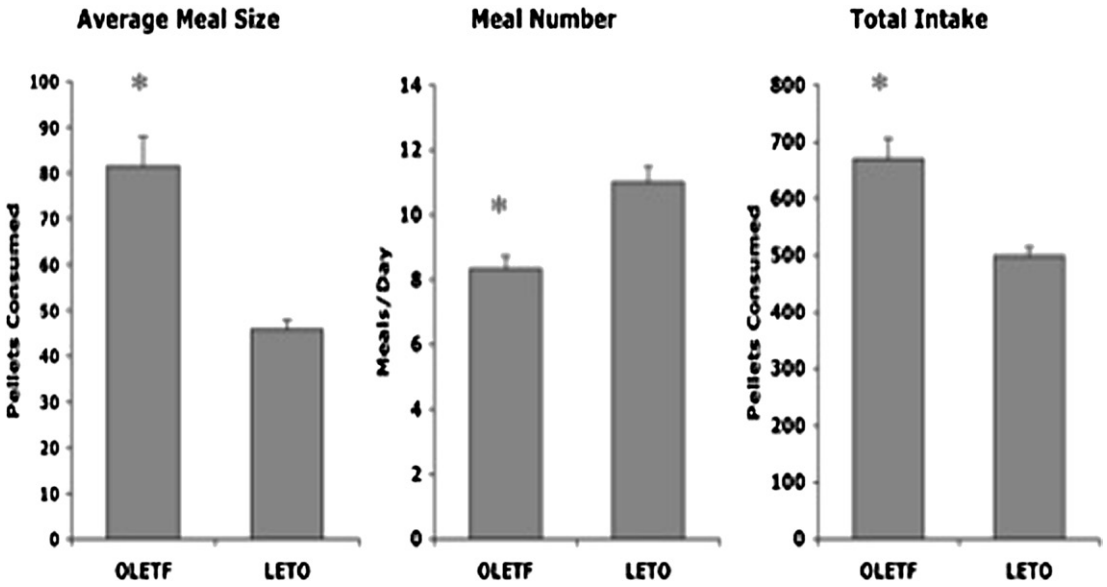

FIGURE 1 Meal patterns in OLETF rats lacking CCK-1 receptors and control LETO rats over a 24-h period. OLETF rats consume much larger spontaneous meals and have a significant reduction in meal number. The reduction in meal number is not sufficient to prevent a significant overall hyperphagia (modified from Ref. 22).

The feeding inhibitory effects of both exogenous and endogenous CCK are predominantly mediated via activation of the CCK-1R on vagal afferents. Vagal afferents nerve terminals are found in the wall of the gastrointestinal tract, in the muscle and mucosa layers. Within the mucosa, terminals are located in the lamina propria close to the basal membrane of I enteroendocrine cells expressing CCK.[2] Local CCK release or exogenous local arterial CCK administration activates vagal afferent CCK-1R, triggering vagal discharge and increase in vagal activity in the form of action potentials. CCK-induced increases in vagal afferent activity are seen in both gastric and duodenal vagal afferents.[27,29] Afferent fibers that respond to CCK are also load sensitive; increases in intraluminal volume also activate these fibers and having been activated by CCK, the identical load produces greater activation (Fig. 2). These interactions provide a basis for synergy between intraluminal load and CCK in inhibiting food intake.

CCK-induced vagal afferent activity results in the modulation of secondary neurons within the nucleus of solitary tract (NTS), the central site of termination of vagal afferents. The feeding inhibitory action of CCK requires the activation of hindbrain NMDA-type glutamate receptors.[34] The phenotypes of the neurons activated by CCK within the NTS include both tyrosine hydroxylase expressing and proopiomelanocortin (POMC) expressing neurons. The necessity of melanocortin signaling in CCK satiety has been shown, as CCK fails to inhibit food intake in mice in lacking MC4R or in mice in which brainstem MC4R are pharmacologically blocked.[12] CCK-induced neural activity at the level of the brainstem is sufficient for mediating CCK satiety, as CCK is effective in decerebrate rats where the hindbrain and forebrain are surgically disconnected.[17] Functional gastrointestinal responses to CCK are mediated via vagal efferents and sympathetic nervous system activation as well as hormonal mechanisms. Neurons in the dorsal motor nucleus (DMV) receive information from the NTS and the midbrain and project back to the GI tract through vagal efferent to modulate gastric functions such as gastric emptying and secretions. Thus, both vagal afferent and efferent probably participate in CCK-induced satiety.[21]

CCK-INDUCED CONTROL OF VAGAL AFFERENT GENE EXPRESSION

In addition to activating vagal afferent fibers, CCK seems to be play a major role in orchestrating gene expression within nodose ganglion neurons and, in this way, modulates vagal afferent sensitivity to a variety of stimuli involved in feeding control. Contrary to long-term regulating signals such as circulating leptin, CCK does not have suppressible tonic activity; thus, the decrease in signaling in a fasted state is not expected to stimulate food intake. However, the absence or presence of food in the gut triggers changes in G-protein-coupled receptors and neurotransmitter expression in vagal afferent neurons that modulate the sensitivity to GI signals. Vagal afferent neurons innervating the stomach and duodenum are found in mid and caudal areas of the nodose ganglion and express receptors for anorexigenic peptides such as CCK, leptin, PYY3–36 and also express receptors for orexigenic signals like orexin, melanin-concentrating hormone (MCH) and cannabinoids receptor.[10] These different receptors are

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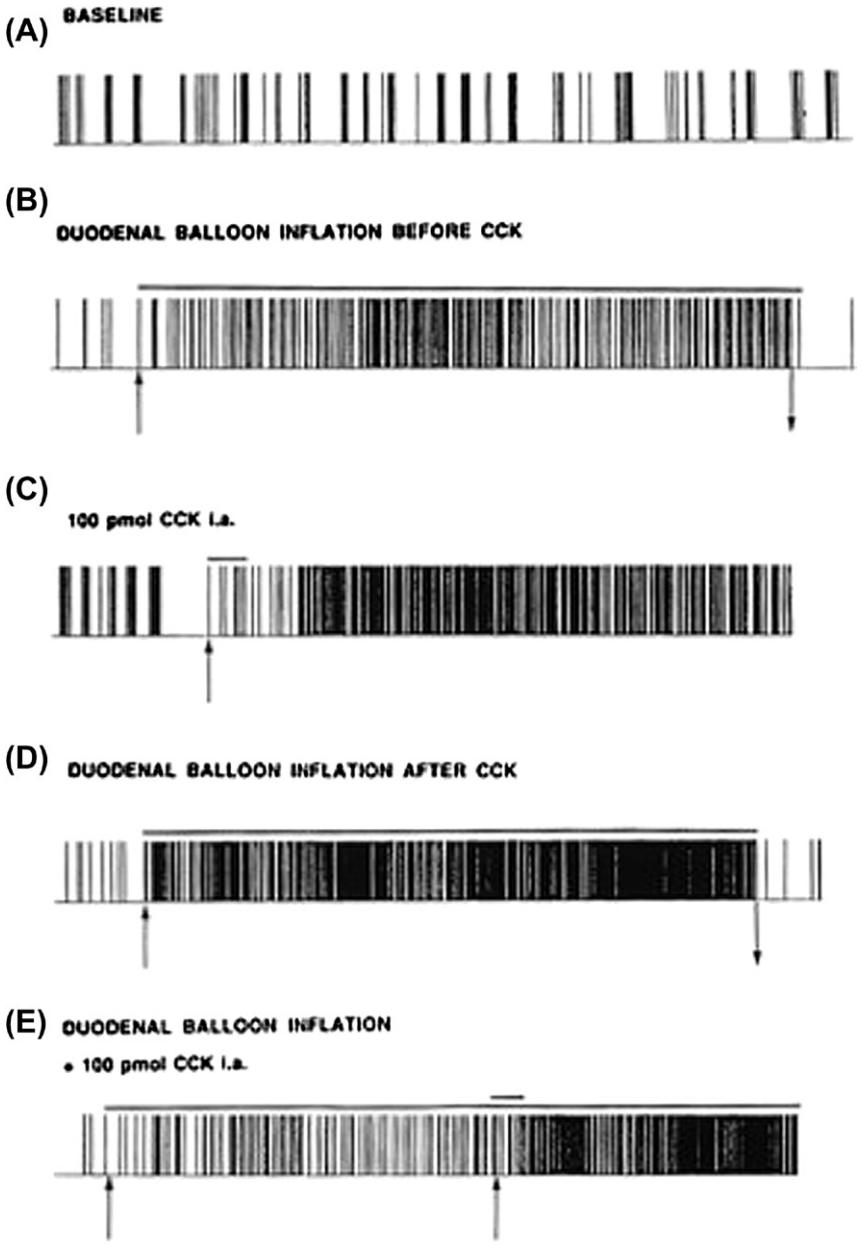

FIGURE 2 Vagal afferent activity in a single fiber innervating the proximal duodenum. (A) Baseline activity. (B) Activity increase in response to intraluminal volume. (C) Activity increase in response to local arterial CCK administration. (D) Activity increase to same intraluminal volume after activation by CCK at a time when activity had returned to baseline levels. E. Increased activity to simultaneous CCK and lintraluminal volume. CCK both activates vagal afferents and sensitizes the afferents to intraluminal volume stimuli.

translocated to the nerve terminals where they can be activated by their respective ligands. In rats, feeding status can influence receptor expression at the mRNA and protein levels through a CCK-dependent pathway. For example, fasting of ≥12 hours in rats leads to an increase in nodose ganglion CB1 receptor expression.[4] CB1 expression decreases 2 h after refeeding and is virtually lost after 5 h. The effect of feeding can be mimicked by exogenous CCK administration and blocked by a CCK-1R antagonist. Likewise, nodose ganglion MCH1R expression increases with fasting and decrease with feeding. Similar to CB1, MCH1R mRNA is undetectable 5 h after refeeding.[5] Administration of CCK has the same effect as feeding on MCH1R expression and feeding-induced decreases in MCH1R occur via a CCK-1R mediated mechanism. Conversely, Y2R expression is decreased 5-fold in vagal afferent neurons of fasted rats compared with that of animals fed ad libitum.[3] In mice, PYY3–36 administration induces fos activation in neurons within the brainstem; this effect is reduced in CCK-1R knockout mice, consistent with a role for CCK in determining Y2R signaling.

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Feeding status also influences the expression of neuropeptides in vagal afferent neurons.[10] In rats, expression of the appetite stimulating neuropeptide MCH increases with fasting. Vagal afferent neurons express MCH and MCH1R; the local release of MCH may stimulate MCH1R to promote appetite.[5] This positive feedback loop is terminated with refeeding or CCK injection. By contrast, expression of the peptide cocaine and amphetamine related transcript (CART) in these neurons is associated with a fed state; upregulation of CART is induced by a CCK-dependent pathway.[8] In cultured neurons, treatment with CART stimulates Y2R and CART production but has no effect on CB1, MCHR1 and MCH. Moreover, incubation with CART siRNA abolishes CCK-induced increase in Y2R and CART. CART seems to mediate CCK anorexigenic signaling and to be a part of an autocrine positive feedback loop promoting satiety.

Such results show that anorexigenic and orexigenic factors interact on the same pathways at the level of vagal affertent neurons to modulate satiety signaling, and neurochemical phenotype of these neurons depends on energy intake and encodes the presence or absence of nutrient ingestion over the previous 24- to 48-h period. CCK controls the changes in expression of G-protein-coupled receptors and peptide neurotransmitters, and its effect on orexigenic signal receptors can contribute to its action as a satiety signal. Thus, CCK is not only involved in the acute regulation of meal size, but it can also influence the capacity of vagal afferent neurons to respond to other neurohormonal signals (Fig. 3).

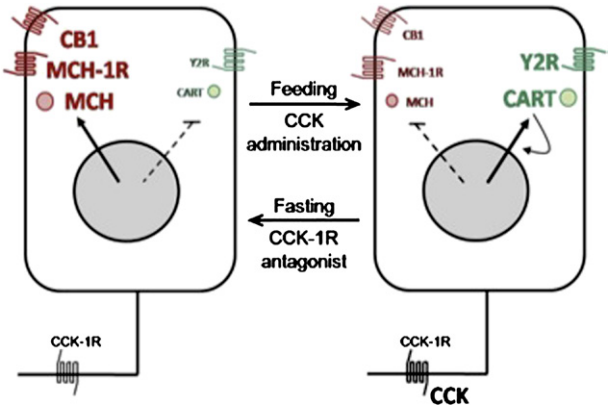

FIGURE 3 In the absence of CCK signaling (fasted or fed in the presence of CCK-1R antagonist), MCH1R, CB1, and MCH expressions are upregulated, whereas CART and Y2R expressions are downregulated. CCK binding to CCK-1R results in an increase in Y2R and CART with CART promoting Y2R and its own synthesis. CCK-1R activation also leads to the downregulation of MCH1R, CB1, and MCH expressions (adapted from Ref. 10).

FUNCTIONAL RESPONSES TO PERIPHERAL CCK IN DIFFERING METABOLIC STATES

The potency and efficacy for peripheral CCK to inhibit food intake depend on the metabolic status. CCK is less effective under conditions of food deprivation than in ad lib feeding, and CCK is less effective in rats during the dark cycle when the majority of feeding occurs. These state-induced changes in the feeding inhibitory efficacy of CCK have been proposed to result from alteration in leptin signaling. Leptin levels are low in response to food deprivation or before nighttime feeding. Leptin affects food intake through a mechanism dependent upon reduction in meal size and some aspects of this action appear to depend on leptin/CCK interactions. Exogenously administered leptin at doses that alone do not affect food intake enhances the ability of CCK to both inhibit feeding and induce neural activation in CCK activated NTS pathways as measured by the presence of the immediate early gene c-Fos or electrophysiolgocial activity.[11,28]

The feeding inhibitory actions of peripheral CCK are also modulated by estrogen.[14] In female rats and mice, the amounts and patterns of food intake vary across the estrous cycle. Food intake is lowest at time points corresponding to high estrogen levels and these decreases are expressed as decreases in meal size. This effect seems to be mediated by altered sensitivity to CCK. In ovariectomized females, CCK is more effective in reducing food intake when females are estrogen primed, and sensitivity to CCK varies across the estrous cycle. Further, CCK antagonists prevent the decreases in food intake that correspond to high estrogen levels.

Chronic consumption of a high-fat (HF) diet also leads to a decreased response to CCK characterized by a reduction in fos activation in NTS neurons after challenge with lipids or CCK and a decrease in CCK-induced anorexia.[6] The presence of fat in the diet seems to lower lipid-induced fos in the NTS independently of caloric density. The pathway by which HF diet alters CCK signaling is unknown. Reduced leptin signaling has been hypothesized to impair CCK action leading to the development of hyperphagia. In rats, leptin signaling is required for normal food intake. Knocking down Ob-r in the hindbrain results in hyperphagia, probably mediated by a reduction in leptin potentiation of CCK signaling. In diet-induced obese rats, vagal leptin signaling is impaired before the appearance of central leptin resistance and coincides with an increase in food intake and weight gained.[7]

Although it is not clear whether the decrease in CCK sensitivity precedes or follows diet-induced hyperphagia, impairment in CCK signaling results in an increase in meal size and alteration in meal patterning eventually leading to an increase in body weight. OLETF rats that have a defect in the CCK-1R gene are hyperphagic and obese. Their increase in daily food intake results from an increase in meal size combined with a lack of a compensatory decrease in meal

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frequency; when put on an HF diet, they increase their rates of body weight gain compared with control rats. Similarly, HF diet access increases the meal size deficit in CCK-1R$^{-/-}$ mice. Given the importance of CCK in determining the sensitivity to other peripheral signals in vagal afferent neurons, the absence of CCK signaling may lead to an alteration in the expression of receptors and neurotransmitters involved in food intake control promoting orexigenic signaling and contributing to the increased deficits to high-fat feeding in the absence of CCK signaling.

SUMMARY

CCK is an important modulator of food intake at the level of individual meals. Nutrient-induced CCK release is a major controller of meal size. In the absence of CCK signaling, satiety is delayed and larger meals are consumed. CCK satiety signaling is mediated by vagal afferent fibers. CCK activates load sensitive vagal afferent fibers innervating the stomach and proximal intestine resulting in altered neural activity at the level of the brainstem nucleus of the solitary tract. CCK not only alters vagal afferent activity but it also modulates the expression of vagal afferent orexigenic and anorexigenic peptides. These actions can modulate the ability of vagal afferents to respond to a variety of other feedback signals.

REFERENCES

1. Antin J, Gibbs J, Holt J, Young RC, Smith GP. Cholecystokinin elicits the complete behavioral sequence of satiety in rats. *J Comp Physiol Psychol* 1975;**89**:784–90.
2. Berthoud HR, Patterson LM. Anatomical relationship between vagal afferent fibers and CCK-immunoreactive entero-endocrine cells in the rat small intestinal mucosa. *Acta Anat (Basel)* 1996;**156**:123–31.
3. Burdyga G, de Lartigue G, Raybould HE, Morris R, Dimaline R, Varro A, et al. Cholecystokinin regulates expression of Y2 receptors in vagal afferent neurons serving the stomach. *J Neurosci* 2008;**28**:11,583–92.
4. Burdyga G, Lal S, Varro A, Dimaline R, Thompson DG, Dockray GJ. Expression of cannabinoid CB1 receptors by vagal afferent neurons is inhibited by cholecystokinin. *J Neurosci* 2004;**24**:2708–15.
5. Burdyga G, Varro A, Dimaline R, Thompson DG, Dockray GJ. Feeding-dependent depression of melanin-concentrating hormone and melanin-concentrating hormone receptor-1 expression in vagal afferent neurones. *Neuroscience* 2006;**137**:1405–15.
6. Covasa M, Grahn J, Ritter RC. High fat maintenance diet attenuates hindbrain neuronal response to CCK. *Regul Pept* 2000;**86**:83–8.
7. de Lartigue G, Barbier de la Serre C, Espero E, Lee J, Raybould HE. Diet-induced obesity leads to the development of leptin resistance in vagal afferent neurons. *Am J Physiol Endocrinol Metab* 2011;**301**:E187–95.
8. De Lartigue G, Dimaline R, Varro A, Raybould H, De la Serre CB, Dockray GJ. Cocaine- and amphetamine-regulated transcript mediates the actions of cholecystokinin on rat vagal afferent neurons. *Gastroenterology* 2010;**138**:1479–90.
9. Dockray GJ. Cholecystokinin and gut-brain signalling. *Regul Pept* 2009;**155**:6–10.
10. Dockray GJ. The versatility of the vagus. *Physiol Behav* 2009;**97**:531–6.
11. Emond M, Schwartz GJ, Ladenheim EE, Moran TH. Central leptin modulates behavioral and neural responsivity to CCK. *Am J Physiol* 1999;**276**:R1545–9.
12. Fan W, Ellacott KL, Halatchev IG, Takahashi K, Yu P, Cone RD. Cholecystokinin-mediated suppression of feeding involves the brainstem melanocortin system. *Nat Neurosci* 2004;**7**:335–6.
13. Funakoshi A, Miyasaka K, Jimi A, Kawanai T, Takata Y, Kono A. Little or no expression of the cholecystokinin-A receptor gene in the pancreas of diabetic rats (Otsuka Long-Evans Tokushima Fatty = OLETF rats). *Biochem Biophys Res Commun* 1994;**199**:482–8.
14. Geary N. Estradiol, CCK and satiation. *Peptides* 2001;**22**:1251–63.
15. Gibbs J, Young RC, Smith GP. Cholecystokinin decreases food intake in rats. *J Comp Physiol Psychol* 1973;**84**:488–95.
16. Gibbs J, Young RC, Smith GP. Cholecystokinin elicits satiety in rats with open gastric fistulas. *Nature* 1973;**245**:323–5.
17. Grill HJ, Smith GP. Cholecystokinin decreases sucrose intake in chronic decerebrate rats. *Am J Physiol* 1988;**254**:R853–6.
18. Kissileff HR, Pi-Sunyer FX, Thornton J, Smith GP. C-terminal octapeptide of cholecystokinin decreases food intake in man. *Am J Clin Nutr* 1981;**34**:154–60.
19. Liddle RA, Goldfine ID, Rosen MS, Taplitz RA, Williams JA. Cholecystokinin bioactivity in human plasma. Molecular forms, responses to feeding, and relationship to gallbladder contraction. *J Clin Invest* 1985;**75**:1144–52.
20. Moran TH, Ameglio PJ, Schwartz GJ, McHugh PR. Blockade of type A, not type B, CCK receptors attenuates satiety actions of exogenous and endogenous CCK. *Am J Physiol* 1992;**262**:R46–50.
21. Moran TH, Baldessarini AR, Salorio CF, Lowery T, Schwartz GJ. Vagal afferent and efferent contributions to the inhibition of food intake by cholecystokinin. *Am J Physiol* 1997;**272**:R1245–51.
22. Moran TH, Katz LF, Plata-Salaman CR, Schwartz GJ. Disordered food intake and obesity in rats lacking cholecystokinin A receptors. *Am J Physiol* 1998;**274**:R618–25.
23. Moran TH, Norgren R, Crosby RJ, McHugh PR. Central and peripheral vagal transport of cholecystokinin binding sites occurs in afferent fibers. *Brain Res* 1990;**526**:95–102.
24. Moran TH, Shnayder L, Hostetler AM, McHugh PR. Pylorectomy reduces the satiety action of cholecystokinin. *Am J Physiol* 1988;**255**:R1059–63.
25. Nilaweera KN, Giblin L, Ross RP. Nutrient regulation of enteroendocrine cellular activity linked to cholecystokinin gene expression and secretion. *J Physiol Biochem* 2010;**66**:85–92.
26. Perez C, Sclafani A. Cholecystokinin conditions flavor preferences in rats. *Am J Physiol* 1991;**260**:R179–85.
27. Schwartz GJ, McHugh PR, Moran TH. Gastric loads and cholecystokinin synergistically stimulate rat gastric vagal afferents. *Am J Physiol* 1993;**265**:R872–6.
28. Schwartz GJ, Moran TH. Leptin and neuropeptide y have opposing modulatory effects on nucleus of the solitary tract neurophysiological responses to gastric loads: implications for the control of food intake. *Endocrinology* 2002;**143**:3779–84.
29. Schwartz GJ, Tougas G, Moran TH. Integration of vagal afferent responses to duodenal loads and exogenous CCK in rats. *Peptides* 1995;**16**:707–11.

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30. Smith G, Jerome C, Norgren R. Afferent axons in the abdominal vagus mediate the satiety effects of cholecystokinin in rats. *Am J Physiol* 1985;**249**:R638–41.
31. Smith GT, Moran TH, Coyle JT, Kuhar MJ, O'Donahue TL, McHugh PR. Anatomic localization of cholecystokinin receptors to the pyloric sphincter. *Am J Physiol* 1984;**246**:R127–30.
32. West DB, Fey D, Woods SC. Cholecystokinin persistently suppresses meal size but not food intake in free-feeding rats. *Am J Physiol* 1984;**246**:R776–87.
33. West DB, Greenwood MR, Sullivan AC, Prescod L, Marzullo LR, Triscari J. Infusion of cholecystokinin between meals into free-feeding rats fails to prolong the intermeal interval. *Physiol Behav* 1987;**39**:111–5.
34. Wright J, Campos C, Herzog T, Covasa M, Czaja K, Ritter RC. Reduction of food intake by cholecystokinin requires activation of hindbrain NMDA-type glutamate receptors. *Am J Physiol Regul Integr Comp Physiol* 2011;**301**:R448–55.

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Chapter 145

CRH

D. Richard and C. Lopez

ABSTRACT

The corticotropin-releasing hormone (CRH) system is involved in the control of food intake and energy expenditure. CRH and its paralogs urocortins 1, 2, and 3 are known for their hypophagic effects, which are mediated through the CRH receptors 1 and 2 (CRHR1, CRHR2). They are responsible for severe stress-induced anorexia. Additionally, CRH and urocortin 1 are also capable of thermogenic effects exerted on brown adipose tissue (BAT) via the sympathetic nervous system (SNS). The complexity of the brain CRH system renders difficult any precise delineation of this system's action in energy balance regulation. Despite its anorectic and thermogenic potential, the CRH system, when activated, can also be anabolic depending on the receptor and the brain regions involved. The CRH system could be behind anxiety/stress-induced obesity. Indeed, CRH stimulates the secretion of corticosteroids, whose effect on food intake and thermogenesis tends to be obesogenic. Meanwhile, the activation of the CRHR1 in the nucleus accumbens (NAc) stimulates the motivation to ingest palatable food.

INTRODUCTION

Evidence has accumulated throughout the years to suggest the involvement of the brain CRH system in the regulation of energy balance in obesity.[9,12,18,30] CRH and urocortins 1, 2, and 3, via actions mediated by CRHR1 and CRHR2, are capable of producing catabolic effects by reducing food intake and increasing energy expenditure. CRH is responsible for the anorectic effect of stress.[9,30] However, there are results supporting the view that peptides of the CRH familly could be involved in stress-induced obesity either by activating the pituitary–adrenal axis[34] or by influencing the rewarding effects of food.[3]

BRAIN DISTRIBUTION OF CRH, UROCORTINS, AND CRH RECEPTOR

CRH

CRH is a 41-amino-acid peptide expressed in neurons widely distributed throughout the mammalian brain.[23] It is highly synthesized in the paraventricular hypothalamic nucleus (PVH) and is also found in large amounts in extrahypothalamic regions, such as the olfactory bulb, bed nucleus of the stria terminalis (BNST), central nucleus of amygdala (CeA), geniculate nucleus, Barington's nucleus, dorsal vagal complex, and inferior olive. The broad distribution of the CRH neurons conforms to the many expected effects of the peptide including those on the food intake and energy expenditure.[9,12,18,30] The PVH CRH neurons project to the median eminence to stimulate the secretion of the adrenocorticotropic hormone (ACTH), which in turn leads to the adrenal secretion of corticosterone or cortisol. CRH represents the main controller of the basal and stress-induced activation of the pituitary–adrenal axis; CRH-knockout mice exhibit pituitary–adrenal hypoactivity.[25]

Urocortins

Urocortins 1, 2, and 3, which show particularly high selectivities for the CRHR2 receptor, are expressed in restricted areas of the brain.[19,29,35] Urocortin I, a 40-amino-acid peptide, is highly expressed in the Edinger–Westphal nucleus and also found in the supraoptic and hypoglossal nuclei.[35] Urocortin 2, a 38-amino-acid peptide that selectively binds to the CRH_2 receptor with no appreciable affinity for the CRHR1 receptor, is expressed in restricted brain areas, including the magnocellular division of the PVH, the arcuate nucleus (ARC) and the locus coeruleus.[29] Urocortin 3, a 38-amino-acid peptide, which, similar to urocortin 2, selectively binds to the CRHR2, is expressed in discrete brain areas including the BNST, the medial nucleus of amygdala, median preoptic nucleus, and brainstem.[19] None of the urocortin neurons projects to the pituitary gland to physiologically control the pituitary–adrenal axis. Urocortin 1, which binds to both the CRHR1 and CRHR2 receptor with a high affinity, activates the pituitary–adrenal axis and elicits anxiety-like behaviors when injected into the brain ventricles.[24]

CRH Receptors and CRH-Binding Protein

CRHR1, for which CRH (in particular ovine CRH) and urocortin have high affinity, is widely distributed in the brain

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00145-7
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with the highest densities of expression observed in cortical, hypothalamic, limbic, and cerebellar regions. Within the PVH, CRHR1 mRNA can only be detected after stressful stimuli.[31] CRHR2, on which urocortins II and III act as selective endogenous ligands, is restrictedly expressed in the olfactory bulb, lateral septum (LS), ventromedial hypothalamic nucleus (VMH), medial and posterior cortical nuclei of the amygdala, mesensephalic raphe nucleus, nucleus of the solitary tract, and area postrema.[20]

In addition to binding to its two receptors, CRH and urocortin also have a noticeable affinity for the CRH-binding protein (CRH-BP), which is widely distributed throughout the brain.[28] In the rat, the protein is expressed in the cortex, amygdale, and hypothalamus. Most brain regions of CRH-BP expression are also sites of CRH synthesis or release, supporting the view that CRH-binding protein could increase the availability of CRH or urocortins for the CRH receptors.

CRH, UROCORTINS, AND ENERGY BALANCE REGULATION

CRH and urocortins are peptides capable of influencing food intake and energy expenditure,[9,12,18,30] the two inescapable components of energy balance regulation. When injected centrally, CRH decreases food intake and increases energy expenditure, whereas the effects of urocortins, especially urocortins 2 and 3, are mainly exerted on food intake. Brain injection of CRH also lowers the body weight threshold at which food-deprived rats start to hoard food in a home cage environment, supporting the idea that CRH could be a germane regulator of energy balance;[5] the body weight threshold at which food-deprived rats start to hoard food represents a surrogate of the body weight set point. Further, the observations (i) that food deprivation decreases brain CRH expression (at least in the PVH)[4] and (ii) that chronically administered CRH[32] and rat/human $CRH_{6–33}$, which dissociates CRH or urocortin from CRH-BP and increases endogenous brain levels of "free" CRH or urocortin, prevent weight gain in obese Zucker rats with little effect in lean animals,[15] speak in favor of a genuine role for peptides of the CRH family in energy balance regulation. However, the exact role of the CRH in energy balance, which is entangled by a complex network of CRH/urocortin neurons and receptors, needs to be fully delineated.

Sites and Mechanisms of the Anorectic and Thermogenic Actions of CRH

Both CRHR1 and CRHR2 could be involved in the effects of CRH and urocortins on food intake and energy expenditure, which complexifies the delineation of the sites and mechanisms whereby peptides of the CRH family exert their effect on the energy balance determinants.[9,12,18,30] Evidence keeps accumulating to emphasize the importance of the CRHR2 in mediating the anorectic effects of CRH and urocortins. CRHR2 agonism would reduce food intake without implicating malaise-like behaviors such as those that could be due to the CRHR1 agonism, which is known to produce interoceptive stress signs associated with anorectic effects.[10] Both urocortins 2 and 3 are capable of anorectic effects when injected centrally, whereas urocortin 2 seems to be more efficient than urocortin 3 in inducing hypophagia when injected in the periphery.[10] The implication of CRHR2 in the control of food intake is also supported by the changes in CRHR2 mRNA levels, which inversely vary with appetite. The expression of the VMH CRHR2 mRNA is decreased in obese, diabetic, and food-deprived rats.[30] It is induced by the intracerebroventricular infusion of leptin.[16] The VMH could represent the main physiological site for the CRHR2 receptor-mediated anorectic effect of the CRH-related peptides. In this regard, this hypothalamic nucleus was demonstrated to be an important site for the anoretic effect of urocortin 3.[7,11] Within the VMH, CRHR2 is expressed on glutamatergic excitatory neurons projecting to the ARC and on which urocortin 3 exerts a stimulating effect triggering proopiomelanocortin (POMC) expression.[7] The LS could also represent a site for the anorectic action of the CRH and CRH-related peptides.[21,26]

The role of CRHR1 in the feeding behavior remains unclear. The observation that urocortin does not elicit an acute anorectic response in CRHR1 ablated mice suggests that CRHR1 plays a role in the anorectic effects of the CRH-related peptides.[30] CRHR1 could mediate the anorectic effects that would be nonspecific and secondary to the anxiety-, fear- and stress-like behaviors (see below).

CRH and urocortin stimulate energy expenditure by increasing BAT thermogenesis.[30] This effect seems to be linked to the stimulation of CRHR1. In fact, the CRHR2 antagonist, antisauvagine-30 proved to be inefficient to block the thermogenic effect of a central injection of urocortin 1.[8] Moreover, the CRHR2 agonism could oppose the stimulating effect of the CRHR1 agonism on BAT thermogenesis; CRHR2-ablated mice exhibit a robust increase in basal BAT thermogenesis linked to an increased basal SNS outflow to BAT.[2] Additionally, CRHR2-mutant mice consume substantially more food when fed a high-fat diet with a reduced feeding efficiency and an increased BAT thermogenic capacity. The idea that the CRHR2 agonism blunts BAT thermogenesis is somewhat in contradiction with the observation that urocortin 3 stimulates ARC POMC neurons when injected in the VMH.[7] The stimulation of those neurons generally leads to the stimulation of SNS-BAT thermogenesis concomitant to a reduction in food intake.

The brain site of the CRHR1-mediated action on BAT thermogenesis is yet to be ascertained. There nonetheless is

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sound evidence for the existence of a circuit involving the preoptic area (POA), dorsomedial hypothalamic nucleus, and raphe pallidus. It has indeed been suggested that CRH in the POA similar to prostaglandins could activate SNS-mediated BAT thermogenesis.[6] The POA expresses the CRHR1 and also comprises melanocortin-4 receptor neurons connected to BAT.[33] The role of the PVH in mediating the CRH and urocortin thermogenic effect is less certain as controversial effects have emerged from two independent studies.[6,17] That the PVH does not apparently express the CRHR1 except under stress stimulation[30] could explain discrepant sets of results regarding CRHR1 agonism-induced BAT thermogenesis.

A role for the CRH system in the anorectic action of leptin has been suggested. Some investigations have reported that the hypophagic action of a central infusion of leptin be blocked by CRH receptor antagonists.[30] However, it is as yet unclear in those studies if the CRH blockade prevented a leptin-mediated hypophagic action or if it curtails a nonspecific stress-related anorectic effect resulting from an acute central injection of large doses of leptin. Incidentally, leptin is recognized for its antistress effects.[30] CRH has also been suggested to mediate leptin-induced brown fat thermogenesis.[22]

CRH AND STRESS-INDUCED ANOREXIA

The CRH system coordinates a wide range of stress responses and evidence keeps accumulating that CRH is responsible for stress-induced anorexia.[9,30] CRH receptor antagonists block the anorexia associated with stressful conditions known to activate the CRH system, including treadmill running and restraint.[30] CRH antagonism also blocks the hypophagic effect of both estradiol and caffeine.[30] The observation that CRH-knockout mice exhibit indistinguishable anorectic responses to restraint stress suggests that alternative pathways could compensate for the long-term CRH deficiency.[30]

Stress-induced anorexia has long been believed to be solely mediated by the CRHR1.[30] Selective CRHR1 antagonists block the anorexia induced by emotional stress. There are, however, results suggesting that the CRHR2 in the LS and BNST could also be involved.[1,26] Restraint stress has been reported to activate neurons harboring the LS CRHR2.[21]

CRH AND STRESS-INDUCED OBESITY

Paradoxically, the CRH system could also be involved in stress-induced obesity. Stress-induced obesity could, in particular circumstances, prevail over stress-induced anorexia as the stimulation of the pituitary–adrenal axis and that of some brain appetitive behaviors for rewarding foods by CRHR1 agonism might favor obesity development. The metabolic response to stress depends on its severity and duration; severe long-lasting stressful events generally create catabolic responses.[9,30] It also depends on the way individuals respond to or cope with stressful events, which is determined by many factors.

The activation of the pituitary–adrenal axis, which represents the most recognized and studied functions of the CRH system, could *per se* promote obesity. Glucocorticoids ensuing from such activation are indeed anabolic by stimulating fat intake, reducing thermogenesis and promoting abdominal fat deposition.[34] The CRH action at the levels of the pituitary–adrenal is mediated by the CRHR1, whose expression is repressed by CRH and glucocorticoids.[30] In laboratory rodents, increase in corticosteroid levels enhances fat deposition by stimulating food intake and the preference for fat food while likely inhibiting BAT thermogenesis.[30] The activation of the brain CRH system in severe stressful events generally overwhelms the effects of the enhanced secretion of corticosteroids on energy balance; murine and rat studies have clearly emphasized the reducing effect of severe stress on fat deposition (see above).

A parallel way through which stress or the activation of the CRH system might sustain obesity is by increasing the value of or the motivation for palatable sweet/fat food through actions selective to certain brain regions.[3] CRH when microinjected in the NAc[27] have been reported to trigger the motivation for food. This action resembles that CRH (via CRHR1) also has to mediate the motivation for drugs in drug-dependent animals.[14] Increasing the motivation to consume would have the ability to extinguish stress responses associated with, for instance, alcohol, drug, or food withdrawal. Incidentally, ingestion of highly palatable food has been reported to reduce stress in rats.[13] Similarly, the high frequency of food ingestion in fa/fa Zucker rats would also seem to prevent the neurogenic-stress response led to by food deprivation in this the leptin-deficient rat model.[30] Leptin can also be seen as an antistress hormone. In obese mice, leptin downregulates the expression of CRH in the paraventricular hypothalamic nucleus,[30] which is consistent with the apparent ability of leptin to reduce the hypothalamic–pituitary–adrenal axis activity.

CONCLUSIONS

The role of the CRH system in energy balance regulation and obesity is complex. CRH and its paralogs urocortins 1, 2, and 3, through effects mediated by CRHR1 and CRHR2, are capable of hypophagic and thermogenic effects. The CRH system plays a major role in stress-induced anorexia. The sites of the anorectic and thermogenic actions of CRH and urocortins could be the LS, VMH, and the POA

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(thermogenic action), which express CRHR1 (POA) and CRHR2 (LS and VMH). Growing evidence tends to support a role for the CRHR1 agonism in stress-induced obesity. The pituitary and the NAc CRHR1-mediated potentiation of the "incentive wanting" to eat could be site-specific action of the CRH system to promote obesity and thereby to oppose its (nonetheless predominant) catabolic effects.

REFERENCES

1. Bakshi VP, Newman SM, Smith-Roe S, Jochman KA, Kalin NH. Stimulation of lateral septum CRF2 receptors promotes anorexia and stress-like behaviors: functional homology to CRF1 receptors in basolateral amygdala. *J Neurosci* 2007;**27**:10,568–77.
2. Bale TL, Anderson KR, Roberts AJ, Lee KF, Nagy TR, Vale WW. Corticotropin-releasing factor receptor-2-deficient mice display abnormal homeostatic responses to challenges of increased dietary fat and cold. *Endocrinology* 2003;**144**:2580–7.
3. Berridge KC, Ho CY, Richard JM, DiFeliceantonio AG. The tempted brain eats: pleasure and desire circuits in obesity and eating disorders. *Brain Res* 2010;**1350**:43–64.
4. Brady LS, Smith MA, Gold PW, Herkenham M. Altered expression of hypothalamic neuropeptide mRNAs in food-restricted and food-deprived rats. *Neuroendocrinology* 1990;**52**:441–7.
5. Cabanac M, Richard D. Acute intraventricular CRF lowers the hoarding threshold in male rats. *Physiol Behav* 1995;**57**:705–10.
6. Cerri M, Morrison SF. Corticotropin releasing factor increases in brown adipose tissue thermogenesis and heart rate through dorsomedial hypothalamus and medullary raphe pallidus. *Neuroscience* 2006;**140**:711–21.
7. Chen P, Vaughan J, Donaldson C, Vale W, Li C. Injection of urocortin 3 into the ventromedial hypothalamus modulates feeding, blood glucose levels, and hypothalamic POMC gene expression but not the HPA axis. *Am J Physiol Endocrinol Metab* 2010;**298**:E337–45.
8. Cullen MJ, Ling N, Foster AC, Pelleymounter MA. Urocortin, corticotropin releasing factor-2 receptors and energy balance. *Endocrinology* 2001;**142**:992–9.
9. Diz-Chaves Y. Ghrelin, appetite regulation, and food reward: interaction with chronic stress. *Int J Pept* 2011;**2011**:898,450.
10. Fekete E, Zhao Y, Szucs A, Sabino V, Cottone P, Rivier J, et al. Systemic urocortin 2, but not urocortin 1 or stressin(1)-A, suppresses feeding via CRF(2) receptors without malaise and stress. *Br J Pharmacol* 2011;**164**:1959–75.
11. Fekete EM, Inoue K, Zhao Y, Rivier JE, Vale WW, Szucs A, et al. Delayed satiety-like actions and altered feeding microstructure by a selective type 2 corticotropin-releasing factor agonist in rats: intra-hypothalamic urocortin 3 administration reduces food intake by prolonging the post-meal interval. *Neuropsychopharmacology* 2007;**32**:1052–68.
12. Fekete EM, Zorrilla EP. Physiology, pharmacology, and therapeutic relevance of urocortins in mammals: ancient CRF paralogs. *Front Neuroendocrinol* 2007;**28**:1–27.
13. Foster MT, Warne JP, Ginsberg AB, Horneman HF, Pecoraro NC, Akana SF, et al. Palatable foods, stress, and energy stores sculpt corticotropin-releasing factor, adrenocorticotropin, and corticosterone concentrations after restraint. *Endocrinology* 2009;**150**: 2325–33.
14. George O, Le Moal M, Koob GF. Allostasis and addiction: role of the dopamine and corticotropin-releasing factor systems. *Physiol Behav* 2011.
15. Heinrichs SC, Lapsansky J, Behan DP, Chan RK, Sawchenko PE, Lorang M, et al. Corticotropin-releasing factor-binding protein ligand inhibitor blunts excessive weight gain in genetically obese Zucker rats and rats during nicotine withdrawal. *Proc Natl Acad Sci USA* 1996;**93**:15,475–80.
16. Huang Q, Timofeeva E, Richard D. Regulation of corticotropin-releasing factor and its types 1 and 2 receptors by leptin in rats subjected to treadmill running-induced stress. *J Endocrinol* 2006;**191**:179–88.
17. Kotz CM, Wang C, Levine AS, Billington CJ. Urocortin in the hypothalamic PVN increases leptin and affects uncoupling proteins-1 and -3 in rats. *Am J Physiol Regul Integr Comp Physiol* 2002;**282**: R546–51.
18. Kuperman Y, Chen A. Urocortins: emerging metabolic and energy homeostasis perspectives. *Trends Endocrinol Metab* 2008;**19**: 122–9.
19. Lewis K, Li C, Perrin MH, Blount A, Kunitake K, Donaldson C, et al. Identification of urocortin III, an additional member of the corticotropin-releasing factor (CRF) family with high affinity for the CRF2 receptor. *Proc Natl Acad Sci USA* 2001;**98**:7570–5.
20. Lovenberg TW, Liaw CW, Grigoriadis DE, Clevenger W, Chalmers DT, De Souza EB, et al. Cloning and characterization of a functionally distinct corticotropin-releasing factor receptor subtype from rat brain. *Proc Natl Acad Sci USA* 1995;**92**:836–40.
21. Martin J, Timofeeva E. Intermittent access to sucrose increases sucrose-licking activity and attenuates restraint stress-induced activation of the lateral septum. *Am J Physiol Regul Integr Comp Physiol* 2010;**298**:R1383–98.
22. Masaki T, Yoshimichi G, Chiba S, Yasuda T, Noguchi H, Kakuma T, et al. Corticotropin-releasing hormone-mediated pathway of leptin to regulate feeding, adiposity, and uncoupling protein expression in mice. *Endocrinology* 2003;**144**:3547–54.
23. Merchenthaler I, Vigh S, Petrusz P, Schally AV. Immunocytochemical localization of corticotropin-releasing factor (CRF) in the rat brain. *Am J Anat* 1982;**165**:385–96.
24. Moreau JL, Kilpatrick G, Jenck F. Urocortin, a novel neuropeptide with anxiogenic-like properties. *Neuroreport* 1997;**8**: 1697–701.
25. Muglia LJ, Bethin KE, Jacobson L, Vogt SK, Majzoub JA. Pituitary–adrenal axis regulation in CRH-deficient mice. *Endocr Res* 2000;**26**:1057–66.
26. Ohata H, Shibasaki T. Involvement of CRF2 receptor in the brain regions in restraint-induced anorexia. *Neuroreport* 2011;**22**:494–8.
27. Pecina S, Schulkin J, Berridge KC. Nucleus accumbens corticotropin-releasing factor increases cue-triggered motivation for sucrose reward: paradoxical positive incentive effects in stress? *BMC Biol* 2006;**4**:8.
28. Potter E, Behan DP, Linton EA, Lowry PJ, Sawchenko PE, Vale WW. The central distribution of a corticotropin-releasing factor (CRF)-binding protein predicts multiple sites and modes of interaction with CRF. *Proc Natl Acad Sci USA* 1992;**89**:4192–6.
29. Reyes TM, Lewis K, Perrin MH, Kunitake KS, Vaughan J, Arias CA, et al. Urocortin II: a member of the corticotropin-releasing factor (CRF) neuropeptide family that is selectively bound by type 2 CRF receptors. *Proc Natl Acad Sci USA* 2001;**98**:2843–8.

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30. Richard D, Lin Q, Timofeeva E. The corticotropin-releasing factor family of peptides and CRF receptors: their roles in the regulation of energy balance. *Eur J Pharmacol* 2002;**440**:189–97.
31. Richard D, Rivest R, Naimi N, Timofeeva E, Rivest S. Expression of corticotropin-releasing factor and its receptors in the brain of lean and obese Zucker rats. *Endocrinology* 1996;**137**:4786–95.
32. Rohner-Jeanrenaud F, Walker CD, Greco-Perotto R, Jeanrenaud B. Central corticotropin-releasing factor administration prevents the excessive body weight gain of genetically obese (fa/fa) rats. *Endocrinology* 1989;**124**:733–9.
33. Song CK, Vaughan CH, Keen-Rhinehart E, Harris RB, Richard D, Bartness TJ. Melanocortin-4 receptor mRNA expressed in sympathetic outflow neurons to brown adipose tissue: neuroanatomical and functional evidence. *Am J Physiol Regul Integr Comp Physiol* 2008;**295**:R417–28.
34. Spencer SJ, Tilbrook A. The glucocorticoid contribution to obesity. *Stress* 2011;**14**:233–46.
35. Vaughan J, Donaldson C, Bittencourt J, Perrin MH, Lewis K, Sutton S, et al. Urocortin, a mammalian neuropeptide related to fish urotensin I and to corticotropin-releasing factor. *Nature* 1995;**378**:287–92.

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Chapter 146

Enterostatin

Charlotte Erlanson-Albertsson

ABSTRACT

Enterostatin is a pentapeptide that specifically suppresses fat intake or high-fat intake as opposed to high-carbohydrate diets. As opposed to most regulatory peptides, enterostatin is formed in the lumen of the intestine and hence is comparable with food-derived peptides. The mechanism for the decreased fat intake is dependent on cholecystokinin pathways, opiate pathways, and melanocortin pathways. Enterostatin also displays metabolic effects, such as inhibition of insulin secretion, lowering of serum cholesterol levels, and enhancement of memory. An enterostatin-deficient mouse has elevated plasma cholesterol levels, pointing to the role of enterostatin in reducing blood lipids. The receptor for enterostatin is the β-subunit of F1-ATPase being localized to the plasma membrane in target cells. Enterostatin and fatty acids cause an increased expression of the receptor on the plasma membrane. Enterostatin may work as a feedback signal in promoting the regulation of fat intake and use of fat as energy substrate.

Peptides are small molecules and can therefore act as regulators in many different ways. Enterostatin is a small peptide comprising five amino acids (Table 1). The structure of this peptide is such that it resembles opiate-like peptides by having two proline residues in positions 2 and 4.[7] Moreover, it is produced in large quantities in the lumen of the intestine, thus being different from most regulatory peptides that are produced in endocrine cells and secreted directly into the blood stream (Fig. 1). Enterostatin instead resembles food-derived peptides that are contained in food proteins and are released during digestion of food proteins, for example, beta-casomorphin, which is formed during the digestion of milk casein (see the Opioid Peptides section of this book). Peptides such as enterostatin may have weak interactions with many different systems, but by virtue of the high concentrations at which these peptides exist, they can influence regulatory systems. In view of these properties, it was perhaps not surprising that the enterostatin knockout mouse displays a high level of stress and elevated levels of blood cholesterol compared with that of their normal littermates (Fig. 2).[19]

Enterostatin is released from pancreatic procolipase during digestion of fat and was first described as a peptide that promotes satiety for fat (Fig. 1). After the initial discovery, many other properties were described, such as the ability to regulate insulin secretion, lower blood cholesterol, improve memory, act antianalgesic, increase thermogenesis, and inhibit angiogenesis. This chapter describes the properties of enterostatin and its suggested mechanism of action. Several reviews have been written on enterostatin, among others.[7]

THE DISCOVERY OF ENTEROSTATIN

The first discovery of enterostatin as a feeding related peptide was in 1988 when during immunization of rabbits with enterostatin the animals lost their appetite. The basis for immunization of rabbits with enterostatin was our observation that all colipase molecules produced by the pancreas were secreted in the form of procolipase molecules. This was strange because colipase is not an enzyme that needs to be protected like other enzymes of the exocrine pancreas such as trypsin or phospholipase. We therefore wondered whether enterostatin was indeed released *in vivo* as we had found *in vitro*. With the knowledge that these rabbits did not want to eat, we started systematic feeding studies in rats and found that enterostatin decreased food intake after intraperitoneal administration.[6] It was very difficult to get this article published, but I was lucky to be invited to write a chapter in the French Journal *Biochimie*, in an issue devoted to professor Pierre Desnuelle, a grand French scientist who died in 1985 and with whom I worked during my postdoctoral scholarship in Marseille in 1972–1973.This was the first publication on the effect of enterostatin, which was then named procolipase activation peptide.[6]

CHEMISTRY OF ENTEROSTATIN AND TISSUE DISTRIBUTION

Enterostatin is a pentapeptide containing two proline residues with an amino acid in between. The structure has been conserved throughout the evolution (Table 1). It is produced through tryptic cleavage of procolipase after secretion into the intestinal lumen (Fig. 1). Enterostatin and its precursor procolipase have been identified in the pancreas, the stomach, and in the central nervous system. By using a specific

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00146-9
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TABLE 1 Sequence of Enterostatin from Various Species

Species	Sequence
Human	APGPR
Rat	APGPR
Mouse	APGPR
Rabbit	APGPR
Dog	VPDPR
Chicken	APGPR
Pig	VPDPR
Horse	VPDPR
Hagfish	APGR
Myocco	APDPR
Spermophilus	APDPR
Canfa	VPDPR

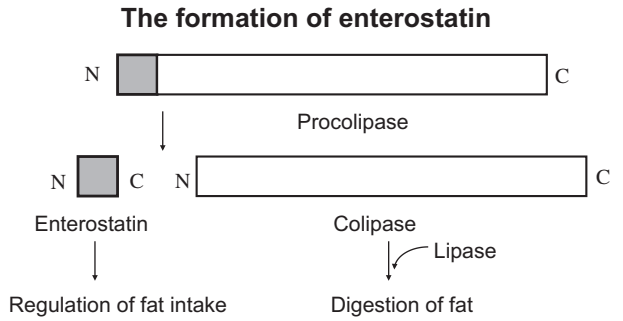

FIGURE 1 Enterostatin is a pentapeptide that is cleaved from the amino terminus of pancreatic procolipase in the intestinal lumen. It is formed in micromolar concentrations representing a high concentration. Although colipase acts as protein cofactor for pancreatic lipase during fat digestion, enterostatin regulates fat intake and fat metabolism in a feedback system.

immune assay, enterostatin was identified in the intestinal content of the rat at a concentration of 1.42×10^{-6} M, which is high for a biologically active peptide. The release of enterostatin into the intestinal content occurs parallel to the release of pancreatic lipase and colipase and is stimulated by CCK.[7] A high-fat diet significantly increases the level of enterostatin in the intestine to 3.86×10^{-6} M already by 24 h after the start of a high-fat diet regimen, indicating a rapid response of enterostatin expression after high-fat feeding.[7]

In the intestinal lumen, enterostatin is degraded within 30 min by epithelial brush border peptidases, mainly eliminating the C-terminal arginine residue. In spite of peptide degradation during normal conditions, intact transepithelial passage of enterostatin occurs in intact form.[7] It was concluded that although immunoreactive enterostatin exhibits a low apparent permeability coefficient in rabbit ileum, the high luminal concentration of enterostatin may be sufficient for the production of biological effects after absorption into the serum.

In the gastrointestinal tract, enterostatin is present also in the gastric mucosa, secreted by chief cells.[7] Cloning of gastric procolipase revealed that this molecule is identical to the pancreatic procolipase. Enterostatin is present in gastric juice, with pepsin and acid being responsible for the cleavage of gastric procolipase into enterostatin and colipase. The role of gastric colipase is unknown but may be important to prepare lipase-catalyzed fat digestion already in the stomach. Enterostatin is also present in endocrine cells in the antral part of the stomach and in the small intestine of the rat.[7] Its immunoreactive cells are more frequently found in the antrum and duodenum and become gradually fewer toward the distal small intestine. In some of the labeled endocrine cells, a coexistence of enterostatin with serotonin was revealed by immunocytochemical double staining, implying that the cells were enterochromaffin cells. Serotonin may be important in the mechanism of action of enterostatin.

Enterostatin has also been identified in the brain in the form of procolipase.[33,27] Procolipase and enterostatin immunoreactivity is seen in PVN, ARC, supraoptic nuclei, amygdala, and dorsal median thalamus.[33] These regions are associated with the regulation of food intake and energy expenditure. Procolipase is seen as dense particles in the cytoplasm, whereas enterostatin immunoreactivity occurs in nerve fibers. This suggests that procolipase is produced and processed in the neuronal cell body, whereas enterostatin is transported down the nerve fiber to be released at the nerve terminal.[33] Because enterostatin is particularly active when injected into the amygdala, the endogenous production of enterostatin in this nucleus suggests that this could be important for the regulation of fat intake. Enterostatin is also present in cells lining the third ventricle, where it may be secreted into the cerebrospinal fluid, as confirmed by Imamura et al.[8]

Hypothalamic procolipase expression is stimulated by a high-fat diet.[27] Because diets rich in fat are energy dense, the stimulation of hypothalamic procolipase seems to be an adequate event to induce satiety and energy balance during fat intake. Fasting overnight causes a 3-fold downregulation of hypothalamic procolipase, which is in agreement with the activity of a satiating agent. There is no expression of classical lipase in the hypothalamus, supporting a specific role of procolipase in the hypothalamus, not related to lipid digestion.[27]

With the immunoreactive method, serum enterostatin has been identified and measured in normal and morbidly obese human subjects.[7] Normal subjects display a biphasic release of enterostatin in the circulation postprandially, whereas morbidly obese patients have a weaker response. The concentration of enterostatin is approximately 10×10^{-9} M, suggesting that enterostatin may be absorbed

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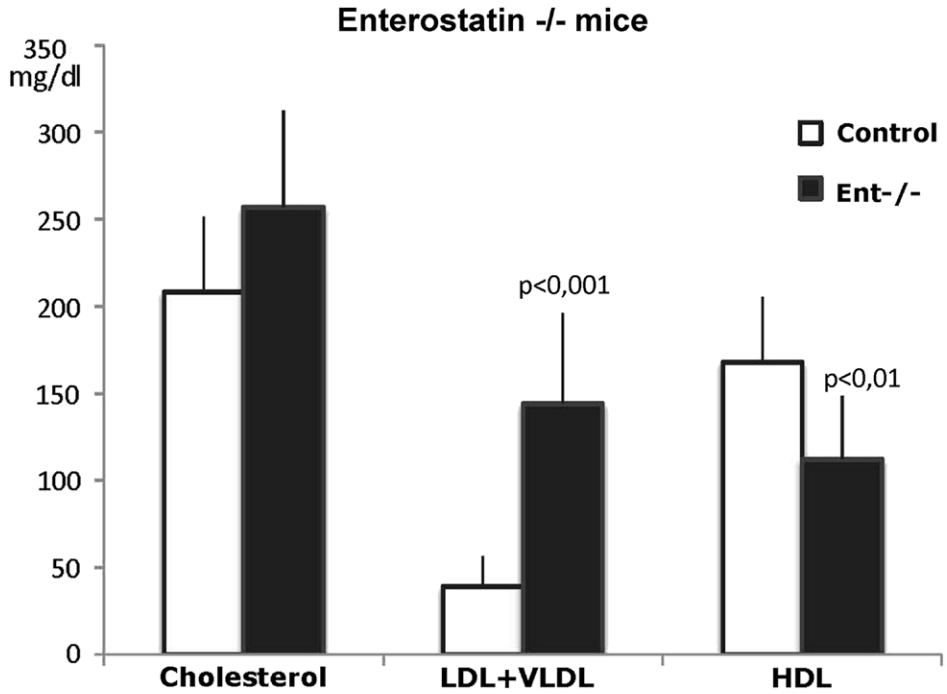

FIGURE 2 Enterostatin affects not only fat intake but also fat metabolism by regulating the use of fat as an energy substrate. The enterostatin-deficient mouse (Ent −/−) displays a normal fat intake but has higher LDL+VLDL blood cholesterol levels and lower HDL cholesterol levels.[19]

through the intestine. At least three different proteins bind enterostatin with a molecular size of 300, 205, and 60 kDa, albumin being one possible binding protein.[32] The presence of enterostatin-binding protein(s) in the circulation retards the uptake of enterostatin into the brain or other local target tissues and may also prevent a rapid degradation.

In rat serum, enterostatin increases postprandially after a meal.[17] The magnitude of response is dependent on the type of meal given. With high-fat and high-fat/high-sucrose meals, the response is greater in magnitude and duration than that after consuming a low-fat meal.[17] Plasma enterostatin concentrations after high-fat feeding are similar to those after intravenous administration of exogenous enterostatin known to inhibit high-fat food intake. Gel chromatography of pooled postprandial plasma extracts from this study followed by high-performance liquid chromatography analysis showed that plasma enterostatin was identical to synthetic enterostatin. Affinity crosslinking of plasma proteins with ^{125}I-enterostatin on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed by autoradiography, revealed a single band with a molecular weight of about 66 kDa, indicating the presence of a potential enterostatin-binding protein in plasma.

THE RECEPTOR FOR ENTEROSTATIN

The receptor for enterostatin is the β subunit of F1-ATPase,[2,22] sequenced through MALDI-TOF analysis. By the surface plasmon resonance technique, the binding constant between ligand and receptor is 1.7×10^{-7} M and with an aqueous two-phase system equal to 1.5×10^{-7} M.[3] Because enterostatin occurs at a concentration of 10^{-6} M, for instance, in the intestinal lumen, a binding to the receptor should be possible.

The enterostatin receptor (β-subunit of F1-ATPase) is localized in the plasma membranes of liver cells and in amygdala.[22] It is also present in INS-1 cells, where enterostatin and fatty acids cause an upregulation of the expression of the receptor.[16] The observation that fat components such as fatty acids cause an increased expression of the enterostatin receptor may explain the need to feed animals high-fat diets before they respond to enterostatin.[13] High-fat diets are known to increase the expression of procolipase/enterostatin.[7,26] Thus, high-fat diets cause an increased expression both of enterostatin and its receptor.

The identity and targeting of the F1-ATPase β-subunit in the plasma membrane[22] and not as expected in the mitochondria as part of the ATP synthase complex[2] is not a unique phenomenon of enterostatin action. There are several reports on the presence of the F1-ATPase β-subunit in the plasma membranes of a variety of cell types, including adipocytes, hepatocytes, and various tumor cell lines[5] and the targeting of signals to this protein. In the plasma membrane, the F1-ATPase β-subunit is probably complexed with different subunits than when it is present in the mitochondria. Different functions have been described. The targeting of enterostatin to the F1-ATPase β-subunit in the plasma

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membrane seems to be a possible mechanism for enterostatin to affect various cellular processes.

The intracellular mechanism once targeted by the enterostatin receptor includes various downstream events. In insulin-producing cells (INS-1 cells), enterostatin targets the F1-ATPase β-subunit with a simultaneous perturbation of ATP production, increase thermogenesis, and reduced insulin secretion.[2] Berger et al[2] suggested that the inhibition of insulin secretion observed in INS-1 cells results from perturbed ATP production and increased thermogenesis, ATP being important to stimulate insulin secretion.

Another intracellular event is an increased activation of AMP-activated kinase (AMPK) as observed during the stimulation of fatty acid oxidation in myocytes by enterostatin. AMP-activated kinase is recognized to have a central role in regulating energy balance during starvation, activating pathways to extract energy from the energy stores of the body, and at the same time inhibiting storage of energy. When activated, AMP-activated kinase leads to increased fatty acid oxidation in the muscle. Enterostatin causes a phosphorylation and subsequent activation of AMP-activated kinase. This is in agreement with the properties of a satiety peptide that not only acts to stop food intake but also acts to recruit energy from energy stores while food intake is suppressed.

Still another intracellular pathway for enterostatin is the activation of cyclic AMP and subsequent activation of MAP kinase ERK pathways.[24] This occurs in GT1–7 murine hypothalamic neuronal cells in culture. In these studies, enterostatin causes a reduced expression of AgRP after 1 hour of incubation.[24] Because AgRP stimulates fat intake, the decreased expression by enterostatin seems a possible explanation for the promotion of satiety for fat (Fig. 3). Initially, enterostatin increased the levels of cAMP, the downstream intracellular event being an activation of the MAP kinase pERK pathway. The activation of the MAP kinase correlates with a decreased expression of AgRP expression. The GT1–7 cells possess the F1-ATPase β-subunit protein, that is, the enterostatin receptor in the plasma membrane, where it is associated with lipid rafts. It was hence suggested that a perturbation of the ATP/ADP occurs by enterostatin, as observed in INS-1 cells,[2] which then increases cAMP concentrations and starts the downstream cell signaling. Thus, the activation of cAMP could initiate both acute events such as the oxidation of energy substrate molecules and more long-term events such as the regulation of hunger/satiety hormone expression.

Another component that is important for the enterostatin response is the presence of CCK-A receptors (Fig. 3). This occurs for the effects of enterostatin on food intake,[12] on memory,[20] and on blood cholesterol.[29] CCK is released by dietary fat reaching the intestine and reduces food intake through a CCK-A receptor. CCK causes a reduction in meal size and is dependent on afferent vagal nerves from the gastrointestinal tract to hypothalamic and extrahypothalamic forebrain regions to reduce food intake in a similar way as enterostatin. The interaction of enterostatin with the CCK-induced signaling is based on studies in the OLEFTA rat, which lacks CCK-A receptors.[12] This rat is unresponsive to enterostatin in contrast to the control LETO rat, where fat intake is reduced by 23% after enterostatin administration. Further support for the involvement of CCK-A receptor is the observation that lorglumide, a receptor antagonist specific for the CCK-A receptor, totally abolishes the enterostatin effect even though there is no binding of enterostatin to the CCK-A receptor, neither peripherally nor centrally. There are therefore several

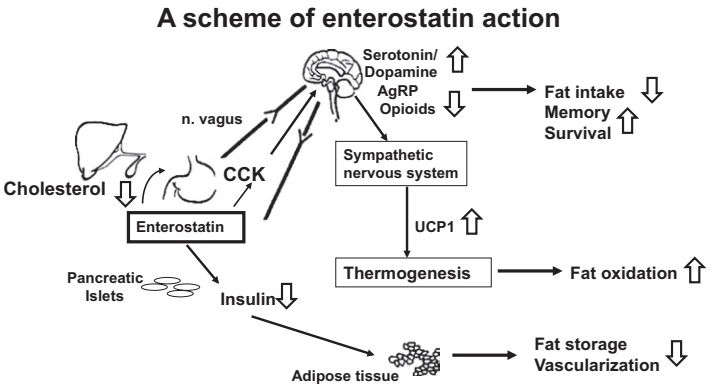

FIGURE 3 Effects of enterostatin: enterostatin is released from the intestine, where it activates CCK-dependent pathways to promote satiety, acting both peripherally to decrease gastric emptying and centrally to release serotonin. In this pathway, the vagal afferent nerves from the intestine to the brain is crucial. Regulation of fat intake by enterostatin further involves the stimulation of dopamine, suppression of AgRP, and suppression of the opioid system. In the islets, enterostatin inhibits insulin secretion, which is important for decreasing fat storage in the adipose tissue. At the same time, enterostatin stimulates thermogenesis through the activation of the sympathetic system and induction of UCP1, important for stimulation of fat oxidation. Low cholesterol levels occur through a CCK-dependent pathway. Enhanced memory by enterostatin is also dependent on CCK-pathways.

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possibilities to explain the enterostatin dependence on the CCK system.

One possible scenario is the influence of enterostatin on CCK-A-receptors present on vagal afferent neurons from the gastrointestinal tract to the brain and higher centers. This is further supported by the observation that vagal afferent neurons are necessary for the mediation of the reduction in fat intake by enterostatin. Another possibility is the release of CCK by enterostatin. The effect of enterostatin to suppress food intake during a longer period than CCK could be explained by a continued release of CCK in response to enterostatin. In the Sprague–Dawley rat, in which procolipase levels were increased through the administration of plant thylakoids,[1] there was also a strong release of CCK suggesting a parallel release between CCK and enterostatin, although the order of release is not known.

MECHANISM OF ENTEROSTATIN IN THE REGULATION OF FOOD INTAKE AND BODY WEIGHT REGULATION

The first experiments showing a specific effect of enterostatin as a regulator of fat intake were performed in Osborne–Mendel rats, rats that are specifically prone to eat fat.[7] In these, there was a dose-dependent reduction in fat intake but not on either protein or carbohydrate intake. In another experimental situation, Sprague–Dawley rats are given a free choice of a low-fat diet (5.2% fat by weight; 14.1% by energy) and a high-fat diet (17.8% fat by weight; 32.8% by energy) in separate containers. After injection of enterostatin into the lateral ventricle, the rats selectively decreased the intake of the high-fat diet by 45% ($p<0.005$), while the intake of the low-fat diet was unaffected compared with that in saline injected controls.[7] It was concluded that the effect of enterostatin to selectively inhibit fat intake is not specific to any special strain of rat. The experiments suggested that enterostatin could act as a feedback signal to regulate the intake of fat (Fig. 3). Dietary fat intake is also reduced after intragastric, intraduodenal, and intraperitoneal administration of enterostatin. The central administration of enterostatin near the celiac artery into the carotid artery likewise gives an immediate effect on feeding at low doses of enterostatin. The most sensitive nuclei to respond to enterostatin response are the PVN and amygdala, where enterostatin decreases meal size and duration.

The response in the gastrointestinal tract is dependent on vagal afferent signaling pathways, whereas the central effect is independent of vagal signaling, suggesting a local transmission of enterostatin signaling close to its production in the brain. By contrast, after intravenous administration of enterostatin a delayed effect on food intake is observed,[18] indicating that the targeting of enterostatin to its receptor protein in this situation is not an immediate process. Enterostatin binds to plasma proteins in the circulating blood, which might influence the response. Whether enterostatin is taken up by the blood–brain barrier to reach brain nuclei[10] after intravenous administration or whether it reaches a peripheral target is not known.

In another type of long-term treatment, rats are given a two-choice diet between high-fat diet and low-fat diet. After adaptation, the animals are chronically infused with enterostatin for 9 days (0.5 μg/h) or artificial cerebrospinal fluid (CSF) into the lateral ventricle of rats. Enterostatin reduces the intake of the high-fat diet with the maximum depression on day 4, without any compensatory increase in low-fat food intake.[7] The body weight of enterostatin-infused rats declines significantly and is associated with a reduction in fat pad and liver weight compared with that of the CSF-infused control rats.[7] Serum triglycerides and insulin are likewise decreased, whereas corticosterone is elevated in enterostatin-infused rats. These studies thus show that enterostatin reduces fat intake in long-term studies and that there is no overeating of the low-fat diet to compensate for the reduced food intake.

The appetite-regulating effect of enterostatin may occur through an involvement of serotonin-dependent pathways. In the presence of a serotonin receptor antagonist (5HT1b), the response of enterostatin is completely abolished.[14] Because enterostatin does not interact directly with a serotonin receptor, the mechanism of action could involve the ability of enterostatin to release serotonin.[9] Serotonin is released by 300% after enterostatin infusion into the lateral hypothalamic area, reaching a maximum 40 min after enterostatin administration. Serotonin may release the satiety hormone melanocyte-stimulating hormone (α-MSH) in the arcuate nucleus to suppress food intake.[11] In melanocortin receptor 4 (MC4R) knockout mice, enterostatin fails to influence satiety. Also the receptor antagonist SHU9119 essentially abolishes the inhibitory effect of enterostatin on food intake, suggesting that the melanocortin pathway is involved in the mechanism of enterostatin in suppressing fat intake.[11] Along the same lines, the long-term effect of enterostatin to reduce appetite for fat could be explained by the ability of enterostatin to decrease the expression of AgRP, a hunger signal specific for fat and interacting with the melanocortin receptor 4 in the amygdala and in the hypothalamus.[11]

There are also studies to suggest that enterostatin promotes the reuptake of dopamine to increase central striatal dopamine in rat.[34] This occurs only after 10 days of high-fat feeding and not after three days of high-fat feeding. High-fat feeding is known to impair the response of dopamine, and hence, enterostatin could act to prevent this downregulation of dopamine in this way promoting satiety for fat

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intake.[34] Thus, it seems that enterostatin may act to enhance both serotonin and dopamine at different sites in the brain.

The appetite-regulating effect of enterostatin may also occur through an involvement of opioid-dependent pathways. This idea came through because of the similarity in the structure between beta-casomorphin, a digest product of casein with the structure YPFPGPI, and enterostatin with the structure APGPR, both peptides containing proline residues, each bridged by an amino acid. Although beta-casomorphin stimulates fat intake, this effect is inhibited by enterostatin. However, at high doses of enterostatin, there is a stimulation of fat intake, suggesting that the response occurs through two different receptors, one high-affinity receptor and one low-affinity receptor. Beta-casomorphin binds to the F1-ATPase β-subunit, and it was suggested that enterostatin stimulates the binding of beta-casomorphin to the low-affinity receptor by inducing a conformational change in the receptor.[22] The enterostatin response may also occur through the interaction of either the kappa or the mu receptor pathway, although there is no direct binding to these receptors. The kappa-opioid pathway is important for the specific regulation of fat intake, whereas the mu-opioid pathway is more related to basal preferences.

The unusual property of enterostatin is that the main formation of enterostatin occurs in the lumen of the intestine, where food digestion occurs and where other food-derived peptides might form. This property justified experiments in which administration of enterostatin was mixed with food. In one such study, female NMRI mice were fed a high-fat diet with and without enterostatin (116 μg added per gram food) for 25 days, during which body weight and food intake were measured.[25] Food intake and body weight were significantly reduced in the animals receiving enterostatin added to food, showing that the action of enterostatin could really take place through the intestine. In these experiments, brown adipose uncoupling protein expression was measured and found to be elevated in the enterostatin-treated animals. This suggests that the decreased body weight is not only a consequence of a decreased food intake but also an increased diet-induced thermogenesis[25] (Fig. 3). The achievement of energy balance is dependent on the ambient temperature. At a temperature (24 °C), below the isothermal temperature for the mice (27 °C), the animals could increase energy expenditure and stay in energy balance when fed a high-fat diet, whereas at an ambient temperature of 27 °C, the animals failed to increase their expression of uncoupling proteins even during enterostatin treatment and hence became obese during high-fat feeding.[25] For enterostatin to increase energy expenditure, the ambient temperature must be lower than the isothermal temperature.[26]

Whether enterostatin has any importance for human body weight regulation and metabolism is not known. By investigation of the enterostatin precursor gene, procolipase including its promotor, it was found that reduced insulin secretion after an oral glucose test in nondiabetic humans was significantly associated with procolipase gene polymorphism.[31] This observation suggests that the procolipase/enterostatin gene might be a candidate gene for development of type-2 diabetes.[31]

The fact that fatty acid oxidation by enterostatin may be important under extreme conditions has been suggested by mice living in constant darkness.[35] In mice experimentally adapted to living in the dark 24 h a day, an increased expression of the procolipase gene is observed. This is increased not only in the gastrointestinal tract but also in the liver, brain, and adipose tissue, thus also showing ectopic expression.[35] Animals living in the dark must be able to recruit energy from their energy stores. It is concluded that these animals have an enhanced fatty acid oxidation and also a curbed appetite though the action of enterostatin/procolipase being overexpressed. Enterostatin/procolipase may be important in hibernating animals to sustain the burning of fat.

EFFECTS OF ENTEROSTATIN OTHER THAN FEEDING EFFECTS

Enterostatin displays various effects, including lowering of blood cholesterol, reduction of angiogenesis, regulation of protein trafficking, regulation of analgesia, and stimulation of memory (Table 2). Whether these are essential for feeding effects is not known but may be due to an interaction of enterostatin with several hormone systems or receptors, remembering the ability of small molecules to interact both specifically and nonspecifically with various receptors that by virtue of quantity can result in significant effects.

The effect of enterostatin to reduce blood cholesterol seems to be important because enterostatin knockout mice show significantly increased serum cholesterol levels.[19] This reduction in cholesterol levels occurs independent of the food suppressive effect of enterostatin and is dependent on cholecystokinin action.[29] The mechanism may be a stimulation of bile salt excretion into the feces by enterostatin, and after this, a conversion of cholesterol to bile salt resulting in reduced serum cholesterol levels takes place.[28]

Enterostatin also reduces angiogenesis, which may be important in reducing fat tissue[23] (Fig. 3). Adipose tissue is a highly vascularized tissue that has the capacity to expand throughout life. Some studies suggest that blocking of angiogenesis in the adipose tissue can help to induce loss of fat mass. By using angiogenic blockers in obese mice, loss of body fat has been induced. In human obesity, several angiogenic factors are increased in the circulation, including VEGF A, C, and D as well as the inflammatory cytokine interleukin-6 and insulin. In liver cells (Hep G2 cells), enterostatin inhibits angiogenesis through the suppression of angiogenic factors such as VEGF.[23] It is possible therefore

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TABLE 2 Properties of Enterostatin

Effects	Reference
Satiety for fat	7
Receptor: Beta-subunit F1-ATPase	2,22
Receptor increased by high-fat diet	15
Reduction of insulin	2
Reduction of serum cholesterol	29,18
Stimulation of fatty oxidation and thermogenesis	4,25
Promotion of memory	20
Promotion of antianalgesia	30
Inhibition of angiogenesis	23
Regulation of protein trafficking	21

that this antiangiogenic effect of enterostatin may contribute to the loss of adipose tissue that is observed after enterostatin administration. The release of fatty acids increases the availability for fatty acids to be oxidized.

Enterostatin can also act as a regulator of protein trafficking.[21] This could explain the inhibition of insulin secretion, where enterostatin causes a downregulation of the proteins needed for insulin sorting, such as dynamin2 (Fig. 3). Hence, enterostatin by virtue of its structure or small size could act as a regulator in many different ways.

Enterostatin has an antianalgesic effect in mice, suggesting that enterostatin acts as an antiopiate[28] (Fig. 3) (see the Antiopioid chapter in the Opioid Peptide section of this book). There is no direct binding of enterostatin to the mu receptor to explain the antianalgesic effect. Instead, this occurs through a CCK-receptor-dependent action, the CCK-receptor antagonist Lorglumide being able to totally abolish the antianalgesic effect of enterostatin.[29] Taken together, a variety of central actions of enterostatin, such as anorexia and antianalgesia seem to be mediated through a CCK-dependent mechanism.

Enterostatin has been reported to stimulate memory in mice after both central and oral administration shown in a step-through-type passive avoidance test.[20] In this, test mice are made to enter a dark room and are subjected to an electric shock. The latency to enter this room is measured with and without enterostatin. The memory-enhancing activity of enterostatin is completely blocked by a CCK-receptor antagonist lorglumide. Because enterostatin does not interact with the CCK receptor, enterostatin may release CCK, which activates the receptor. Several anorexic peptides have been shown to enhance memory, such as CCK, leptin, and CRH (discussed elsewhere in this book); thus, enterostatin is another example of such an activity. Because enterostatin has an effect through oral administration, it is possible that food that releases enterostatin may promote memory.

ACKNOWLEDGMENTS

The author's research on enterostatin and the regulation of fat intake has been possible through grants from the Swedish Medical Research Council (VR), Vinnova, FORMAS, and Crafoord Foundation, to whom the author expresses gratitude.

REFERENCES

1. Albertsson PA, Kohnke R, Emek SC, Mei J, Rehfeld JF, Akerlund HE, et al. Chloroplast membranes retard fat digestion and induce satiety: effect of biological membranes on pancreatic lipase/co-lipase. *Biochem J* 2007;**401**:727–33.
2. Berger K, Sivars U, Winzell MS, Johansson P, Hellman U, Rippe C, et al. Mitochondrial ATP synthase—a possible target protein in the regulation of energy metabolism in vitro and in vivo. *Nutr Neurosci* 2002;**5**:201–10.
3. Berger K, Winzell MS, Mei J, Erlanson-Albertsson C. Enterostatin and its target mechanisms during regulation of fat intake. *Physiol Behav* 2004;**83**:623–30.
4. Bray GA. Afferent signals regulating food intake. *Proc Nutr Soc* 2000;**59**:373–84.
5. Champagne E, Martinez LO, Collet X, Barbaras R. Ecto-F1Fo ATP synthase/F1 ATPase: metabolic and immunological functions. *Curr Opin Lipidol* 2006;**17**:279–84.
6. Erlanson-Albertsson C, Larsson A. A possible physiological function of pancreatic pro-colipase activation peptide in appetite regulation. *Biochimie* 1988;**70**:1245–50.
7. Erlanson-Albertsson C, York D. Enterostatin—a peptide regulating fat intake. *Obes Res* 1997;**5**:360–72.
8. Imamura M, Sumar N, Hermon-Taylor J, Robertson HJ, Prasad C. Distribution and characterization of enterostatin-like immunoreactivity in human cerebrospinal fluid. *Peptides* 1998;**19**:1385–91.
9. Koizumi M, Kimura S. Enterostatin increases extracellular serotonin and dopamine in the lateral hypothalamic area in rats measured by in vivo microdialysis. *Neurosci Lett* 2002;**320**:96–8.
10. Koizumi M, Nakanishi Y, Sato H, Morinaga Y, Ido T, Kimura S. Uptake across the blood–brain barrier and tissue distribution of enterostatin after peripheral administration in rats. *Physiol Behav* 2002;**77**:5–10.
11. Lin L, Park M, York DA. Enterostatin inhibition of dietary fat intake is modulated through the melanocortin system. *Peptides* 2007;**28**:643–9.
12. Lin L, Thomas SR, Kilroy G, Schwartz GJ, York DA. Enterostatin inhibition of dietary fat intake is dependent on CCK-A receptors. *Am J Physiol Regul Integr Comp Physiol* 2003;**285**:R321–8.
13. Lin L, York DA. Chronic ingestion of dietary fat is a prerequisite for inhibition of feeding by enterostatin. *Am J Physiol* 1998;**275**:R619–23.
14. Lin L, York DA. Amygdala enterostatin induces c-Fos expression in regions of hypothalamus that innervate the PVN. *Brain Res* 2004;**1020**:147–53.
15. Lindqvist A, Berger K, Erlanson-Albertsson C. Enterostatin up-regulates the expression of the beta-subunit of F[F(o)-ATPase in the plasma membrane of INS-1 cells. *Nutr Neurosci* 2008;**11**:55–60.

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16. Lindqvist A, Berger K, Erlanson-Albertsson C. Enterostatin upregulates the expression of the beta subunit of F1F0-ATPase in the plasma membranes of INS1-cells. *Nutr Neurosci* 2008;**11**:1–6.
17. Mei J, Sorhede-Winzell M, Erlanson-Albertsson C. Plasma enterostatin: identification and release in rats in response to a meal. *Obes Res* 2002;**10**:688–94.
18. Mei J, Erlanson-Albertsson C. Effect of enterostatin given intravenously and intracerebroventricularly on high-fat feeding in rats. *Regul Pept* 1992;**41**:209–18.
19. Miller R, D'Agostino D, Erlanson-Albertsson C, Lowe ME. Enterostatin deficiency increases serum cholesterol but does not influence growth and food intake in mice. *Am J Physiol Endocrinol Metab* 2009;**297**:E856–65.
20. Ohinata K, Sonoda S, Shimano T, Yoshikawa M. Enterostatin (APGPR) enhances memory consolidation in mice. *Peptides* 2007;**28**:719–21.
21. Park M, Farrell J, Lemmon K, York DA. Enterostatin alters protein trafficking to inhibit insulin secretion in Beta-TC6 cells. *Peptides* 2009;**30**:1866–73.
22. Park M, Lin L, Thomas S, Braymer HD, Smith PM, Harrison DH, et al. The F1-ATPase beta-subunit is the putative enterostatin receptor. *Peptides* 2004;**25**:2127–33.
23. Park M, Lyons III J, Oh H, Yu Y, Woltering EA, Greenway F, et al. Enterostatin inhibition of angiogenesis: possible role of pAMPK and vascular endothelial growth factor A (VEGF-A). *Int J Obes (Lond)* 2008.
24. Park M, Oh H, York DA. Enterostatin affects cyclic AMP and ERK signaling pathways to regulate Agouti-related protein (AgRP) expression. *Peptides* 2009;**30**:181–90.
25. Rippe C, Berger K, Boiers C, Ricquier D, Erlanson-Albertsson C. Effect of high-fat diet, surrounding temperature, and enterostatin on uncoupling protein gene expression. *Am J Physiol Endocrinol Metab* 2000;**279**:E293–300.
26. Rippe C, Berger K, Mei J, Lowe ME, Erlanson-Albertsson C. Effect of long-term high-fat feeding on the expression of pancreatic lipases and adipose tissue uncoupling proteins in mice. *Pancreas* 2003;**26**:e36–42.
27. Rippe C, Erlanson-Albertsson C, Lindqvist A. Consequences of metabolic challenges on hypothalamic colipase and PLRP2 mRNA in rats. *Brain Res* 2007;**1185**:152–7.
28. Takenaka Y, Nakamura F, Yamamoto T, Yoshikawa M. Enterostatin (VPDPR) and its peptide fragment DPR reduce serum cholesterol levels after oral administration in mice. *Biosci Biotechnol Biochem* 2003;**67**:1620–2.
29. Takenaka Y, Shimano T, Mori T, Hou IC, Ohinata K, Yoshikawa M. Enterostatin reduces serum cholesterol levels by way of a CCK(1) receptor-dependent mechanism. *Peptides* 2008;**29**:2175–8.
30. Takenaka Y, Shimano T, Yamada Y, Yoshida M, Ohinata K, Yoshikawa M. Enterostatin (APGPR) suppresses the analgesic activity of morphine by a CCK-dependent mechanism. *Peptides* 2008;**29**:559–63.
31. Weyrich P, Albet S, Lammers R, Machicao F, Fritsche A, Stefan N, et al. Genetic variability of procolipase associates with altered insulin secretion in non-diabetic Caucasians. *Exp Clin Endocrinol Diabetes* 2009;**117**:83–7.
32. Wu YJ, Hughes D, Lin L, Braymer DH, York DA. Comparative study of enterostatin sequence in five rat strains and enterostatin binding proteins in rat and chicken serum. *Peptides* 2002;**23**:537–44.
33. York DA, Lin L, Thomas SR, Braymer HD, Park M. Procolipase gene expression in the rat brain: source of endogenous enterostatin production in the brain. *Brain Res* 2006;**1087**:52–9.
34. York DA, Teng L, Park-York M. Effects of dietary fat and enterostatin on dopamine and 5-hydroxytrytamine release from rat striatal slices. *Brain Res* 2010;**1349**:48–55.
35. Zhang J, Kaasik K, Blackburn MR, Lee CC. Constant darkness is a circadian metabolic signal in mammals. *Nature* 2006;**439**:340–3.

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Chapter 147

Galanin

Jessica R. Barson, Irene Morganstern and Sarah F. Leibowitz

ABSTRACT

Galanin (GAL) plays an important role in stimulating ingestive behavior, acting particularly through the paraventricular nucleus of the hypothalamus (PVN). This peptide exhibits a special relationship with dietary fat and alcohol, stimulating the ingestion of these two substances and being stimulated by them. A large number of signaling molecules interact with GAL, including hormones, neuropeptides, and monoamines, but lipids and inflammatory cytokines show a particularly strong relationship with GAL, possibly mediating the interaction of fat and ethanol with this peptide. Although GAL may have provided an evolutionary advantage by specifically promoting intake of the most energy-efficient macronutrient, fat, it has taken on a nonhomeostatic role in the present-day environment, promoting not only overconsumption of dietary fat but also of the drug alcohol.

INTRODUCTION

Galanin (GAL) is a 29–30 amino acid peptide that is well-conserved among species, including rats and humans.[26] It is widely distributed throughout the brain, including the emotion-regulating amygdala and the feeding-regulating hypothalamus.[4] Within the hypothalamus, GAL is expressed in many neuronal populations, including the paraventricular nucleus (PVN), dorsomedial nucleus, and arcuate nucleus. This peptide is involved in many physiological and psychological processes, including pain, epilepsy, and depression; however, it is also important in ingestive behavior, which is the focus of this review.

EFFECTS OF GALANIN INJECTION ON INGESTIVE BEHAVIOR

The peptide GAL stimulates feeding, primarily through its actions within the hypothalamus. The first study to examine these effects found that acute hypothalamic injection of GAL stimulated food intake in rats.[24] Although this effect is smaller than that seen with the potent orexigenic hypothalamic peptide, neuropeptide Y, it is robust and has since been replicated in multiple studies.[26] This stimulatory effect of GAL on food intake is strongest with injection in the medial parvocellular division of the PVN and sharply diminishes in magnitude as the injection site moves to the lateral PVN and then beyond this nucleus in all directions.[26] Galanin can, however, increase food intake with injection in a limited number of nuclei outside of the hypothalamus, including the central nucleus of the amygdala and, under certain conditions, in the hindbrain nucleus of the solitary tract and the lateral parabrachial nucleus.[4]

The specific orexigenic effects of GAL injection have primarily been examined with the macronutrients and with ethanol. With regard to macronutrients, GAL's effects are most strongly linked to fat. Although injections do not affect the preference for pure fat over carbohydrate, protein, or laboratory chow, the GAL-induced feeding response is stronger and more prolonged in subjects maintained on a high-fat (compared with those on a low-fat) diet and also in strains of rats that naturally prefer fat.[4,26] The intake of fat is reduced by PVN injection of the general GAL antagonists, C7, galantide, and M40 and by repeated PVN injections of antisense oligonucleotides to GAL mRNA, which decrease GAL peptide levels.[26] Chronic injections of GAL cause rats to overeat and eventually become overweight but only if the diet contains at least 30% fat.[26] This special relationship with fat is consistent with the idea that GAL may be involved in the large meal size and overeating that occurs when fat-rich foods are available.

Of other ingested substances examined for their relationship with GAL, alcohol intake shows the most robust stimulation by GAL injection. In rats, injection of GAL into the PVN or adjacent third ventricle enhances the drinking of ethanol at a variety of concentrations, in the dark or light and with or without food concurrently available.[4] The opposite effect occurs with injection of the GAL antagonist M40 into these regions.[4] and peripheral injection of the specific GAL 3 receptor antagonist SNAP 37889 decreases operant responding for ethanol.[2] As with fat, the GAL-induced ethanol drinking response is strongest with injection in the PVN and in fact has not been found to occur with injection in other areas of the hypothalamus or outside of the

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00147-0
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hypothalamus.[4] Aside from ethanol and fat, the effect of GAL injection on the ingestion of other substances has only been reported in one study which found operant responding for both sucrose and saccharin to be decreased by the same peripheral GAL 3 receptor antagonist that decreased operant responding for ethanol.[2]

Endogenous GAL also drives fat and ethanol intake. Gene expression and GAL peptide levels are increased in the anterior parvocellular region of the PVN during food deprivation, when the drive for fat intake is exacerbated.[4] In free-feeding rats, hypothalamic GAL reaches peak levels during the middle of the feeding cycle, when the preference for fat naturally rises.[26] Elevated hypothalamic GAL has also been described in strains of rodents, both rats and mice, which have a strong, natural preference for fat, even if they do not have access to a high-fat diet. These include multiple lines of inbred mice, obese Zucker rats, and Brattleboro (di/di) rats.[4] Interestingly, Brattleboro rats drink more ethanol than controls do.[29]

GENETIC MANIPULATION OF GALANIN

Genetic manipulation of GAL in mice has yielded further support for the stimulatory effect of this peptide on fat and ethanol intake and has further confirmed the specificity of this relationship. Mice with deletions of the GAL gene consume less fat both with chronic access and in acute meal tests, and they show a decreased preference for fat compared with that of littermate wild-type controls.[4] Mice missing the GAL 1 receptor also consume less fat than wild-type mice do.[4] By contrast, mice that overexpress GAL consume more of a high-fat diet compared with wild-type controls.[4] Similarly, GAL knockout mice drink less ethanol and show a lower preference for ethanol than wild-type mice do, whereas GAL overexpressors drink more and show a higher preference.[4] These changes in ingestive behavior seem to be specific to dietary fat and ethanol. Neither GAL knockout nor GAL overexpressing mice show differences in sucrose or quinine preference,[4] and GAL knockout mice also show no difference in overall cocaine self-administration.[10] Further, despite substantial evidence that hypothalamic GAL injection stimulates the intake of laboratory chow, GAL knockouts and overexpressors consume chow at the same levels as wild-type mice and consequently have similar body weight when maintained on a low-fat diet.[21] Therefore, studies with genetically manipulated mice suggest that GAL drives ingestive behavior specifically of fat and ethanol.

EFFECTS OF INGESTIVE BEHAVIOR ON GALANIN

In addition to driving fat and ethanol intake, GAL expression and levels are also increased by the intake of these substances, suggesting that GAL exists as part of a nonhomeostatic, positive feedback loop that can promote excessive fat or ethanol intake. With regard to the macronutrients, consumption of a diet high in fat compared with one high in carbohydrates or a balanced, control diet for 1–4 weeks stimulates gene expression and peptide production of GAL in the PVN.[3,4] This is seen particularly in the anterior and middle parvocellular regions of this nucleus, where GAL is positively correlated with the amount of fat consumed.[3,4] Moreover, outbred rats made obese by their diet also have elevated GAL expression in the PVN, although only if the diet contains a sufficient amount of fat rather than carbohydrate.[4] Conversely, pharmacological blockade of fat oxidation reduces the PVN expression of GAL and stimulates PVN gene expression of the GAL 1 receptor.[4] Thus, in addition to stimulating fat ingestion, the intake of dietary fat, through signals arising from fat oxidation, itself stimulates the production of GAL.

These effects of dietary fat intake in adults can also be seen in the offspring of dams that consumed a fat-rich diet during pregnancy. Gene expression and peptide levels of GAL in the PVN are elevated at birth and through adulthood in rats prenatally exposed to a diet high in fat as compared with a carbohydrate-rich or balanced, control diet.[5,11] This effect is probably specific to the macronutrient fat, as it does not occur with prenatal exposure to a high-carbohydrate, low protein diet compared with a balanced diet.[7] The effect of fat seems to occur by stimulating the proliferation and differentiation of developing neurons that ultimately express GAL in the PVN.[11] As expected from animals with higher levels of GAL, these fat-exposed offspring also show a higher preference for fat after puberty.[11] Thus, exposure to fat *in utero* reprograms the brain to express GAL at higher levels over the long term which can then lead animals to ingest excessive amounts of dietary fat.

In addition to fat, the ingestion of ethanol also stimulates GAL expression and levels in the PVN. This occurs in adults with chronic drinking or acute ethanol injection and, like fat, is seen preferentially in the anterior and medial portions of the PVN.[4,12] Ethanol drinking also correlates with hypothalamic gene expression of the GAL 1 receptor.[4] These studies suggest that GAL functions in a positive feedback loop with ethanol, just as it does with fat.

ROLE OF GALANIN RECEPTORS

The peptide GAL can interact with any of three G-protein-coupled receptors, GalR1, GalR2, or GalR3, all of which are expressed within the hypothalamus.[32,33] Although GalR1 and GalR3 are believed to be G_i/G_o-coupled receptors, GalR2 seems to be coupled to a G_q/G_i-mediated pathway.[32,33] The evidence to date suggests that it is the GalR1 that mediates the role of GAL in fat intake, whereas GalR1 or GalR3 may mediate its role in ethanol intake. Mutant mice lacking the GalR1 gene consume fewer calories than

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wild-type mice when fed a high-fat rather than low-fat diet,[4] and ventricular injection of the specific GalR1 agonist M617 stimulates outbred rats to ingest more high-fat milk.[30] Expression of GalR1 is also higher in the PVN of fat-preferring Brattleboro rats[25] as well as after one week of high-fat compared with balanced diet intake in outbred rats.[3] By contrast, food intake is no different between GalR2 knockout and wild-type mice,[18] and intake of neither high-fat milk nor cookie mash is altered by ventricular injection of any of the several GalR2 agonists.[30] With ethanol intake, GalR1 expression in the hypothalamus correlates with ethanol drinking.[4] In support of the role of the GalR3, peripheral injection of the GalR3 antagonist SNAP 37889 decreases operant responding for ethanol, although it also reduces it for sucrose and saccharin.[2] Although more studies need to be conducted on the role of the GAL receptors in ingestive behavior, the available evidence supports a strong role of GalR1 in fat and possibly ethanol intake and also points to a role for GalR3 in ethanol intake.

EFFECTS OF HORMONES ON GALANIN

The positive feedback loop between fat intake and GAL may be mediated, in part, by reproductive hormones. In female rats, fat ingestion stimulates circulating levels of estrogen and progesterone.[4] In addition, it increases GAL levels not only in the PVN but also in the median eminence, the medial preoptic nucleus just anterior to the PVN and in the nearby anterior pituitary. Notably, in ovariectomized females, GAL expression and levels in the hypothalamus and anterior pituitary are stimulated by injection of estradiol in a dose- and time-dependent manner, and they are stimulated even further by progesterone replacement after estrogen priming.[4] Also, in intact females, GAL peptide levels increase in the anterior parvocellular PVN, median eminence and medial preoptic nucleus during proestrus, a time when estrogen and progesterone levels naturally increase.[4] Notably, the preference for fat also increases in females during proestrus and is found to be greater in female rats with higher GAL than in males.[4] Although the regulation of GAL by estrogen and progesterone has received a great deal of attention, GAL may also be regulated by reproductive hormones in males. Hypothalamic GAL gene expression in castrated male rats is increased by treatment with either estrogen or testosterone.[14]

The relationship between fat intake, sex steroids, and GAL can also be seen in female rats during puberty. Although GAL levels steadily increase in the hypothalamus starting as early as day 15 of gestation, greater levels are not seen in females compared with that of males until puberty.[17] It is during this time (around day 40 in rats) that females begin to show an increase in their preference for fat.[4] In rats examined at different ages before (day 30) and after (days 45 and 60) puberty, this rise in fat intake is associated with a marked increase, from days 30 to 45, in the levels of estradiol and progesterone and also in GAL mRNA or peptide levels, specifically in the PVN and medial preoptic nucleus but not in other hypothalamic areas.[4] This positive relationship between increased fat intake, sex steroids, and GAL across ages is also evident in pubertal rats that naturally prefer fat (>25% of total diet) compared with those consuming little fat (<15%) or rats that reach puberty at an early age (days 30–34) compared with those that are late (days 37–40). These rats with early puberty onset exhibit a strong fat preference 3–4 days before vaginal opening, which is positively related to steroid levels, GAL, fat intake and also with body fat accrual after puberty.[4] These findings suggest that one signal for puberty onset may involve a surge in steroids that stimulate GAL to increase fat intake in females.

INTERACTIONS BETWEEN GALANIN AND OTHER OREXIGENIC TRANSMITTERS

The stimulatory effects of PVN GAL on fat intake may occur through interaction with any of a number of other orexigenic neurochemicals, including neuropeptide Y (NPY), norepinephrine (NE), dopamine (DA), and the opioid peptide enkephalin (ENK). Although NPY is produced in many areas of the brain, it exerts its potent feeding effects predominantly through transcription in the arcuate nucleus and release within the hypothalamus.[26] In contrast to GAL, NPY is more closely linked with carbohydrates rather than fat. Injection of NPY into the PVN stimulates ingestion of a high-carbohydrate diet in preference to a high-fat or high-protein diet,[27] while ingestion of a carbohydrate-rich diet increases levels of NPY specifically in the parvocellular area of the PVN.[23,34] Although GAL has been found to stimulate NPY secretion from primary hypothalamic neurons in culture,[9] inhibitory NPY axons projecting from the arcuate nucleus also surround and make contact with GAL cells in the PVN.[22] These findings imply that GAL stimulates NPY but that NPY in turn inhibits GAL. This negative feedback by NPY suggests that the ingestion of carbohydrates by stimulating NPY may, by inhibition of GAL, causes a reduction in subsequent fat intake. Notably, the same high-carbohydrate meal that stimulates NPY levels in the PVN also reduces GAL in this nucleus.[34] Further, Brattleboro di/di rats, which consume more fat and less carbohydrate and have higher expression of PVN GAL, also have lower NPY levels in the PVN.[6]

With NE, PVN GAL may work in part by stimulating this neurochemical. The injection of NE in the PVN produces a rapid feeding response in satiated rats,[4] which is comparable in magnitude to that induced by GAL.[4] With both GAL and NE, the PVN is the nucleus that yields the strongest effect on feeding, as most other brain areas are generally unresponsive.[4] In addition to GAL coexisting

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with NE in cells of the PVN, GAL also seems to stimulate NE release. Injection of GAL into the PVN increases levels of NE in this nucleus by nearly 90%, whereas GAL-induced feeding is inhibited by an α_2-noradrenergic receptor antagonist or a compound that inhibits NE synthesis.[4] The stimulatory effect of GAL on food intake may thus be due, in part, to downstream effects on NE release.

Another downstream effect of GAL activity may be an increase in mesolimbic DA levels. The ventral tegmental area, which contains the dopaminergic cell bodies that project to the nucleus accumbens (NAc), expresses all three GAL receptor types, and GAL binding in this area has been demonstrated with autoradiography.[4] Local application of GAL in the ventral tegmental area enhances the accumulation of DA metabolites in the striatum, indicating an increase in the rate of DA synthesis.[4] Importantly, PVN injection of GAL increases extracellular levels of DA in the NAc shell, and it does so only in animals that, on separate test days, show a feeding response to PVN GAL.[4] This DA conveys signals of the incentive-motivational properties of a reward such as a meal high in fat or a drink of ethanol.[4] These results suggest that GAL in the PVN stimulates ingestive behavior, in part, by increasing accumbal DA levels. Interestingly, just as ingestion of a high-fat diet or ethanol stimulates GAL in the PVN, they also increase levels of DA in the NAc shell.[4] Therefore, GAL may stimulate NAc DA release, to enhance not just the consumption of fat and ethanol but also the motivational aspects of their consumption.

Evidence also strongly suggests that GAL stimulates ENK within the hypothalamus. As with GAL, ENK has its strongest feeding effects in the PVN, and the injection of ENK analogs stimulates consumption of a high-fat over a high-carbohydrate diet as well as ingestion of ethanol over food or water.[4] Administration of ENK analogs into the PVN, much like GAL, also increases levels of DA in the NAc shell.[4] Similar to that occurring with NE, injection of GAL just dorsal to the PVN stimulates expression of ENK in this nucleus,[4] which may occur through GalR1-induced inhibition of the many γ-aminobutyric acid-containing neurons in the PVN.[3] Galanin-induced fat intake in particular seems to be driven by its effects on PVN ENK. Not only can fat ingestion induced by GAL injection be blocked by pretreatment with peripheral or central injection of the general opioid antagonist, naloxone, or by a mu-opioid receptor antagonist[4] but also fat intake induces the co-expression of GAL and ENK within the same parvocellular neurons of the PVN.[3] This suggests that, within the hypothalamus, GAL stimulates and may act in concert with PVN ENK.

Outside of the hypothalamus, the relationship between GAL and ENK is less straightforward. In mice lacking the GAL gene, drug reward is decreased[28] while symptoms of withdrawal from the general opioid agonist morphine are increased.[4] In contrast, morphine withdrawal is lessened in GAL overexpressing mice or by centrally administered GAL or the GAL agonist, galnon, in wild-type mice.[4] These differences seem to result from transcriptional changes in mesolimbic regions, including the NAc and amygdala where, for example, morphine increases CREB phosphorylation in GAL knockout but not in wild-type mice.[19] Although GAL outside of the hypothalamus may antagonize the effects of the opioids, it may also simply be acting to replace them.

CLOSE RELATIONSHIP BETWEEN GALANIN AND CIRCULATING LIPIDS

The particular relationship of GAL with fat and ethanol may be mediated in part by circulating lipids, specifically triglylcerides (TG). Fat and ethanol both increase levels of circulating TG when taken acutely or chronically.[4] With fat, this occurs in direct proportion to the amount consumed, with TG levels rising higher and remaining elevated for longer periods after fat-rich meals[4] and with diets higher in saturated compared with unsaturated fat.[3] Behaviorally, there is also a close, positive relationship between fat and ethanol ingestion that, in turn, may be associated with circulating TGs. Rats maintained on a high-fat diet or exhibiting a preference for fat are found to consume more ethanol.[4] Similarly, injection of ethanol preferentially stimulates ingestion of fat relative to carbohydrate.[4] Clinical studies show that fat intake is greater in ethanol drinkers, with bingeing on fat-rich foods associated with high rates of alcohol use disorder and drinkers on a fat-rich diet compared with a carbohydrate-rich diet exhibiting shorter periods of abstinence from ethanol.[4] In support of the idea that TG mediates this relationship, there is evidence that ethanol consumption is stimulated by the lipid emulsion Intralipid, which increases TG levels[4] and is reduced by the fibrate drug, gemfibrozil, which lowers them.[4] In addition, whereas dietary fat and ethanol both elevate TG levels, their consumption together produces a considerably larger increase in TGs than does the ingestion of ethanol with a low-fat diet or of a fat-rich diet without the intake of ethanol.[4]

This interaction between fat, ethanol, and TGs may operate centrally through effects on PVN GAL. In recent studies, GAL in the PVN has been found to be strongly, positively related to circulating levels of TG.[4] This relationship with GAL is robust, seen in different models of dietary obesity and even with acute exposure to a high-fat diet, supporting its independence from changes in body weight and adiposity-related hormones. Further, it is seen with both acute and chronic ingestion of a diet high in saturated, compared with unsaturated fat[3] suggesting that GAL is particularly associated with saturated fatty acids. Injection of Intralipid, which increases TG levels, also stimulates GAL expression in the PVN.[4] Although TGs themselves may not have a direct effect on GAL gene expression, fatty acids from the hydrolysis of TGs can cross the blood

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brain of barrier to exert central effects. Although not yet examined with GAL, short-chain fatty acids have been shown to regulate gene expression of ENK[4] that, as described above, acts in close relation to GAL. Although fatty acids from TGs may stimulate GAL, this relationship may also work in reverse, with PVN GAL increasing TG levels. Rats and mice that exhibit a preference for fat, compared with those preferring carbohydrate, have higher levels of circulating TGs in addition to elevated GAL in the PVN.[4] Further, transgenic mice that overexpress GAL have a ten-fold increase in circulating TG levels, even when maintained on a low-fat chow diet and not overeating this diet.[4] The relationship between TGs and GAL, just as with fat or ethanol and GAL, may therefore, be bidirectional, forming part of a positive feedback loop that can lead to escalating fat or ethanol intake.

ROLE OF INFLAMMATION IN INCREASED GALANIN

The relationship of GAL with fat and ethanol may also be associated with inflammation, specifically inflammatory cytokines. Levels of interleukin-1 beta (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF-α) have been found to increase in the hypothalamus after chronic high-fat feeding[13] or acute ethanol administration.[15] Not only are receptors for these cytokines transcribed within the PVN[8] but the parvocellular PVN, where GAL is most significantly affected by fat and ethanol, is also particularly stimulated by the cytokines.[16] Compellingly, GAL gene expression in cultured cells is stimulated by the application of IL-1β or TNF-α.[1] indicating that cytokines induced by fat or ethanol may directly stimulate GAL transcription. An increase in PVN GAL may be necessary for proper responding to cellular damage induced by fat or ethanol ingestion. The normal neurogenic inflammatory response to injury or TNF-α application to the skin is absent or greatly diminished in GAL knockout compared with wild-type mice.[31] Although still preliminary, this evidence points to a specific role for inflammation and inflammatory cytokines in mediating the stimulation of GAL by dietary fat and ethanol.

CLINICAL SUPPORT

Although far less numerous than the animal studies, several clinical studies have also examined GAL in relation to fat and alcohol, and they generally support the animal work. Consistent with the increase in GAL after fat intake or potentially with its stimulation of fat intake, GAL peptide is elevated in the serum of obese menopausal women, whereas it is reduced in the cerebrospinal fluid of recovered anorexic women.[4] Just as with animals, however, it does not seem to be directly related to body weight *per se*. No allelic differences in the GAL or GalR1 genes have been found in obese children or adolescents compared with healthy underweight young adults, and, perhaps not surprisingly, no association has been detected between obese phenotypes and GalR2.[4] In support of the idea that circulating TG may be related to an increase in GAL, the Rs2187331 allele, which resides in promoter regions of the GAL gene, has recently been associated with increased circulating levels of TG.[4] Emerging evidence also supports a role for GAL in alcohol intake. Single-nucleotide polymorphisms on the GAL and GalR3 genes have been associated with alcoholism in adult men.[4] Intriguingly, a recent study has found that the levels of serum GAL are lowest immediately after ethanol discontinuation in alcohol-dependent patients and are negatively correlated with alcohol craving.[20] Although these results may suggest that alcohol intake can inhibit GAL, they could also indicate that drinking, by potently stimulating GAL release, may in long-time drinkers cause a depletion of GAL stores.

SUMMARY AND CONCLUSIONS

The peptide GAL plays an important role in stimulating ingestive behavior, largely acting through the hypothalamus and particularly through the parvocellular PVN. Injection of GAL into this nucleus stimulates the intake of diets high in fat and also the drinking of ethanol, whereas GAL antagonists inhibit these behaviors. Endogenously, hypothalamic GAL expression and levels increase during fasting and in the middle of the feeding cycle when the preference for fat is higher, and they are also elevated in rats and mice that prefer fat. Genetic knockout of GAL diminishes fat and ethanol intake, while overexpression specifically increases it. In turn, the intake of fat or ethanol, whether acute or chronic, during adulthood or *in utero*, further stimulates PVN GAL, acting through a positive feedback loop than can escalate fat and ethanol intake. These effects seem to occur largely through the GalR1, but the effects with ethanol may also be through the GalR3. A large number of signaling molecules interact with GAL, including hormones, neuropeptides, monoamines, lipids, and cytokines. In females, estrogen and progesterone, which are increased during proestrus and at puberty, stimulate hypothalamic GAL and, in turn, increase fat intake. Galanin stimulates the potently orexigenic NPY but is itself inhibited by it, which may be one mechanism for curtailing the positive feedback between GAL and fat intake. Instead, PVN GAL may work in part by stimulating the release of hypothalamic NE and accumbal DA, which can increase the motivational aspects of fat and ethanol intake. In addition, GAL stimulates the opioid peptide ENK within the PVN and may act in concert with ENK in response to fat intake. The specific relationship of GAL with fat and ethanol suggests that these two ingested substances share a special relationship. Indeed, the intake of one can increase the intake of the other, and this may

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occur through stimulation of the circulating lipids, TGs. Not only does an increase in TGs lead to higher expression of PVN GAL, but also higher GAL may lead to higher TGs, suggesting that a bidirectional relationship exists between these two molecules. In addition to lipids, inflammatory cytokines may also stimulate PVN GAL. Levels of cytokines are increased in the hypothalamus after high-fat or ethanol exposure, and they have been shown in culture to stimulate GAL expression. Although the majority of this work has been conducted in rats and mice, clinical studies largely support these findings. In an environment with limited food availability, GAL may have provided an evolutionary advantage by promoting the intake of fat, which is the most energy efficient of the macronutrients. In the present-day environment, where fat-rich foods are abundant and alcohol is widely available, GAL has taken on a non-homeostatic role, promoting not only overconsumption of dietary fat but also of the drug alcohol.

ACKNOWLEDGMENTS

This research was supported by USPHS grants AA12882 and DA21518.

REFERENCES

1. Ait-Ali D, Turquier V, Grumolato L, Yon L, Jourdain M, Alexandre D, et al. The proinflammatory cytokines tumor necrosis factor-alpha and interleukin-1 stimulate neuropeptide gene transcription and secretion in adrenochromaffin cells via activation of extracellularly regulated kinase 1/2 and p.38 protein kinases, and activator protein-1 transcription factors. *Mol Endocrinol* 2004;**18**:1721–39.
2. Ash BL, Zanatta SD, Williams SJ, Lawrence AJ, Djouma E. The galanin-3 receptor antagonist, SNAP 37889, reduces operant responding for ethanol in alcohol-preferring rats. *Regul Pept* 2011;**166**:59–67.
3. Barson JR, Chang GQ, Poon K, Morganstern I, Leibowitz SF. Galanin and the orexin 2 receptor as possible regulators of enkephalin in the paraventricular nucleus of the hypothalamus: relation to dietary fat. *Neuroscience* 2011;**193**:10–20.
4. Barson JR, Morganstern I, Leibowitz SF. Galanin and consummatory behavior: special relationship with dietary fat, alcohol and circulating lipids. *EXS* 2010;**102**:87–111.
5. Beck B, Kozak R, Moar KM, Mercer JG. Hypothalamic orexigenic peptides are overexpressed in young Long-Evans rats after early life exposure to fat-rich diets. *Biochem Biophys Res Commun* 2006;**342**:452–8.
6. Beck B, Max JP. Neuropeptide Y in the arcuato-paraventricular pathway and diet selection in the vasopressin-deficient Brattleboro rat. *Brain Res Bull* 2008;**76**:454–7.
7. Bellinger L, Sculley DV, Langley-Evans SC. Exposure to undernutrition in fetal life determines fat distribution, locomotor activity and food intake in ageing rats. *Int J Obes (Lond)* 2006;**30**:729–38.
8. Benrick A, Schele E, Pinnock SB, Wernstedt-Asterholm I, Dickson SL, Karlsson-Lindahl L, et al. Interleukin-6 gene knockout influences energy balance regulating peptides in the hypothalamic paraventricular and supraoptic nuclei. *J Neuroendocrinol* 2009;**21**:620–8.
9. Bergonzelli GE, Pralong FP, Glauser M, Cavadas C, Grouzmann E, Gaillard RC. Interplay between galanin and leptin in the hypothalamic control of feeding via corticotropin-releasing hormone and neuropeptide Y. *Diabetes* 2001;**50**:2666–72.
10. Brabant C, Kuschpel AS, Picciotto MR. Locomotion and self-administration induced by cocaine in 129/OlaHsd mice lacking galanin. *Behav Neurosci* 2010;**124**:828–38.
11. Chang GQ, Gaysinskaya V, Karatayev O, Leibowitz SF. Maternal high-fat diet and fetal programming: increased proliferation of hypothalamic peptide-producing neurons that increase risk for overeating and obesity. *J Neurosci* 2008;**28**:12107–19.
12. Chang GQ, Karatayev O, Ahsan R, Avena NM, Lee C, Lewis MJ, et al. Effect of ethanol on hypothalamic opioid peptides, enkephalin, and dynorphin: relationship with circulating triglycerides. *Alcohol Clin Exp Res* 2007;**31**:249–59.
13. De Souza CT, Araujo EP, Bordin S, Ashimine R, Zollner RL, Boschero AC, et al. Consumption of a fat-rich diet activates a proinflammatory response and induces insulin resistance in the hypothalamus. *Endocrinology* 2005;**146**:4192–9.
14. Delemarre-van de Waal HA, Burton KA, Kabigting EB, Steiner RA, Clifton DK. Expression and sexual dimorphism of galanin messenger ribonucleic acid in growth hormone-releasing hormone neurons of the rat during development. *Endocrinology* 1994;**134**:665–71.
15. Emanuele NV, LaPaglia N, Kovacs EJ, Emanuele MA. The impact of burn injury and ethanol on the cytokine network of the mouse hypothalamus: reproductive implications. *Cytokine* 2005;**30**:109–15.
16. Ferri CC, Ferguson AV. Interleukin-1 beta depolarizes paraventricular nucleus parvocellular neurones. *J Neuroendocrinol* 2003;**15**:126–33.
17. Gabriel SM, Kaplan LM, Martin JB, Koenig JI. Tissue-specific sex differences in galanin-like immunoreactivity and galanin mRNA during development in the rat. *Peptides* 1989;**10**:369–74.
18. Gottsch ML, Zeng H, Hohmann JG, Weinshenker D, Clifton DK, Steiner RA. Phenotypic analysis of mice deficient in the type 2 galanin receptor (GALR2). *Mol Cell Biol* 2005;**25**:4804–11.
19. Hawes JJ, Brunzell DH, Narasimhaiah R, Langel U, Wynick D, Picciotto MR. Galanin protects against behavioral and neurochemical correlates of opiate reward. *Neuropsychopharmacology* 2008;**33**:1864–73.
20. Heberlein A, Muschler M, Frieling H, Lenz B, Wilhelm J, Groschl M, et al. Decreased galanin serum levels are associated with alcohol-craving during withdrawal. *Prog Neuropsychopharmacol Biol Psychiatry* 2011;**35**:568–72.
21. Hohmann JG, Krasnow SM, Teklemichael DN, Clifton DK, Wynick D, Steiner RA. Neuroendocrine profiles in galanin-overexpressing and knockout mice. *Neuroendocrinology* 2003;**77**:354–66.
22. Horvath TL, Naftolin F, Leranth C, Sahu A, Kalra SP. Morphological and pharmacological evidence for neuropeptide Y-galanin interaction in the rat hypothalamus. *Endocrinology* 1996;**137**:3069–78.
23. Jhanwar-Uniyal M, Beck B, Jhanwar YS, Burlet C, Leibowitz SF. Neuropeptide Y projection from arcuate nucleus to parvocellular division of paraventricular nucleus: specific relation to the ingestion of carbohydrate. *Brain Res* 1993;**631**:97–106.
24. Kyrkouli SE, Stanley BG, Leibowitz SF. Galanin: stimulation of feeding induced by medial hypothalamic injection of this novel peptide. *Eur J Pharmacol* 1986;**122**:159–60.
25. Landry M, Aman K, Burlet A, Hokfelt T. Galanin-R1 receptor mRNA expression in the hypothalamus of the Brattelboro rat. *Neuroreport* 1999;**10**:2823–7.

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26. Leibowitz SF. Hypothalamic galanin and ingestive behavior: relation to dietary fat, alcohol, and circulating lipids. In: Kastin A, editor. *The handbook of biologically active peptides*. Burlington, MA: Academic Press; 2006.
27. Morley JE, Levine AS, Gosnell BA, Kneip J, Grace M. Effect of neuropeptide Y on ingestive behaviors in the rat. *Am J Physiol* 1987;**252**:R599–609.
28. Narasimhaiah R, Kamens HM, Picciotto MR. Effects of galanin on cocaine-mediated conditioned place preference and ERK signaling in mice. *Psychopharmacology (Berl)* 2009;**204**:95–102.
29. Rigter H, Crabbe J. Ethanol preference in homozygous diabetes insipidus (Brattleboro) rats: effect of vasopressin fragments. *Ann NY Acad Sci* 1982;**394**:663–71.
30. Saar I, Runesson J, McNamara I, Jarv J, Robinson JK, Langel U. Novel galanin receptor subtype specific ligands in feeding regulation. *Neurochem Int* 2011;**58**:714–20.
31. Schmidhuber SM, Starr A, Wynick D, Kofler B, Brain SD. Targeted disruption of the galanin gene attenuates inflammatory responses in murine skin. *J Mol Neurosci* 2008;**34**:149–55.
32. Smith KE, Forray C, Walker MW, Jones KA, Tamm JA, Bard J, et al. Expression cloning of a rat hypothalamic galanin receptor coupled to phosphoinositide turnover. *J Biol Chem* 1997;**272**:24,612–6.
33. Smith KE, Walker MW, Artymyshyn R, Bard J, Borowsky B, Tamm JA, et al. Cloned human and rat galanin GALR3 receptors. Pharmacology and activation of G-protein inwardly rectifying K+ channels. *J Biol Chem* 1998;**273**:23,321–6.
34. Wang J, Dourmashkin JT, Yun R, Leibowitz SF. Rapid changes in hypothalamic neuropeptide Y produced by carbohydrate-rich meals that enhance corticosterone and glucose levels. *Brain Res* 1999;**848**: 124–36.

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Chapter 148

Ghrelin

Omar Al-Massadi, Kristy M. Heppner, Rubén Nogueiras, Diego Pérez-Tilve and Matthias H. Tschöp

ABSTRACT

Ghrelin is the only known circulating hormone with strong orexigenic activity. No other peripheral endocrine agent has comparable effects on hunger and energy balance. Although the first evidence of the contribution of ghrelin to the control of food intake was obtained soon after its discovery, the precise mechanisms whereby ghrelin regulates feeding have remained largely unknown. However, recent reports have begun to provide novel insights into the effects of ghrelin on feeding behavior and their neuroanatomical pathways and molecular mechanisms underlying such effects. Because of this, the current view about the role of ghrelin in the control of food intake is substantially more complex than that initially expected. This chapter summarizes the role of ghrelin in regulating multiple aspects of feeding behavior.

GHRELIN REGULATES FEEDING BEHAVIOR ACROSS SPECIES

Although ghrelin was discovered as the endogenous ligand for an "orphan" G protein-coupled receptor (GPCR) involved in the secretion of growth hormone, the growth hormone (GH) secretagog receptor (GHSR-1a), it was soon shown that, in addition to its effect as a GH secretagog, ghrelin plays a role in the control of energy balance. Thus, the administration of ghrelin, either directly into the brain or in the periphery, induces a solid increase in orexigenic activity that results in body weight gain if the treatment is maintained chronically.[17]

One reason that ghrelin receives substantial recognition as a potentially important coregulator of energy balance is that its genomic sequence and its effect on food intake are well preserved across numerous species.[17] Ghrelin influences feeding-related behavior in several species including non-mammals such as goldfish and chicken,[14] and different species of mammals including species with commercial value for the food industry such as sheep, cattle,[24] and swine.[9] Although ghrelin increases feeding in most of the species tested, intracerebroventricular injection of ghrelin reduces food intake in chicken and Japanese quail. Interestingly, peripheral administration of relatively low doses of ghrelin increases feeding in quail.[14] Altogether, these results suggest that ghrelin contributes to the regulation of feeding behavior by multiple mechanisms and through both central and peripheral sites of action.

The role of ghrelin in the control of feeding behavior relates to more than an actual increase of ingested calories. For example, although Siberian hamsters do not develop a marked increase in food intake after ghrelin administration, they exhibit significantly increased foraging and food hoarding behavior.[3] Another activity intimately associated with feeding behavior regulated by ghrelin is olfaction. The olfactory bulb expresses ghrelin receptors and intracerebroventricular administration of ghrelin decreases odor detection thresholds and increases sniffing frequency in rats. In humans, intravenous infusion of ghrelin enhances sniffing without affecting the pleasantness ratings of odors.[28] Finally, ghrelin is involved in the control of digestive processes such as gut motility and gastric acid secretion that may condition feeding behavior. See the chapter on ghrelin in the Gastrointestinal Peptides section of this book, as well as the Brain, Endocrine, and Cardiovascular Peptides sections.

Importantly, ghrelin administration in clinical studies causes an increase in food intake or hunger sensation in both healthy human volunteers and patients with feeding disorders such as anorexia nervosa.[35] In humans, ghrelin stimulates food intake in both lean and obese subjects, although obese but not lean patients report an increase in the palatability of food after ghrelin infusion.[11] These findings are of substantial importance because there are many known peripherally circulating satiety hormones (e.g. cholecystokinin, PYY, leptin, GLP-1, oxyntomodulin) but only one hunger-inducing hormone.

Ghrelin is still the only known circulating peptide with considerable orexigenic potency. However the specific aspects of feeding behavior that are physiologically regulated by changes in plasma ghrelin levels are still being investigated.

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00148-2
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THE GROWTH HORMONE SECRETAGOG RECEPTOR GHSR-1A MEDIATES GHRELIN-INDUCED FOOD INTAKE

Results obtained from a series of studies with the endogenous GHSR-ligand ghrelin are consistent with earlier reports published throughout the previous decade, in which several synthetic ligands of the GHSR-1a were found to increase food intake in rodents. This list includes for example, growth hormone releasing peptide-2 (GHRP-2), ipamorelin, GHRP-6, hexarelin, and several other GHSR-1a ligands.

GHSR-1a-KO mice treated with ghrelin do not experience an increase in food intake when compared with wildtype littermates, indicating that GHSR-1a is the essential mediator of this pathway for this phenomenon.[17] The GHSR-1a expression pattern as identified in rodents includes many brain areas involved in the control of food intake.[17] More evidence for the important role of the GHSR-1a receptor in the control of food intake comes from genetically modified rats that overexpress antisense mRNA against the GHSR-1a under the control of the promoter for tyrosine hydroxylase (TH). These rats lack GHSR-1a selectively in the hypothalamic arcuate nucleus and do not respond to GHS with increased food intake as observed in their wildtype littermates.[17] Overall, these transgenic rats show less food intake and lower body weight as well as fat levels in the first weeks after weaning indicating that the GHSR-1a might have an endogenous role in energy balance regulation.

In addition to the interaction with ghrelin, GHSR-1a activity is modulated by heterodimerization with other GPCRs.[15,23] Hypothalamic neuropeptide Y (NPY)/agouti-related peptide (AgRP) neurons coexpress GHSR-1a and melanocortin-3 receptor (MC3R). The MC3R promotes orexigenic activity acting as an inhibitory autoreceptor on proopiomelanocortin (POMC) neurons.[6] *In vitro* studies confirmed that GHSR-1a heterodimerizes with MC3R in neurons of the arcuate nucleus of the hypothalamus (ARC).[23] Functional studies showed that MC3R ligand (alpha-MSH) binding to MC3R-GHSR-1a heterodimer enhances the downstream signaling of MC3R. By contrast, the heterodimer complex diminishes the basal constitutive activity of GHSR-1a. GHSR-1a also heterodimerizes with the dopamine receptor subtype-2 (DRD2) in hypothalamic neurons.[15] To explore the function of the GHSR-1a-DRD2 heterodimer, wildtype, ghrelin−/−, and ghsr−/− mice were treated with a DRD2 agonist, cabergoline. Interestingly, the anorexigenic effect of cabergoline occurred in both wildtype and ghrelin−/− mice; however, a decrease in food intake was not observed in ghsr−/− mice indicating that GHSR is essential for DRD2-induced anorexia. Further, pharmacological GHSR-1a antagonism blocks dopamine signaling in neurons coexpressing DRD2 and GHSR-1a.[15] Mice pretreated with the GHSR-1a antagonist did not experience a decrease in food intake on administration of cabergoline. Taken together, these studies provide novel insight into GHSR-1a receptor signaling and highlight an important interplay between multiple GPCR signaling pathways. Importantly, they implicate a new approach to modulate GHSR-1a activity, which can ultimately lead to the development of a new generation of pharmacotherapies.

GHRELIN TARGETS THE HYPOTHALAMUS TO REGULATE FEEDING

Although the main source of circulating plasma ghrelin is the stomach, and this peripherally secreted ghrelin can cross the blood–brain barrier to target centers in the brain involved in the control of feeding, there is evidence that ghrelin is produced by neurons in the hypothalamus. Some of these ghrelinergic neurons are located in the ventral–lateral part of the arcuate nucleus[17] and in the interstitial areas among the dorsomedial hypothalamus (DMH), ventromedial hypothalamus (VMH), and the walls of the third ventricle. Ghrelin immunopositive fibers were identified in close proximity to NPY-, POMC-, thyroid hormone releasing hormone (TRH)-, and corticotropin-releasing hormone (CRH)-immunoreactive cells, as well as within nuclei that control food intake such as the lateral hypothalamus (LH) and the paraventricular hypothalamus (PVH). Thus, in addition to the blood-borne ghrelin, hypothalamic ghrelin may contribute to regulatory circuits controlling energy homeostasis.

Two major hypothalamic pathways are targeted by ghrelin to control food intake. Ghrelin action increases the activity of orexigenic NPY/AgRP neurons that control feeding in concerted action with the anorectic POMC/cocaine-activated related transcript (CART) neurons. Ghrelin attenuates the melanocortinergic anorexigenic signals, by increasing the release of the melanocortin receptor inverse agonist, AgRP, and indirectly by decreasing the discharge of alpha-melanocyte stimulating hormone (MSH) from POMC neurons onto melanocortinergic receptor neurons in the PVN. Simultaneously, ghrelin increases the expression of the orexigenic NPY and AgRP in hypothalamic neurons in the arcuate nucleus.[17]

Ghrelin reaches at least a certain number of neurons expressing GHSR-1a that are located in mediobasal parts of the hypothalamic arcuate nucleus that are targeted as well by leptin. Such an action seems to be comodulated by a population of mostly bipolar shaped specifically ghrelin expressing neurons that coinfluence the firing rats of the orexigenic NPY/AgRP neurons and the anorexigenic POMC neurons, both of which are a target for ghrelin. This model is consistent with the effect of ghrelin on energy balance, neuropeptide expression, and neuronal activity and points to a combined

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role for ghrelin as an afferent nutrient sensor and a central energy balance regulator. Both leptin and ghrelin have been clearly shown to modulate the electrophysiological activity of NPY/AgRP neurons, and leptin reduces the ghrelin-triggered increase in calcium concentration in NPY cells.[16] Although ghrelin induces pacemaker activity in arcuate neurons containing NPY and AgRP, leptin suppresses such activity.[32] Similar opposing action occurs in POMC neurons in which leptin increases the expression of POMC mRNA and the neuronal excitability,[6] whereas ghrelin reduces the activity of the POMC neurons by increasing the inhibitory release of gamma amino butyric acid (GABA) onto these neurons.[7] These opposing neurophysiological effects further support the notion that ghrelin and leptin may be endogenous opponents in the regulation of energy balance.

Binding of ghrelin to NPY/AgRP neurons in the arcuate nucleus induces an increase in firing activity,[17] membrane excitability,[32] and expression of NPY and AgRP. The potency of ghrelin to modulate POMC neurons is weaker than its effect on NPY/AgRP neurons. POMC expression is not changed after intracerebroventricular infusion of ghrelin for 72 h, whereas this treatment raises both NPY and AgRP mRNA levels.[17] Ghrelin receptors are not colocalized on POMC neurons, although ghrelin induces a decrease in POMC neuronal firing rate.[7] However, this effect disappears, or is reversed, by adding a Y1 receptor antagonist and an inhibitor of GABA release, suggesting that the effect on POMC neurons is indirect and mediated via the activation of GABAergic NPY/AgRP neurons. However, the integrity of the melanocortin signaling pathway seems to be essential for ghrelin to exert its effect on food intake and adiposity. Thus, ghrelin loses its orexigenic effect in an obese mouse model with ectopic overexpression of the melanocortin receptors inverse agonist AgRP as well as in MC3R and MC4R-deficient mice.[5]

Although the increase in food intake after central administration of ghrelin can be completely prevented in mice lacking both AgRP and NPY, ghrelin-induced adiposity is not exclusively dependent on NPY, because ghrelin increases fat content of NPY deficient mice identical to its effects in wildtype mice.[30] Ghrelin is also involved in anxiety-like behavior in rodents. In mice, both intracerebroventricular and intraperitoneal administration of ghrelin potently induces anxiogenic activities via mechanisms involving the hypothalamic–pituitary–adrenal axis. In addition, peripherally administered ghrelin significantly increases CRH mRNA expression in the hypothalamus and administration of a CRH receptor antagonist actually inhibits ghrelin-induced anxiogenic effects. These findings suggest that ghrelin may have a role in mediating neuroendocrine and behavioral responses to stressors and may not only directly stimulate hunger and appetite but also indirectly affect energy relevant behavior via effects on anxiety.[2]

GHRELIN TARGETS INTRACELLULAR FUEL-SENSING PATHWAYS IN HYPOTHALAMIC NEURONS TO CONTROL FEEDING

Recent studies have provided evidence of the intracellular mechanisms activated in neurons in response to ghrelin. Interestingly, some of those mechanisms involve the control of intracellular fuel availability through the activity of AMP-activated protein kinase (AMPK). AMPK is a serine/threonine kinase implicated in the regulation of multiple metabolic pathways that acts as an intracellular nutrient sensor of energy and nutrients. AMPK is activated when energy is depleted and induces catabolic processes to generate adenosine triphosphate (ATP) through the oxidation of fatty acids and mitochondrial biogenesis.[21] Thus, ghrelin increases AMPK activity in the VMN, resulting in the decrease of intracellular malonyl coenzyme-A levels. Low malonyl coenzyme-A levels lead to the reduction of the de novo fatty acid synthesis and an increase of carnitine-palmitoyl transferase I (CPT1) activity.[21] Accordingly, ghrelin administration reduces malonyl coenzyme-A and increases CPT1 activity in the hypothalamus.

The increase in mitochondrial activity in hypothalamic neurons resulting from the increased CPT1 activity and concomitant fatty acid beta-oxidation is a key component for ghrelin-induced hyperphagia. Conversely, the impairment of mitochondrial function in hypothalamic neurons leads to the reduction of the ghrelin orexigenic action.[1] The increase in mitochondrial fatty acid beta-oxidation is accompanied by the increase in the production of reactive oxygen species (ROS). Uncoupling proteins (UCPs), located within the inner membrane of the mitochondria, prevent excessive ROS production by reducing the proton gradient across the mitochondrial membrane. Hypothalamic cells involved in the generation of the orexigenic effect of ghrelin, such as the NPY/AgRP neurons in the arcuate nucleus, express the uncoupling protein UCP2. Ghrelin administration increases UCP2 gene expression in the hypothalamus, which is an effect that correlates with the increase in mitochondrial respiration. Consistently, the orexigenic response to the administration of ghrelin is blunted in UCP2-deficient mice.[1]

One of the mechanisms whereby ghrelin activates AMPK in the hypothalamus involves the sirtuin 1 and p53 (Sirt1/p53) pathway.[33] Sirt1 is an NAD+-dependent deacetylase that is activated during negative energy balance by the tumor-suppressor protein p53. Sirt1 is implicated in the central and peripheral metabolic control of lipid metabolism as well as glucose homeostasis in peripheral tissues. The role of Sirt1/p53 in the activation of AMPK induced by ghrelin is evidenced by the suppression of ghrelin-induced AMPK phosphorylation when Sirt1 activity is blocked by the use of either a pharmacological or genetic approach. In addition to

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the blunted AMPK activity, the suppression of Sirt1 leads as well to decreased orexigenic response induced by ghrelin, which correlates with the blunted increase in the expression of transcription factors such as brain-specific homeobox (BSX), forkhead box 01 (Fox01), and cAMP response element binding protein (CREB), or orexigenic neuropeptides such as NPY and AgRP.

The control of gene expression in NPY/AgRP neurons by ghrelin requires the hypothalamic transcription factor BSX. The pharmacological inhibition of CPT1 blocks ghrelin-induced increase in hypothalamic β-oxidation and impairs the BSX-dependent upregulation of NPY and AgRP expression. Other transcription Fox01 and CREB contribute to the control of the gene expression of these neuropeptides by ghrelin.[18] Altogether, these results suggest that the control of intracellular fuel-sensing mechanisms by ghrelin contributes to the control of the transcriptional activity in hypothalamic neurons involved in the control of energy balance.

Although the hypothalamic arcuate nucleus contributes significantly to the orexigenic effect of ghrelin, the PVN seems to be an important site of ghrelin action as well. Intracerebroventricular administration of either ghrelin or the GHSR-1a agonist GHRP-6 induces c-fos expression in PVN neurons[19] and injection of ghrelin directly in the PVN increases food intake.[17] Interestingly, ghrelin immunoreactive neurons have been detected in the PVN indicating that this nucleus is a source of ghrelin as a neuropeptide.[26] The orexigenic activity of ghrelin in the PVN is mediated, at least in part, by endocannabinoids and this effect can be abolished by systemic pretreatment with the cannabinoid receptor antagonist SR141716.[31] The lateral hypothalamus (LH) may be involved as well in ghrelin-induced hyperphagia via the activation of the orexin pathways. Ghrelin treatment increases c-fos expression in orexin expressing neurons in the LH and the increase of food intake induced by ghrelin can be partially blocked by the coadministration of an antiserum against orexin A.[29] The role of orexin as a mediator of the ghrelin-induced hyperphagia was confirmed in orexin A-deficient mice, which show reduced effectiveness of ghrelin in increasing food intake when compared with wildtype littermates.[29]

GHRELIN TARGETS EXTRA-HYPOTHALAMIC AREAS TO REGULATE FEEDING

Although the role of the hypothalamus mediating the increase on food intake induced by ghrelin has been clearly established, multiple CNS regions contribute to the control of feeding behavior by ghrelin. The brainstem is one of those areas, consistent with its role mediating the feeding response of other afferent signals such as cholecystokinin, GLP-1, and leptin. Thus, the administration of ghrelin into the fourth ventricle or directly on the dorsal vagal complex, results in an hyperphagic response of similar magnitude to the one obtained in the third ventricle, measured as total amounts of ingested food as well as by meal pattern analysis.[12] The administration of ghrelin or GHRP-6 increases the number of c-fos positive cells in nucleus of the brainstem such as the nucleus of the solitary tract (NTS) or the area postrema (AP).[19] This is consistent with the expression pattern of the GHSR-1a, which is localized in neurons of the NTS, the dorsal motor nucleus of the vagus (DMNV), and the rostral ventrolateral medulla (RVLM).[20]

Ghrelin acts in multiple areas of the brain not traditionally associated with the homeostatic control of feeding such as the hippoccampus, dorsal nucleus of the raphe, ventral tegmental area (VTA), nucleus accumbens, amygdala, prefrontal cortex nucleus, and the olfactory bulb. As a result of this, ghrelin regulates multiple aspects associated with feeding behavior such as food reward, stress-induced feeding, incentive motivation, addictive behavior associated with the consumption of high calorie diets, alcohol, or drugs, and with learning and memory responses associated with feeding in both rodents and in humans.[8] Studies in human subjects support the involvement of ghrelin in the control of complex behaviors associated with feeding. Hence, intravenous infusion of ghrelin in humans increases the activity in areas of the brain involved in reward, such as the striatum, amygdala, insula, and orbitofrontal cortex. Moreover, ghrelin administration increases the sniffing intensity and even increases the willingness to spend more money on food instead of nonedible items,[8] which suggests that ghrelin increases the perceived value of food in humans.

GHRELIN TARGETS VISCERAL AFFERENTS TO REGULATE FEEDING

There is experimental evidence that in addition to the humoral distribution, ghrelin may control feeding by acting on vagal afferent nerves. Supporting this, GHSR-1a is expressed in neurons of the nodose ganglia that project to the stomach.[17] Further, some reports have shown that ghrelin has no effect on food intake in human subjects that have undergone surgical procedures involving vagotomy. However, experiments performed in rats have been inconclusive. Therefore, although a report showed that elimination of the gastric vagal afferent signals abolished ghrelin-induced feeding, others reported the lack of a significant role of the vagal afferents in the control of feeding by ghrelin.[4]

PERIPHERAL GHRELIN LEVELS ARE REGULATED BY FEEDING AND NUTRITIONAL STATUS

Ghrelin secretion is clearly regulated by feeding and fasting in many species through multiple mechanisms including

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hormonal, nutrient, and neural signals. The complex and multifactorial control of ghrelin secretion further supports the notion that ghrelin is a hormone with a strong link to ingestive behavior.

The fluctuations in circulating ghrelin correspond to the measurement of both acyl and des-acyl-ghrelin. Acyl-ghrelin circulates in low concentrations, and this limits the understanding of the mechanisms controlling its secretion. Improvements in the methods of detection of acyl-ghrelin and the discovery and characterization of the enzyme responsible of the acylation of ghrelin have provided new tools to study the factors underlying the control of circulating acyl-ghrelin concentrations.

Ghrelin is acylated by the action of a specific enzyme named ghrelin O-acyltransferase (GOAT) (see the chapter on this enzyme in the Peptide Biosynthesis/Processing section of the book). This specificity is manifested by the observation that mice lacking GOAT are devoid of the acylated form of ghrelin. Like ghrelin, GOAT is regulated by nutritional status. Thus, fasting leads to the decrease of the gene expression of gastric GOAT in mice.[25] Interestingly, although total circulating concentrations of ghrelin increase during fasting, this is a result of an increase in the des-acyl form of ghrelin, whereas circulating acyl-ghrelin is unchanged. This indicates that fasting regulates ghrelin activity by the inhibition of ghrelin acylation, independently of the control of total ghrelin secretion.

GOAT is able to use dietary lipids as a substrate to acylate ghrelin, which was shown when mice were found to have circulating ghrelin acylated with a C7-FA side chain after being fed a diet rich in nonendogenously produced C7-chain FAs.[25] When wildtype mice are fed this diet, they experience an increase in body weight and fat mass. Interestingly, GOAT knockout mice fed the same diet do not gain weight or fat mass; however, the improved body composition does not result from a decreased food intake but most probably is a result of increased energy expenditure in the GOAT knockout mice. Transgenic mice that express human GHRL and human MBOAT4 genes in the liver under the control of the human APOE (encoding apolipoprotein E) promoter have significantly higher acyl-ghrelin levels compared with wildtype control mice when fed a medium-chain triglyceride (MCT)-rich diet. The transgenic mice experience a gain in body weight and adiposity, but this is independent of food intake. In addition to genetically manipulating the GOAT/ghrelin system, the development of a pharmacological inhibitor for GOAT has further facilitated examination of the role of ghrelin acylation in mediating food intake. Treatment with the inhibitor for one month prevents a significant weight gain and adiposity in mice fed an MCT-rich high-fat diet, yet no difference in food intake is observed. The resistance to weight gain observed in wildtype mice treated with the inhibitor is not apparent in ghrelin knockout mice that were subjected to the treatment, confirming that the metabolic improvements are mediated by acyl-ghrelin inhibition. Together, these results show that chronic inhibition of ghrelin acylation results in metabolic improvement that is independent of food intake. Further, specific acyl-ghrelin antagonism may be a novel obesity therapeutic.

Because the fluctuations in both insulin and glucose levels are crucial for the control energy balance, it is obvious to suggest that both insulin and glucose may play a role in the control of circulating ghrelin concentrations. Indeed studies in humans involving hyperinsulinemic euglycemic clamps suggest a determinant role of insulin in the control of ghrelin levels. However, studies in streptozotozin-induced diabetic rats have shown that ghrelin levels can be suppressed after a meal in the absence of insulin,[13] indicating that a calorie-dependent but insulin-independent mechanism may control ghrelin levels in response to ingestive behavior.

The nutrients that contribute to the regulation of ghrelin secretion after a meal do not necessarily act from the lumen of the stomach. Thus, the decrease in plasma ghrelin-induced by the delivery of glucose directly in the stomach of rats is blunted when the pylorus is closed, in contrast to the situation when it is open. Consistent with these findings, the decrease of plasma ghrelin also occurs after the administration of nutrients directly into the duodenum and jejunum.[22] This indicates that nutrients either need to be absorbed distal from the stomach to act on ghrelin secreting cells via circulating blood, or nutrient-sensing mechanisms in the upper intestine may trigger signals that lead to the reduction of plasma ghrelin.

In addition to glucose, aminoacids are very effective in reducing circulating ghrelin concentrations. By contrast, the administration of an isocaloric amount of lipids is less effective reducing ghrelin in comparison with the administration of glucose or amino acids,[22] suggesting that in addition to the calorie load, specific mechanisms involved in the nutrient-sensing contribute to the regulation of ghrelin levels. Some of those mechanisms may involve neural signals. The increase of ghrelin levels in response to fasting requires the integrity of vagal efferents from the CNS, and the intragastric administration of nutrients in vagotomized animals fails to decrease ghrelin levels.[34] These results suggest that a regulatory feedback loop between the gut and the stomach, independent of the CNS, cannot be excluded as a potential mechanism for the regulation of ghrelin levels in response to a meal. In addition to vagal signals, the sympathetic nervous system contributes to the control of ghrelin secretion via beta 1 adrenergic receptor signaling, because the increase in the activity of these receptors leads to the increase in ghrelin levels.

In addition to the regulation of plasma ghrelin concentrations by the caloric changes associated with fasting and

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feeding, there is a distinctive pattern of secretion that correlates with the time of the meals, with three premeal peaks and a nightly increase in humans. The increase in ghrelin secretion may be under the control of the similar neural mechanisms underlying the nutrient independent increase in insulin secretion observed immediately before a meal, known as the "cephalic phase" of insulin secretion. Thus, when rats are trained to consume their daily calories only during an artificial fixed 4-h meal schedule, circulating ghrelin increases before the expected meal.[10] This suggests that, similarly to insulin, ghrelin secretion may have as well a "cephalic phase." This increase in ghrelin levels before the meal suggests that ghrelin may be involved in processes linked to the initiation of meals. Conversely, the decrease in ghrelin secretion from the stomach can be evoked with "tease" feeding (presentation but not access to food) in rats, suggesting that the decrease in ghrelin secretion after feeding may not require an increase in calorie availability.[27] Interestingly, the decrease in ghrelin secretion can be prevented by surgical or pharmacological disruption of the vagal function. This suggests that signals other than nutrient-sensing dependent mechanisms contribute to the control of ghrelin secretion and highlight the contribution of neural signals in the control of ghrelin secretion by feeding.

REFERENCES

1. Andrews ZB, Liu ZW, Walllingford N, Erion DM, Borok E, Friedman JM, et al. UCP2 mediates ghrelin's action on NPY/AgRP neurons by lowering free radicals. *Nature* 2008;**454**:846–51.
2. Asakawa A, Inui A, Kaga T, Yuzuriha H, Nagata T, Fujimiya M, et al. A role of ghrelin in neuroendocrine and behavioral responses to stress in mice. *Neuroendocrinology* 2001;**74**:143–7.
3. Bartness TJ, Keen-Rhinehart E, Dailey MJ, Teubner BJ. Neural and hormonal control of food hoarding. *Am J Physiol Regul Integr Comp Physiol* 2011;**301**:R641–55.
4. Berthoud HR. Vagal and hormonal gut-brain communication: from satiation to satisfaction. *Neurogastroenterol Motil* 2008;**20**(Suppl. 1): 64–72.
5. Chen HY, Trumbauer ME, Chen AS, Weingarth DT, Adams JR, Frazier EG, et al. Orexigenic action of peripheral ghrelin is mediated by neuropeptide Y and agouti-related protein. *Endocrinology* 2004;**145**:2607–12.
6. Cowley MA, Smart JL, Rubinstein M, Cerdan MG, Diano S, Horvath TL, et al. Leptin activates anorexigenic POMC neurons through a neural network in the arcuate nucleus. *Nature* 2001;**411**:480–4.
7. Cowley MA, Smith RG, Diano S, Tschop M, Pronchuk N, Grove KL, et al. The distribution and mechanism of action of ghrelin in the CNS demonstrates a novel hypothalamic circuit regulating energy homeostasis. *Neuron* 2003;**37**:649–61.
8. Dickson SL, Egecioglu E, Landgren S, Skibicka KP, Engel JA, Jerlhag E. The role of the central ghrelin system in reward from food and chemical drugs. *Mol Cell Endocrinol* 2011;**340**:80–7.
9. Dong XY, Xu J, Tang SQ, Li HY, Jiang QY, Zou XT. Ghrelin and its biological effects on pigs. *Peptides* 2009;**30**:1203–11.
10. Drazen DL, Vahl TP, D'Alessio DA, Seeley RJ, Woods SC. Effects of a fixed meal pattern on ghrelin secretion: evidence for a learned response independent of nutrient status. *Endocrinology* 2006;**147**: 23–30.
11. Druce MR, Wren AM, Park AJ, Milton JE, Patterson M, Frost G, et al. Ghrelin increases food intake in obese as well as lean subjects. *Int J Obes Relat Metab Disord* 2005;**29**:1130–6.
12. Faulconbridge LF, Cummings DE, Kaplan JM, Grill HJ. Hyperphagic effects of brainstem ghrelin administration. *Diabetes* 2003;**52**: 2260–5.
13. Gelling RW, Overduin J, Morrison CD, Morton GJ, Frayo RS, Cummings DE, et al. Effect of uncontrolled diabetes on plasma ghrelin concentrations and ghrelin-induced feeding. *Endocrinology* 2004;**145**:4575–82.
14. Kaiya H, Furuse M, Miyazato M, Kangawa K. Current knowledge of the roles of ghrelin in regulating food intake and energy balance in birds. *Gen Comp Endocrinol* 2009;**163**:33–8.
15. Kern A, Albarran-Zeckler R, Walsh HE, Smith RG. Apo-ghrelin receptor forms heteromers with DRD2 in hypothalamic neurons and is essential for anorexigenic effects of DRD2 agonism. *Neuron* 2012;**73**: 317–32.
16. Kohno D, Gao HZ, Muroya S, Kikuyama S, Yada T. Ghrelin directly interacts with neuropeptide-Y-containing neurons in the rat arcuate nucleus: Ca^{2+} signaling via protein kinase A and N-type channel-dependent mechanisms and cross-talk with leptin and orexin. *Diabetes* 2003;**52**:948–56.
17. Kojima M, Kangawa K. Ghrelin: structure and function. *Physiol Rev* 2005;**85**:495–522.
18. Lage R, Vazquez MJ, Varela L, Saha AK, Vidal-Puig A, Nogueiras R, et al. Ghrelin effects on neuropeptides in the rat hypothalamus depend on fatty acid metabolism actions on BSX but not on gender. *FASEB J* 2010;**24**:2670–9.
19. Lawrence CB, Snape AC, Baudoin FM, Luckman SM. Acute central ghrelin and GH secretagogues induce feeding and activate brain appetite centers. *Endocrinology* 2002;**143**:155–62.
20. Lin Y, Matsumura K, Fukuhara M, Kagiyama S, Fujii K, Iida M. Ghrelin acts at the nucleus of the solitary tract to decrease arterial pressure in rats. *Hypertension* 2004;**43**:977–82.
21. Lopez M, Lage R, Saha AK, Perez-Tilve D, Vazquez MJ, Varela L, et al. Hypothalamic fatty acid metabolism mediates the orexigenic action of ghrelin. *Cell Metab* 2008;**7**:389–99.
22. Overduin J, Frayo RS, Grill HJ, Kaplan JM, Cummings DE. Role of the duodenum and macronutrient type in ghrelin regulation. *Endocrinology* 2005;**146**:845–50.
23. Rediger A, Piechowski CL, Yi CX, Tarnow P, Strotmann R, Gruters A, et al. Mutually opposite signal modulation by hypothalamic heterodimerization of ghrelin and melanocortin-3 receptors. *J Biol Chem* 2011;**286**:39623–31.
24. Roche JR, Blache D, Kay JK, Miller DR, Sheahan AJ, Miller DW. Neuroendocrine and physiological regulation of intake with particular reference to domesticated ruminant animals. *Nutr Res Rev* 2008; **21**:207–34.
25. Romero A, Kirchner H, Heppner K, Pfluger PT, Tschop MH, Nogueiras R. GOAT: the master switch for the ghrelin system? *Eur J Endocrinol* 2010;**163**:1–8.
26. Sato T, Fukue Y, Teranishi H, Yoshida Y, Kojima M. Molecular forms of hypothalamic ghrelin and its regulation by fasting and 2-deoxy-D-glucose administration. *Endocrinology* 2005;**146**:2510–6.

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27. Seoane LM, Al-Massadi O, Caminos JE, Tovar SA, Dieguez C, Casanueva FF. Sensory stimuli directly acting at the central nervous system regulate gastric ghrelin secretion. An ex vivo organ culture study. *Endocrinology* 2007;**148**:3998–4006.
28. Tong J, Mannea E, Aime P, Pfluger PT, Yi CX, Castaneda TR, et al. Ghrelin enhances olfactory sensitivity and exploratory sniffing in rodents and humans. *J Neurosci* 2011;**31**:5841–6.
29. Toshinai K, Date Y, Murakami N, Shimada M, Mondal MS, Shimbara T, et al. Ghrelin-induced food intake is mediated via the orexin pathway. *Endocrinology* 2003;**144**:1506–12.
30. Tschop M, Statnick MA, Suter TM, Heiman ML. GH-releasing peptide-2 increases fat mass in mice lacking NPY: indication for a crucial mediating role of hypothalamic agouti-related protein. *Endocrinology* 2002;**143**:558–68.
31. Tucci SA, Rogers EK, Korbonits M, Kirkham TC. The cannabinoid CB1 receptor antagonist SR141716 blocks the orexigenic effects of intrahypothalamic ghrelin. *Br J Pharmacol* 2004;**143**:520–3.
32. van den Top M, Lee K, Whyment AD, Blanks AM, Spanswick D. Orexigen-sensitive NPY/AgRP pacemaker neurons in the hypothalamic arcuate nucleus. *Nat Neurosci* 2004;**7**:493–4.
33. Velasquez DA, Martinez G, Romero A, Vazquez MJ, Boit KD, Dopeso-Reyes IG, et al. The central sirtuin 1/p.53 pathway is essential for the orexigenic action of ghrelin. *Diabetes* 2011;**60**:1177–85.
34. Williams DL, Grill HJ, Cummings DE, Kaplan JM. Vagotomy dissociates short- and long-term controls of circulating ghrelin. *Endocrinology* 2003;**144**:5184–7.
35. Yi CX, Heppner K, Tschop MH. Ghrelin in eating disorders. *Mol Cell Endocrinol* 2011;**340**:29–34.

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Chapter 149

GLP-1

Gustavo Pacheco-López and Wolfgang Langhans

ABSTRACT

Glucagon-like peptide (7–36) amide (GLP-1) is mainly produced by enteroendocrine cells in the small intestine and by a small set of neurons in the hindbrain. Exogenous and endogenous peripheral and central GLP-1 inhibits eating. Peripheral GLP-1 can inhibit eating through a paracrine action on vagal afferents or an endocrine action on the brain. Because of the short biological half-life of GLP-1, the endocrine action may be relevant only after substantial stimulation of GLP-1 release or pharmacological interventions. Although hindbrain circuitries alone can mediate the satiating effect of hindbrain GLP-1 receptor (GLP-1R) stimulation, rostral brain areas may also be involved. Peripheral or central GLP-1R activation can cause conditioned flavor avoidance, but GLP-1's satiating and avoidance effects can be dissociated. Finally, enhanced GLP-1 release contributes to the antiobesity and antidiabetic effects of bariatric surgery. In summary, GLP-1 analogs may be a promising therapeutic option to fight obesity and type-II diabetes.

GENERAL

Glucagon-like-peptide (7–36) amide (GLP-1) results from posttranslational processing of proglucagon in the gut and the brain.[15] Proglucagon is encoded by the proglucagon gene, which has been identified in all mammals and in a variety of other vertebrates, suggesting an early origin in evolution and a prominent role for the encoded peptides. A discreet set of neurons in the nucleus tractus solitarii (NTS)[29] and enteroendocrine L-cells located in the distal jejunum and ileum are the main producers of GLP-1.[15] Dietary carbohydrates and fats potently stimulate peripheral GLP-1 secretion, in part by activating taste and other G-protein-coupled receptors expressed on enteroendocrine cells. These cells can therefore detect the presence of nutrients in the intestinal lumen. In addition, a neural feed-forward loop from the proximal to the distal small intestine may be involved in GLP-1 release.[15] GLP-1 has also been identified in sweet- and umami-taste receptor cells in the oral cavity,[21] suggesting that GLP-1 signaling might also be involved in taste perception. Indirect evidence suggests that eating also triggers the release of brain GLP-1, but interoceptive stress seems to be another potent stimulus.[32] GLP-1 receptors (GLP-1R) are widely distributed in peripheral organs such as the pancreatic islets and the whole gastrointestinal (GI) tract,[15] and in the brain, where they are found in several hindbrain and forebrain sites,[29] including areas that are implicated in the control of food intake and regulation of energy balance such as the area postrema (AP), NTS, hypothalamus, amygdala, and in the mesolimbic reward system.

GLP-1 is probably best known for its potent incretin effect, that is, for the stimulation of glucose-induced insulin release,[15] and for the inhibition of gastric emptying that contributes to the ileal brake mechanism.[15] These and other effects of GLP-1 are discussed in other chapters of this volume. This chapter focuses on the inhibiting effect of GLP-1 on eating.[3,11,21,33]

PERIPHERAL GLP-1

General

Peripheral, systemic administration of native GLP-1 or of potent, long-acting natural or synthetic GLP-1 analogs, such as exendin-4 (Ex-4) or liraglutide, inhibits eating in many species, including humans.[3,11,21,33] Ex-4 is a naturally occurring peptide resistant to rapid enzymatic degradation by dipeptidyl peptidase-4 (DPP-4). The synthetic GLP-1 analog liraglutide also resists enzymatic degradation, and its renal excretion is delayed because it also binds to albumin in plasma. Chronic peripheral administrations of GLP-1 or its analogs reduce body weight and improve glucose metabolism in overweight individuals, suggesting that GLP-1 offers a promising therapeutic option to treat obesity and type-II-diabetes (T2D). GLP-1R activation reduces food intake by primarily decreasing meal size,[23] indicating that GLP-1 enhances satiation. Some studies report an additional effect of GLP-1R activation on the duration of the intermeal interval.[3,33] Interestingly, rats adapted to restricted feeding display an increase in GLP-1 secretion about 90 min before the expected food access. Blocking the effect of this preprandial GLP-1 release by GLP-1R antagonism decreases subsequent food intake, indicating that the preprandial spike

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00149-4
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in circulating GLP-1 is anticipatory and facilitates handling of the expected nutrient load.[31] Chronic maintenance on, and even brief exposure to, a high fat (HF) diet reduces the acute eating-inhibitory effect of Ex-4, indicating that HF diet exposure compromises the response to GLP-1R activation. Also, intraperitoneally (ip) injected GLP-1 fails to reduce food intake in leptin receptor-deficient Koletsky rats, whereas leptin enhances the satiating effect of ip GLP-1.[34] These findings indicate that leptin receptor signaling is necessary for, and that leptin enhances, the satiating effect of GLP-1, similar to its synergistic satiating effect with other gastrointestinal satiation signals such as cholecystokinin and gastric distension. Some evidence even indicates that GLP-1 acts as a downstream mediator of leptin's inhibitory effect on eating.[3] Whether the decreased sensitivity of HF diet-fed rats to ip GLP-1 is related to the leptin resistance usually induced by HF feeding awaits further investigation.

Peripheral and central administrations of GLP-1 and GLP-1R agonists have also been shown to inhibit water intake in several species. This raises the question of a possible interaction or even crossdependence between GLP-1's eating- and drinking-inhibitory actions. A more recent study[19] showed, however, that GLP-1, Ex-4, and liraglutide all suppressed water intake after ip administration in both the presence and absence of food, indicating that the antidipsogenic effect of GLP-1R activation is independent of the inhibition of eating.

Site and Mode of Action

The reduction of food intake after ip administration of native GLP-1 or GLP-1R agonists may result in part from a peripheral action because peripheral administration of albumin-conjugated GLP-1 that probably does not cross the blood–brain barrier still reduced food intake. Williams et al. showed that the effect of ip injected GLP-1 could be blocked by ip, but not by intracerebroventricular (icv), administration of the GLP-1R antagonist exendin-9 (Ex-9).[35] We reported that the satiating effect of IP infused GLP-1 depends on intact abdominal vagal afferents.[23] Interestingly, however, GLP-1 infused into the hepatic portal vein reduces food intake in rats after subdiaphragmatic vagal deafferentation (SDA) or sham surgery,[23] although the threshold dose for a reliable reduction of meal size and duration is about ten times higher after ip than after hepatic portal vein infusion. These findings suggest that GLP-1R agonists can inhibit eating through activation of at least two partially separate pathways: intravenously infused GLP-1 presumably through an endocrine action on the brain, and ip administered GLP-1 through a paracrine action on vagal afferents.

Unpublished data from our laboratory (Punjabi et al., in preparation) indicate that GLP-1 infused into the hepatic portal vein or ip at doses that effectively inhibit eating do not generate learned aversive behaviors. Therefore, at least under our experimental conditions, that is, in response to remotely controlled hepatic portal vein or ip administrations, GLP-1 can induce satiation independent of aversion.

Although the satiating effect of ip administered native GLP-1 may be primarily mediated by peripheral, presumably vagal afferent GLP-1Rs,[23] ip injected long-acting GLP-1R agonists, such as Ex-4 and liraglutide, can also recruit central nervous system (CNS) GLP-1Rs. This seems reasonable given these compounds' stability and the observation that Ex-4 can cross the blood–brain barrier.[3] Administration of the GLP-1R antagonist Ex-9 into the third cerebral ventricle attenuates the eating-inhibitory effect of ip Ex-4 or liraglutide, in particular at 6 and 24 h after administration.[16] Moreover, much higher doses of Ex-4 and liraglutide are needed to inhibit eating in SDA rats than in sham-operated control rats. Thus, GLP-1R expressed on abdominal vagal afferents and CNS GLP-1R seems to mediate the inhibition of eating after ip Ex-4 and liraglutide.[16] Most of the CNS GLP-1Rs, however, are located behind the blood–brain barrier, and circulating GLP-1 may not be able to reach them. Orskov et al. (1996) in *Diabetes* addressed this question in a study using radioactively labeled GLP-1 and autoradiography and concluded that only GLP-1R in the area postrema (AP) and subfornical organ (SFO) are accessible to circulating GLP-1. The AP and SFO are circumventricular organs with a very leaky blood–brain barrier. However, intravenous injection of albumin-conjugated GLP-1, which is unable to cross the blood–brain barrier, induced an increased expression of c-Fos in the AP, NTS, rostral ventrolateral medulla, parabrachial nucleus, and hypothalamic paraventricular nucleus (PVN),[3,29] suggesting that some of these areas are indirectly activated after binding of GLP-1 to GLP-1R in the AP and SFO. Quantification of influx by the very sensitive multiple time-regression analysis method, provides evidence that intravenous GLP-1 may enter brain parenchyma by a nonsaturable mechanism. In general, the results are consistent with findings from our group suggesting that AP GLP-1R are involved in mediating the satiating effect of native GLP-1 infused into the hepatic portal vein.[4] Whether circulating GLP-1 recruits hindbrain GLP-1 neurons for its inhibiting effect on eating is presently unknown. If so, it can do so only indirectly because the hindbrain GLP-1 neurons do not express GLP-1Rs.[29]

ENDOGENOUS PERIPHERAL GLP-1

Endogenous intestinal GLP-1 has been implicated in meal termination (i.e., satiation) because (1) the presence of nutrients in the small intestine stimulates the release of intestinal GLP-1, (2) exogenous GLP-1 inhibits eating by primarily reducing meal size (see above) and (3) ip injection of the specific GLP-1R antagonist Ex-9 stimulates eating under some conditions,[35] indicating that satiation can be delayed when

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endogenous GLP-1 signaling is blocked. After secretion into the blood stream, GLP-1 is rapidly degraded into GLP-1 (9–36) by DPP-4, accounting for the short biological half-life of GLP-1 of <5 min. This may limit GLP-1's potential as an endocrine satiating signal unless GLP-1 acts close to the site of its release (for instance in the hepatic portal vein) or when circulating concentrations are substantially increased.

In addition to a possible endocrine mechanism of action, gut hormones can signal the brain through a paracrine effect on afferent nerves, with the vagus being the most common neural route of peripheral to central signaling. After the secretion from intestinal L-cells GLP-1 diffuses across the basal lamina and enters the lamina propria of the mucosa, where vagal afferents terminate and where the highest concentration of active GLP-1 can be expected before its enzymatic degradation by DPP-4 (Fig. 1). Intestinal lymph reflects the composition of the interstitial fluid of the mucosa fairly well. As a result, the intestinal lymphatic GLP-1 concentration increases substantially in response to intragastric nutrient infusions in anesthetized rats and in response to normal meals in freely moving rats (Arnold et al., 2012, *Am J Physiol*). Peptides administered ip accumulate in the intestinal lymph and, hence, presumably target the vagal afferents terminating in the wall of the small intestine. Administration ip should therefore be a good model to examine the paracrine satiating effect of endogenous GLP-1.[21] In addition to the failure of ip infused GLP-1 to inhibit eating in rats after SDA,[23] other findings indirectly support a role of abdominal vagal afferents in the satiating effect of ip administered, and possibly also endogenous, GLP-1: vagal afferent neurons in the nodose ganglion express the GLP-1 receptor gene, and intravenous administration

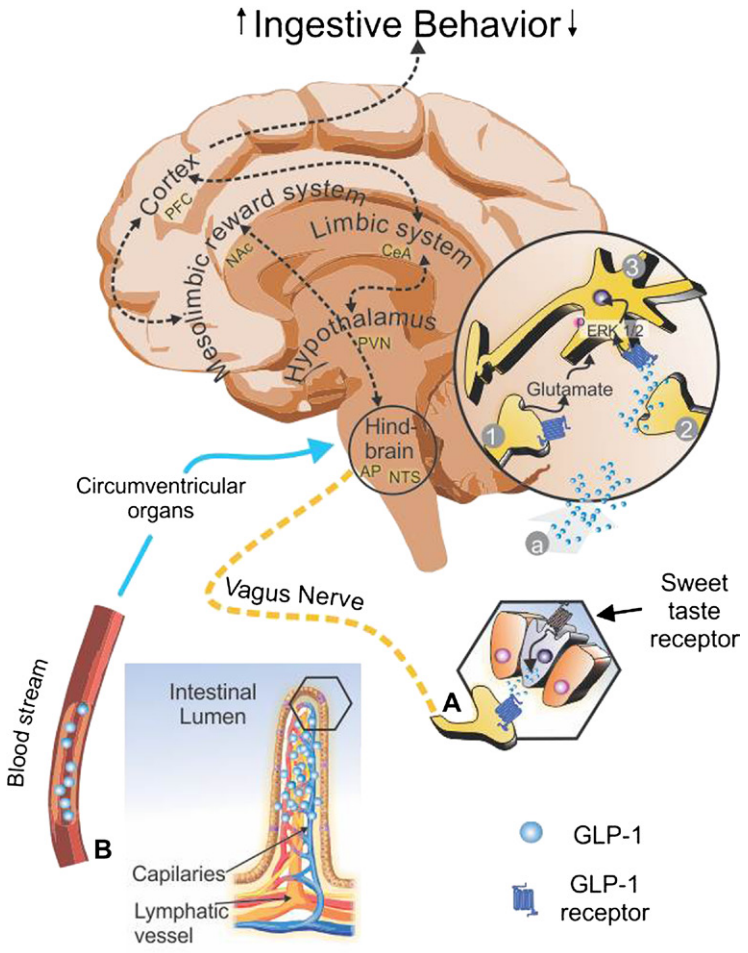

FIGURE 1 Scheme of GLP-1 satiation. Intestinal luminal nutrients trigger GLP-1 release through taste receptor mechanisms present in enteroendocrine L-cells. GLP-1 can inhibit eating (A) through a paracrine action on GLP-1 receptors (GLP-1Rs) on intestinal vagal afferents that connect the gut to the nucleus tractus solitarii (NTS) in the hindbrain; and (B) through an endocrine action on the brain, provided that sufficient GLP-1 escapes inactivation by dipeptidyl peptidase-4 to act through circumventricular organs such as the area postrema (AP) and perhaps elsewhere. (a) Peripheral GLP-1 crossing the blood–brain barrier, (1) pre-synaptic glutamatergic neurotransmission, (2) GLP-1ergic neuromodulation, and (3) post-synaptic activation. Although hindbrain processing of the GLP-1 signal seems to be sufficient to inhibit eating, rostral brain areas such as the hypothalamic paraventricular nucleus (PVN), the nucleus accumbens (NAc) and the central nucleus of the amygdala (CeA) may also be involved. See the text for further details. See color plate 41.

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of GLP-1 that produces high physiological plasma concentrations activates ventral gastral vagal afferents in multiunit recordings. Moreover, exenatide, another synthetic GLP-1R agonist, activates duodenal intramural neurons and the dorsal motor nucleus of the vagus.[3,11,21] The food intake reducing effects of GLP-1R activation do not require an intact common hepatic branch of the vagus, suggesting that the abdominal vagal afferent fibers involved do actually originate in the GI tract itself. Interestingly, visceral sensory afferents provide direct glutamatergic input to hindbrain GLP-1 neurons. Endogenous peripheral – and ip adminstered—GLP-1 could therefore, modulate hindbrain GLP-1 neuronal activity through glutamatergic inputs. Whether endogenous peripheral GLP-1 induces satiation through an endocrine or paracrine action is still unresolved, and current research also focuses on this question. Overall, however, these results suggest that GLP-1R activation can inhibit eating through receptor populations in at least two distinct locations, that is, on abdominal, presumably intestinal, vagal afferents and in the brain (Fig. 1).

CENTRAL NERVOUS SYSTEM GLP-1

GLP-1 expressing neurons are located primarily in the NTS.[29] Microinfusions of GLP-1 directly into the cerebral ventricles have long been known to inhibit eating.[26,30] The effect is usually acute and transient, and it could be antagonized by the GLP-1R antagonist Ex-9. Ex-9 alone also stimulates eating in satiated rats, indicating that central GLP-1 is an endogenous short-term control of eating. Repetitive icv GLP-1 or Ex-9 administrations also change body weight,[3] suggesting that central GLP-1 contributes to the regulation of energy homeostasis as well. Results showing that viral-mediated downregulation of proglucagon gene expression in the caudal NTS causes hyperphagia and increased fat accumulation in response to HF diet exposure[2] support this view.

GLP-1Rs are expressed in many areas of the brain. Particularly high GLP-1R densities occur in the hypothalamus and in the brainstem, whereas no GLP-1Rs are found in the cortex and cerebellum. Hindbrain GLP-1 neurons project to the hypothalamic PVN and dorsal medial nucleus, two areas implicated in the control of eating. A projection from NTS GLP-1 neurons to the NAc also seems to be involved in the control of eating,[6] most probably related to the forebrain processing of food reward. GLP-1 icv induces c-Fos expression in the PVN and central nucleus of the amygdala (CeA),[30] suggesting that these areas are involved in the satiating effect of central GLP-1. Consistent with a role of PVN GLP-1Rs in the mediation of the satiating effect of GLP-1, direct intra-PVN injections of GLP-1 reduce food intake without inducing aversion or affecting locomotion.[20]

However, icv administered GLP-1 clearly has aversive effects in other studies.[17,32] The lowest doses that produce a conditioned taste aversion (CTA) and an inhibition of eating were identical. Also, icv GLP-1 and IP injections of lithium chloride (LiCl), a model substance to induce "malaise" and CTA, produce a similar pattern of neuronal activation in the brain. Finally, GLP-1R antagonism blocks the aversive effect of peripherally administered LiCl, suggesting that CNS GLP-1R activation contributes to LiCl-induced CTA.[27] Similar to the results obtained with peripheral GLP-1 administration (see above), the aversive and satiating effects of central GLP-1R activation can be dissociated. Bilateral lesions of the PVN prevent the eating-inhibitory but not the aversive effect of icv GLP-1, whereas bilateral lesions of the CeA produce the opposite results.[32] In another study, lateral and fourth ventricular GLP-1 administrations both reduce food intake, but only lateral ventricular GLP-1 induces an aversion, an effect that could be reproduced by the direct administration of GLP-1 into the CeA.[17] Further, blockade of amygdaloid GLP-1R prevents the CTA in response to ip injections of LiCl, suggesting that amygdaloidal GLP-1R activation contributes to taste aversion learning.[17] More recently, GLP-1 significantly reduced food intake (1, 2, and 24 h posttreatment) but did not induce a CTA after microinjection into the NAc core.[6] All these results indicate that at least partly separate neuroanatomical pathways mediate the satiating and aversive effects of central GLP-1, and that the CeA is involved in GLP-1-induced taste aversion learning. The administration icv of GLP-1 or GLP-1R agonists may therefore activate both pathways. The exact circumstances determining which of the pathways prevail remain to be identified.

In addition to midbrain and forebrain GLP-1Rs hindbrain GLP-1Rs seem to be involved in the control of eating and regulation of energy balance. Hayes et al.[13] showed that peripheral as well as intra-fourth ventricular administration of the GLP-1R agonist Ex-4 reduces glucose solution intake in chronic supracollicular decerebrate rats and sham-operated rats similarly, indicating that hindbrain processing of the signal derived from GLP-1R activation is sufficient for the observed effects. The hindbrain GLP-1R activation increases PKA and MAPK activity and decreases phosphorylation of AMPK in NTS. Further experiments showed that PKA and MAPK signaling contribute to the eating-inhibitory and body weight decreasing effects of Ex-4, suggesting that hindbrain GLP-1R activation decreases food intake and body weight through a coordinated PKA-mediated suppression of AMPK and activation of MAPK.[12] The same group also showed that direct medial NTS injection, but not intrafourth ventricular administration, of the GLP-1R antagonist Ex-9 stimulates eating in rats, and blockade of hindbrain GLP-1R also antagonizes satiation induced by nutrient preload consumption or artificial gastric distension, but not intraduodenal nutrient

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delivery. These findings implicate activation of hindbrain GLP-1Rs in physiological satiation and suggest that gastric distension, perhaps through a vagal afferent mechanoreceptor signal, activates the hindbrain GLP-1 system. In contrast, intraduodenal nutrient infusions presumably also stimulate intestinal GLP-1 release and, hence, recruit additional pathways that are not antagonized by hindbrain GLP-1R blockade. Nevertheless, these findings are consistent with the idea that nutrient consumption induces satiation via release of both peripheral and central GLP-1, and that both can act on hindbrain GLP-1R to influence eating.[10]

GLP-1 IN OBESITY THERAPY

Roux-en-Y Gastric Bypass (RYGB)

Bariatric surgery, in particular RYGB, efficiently decreases excess body weight and leads to a rapid improvement of insulin sensitivity, with complete remission of T2D in up to 80% of the patients. Most remissions occur almost immediately after surgery and before significant weight loss has taken place. Several reports from studies in humans and laboratory animal models indicate that RYGB substantially increases postprandial circulating GLP-1 and peptide tyrosine–tyrosine (PYY) levels,[22] and that this increase is a major contributor to the obesity and T2D-curbing effects of RYGB. Such findings promote GLP-1 analogs as promising compounds for the pharmacotherapy of T2D and obesity. There are, however, reports that some patients develop mood disorders and that the suicide rate may increase after RYGB.[28] Although patients' well-being apparently tends to improve with exenatide treatment,[7] it is prudent to examine whether psychological problems after RYGB could somehow be related to GLP-1 neurobiology, in particular because some evidence indicates that icv GLP-1 administration increases anxiety in rodents.[8]

GLP-1 and GLP-1 Analogs

Because active, circulating GLP-1 is rapidly degraded by the enzyme DPP-4, pharmacological treatment strategies mainly employ analogs of GLP-1, such as Ex-4 (and exenatide) or liraglutide. Ex-4, a potent and long-acting natural GLP-1R agonist isolated from the saliva of the Gila monster, is a 39-amino acid peptide that shares 53% homology with native GLP-1. Because Ex-4 is not metabolized by DPP-4 it has a much longer plasma half-life than GLP-1.[15] Ex-4 potently reduces food intake, inhibits gastric emptying and improves glucoregulation. Although the strong eating-inhibitory effect of Ex-4 may carry the risk of undesirable side effects such as nausea or general malaise,[1] exenatide, a synthetic Ex-4 (marketed as Byetta) has been approved (FDA, 04-2005) for the treatment of T2D.

The other currently commercially available GLP-1R agonist, liraglutide, is a synthetic GLP-1 analog that shows 97% homology with the native peptide and features an even longer biological half-life than Ex-4. Also liraglutide has been approved as an anti-diabetic drug (FDA, 01-2010). It must be administered only once a day instead of twice daily for Ex-4. Current research aims at the development of other, even more effective GLP-1 agonists that could perhaps be administered only once a week. Despite the obvious therapeutic potential of GLP-1R agonists, some potential risk factors with chronic administration deserve attention. Chronic administration of liraglutide or Ex-4 in laboratory animals was associated with an increased incidence of thyroid cancer and the occurrence of cell lesions, respectively. So far, such effects were not observed in humans, but further studies are required to better judge on the relationship between potential risks and beneficial actions.[18] Another therapeutic option is to enhance intestinal absorption of orally administered GLP-1 by a substance called sodium *N*-[8-(2-hydroxybenzoyl)amino] caprylate (SNAC). SNAC effectively delivers peptides of low oral bioavailability and should produce high intestinal tissue and hepatic portal vein GLP-1 concentrations, hence copying the normal route of delivery after GLP-1 release from enteroendocrine cells into the interstitial fluid of the mucosal lamina propria. A single oral administration of such an SNAC-GLP-1 preparation produced much higher short-term increases in systemic plasma concentrations of active GLP-1 than a meal, decreased food intake and improved glycemic control by enhancing glucose-stimulated insulin release.[24,25] These findings suggest that the SNAC-GLP-1 preparation did in fact produce high local GLP-1 concentrations at the critical splanchnic receptor sites. SNAC preparations of satiating peptides may therefore be another viable option for the pharmacotherapy of T2D and obesity.

DPP-IV Inhibitors

As an alternative to enhancing GLP-1R activation by administering GLP-1 analogs, degradation of the native endogenous peptide can be delayed by inhibiting DPP-4. DPP-4 is a membrane-bound enzyme on capillary endothelia also present in the blood in soluble form. DPP-4 inhibitors such as PF-734200 or sitagliptin decrease prandial increases in circulating glucose mainly by increasing circulating GLP-1 levels.[9] Although DPP-4 inhibitors seem to hold potential for the treatment of T2D, they have scarcely any effect on eating. This is presumably because DPP-4 activates PYY, another eating-inhibitory peptide. The effectiveness of DPP-4 inhibitors for the treatment of obesity is therefore limited. Also, although inhibitor-based therapies are often considered less risky, some side effects such as headache, constipation, diarrhea, etc., have been reported in relation to DPP-4 inhibitor administration.[9,14]

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New GLP-1 Co-agonists

GLP-1 may also be promising as part of novel chimeric molecules developed via chemical connection of peptides using polyethylene glycol covalent bonds (i.e., PEGylation). The administration of such a PEGylated peptide combining GLP-1 and pancreatic glucagon agonist activity was shown to normalize adiposity and glucose tolerance in diet-induced obese mice.[5] The reduction of body weight in this study was achieved by a loss of body fat resulting from decreased food intake and increased energy expenditure. Such novel pharmacological "cocktail" approaches to treat obesity and T2D may eventually be possible with custom-tailored combinations of two or more gut-brain peptides in a single molecule. A sound knowledge about the mechanisms underlying the physiological effects of each single peptide is necessary to develop such compounds and to minimize adverse side effects.

CONCLUSIONS AND PERSPECTIVES

This chapter aimed at summarizing and assessing the available evidence for a role of GLP-1 in the control of eating and the underlying mechanisms of its action. Several lines of reasoning implicate both peripheral and CNS GLP-1 in the control of eating. Peripheral GLP-1 stems primarily from enteroendocrine cells in the small intestine; it can inhibit eating through a paracrine and an endocrine action. GLP-1Rs on abdominal, primarily intestinal, vagal afferents most likely mediate the paracrine action, whereas GLP-1Rs in the AP and NTS seem to mediate the endocrine action, which presumably requires significant increases in circulating GLP-1. Because of the short biological half-life of native GLP-1 such increases may occur only when GLP-1 release is substantially stimulated or after pharmacological interventions. Whether peripheral GLP-1 has an additional endocrine satiating effect mediated by GLP-1R on vagal afferents terminating in the wall of the hepatic portal vein remains to be thoroughly examined. A small set of NTS neurons primarily produces central GLP-1. Its inhibitory effect on eating can be mediated solely through hindbrain circuitries, but may also recruit midbrain and forebrain areas such as the PVN, the NAc, and the CeA. It remains to be determined whether or not peripheral GLP-1 ultimately recruits the hindbrain GLP-1 system for its satiating action. The effects of peripheral and central GLP-1R activation on eating may include an aversive component. The satiating and aversive actions of GLP-1 can, however, be dissociated, even with respect to the brain areas involved. Under which conditions one of the two actions prevails, or when they may coexist, needs to be characterized. Strong evidence indicates that endogenous GLP-1 contributes to the striking T2D- and obesity-curbing effects of RYGB. This and the fact that GLP-1 reduces food intake and body weight also in obese individuals spurred substantial interest into the therapeutic potential of GLP-1. Despite some open questions, GLP-1R agonists currently provide promising treatment options.

REFERENCES

1. Barrera JG, D'Alessio DA, Drucker DJ, Woods SC, Seeley RJ. Differences in the central anorectic effects of glucagon-like peptide-1 and exendin-4 in rats. *Diabetes* 2009;**58**:2820–7.
2. Barrera JG, Jones KR, Herman JP, D'Alessio DA, Woods SC, Seeley RJ. Hyperphagia and increased fat accumulation in two models of chronic CNS glucagon-like peptide-1 loss of function. *J Neurosci* 2011;**31**:3904–13.
3. Barrera JG, Sandoval DA, D'Alessio DA, Seeley RJ. GLP-1 and energy balance: an integrated model of short-term and long-term control. *Nat Rev Endocrinol* 2011;**7**:507–16.
4. Baumgartner I, Pacheco-Lopez G, Ruttimann EB, Arnold M, Asarian L, Langhans W, et al. Hepatic-portal vein infusions of glucagon-like peptide-1 reduce meal size and increase c-Fos expression in the nucleus tractus solitarii, area postrema and central nucleus of the amygdala in rats. *J Neuroendocrinol* 2010;**22**:557–63.
5. Day JW, Ottaway N, Patterson JT, Gelfanov V, Smiley D, Gidda J, et al. A new glucagon and GLP-1 co-agonist eliminates obesity in rodents. *Nat Chem Biol* 2009;**5**:749–57.
6. Dossat AM, Lilly N, Kay K, Williams DL. Glucagon-like peptide 1 receptors in nucleus accumbens affect food intake. *J Neurosci* 2011;**31**:14,453–7.
7. Grant P, Lipscomb D, Quin J. Psychological and quality of life changes in patients using GLP-1 analogues. *J Diabetes Complications* 2011;**25**:244–6.
8. Gulec G, Isbil-Buyukcoskun N, Kahveci N. Effects of centrally-injected glucagon-like peptide-1 on pilocarpine-induced seizures, anxiety and locomotor and exploratory activity in rat. *Neuropeptides* 2010;**44**:285–91.
9. Gustavson SM, Dai H, Preston GM, Somayaji V, Hirshberg B, Calle RA. Effects of multiple doses of the DPP-IV inhibitor PF-734200 on the relationship between GLP-1 and glucose in subjects with type 2 diabetes mellitus. *Diabetes Res Clin Pract* 2011;**91**:e45–9.
10. Hayes MR, Bradley L, Grill HJ. Endogenous hindbrain glucagon-like peptide-1 receptor activation contributes to the control of food intake by mediating gastric satiation signaling. *Endocrinology* 2009;**150**:2654–9.
11. Hayes MR, De Jonghe BC, Kanoski SE. Role of the glucagon-like-peptide-1 receptor in the control of energy balance. *Physiol Behav* 2010;**100**:503–10.
12. Hayes MR, Leichner TM, Zhao S, Lee GS, Chowansky A, Zimmer D, et al. Intracellular signals mediating the food intake-suppressive effects of hindbrain glucagon-like peptide-1 receptor activation. *Cell Metabolism* 2011;**13**:320–30.
13. Hayes MR, Skibicka KP, Grill HJ. Caudal brainstem processing is sufficient for behavioral, sympathetic, and parasympathetic responses driven by peripheral and hindbrain glucagon-like-peptide-1 receptor stimulation. *Endocrinology* 2008;**149**:4059–68.
14. Herman GA, Mistry GC, Yi B, Bergman AJ, Wang AQ, Zeng W, et al. Evaluation of pharmacokinetic parameters and dipeptidyl peptidase-4 inhibition following single doses of sitagliptin in healthy, young Japanese males. *Br J Clin Pharmacol* 2011;**71**:429–36.

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15. Holst JJ. The physiology of glucagon-like peptide 1. *Physiol Rev* 2007;**87**:1409–39.
16. Kanoski SE, Fortin SM, Arnold M, Grill HJ, Hayes MR. Peripheral and central GLP-1 receptor populations mediate the anorectic effects of peripherally administered GLP-1 receptor agonists, liraglutide and exendin-4. *Endocrinology* 2011;**152**:3103–12.
17. Kinzig KP, D'Alessio DA, Seeley RJ. The diverse roles of specific GLP-1 receptors in the control of food intake and the response to visceral illness. *J Neurosci* 2002;**22**:10,470–6.
18. Madsbad S, Kielgast U, Asmar M, Deacon CF, Torekov SS, Holst JJ. An overview of once-weekly glucagon-like peptide-1 receptor agonists—available efficacy and safety data and perspectives for the future. *Diabetes Obes Metab* 2011;**13**:394–407.
19. McKay NJ, Kanoski SE, Hayes MR, Daniels D. Glucagon-like peptide-1 receptor agonists suppress water intake independent of effects on food intake. *Am J Physiol Regul Integr Comp Physiol* 2011;**301**:R1755–64.
20. McMahon LR, Wellman PJ. PVN infusion of GLP-1-(7–36) amide suppresses feeding but does not induce aversion or alter locomotion in rats. *Am J Physiol* 1998;**274**:R23–9.
21. Punjabi M, Arnold M, Geary N, Langhans W, Pacheco-Lopez G. Peripheral glucagon-like peptide-1 (GLP-1) and satiation. *Physiol Behav* 2011;**105**:71–6.
22. Rhee NA, Vilsbøll T, Knop FK. Current evidence for a role of GLP-1 in Roux-en-Y gastric bypass induced remission of type 2 diabetes. *Diabetes Obes Metabolism* 2011.
23. Ruttimann EB, Arnold M, Hillebrand JJ, Geary N, Langhans W. Intrameal hepatic portal and intraperitoneal infusions of glucagon-like peptide-1 reduce spontaneous meal size in the rat via different mechanisms. *Endocrinology* 2009;**150**:1174–81.
24. Steinert RE, Poller B, Castelli MC, Drewe J, Beglinger C. Oral administration of glucagon-like peptide 1 or peptide YY 3-36 affects food intake in healthy male subjects. *Am J Clin Nutr* 2010;**92**:810–7.
25. Steinert RE, Poller B, Castelli MC, Friedman K, Huber AR, Drewe J, et al. Orally administered glucagon-like peptide-1 affects glucose homeostasis following an oral glucose tolerance test in healthy male subjects. *Clin Pharmacol Therapeutics* 2009;**86**:644–50.
26. Tang-Christensen M, Larsen PJ, Goke R, Fink-Jensen A, Jessop DS, Moller M, et al. Central administration of GLP-1-(7-36) amide inhibits food and water intake in rats. *Am J Physiol* 1996;**271**:R848–56.
27. Thiele TE, Seeley RJ, D'Alessio D, Eng J, Bernstein IL, Woods SC, et al. Central infusion of glucagon-like peptide-1-(7–36) amide (GLP-1) receptor antagonist attenuates lithium chloride-induced c-Fos induction in rat brainstem. *Brain Res* 1998;**801**: 164–70.
28. Tindle HA, Omalu B, Courcoulas A, Marcus M, Hammers J, Kuller LH. Risk of suicide after long-term follow-up from bariatric surgery. *Am J Med* 2010;**123**:1036–42.
29. Trapp S, Hisadome K. Glucagon-like peptide 1 and the brain: central actions-central sources? *Auton Neurosci* 2011;**161**:14–9.
30. Turton MD, O'Shea D, Gunn I, Beak SA, Edwards CM, Meeran K, et al. A role for glucagon-like peptide-1 in the central regulation of feeding. *Nature* 1996;**379**:69–72.
31. Vahl TP, Drazen DL, Seeley RJ, D'Alessio DA, Woods SC. Meal-anticipatory glucagon-like peptide-1 secretion in rats. *Endocrinology* 2010;**151**:569–75.
32. van Dijk G, Thiele TE. Glucagon-like peptide-1 (7–36) amide: a central regulator of satiety and interoceptive stress. *Neuropeptides* 1999;**33**:406–14.
33. Williams DL. Minireview: finding the sweet spot: peripheral versus central glucagon-like peptide 1 action in feeding and glucose homeostasis. *Endocrinology* 2009;**150**:2997–3001.
34. Williams DL, Baskin DG, Schwartz MW. Leptin regulation of the anorexic response to glucagon-like peptide-1 receptor stimulation. *Diabetes* 2006;**55**:3387–93.
35. Williams DL, Baskin DG, Schwartz MW. Evidence that intestinal glucagon-like peptide-1 plays a physiological role in satiety. *Endocrinology* 2009;**150**:1680–7.

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Chapter 150

Glucagon

Nori Geary

ABSTRACT

Glucagon is a peptide hormone synthesized and secreted by the pancreatic α-cells. The glucagon receptor is a G-protein-coupled, seven-transmembrane domain receptor and is expressed in the liver, pancreatic islets, heart, adipose tissue, and, less abundantly, in other tissues. Glucagon is secreted when blood glucose concentrations decrease, and it rapidly increases hepatic glucose production, through glycogenolysis for the first approximately 3 h and both glycogenolysis and gluconeogenesis thereafter. Glucagon has several effects on lipid metabolism, notably stimulation of hepatic fatty acid oxidation and ketone synthesis. Glucagon is also secreted during stress, such as during exercise, and contributes to the maintenance of metabolic fuel homeostasis in these situations. Glucagon is secreted during most meals, and prandial glucagon secretion contributes to meal-ending satiation. Plasma glucagon concentrations are chronically elevated in diabetes and contribute to its pathophysiological consequences. The therapeutic potential of glucagon in several of the areas mentioned is the focus of current research.

INTRODUCTION

Glucagon research has a long history.[19] In their initial description of the discovery of insulin in 1921, Banting and Best[3] noted that their pancreatic extracts had hyperglycemic and hypoglycemic properties. Only 2 years later, Murlin et al.[22] isolated pancreatic extracts that had only hyperglycemic activity and named the putative active molecule glucagon. Glucagon was isolated to the pancreatic α-cells by Sutherland and de Duve in 1948,[28] the amino acid sequence of glucagon was established by Bromer et al. in 1956,[4] and a radioimmunoassay was developed by Unger et al. in 1959.[29] Consistent with its long history and potent biological effects, glucagon physiology and pathophysiology have been studied extensively in mice, rats, dogs, pigs, nonhuman primates, and, of course, humans. The good correspondence of most results in these diverse animal models with those in humans indicates the high translational utility of animal research on glucagon. Progress in glucagon physiology and pathophysiology through the mid-1990s was reviewed in detail in three volumes of the Handbook of Experimental Pharmacology.[16–18]

GLUCAGON AND THE GLUCAGON RECEPTOR

Posttranslational processing of the glucagon gene product proglucagon, a 160-amino-acid polypeptide, produces a variety of bioactive peptide hormones. This processing is tissue-specific because of the differential expression of the prohormone convertase PC2 (or neuroendocrine convertase 2, NEC2) in the pancreatic α-cells and PC1/3 elsewhere, respectively (see the Peptide Biosynthesis/Processing section of the book). Glucagon, human proglucagon 33–61, is synthesized and released mainly from the pancreatic α-cells and acts almost exclusively on the glucagon receptor, which recognizes glucagon's C-terminal. The exception is that glucagon has pharmacological and perhaps physiological actions on gastrointestinal smooth muscles that are mediated by an unknown receptor that recognizes glucagon's N-terminal. The major residues of glucagon synthesis, that is, proglucagon 1–30 (GRPP, glicentin-related polypeptide), proglucagon 64–69 (intervening peptide-1), and proglucagon 72–158 (MPGF, major proglucagon fragment), seem to be inactive. The glucagon gene and its posttranslational products are highly conserved in the vertebrate lineage. Rat, mouse, and human glucagons are identical.

Intestinal L cells produce glicentin (proglucagon 1–69), which seems to be inactive, as well as glucagon-like peptide-1 (GLP-1; proglucagon 78–107amide), GLP-2 (proglucagon 126–158), and oxytomodulin (proglucagon 33–69), all of which are active. Small amounts of GLP-1 may also result form cleavage of MPGF in the α-cells. As the proglucagon amino acid sequences of these peptides suggest, only oxyntomodulin has structural homology to glucagon and mimics glucagon's bioactivity. Oxyntomodulin, however, stimulates hepatic glucose production about an order of magnitude less potently than does glucagon, presumably because its C-terminal extension interferes with receptor binding. The actions of GLP-1 and GLP-2 are independent of those of glucagon. L cells also produce glucagon in physiologically relevant amounts if the pancreatic α-cells do not, for example, after pancreotomy. Finally, a circumscribed set of neurons in the nucleus tractus solitarius produces GLP-1,

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00150-0
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but whether glucagon is produced or acts in the CNS remains controversial.

The human glucagon receptor is a 485-amino-acid protein with a 143-amino-acid extracellular N-terminal. Amino acids 80–142 seem crucial for specific recognition of glucagon, and other sites in the N terminal as well as other extracellular domains seem to determine the receptor conformation that permits binding. The structure of the glucagon receptor, like that of glucagon, is highly conserved. The N-terminal sequence of the glucagon receptor is 97% identical in rats and humans. Glucagon receptors are expressed densely in the liver and also in physiologically relevant numbers in the pancreatic islets, kidney, heart, adipose tissue, gastrointestinal tract, and, possibly, brain. The glucagon receptor is a member of a distinct family of G-protein-coupled seven transmembrane domain receptors, whose activation leads to activation of membrane-bound adenyl cyclase, which increases intracellular cyclic adenosine monophosphate (cAMP) and via cAMP increases inositol 1,4,5-triphsophate (InsP3) or Ca^{2+}. Work on glucagon's intracellular signaling pathway led to Nobel Prizes to Earl Sutherland in 1971, for cAMP signaling, and to Martin Rodbell in 1994, for G-protein-coupled receptor signaling. This remains the canonical glucagon-signaling pathway, although additional mechanisms may contribute in some tissues. For example, some results indicate that some elevation of intracellular InsP3 and Ca^{2+} may occur independent of cAMP in the liver.

Glucagon itself is a prohormone in some target tissues, including liver and heart, where glucagon 19–29, or miniglucagon, is produced by endopeptidases that attack the arginine doublet at positions 17 and 18 of glucagon. Miniglucagon inhibits the hepatic Ca^{2+} pump 2–3 orders of magnitude more potently than glucagon and also is a component of the positive inotropic effect of glucagon in the heart.

GLUCAGON SECRETION

Glucagon is secreted tonically as plasma glucose concentrations decrease during fasting or in hypoglycemia and phasically during sudden-onset hypoglycemia, during meals (except pure carbohydrate meals), and during certain physiological stresses, including exercise. The increase in glucagon secretion produced by decreasing plasma glucose levels is in large part an indirect effect caused by release from a number of inhibitory signals that are tonically active during euglycemia, with some contribution of a direct inhibitory action of glucose.[11,20] The most important of the inhibitory signals that tonically inhibit glucagon secretion are related to local, intra-islet secretions. Falling plasma glucose decreases the secretion of insulin, zinc, and GABA by the pancreatic β-cells. Insulin and zinc act on ATP-sensitive K^+ channels to hyperpolarize the α-cell membrane. GABA, acting via cognate receptors on the α-cell membrane, also produces α-cell hyperpolarization. Decreases in plasma glucose concentratons are also sensed in the ventromedial hypothalamus and other brain sites, leading to increases in sympathetic and parasympathetic neural activity, both of which can stimulate glucagon secretion. Increased epinephrine secretion by the adrenal medulla also participates. The autonomic nervous system is also the main mediator of stress-related and prandial glucagon secretion. Further controls include somatostatin and GLP-1, both of which act on receptors on the α-cell membrane to inhibit glucagon secretion, and various plasma amino acids, which stimulate glucagon secretion (and presumably participate in the increase in glucagon secretion afater ingestion of high-protein meals). Although glucagon secretion is pulsatile, pulsatility has not been shown to affect glucagon's potency *in vivo*.

PHYSIOLOGICAL EFFECTS

Glucagon has a panoply of physiological effects,[13,16–18] a few of which are briefly reviewed here.

Hepatic Glucose Production

Glucagon phasically and tonically stimulates hepatic glucose output.[6,7,11,23,25] The phasic effect results entirely from the stimulation of glycogenolysisis. Over its physiological range of approximately 0–200 ng/L plasma, glucagon has a relatively linear effect on hepatic glucose production, which can vary approximately 10-fold, between 2.5 and 25 μmol/kg/min.[25] The increase in glycogenolysis is mediated by glucagon-stimulated increases in cAMP in the hepatocytes, which activate glycogen phosphorylase and inhibit glycogen synthase. Maximal hepatic glycogenolysis is sustained only for approximately 15 min, however, after which the rate of glycogenolysis decreases to approximately one-third maximal over approximately 3 h. Hepatic gluconeogenesis increases beginning around 3 h after the initial increase in glucagon. This is mediated by slowly developing increases in the activity of pyruvate carboxylase and other gluconeogenic enzymes and by upregulation of several genes. Glycogenolysis and gluconeogenesis seem to contribute quantitatively similar amounts to the tonic stimulation of hepatic glucose production that follows an overnight fast.

Lipid Metabolism

Glucagon and insulin cooperate in the regulation of hepatic ketogenesis. Insulin inhibits adipose tissue lipolysis, thus increasing the supply of nonesterified fatty acids (NEFA) to the liver, which is necessary for ketogenesis. An increase in the liver's capacity for fatty acid oxidation and concomitant decrease in hepatic lipogenesis is also required, which is produced by an increase in the glucagon:insulin ratio.

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Ketogenesis results from β-oxidation of fatty acids in the hepatic mitochondria, which is regulated by the activity of carnitine palmitytol transferase I, which catalyzes uptake of fatty acid acyl-CoA into the mitochondria. In turn, malonyl-CoA, the first committed intermediate in the conversion of glucose into fat, acts to negatively regulate carnitine palmitytol transferase I, such that the fall in malonyl-CoA that accompanies the increase in the glucagon:insulin ratio occurring during the transition from the fed to the fasted state directs the flow of fatty acids into ketogenesis.[21] Ketones provide an alternate source of metabolic fuel for otherwise glucose-dependent tissues.

Numerous *in vitro* studies indicate that glucagon acts on glucagon receptors on adipocytes to reduce lipolysis and decrease nonesterified fatty acid release.[13] Attempts to document these phenomena *in vivo*, however, have met with mixed success,[10] probably partly because of the complex interactions between the effects of insulin and glucagon on lipid metabolism.

Glucagon causes the movement of plasma cholesterol and triglycerides into the platelet fraction of the blood. Glucagon also reduces the synthesis of triglycerides and apolipoprotein E in the liver and increases the binding capacity of low-density lipoprotein receptors. In this case as well, the normal physiological relevance of these effects remains unresolved.

Bile Acid Metabolism

A study by Song and Chiang[27] indicates that glucagon down regulates hepatic expression of cholesterol 7-alpha-monooxygenase, the rate-limiting enzyme in bile acid synthesis. This suggests a role for glucagon in the regulation of bile acid metabolism and provides another potential mechanism for its control of plasma cholesterol.

Cardiac Effects

Glucagon stimulates the release of cAMP in the myocardium, thus leading to positive inotropic and chronotropic effects.[30] Because this action does not require a beta-1 adrenoceptor stimulation, glucagon is used to treat overdoes of beta-blockers and other drugs. It may also be used for this purpose in shock, but does not function as effectively as catecholamine treatment in this situation.

Satiation

As mentioned above, glucagon secretion increases during meals, except during very high-carbohydrate meals. The demonstration that antagonizing circulating glucagon during meals increased meal size implicates prandial glucagon secretion in normal meal-ending satiation.[14] This, incidentally, was the first use of an antagonist strategy to identify a normal endocrine control of eating. A subsequent study showed that intravenous infusion of a physiological glucagon dose was sufficient to hasten satiation in normal weight men.[9] The satiating effect of glucagon occurs in the liver or portal vein and is relayed to the brain via vagal afferents.

PATHOPHYSIOLOGY

Diabetes Mellitus

Glucagon has been implicated in the pathophysiologies of both type 1 and type 2 diabetes mellitus (T1DM and T2DM, respectively).[1,2,6,7,12,26] The normal or near-normal fasting plasma glucagon concentrations found in patients with T2DM represent marked hyperglucagonemia relative to the elevated concentrations of plasma glucose and insulin, both of which normally inhibit glucagon secretion, and lead to increased hepatic glucose production. Similarly, carbohydrate ingestion fails to inhibit glucagon secretion after meals, leading to exaggerated prandial glucagonemia and continued hepatic glucose production. The glucagon response to protein ingestion is also exaggerated. Thus, disturbances in glucose-controlled and prandial glucagon secretion are an important component of the pathophysiology of T2DM, and their control may represent a therapeutic option. The mechanisms underlying these disturbances, unfortunately, remain obscure.

Relative hyperglucagonemia has also been implicated in the pathophysiology of T1DM. Lee et al.[15] indicate that this contribution is not merely related to unrestrained glucose production, as described above. These investigators found, as others had reported, that streptozotocin, which is toxic to the pancreatic β-cells and produces a model of T1DM, fails to elicit hyperglycemia in transgenic mice lacking the glucagon receptor. In addition, and very surprisingly, they found that these mice display normal glucose tolerance, suggesting that the lack of glucagon signaling somehow increases the ability of liver and muscle to take up glucose in the absence of insulin. In so far as muscle normally fails to express glucagon receptors, the mechanism must be at least in part indirect. One possibility is that the markedly increased plasma GLP-1 levels in the diabetic, glucagon-receptor knockout mice contribute.

Glucagonoma

Glucagonomas are rare, slow-growing, malignant, glucagon-secreting tumors of the pancreatic islets. Fasting plasma glucagon concentrations are elevated 2- to 200-fold. The commonest presenting sign is necrolytic migratory erythematotic rash. Mild T2DM, weight loss, and anorexia are typical. Metastases occur in the liver, bone, lymph glands, and other tissues. Because glucagonomas are rarely detected early, management is challenging. A combination of dermatologic

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treatments, tumor-reducing surgery, hepatic artery embolization, and antagonism of the effects of glucagon with somatostatin analogs usually effectively reverses the signs and symptoms of glucagonoma.[8]

THERAPEUTIC USE

Hypoglycemia

The longest-standing therapeutic application of glucagon is in the treatment of acute hypoglycemic episodes that are too severe to permit the patient to ingest glucose orally and if intravenous glucose infusion therapy is not available. It is physiologically benign, with glucagon hypersensitivity very rarely observed. Thus, glucagon can be used even when diagnosis of hypoglycemia is uncertain, as in anaphylaxis, or when its cause is unknown. Because glucagon leads to catecholamine release, pheochromocytoma is a contraindication.

Gastrointestinal Radiology and Esophageal Foreign Body Impaction (EFBI)

Because glucagon relaxes gastrointestinal smooth muscle, especially esophageal smooth muscle, a second standard use of glucagon is to relax the gastrointestinal tract before radiologic examination. Again, the main contraindication for this use of glucagon is pheochromocytoma.

Glucagon's effect to relax the esophagus has led to its use in emergency medicine to treat EFBI. A recent review,[2] however, indicates that glucagon is not an effective therapy for EFBI and, because it can elicit vomiting, increases the risks of aspiration and esophageal perforation.

Diabetes

The emerging role of relative hyperglucagonemia in diabetes mellitus described above has led to speculation as to the usefulness of glucagon antagonism in diabetes treatment, and promising results have been reported in animal models of both T1DM and T2DM.[1,6,7,12,15,25,26]

Obesity

Two studies[5,24] suggest that formulations with both glucagon and GLP-1 receptor-agonist properties reduce body weight in rodent models. Changes in both eating and energy expenditure contribute to the effects. Future work will have to address the conundrum that GLP-1 agonism plus glucagon antagonism appear beneficial in T2DM, which is most frequently caused by obesity, whereas GLP-1 agonism plus glucagon antagonism seem beneficial in obesity.

REFERENCES

1. Ali S, Drucker DJ. Benefits and limitations of reducing glucagon action for the treatment of type 2 diabetes. *Am J Physiol* 2009;**296**:E415–21.
2. Arora S, Galich P. Myth: glucagon is an effective first-line therapy for esophageal foreign body impaction. *CJEM* 2009;**11**:169–71.
3. Banting FG, Best CH. The internal secretion of the pancreas. *J Lab Clin Med* 1921–1922;**7**:251–66.
4. Bromer WW, Sinn LG, Staub A, Behrens OK. The amino acid sequence of glucagon. *J Am Chem Soc* 1956;**78**:3858–9.
5. Day JW, Ottaway N, Patterson JT, Gelfanov V, Smiley D, Gidda J, et al. A new glucagon and GLP-1 co-agonist eliminates obesity in rodents. *Nat Chem Biol* 2009;**5**:749–57.
6. Dunning BE, Gerich JE. The role of alpha-cell dysregulation in fasting and postprandial hyperglycemia in type 2 diabetes and therapeutic implications. *Endocr Rev* 2007;**28**:253–83.
7. Edgerton DS, Cherrington AD. Glucagon as a critical factor in the pathology of diabetes. *Diabetes* 2011;**60**:377–80.
8. Frankton S, Bloom SR. Gastrointestinal endocrine tumours. Glucagonomas *Baillieres Clin Gastroenterol* 1996;**10**:697–705.
9. Geary N, Kissileff HR, Pi-Sunyer FX, Hinton V. Individual, but not simultaneous, glucagon and cholecystokinin infusions inhibit feeding in men. *Am J Physiol* 1992;**262**:R975–80.
10. Gravholt CH, Moller N, Jensen MD, Christiansen JS, Schmitz O. Physiological levels of glucagon do not influence lipolysis in abdominal adipose tissue as assessed by microdialysis. *J Clin Endocrinol Metab* 2001;**86**:2085–9.
11. Gromada J, Franklin I, Wollheim CB. α-cells of the endocrine pancreas: 35 years of research but the enigma remains. *Endocr Rev* 2007;**28**:84–116.
12. Gu W, Winters KA, Motani AS, Komorowski R, Zhang Y, Liu Q, et al. Glucagon receptor antagonist-mediated improvements in glycemic control are dependent on functional pancreatic GLP-1 receptor. *Am J Physiol* 2010;**299**:E624–32.
13. Habegger KM, Heppner KM, Geary N, Bartness TJ, DiMarchi R, Tschöp MH. The metabolic actions of glucagon revisited. *Nat Rev Endocrinol* 2010;**6**:689–97.
14. Langhans W, Zeiger U, Scharrer E, Geary N. Stimulation of feeding in rats by intraperitoneal injection of antibodies to glucagon. *Science* 1982;**218**:894–6.
15. Lee Y, Wang MY, Du XQ, Charron MJ, Unger RH. Glucagon receptor knockout prevents insulin-deficient type 1 diabetes in mice. *Diabetes* 2011;**60**:391–7.
16. Lefèbvre PJ, editor. *Glucagon I*. Berlin, Heidelberg, New York and Tokyo: Springer-Verlag; 1983.
17. Lefèbvre PJ, editor. *Glucagon II*. Berlin, Heidelberg, New York and Tokyo: Springer-Verlag; 1983.
18. Lefèbvre PJ, editor. *Glucagon III*. Berlin, Heidelberg, New York and Tokyo: Springer-Verlag; 1996.
19. Lefèbvre PJ. Early milestones in glucagon research. *Diabetes Obes Metabol* 2011;**13**(Suppl. 1):1–4.
20. MacDonald PE, De Marinis YZ, Ramracheya R, Salehi A, Ma X, Johnson PR, et al. A K ATP channel-dependent pathway within alpha cells regulates glucagon release from both rodent and human islets of Langerhans. *PLoS Biol* 2007;**5**:e143.
21. McGarry JD, Woeltje KF, Kuwajima M, Foster DW. Regulation of ketogenesis and the renaissance of carnitine palmitoyltransferase. *Diabetes Metab Rev* 1989;**5**:271–84.

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22. Murlin JR, Clough HD, Gibbs CBF, Stokes AM. Aqueous extracts of the pancreas. I. Influence on the carbohydrate metabolism of depancreatized animals. *J Biol Chem* 1923;**56**:253–96.
23. Nuttall FQ, Ngo A, Gannon MC. Regulation of hepatic glucose production and the role of gluconeogeneisis in humans. *Diabetes Metab Res Rev* 2008;**24**:438–58.
24. Pocai A, Carrington PE, Adams JR, Wright M, Eiermann G, Zhu L, et al. Glucagon-like peptide 1/glucagon receptor dual agonism reverses obesity in mice. *Diabetes* 2009;**58**:2258–66.
25. Ramnanan CJ, Edgerton DS, Kraft G, Cherrington AD. Physiologic action of glucagon on liver glucose metabolism. *Diab Obes Metabl* 2011;**13**(Suppl. 1):118–25.
26. Shat P, Vella A, Basu A, Schwenk WF, Rizza RA. Lack of suppression of glucagon contributes to postprandial hyperlycemia in subjects with type 2 diabetes mellitus. *J Clin Endocrinol Metab* 2000;**85**:4053–9.
27. Song KH, Chiang JY. Glucagon and cAMP inhibit cholesterol 7alpha-hydroxylase (CYP7A1) gene expression in human hepatocytes: discordant regulation of bile acid synthesis and gluconeogenesis. *Hepatology* 2006;**43**:117–25.
28. Sutherland EW, de Duve C. Origin and distribution of the hyperglycemic-glycogenolytic factor of the pancreas. *J Biol Chem* 1948;**175**:663–74.
29. Unger RH, Eisentraut AM, McCall MS, Keller S, Lanz HC, Madison LL. Glucagon antibodies and their use for immunoassay for glucagon. *Proc Soc Exp Biol Med* 1959;**102**:621–3.
30. White CM. A review of potential cardiovascular uses of intravenous glucagon administration. *J Clin Pharmacol* 1999;**39**:442–7.

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Chapter 151

Insulin

Bernadette E. Grayson and Stephen C. Woods

ABSTRACT

The pancreatic hormone, insulin, regulates circulating glucose levels, and in addition, it has a close relationship with the central nervous system to control food intake and body weight. Circulating insulin levels are directly associated with the levels of adiposity. Although most of its peripheral effects are anabolic, the central actions of insulin are specifically catabolic. These central effects of insulin are mediated largely through the arcuate nucleus of the hypothalamus, an important brain locale for modulation of food intake and body weight.

INTRODUCTION

Insulin is an approximately 5.8-kDa peptide hormone synthesized by the β-cells of the islets of Langerhans in the endocrine pancreas. Insulin has diverse functions *in vivo* which include regulating glucose and lipid metabolism, controlling food intake, maintaining long-term body weight, and influencing basic cell growth and replication. The actions of insulin first came to light in 1889 when Minkowski and von Mering found that the removal of the pancreas from a dog produces diabetes, a disease resulting from insufficient levels and/or action of insulin in the body. However, it was not until the work of Banting, Best, and Macleod that insulin was purified and its fundamental importance made evident. The role of insulin in the central nervous system (CNS) control of food intake and body weight in the context of its other roles is the focus of this review.

GLUCOREGULATORY ACTIONS OF INSULIN

Simply put, the primary role of insulin is to control the levels of circulating glucose by sequestering glucose into cells after meal ingestion. In response to a meal, the levels of glucose and other nutrients increase in the circulation. After entering the β-cells through facilitated diffusion via the glucose transporter, GLUT2, intracellular glucose directly stimulates glycolysis, increasing the intracellular ATP:ADP ratio. Consequent closure of the ATP-sensitive potassium channels (K_{ATP}) results in depolarization of the β-cell membrane, triggering calcium entry via voltage-dependent calcium channels and initiates a signal transduction cascade culminating in discharge of the releasable pool of insulin into circulation. Insulin secretion is consequently directly proportional to the concentration of glucose reaching the β-cell.

INSULIN ACTIONS

Insulin, with a half-life of only 2–3 min, exerts only a transient effect when secreted. Its major peripheral actions are in the liver, adipose tissue, and skeletal muscle although insulin receptors (IR) are ubiquitously found on most tissues including much of the CNS.[19] The IR is a transmembrane tyrosine kinase receptor with two extracellular α subunits and two transmembrane β subunits. Binding of insulin to its receptor initiates phosphorylation of catalytic substrates, which trigger two major signaling cascades: the phosphatidylinositol 3-OH kinase (PI3K) pathway and the mitogen-activated protein kinase (MAPK) pathway. Activity in these pathways results in translocation of tissue-specific glucose transporters (GLUTs), primarily GLUT2 in liver and GLUT4 in skeletal muscle and adipocytes, from the cytoplasm to the plasma membrane. Via these GLUTs, insulin stimulates an increase of glucose uptake by the cell. Because of this primary action of insulin, pharmacologic insulin administration results in decreased blood glucose and is used in the treatment of diabetic individuals who are unable to produce insulin in sufficient quantities to reduce blood glucose to a normal, low level. Insulin administration to non-diabetics results in hypoglycemia.

In the pancreatic islets, insulin inhibits the secretion of pancreatic glucagon, a hormone from α-cells that directly opposes the actions of insulin by increasing blood glucose, primarily by stimulating hepatic glucose production and secretion. In the liver, insulin stimulates glycogen synthesis and storage and inhibits the breakdown of glycogen, and at the same time inhibits the formation of *de novo* glucose by gluconeogenesis. In adipose tissue, insulin promotes the uptake of fatty acids by adipocytes and their conversion to stored triglycerides. Hence,

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00151-2
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in the periphery, insulin is primarily anabolic, promoting a build-up of stored energy in many tissues. This is in direct contrast to the overriding catabolic action of insulin in the CNS.

INSULIN SECRETION

Fasting insulin levels in the plasma are low and stable (basal condition), and they increase concomitant with meal ingestion (prandial condition) or glucose administration (stimulated condition). Lean individuals have lower levels of basal, prandial, and stimulated concentrations of insulin, and obese individuals, particularly those with increased levels of visceral adiposity, maintain higher concentrations of basal insulin and they secrete proportionally more insulin in response to a standard meal[2] (Fig. 1). Because obese individuals require more insulin to maintain normoglycemia, they are considered "insulin resistant," and the term also refers to an inability of insulin to elicit glucose uptake in a tissue-specific manner.

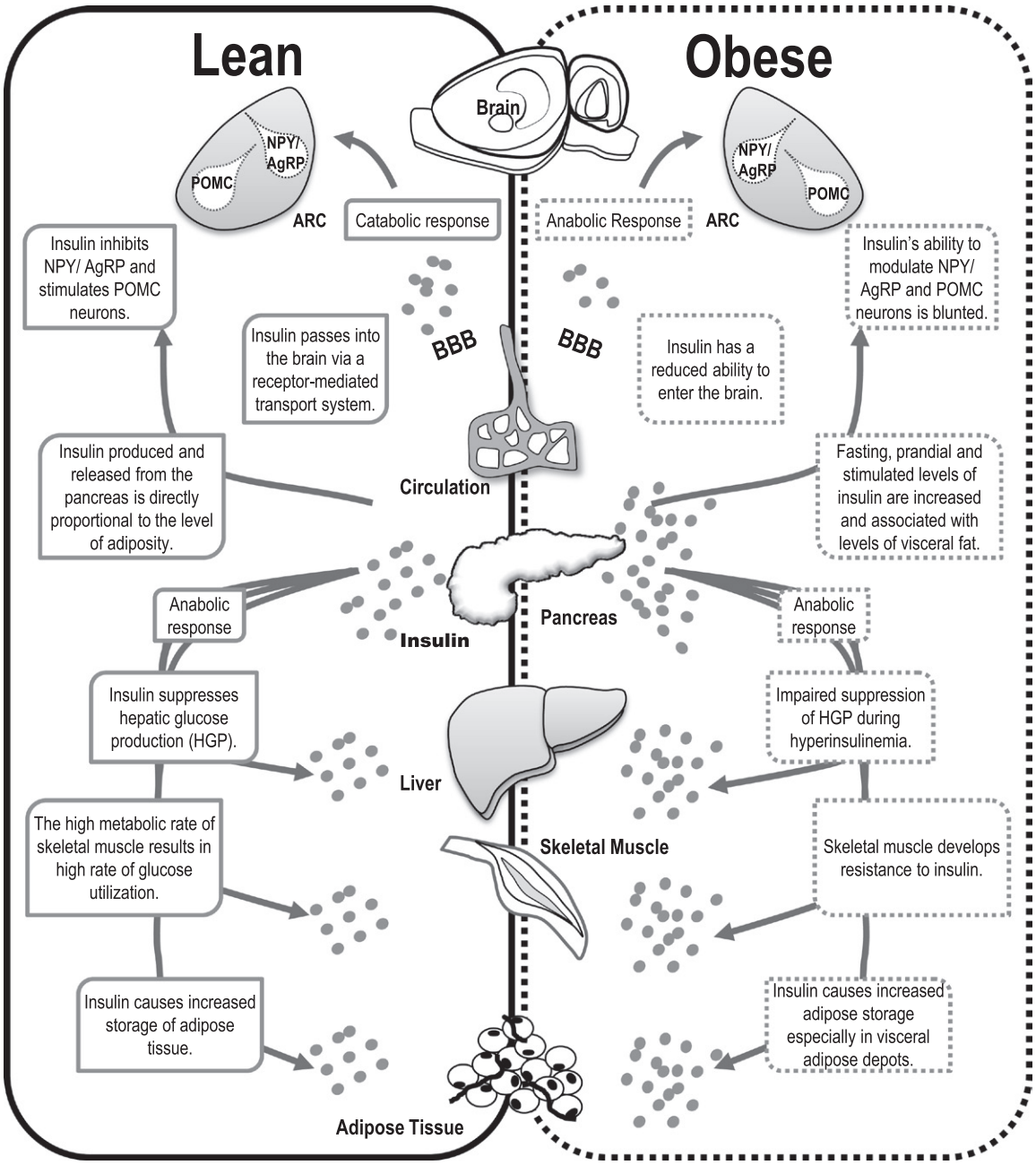

FIGURE 1 Central and peripheral activities of insulin in lean and obese states. Overall actions on the brain, pancreas, liver, skeletal muscle, and adipose tissue in lean and obese individuals (BBB, blood–brain barrier).

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Because its secretion is in direct proportion to the amount of body fat, and because it is able to penetrate the blood–brain barrier (BBB) from the circulation, insulin is considered an adiposity signal that provides information to the CNS that contributes to the homeostatic regulation of body fat. When an individual is food-restricted or voluntarily diets and loses weight, less insulin is secreted and reaches the brain; and the catabolic tone of the hypothalamus is consequently reduced. Food intake increases in this circumstance until body weight and the central insulin signal are restored to the predieting level. In instances of forced-overfeeding or voluntary overconsumption of nutrients, adiposity and the insulin signal rise, increasing the catabolic tone of the hypothalamus and eliciting hypophagia until the excess weight is lost (Fig. 1). Thus, the amount of insulin, together with that of leptin (an adipose-derived hormone),[16] directly correlates with adiposity and conveys a key index of the degree of peripheral adipose stores.

INSULIN AND THE BRAIN

The brain has an obligate need for glucose as a fuel source, and it uses upwards of 25% of total circulating glucose throughout the day. Unlike most tissues, the brain is exempt from the direct need for insulin to uptake glucose from the extracellular fluid. Rather, in the CNS, GLUT3 is located on the plasma membrane of neurons and GLUT1 is expressed by nonneuronal cells such as glia and ependymal cells. Both transporters are insulin-independent and allow glucose uptake in the CNS.[26] Thus, rather than stimulating glucose uptake by brain cells, insulin functions as a hormonal messenger to the CNS regarding peripheral nutrition and stored fat.

The BBB is a physical and functional wall created by endothelial cells riveted with tight junctions surrounding brain capillaries and buffered by a layer of astroglia. The BBB prevents the entry of most large molecules from the circulation into the brain interstitial fluid. Many molecules that are needed by brain cells, such as glucose and amino acids, therefore gain entry to the brain via specific molecular transport systems, and this is also true for insulin. A saturable, IR-mediated transport system on brain capillary endothelial cells moves insulin through the cell and releases it into brain interstitial fluid biologically intact.[3] Some of the insulin diffuses into the cerebrospinal fluid (CSF) where its levels can be determined and reflect insulin activity in the brain.[28] CSF insulin therefore correlates with plasma insulin and secondarily with body adiposity[34] (Fig. 1).

There are important consequences based on the entry of insulin into the brain being IR mediated. For one, the transport system can become saturated. Thus, as circulating insulin increases, the entry of insulin does not increase beyond the point of saturation.[3] Another consequence is that insulin transport into the CNS is regulated. As an example, it is decreased in fasting and weight loss, and increased after meals.[31] Animals with compromised insulin receptors have reduced ability of insulin to enter the brain. Although most brain areas express some IR, many brain regions have particularly high levels. The olfactory bulb densely expresses IR, as does the cortex, hippocampus and regions of the hypothalamus, the latter being important for the integrative control of food intake and body weight regulation.[6,15]

When exogenous insulin is administered directly into the brain, near or directly within the mediobasal hypothalamus, animals behave as if they have excess fat in the body; they eat less food and lose weight[6,29] (Fig. 1). This response is dose dependent; has been documented in numerous species including mice, rats, sheep, and nonhuman primates; and the hypophagia is not secondary to illness.[11] Conversely, if the insulin signal is reduced in the brain, either by local administration of specific insulin antibodies[22] or else by compromising the action of the IR locally in the mediobasal hypothalamus,[17] animals overeat and gain weight. When insulin levels in the brain are held constant for long intervals by means of slow, steady local infusions, body weight is maintained and defended at a level determined by the dose of insulin administered.[10] Although insulin has not been administered directly into the brain of humans, it has been administered intranasally to humans with a consequent increase of insulin in the cerebrospinal fluid. Humans administered insulin intranasally eat less food and lose body fat[18] Males are more sensitive to the intranasal insulin administration than females consistent with an observed sex difference in rats.[13] This basic sex difference is consistent with the observation that, in general, males have higher levels of visceral adiposity and concomitant higher levels of circulating insulin than females.

INSULIN AND THE ARC

The hypothalamic arcuate nucleus (ARC) borders the floor of the third cerebral ventricle near the median eminence and contains two classes of neurons that project to other brain areas. One class is considered catabolic, eliciting decreased food intake when stimulated and loss of body weight and body fat when stimulation is prolonged. Primary neurotransmitters of these neurons include the peptides, α-melanocyte-stimulating hormone (αMSH, a cleavage product of proopiomelanocortin, or POMC) and cocaine and amphetamine-related transcript (CART). The other class of ARC neurons is anabolic, and when stimulated these neurons elicit increased food intake and body weight. These neurons secrete the neurotransmitters, neuropeptide Y (NPY) and agouti-related peptide (AgRP). αMSH is an agonist of melanocortin 3 and 4 receptors, MC3/4R, whereas AgRP is an inverse agonist/antagonist at those

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same MC3/4R. The role of these peptides in the control of food intake is the subject of other chapters in this volume and is reviewed only briefly here. Insulin acts on IR in the ARC to elicit inhibition of NPY/AgRP neurons and stimulation of αMSH/CART neurons, and its local administration into or near the ARC causes anorexia and weight loss. This dual anabolic/catabolic circuitry of the ARC is sensitive to numerous other metabolic signals in addition to insulin, including leptin, meal-elicited satiation signals such as cholecystokinin (CCK, see below), and nutrients including glucose and some fatty acids and amino acids.[14,20] The outputs of these ARC neurons are projections to other areas in the hypothalamus important in the homeostatic regulation of body weight, such as the paraventricular nuclei (PVN), dorsomedial nuclei (DMH) and lateral hypothalamic areas (LHA). Thus, the ARC is a site of integration of myriad metabolic signals, with the output projecting to areas that influence autonomic, hypothalamus–pituitary–adrenal (HPA), and ingestive responses in many parts of the brain.

Insulin binds to IR on NPY/AgRP neurons, inhibiting both the expression and secretion of both transmitters. Conversely, insulin stimulates the activity of a subset of αMSH-containing neurons, leading to reduced activity that is partially dependent on the melanocortin receptor system because insulin's catabolic action is greatly reduced in the presence of MC3/4R antagonists.[8] The binding of insulin to its receptor on ARC neurons uses the same PI3K pathway as in peripheral tissues, and local administration of drugs that block the PI3K pathway prevents the catabolic actions of insulin.[24] The administration of insulin mimetics that bypass the extracellular portion of the insulin receptor, stimulating the intracellular insulin signaling cascade directly, reduces food intake and body weight, and suppresses hepatic glucose production.[1] These findings reveal the importance of insulin signaling to the normal regulation of energy homeostasis, because disruption of insulin signaling cascades within the hypothalamus can lead to hyperphagia, obesity, and glucose dysregulation.

INTERACTION OF BRAIN INSULIN WITH GUT HORMONES

The homeostatic regulation of body weight relies on control over food intake, and this is accomplished by the brain's influencing meal size as opposed to meal initiation.[33] Meal size and the phenomenon of satiation (fullness, in humans) are governed in part by gastrointestinal (GI) hormones that are secreted in response to the food entering the GI tract during meals. As an example, CCK is secreted in response to nutrients (especially lipids) entering the duodenum, and it acts locally on its receptors on nearby sensory nerve endings that transmit a signal to the brain via the vagus nerve and contribute to the process of satiation.[23] The administration of CCK to humans and rodents during meals results in ingestion of fewer calories than occurs in controls; and administration of CCK antagonists results in consumption of larger meals. The efficacy of the combined satiation signal generated by CCK and other GI peptides such as glucagon-like peptide-1 (GLP-1)[4] and peptide YY (PYY)[5] can be modulated by insulin and leptin. When these adiposity signals are elevated, they act in the brain to increase sensitivity to CCK and other satiation signals[27,32] (Fig. 1). Thus, when an individual has gained a little weight (fat) and consequently has increased secretion of insulin and leptin, meals that are initiated will end sooner because of an enhanced satiation signal, contributing to a homeostatic reduction of body weight. Conversely, if the insulin signal is reduced in the brain, animals eat larger meals and tend to gain weight over time.[22] The interaction of satiation signals and adiposity signals provides a robust negative feedback mechanism that opposes changes of body weight.

Molecular-genetic tools have been used in recent years to gain new insights into the actions of insulin in the CNS at a cellular and neuronal-circuit level. Using cre/loxP technology, Brüning et al. originally created neuron-specific IR knockout (NIRKO) mice.[9] These animals have obesity, hyperleptinemia, hyperinsulinemia, and mild insulin resistance, and the females are hyperphagic. Selective decrease of IR by use of an antisense oligonucleotide in predominantly NPY/AgRP neurons in the ARC results in a rapid onset of hyperphagia and increased adiposity as well as suppression of endogenous glucose production.[25] Deletion of IR specifically in AgRP ($IR\Delta^{AGRP}$) or POMC ($IR\Delta^{POMC}$) neurons in the ARH[7] results in no alterations in food intake, energy expenditure, or adiposity in comparison with that of controls. However $IR\Delta^{AGRP}$ animals do exhibit a selective reduction in hepatic glucose production under hyperinsulinemic–euglycemic clamp conditions. Selective deletion of IR from VMH neurons under the SF-1 promoter ($SF\text{-}1\Delta^{IR}$) results in diminished PI3K signaling in SF-1 neurons leading to overall protection from diet-induced leptin resistance, weight gain, adiposity, and impaired glucose tolerance whereas over-expression results in driving PI3K signaling pathways.[21] Thus, insulin acts on several neuronal types and areas of the hypothalamus, stimulating some circuits and inhibiting others, with a net catabolic effect of decreased food intake and body fat, increased energy expenditure, and reduced hepatic glucose production.

PATHOPHYSIOLOGIC IMPLICATIONS OF BRAIN INSULIN DYSFUNCTION

There are two categories of diabetes mellitus. Type I or insulin-deficiency diabetes mellitus (IDDM) is an autoimmune disease in which pancreatic β-cells are destroyed.

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Symptoms of insulin deficiency include hyperglycemia, glycosuria, and other metabolic problems, as well as extreme hyperphagia, and patients with IDDM require insulin maintenance throughout their lives. When insulin is added into the CNS of animals with IDDM, the hyperphagia is eliminated without any improvement of the peripheral metabolic symptoms, showing that the hyperphagia is a consequence of absent brain insulin in IDDM.[30]

Type II diabetes mellitus or noninsulin dependent diabetes mellitus (NIDDM) occurs when target tissues such as skeletal muscle become resistant to insulin's action. It is most commonly associated with obesity, fatter individuals having a greater degree of insulin resistance; and losing weight is typically accompanied by improved insulin sensitivity. Patients with NIDDM and mild insulin resistance can take insulin-sensitizing drugs that enhance the body's ability to respond to its own insulin. When those compounds are no longer effective, patients with NIDDM often must take exogenous insulin to maintain health. Obese individuals are also resistant to insulin's ability to reduce blood glucose and make other metabolic improvements, and the brain of these animals is also resistant to the ability of insulin to reduce food intake and body weight.[12] The important point is that the brain insulin system has a major role in both types of diabetes mellitus.

CONCLUSIONS

The insulin system consists of insulin originating from the pancreas and ubiquitously expressed insulin receptors throughout the body. Insulin is transported via the plasma throughout the body including the brain. Peripherally, insulin provides a conduit for glucose sequestration into cells. Along with leptin and perhaps other molecules, insulin acts in the brain to signal the amount of adiposity stored in the body. Through its integration with other hormonal signals, insulin is a potent catabolic influence reducing energy intake and storage. Insulin resistance, either centrally or peripherally, leads to global and local dysfunction.

ACKNOWLEDGMENTS

S. C. Woods is supported by NIH DK 017844. B. E. Grayson is supported by NIH 1F32 HD 068103.

REFERENCES

1. Air EL, Strowski MZ, Benoit SC, Conarello SL, Salituro GM, Guan XM, et al. Small molecule insulin mimetics reduce food intake and body weight and prevent development of obesity. *Nat Med* 2002;**8**:179–83.
2. Bagdade JD, Bierman EL, Porte Jr D. The significance of basal insulin levels in the evaluation of the insulin response to glucose in diabetic and nondiabetic subjects. *J Clin Invest* 1967;**46**:1549–57.
3. Banks WA, Jaspan JB, Huang W, Kastin AJ. Transport of insulin across the blood–brain barrier: saturability at euglycemic doses of insulin. *Peptides* 1997;**18**:1423–9.
4. Barrera JG, Jones KR, Herman JP, D'Alessio DA, Woods SC, Seeley RJ. Hyperphagia and increased fat accumulation in two models of chronic CNS glucagon-like peptide-1 loss of function. *J Neurosci* 2011;**31**:3904–13.
5. Batterham RL, Cowley MA, Small CJ, Herzog H, Cohen MA, Dakin CL, et al. Gut hormone PYY(3-36) physiologically inhibits food intake. *Nature* 2002;**418**:650–4.
6. Belgardt BF, Bruning JC. CNS leptin and insulin action in the control of energy homeostasis. *Ann N Y Acad Sci* 2010;**1212**:97–113.
7. Belgardt BF, Okamura T, Bruning JC. Hormone and glucose signalling in POMC and AgRP neurons. *J Physiol* 2009;**587**:5305–14.
8. Benoit SC, Air EL, Coolen LM, Strauss R, Jackman A, Clegg DJ, et al. The catabolic action of insulin in the brain is mediated by melanocortins. *J Neurosci* 2002;**22**:9048–52.
9. Brüning JC, Gautam D, Burks DJ, Gillette J, Schubert M, Orban PC, et al. Role of brain insulin receptor in control of body weight and reproduction. *Science* 2000;**289**:2122–5.
10. Chavez M, Kaiyala K, Madden LJ, Schwartz MW, Woods SC. Intraventricular insulin and the level of maintained body weight in rats. *Behav Neurosci* 1995;**109**:528–31.
11. Chavez M, Seeley RJ, Woods SC. A comparison between the effects of intraventricular insulin and intraperitoneal LiCl on three measures sensitive to emetic agents. *Behav Neurosci* 1995;**109**:547–50.
12. Clegg DJ, Gotoh K, Kemp C, Wortman MD, Benoit SC, Brown LM, et al. Consumption of a high-fat diet induces central insulin resistance independent of adiposity. *Physiol Behav* 2011;**103**:10–6.
13. Clegg DJ, Riedy CA, Smith KA, Benoit SC, Woods SC. Differential sensitivity to central leptin and insulin in male and female rats. *Diabetes* 2003;**52**:682–7.
14. Cota D, Proulx K, Seeley RJ. The role of CNS fuel sensing in energy and glucose regulation. *Gastroenterology* 2007;**132**:2158–68.
15. Elmquist JK, Coppari R, Balthasar N, Ichinose M, Lowell BB. Identifying hypothalamic pathways controlling food intake, body weight, and glucose homeostasis. *J Comp Neurol* 2005;**493**:63–71.
16. Friedman JM. The function of leptin in nutrition, weight, and physiology. *Nutr Rev* 2002;**60**:S1–14: discussion S68-84, 85–17.
17. Grillo CA, Tamashiro KL, Piroli GG, Melhorn S, Gass JT, Newsom RJ, et al. Lentivirus-mediated downregulation of hypothalamic insulin receptor expression. *Physiol Behav* 2007;**92**:691.
18. Hallschmid M, Benedict C, Schultes B, Fehm HL, Born J, Kern W. Intranasal insulin reduces body fat in men but not in women. *Diabetes* 2004;**53**:3024–9.
19. Hill JM, Lesniak MA, Pert CB, Roth J. Autoradiographic localization of insulin receptors in rat brain: prominence in olfactory and limbic areas. *Neuroscience* 1986;**17**:1127–38.
20. Jordan SD, Konner AC, Bruning JC. Sensing the fuels: glucose and lipid signaling in the CNS controlling energy homeostasis. *Cell Mol Life Sci* 2010;**67**:3255–73.
21. Klockener T, Hess S, Belgardt BF, Paeger L, Verhagen LAW, Husch A, et al. High-fat feeding promotes obesity via insulin receptor/PI3K-dependent inhibition of SF-1 VMH neurons. *Nat Neurosci* 2011;**14**:911.
22. McGowan MK, Andrews KM, Grossman SP. Chronic intrahyphothalamic infusions of insulin or insulin antibodies alter body weight and food intake in the rat. *Physiol Behav* 1992;**51**:753–66.

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23. Moran TH, Kinzig KP. Gastrointestinal satiety signals II. Cholecystokinin. *Am J Physiol* 2004;**286**:G183–8.
24. Niswender KD, Morrison CD, Clegg DJ, Olson R, Baskin DG, Myers Jr MG, et al. Insulin activation of phosphatidylinositol 3-kinase in the hypothalamic arcuate nucleus: a key mediator of insulin-induced anorexia. *Diabetes* 2003;**52**:227–31.
25. Obici S, Zhang BB, Karkanias G, Rossetti L. Hypothalamic insulin signaling is required for inhibition of glucose production. *Nat Med* 2002;**8**:1376–82.
26. Rayner DV, Thomas ME, Trayhurn P. Glucose transporters (GLUTs 1–4) and their mRNAs in regions of the rat brain: insulin-sensitive transporter expression in the cerebellum. *Can J Physiol Pharmacol* 1994;**72**:476–9.
27. Riedy CA, Chavez M, Figlewicz DP, Woods SC. Central insulin enhances sensitivity to cholecystokinin. *Physiol Behav* 1995;**58**:755–60.
28. Schwartz MW, Bergman RN, Kahn SE, Taborsky Jr GJ, Fisher LD, Sipols AJ, et al. Evidence for uptake of plasma insulin into cerebrospinal fluid through an intermediate compartment in dogs. *J Clin Invest* 1991;**88**:1272–81.
29. Schwartz MW, Woods SC, Porte DJ, Seeley RJ, Baskin DG. Central nervous system control of food intake. *Nature* 2000;**404**:661–71.
30. Sipols AJ, Baskin DG, Schwartz MW. Effect of intracerebroventricular insulin infusion on diabetic hyperphagia and hypothalamic neuropeptide gene expression. *Diabetes* 1995;**44**:147–51.
31. Strubbe JH, Porte DJ, Woods SC. Insulin responses and glucose levels in plasma and cerebrospinal fluid during fasting and refeeding in the rat. *Physiol Behav* 1988;**44**:205–8.
32. Williams DL, Baskin DG, Schwartz MW. Hindbrain leptin receptor stimulation enhances the anorexic response to cholecystokinin. *Am J Physiol Regul Integr Comp Physiol* 2009;**297**:R1238–46.
33. Woods SC. The control of food intake: behavioral versus molecular perspectives. *Cell Metab* 2009;**9**:489–98.
34. Woods SC, Porte Jr D. Relationship between plasma and cerebrospinal fluid insulin levels of dogs. *Am J Physiol* 1977;**233**:E331–4.

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Chapter 152

Leptin

Martin G. Myers Jr. and Megan Greenwald-Yarnell

ABSTRACT

Leptin is an adipocyte-derived polypeptide hormone that acts via its receptor (LEPR-B) to inhibit feeding and regulate other aspects of energy balance. Alterations in leptin levels during changes in nutritional status regulate feeding and energy expenditure; low leptin concentrations in states such as starvation promote increased hunger and decreased energy expenditure. The major sites of leptin action are in the brain. Although much work has focused on the hypothalamic arcuate nucleus (ARC), leptin controls feeding via a broadly distributed network of LEPR-B neurons in regions that include the hypothalamus, midbrain, and brainstem. The roles for specific LEPR-B signaling pathways in leptin action have been defined, with each mediating distinct aspects of the control of feeding. The apparently reduced anorectic efficacy of leptin in states of nutritional surfeit and obesity remains a major area of investigation and may result from multiple mechanisms, including processes that interfere with LEPR-B signaling.

LEPTIN AND FEEDING BEHAVIOR

Leptin was identified through the study of the spontaneous loss-of-function mutation (*obese* or Lep^{ob} allele) in mice that causes hyperphagia and obesity in the homozygous ($Lep^{ob/ob}$) state.[8] As implied by the elegant parabiosis studies of Coleman,[3] and later proven by the positional cloning of Lep^{ob},[35] the gene encodes a secreted polypeptide. This protein was named leptin (Greek: *leptos* = thin), because injection of leptin into obese $Lep^{ob/ob}$ animals resulted in a profound weight loss. Physiologically, leptin, which is produced primarily by adipocytes, communicates the status of body energy stores from adipocytes to the brain to control the intake and expenditure of calories.[2,8] Leptin treatment of $Lep^{ob/ob}$ animals results in decreased caloric intake and increased energy expenditure. Leptin is a crucial controller of many central nervous system (CNS) peptides that regulate feeding and energy expenditure.[6,26]

THE ABSENCE OF LEPTIN ACTION

Because of the decades-long existence of $Lep^{ob/ob}$ mice, there is a long-standing literature regarding the phenotype of mice with loss-of-function mutations for leptin (and mice null for the leptin receptor (LEPR; $Lepr^{db/db}$ mice)).[8,28] These $Lep^{ob/ob}$ and $Lepr^{db/db}$ mice have similar phenotypes of hyperphagia, low metabolic rate, and consequent obesity. Additionally, $Lep^{ob/ob}$ and $Lepr^{db/db}$ mice display a stereotypical neuroendocrine starvation response (even in the face of nutrient excess and obesity) that is characterized by hypothyroidism, hypothalamic infertility, decreased growth, and decreased immune function.[2] In $Lep^{ob/ob}$, but not in $Lepr^{db/db}$, mice exogenous leptin therapy reverses all these phenotypes. Similar phenotypes have been observed in rare human patients null for leptin or its receptor.[7] Thus, leptin action not only suppresses feeding, but it also increases metabolic rate and permits neuroendocrine energy expenditure; absent leptin action results in an increased drive to feed and initiates a series of energy-sparing events.

LEPTIN PRODUCTION AND THE CONTROL OF CIRCULATING LEPTIN

Leptin is produced primarily by adipocytes.[8] Although no tissue-specific null mice have been generated to probe the physiologic relevance of leptin production by specific tissues, the phenotypes of lipodystrophic patients and rodent models (which lack adipose tissue) are remarkably similar to the phenotype of leptin deficiency (with the exception of adipose mass).[21] Lipodystrophy is characterized by low circulating leptin concentrations, increased feeding, decreased metabolic rate, and neuroendocrine dysfunction similar to that observed in starvation or genetic leptin deficiency. Further, most of these phenotypes are partially or completely remedied by treatment of lipodystrophic patients or animals with exogenous leptin,[21] suggesting that adipose tissue is the main source of circulating leptin. The placenta also produces substantial quantities of leptin, although the physiologic role for the resulting increased leptin concentrations in pregnancy remains relatively poorly understood.[16]

In adipocytes, leptin production is regulated by nutritional status: generally speaking, increased stored triglyceride results in greater leptin production, whereas decreased stored fat diminishes leptin.[8] The regulation of

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00152-4
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leptin production by body fat stores positions leptin well to function as an indicator of body energy stores. Thus, in times of inadequate energy stores (e.g., starvation) leptin levels decrease; these low leptin levels enhance appetite and decrease energy use, whereas leptin treatment reverses these effects.[1,25] Conversely, with adequate energy stores, appropriate leptin levels moderate the drive to eat and permit the use of energy.

Leptin secretion is constitutive: Leptin is synthesized and extruded into the secretory pathway for release by mass action, as opposed to being packaged into secretory vesicles for release in response to an acute stimulus.[24] Thus, although there is modest diurnal variation in circulating leptin concentrations, leptin secretion and circulating leptin concentrations depend primarily on the rates of transcription and translation of the leptin mRNA dictated by adipose energy stores, and circulating leptin concentrations vary little on a minute-to-minute or hour-to-hour basis.[1] That circulating leptin concentrations change only after prolonged alterations in nutritional status predicts that leptin acts to set the chronic tone of the feeding and neuroendocrine systems, as opposed to acutely regulating the activity of the neural circuits by which it acts (short-term regulation is probably accomplished by more acutely regulated factors, such as insulin and ghrelin).

LEPTIN RECEPTORS

Multiple LEPR isoforms exist; all are products of a single *Lepr* gene and result from alternative mRNA splicing and/or proteolytic processing.[24,28] LEPR isoforms fall into three classes: secreted, short, and long. The secreted forms are alternative splice products (e.g., the murine LEPR-E) or proteolytic cleavage products of membrane-bound LEPR forms. These secreted receptors contain only extracellular domains (including the leptin binding site) and bind circulating leptin with an affinity similar to that of cell-associated LEPRs. Short (LEPR-A, LEPR-C, LEPR-D, etc.) and long form (LEPR-B) receptors contain identical extracellular and transmembrane domains and the same first 29 intracellular amino acids, subsequently diverging in sequence secondary to alternative splicing of the *Lepr* 3′-exons.[24,28] LEPR-B is highly conserved among species and possesses an intracellular domain of approximately 300 residues.

Although the function of the short LEPR forms remains unclear, LEPR-B is crucial for leptin action. Indeed, the originally described $Lep^{db/db}$ mice lack only LEPR-B (as a consequence of a mutation that results in missplicing of the LEPR-B-encoding mRNA) but exhibit a phenotype similar to that of leptin-deficient $Lep^{ob/ob}$ animals and of $Lepr^{db3J/db3J}$ mice (which are deficient in all LEPR isoforms).[24,28]

The concentration and bioavailability of circulating leptin are also modulated by the presence of circulating LEPR-extracellular domain that is devoid of transmembrane or intracellular components (that cleaved from cell surface LEPRs, along with LEPR-E). Although the acute elevation of free extracellular LEPR, as by injection, can decrease leptin bioavailability, chronic increases in circulating LEPR stabilize leptin in circulation, and may serve to increase the amount of active, as well as total, leptin in the circulation;[34] mice that overexpress LEPR-E are leaner with reduced food intake.[15]

SITES OF LEPTIN ACTION

Most leptin effects on energy balance are attributable to effects in the CNS. CNS administration of leptin recapitulates the anorectic and neuroendocrine effects of peripherally delivered leptin.[8] Further, brain-specific transgenic expression of LEPR-B rescues the anorectic and neuroendocrine phenotypes of $Lepr^{db3J/db3J}$ mice, and brain-specific disruption of *Lepr* increases feeding and adiposity.[24,28] Much attention has been paid to two distinct populations of LEPR-B-expressing neurons in the arcuate nucleus (ARC) in the mediobasal hypothalamus: neurons that express proopiomelanocortin (POMC), and neurons that coexpress neuropeptide Y (NPY) and agouti-related peptide (AgRP).[6,26] POMC is processed to α-melanocyte stimulating hormone (αMSH) in the ARC POMC neuron. αMSH mediates a powerful anorectic (appetite-suppressing) signal; LEPR-B stimulates the expression of POMC and activates the POMC neuron. AgRP is an antagonist/inverse agonist of central melanocortin signaling, and NPY is itself an orexigenic (appetite-stimulating) peptide that also acts to suppress the central growth and reproductive axes. Leptin inhibits the NPY/AgRP neurons and suppresses expression of these neuropeptides, stimulates the production of anorectic neuropeptides, and suppresses levels of orexigenic peptides in this circuit. Conversely, when leptin action is decreased or deficient (e.g., starvation, $Lep^{ob/ob}$ or $Lepr^{db/db}$ mice), appetite is stimulated via the suppression of anorectic neuropeptides (e.g., POMC-derived peptides) and by increased expression of orexigenic peptides (e.g., NPY and AgRP).

Genetic ablation of LEPR-B from POMC and AgRP neurons either individually or collectively produces only a small increase in body weight and does not significantly change many other parameters that are perturbed in $Lepr^{db/db}$ mice,[29] indicating the importance of LEPR-B signaling in other neurons for leptin action. Indeed, many LEPR-B-expressing neurons reside in a number of brain regions associated with the regulation of energy balance, including various hypothalamic, midbrain, and brainstem nuclei.[19]

Within the hypothalamus, the ARC, ventromedial hypothalamic nucleus (VMH), dorsomedial hypothalamic nucleus (DMH), ventral premammilary nucleus (PMv), lateral hypothalamic area (LHA), and preoptic area (POA) contain large numbers of LEPR-B neurons (Figure 1).[19]

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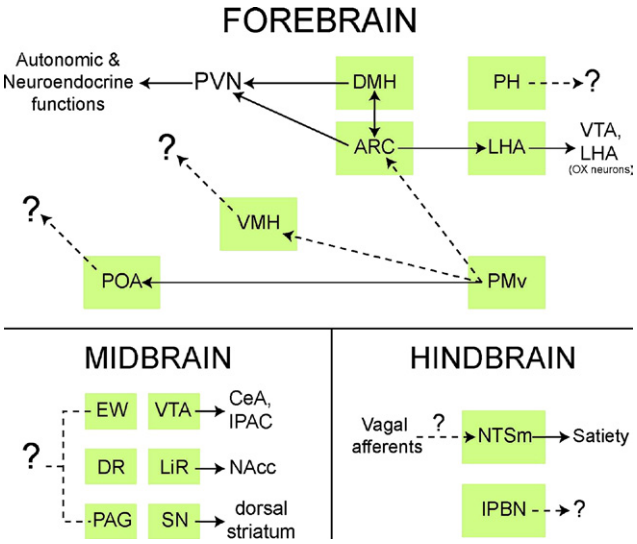

FIGURE 1 A distributed network of LEPR-B neurons mediates leptin action. Brain areas containing substantial numbers of LEPR-B neurons in the forebrain, midbrain, and hindbrain are designated by grey boxes. The known inputs, targets, and functions of the LEPR-B neurons in each area are identified, where known, but our understanding of many components of this network of leptin-responsive neurons remains incomplete, and some connections remains speculative (dashed lines). PVN, paraventricular nucleus; POA, preoptic area; DMH, dorsomedial nucleus of the hypothalamus; ARC, arcuate nucleus; VMH, ventromedial nucleus of the hypothalamus; PH, posterior hypothalamus; LHA, lateral hypothalamic area; PMv, ventral premammillary nucleus; EW, Edinger–Westphal nucleus; DR, dorsal raphe; PAG, periaqueductal gray; VTA, ventral tegmental area; LiR, linear raphe; SN, substantia nigra; NTSm, medial nucleus of the tractus solitarius; lPBN, lateral parabrachial nucleus.

VMH-specific LEPR-B deletion leads to a modest metabolic phenotype but fails to recapitulate most of the *Lepr*$^{db/db}$ phenotype.[5] Leptin action via LHA LEPR-B neurons modulates local neurons that express orexin (aka hypocretin), as well as innervating the midbrain ventral tegmental area (VTA).[13] Deletion of LEPR-B from neurotensin-containing LHA neurons also produces only a modest increase in body weight and fat mass, along with a decrease in energy expenditure that is associated with alterations in orexin and VTA function.

Within the midbrain, LEPR-B neurons lie in the VTA, substantia nigra (SN) and several nearby midline nuclei.[19] Most of the VTA and SN LEPR-B neurons contain the neurotransmitter dopamine (DA).[9,11] Although leptin administration to the midbrain decreases food intake, deletion of LEPR-B from DA neurons produces no detectable change in parameters of energy balance; indeed, some lines of evidence suggest a potential role for these neurons in the regulation of anxiety-like behaviors, rather than feeding.[11,14] Although the dorsal raphe (DR) also contains a population of LEPR-B-expressing neurons, these neurons do not (as previously reported) contain serotonin, and thus, genetic deletion of LEPR-B in serotonergic neurons produces no effect on energy balance.[12]

LEPR-B is also found in the hindbrain, primarily within the medial nucleus tractus solitarius (mNTS) and lateral parabrachial nucleus (lPBN).[19] Although partial genetic deletion of LEPR-B from the hindbrain produces little phenotype, viral-mediated RNAi knockdown of LEPR-B in the area of the NTS alters meal size and slightly increases body weight, suggesting some role for direct hindbrain leptin action in the regulation of energy balance.[10,27]

Thus, no single discrete site of leptin action has yet been revealed to mediate the majority of leptin action on energy balance. Several larger groups of neurons play a crucial role, however. Deletion of LEPR-B from the entire hypothalamus promotes severe obesity rivaling that observed in *Lepr*$^{db/db}$ animals.[22] Similarly, deletion of LEPR-B from neurons that express the vesicular GABA transporter (vGAT) produces dramatic hyperphagic obesity.[31] LEPR-B/vGAT neurons lie primarily in the hypothalamus and comprise most LEPR-B neurons in the ARC, DMH, and LHA, suggesting that the LEPR-B neurons in these nuclei mediate crucial components of leptin action on energy balance. Interestingly, although LEPR-B/vGAT neurons are distinct from POMC neurons, they participate in the regulation of POMC neurons, suggesting that hypothalamic melanocortin action is regulated by the concerted action of a distributed set of LEPR-B neurons, as are most other leptin effects.

LEPR-B SIGNALING PATHWAYS AND THE CONTROL OF ENERGY BALANCE

The initially observed homology of LEPR-B with other type I cytokine receptors (e.g., the gp130 subunit of the IL-6 receptor family) suggested that leptin binding to LEPR-B activates Jak kinases to mediates cytokine receptor-like signals.[24,28] Jak2 represents the Jak kinase primarily involved in signaling by the intracellular domain of LEPR-B.[24] All functional cytokine receptors contain a proline-rich "Box 1" motif that is required for Jak kinase interaction and activation; additional less-conserved sequences (sometimes referred to as "Box 2") COOH-terminal to Box 1 are also important for Jak kinase interactions and likely function in Jak kinase isoform selectivity.[24] Intracellular residues 31–36 of LEPR-B (i.e., sequences immediately downstream of the alternative splice junction after amino acid 29) represent Box 2 and are required for Jak2 activation. This motif is absent from all described short LEPR isoforms, explaining the inability of these molecules to mediate leptin action in *Lepr*$^{db/db}$ animals. Figure 2

There are three conserved tyrosine residues on the intracellular domain of LEPR-B (Tyr_{985}, Tyr_{1077}, and Tyr_{1138}), all of which are phosphorylated to mediate downstream signaling.[24] In human LEPR-B, there exist two additional

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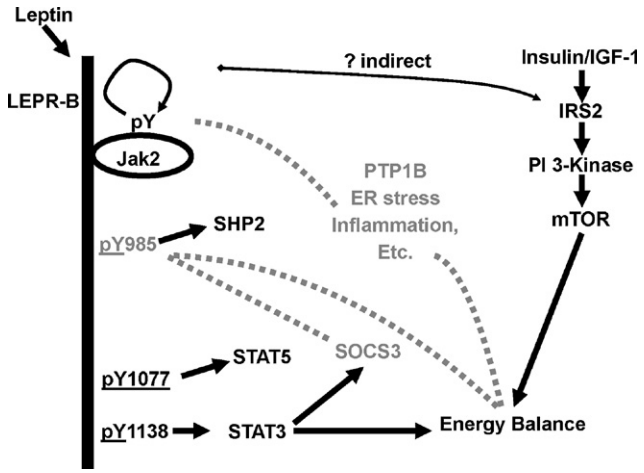

FIGURE 2 LEPR-B signaling, inhibitory pathways, and the control of energy balance. Leptin binding to LEPR-B activates the associated Jak2 tyrosine kinase, resulting in the tyrosine phosphorylation (pY) and activation of Jak2 and subsequently the phosphorylation of LEPR-B on Tyr_{985}, Tyr_{1077}, and Tyr_{1138}. Tyr_{1138} recruits STAT3 to mediate its transcriptional activation; STAT3 is also an important mediator of energy balance regulation during leptin action. Tyr_{1077} mediates the majority of STAT5 recruitment and activation, while Tyr_{985} recruits SHP-2. LEPR-B also engages inhibitory (gray/dashed lines) signals: one transcriptional target of STAT3 is the inhibitory SOCS3, which binds Tyr_{985} to feedback on and attenuate LEPR-B signaling. Other cellular processes that may inhibit LEPR-B to promote obesity/impair the control of energy balance include protein tyrosine phosphatases (such as PTP1B), ER stress, and inflammatory signaling pathways. Insulin-like signals that act via IRS2, PI3K and mTOR also contribute to the control of energy balance, although it is not clear whether these act primarily downstream of LEPR-B, or rather provide a converging/supporting signal resulting from the much stronger activation of these pathways by the insulin and IGF-1 receptors.

intracellular tyrosine residues, although little is known about their potential function (or lack thereof).[28] There are thus four known sites for the initiation of intracellular signaling by LEPR-B: those originating directly from Jak2, along with those stemming from Tyr_{985}, Tyr_{1077}, and Tyr_{1138} of LEPR-B. In cultured cells, Tyr_{985} interacts with SHP-2 (aka PTPN11) to mediate the majority of extracellular regulated kinase (ERK) activation; the remainder of ERK signaling is mediated by Jak2 independently of tyrosine phosphorylation sites on LEPR-B.[24] Tyr_{985} also binds to the suppressor of cytokine signaling-3 (SOCS3). Tyr_{1077} recruits the signal transducer and activator of transcription (STAT)-5, to control the majority of LEPR-B-mediated STAT5-dependent transcriptional events in cultured cells. Tyr_{1138} recruits STAT3.[24] In addition to mediating a variety of positive signals, the LEPR-B Tyr_{1138}→STAT3 pathway induces SOCS3 expression; SOCS3 interacts with phosphorylated Tyr_{985} of LEPR-B to limit LEPR-B signaling. This LEPR-B Tyr_{1138} →STAT3→SOCS3→Tyr_{985} pathway represents a major feedback loop to inhibit LEPR-B signaling.[24]

In cultured cells, Jak2 tyrosine phosphorylation during LEPR-B stimulation may mediate some signals independently of tyrosine phosphorylation sites on LEPR-B, but most Jak2 tyrosine phosphorylation sites studied to date function to regulate Jak2 activity rather than mediating downstream signaling.[23] Indeed, Jak2-dependent leptin signals fail to modulate parameters of energy balance in the absence of LEPR-B tyrosine phosphorylation sites, suggesting a crucial role for LEPR-B tyrosine phosphorylation in the control of feeding and energy expenditure.[23] By contrast, mice in which LEPR-B is replaced by a mutant that contains a substitution mutation of Tyr_{1138}, which abrogates LEPR-B→STAT3 signaling, display hyperphagia and decreased energy expenditure, resulting in massive early-onset obesity; similar hyperphagia and obesity have been observed in mice null for STAT3 in the brain. Thus, the LEPR-B Tyr_{1138}→STAT3 signal is central to the regulation of body weight by leptin.[24] By contrast, mutation of Tyr_{985} produces lean mice, consistent with a role for the Tyr_{985}/SOCS3 pathway in feedback inhibition/LEPR-B signal attenuation *in vivo*, as in cultured cells.[24]

Many insulin-like signals (including insulin receptor substrate (IRS)-2, phosphatidylinositol 3′-kinase (PI3K), and mammalian target of rapamycin (mTOR)) are modulated by leptin in the brain, and are required for the full efficacy of leptin action *in vivo*.[4,20] Delineating the direct pathway from LEPR-B to these signals in cultured cells has not been straightforward, however, and PI3K and mTOR are regulated oppositely by leptin in different neuronal populations.[30,32] Because blockade of these pathways does not interfere with melanocortin action, they are required in neurons upstream of the melanocortin-responsive neurons crucial for the control of energy balance.[4,20] Hence, although the integrity of these pathways in the hypothalamus, and even in subsets of LEPR-B neurons, is crucial for energy balance, leptin may modulate these pathways indirectly and/or these pathways may primarily function to relay insulin signals to leptin-regulated neural pathways in support of leptin action. Similarly, the cellular energy sensing AMP-dependent protein kinase (AMPK), which interferes with leptin action, may not be controlled by LEPR-B directly, but may serve to integrate additional physiologic data with the leptin signal in the leptin-regulated neural circuitry.[17,33]

"LEPTIN RESISTANCE" AND CELLULAR MECHANISMS THAT IMPAIR LEPR-B SIGNALING

More than one quarter of adult Americans are obese, and the incidence of obesity continues to increase in the U.S. and other industrialized nations.[18] Obesity is a major risk factor for Type 2 diabetes, cardiovascular disease, and some forms of cancer. Because administration of leptin to $Lep^{ob/ob}$ animals and other lean rodents acutely decreases food intake

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and body weight, resulting in the loss of fat mass, leptin was initially hailed as a potential cure for obesity.[8] With the exception of rare human patients with genetic leptin deficiency, however, circulating leptin concentrations correlate with BMI and total body fat mass, and most obese individuals have elevated circulating leptin concentrations, but the abundant leptin in obesity fails to restore body weight and adiposity to normal levels. This, along with the minimal anorectic response to even heroic doses of exogenous leptin in many obese humans and rodent, has given rise to the notion of "leptin resistance," where the efficacy of leptin is diminished in states of obesity and/or nutritional surfeit.[18] Since leptin levels are expected to be increased in proportion to adipose mass in obesity, defining leptin resistance as obesity in the face of hyperleptinemia seems rather tautological, however, although (practically speaking) it will be useful to understand the limited response to leptin in obesity.

Although it is not clear whether they are primary to the genesis of obesity, or rather secondary and permissive, it is clear that many mechanisms limit leptin action in obese or overnourished animals. The proposed mechanisms of the so-called leptin resistance are many and range from the trivial (leptin action is near maximal at levels observed in obesity, and additional leptin fails to appreciably increase leptin action), to decreased leptin transport into the CNS, to cellular mechanisms that limit LEPR-B signaling. Although each of these processes may play a role, a substantial amount of genetic evidence support the notion that feedback pathways (including Tyr_{985}/SOCS3), tyrosine phosphatase action (e.g., via PTP1B/PTPN1), ER stress, inflammation, and many other cellular process activated during increased feeding or obesity impair LEPR-B signaling to decrease the efficacy of endogenous and exogenous leptin.[18] Thus, leptin action must be assessed in young lean subjects to determine whether a particular lesion that promotes obesity actually interferes with leptin action *per se*, lest these mechanisms confound the results. Clearly, however, although the term "leptin resistance" is used to convey numerous concepts and does not imply a specific mechanism, understanding genetic and cellular mechanisms that may interfere with leptin action will be crucial, as they may represent potential targets for therapy in obesity.

REFERENCES

1. Ahima RS, Prabakaran D, Flier JS. Postnatal leptin surge and regulation of circadian rhythm of leptin by feeding. Implications for energy homeostasis and neuroendocrine function. *J Clin Invest* 1998;**101**:1020–7.
2. Ahima RS, Saper CB, Flier JS, Elmquist JK. Leptin regulation of neuroendocrine systems. *Front Neuroendocrinol* 2000;**21**:263–307.
3. Coleman DL, Hummel KP. Effects of parabiosis of normal with genetically diabetic mice. *Am J Physiol* 1969;**217**:1298–304.
4. Cota D, Proulx K, Smith KA, Kozma SC, Thomas G, Woods SC, et al. Hypothalamic mTOR signaling regulates food intake. *Science* 2006;**312**:927–30.
5. Dhillon H, Zigman JM, Ye C, Lee CE, McGovern RA, Tang V, et al. Leptin directly activates SF1 neurons in the VMH, and this action by leptin is required for normal body-weight homeostasis. *Neuron* 2006;**49**:191–203.
6. Elmquist JK, Elias CF, Saper CB. From lesions to leptin: hypothalamic control of food intake and body weight. *Neuron* 1999;**22**:221–32.
7. Farooqi IS, O'Rahilly S. Monogenic human obesity syndromes. *Recent Prog Horm Res* 2004;**59**:409–24.
8. Friedman JM, Halaas JL. Leptin and the regulation of body weight in mammals. *Nature* 1998;**395**:763–70.
9. Fulton S, Pissios P, Manchon RP, Stiles L, Frank L, Pothos EN, et al. Leptin regulation of the mesoaccumbens dopamine pathway. *Neuron* 2006;**51**:811–22.
10. Hayes MR, Skibicka KP, Leichner TM, Guarnieri DJ, DiLeone RJ, Bence KK, et al. Endogenous leptin signaling in the caudal nucleus tractus solitarius and area postrema is required for energy balance regulation. *Cell Metab* 2010;**11**:77–83.
11. Hommel JD, Trinko R, Sears RM, Georgescu D, Liu ZW, Gao XB, et al. Leptin receptor signaling in midbrain dopamine neurons regulates feeding. *Neuron* 2006;**51**:801–10.
12. Lam DD, Leinninger GM, Louis GW, Garfield AS, Marston OJ, Leshan RL, et al. Leptin does not directly affect CNS serotonin neurons to influence appetite. *Cell Metab* 2011;**13**:584–91.
13. Leinninger GM, Opland D, Jo YH, Faouzi M, Christensen L, Cappelluci L, et al. Leptin action via neurotensin neurons controls orexin, the mesolimbic dopamine system and energy balance. *Cell Metab* 2011;**14**:313–23.
14. Liu J, Perez SM, Zhang W, Lodge DJ, Lu XY. Selective deletion of the leptin receptor in dopamine neurons produces anxiogenic-like behavior and increases dopaminergic activity in amygdala. *Mol Psychiatry* 2011.
15. Lou PH, Yang G, Huang L, Cui Y, Pourbahrami T, Radda GK, et al. Reduced body weight and increased energy expenditure in transgenic mice over-expressing soluble leptin receptor. *PLoS One* 2010;**5**:e11669.
16. Masuzaki H, Ogawa Y, Sagawa N, Hosoda K, Matsumoto T, Mise H, et al. Nonadipose tissue production of leptin: leptin as a novel placenta-derived hormone in humans. *Nat Med* 1997;**3**:1029–33.
17. Minokoshi Y, Alquier T, Furukawa N, Kim YB, Lee A, Xue B, et al. AMP-kinase regulates food intake by responding to hormonal and nutrient signals in the hypothalamus. *Nature* 2004;**428**:569–74.
18. Myers Jr MG, Leibel RL, Seeley RJ, Schwartz MW. Obesity and leptin resistance: distinguishing cause from effect. *Trends Endocrinol Metab* 2010;**21**:643–51.
19. Myers Jr MG, Munzberg H, Leinninger GM, Leshan RL. The geometry of leptin action in the brain: more complicated than a simple ARC. *Cell Metab* 2009;**9**:117–23.
20. Niswender KD, Morton GJ, Stearns WH, Rhodes CJ, Myers Jr MG, Schwartz MW. Intracellular signalling key enzyme in leptin-induced anorexia. *Nature* 2001;**413**:794–5.
21. Oral EA, Simha V, Ruiz E, Andewelt A, Premkumar A, Snell P, et al. Leptin-replacement therapy for lipodystrophy. *New Engl J Med* 2002;**346**:570–8.
22. Ring LE, Zeltser LM. Disruption of hypothalamic leptin signaling in mice leads to early-onset obesity, but physiological adaptations

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in mature animals stabilize adiposity levels. *J Clin Invest* 2010;**120**: 2931–41.
23. Robertson S, Ishida-Takahashi R, Tawara I, Hu J, Patterson CM, Jones JC, et al. Insufficiency of Janus kinase 2-autonomous leptin receptor signals for most physiologic leptin actions. *Diabetes* 2010;**59**:782–90.
24. Robertson SA, Leinninger GM, Myers Jr MG. Molecular and neural mediators of leptin action. *Physiol Behav* 2008;**94**:637–42.
25. Rosenbaum M, Murphy EM, Heymsfield SB, Matthews DE, Leibel RL. Low dose leptin administration reverses effects of sustained weight-reduction on energy expenditure and circulating concentrations of thyroid hormones. *J Clin Endocrinol Metab* 2002;**87**:2391–4.
26. Schwartz MW, Woods SC, Porte Jr D, Seeley RJ, Baskin DG. Central nervous system control of food intake. *Nature* 2000;**404**:661–71.
27. Scott MM, Williams KW, Rossi J, Lee CE, Elmquist JK. Leptin receptor expression in hindbrain Glp-1 neurons regulates food intake and energy balance in mice. *J Clin Invest* 2011;**121**:2413–21.
28. Tartaglia LA. The leptin receptor. *J of Bcal Ctry* 1997;**272**:6093–6.
29. van de WE, Leshan R, Xu AW, Balthasar N, Coppari R, Liu SM, et al. Collective and individual functions of leptin receptor modulated neurons controlling metabolism and ingestion. *Endocrinology* 2008;**149**:1773–85.
30. Villanueva EC, Munzberg H, Cota D, Leshan RL, Kopp K, Ishida-Takahashi R, et al. Complex regulation of mammalian target of rapamycin complex 1 in the basomedial hypothalamus by leptin and nutritional status. *Endocrinology* 2009;**150**:4541–51.
31. Vong L, Ye C, Yang Z, Choi B, Chua Jr S, Lowell BB. Leptin action on GABAergic neurons prevents obesity and reduces inhibitory tone to POMC neurons. *Neuron* 2011;**71**:142–54.
32. Xu AW, Kaelin CB, Takeda K, Akira S, Schwartz MW, Barsh GS. PI3K integrates the action of insulin and leptin on hypothalamic neurons. *J Clin Invest* 2005;**115**:951–8.
33. Yang Y, Atasoy D, Su HH, Sternson SM. Hunger states switch a flip-flop memory circuit via a synaptic AMPK-dependent positive feedback loop. *Cell* 2011;**146**:992–1003.
34. Zhang J, Scarpace PJ. The soluble leptin receptor neutralizes leptin-mediated STAT3 signalling and anorexic responses in vivo. *Br J Pharmacol* 2009;**158**:475–82.
35. Zhang Y, Proenca R, Maffei M, Barone M, Leopold L, Friedman JM. Positional cloning of the mouse obese gene and its human homologue. *Nature* 1994;**372**:425–32.

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Chapter 153

Melanocortins

Joram D. Mul and Roger A.H. Adan

ABSTRACT

The central melanocortin (MC) system consists of neurons that express the POMC-derived peptides α-melanocyte-stimulating hormone (α-MSH), β-MSH, and γ-MSH, which are endogenous melanocortin-receptor 3 (MC3R) or MC4R agonists, and neurons that express AgRP, an MC4R inverse agonist. Downstream, subpopulations of neurons express MC3R and/or MC4R. The activity of the MC system plays a key role in the regulation of energy homeostasis. Mutations in MC system components, in either humans or rodents, result in obesity by affecting several aspects of energy homeostasis including ingestive behavior, energy expenditure, insulin sensitivity, and adipose metabolism. Here, we aim to provide an update of how the MC system modifies ingestive behavior. We first describe how ingestive behavior is altered in humans and rodents with mutations in the MC system followed by a description of how the MC system modulates the different neural circuits involved in feeding.

THE MELANOCORTIN SYSTEM

To maintain a stable body weight, one has to balance energy intake or ingestive behavior (by eating and drinking) and energy expenditure (by exercise, basal metabolism, and thermogenesis). Recent advances in scientific research have shown a large number of neuronal effectors to be essential for the central regulation of ingestive behavior. Perhaps one of the best-characterized neuronal pathways in the regulation of energy homeostasis is the melanocortin (MC) system.[1,8,12,28] The central MC system includes two distinct subpopulations of neurons in the arcuate nucleus expressing agouti-gene-related protein (*AgRP*) or proopiomelanocortin (*POMC*), as well as a subpopulation of neurons in the brainstem expressing *POMC*, and downstream targets of these neurons expressing melanocortin-3 and/or melanocortin-4 receptors (*MC3R* and *MC4R*).[1,8,12,28] Processing of the POMC precursor generates several products, including adrenocorticotropin (ACTH), the opioid receptor agonist β-endorphin, and the endogenous MCR3/4 agonists α-melanocyte-stimulating hormone (α-MSH), β-MSH, and γ-MSH.[1,8,12,28] AgRP functions as an inverse agonist on constitutively active MC3Rs and MC4Rs.[1,8,12,28] Out of the five MC receptors, all of which are G-protein-coupled receptors, MC3R and MC4R are the main brain MCRs.[1,8,12,28]

Leptin, a key modulator of energy homeostasis, strongly regulates neuropeptidergic circuits in the arcuate nucleus of the hypothalamus to modulate food intake and energy expenditure.[25] Within the arcuate nucleus, leptin activates neurons expressing *POMC* but inhibits neurons expressing *AgRP*.[25] During a positive state of energy balance, high levels of leptin inhibit POMC neurons and stimulate AgRP neurons, whereas a negative state of energy balance results in the opposite situation. The unique presence of endogenous agonists and an inverse agonist acting within the same receptor system indicates that the activity of the central MC system is tightly regulated and implicates the importance of the MC system in regulating energy homeostasis. Although the activity of the MC system also affects energy expenditure, insulin sensitivity, adipose metabolism, blood pressure, heart rate, natriuresis, and erectile function,[8,28] this chapter mainly focuses on how and via which neural circuits the central MC system modulates ingestive behavior.

INGESTIVE BEHAVIOR IN HUMANS WITH MUTATIONS IN THE MC SYSTEM

Genetic Variance

Single-nucleotide polymorphisms in *MC4R* or genetic variances near *MC4R* are highly associated with increased body mass index (BMI), adipose mass, early-onset obesity, and risk to develop obesity.[26] Moreover, a plethora of mutations in *MC4R*, predominantly with a negative effect on MC4R function, have been reported to date in humans.[28] This makes haploinsufficiency of *MC4R* the most common monogenic cause of severe human obesity, accounting for approximately 4% of all cases.[8,12,28] However, strong variation in the prevalence of MC4R haploinsufficiency levels is observed depending on ethnic origin, grade of obesity, and obesity onset of the population screened.[28] Human genomewide association

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00153-6
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studies (GWAS) show that variation at the *MC4R* locus also explains some of the variability in BMI in the general population. In addition, a GWAS analysis has also shown that variation near *POMC* affects susceptibility for obesity.[26]

Mutations in MC4R

Loss-of-function mutations in *MC4R* result in hyperphagia, hyperinsulinemia, and increased mass and lean body mass.[11] However, the hyperphagia seemed to subside with age in children carrying a heterozygous loss-of-function mutation.[11] In general, heterozygous carriers for loss-of-function mutations in *MC4R* show an intermediate metabolic phenotype compared with homozygous or wildtype carriers, suggesting that the regulation of energy balance in humans is sensitive to the variation in functional MC4R levels.[11]

Mutations in MC3R

In addition to *MC4R*, some humans carry mutations in *MC3R*. A primary study showed that a locus on human chromosome 20q encoding *MC3R* is linked to BMI, subcutaneous fat mass, and glucose metabolism.[23] Subsequently, numerous mutations were discovered in *MC3R*, which affect a variety of phenotypes, including fat storage, BMI, lipid metabolism, glucose metabolism, and ingestive behavior.[23] However, despite a clear obese metabolic phenotype in MC3R-deficient mice (discussed below), a similar striking phenotype for humans carrying a loss-of-function mutation in *MC3R* has not yet been found.[23]

Mutations in POMC

Humans with mutations in *POMC*, which either interfered with the synthesis of α-MSH and ACTH or abolished *POMC* translation, are hyperphagic and obese.[16] The usual suspect for causing these POMC-deficient phenotypes is α-MSH, which is the main peptide for signaling through the MC4R receptor. However, disruption of β-MSH function in humans also results in an obese phenotype, supporting a significant role for β-MSH in the regulation of human body weight[3]; ingestive behavior was not analyzed. Although rodents do not process endogenous β-MSH, in contrast to humans, administration of exogenous β-MSH to rats decreases ingestive behavior in rodents, whereas the abovementioned mutant form of β-MSH does not.[3]

Mutations in AGRP

Loss-of-function mutations in *AgRP* have not yet been reported in humans. However, several polymorphisms in *AgRP* are associated with leanness and differences in macronutrient intake.[17] Moreover, the results regarding these polymorphisms seem to be parallel to the results observed in genetic rodent models with loss of AgRP function, as they negatively influence energy balance.[17]

INGESTIVE BEHAVIOR IN RODENT MODELS WITH DISRUPTION OF MC4R FUNCTION

A major breakthrough promoting the involvement of MC4R in the regulation of body weight was the discovery that agouti was an MC4R antagonist. Two naturally occurring obese mouse models, *lethal yellow* (A^y) and *viable yellow* (A^{vy}), show central ectopic overexpression of *agouti*, which is normally expressed in the skin to antagonize MC1R. The overexpression of agouti results in obesity because agouti in the brain blocks MC4R function.[10]

Using embryonic stem cell manipulation, Huszar and colleagues generated mice lacking MC4R ($MC4R^{-/-}$) on a mixed 129/Sv and C57BL/6J background and showed that chronic full-body MC4R-deficiency in the mouse results in a late-onset obesity, hallmarked by hyperphagia, reduced locomotor activity, increased body length, hyperleptinemia, and hyperinsulinemia.[14] Heterozygous male and female $MC4R^{-/-}$ mice showed an intermediate phenotype, showing a gene dosage effect of MC4R-ablation on body weight regulation.[14] The intermediate phenotype observed in $MC4R^{+/-}$ mice is in agreement with the intermediate phenotype observed in humans carrying a heterozygous loss-of-function mutation.[11,14] The observation that loss of one functional MC4R allele results in an intermediate phenotype is unusual for G protein-coupled receptors. This observation is, in addition to having endogenous agonists and an inverse agonist working on the same receptor system, another unique feature of the MC system.[8] To investigate whether the hyperphagia in these MC4R-deficient mice is the main contributor for their obesity, a subsequent study comparing pair-fed female $MC4R^{-/-}$ mice with wildtype controls observed that the body weight of the pair-fed female $MC4R^{-/-}$ mice was intermediate between wildtype and non-pair-fed $MC4R^{-/-}$ mice, suggesting that changes in feeding are not solely responsible for the obese phenotype.[27] However, body weights of male $MC4R^{-/-}$ mice used in the same pair-feeding paradigm were not significantly different from wildtype mice.[27] Further, ambulatory activity of *ad libitum*-fed female $MC4R^{-/-}$ mice was normal, whereas ambulatory activity of *ad libitum*-fed male $MC4R^{-/-}$ mice was significantly lower compared with that of wildtype controls, predominantly during the dark phase.[27] These observations indicate that MC4R-deficiency in this specific mouse line has significant gender-specific effects on body weight regulation.

The majority of genetic models of the MC system are in mice. However, a rat model on a Wistar background with chronic full-body functional loss of MC4R ($MC4R^{K314X/K314X}$)

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has been generated and characterized.[20] MC4R$^{K314X/K314X}$ rats show early-onset obesity, hallmarked by hyperphagia, reduced locomotor activity, increased body length, hyperleptinemia, and hyperinsulinemia.[20] Although not tested for all the aforementioned parameters, MC4R$^{+/K314X}$ rats show an intermediate phenotype.[20]

Nutrient Sensing

Using the same genetic line of MC4R$^{-/-}$ mice, MC4R$^{-/-}$ mice that are exposed to nutritional deprivation lose equal amounts of body weight as wildtype controls and after resumption of *ad libitum* feeding quickly return to their premalnutrition body weight.[5] This indicates that MC4R is not necessary for sensing nutritional deficits or for producing the homeostatic response that returns body weight to the original set point level after an involuntary negative energy balance. However, when fed a moderate-fat (25%) diet, the same study also showed that MC4R$^{-/-}$ mice have a marked increase in body weight gain. Analysis of the feeding behavior of these mice switching between a standard low-fat diet and the moderate-fat diet reveals that wildtype controls adjust their food intake according to the increased amount of calories in the moderate-fat diet (i.e., they remain isocaloric compared with the low-fat diet), whereas MC4R$^{-/-}$ mice lack this adjustment. In addition, MC4R$^{-/-}$ mice become even more hyperphagic compared with wildtype controls on the moderate-fat diet compared with the hyperphagia observed with the low-fat diet.[5] Thus, MC4R does not seem to be critical for nutrient sensing, but loss of MC4R function dysregulates the adequate metabolic response to handle increases in caloric density.

Feed Efficiency

When male or female MC4R$^{-/-}$ mice are fed a moderate-fat diet (without choice), the feed efficiency (weight gain per kilocalories ingested) is significantly increased compared with that of wildtype controls on a moderate-fat diet or compared with that of MC4R$^{-/-}$ mice fed the low-fat diet.[5] MC4R$^{-/-}$ mice have increased feed efficiency because they do not adjust their feeding behavior regarding the increased caloric content of the moderate-fat diet and because they are unable to increase their energy expenditure in response to the moderate-fat diet.[5] Moreover, MC4R$^{-/-}$ mice display normal activity (wheel turns) compared with that of wildtype mice when housed in wheel cages with low-fat diet. However, on a moderate-fat diet, wildtype mice increase their wheel turns, which may be important to burn excess calories, whereas MC4R$^{-/-}$ mice do not increase their number of wheel turns.[5]

On a low-fat diet, no difference in feed efficiency is observed between MC4R$^{-/-}$ and wildtype mice, either male or female.[5] In MC4R$^{K314X/K314X}$ and MC4R$^{+/K314X}$ rats, feed efficiency (weight gain per effective kcal ingested (ingestion minus secretion)) is equal as well as when fed a low-fat diet.[20] Further, locomotor activity measured during 24-h after a 24-h acclimatization period using a home-cage monitoring system is decreased in MC4R$^{K314X/K314X}$ rats compared with that of wildtype controls.[20] The latter observations suggest that excess body weight gain on a low-fat diet in MC4R-deficient rats results from hyperphagia and reduced locomotor activity, whereas the contribution of the energy expenditure component to the obese phenotype of this rat model still remains to be studied in detail. In addition, it has been proposed that the reduced locomotor activity observed in MC4R-deficient rodent models results from the finding that the measurements are performed shortly after introduction to the 'novel' environment, suggesting that the hypoactive phenotype might be stress related.[5,20,27] However, an additional study has shown that MC4R$^{-/-}$ mice exhibit a hypoactive phenotype during the dark phase over a period of 8 days,[29] suggesting that these different observations regarding locomotor activity are related to the technique used to measure activity (i.e., home-cage monitoring versus wheel running cages).

Response to Orexigenic and Anorectic Peptides

Because MC4R-deficient mice display prominent hyperphagia, it was hypothesized that MC4R function modulates food intake by altering the postingestive responsiveness. However, Vaughan et al. showed that MC4R-deficiency-induced hyperphagia does not result from diminished responsiveness to a nutrient preload or the gut satiety hormones cholecystokinin (CCK) or bombesin.[31] In addition, anorexia induced by peripherally administered CCK is equal between MC4R$^{-/-}$, MC4R/MC3R double knockout and wildtype mice.[2] These studies suggest that at least CCK or bombesin function does not seem to be affected by MC4R signaling. However, other studies have shown that MC4R is required for early CCK action.[28] Further, obese MC4R$^{-/-}$ mice have feeding responses similar to central neuropeptide-Y, peptide-YY$_{3-36}$, orexin-A, orexin-B, and urocortin administration compared with that of wildtype mice.[19] Peripheral leptin treatment does not affect ingestive behavior in obese MC4R$^{-/-}$ mice compared with that in wildtype controls, whereas young lean MC4R$^{-/-}$ mice or lean adult MC4R$^{-/-}$ mice pair fed to wildtype mice are sensitive to the anorectic effects of central leptin, albeit sensitivity is reduced compared with that in control mice.[19]

Motivational Aspects of Feeding, Meal Size, and Meal Frequency

When investigating motivational aspects of feeding behavior in MC4R-deficient mice, Vaughan and colleagues observed

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in an initial study that MC4R$^{-/-}$ mice are not hyperphagic compared with control mice and lose weight when they work for food pellets in operant chambers using a procurement-consummatory dual cost foraging protocol.[32] Surprisingly, this weight loss is not accompanied by a compensational increase in feeding behavior. Further, meal size and meal frequency seem to be normal in MC4R$^{-/-}$ mice compared with that of wildtype mice during this behavioral paradigm.[32] However, during the 3- to 4-week intertesting phase, mice resided in their home cage and although *ad libitum* food intake was not measured during that period, body weights recover to their original level or even higher as indicated by the body weights at the start of their next experimental testing phase.[32] Further, MC4R$^{-/-}$ mice work for normal amounts of food and increase their work efforts for pellets on more stringent lever pressing ratios, indicating that the mice work more at a higher unit cost. Therefore, it is unlikely that the hypoactivity of MC4R$^{-/-}$ mice underlies the inability to compensate for the body weight loss as hypoactive leptin-deficient mice are able to sustain hyperphagia during the same behavioral paradigm.[32] To investigate the motivational aspects of feeding behavior in MC4R$^{-/-}$ mice in more detail, the same group subsequently studied MC4R$^{-/-}$ mice in operant chambers in a closed economy-feeding paradigm under a fixed ratio (FR) or progressive ratio (PR) and looked at their meal patterns. This time MC4R$^{-/-}$ mice are hyperphagic in an FR3 schedule (3 lever presses to earn a pellet).[30] Moreover, MC4R$^{-/-}$ mice exhibit a higher level of motivation to work for food compared with that of control mice, indicated by higher breakpoint values.[30] Finally, MC4R$^{-/-}$ mice have increased mean meal size and decreased meal frequency during this closed economy-feeding paradigm.[30] The striking differences between these two studies might result from the different lever set-ups used (two lever-system versus one lever), the different consequences of not responding over time, or a combination of both. A third and more recent article from this group showed with a closed economy food demand paradigm (mice were in chambers 23 h per day) in self-administration chambers that MC4R$^{-/-}$ mice are hyperphagic at a low fixed unit price (FUP2; two nose pokes per pellet) for food.[2] However, during the similar food demand paradigm, now at a much higher fixed unit price (FUP50; 50 nose pokes per pellet), MC4R$^{-/-}$ mice are hypophagic compared with wildtype mice, indicating increased elasticity of food demand.[2] Moreover, the mean meal frequency does not differ significantly between MC4R$^{-/-}$ and wildtype mice when increasing the fixed unit price, whereas the mean meal size of MC4R$^{-/-}$ mice drops from overeating approximately 4 pellets per meal compared with control mice at FUP2 to undereating approximately 3 pellets per meal at FUP50.[2] Thus, these three studies indicate that MC4R$^{-/-}$ mice are hyperphagic when the pellets can be obtained with minimal efforts, that is, approximately 10 responses or less per pellet. By contrast, when costs per unit increase further if a procurement-consummatory dual cost foraging protocol is used, the hyperphagia disappears or even changes to hypophagia.[2]

Nutrient Preference

During a home-cage food-choice paradigm, MC4R$^{K314X/K314X}$ rats show increased preference for fat and a decreased preference for carbohydrates.[20] The latter observation fits with the carbohydrate to fat preference shift observed after acute pharmacological blockade of MC3/4R signaling.[20]

Summary

The above-described general phenotypes observed in MC4R-deficient rodent models are rather similar to the phenotypes observed in humans with loss-of-function mutations in *MC4R* or *POMC*,[11,14,16] suggesting that these rodent models are *bona fide* tools to study the effects of dysregulated MC4R function in humans. However, the hyperphagia observed in children carrying a heterozygous loss-of-function mutation seem to subside with age,[11] a phenomenon that is not observed in rodents. Overall, observations in obese MC4R-deficient rodent models have led to the conception that excess weight gain in MC4R$^{-/-}$ rodents predominantly results from increased ingestive behavior, changes in energy expenditure, and reduced locomotor activity. These observations imply that MC4R is important for the limitation of ingestive behavior and the stimulation of energy expenditure.[1,8,12,28]

INGESTIVE BEHAVIOR IN RODENT MODELS WITH DISRUPTION OF MC3R FUNCTION

General Phenotypes

Using embryonic stem cell manipulation two laboratories generated mice lacking MC3R (MC3R$^{-/-}$) on a mixed 129/Sv and C57BL/6J background. Male full-body MC3R$^{-/-}$ on low- or moderate-fat diets show no clear body weight phenotype despite hypophagia, increased body fat content, increased fat mass, and lower lean mass compared with wildtype siblings.[4,6] Moreover, MC3R$^{-/-}$ mice display stunted growth, hyperleptinemia, and mild hyperinsulinemia.[6] Female MC3R$^{-/-}$ mice, maintained on a moderate- or high-fat diet, showed normal or increased body weight growth, respectively, and normal food intake.[4,6] Although full-body MC3R$^{-/-}$ mice do not display clear feeding and body weight phenotypes, a recent study using an MC3R partial agonist reduced food intake in MC4R$^{-/-}$ mice suggesting

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that MC3R plays a small but significant role in the regulation of ingestive behavior.[15] Analysis of energy expenditure and ambulatory activity, on either low-fat or high-fat diet, do not reveal consistent phenotypes in male or female full-body MC3R$^{-/-}$ mice.[4,6]

Nutrient Sensing

As previously mentioned for MC4R$^{-/-}$ mice, MC3R$^{-/-}$ mice that are exposed to nutritional deprivation also lose equal amounts of body weight, and after resumption of *ad libitum* feeding quickly return to their premalnutrition body weight compared with controls, indicating that both MC3R and MC4R are not necessary for sensing nutritional deficits or for producing the homeostatic response that returns body weight to the original set point level.[5]

Feed Efficiency

Although male MC3R$^{-/-}$ mice maintained on a regular chow diet are hypophagic, they show increased feed efficiency.[6] In addition, female MC3R$^{-/-}$ mice, showing normal food intake on a low or high-fat diet, also show increased feed efficiency.[6]

Summary

The main phenotype of MC3R$^{-/-}$ mice is an increase in feed efficiency. This suggests that the increased feed efficiency, and not changes in ingestive behavior, is underlying the increased adipose mass in MC3R$^{-/-}$ mice. Although humans with mutations in *MC3R* show phenotypes that are reminiscent of the *Mc3r* knockout mouse model, the effect of MC3R function on feed efficiency in humans remains to be studied in more detail.

INGESTIVE BEHAVIOR IN RODENT MODELS WITH DISRUPTION OF AGRP FUNCTION

Using embryonic stem cell manipulation Qian et al. generated mice lacking AgRP (AgRP$^{-/-}$) on a mixed 129/Sv and C57BL/6J background and observed that AgRP$^{-/-}$ mice display normal food intake, body weight gain, and energy expenditure.[22] However, a subsequent study that compared younger (~3 months old) and older (~6-months old) AgRP$^{-/-}$ mice found that ~6-month-old AgRP$^{-/-}$ mice display an age-dependent lean phenotype predominantly resulting from metabolic changes because food intake is still normal,[33] indicating that AgRP-deficiency does result in a metabolic phenotype albeit at a later age. Moreover, a set of very elegant studies have shown that toxin-induced ablation of AgRP neurons during embryogenesis results in compensatory adaptations resulting in relative normal energy homeostasis compared with that in wildtype controls, whereas ablation of AgRP neurons postembryogenesis results in a lean phenotype hallmarked by decreased caloric intake or even death.[13,18] AgRP neurons coexpress γ-aminobutyric acid (GABA), which inhibits POMC neuron function.[34] Wu and Palmiter showed that the loss of AgRP neuron-specific γ-aminobutyric acid (GABA) expression induces the hypophagia and severe loss of body weight after ablation of AgRP neurons during adulthood, whereas chronic blockade of MC4R is inadequate to rescue these phenotypes.[34] Finally, transgenic overexpression of *AgRP* also results in obesity hallmarked in part by hyperphagia.[21]

INGESTIVE BEHAVIOR IN RODENT MODELS WITH DISRUPTION OF POMC FUNCTION

Using embryonic stem cell manipulation Yaswen et al. generated mice lacking POMC (POMC$^{-/-}$) on a mixed 129/Sv and C57BL/6J background and showed that POMC deficiency in the mouse results in obesity resulting in part from hyperphagia.[35] Further, POMC$^{-/-}$ mice are unable to properly adjust their energy intake and expenditure as wildtype mice do when switched to a high-fat diet.[35]

POMC$^{-/-}$ mice also display corticosterone (CORT) deficiency.[7] Moreover, rescuing plasma CORT concentrations in POMC$^{-/-}$ mice even further elevates the preexisting hyperphagia, whereas no effect of CORT administration on ingestive behavior is observed in wildtype mice.[7] This suggests that CORT deficiency observed in POMC$^{-/-}$ mice might partially mask the adverse metabolic consequences induced by deficiency of POMC-derived peptides.[7]

PHARMACOLOGICAL STUDIES WITH MCR LIGANDS MODULATING INGESTIVE BEHAVIOR

A vast collection of data is available regarding pharmacological manipulation of the MC system. Some excellent reviews address this subject.[1,8,12,28] Briefly, acute central administration of (nonspecific) MC3/4R agonists results in decreased feeding, whereas (nonspecific) MC3/4R antagonists increase food intake. Taken together, pharmacological manipulation of the MC system clearly indicates that the MC system plays an important role in modulating ingestive behavior in rodents. Further, observations regarding meal size, meal duration, and modulation of MC system function when food intake is expected suggest that the MC system is rather involved in meal termination rather than meal initiation.[1]

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BRAIN SITES WHERE MC RECEPTOR SIGNALING AFFECTS INGESTIVE BEHAVIOR

Sites of Melanocortin Signaling

POMC expression is mainly found in the neurons of the arcuate nucleus, where it is coexpressed with the anorectic peptide cocaine amphetamine-related transcript (CART).[8] These POMC/CART neurons project fibers to several nuclei in forebrain, midbrain, and hindbrain.[8] The brainstem also contains a local smaller subset of *POMC*-expressing neurons.[8] Neurons coexpressing *Npy* and *AgRP* are also predominantly found in the arcuate nucleus, and these neurons project fibers to a subset of hypothalamic and septal brain regions identical to those containing dense POMC innervation.[8] Finally, a striking difference between the projections of the POMC/CART and NPY/AgRP neurons, in the rat at least, is that the NPY/AgRP neurons seem to be much less widely distributed.[8]

Mc4r is expressed in many brain regions, which are located in the hypothalamus, forebrain, and hindbrain. Moreover, the paraventricular nucleus (PVN), dorsomedial hypothalamic nucleus (DMH), and medial preoptic area were identified as primary sites of MC4R action using MCR ligands.[1] However, MCR-ligand-induced modulation of feeding in the PVN, in either a positive or negative direction, is only observed under conditions where food intake is stimulated by either the onset of darkness, fasting, or central NPY administration.[1] These observations have generated the notion that MC4R signaling in the PVN is important for meal termination but not for meal initiation.[1] More detailed analysis of the PVN neurons expressing *Mc4r* has shown that a subpopulation of these neurons express corticotropin-releasing hormone (CRH) or thyrotropin-releasing hormone. These hormones, especially CRH, seem to function downstream of MC4R signaling in the PVN and contribute to the mechanisms by which the MC system mediates ingestive behavior.[1]

MC4R signaling in the DMH also affects ingestive behavior. For example, the administration of [norleucine;^{4} D-phenylalanine^7]-α-MSH to the DMH decreases food intake, whereas AgRP administration functions in opposite direction.[1] Further, AgRP administration to the DMH increases sucrose intake, suggesting that MC4R signaling in the DMH modulates the palatability of a diet.[1]

In the brainstem, *Mc4r* is expressed in the dorsal motor nucleus of the vagus nerve and in the nucleus of the solitary tract (NTS), both being important brain sites for autonomic control.[8] Several pharmacological studies have shown that administration of an MC4R agonist or antagonist in the fourth ventricle or into the dorsal vagal complex (DVC) mediates similar effects on food intake as comparable with lateral ventricle administration.[1] In addition, the anorexigenic effects of MTII, an MC3/4R nonspecific agonist, in the fourth ventricle resulted from a reduction in meal size, and not meal frequency.[1] Finally, MC4R signaling in the brainstem is important for the inhibition of food mediated by CCK.[28] Other brain regions that express *Mc4r* are the central amygdala (CeA) and the lateral hypothalamus (LH). Administration of the agonist NDP-MSH into the CeA does not modify food intake, whereas the administration of the antagonists AgRP or HS014 increases food intake.[1] However, the orexigenic effect is not as strong as observed for administration to the PVN, DMH, or DVC. In the LH, administration of AgRP seems to modify activity of orexin neurons or gene expression of the orexigenic peptide melanin-concentrating hormone,[1] but it remains unclear what the function of these neurons and/or genes is related to AgRP-induced hyperphagia. Finally, administration of an MC4R agonist into the LH, arcuate nucleus, ventromedial hypothalamus (VMH), or nucleus accumbens did not or very modestly modify food intake. In sum, although many brain regions express *Mc4r*, the PVN, DMH, and DVC/NTS seem to be the predominant regions modulating the effects of the MC system on ingestive behavior. Further studies need to be performed to clarify the function of the other brain regions where MC4R activity can be modulated.

In rodents, central expression of *Mc3r* is more moderate compared with the wide expression of *Mc4r*. Strongest expression of MC3R is observed in the hypothalamus, predominantly in the arcuate nucleus and the ventromedial hypothalamus.[23] Other central brain regions that express *Mc3r* include the medial habenula, the ventral tegmental area, and the raphe.[23] An interesting absentee in this list is the NTS as *Mc3r* expression is not observed or is very limited in the hindbrain.[23,28] Intracerebroventricular or peripheral administration of the stabilized γ-MSH analog D-Trp^8-γ-MSH, an agonist with significant selectivity for MC3R over MC4R, increases food intake.[23] In addition, a study using JRH322-18, a new tetrapeptide compound which has a mixed MC3R partial agonist/antagonist and MC4R agonist function, indicates that central MC3R plays a small but significant role in the regulation of food intake.[15]

Conditional Mutant Models of Melanocortin Function

Because lifelong loss of gene expression in genetic rodent models can result in developmental compensatory effects and acute injections only have short-lasting effects, a preferred genetic approach, to date only used in mice, is the Cre-lox system, which uses Cre recombinase to locally activate a gene of interest in mice carrying a lox-stop-lox allele for that particular gene of interest. Using this system, reactivation of *Mc4r* in mice carrying a loss-of-function allele for *Mc4r* specifically in PVN and a subpopulation

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of MC4R neurons in the CeA normalizes the hyperphagia and reduced body weight, whereas this specific strategy does not affect energy expenditure.[1,12] Reexpression of MC4Rs in cholinergic neurons (ChAT-Cre, loxTB MC4R mice) modestly reduces body weight gain without altering food intake and is sufficient to normalize energy expenditure, whereas restoration of MC4R expression in brainstem neurons including those in the dorsal motor nucleus of the vagus (Phox2b-Cre, loxTB MC4R mice) does not rescue the obese nor the hyperphagic phenotype in MC4R knockout mice.[24]

Viral Manipulation of Melanocortin Function

A different but complementary approach to the above-mentioned Cre-lox system is the use of adeno-associated virus (AAV)-mediated expression. AAV-mediated expression of *Agouti* to inhibit MC signaling in the PVN results in increased food intake and body weight.[1] In studies with AAV-mediated overexpression of AgRP, overexpression in the PVN, LH, and VMH increases body weight predominantly by increasing meal size.[9]

CONCLUDING REMARKS

Functional disruption of the central MC system (e.g., at the level of *AgRP*, *POMC*, *Mc3r*, or *Mc4r*) reveals a wide variety of metabolic phenotypes. Increased body weight is often observed in both rodents and humans with mutations in the central MC system, characterized by hyperphagia, changes in meal size, food preference, and energy expenditure. These changes seem to be mediated by distinct brain sites expressing MC receptors. So far, the PVN seems to be an especially important brain site for MCR-mediated hyperphagia. However, further research is warranted to unravel how the MC system exactly modulates the energy balance through changes in energy expenditure and ingestive behavior.

REFERENCES

1. Adan RA, Tiesjema B, Hillebrand JJ, la Fleur SE, Kas MJ, de Krom M. The MC4 receptor and control of appetite. *Br J Pharmacol* 2006;**149**:815–27.
2. Atalayer D, Robertson KL, Haskell-Luevano C, Andreasen A, Rowland NE. Food demand and meal size in mice with single or combined disruption of melanocortin type 3 and 4 receptors. *Am J Physiol Regul Integr Comp Physiol* 2010;**298**:R1667–74.
3. Biebermann H, Castaneda TR, van Landeghem F, von Deimling A, Escher F, Brabant G, et al. A role for beta-melanocyte-stimulating hormone in human body-weight regulation. *Cell Metab* 2006;**3**:141–6.
4. Butler AA, Kesterson RA, Khong K, Cullen MJ, Pelleymounter MA, Dekoning J, et al. A unique metabolic syndrome causes obesity in the melanocortin-3 receptor-deficient mouse. *Endocrinology* 2000;**141**:3518–21.
5. Butler AA, Marks DL, Fan W, Kuhn CM, Bartolome M, Cone RD. Melanocortin-4 receptor is required for acute homeostatic responses to increased dietary fat. *Nat Neurosci* 2001;**4**:605–11.
6. Chen AS, Marsh DJ, Trumbauer ME, Frazier EG, Guan XM, Yu H, et al. Inactivation of the mouse melanocortin-3 receptor results in increased fat mass and reduced lean body mass. *Nat Genet* 2000;**26**:97–102.
7. Coll AP, Challis BG, Lopez M, Piper S, Yeo GS, O'Rahilly S. Proopiomelanocortin-deficient mice are hypersensitive to the adverse metabolic effects of glucocorticoids. *Diabetes* 2005;**54**:2269–76.
8. Cone RD. Anatomy and regulation of the central melanocortin system. *Nat Neurosci* 2005;**8**:571–8.
9. de Backer MW, la Fleur SE, Adan RA. Both overexpression of agouti-related peptide or neuropeptide Y in the paraventricular nucleus or lateral hypothalamus induce obesity in a neuropeptide- and nucleus specific manner. *Eur J Pharmacol* 2011;**660**:148–55.
10. Fan W, Boston BA, Kesterson RA, Hruby VJ, Cone RD. Role of melanocortinergic neurons in feeding and the agouti obesity syndrome. *Nature* 1997;**385**:165–8.
11. Farooqi IS, Keogh JM, Yeo GS, Lank EJ, Cheetham T, O'Rahilly S. Clinical spectrum of obesity and mutations in the melanocortin 4 receptor gene. *New Engl J Med* 2003;**348**:1085–95.
12. Garfield AS, Lam DD, Marston OJ, Przydzial MJ, Heisler LK. Role of central melanocortin pathways in energy homeostasis. *Trends Endocrinol Metab* 2009;**20**:203–15.
13. Gropp E, Shanabrough M, Borok E, Xu AW, Janoschek R, Buch T, et al. Agouti-related peptide-expressing neurons are mandatory for feeding. *Nat Neurosci* 2005;**8**:1289–91.
14. Huszar D, Lynch CA, Fairchild-Huntress V, Dunmore JH, Fang Q, Berkemeier LR, et al. Targeted disruption of the melanocortin-4 receptor results in obesity in mice. *Cell* 1997;**88**:131–41.
15. Irani BG, Xiang Z, Yarandi HN, Holder JR, Moore MC, Bauzo RM, et al. Implication of the melanocortin-3 receptor in the regulation of food intake. *Eur J Pharmacol* 2011. http://dx.doi.org/10.1016/j.ejphar.2010.10.101.
16. Krude H, Biebermann H, Luck W, Horn R, Brabant G, Gruters A. Severe early-onset obesity, adrenal insufficiency and red hair pigmentation caused by POMC mutations in humans. *Nat Genet* 1998;**19**:155–7.
17. Loos RJ, Rankinen T, Rice T, Rao DC, Leon AS, Skinner JS, et al. Two ethnic-specific polymorphisms in the human Agouti-related protein gene are associated with macronutrient intake. *Am J Clin Nutr* 2005;**82**:1097–101.
18. Luquet S, Perez FA, Hnasko TS, Palmiter RD. NPY/AgRP neurons are essential for feeding in adult mice but can be ablated in neonates. *Science (New York, NY)* 2005;**310**:683–5.
19. Marsh DJ, Hollopeter G, Huszar D, Laufer R, Yagaloff KA, Fisher SL, et al. Response of melanocortin-4 receptor-deficient mice to anorectic and orexigenic peptides. *Nat Genet* 1999;**21**:119–22.
20. Mul JD, van Boxtel R, Bergen DJM, Brans M, Brakkee J, Toonen PW, et al. Melanocortin receptor 4-deficiency affects body weight regulation, grooming behavior, and substrate preference in the rat. *Obesity (Silver Spring)* 2011; in press.
21. Ollmann MM, Wilson BD, Yang YK, Kerns JA, Chen Y, Gantz I, et al. Antagonism of central melanocortin receptors in vitro and in vivo by agouti-related protein. *Science (New York, NY)* 1997;**278**:135–8.
22. Qian S, Chen H, Weingarth D, Trumbauer ME, Novi DE, Guan X, et al. Neither agouti-related protein nor neuropeptide Y is critically required for the regulation of energy homeostasis in mice. *Mol Cell Biol* 2002;**22**:5027–35.

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23. Renquist BJ, Lippert RN, Sebag JA, Ellacott KL, Cone RD. Physiological roles of the melanocortin MC(3) receptor. *Eur J Pharmacol* 2011. http://dx.doi.org/doi:10.1016/j.ejphar.2010.12.025.
24. Rossi J, Balthasar N, Olson D, Scott M, Berglund E, Lee CE, et al. Melanocortin-4 receptors expressed by cholinergic neurons regulate energy balance and glucose homeostasis. *Cell Metab* 2011;**13**:195–204.
25. Schwartz MW, Woods SC, Porte Jr D, Seeley RJ, Baskin DG. Central nervous system control of food intake. *Nature* 2000;**404**:661–71.
26. Speliotes EK, Willer CJ, Berndt SI, Monda KL, Thorleifsson G, Jackson AU, et al. Association analyses of 249,796 individuals reveal 18 new loci associated with body mass index. *Nat Genet* 2010;**42**:937–48.
27. Ste Marie L, Miura GI, Marsh DJ, Yagaloff K, Palmiter RD. A metabolic defect promotes obesity in mice lacking melanocortin-4 receptors. *Proc Natl Acad Sci U S A* 2000;**97**:12339–44.
28. Tao YX. The melanocortin-4 receptor: physiology, pharmacology, and pathophysiology. *Endocr Rev* 2010;**31**:506–43.
29. Tucker K, Overton JM, Fadool DA. Kv1.3 gene-targeted deletion alters longevity and reduces adiposity by increasing locomotion and metabolism in melanocortin-4 receptor-null mice. *Int J Obes (Lond)* 2008;**32**:1222–32.
30. Vaughan C, Moore M, Haskell-Luevano C, Rowland NE. Food motivated behavior of melanocortin-4 receptor knockout mice under a progressive ratio schedule. *Peptides* 2006;**27**:2829–35.
31. Vaughan CH, Haskell-Luevano C, Andreasen A, Rowland NE. Effects of oral preload, CCK or bombesin administration on short term food intake of melanocortin 4-receptor knockout (MC4RKO) mice. *Peptides* 2006;**27**:3226–33.
32. Vaughan CH, Moore MC, Haskell-Luevano C, Rowland NE. Meal patterns and foraging in melanocortin receptor knockout mice. *Physiol Behav* 2005;**84**:129–33.
33. Wortley KE, Anderson KD, Yasenchak J, Murphy A, Valenzuela D, Diano S, et al. Agouti-related protein-deficient mice display an age-related lean phenotype. *Cell Metab* 2005;**2**:421–7.
34. Wu Q, Palmiter RD. GABAergic signaling by AgRP neurons prevents anorexia via a melanocortin-independent mechanism. *Eur J Pharmacol* 2011;**660**:21–7.
35. Yaswen L, Diehl N, Brennan MB, Hochgeschwender U. Obesity in the mouse model of pro-opiomelanocortin deficiency responds to peripheral melanocortin. *Nat Med* 1999;**5**:1066–70.

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Chapter 154

NPY

Simona Perboni, Marzia Vignoni and Akio Inui

ABSTRACT

Feeding behavior is tightly regulated by peptidergic transmission within the hypothalamus.

Neuropeptide Y (NPY) is one of the most abundant and most potent known orexigenic peptides found in the brain. It has been shown that NPY plays a pivotal role in the control of food intake and body weight in the hypothalamus. Peripheral and central signals of energy balance are integrated within the ARC both via the NPY and melanocortin systems. Therefore, the actions of NPY extend throughout the hypothalamus and affect the heterogeneous population of neurons, and many of them are associated with the regulation of energy homeostasis. NPY can act on several Y receptors, but only Y1, Y2, and Y5 receptors are implicated in the ARC feeding circuitry. Ligands for Y receptors may be of benefit for the treatment of obesity.

NPY AND THE CENTRAL REGULATION OF FOOD INTAKE

The central nervous system (CNS) has developed a meticulously interconnected circuitry to keep us fed and in adequate nutritional state. Specific areas in the hypothalamus and in the brainstem are important in coordinating signaling molecules, such as the hormones insulin and leptin, which circulate in proportion to body fat mass and are critical for normal energy homeostasis. Peripheral and central signals of energy balance are integrated within the arcuate nucleus (ARC) both via the Neuropeptide Y (NPY) and melanocortin systems. ARC neurons in turn send dense projections to various hypothalamic neurons, including the paraventricular nucleus (PVN), the ventromedial nucleus (VMN), the lateral hypothalamic area (LHA), and the dorsomedial nucleus (DMN). Therefore, the actions of NPY extend throughout the hypothalamus and affect a diverse and heterogeneous population of neurons,[26] many of them associated with the regulation of energy homeostasis or mediating food reward, such as in the nucleus of the tractus solitarius, the nucleus accumbens, and the ventral tegmental area.[28]

NEUROPEPTIDE Y AND FEEDING

Feeding behavior is tightly regulated by peptidergic transmission within the hypothalamus. NPY is one of the most abundant and most potent known orexigenic peptides found in the brain.[1] It plays a pivotal role in the control of food intake and body weight within the hypothalamus. The feeding stimulatory effect of NPY is approximately 500 times more potent on a molar basis than norepinephrine.[15] Central administration of NPY by intracerebroventricular injection or its direct injection into specific hypothalamic sites, especially the perifornical area (PFA), the PVN, and the VMN stimulates robust feeding responses in a dose-dependent manner. Chronic administration of NPY produces a sustained hyperphagia and increased body weight leading to obesity.[6,14] NPY stimulates appetite and consummatory ingestive behaviors under a variety of conditions.[7] In several animal models of obesity, the levels of NPY gene expression and NPY itself are increased. Similarly, increases in levels of NPY gene expression and in peptide itself precede hyperphagic behavior and are directly correlated with conditions of increased energy deprivation and energy demand.[27] In effect, food deprivation is one of the most important factors that increase the expression of hypothalamic NPY. The NPY release is enhanced immediately before the onset of feeding and gradually decreases as food intake continues. The actions of the NPY, even fasting-induced refeeding, can be blocked by the pharmacological application of NPY antagonists.[31] NPY produces a shift to positive energy balance by increasing food intake, by decreasing energy expenditure primarily with a reduction in thermogenesis in brown adipose tissue and by facilitating fat deposition in white adipose tissue partly through an increased insulin activity. Taken together, these studies show that NPY administration is directly correlated with a state of positive energy balance.[15]

Prostaglandin (PG) D(2), the most abundant PG in the CNS, stimulates food intake after intracerebroventricular administration in mice. The orexigenic activity of PGD(2) is also abolished by an antagonist for NPY Y1 receptor. PGD(2) may stimulate food intake through central DP(1)

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00154-8
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receptor coupled to the NPY system.[29] Moreover, the complement C5a stimulates food intake by activating the orexigenic PGD(2) system. The orexigenic activity of C5a is inhibited by an antagonist for NPY Y1 receptor, which is activated downstream of the PGD(2)–DP(1) system. These results suggest that C5a stimulates food intake via a PGD(2)- and NPY-dependent mechanism. C5a is the first example of orexigenic peptides acting through the PGD(2)–NPY system in the CNS.[30] As described in the nesfatin chapters in the Brain and Gastrointestinal Peptides sections of this book, nesfatin-1-induced inhibition of feeding might be mediated through the inhibition of orexigenic NPY neurons in ARC.[32]

NPY IN THE ARC NUCLEUS

The ARC is an important hypothalamic nucleus in the control of appetite and plays a primary role in the interaction between peripheral organs and the brain. The main source of NPY within the hypothalamus is from specialized neurons in the ARC.[1] Two distinct subsets of neurons are involved in the control of food intake in the ARC.[24] One group contains neurons expressing the orexigenic peptides, NPY and agouti-related peptide (AgRP).[24] The other group expresses anorexigenic peptides derived from proopiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART); POMC is a precursor to the anorectic alpha melanocyte stimulating hormone (α-MSH). Activation of these neurons has the effect of decreasing food intake and increasing energy expenditure.[24] The arcuate POMC and NPY/AgRP neurons constitute a functional unit in which neural inputs to NPY/AgRP cells may rapidly affect both NPY/AgRP and POMC neurons. NPY neurons provide a dominant inhibitory tone onto POMC. The majority of POMC neurons are contacted by terminals containing both γ-aminobutyric acid (GABA) and NPY and express the Y1 receptor on the soma. Fasting and weight loss lead to the activation of anabolic effectors and inhibition of catabolic effectors in the brain, increasing food intake. A decrease in the release of the POMC-derived peptide γ3-MSH and an increased release of the AgRP are seen in rats in response to fasting. Over time, accumulation of excess calories causes weight gain until body fat content is restored to its original value.[5]

PERIPHERAL SIGNALING INVOLVED IN THE REGULATION OF FOOD INTAKE IN THE ARC

The central representation of the metabolic state is mediated in part by the levels of distinct circulating factors, including insulin, leptin and ghrelin.[13] During and after a meal, signals in the periphery, including taste signals from the oral cavity, gastric distension, and humoral signals from the gastrointestinal tract are generated. These afferent signals are transmitted mainly by the vagus nerve and by the sympathetic nervous system to the hindbrain, particularly the nucleus of the solitary tract. This brain region communicates with higher brain areas such as the hypothalamus and the cerebral cortex. As stated above, the ARC is a prominent region in the hypothalamus for integrating these peripheral signals. This information is then relayed to various second-order hypothalamic targets.[5]

Insulin was the first circulating signal proposed to be involved in energy homeostasis. Insulin receptors are present on ARC NPY neurons and insulin can act locally to inhibit its neuronal firing. However, in overweight animals, these neurons are activated by insulin resistance, suggesting that, in obese animals, insulin action at ARC NPY neurons can contribute to positive energy state of the animal.[15] Leptin reaches the ARC within the hypothalamus, directly affecting neurons which coexpress POMC/CART or NPY/AgRP. For example, leptin depolarizes POMC neurons and simultaneously hyperpolarizes the soma of neuropeptide Y/GABA neurons, reducing the release from these terminals. This diminished release of GABA disinhibits POMC neurons. The POMC–CART- and NPY–AGRP-containing neurons, which are regulated in an opposing manner by leptin, project further to other brain centers. These include the VMH and DMN, which also express NPY, and the PVN and the LHA, which express melanin concentrating hormone (MCH). The LHA and other brain areas communicate with the cerebral cortex, where feeding behavior is finally coordinated.[21] There are functional and anatomical connections between ghrelin and NPY signaling. Ghrelin acts directly on ARC NPY neurons to depolarize them and increase their spontaneous firing frequency.[19] Conversely, ARC POMC neurons are indirectly inhibited by ghrelin. The ghrelin-mediated increase in the activity of ARC NPY neurons that innervate the POMC neurons results in an inhibition of the latter. This effect is GABA mediated. In addition, the ARC POMC neuron expresses functional Y1 and Y2 receptors, which inhibit the POMC neuron when activated by NPY.[5] The involvement of the Y5 receptor has been implicated in the regulation of spontaneous release of alpha-MSH from POMC neurons, further supporting the NPY-mediated inhibition of the melanocortin system.[26] Taken together, ghrelin, by activating ARC NPY neurons and indirectly suppressing the activity of ARC POMC neurons, will further reinforce the NPY/AgRP dominant state. These observations show that elevated peripheral levels of ghrelin can induce food intake by the activation of orexigenic NPY-mediated signaling, which also results in the concomitant inhibition of the anorexigenic pathways.[5]

NPY RECEPTORS IN THE ARC

As described in the NPY chapter of the Brain Peptides section of the book, NPY can act on several Y receptors, but only Y1, Y2, and Y5 receptors are implicated in the ARC

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feeding circuitry. There is a distinct division in the location of the Y1 and Y2 receptors on the ARC neurons with Y1 located only at the postsynaptic membrane, whereas Y2 receptors are found post-synaptically and at the presynaptic terminal. Further, Y5 receptor activation can depress ARC synaptic transmission.[5]

As mentioned above, NPY has long projections, and all hypothalamic nuclei receive NPY-ergic projections to some extent. The actions of NPY are almost always inhibitory, however, depending on the site of its action this may lead directly to a postsynaptic inhibition or to presynaptic effects in which excitatory inputs are suppressed. Thus, the overall or net effect of NPY in each hypothalamic region does not result from the same action. For example, although NPY can directly inhibit to suppress anorexigenic tone, NPY inhibits excitatory orexin neurons in the LHA as part of a short negative feedback loop with ARC NPY neurons.[26]

THE ROLE OF NPY IN THE DMN

NPY can also be produced by hypothalamic DMN neurons, though only transiently during certain states of negative energy balance. Interestingly, NPY gene expression in the DMN increases in response to chronic food restriction and long-term exercise[3] but not to acute food deprivation.[2] This finding suggests that, unlike arcuate NPY neurons, DMN-NPY neurons may play an important role in maintaining energy homeostasis only in response to long-term alterations in energy intake or expenditure.[3] Unlike NPY in the ARC, the regulation of NPY gene expression in the DMN is independent of leptin changes.[2] The elevation of NPY gene expression in DMN is closely associated with melanocortin and cholecystokinin (CCK) signaling. The α-MSH fibers are probably derived mainly from the ARC, and their presence suggests that they can regulate the NPY expression presynanptically. By contrast, CCK-1 and CCK-2 receptors are located on DMN NPY neurons. The DMN neurons are negatively regulated by α-MSH and CCK signaling, so that their anorectic signals are mediated, at least in part, by an inhibition of DMN NPY neurons.[21] As previously mentioned, neurons in the DMN are able to transiently express NPY, perhaps to maintain hyperphagia in conditions of real or perceived energy demand. In support of this idea, Yang et al.[35] showed that bilateral AAV-mediated NPY overexpression in the DMN of lean rats increases food intake and body weight and exacerbates diet-induced obesity. Conversely, NPY knock-down using adeno-associated-virus (AAV)-RNA in the DMN of genetically obese rats causes reductions in food intake and a significant improvement in obesity and glucose tolerance.[12] The reduced food intake is the result of decreased nocturnal meal size, indicating a more specific role for DMN NPY in meal size modulation, in contrast to the actions of NPY in the PVN where it affects meal frequency.[4]

NPY ACTION IN THE PVN

The PVN has emerged as one of the most significant target of NPY-ergic fibers and its critical role in energy balance regulation is highlighted by studies in which PVN lesions lead to an obesity syndrome characterized by hyperphagia and increased body weight gain. Increased NPY release is seen in the PVN both of food deprived rats and in hyperphagic rodents with streptozotocin induced diabetes.[8] Acute injections of NPY into the PVN can induce a strong feeding response. Sustained local overexpression and release of NPY in the PVN lead to increased food intake via increased meal frequency. Reducing levels of NPY in the PVN with anti NPY antibody injections lead to reduced food intake in rats. NPY modulates PVN neuronal activity by both presynaptic and postsynaptic mechanisms. In the former, NPY inhibits GABA release from a population of GABA-ergic terminals that synapse on a subset of hypocellularity neurons within the PVN. This inhibitory presynaptic action of NPY is reduced in obese Zucker rats compared with that in lean controls, which is consistent with the reduced sensitivity of obese rats to NPY. The postsynaptic effect of NPY is evident specifically at PVN neurons that express the melanocortin 4 receptor (MC4R). Here, NPY inhibits the firing frequency of MC4R-expressing PVN neurons by directly eliciting a membrane hyperpolarization. The presynaptic effect of NPY in the PVN is already prominent very early in postnatal development and is mediated by more than one NPY receptor subtype, that is, Y1, Y2, and Y5. Interestingly, the postsynaptic response seems to be mediated entirely by Y1 receptor.[7]

NPY ACTIONS IN VMN

The VMN is one of the most sensitive targets for NPY-mediated feeding. Microinjections of NPY directly into the VMN robustly increases food intake. Although electrolytic lesions to the VMN produce a hyperphagic phenotype, electrical stimulation of VMN suppresses food intake. This suggests that VMN activity exerts an anorexigenic tone.[5] Although NPY is found within the VMN, its presence is markedly less abundant than in other hypothalamic regions such as ARC and PVN. The density of NPY fibers reaching the core of the VMN is sparse, but the VMN expresses a high density of Y1 receptors. VMN neurons are nearly all glutaminergic, meaning that their output is excitatory to their downstream contacts. NPY reduces the firing activity of VMN by the direct activation of postsynaptic Y1 receptors which hyperpolarizes VMN and reduces their excitability. In addition to this prominent postsynaptic action, NPY also acts at the presynaptic terminals on VMN neurons inhibiting the synaptic transmission. Although NPY suppresses both excitatory glutamatergic and inhibitory GABA-ergic synaptic inputs to NPY-sensitive VMN

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neurons, the suppression of excitatory input is the dominant action. The NPY-mediated inhibition of VMN neurons may have significant consequences for energy balance regulation. Although NPY acts on a wider population of neurons, it seems to preferentially affect neurons expressing the signaling form of the leptin receptor. Interestingly, these neurons are inhibited by increasing glucose levels, whereas VMN neurons that respond to glucose are excited by elevated glucose levels.[20]

NPY ACTION IN THE LHA

LHA plays a key role in the hypothalamic regulation of feeding. Acute NPY injections into the perifornical area of LHA strongly induce food intake, but evidence from diffusion studies of NPY injected in his manner does not exclude the possibility that they are the neighboring regions of the LHA that mediates this NPY-induced feeding response. Similarly, long-term ectopic overexpression of NPY in the LHA using AAV injections leads to sustained hyperphagia and weight gain with increased meal size.[12] Two major classes of orexigenic neurons are present in the LHA, the orexin and the MCH neurons. Orexin is generally excitatory to hypothalamic neurons and orexin neurons have feedback connections with ARC NPY neurons. Orexin increases NPY tone in the ARC by both increasing NPY mRNA expression and by increasing neuronal activity of NPY neurons. In turn, ARC NPY neurons also innervate orexin LHA neurons, which are inhibited by NPY release. Like orexin neurons, MCH neurons of the LHA also receive innervation from NPY-containing fibers and express mRNA for the Y5 receptor.[9] LHA neurons are directly inhibited by the application of NPY, as evidenced by the NPY-mediated membrane hyperpolarization and reduced firing rate. Intracerebroventricular infusion of MCH leads to a significant increase in NPY mRNA, suggesting a feedback circuit between NPY and MCH neurons similar to that seen between NPY and orexin neurons. Interestingly, NPY does not elicit the major orexigenic system in LHA. This might imply that the LHA orexigenic system acts in part by helping to initially activate the ARC NPY system, and once activated, the NPY neurons in turn suppress the LHA neurons.[34]

NPY AND FEEDING BEHAVIOR

It seems that we can no longer think of NPY as only a potent orexigenic agent that acts to increase food intake in all circumstances. There is a more complex role of NPY in organizing feeding behavior than is apparent when only the total amount of food consumed is measured.[33] Both NPY microinjections and injections of Y1 receptor agonist in the third ventricle of Siberian hamster markedly increase foraging and food hoarding to a greater extent than food intake.[7] Conversely, Y5 receptor agonists stimulate food intake to a greater degree than foraging or hoarding.[6] Accordingly with these results, NPY microinjections into the PVN and perifornical area (PFA) of the hypothalamus can alter the ingestive behavior of foraging and food hoarding in starving rats. Specifically, NPY injected into the PVN or PFA increases food hoarding to a greater extent than foraging or food intake. Moreover, the NPY Y1 receptor seems to play a role in controlling food hoarding through the PVN and PFA on the basis of the ability of an Y1 receptor antagonist to inhibit increases in food hoarding in refed rats.[6,22] Therefore, understanding the NPY-mediated control of human foraging and food storing (hoarding) could provide an additional target for pharmaceutical or behavioral manipulations in the treatment or prevention of obesity[7]

INSIGHT FROM GENETIC MODELS

Research investigating the effects of genetic manipulation of NPY expression and feeding behavior belies the complexity of food intake regulation. For example, mice engineered to overexpress central NPY do not display increased feeding, and NPY knockout mice on a mixed background have normal food intake and body weight and are able to respond to fasting with hyperphagia.[17,18,31] C57BL/6 NPY−/− mice even develop mild obesity when access to food is unrestricted. Concomitant knockout of NPY and AgRP in ARC does not prevent mice from developing to normal body weight or displaying hyperphagia in response to negative energy balance.[18] Similarly, knockout mice for the Y1 receptor do not show an obesity-resistant phenotype and none of the knockout models for a singular receptor produce significant changes in body weight. It is perhaps not surprising that the knockout of any single NPY receptor has such a modest effect on the feeding response to NPY itself, because multiple Y receptors participate in the regulation of food intake. Double knockout involving multiple Y receptors leads to enhanced susceptibility toward diet-induced obesity in mice.[21] Plastic changes to NPY-ergic signaling networks occur throughout development. Neonatal ablation of ARC NPY/AgRP neurons has minimal effects on feeding. However, ablation of these NPY/AgRP neurons in adults causes fatal starvation. Given that melanocortin signaling pathways are not mature in neonates, the loss of NPY neurons does not result in an anorexigenic phenotype in this early stage of development. In adults, however, the potent melanocortin system is established; it is probable that ablation of NPY neurons will cause an imbalance in which excessive melanocortin signaling could lead to anorexia and starvation. Such a situation appears in animal models of cachexia, where the anorexia can be substantially reversed with melanocortin receptor antagonists.[5,27]

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REGULATION OF NPY RECEPTORS IN FEEDING BEHAVIOR

Studies have been devoted to the identification of NPY receptor subtypes responsible for the regulation of energy homeostasis and the development of obesity.[14] Y1, Y2, and Y5 receptors may affect energy homeostasis through different mechanisms, which have been shown by sustained activation of these receptors in the brain.[20] Changes in feeding behavior and energy balance induce a marked plasticity in the Y1 function and expression in specific regions of the hypothalamus, reinforcing the involvement of this receptor in stimulation of food intake.[11] Fasting induces an increase in NPY orexigenic activity and a decrease in Y1 receptor expression in the ARC and PVN in rats. Neither Y2 nor Y5 mRNA levels are affected by fasting. Decreased activity of Y1 in ARC and PVN induced by food deprivation is attenuated by glucose ingestion and counteracted by leptin treatment, supporting the regulation of NPY Y1 system by leptin in the PVN. Activation of central Y5 receptors leads to weight gain and adiposity through a combination of hyperphagic and nutrient-partitioning effects by metabolizing carbohydrate and decreasing energy metabolized per energy ingested. Chronic activation of central Y1 causes weight gain by altering nutrient partitioning alone, and chronic activation of central Y2 causes transient weight loss mainly through hypophagia and has little effect on energy expenditure. Ligands for Y receptors may be of benefit for the treatment of obesity, and there is a promising role of Y2 and Y4 receptor agonists in protecting against diet-induced obesity.

Intracerebroventricular administration of selective Y1 receptor agonist consistently shows orexigenic activity by stimulating food intake and inducing body weight gain. Several studies have shown that Y1 antagonists injected in hypothalamus reduce NPY-induced hyperphagia.[22] A newer antagonist, BMS-193885, shows significant ability to cross the brain–blood barrier and reduce food intake and body weight in rats without any adverse cardiovascular effects. This study highlights the possibility of Y1 antagonism as an antiobesity agent.[21] The peripheral administration of Y2 receptor agonist reduces food intake and promotes fat metabolism in wild-type mice. Blockade of Y2 receptor in the ARC with selective Y2 antagonist leads to increase feeding in satiated rats. This result is consistent with Y2 receptor tonically inhibiting NPY neurons. Thus, an Y2 antagonist would increase NPY neuronal activity resulting in an elevated release of both NPY and AgRP that stimulate food intake in satiated animals. Similar results have been found in targeted deletion of Y2 receptors in NPY neurons in a mouse model with increased food intake and body weight gain, heightened NPY expression, and reduced POMC expression. These results support the anorexigenic role for the Y2 receptor, with its loss leading to disinhibition of NPY neurons.[16,21] Pharmacological stimulation of Y5 receptor leads to potent increase in food intake in rats. This finding leads to the hypothesis that antagonism of this receptor may be a good target for antiobesity treatments. Two Y5 receptor antagonists have completed phase II clinical trials as antiobesity agents. Velneperit in combination with a low calorie diet causes significantly more weight loss than in control subjects, though the total amount is modest. Those treated with velneperit also show decreased waist circumference and improved serum lipid profile.[10] These studies indicate that Y5 antagonism in humans is well tolerated and may produce significant weight loss in obese populations. Numerous Y5 antagonists remain in development as antiobesity agents, with improved oral bioavailability, brain penetrance, and increased effects on food intake and body weight in obese animal models.[10,21] In mice, the Y4 receptor agonist BVD-74D was administered to C57BL/6 J mice that were fed with a normal or high-fat diet, and to fatty liver Shionogi (FLS)-ob/ob mice to clarify the role of the novel Y4 agonist in the regulation of food intake. The novel Y4 receptor agonist BVD-74D shows suppressive effects on food intake, water intake, and weight gain in normal mice fed with normal diets and on food intake in normal mice fed with high-fat diets and in FLS-ob/ob mice. These findings indicate that the Y4 receptor and its agonist would be promising targets for obesity.[23] 7TM Pharma (Horsholm, Denmark) has reported results from its clinical phase I/II study with the compound TM30338 and the planning of its phase II clinical study.[25] TM30338 is a dual NPY Y2–Y4 receptor agonist developed for the treatment of obesity and related diseases. According to 7TM Pharma, single subcutaneous doses of TM30338 administered to healthy obese subjects are safe and well tolerated. Food intake is suppressed compared with that of placebo both once a day and twice a day for multiple days. They also report their previous start on a preclinical development of a selective Y4 receptor agonist peptide TM30339, which has effects on reduction of food intake and weight loss, and their plan to initiate phase I/II studies.

CONCLUSIONS

NPY remains among the most potent orexigenic influences known. Multiple peptide and hormone systems from the periphery and from within the hypothalamus can affect NPY expression and release. It has been difficult to study the critical components of the NPY system because it is integrated within a network of densely redundant system. There are multiple high-affinity receptors for NPY that contribute to the persistence of NPY system over evolution. Given the complexity afforded by diverse peripheral and central energy balance signals and the intricate network

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of redundant NPY receptor systems, future studies of NPY physiology might reconsider the use of other techniques and more sophisticated genetic models. A better understanding the NPY-mediated feeding could provide an additional target for pharmaceutical or behavioral manipulations in the treatment or prevention of obesity.

REFERENCES

1. Allen YS, Adrian TE, Allen JM, Tatemoto K, Crow TJ, Bloom SR, et al. Neuropeptide Y distribution in the rat brain. *Science* 1983;**221**:877–9.
2. Bi S, Robinson BM, Moran TH. Acute food deprivation and chronic food restriction differentially affect hypothalamic NPY mRNA expression. *Am J Physiol Regul Integr Comp Physiol* 2003;**285**:R1030–6.
3. Bi S. Role of dorsomedial hypothalamic neuropeptide Y in energy homeostasis. *Peptides* 2007;**28**:352–6.
4. Chao PT, Yang L, Aja S, Moran TH, Bi S. Knockdown of NPY expression in the dorsomedial hypothalamus promotes development of brown adipocytes and prevents diet-induced obesity. *Cell Metab* 2011;**5**:573–83.
5. Chee MJ, Colmers WF. Y eat? *Nutrition* 2008;**24**:869–77.
6. Dailey MJ, Bartness TJ. Arcuate nucleus destruction does not block food-deprivation-induced increases in food foraging and hoarding. *Brain Res* 2001;**1323**:94–108.
7. Dailey MJ, Bartness TJ. Appetite and consummatory ingestive behaviors stimulated by PVH and perifornical area NPY injections. *Am J Physiol Regul Integr Comp Physiol* 2009;**296**:R877–92.
8. Dube MG, Sahu A, Kalra PS, Karla SP. Neuropeptide Y release is elevated from the microdissected paraventricular nucleus of food-deprived rats: an in vitro study. *Endocrinology* 1992;**2**:684–8.
9. Dube MG, Horvath TL, Kalra PS, Karla SP. Evidence of NPY Y5 receptor involvement in food intake elicited by orexin A in satiated rats. *Peptides* 2000;**10**:1557–60.
10. Erondu N, Gantz I, Musser B, Suryawanshi S, Mallick M, Addy C, et al. Neuropeptide Y5 receptor antagonism does not induce clinically meaningful weight loss in overweight and obese adults. *Cell Metab* 2006;**4**:275–82.
11. Eva C, Serra M, Mele P, Panzica G, Oberto A. Physiology and gene regulation of the brain NPY Y(1) receptor. *Front Neuroendocrinol* 2006;**27**:308–39.
12. Gardiger JV, Kong WM, Ward H, Murphy KG, Dhillo WS, Bloom SR. AAV mediated expression of antisense neuropeptide Y cRNA in the arcuate nucleus of rats results in decreased weight gain and food intake. *Biochem Biol Phys Res Commun* 2005;**4**:1088–93.
13. Hellstrom PM, Geliebter A, Naslund E, Schmidt PT. Peripheral and central signals in the control of eating in normal, obese and binge-eating human subjects. *Br J Nutr* 2004;**92**:S47–57.
14. Inui A. Neuropeptide Y feeding receptors: are multiple subtypes involved? *Trends Pharmacol Sci* 1999;**20**:43–6.
15. Inui A. Transgenic approach to the study of body weight regulation. *Pharmacol Rev* 2000;**52**:35–61.
16. Kaga T, Fujimiya M, Inui A. Emerging functions of neuropeptide Y Y(2) receptors in the brain. *Peptides* 2001;**22**:501–6.
17. Kaga T, Inui A, Okita M, Asakawa A, Ueno N, Kasuga M, et al. Modest overexpression of neuropeptide Y in the brain leads to obesity after high-sucrose feeding. *Diabetes* 2001;**50**:1206–10.
18. Kalra SP, Dube MG, Pu S, Xu B, Horvart TL, Karla PS. Interacting appetite-regulating pathways in the hypothalamic regulation of body weight. *Endocr Rev* 1999;**20**:68–100.
19. Kalra PS, Ueno N, Kalra SP. Stimulation of appetite by ghrelin is regulated by leptin restrain: peripheral and central sites of action. *J Nutr* 2005;**5**:1331–5.
20. Kamiji MM, Inui A. NPY Y2 and Y4 receptors selective ligands: promising anti-obesity drugs? *Curr Top Med Chem* 2007;**7**:1734–42.
21. Kamiji MM, Inui A. Neuropeptide Y receptor selective ligands in the treatment of obesity. *Endocr Rev* 2007;**28**:664–84.
22. Keen-Rhinehart E, Bartness TJ. NPY Y1 receptor is involved in ghrelin- and fasting-induced increases in foraging, food hoarding, and food intake. *Am J Physiol Regul Intregr Comp Physiol* 2007;**292**: R1728–37.
23. Li JB, Asakawa A, Terashi M, Cheng K, Chaolu H, Zoshiki T, et al. Regulatory effects of Y4 receptor agonist (BVD-74D) on food intake. *Peptides* 2010;**31**:1706–10.
24. McMinn JE, Baskin DG, Schwartz MW. Neuroendocrine mechanisms regulating food intake and body weight. *Obes Rev* 2000;**1**:37–46.
25. Melnikowa I, Wages D. Anti-obesity therapies. *Nat Rev Discov* 2006;**5**:369–70.
26. Mercer RE, Chee MJS, Colmers WF. The role of NPY in hypothalamic mediated food intake. *Front Neuroendocrinol* 2001;**32**:398–415.
27. Minor RK, Chang JW, de Cabo R. Hungry for life: how the arcuate nucleus and neuropeptide Y may play a critical role in mediating the benefits of calorie restriction. *Mol Cell Endocrinol* 2009;**299**:79–88.
28. Morton GJ, Cummings DE, Baskin DG, Barsh GS, Schwartz MW. Central nervous system control of food intake and body weight. *Nature* 2006;**443**:289–95.
29. Ohinata K, Takagi K, Biyajima K, Fujiwara Y, Fukumoto S, Eguchi N, et al. Central prostaglandin D(2) stimulates food intake via the neuropeptide Y system in mice. *FEBS Lett* 2008;**582**:679–84.
30. Ohinata K, Takagi K, Biyajima K, Kaneko K, Miyamoto C, Asakawa A, et al. Complement C5a stimulates food intake via a prostaglandin D(2)- and neuropeptide Y-dependent mechanism in mice. *Prostaglandins Other Lipid Mediat* 2009;**90**:81–4.
31. Pate HR, Qi Y, Hawkins EJ, Hileman SM, Elmquist JK, Imai Y, et al. Neuropeptide Y deficiency attenuates responses to fasting and high fat diet in obesity-prone mice. *Diabetes* 2006;**11**:3091–8.
32. Price CJ, Samson WK, Ferguson AV. Nesfatin-1 inhibits NPY neurons in the arcuate nucleus. *Brain Res* 2008;**1230**:99–106.
33. Salter-Venzon D, Watts AG. Site-specific attenuation of food intake but not the latency to eat after hypothalamic injections of neuropeptide Y in dehydrated-anorexic rats. *Am J Physiol Regul Integr Comp Physiol* 2009;**297**:R1813–21.
34. Williams G, Harrold JA, Cutler DJ. The hypothalamus and the regulation of energy homeostasis: lifting the lid on a black box. *Proc Nutr Soc* 2000;**59**:358–96.
35. Yang L, Scott KA, Hyun J, Tamashito N, Tray N, Moran TH, et al. Role of dorsomedial hypothalamic neuropeptide Y in modulating food intake and energy balance. *J Neurosci* 2009;**1**:179–90.

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Chapter 155

Opioids

Blake A. Gosnell, Catherine M. Kotz, Charles J. Billington and Allen S. Levine

ABSTRACT

Opioid agonists and antagonists cause increases and decreases, respectively, in food intake. Based on studies of taste and diet preferences, it seems that opioid peptides play a role in mediating palatability and/or the hedonic aspects of feeding. Studies with selective opioid ligands indicate a role for mu, delta, and kappa receptors, and microinjection studies have identified many brain areas in which opioids influence feeding. Opioid systems can be influenced by the intake of palatable foods and by changes in energy balance. The effects of several other feeding-related peptides may be influenced by or dependent on opioid activity. In humans, genetic variation in mu opioid receptors may explain some variability in preferences for palatable food, and pharmacological approaches that include opioid receptor antagonism may be useful in the treatment of obesity.

EFFECTS OF OPIOIDS ON FEEDING BEHAVIOR

Exogenous and endogenous opioids affect the intake of a variety of foods and liquids. Generally, opioid agonists increase food intake, whereas antagonists reduce intake. After decades of research into the role of opioids in feeding, a few general observations can be made. Agonists, particularly those acting on mu opioid receptors, seem to be particularly important in the hedonic or rewarding aspects of feeding. For example, injections of opioid agonists increase the intake of sweet solutions, and the opioid receptor antagonist naloxone decreases sucrose solution intake much more effectively than water intake. Similarly, agonists and antagonists more readily affect the intake of highly palatable food than that of standard laboratory chow. In taste-reactivity tests, which measure hedonic responses to tastes rather than actual intake, agonists increase, and antagonists decrease positive responses to palatable tastes. Importantly, opioid antagonism does not seem to affect taste perception. Finally, several studies with opioid antagonists suggest that opioids are more involved in the maintenance of feeding than in the initiation of feeding. These findings, based on laboratory animal studies, are generally supported in human studies on the effects of opioid antagonists. Details of many of these general findings can be found in a historical perspective on opioids and feeding,[4] a review of human studies with opioid antagonists,[32] and a more recent review with an emphasis on opioid involvement in food reward.[11] Also, see the Opioid Peptides section of this book.

Given the effectiveness of opioid antagonists in reducing the intake of palatable foods and fluids, Levine and colleagues examined whether continuous naltrexone (NTX) infusions would influence the preference for a high-sucrose diet (vs. a high-starch diet). Although NTX reduced total intake during the 10-day infusion period, preference for the sucrose diet was generally unaffected. However, if rats were given only the starch diet for a 10-day period before the start of NTX infusions, subsequent preference for the sucrose diet was decreased for the duration of the infusions and returned after termination of the infusions.[20] Although this study showed that NTX can affect the re-expression of an unlearned preference, it does not directly address the role of opioids in learned preferences. Sclafani et al. have conducted extensive research on the acquisition and expression of learned flavor preferences. In studies in which a flavor preference was conditioned based on its pairing to either the taste of sucrose or intragastric carbohydrate infusions, naltrexone did not significantly influence the acquisition or the expression of the preference. The authors suggest the possibility of a differential involvement of opioids in unlearned versus learned preferences.[28]

Although the effect of opioid agonists on the intake of sweet foods has been well documented, diet selection studies indicate a role for opioids in macronutrient selection. Taha[27] has written a detailed review and analysis of studies in which the intake and preference for diets differing in fat and carbohydrate content were measured after the administration of opioid agonists (primarily morphine and D-Ala^2, NMe^4, Glyol^5-enkephalin (DAMGO)) and antagonists (primarily

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00155-X
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naloxone and naltrexone). In most situations, agonists cause a preferential increase in fat intake. When several doses are tested, higher doses tend to have a more selective effect on fat intake than do the lower doses. This primary effect, however, is modulated to a degree by baseline diet preferences, such that agonists are less effective in stimulating fat intake in rats with a low baseline fat preference.[27] In contrast to evidence suggesting that opioids preferentially influence fat intake, injections of DAMGO into the ventromedial prefrontal cortex cause a preferential increase in the intake of a high carbohydrate.[23] This effect is observed in both fat-preferring and carbohydrate-preferring rats. Further testing of the effects of opioids injected into other brain sites should provide a clearer picture of the opioid influence on diet selection.

STUDIES FROM GENETIC MANIPULATIONS OR MUTATIONS

Relatively few studies have been conducted with feeding in animals with genetic modifications of the opioid system. In one study, mu-receptor-deficient CXBK mice preferred saccharin less than intact mice.[33] Mice lacking β-endorphin and enkephalin work less for food on a progressive ratio schedule than wildtype mice.[13] Also, mu opioid receptor knockout mice have diminished food-anticipatory activity compared with that of wildtype mice, as measured by schedule-induced running wheel activity before food intake.[15] Davis et al. published a psychogenetic analysis to determine whether variability in the human mu opioid receptor gene (OPRM1) is associated with variability in preference for sweet and fatty foods. In a study of 300 men and women, subjects with the G/G genotype of the functional A118G marker of this gene reported higher preferences for foods with high fat, high sugar, and high sugar and fat than did subjects with the GA and AA genotypes. At another marker of the OPMR1 gene, rs495491, subjects with the G/G genotype reported lower preferences for foods with high fat and high sugar and fat.[8] Thus, genetic manipulations and analyses support the results of pharmacological studies that indicate a role for opioids in mediating food palatability and reward.

FUNCTIONAL RESPONSE OF OPIOIDS TO METABOLIC AND FEEDING STATES

Levels of brain opioid peptides and their receptors can be affected by the intake of palatable foods and by food deprivation. Some of the many studies of feeding-related changes in gene expression have been summarized by Bodnar[4] in a historical perspective. Food deprivation causes an increase in mu opioid receptor (MOR) mRNA in the ventral medial hypothalamus and arcuate nucleus.[2] When a sweetened fat diet is available concurrently with chow, or is presented in an intermittent pattern that also includes intermittent food restriction, the levels of MOR mRNA are decreased in the nucleus of the solitary tract.[3] Similarly, in mice maintained on a high-fat diet, MOR mRNA levels are decreased in the ventral tegmental area (VTA), the nucleus accumbens (NAcc), and the prefrontal cortex. Levels of proenkephalin mRNA are increased in the NAcc, prefrontal cortex, and hypothalamus.[29]

It has been argued that under some conditions, sugar (particularly sucrose and glucose) can be addicting. Two lines of evidence supporting this possibility involve the presentation of sugar in a cyclic pattern that also involves cyclic access to standard laboratory chow. First, this regimen alters mu opioid receptor binding in several brain areas, including an increase in the nucleus accumbens. Second, after a nine-day period of cyclic presentation of chow and glucose, injections of the opioid antagonist naloxone cause somatic signs and neurochemical changes that resemble those observed during opioid withdrawal.[1]

OPIOID LIGANDS AND RECEPTORS: SITES OF ACTION AND NEURAL NETWORKS AFFECTED

Opioid peptides and receptors are distributed in many brain sites, including those known to affect reward and food intake.[18] Endogenous opioid peptides include β-endorphin, methionine- and leucine-enkephalin, dynorphins, and endomorphins. For research purposes, most studies use nonpeptide agonists (e.g., morphine) or peptides that have been modified for increased stability and/or receptor selectivity (e.g., DAMGO). Microinjection studies show that opioid agonists and antagonists can influence food intake when injected into many specific sites throughout the brain.[4,10] The use of selective ligands indicates a role for mu, delta, and kappa receptors. Interestingly, although, mu and kappa receptor activation typically produces opposing physiological effects, which suggests that these two types of opioid receptors contribute via different neural systems to the control of feeding.[30]

Kelley et al. measured Fos-like immunoreactivity to show that stimulation of DAMGO injection into the NAcc increases the intake of a high-fat diet and activated neurons in the lateral hypothalamus (LH), dorsomedial hypothalamic nucleus (DMH), medial preoptic area, supramammillary nucleus, VTA, substantia nigra, and the intermediate nucleus of the solitary tract (iNST).[34] The functional significance of activation of these areas is seen in a second study that combined injections of DAMGO into the NAcc with inactivation of several brain areas via an injection of muscimol, a GABAA agonist. Inactivation of the LH, DMH, VTA, or iNTS blocks feeding elicited by NAcc injections of DAMGO.[31] Thus, feeding produced by opioid activation of

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the NAcc seems to involve areas related to reward, energy balance, and autonomic function.

MacDonald et al. found that feeding induced by unilateral injection of DAMGO into VTA is dose dependently decreased by bilateral injections of the dopamine receptor 1 antagonist SCH 23390 into the NAcc.[22] When DAMGO is injected into NAcc, its orexigenic effects are diminished by SCH 23390 injected into VTA but not by the administration of a dopamine receptor 2 antagonist. Injections of SCH 23390 into NAcc inhibit feeding induced by intra-NAcc DAMGO but not by a delta-opioid agonist. Together, these results indicate the presence of an opioid–dopamine interaction important to feeding within NAcc and VTA. As noted above, opioid injections into several brain sites will produce feeding. By combining injections of DAMGO into one site and naltrexone into a second site, the connections between the paraventricular nucleus of the hypothalamus (PVN), central nucleus of the amygdala (CeA), NTS, NAcc, and VTA have been investigated. Pairs of sites tested include: CeA and NTS, PVN and VTA, NAcc and CeA, PVN and CeA, and NAcc and VTA. The effect was bidirectional in most sites because DAMGO injection in one site enhances feeding and opioid-receptor blockade in the corresponding site pair reduces DAMGO-induced feeding.[19] One exception occurs when DAMGO is injected into PVN and naltrexone in CeA, and vice versa.[9] In this situation, intra-PVN naltrexone blocks the intra-CeA DAMGO effect, but naltrexone injected into CeA (and DAMGO injected into PVN) is ineffective. Together, these studies indicate that feeding is regulated in part by a widely distributed complex central opioid network.

INTERACTIONS WITH OTHER NEUROCHEMICAL SYSTEMS

Opioid systems involved in the control of feeding seem to interact with many peptidergic systems, including neuropeptide Y (NPY) and α-melanocyte-stimulating hormone (α-MSH). Injection of naltrexone into the NTS or CeA decreases the food intake stimulated by injection of NPY into PVN.[17] However, there is only a minor effect of naltrexone on NPY-induced feeding when both compounds are injected into PVN. Naltrexone administered chronically (subcutaneously) via minipump increases NPY mRNA levels in the arcuate nucleus.[16] α-MSH is an agonist at melanocortin (MC) receptors, and agouti-related protein (AgRP) is an endogenous antagonist at MC-3/4 receptors. The combined blockade of mu- and kappa-opioid receptors prevents AgRP-induced feeding.[5] Similarly the long-term feeding (24–48 h) elicited by the SHU-9119, an MC-3/4 antagonist, is reduced by the mu opioid antagonist β-funaltrexamine.[12] In addition, β-endorphin-induced feeding is reduced by melanotan-II, an MC-3/4 agonist.[12]

Orexin neurons may also mediate reward-based feeding via an interaction with opioids. Feeding induced by orexin-A (OxA) is blocked by central opioid antagonist injections.[26] Peripheral naloxone also blocks OxA-induced feeding;[6] however, this effect results from an action on central pathways because naloxone methiodide, a form of naloxone that does not cross the blood–brain barrier, does not block OxA-induced eating.[26] The opioid–orexin interaction also seems to be reciprocal. The injection of an orexin antagonist into the ventral tegmental area blocks the hyperphagic effect of DAMGO injected into the NAcc, and intraaccumbens injection of DAMGO in orexin-deficient mice does not increase food intake.[35] More indirect evidence for orexin–opioid interactions also exists. Like opioids, OxA selectively enhances high-fat consumption when animals are given the choice of high- and low-fat diets.[6] However, also like opioids, when a low-fat diet is the only choice available, OxA enhances consumption.

Opioids may also mediate some of the effects of melanin-concentrating hormone (MCH) on food intake. Injections of MCH into the lateral ventricle increased food intake and the hedonic response to sucrose (taste reactivity); preinjection with selective mu, delta or kappa-opioid antagonists blocks or blunts these effects.[21] A previous report[6] found no effect of systemic naloxone on food intake induced by injections of MCH into the third ventricle and it was suggested that it may be related to the route of injection or nonspecific nature of naloxone.[21]

As mentioned above, it is believed that opioids are involved in maintaining rather than in initiating ingestion. One possible mechanism is via an interaction with the oxytocin (OT) system. OT has been shown to decrease food intake, and OT neuronal activation (as measured by double immunostaining for OT and cFos) is greater at a time associated with the termination of feeding than at a time associated with the initiation of feeding. Treatment with the opioid agonist butorphanol, which causes an increase in food intake, causes a decrease in oxytocin neuronal activation.[24]

Finally, the endogenous cannabinoid system, like the opioid system, is believed to play a role in mediating food reward. The primary evidence supporting an interaction comes from studies that show that combined opioid and cannabinoid antagonists decrease food intake at doses that are ineffective when tested individually.[7] Collectively, the results summarized in this section indicate that opioids interact with a number of broadly distributed neuroregulators that alter feeding.

PHYSIOLOGICAL AND PATHOPHYSIOLOGICAL IMPLICATIONS

Opioids are synthesized in the central and peripheral nervous system and affect both physiology and behavior. They

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involve processes such as learning and memory; nociception; stress and immunological responses; and intestinal, renal, and hepatic functions. The wide distribution of opioids, the variety of receptor subtypes, and involvement in so many other systems suggest that in addition to effects on palatability and food reward, opioids may also indirectly influence feeding via effects on these systems.

The clear demonstration of the effects of opioid compounds on feeding in laboratory animals has led to the efforts to determine whether similar effects are observed in humans. The antagonists naloxone and naltrexone have been found to reduce food intake in normal-weight and obese humans, and reduced bingeing behavior in subjects with bulimia.[32] As has been observed with rats, the antagonists generally reduced food pleasantness but not taste perception. However, opioid antagonists alone have not proven to be effective long-term treatments for obesity, because of side effects and limited weight loss. In one study, functional magnetic resonance imaging was used to evaluate the effects of GSK1521498, a compound that acts as a selective antagonist at mu opioid receptors and in some situations, acts as an inverse agonist. In the open label study of healthy adult males, scans were performed after oral administration of a palatable liquid food or a neutral stimulus. An oral dose of GSK1521498 causes a significant attenuation of the food-induced fMRI activation of the amygdala. This effect is not observed after an oral dose of naltrexone.[25] The development of more selective opioid compounds, therefore, may lead to an effective long-term treatment of obesity. Given the complex neurochemical networks involved in the control of feeding and energy balance, it is also possible that the combination of opioid antagonism with a second drug may offer a better weight loss treatment. For example, the combination of naltrexone and bupropion (a dopamine/norepinephrine reuptake inhibitor) can cause significant weight loss.[14]

CONCLUSIONS

Strong evidence implicates opioids in the control of food intake. Behavioral, neurochemical, and genetic studies suggest that opioids are particularly involved in mediating the hedonic or rewarding properties of food. Opioids participate in a network of areas throughout the brain and interact with many other neurochemically defined systems in areas generally associated with homeostatic, reward, and autonomic function. In short-term studies in humans, opioid antagonists cause a reduction in food intake. However, the usefulness of opioid antagonists in the long-term treatment of obesity will probably depend on the development of more selective antagonists and/or their use in combination with drugs acting on other neurochemical systems.

REFERENCES

1. Avena NM, Rada P, Hoebel BG. Evidence for sugar addiction: behavioral and neurochemical effects of intermittent, excessive sugar intake. *Neurosci Biobehav Rev* 2007;**32**:20–39.
2. Barnes MJ, Primeaux SD, Bray GA. Food deprivation increases the mRNA expression of μ-opioid receptors in the ventral medial hypothalamus and the arcuate nucleus. *Am J Physiol Regul Integr Comp Physiol* 2008;**295**:R1385–90.
3. Bello NT, Patinkin ZW, Moran TH. Opioidergic consequences of dietary-induced binge eating. *Physiol Behav* 2011;**104**:98–104.
4. Bodnar RJ. Endogenous opioids and feeding behavior: a 30-year historical perspective. *Peptides* 2004;**25**:697–725.
5. Brugman S, Clegg DJ, Woods SC, Seeley RJ. Combined blockade of both μ- and κ-opioid receptors prevents the acute orexigenic action of Agouti-related protein. *Endocrinology* 2002;**143**:4265–70.
6. Clegg DJ, Air EL, Woods SC, Seeley RJ. Eating elicited by orexin-a, but not melanin-concentrating hormone, is opioid mediated. *Endocrinology* 2002;**143**:2995–3000.
7. Cota D, Tschop MH, Horvath TL, Levine AS. Cannabinoids, opioids and eating behavior: the molecular face of hedonism? *Brain Res Rev* 2006;**51**:85–107.
8. Davis C, Zai C, Levitan RD, Kaplan AS, Carter JC, Reid-Westoby C, et al. Opiates, overeating and obesity: a psychogenetic analysis. *Int J Obesity* 2011;**35**:1347–54.
9. Giraudo SQ, Billington CJ, Levine AS. Effects of the opioid antagonist naltrexone on feeding induced by DAMGO in the central nucleus of the amygdala and in the paraventricular nucleus in the rat. *Brain Res* 1998;**782**:18–23.
10. Gosnell BA, Levine AS. Stimulation of ingestive behavior by preferential and selective opioid agonists. In: Cooper SJ, Clifton PG, editors. *Drug receptor subtypes and ingestive behavior*. San Diego, CA: Academic Press; 1996. p. 147–66.
11. Gosnell BA, Levine AS. Reward systems and food intake: role of opioids. *Int J Obesity* 2009;**33**:S54–8.
12. Grossman HC, Hadjimarkou MM, Silva RM, Giraudo SQ, Bodnar RJ. Interrelationships between μ opioid and melanocortin receptors in mediating food intake in rats. *Brain Res* 2003;**991**:240–4.
13. Hayward MD, Pintar JE, Low MJ. Selective reward deficit in mice lacking beta-endorphin and enkephalin. *J Neurosci* 2002;**22**:8251–8.
14. Ioannides-Demos LL, Piccenna L, McNeil JJ. Pharmacotherapies for obesity: past, current, and future therapies. *J Obes* 2011;**2011**:179674.
15. Kas MJ, van den Bos R, Baars AM, Lubbers M, Lesscher HM, Hillebrand JJ, et al. Mu-opioid receptor knockout mice show diminished food-anticipatory activity. *Eur J Neurosci* 2004;**20**:1624–32.
16. Kotz CM, Grace MK, Briggs JE, Billington CJ, Levine AS. Naltrexone induces arcuate nucleus neuropeptide Y gene expression in the rat. *Am J Physiol* 1996;**271**:R289–94.
17. Kotz CM, Grace MK, Briggs J, Levine AS, Billington CJ. Effects of opioid antagonists naloxone and naltrexone on neuropeptide Y-induced feeding and brown fat thermogenesis in the rat. Neural site of action. *J Clin Invest* 1995;**96**:163–70.
18. Le Merrer J, Becker JA, Befort K, Kieffer BL. Reward processing by the opioid system in the brain. *Physiol Rev* 2009;**89**:1379–412.
19. Levine AS. The animal model in food intake regulation: examples from the opioid literature. *Physiol Behav* 2006;**89**:92–6.

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20. Levine AS, Grace MK, Cleary JP, Billington CJ. Naltrexone infusion inhibits the development of preference for a high-sucrose diet. *Am J Physiol Regul Integr Comp Physiol* 2002;**283**:R1149–54.
21. Lopez CA, Guesdon B, Baraboi ED, Monge Roffarello B, Hetu M, Richard D. Involvement of the opioid system in the orexigenic and hedonic effects of melanin-concentrating hormone. *Am J Physiol Regul Integr Comp Physiol* 2011;**301**:R1105–11.
22. MacDonald AF, Billington CJ, Levine AS. Alterations in food intake by opioid and dopamine signaling pathways between the ventral tegmental area and the shell of the nucleus accumbens. *Brain Res* 2004;**1018**:78–85.
23. Mena JD, Sadeghian Baldo BA. Induction of hyperphagia and carbohydrate intake by mu-opioid receptor stimulation in circumscribed regions of frontal cortex. *J Neurosci* 2011;**31**:3249–60.
24. Olszewski PK, Levine AS. Central opioids and consumption of sweet tastants: when reward outweighs homeostasis. *Physiol Behav* 2007;**91**:506–12.
25. Rabiner EA, Beaver J, Makwana A, Searle G, Long C, Nathan PJ, et al. Pharmacological differentiation of opioid receptor antagonists by molecular and functional imaging of target occupancy and food reward-related brain activation in humans. *Mol Psychiatry* 2011;**16**:826–35.
26. Sweet DC, Levine AS, Kotz CM. Functional opioid pathways are necessary for hypocretin-1 (orexin-A)-induced feeding. *Peptides* 2004;**25**:307–14.
27. Taha SA. Preference or fat? Revisiting opioid effects on food intake. *Physiol Behav* 2010;**100**:429–37.
28. Touzani K, Bodnar RJ, Sclafani A. Neuropharmacology of learned flavor preferences. *Pharmacol Biochem Behav* 2010;**97**:55–62.
29. Vucetic Z, Kimmel J, Reyes TM. Chronic high-fat diet drives postnatal epigenetic regulation of mu-opioid receptor in the brain. *Neuropsychopharmacol* 2011;**36**:1199–206.
30. Wee S, Koob GF. The role of the dynorphin-κ opioid system in the reinforcing effects of drugs of abuse. *Psychopharmacology* 2010;**210**:121–35.
31. Will MJ, Franzblau EB, Kelley AE. Nucleus accumbens μ-opioids regulate intake of a high-fat diet via activation of a distributed brain network. *J Neurosci* 2003;**23**:2882–8.
32. Yeomans MR, Gray RW. Opioid peptides and the control of human ingestive behavior. *Neurosci Biobehav Rev* 2002;**26**:713–28.
33. Yirmiya R, Lieblich I, Liebeskind JC. Reduced saccharin preference in CXBK (opioid receptor-deficient) mice. *Brain Res* 1988;**438**:339–42.
34. Zhang M, Kelley AE. Enhanced intake of high-fat food following striatal mu-opioid stimulation: microinjection mapping and Fos expression. *Neuroscience* 2000;**99**:267–77.
35. Zheng H, Patterson LM, Berthoud HR. Orexin signaling in the ventral tegmental area is required for high-fat appetite induced by opioid stimulation of the nucleus accumbens. *J Neurosci* 2007;**27**:11,075–82.

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Chapter 156

Orexin

Jon F. Davis and Stephen C. Benoit

ABSTRACT

Orexigenic hypothalamic peptides have been studied extensively for their ability to regulate feeding behavior. The traditional theory regarding orexigenic peptide function suggested that orexigenic peptides produced within the mediobasal hypothalamus exert their effects on feeding through local hypothalamic centers, including the lateral hypothalamus. However, recent reports indicate that orexigenic peptides are also capable of regulating food intake through their actions at extrahypothalamic centers, including endogenous reward circuitry. This observation expands the complexity of feeding behavior and indicates that feeding can be regulated by extra-hypothalamic reward centers. Here, we review work on the hypothalamic orexin system, its functional–anatomical importance, as well as the ability of this system to interact and modulate mesolimbic reward circuitry with respect to food intake. In addition, this chapter will detail the ability of central effector signals that regulate feeding behavior to impact orexin-induced feeding behavior.

INTRODUCTION

The primary focus of this chapter is to review how orexin peptides influence feeding behavior. In particular, this chapter will focus on how orexin peptides produced in the lateral hypothalamus (LH) interact within and outside hypothalamic nuclei to regulate feeding. The traditional contention regarding food intake suggests that peptides capable of influencing feeding behavior exerted their effects primarily by acting within local hypothalamic centers, with a major emphasis on the LH. It is now recognized that orexin acts within extra-hypothalamic centers, including the brain's endogenous reward circuitry, to modulate food intake. This chapter will review studies that detail orexin's input to these areas. Additionally, it has become clear that a variety of centrally and peripherally produced signals are capable of influencing feeding behavior. Here, the ability of such factors will be discussed in relation to their ability to influence orexin function and orexin-induced increases in feeding behavior. Finally, the ability of metabolic status and aspects associated with this parameter, for example, obesity and caloric restriction will be examined regarding the regulation of orexin-induced feeding. To begin our discussion of how this peptide regulates feeding behavior, it is first important to describe the history and origin of orexin.

OREXIN

Orexins (also called hypocretins) are neurotransmitters produced in small neuronal populations within the lateral (LH) and perifornical (PFA) areas of the hypothalamus. The name orexin originated from the Greek root word for appetite, orexis.[27] Orexin peptides are known to regulate arousal, wakefulness, food intake, and reward-related behaviors through their actions in a disturbed set of brain nuclei (for a review, please see the Hypocretin/Orexin chapter in Brain Peptides section of the book). Orexin peptides exist in two forms, both produced from the cleavage of preproorexin: orexin-A (33 amino acids) and orexin-B (28 amino acids). Orexin-A can bind to both orexin-1 receptor (O×1R) and, with lower affinity, orexin-2 receptor (O×2R) while orexin-B has preferential binding affinity for O×2R. Both orexin receptors are G protein-coupled receptor subtypes[27] and are widely distributed throughout the central nervous system.[20] Due to the lack of an effective and commercially available O×2R antagonist, orexin-A signaling on O×1R has been more widely studied and is much better characterized.

Orexin neurons receive reciprocal connections with mediobasal hypothalamic structures implicated in the control of food intake. In particular, orexin-producing neurons in the LH receive input from neuropeptide Y-/agouti-related peptide-expressing neurons of the arcuate nucleus leading to the contention that LH orexin neurons are 'second-order' neurons in the integration processes involved in promoting food intake.[31] However, other evidence suggests that orexin neurons may serve as "first-order" neurons. In this capacity, they are sensors of metabolic status, that are directly modulated by circulating factors such as leptin, glucose, and ghrelin.[3,4,27,28] Orexin neurons probably take on both first- and second-order properties during the complex integrations of neuropeptide and adipostatic signals which exert counter regulatory effects on feeding behavior (i.e. those that increase and decrease food intake). In terms of feeding behavior, numerous reports have described the orexigenic properties of orexin-A on food intake. For example,

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00156-1
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intracerebroventricular (icv) administration of orexin-A increases food intake in rodents.[27] When given a choice, orexin administration in rats will selectively increase intake of preferred diets and more specifically diets that are high in saturated fat.[7] Further, pharmacological antagonism of the orexin-1 receptor is effective in blocking orexin-A-induced hyperphagia and behavioral satiety.[26]

LATERAL HYPOTHALAMUS

As mentioned above, orexin neurons originate in the LH of the hypothalamus and reach many areas of the CNS. The LH has been studied for many years for its ability to regulate "brain stimulation reward" or (BSR). In this model, rats will actively seek out stimulation of the LH[24] and early studies investigating the effects of metabolic factors on BSR indicated that factors that modulate food intake also regulate BSR. More than 20 years after this discovery, orexins were discovered in the LH, and soon after, it was shown that orexin-A possesses the ability to regulate a variety of reward behaviors (for reference please see the Hypocretin/Orexin chapter in Brain Peptides section of the book). These demonstrations were particularly impressive in that (1) they established that a neuropeptide produced in the LH was capable of regulating reward behaviors, thus providing a potential mechanism for BSR and (2) raised the possibility that orexin may be capable of regulating food reward.

MESOLIMBIC SYSTEM

Rewarding behaviors including food reward contain three basic functional components: (1) they induce learning, (2) they produce approach and consummatory behavior, and (3) they induce positive emotions regarding the behavior. Thus, reward and learning overlap to produce optimal conditions for obtaining a particular goal/reward. In rodents, rewarding behaviors are controlled by the mesocorticolimbic system which originates in the ventral mesencepahalon or ventral tegmental area (VTA). Dopaminergic fibers originating in the VTA send dense projections to the nucleus accumbens (Nacc) and the medial wall of the Prefrontal Cortex (mPFC). The projection of dopamine neurons from the VTA to Nacc constitutes the mesolimbic component of this system and the dopamine projection from the VTA to the mPFC make up the mesocortical component of this system. The mPFC along with receiving dopamine input from the VTA sends glutamatergic projections both back to the VTA and to the Nacc thus allowing a cortical interface to be superimposed onto rewarding behaviors. Although it is not the primary focus of this chapter, it should be noted that manipulations of orexin signaling within the mesolimbic system affect many aspects of reward behavior including self administration, relapse, and withdrawal from abused drugs.

OREXIN AND MESOLIMBIC SYSTEM

In terms of orexin regulation of food reward, studies suggest that orexin acts within mesolimbic structures to regulate reward-related appetitive behaviors. For example, orexin neurons in the LH send direct projections to the VTA in the midbrain where dopamine is produced[8] and VTA neurons express both orexin receptor subtypes.[20] The first demonstration that this orexin-VTA connection is physiologically relevant came from investigations of drug withdrawal. These findings suggested that neuroadaptations occurring secondary to psychostimulant exposure are regulated by orexin input into the VTA.[5] Indeed, orexinergic projections to the VTA signal specifically on a majority of dopamine neurons, increase dopaminergic neuron firing rates and activate the mesolimbic pathway.[23] Drug-associated cues activate orexin neurons, and central application of orexin-A reinstates drug-seeking behavior.[12] Moreover, orexin is critical for learning reward–stimulus associations as VTA O×1R antagonism attenuates acquisition of morphine conditioned place preference.[23] These associations are probably mediated by long-term plastic changes occurring within VTA neurons. When an O×1R antagonist is administered into the VTA, behavioral sensitization and neurophysiological changes that are typically associated with chronic cocaine use are blocked.[2] Similar results have been shown with other substances of abuse using O×1R blockade to attenuate operant responding for alcohol and the reinstatement of alcohol- and cocaine-seeking.[14] Further, studies that examine reproductive behavior indicate that orexin regulates the rewarding aspects of male sexual reward.[22]

Orexin has also been studied for its ability to regulate food reward and related behaviors. Orexin-A regulates operant responding for palatable food rewards.[2] Importantly, a seminal set of studies by Zheng and colleagues established that orexin-A action within the mesolimbic system, is necessary for opioid-induced high fat intake.[33] Together, these behavioral studies were the first to provide evidence that orexin has a functional role in the regulation of food reward through its actions within the mesolimbic system. These findings have been extended by examination of the propensity of orexin-A to modulate a variety of forms of food reward; specifically progressive ratio responding for food rewards and reward-based feeding. A progressive ratio (PR) schedule of operant responding, as used in this study, is considered a robust test of motivation to obtain rewards as the animal must work progressively harder for each subsequent food reward. When orexin-A is delivered centrally PR responding for sucrose is augmented. By contrast, blockade of orexin signaling with a pharmacological antagonist specific to the OR×1R reduces PR responding for chocolate.[6] These results suggest that the influence of orexin on feeding may occur through modulation of cognitive and emotional processes such as

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motivation to obtain food. Feeding beyond homeostatic need, sometimes termed "nonhomeostatic feeding" is regulated by the palatability of a particular food and has been hypothesized to be a major contributor to the current increase in obesity. This behavior can be modeled by allowing calorically sated rats to feed selectively from a high fat butter diet. In this way, high fat feeding is driven by unconditioned endogenous regulation of reward-based feeding in contrast to drug-induced overconsumption of food which is commonly used. This behavioral paradigm was developed to model feeding in humans that occurs independent of caloric need. The data we collected using this paradigm indicate that blockade of orexin-A signaling is sufficient to reduce overfeeding from high fat diet under the condition of satiation.[6] The interpretation of these results is that, similar to conditioned responding for food rewards, orexin-A is also capable of regulating unconditioned reward-based high fat feeding. Collectively, these results further support a role for orexin in the modulation of cognitive-emotional processes, which orient an organism to food.

In support of this notion, the orexin system is activated in anticipation of a variety of rewards. In particular, the expectancy of morphine or sweet flavored cereal activates LH orexin neurons.[12] Separate studies have extended these findings by examining the ability of food rewards and restricted feeding to activate LH orexin neurons. Expectation of a chocolate food reward induces neuronal activation within LH orexin neurons and several brain regions implicated in the control of feeding and reward behavior. Importantly, rats trained to expect their daily ration of standard rodent chow also display increased c-Fos in LH orexin neurons. Thus, LH orexin neurons seem to be sensitive to either the expectation of food reward (chocolate) or feeding in general (chow) suggesting that cues associated with not only palatable food but also the anticipation of daily feeding activates orexin-A neurons.[6] One possible reason for this result may lie in the specific activation of other PFA-LH neuropeptide systems, such as melanin-concentrating hormone (see the MCH chapter in Brain Peptides section) also known to be involved in the regulation of feeding and reward-related behaviors. The increased activation of orexin-A expressing neurons under these conditions was further characterized to be selectively significant in the PFA, but not the LH region of the orexin field. This distinction is consistent with a previous results indicating that pharmacologically induced high fat feeding specifically activates PFA orexin neurons.[33] This is in contrast to that observed after expectation of drug-associated cues and may stem from the caloric value of food vs. drugs that contain no calories. It is also possible that the brains endogenous stress system may modulate LH or PFA orexin neurons differentially depending on the level of experience the organism has with the particular reinforcer. Moreover, it has been suggested that nonspecific activation of PFA orexin neurons may be indicative of heightened general arousal (please refer to the Hypocretin/Orexin chapter for more detail). A conditioned meal-feed response to limited chow access or a conditioned reward-based feeding response both probably require a learned increase in general arousal to maximize the animal's ability to benefit from such conditions. Based on these and other findings, the ability for PFA orexin to signal arousal-based activity to selectively act on reward processing may depend more on the specific sites of action (target regions) as opposed to the origin (PFA orexin activation).

OREXIN TARGETS

Identification of neuronal activation within known targets of the orexin system is achieved through visualization of c-Fos in neurons that also express the orexin-1 receptor (OR×1R). Rats trained to expect chocolate display increased c-Fos within the LH, PFA, VTA, mPFC NAcc, and paraventricular thalamic nucleus (PVT). Increased c-Fos immunolabeling in the PFA-LH, PVT, mPFC, and VTA of rats expecting chocolate is consistent with several similar reports investigating neuronal activation in rodents expecting food or drug reinforcers.[12] It is notable that expectancy of chocolate produced the most robust c-Fos response and in particular within the PVT neurons that express OR×1R.[6] The PVT receives a dense orexinergic projection from the LH orexin field, and these data indicate that during times of food reward expectancy, orexin-A targets OR×1R neurons to activate the PVT.

OREXIN AND THE PARAVENTRICULAR THALAMIC NUCLEUS

The paraventricular nucleus of the thalamus (PVT), a midline thalamic nucleus where thalamocortical projections originate, is most closely linked to attention and arousal. The PVT receives dense inputs from LH orexinergic fibers and expresses orexin receptors. Orexin can synapse within the midline thalamus to directly activate cortical-projecting PVT excitatory neurons as another mechanism for which orexin, sensitive to physiological and visceral information can enhance arousal and attentional tone. Studies showing that the PVT mediates reinstatement of extinguished alcohol-seeking and conditioned place aversion during morphine withdrawal[10] have established the possibility that the PVT is also involved in reward-related behaviors. In addition, excitatory PVT projections to the nucleus accumbens (NAcc), a major component of the mesolimbic reward circuit, occur on dopaminergic terminals and increase dopamine efflux independent of VTA function. Several recent studies have also implicated PVT orexin in affecting emotionality and addiction behaviors.[16,17] Taken together, these studies strongly support a role for orexin as a modulator of attention and arousal, to activate the necessary alertness required to promote and enhance goal-oriented, specifically reward-related, behaviors.

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OREXIN AND METABOLIC STATUS

A central theme in the study of ingestive ascribes that factors produced in relation to an organism's state of energy balance (i.e. fed vs. fasted states) travel through the circulation to act centrally on hypothalamic structures to regulate food intake and energy expenditure. Specifically, leptin produced from adipocytes and insulin produced by the pancreas act within key hypothalamic structures contained in the mediobasal hypothalamus to negatively regulate food intake. Conversely, the stomach derived hormone ghrelin also acts within the hypothalamus to increase food intake in times of negative energy balance. One study in obese human patients reported that orexin-A in plasma is lower in obese patients relative to lean controls.[1] Earlier studies examining this phenomenon in rodents used a different approach to assay the central distribution of orexin peptide. In these studies, the central distribution of orexins in the hypothalamus and neighboring areas is higher in obese rats relative to lean controls.[21] The obese rodents used in these studies lacked functional leptin receptors. As leptin is known to negatively regulate orexin expression,[18] increased orexin expression in rodents that lack the ability to sense leptin fits with the conceptual framework regarding metabolic influence of orexin. These results indicate that in the context of disrupted leptin signaling, such as obesity, central orexin tone is increased. In terms of obesity, triglycerides and dietary fat have been reported to stimulate orexin tone.[30] A similar set of studies investigating orexin tone in hypoglycemic rats indicates that orexin-B levels in the hypothalamus are 10-fold higher than in control rats.[4] Together these studies indicate that orexin output is responsive to metabolic status. Reductionist approaches are often used to discern function of a particular gene or peptide. Many times, genetic knockout or ablation techniques are used to generate mutant mice to gain a better understanding a particular genes function. Genetic ablation of orexin neurons in the LH results in hyperphagia and obesity.[11] The interpretation of this result was that central orexin neurons represent a key node for counterregulatory mechanisms to maintain stable energy homeostasis. Thus, although metabolic status is capable of influencing orexin tone, orexin is also a critical factor regulating energy homeostasis.

OREXIN REGULATION BY FEEDING PEPTIDES

Melanocortins

Over the past two decades, factors that act on hypothalamic structures to regulate feeding behavior have continued to emerge. The melanocortin system has been studied extensively for its role in the regulation of food intake. This system is unique in that it possesses both an endogenous agonist and an inverse agonist with opposite effects on feeding behavior. Alpha-melanocyte stimulating hormone (α-MSH) and agouti-related peptide (AgRP) are the primary effector peptides of the melanocortin system (see the Melanocortin/AgRP chapters in Brain Peptides and Ingestive Peptides sections of the book). Increased α-MSH release decreases food intake, whereas increased AgRP drives food consumption. Under basal conditions, the expression of each peptide is regulated by caloric status as downstream targets of the adiposity hormone leptin. A unique feature of melanocortin-induced feeding is the duration in which feeding behavior is affected. When administered centrally, AgRP increases food intake each time an animal takes a meal for a period of five to seven days. The nature of this effect presumes that other neural systems become activated to maintain this increased food intake. In support of this idea, LH orexin neurons become activated 23 hours after AgRP injection[32] indicating that the long lasting effects of AgRP on food intake are correlated with increased orexin tone. In contrast, orexin-A stimulates electrical activity in AgRP neurons that reside in the mediobasal hypothalamus.[29] These results indicate that orexin-induced activation of AgRP neurons is critical for the release of AgRP and the maintenance of long-term energy balance. AgRP neurons also express neuropeptide Y (NPY), a potent inducer of feeding behavior. The increased feeding response elicited by orexin-A is inhibited by pharmacological antagonism of NPY receptors.[13] Thus, NPY seems to be a critical downstream target for orexin-induced feeding. These results describe a reciprocal role regarding the relationship of orexin and AgRP/NPY; the nature of this activation and its subsequent effects on feeding behavior require further investigation.

Ghrelin

Ghrelin is a feeding-related hormone derived in the stomach involved in the homeostatic and hedonic regulation of food intake[25] (see the Ghrelin chapters in several sections of the book). Ghrelin concentrations peak before expected meals, and when administered centrally, ghrelin induces food intake. Ghrelin receptors are present in the LH and when ghrelin is administered centrally LH orexin neurons become activated.[15] The appetite stimulatory effect of ghrelin is suppressed in mice that lack orexin[28] indicating that orexin mediates the effects of ghrelin on food intake. In the context of food reward, mice that form a place preference to high fat diet display elevated c-Fos expression in orexin neurons.[25] Further, ghrelin's ability to stimulate operant performance for high fat diet is attenuated in orexin-deficient mice.[25] Together these data indicate that ghrelin's ability to modulate feeding and food reward behaviors is mediated in part by the LH orexin system.

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Leptin

Leptin is an adipocyte-derived hormone that acts centrally to reduce food intake (see the Leptin chapters in Brain, Endocrine, Ingestive, Gastrointestinal, and Cancer/Anticancer Peptides sections of the book). Leptin acts within the mediobasal and lateral hypothalamus to attenuate food intake. Leptin receptors are expressed in LH orexin neurons,[9] and when leptin is administered systemically, orexin neurons contain P-STAT3 the downstream marker of leptin receptor activation. Early studies investigating the effects of leptin on orexin expression reported that central (third ventricular) administration of leptin attenuated fasting-induced increases in preproorexin.[18] In addition, leptin treatment reduces expression of hypothalamic OR×1R.[18] However, more recent evidence indicates that systemic or direct application of leptin into the LH increases orexin mRNA.[19] The discrepancy in the directionality of orexin expression after leptin administration could be accounted for by the model system used (e.g. rats vs. mice) or the time period in which orexin expression was examined. In the context of counterregulation of feeding behavior, decreases in orexin secondary to leptin administration are in accord with appetite suppressant activity of leptin. More studies are required to determine the exact nature and directionality of leptin's effects on LH orexin expression.

Summary

From this collection of results, several themes emerge. It is clear that orexin can induce feeding behavior. The nature of this effect indicates that orexin may achieve this induction through its actions on mesolimbic reward circuits. Orexin receptors are expressed within neurocircuits that regulate brain dopamine and reward function. The orexin system along with the regions targeted by orexin is activated during anticipation of food rewards and feeding in general. In the context of feeding behavior, orexin seems to facilitate food intake primarily by modulating the motivation to consume food. Given that brain reward regions overlap with homeostatic control circuits in the control of ingestive behavior, it is not surprising that a centrally produced factor such as orexin is capable of influencing feeding behavior through its actions in both systems. Metabolic status, a potent modulator of food intake regulates the effects of orexin on food intake. More specifically, factors that regulate energy homeostasis to exert their influence on food intake do so by modulating orexin function. Overall, these results highlight the propensity of orexin to regulate food intake, neurobiological responses associated with feeding, and the motivation to obtain food rewards. As all these aspects of biology are associated with obesity, these results also underscore the utility of orexin as a laboratory tool to understand better the neurobiology of obesity.

REFERENCES

1. Adam J, Menheere P, van Dielen F, Soeters P, Buurman W, Greve J. Decreased plasma orexin-A levels in obese individuals. *In J Obs* 2002;**26**:274–6.
2. Borgland SL, Chang SJ, Bowers MS, Thompson JL, Vittoz N, Floresco SB, et al. Orexin A/hypocretin-1 selectively promotes motivation for positive reinforcers. *J Neurosci* 2009;**29**:11215–25.
3. Burdakov D, Gerasimenko O, Verkhratsky A. Physiological changes in glucose differentially modulate the excitability of hypothalamic melanin-concentrating hormone and orexin neurons in situ. *J Neurosci* 2005;**25**:2429–33.
4. Cai XJ, Widdowson PS, Harrold J, Wilson S, Buckingham RE, Arch JR, et al. Hypothalamic orexin expression: modulation by blood glucose and feeding. *Diabetes* 1999;**48**:2132–7.
5. Carr D, Kalivas PW. Orexin: a gatekeeper of addiction. *Nat Med* 2006;**12**:274–6.
6. Choi DL, Davis JF, Fitzgerald ME, Benoit SC. The role of orexin-A in food motivation, reward-based feeding behavior and food-induced neuronal activation in rats. *Neuroscience* 2010;**167**:11–20.
7. Clegg DJ, Air EL, Woods SC, Seeley RJ. Eating elicited by orexin-a, but not melanin-concentrating hormone, is opioid mediated. *Endocrinology* 2002;**143**:2995–3000.
8. Fadel J, Deutch AY. Anatomical substrates of orexin-dopamine interactions: lateral hypothalamic projections to the ventral tegmental area. *Neuroscience* 2002;**111**:379–87.
9. Hakansson M, de Lecea L, Sutcliffe J, Yanagisawa M, Meister B. Leptin receptor and STAT-3 immunoreactivities in hypocretin/orexin neurones of the lateral hypothalamus. *J Neuroendocrin* 1999;**11**:653–63.
10. Hamlin AS, Clemens KJ, Choi EA, McNally GP. Paraventricular thalamus mediates context-induced reinstatement (renewal) of extinguished reward seeking. *Eur J Neurosci* 2009;**29**:802–12.
11. Hara J, Beuckmann C, Nambu T, Willie J, Chemelli R, Sinton C, et al. Genetic ablation of orexin neurons in mice results in narcolepsy, hypophagia and obesity. *Neuron* 2001;**30**:345–54.
12. Harris GC, Wimmer M, Aston-Jones G. A role for lateral hypothalamic orexin neurons in reward seeking. *Nature* 2005;**437**:556–9.
13. Ida T, Nakahara K, Kuroiwa T, Fukui K, Nakazato M, Murakami T, et al. Both corticotrophin releasing factor and neuropeptide Y are involved in the effect of orexin (hypocretin) on the food intake in rats. *Neurosci Lett* 2000;**293**:119–22.
14. Lawrence AJ, Cowen MS, Yang HJ, Chen F, Oldfield B. The orexin system regulates alcohol-seeking in rats. *Br J Pharmacol* 2006;**148**:752–9.
15. Lawrence C, Snape A, Baudoin F, Luckman S. Acute central ghrelin and GH Secretagogues induce feeding and activate brain appetite centers. *Endocrinology* 2003;**143**:155–62.
16. Li Y, Li S, Wei C, Wang H, Sui N, Kirouac GJ. Orexins in the paraventricular nucleus of the thalamus mediate anxiety-like responses in rats. *Psychopharmacology (Berl)* 2010;**212**:251–65.
17. Li Y, Wang H, Qi K, Chen X, Li S, Sui N, et al. Orexins in the midline thalamus are involved in the expression of conditioned place aversion to morphine withdrawal. *Physiol Behav* 2011;**102**:42–50.
18. Lopez M, Seoane L, Garcia MC, Lago F, Casanueva FF, Senaris R, et al. Leptin regulation of prepro-orexin and orexin receptor mRNA levels in the hypothalamus. *Biochem Biophys Res Commun* 2000;**269**:41–5.
19. Louis G, Leinninger G, Rhodes C, Myers M. Direct innervation and modulation of orexin neurons by lateral hypothalamic LepRb neurons. *J Neurosci* 2010;**34**:11278–87.

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20. Marcus JN, Aschkenasi CJ, Lee CE, Chemelli RM, Saper CB, Yanagisawa M, et al. Differential expression of orexin receptors 1 and 2 in the rat brain. *J Comp Neurol* 2001;**435**:6–25.
21. Mondal M, Nakazato M, Date Y, Murakami N, Hanada R, Sakata T, et al. Characterization of orexin-A and orexin-B in the microdissected rat brain nuclei and their contents in two obese rat models. *Neurosci Lett* 1999;**273**:45–8.
22. Muschamp JW, Dominguez JM, Sato SM, Shen RY, Hull EM. A role for hypocretin (orexin) in male sexual behavior. *J Neurosci* 2007;**27**:2837–45.
23. Narita M, Nagumo Y, Hashimoto S, Narita M, Khotib J, Miyatake M, et al. Direct involvement of orexinergic systems in the activation of the mesolimbic dopamine pathway and related behaviors induced by morphine. *J Neurosci* 2006;**26**:398–405.
24. Olds J, Milner P. Positive reinforcement produced by electrical stimulation of the septal area and other regions of rat brain. *J Comp Physiol Psychol* 1954;**47**:419–27.
25. Perello M, Sakata I, Birnbaum S, Chuang JC, Osborne-Lawrence S, Rovinsky SA, et al. Ghrelin increases the rewarding value of high-fat diet in an orexin-dependent manner. *Biol Psychiatry* 2010;**67**:880–6.
26. Rodgers RJ, Halford JC, Nunes de Souza RL, Canto de Souza AL, Piper DC, Arch JR, et al. SB-334867, a selective orexin-1 receptor antagonist, enhances behavioural satiety and blocks the hyperphagic effect of orexin-A in rats. *Eur J Neurosci* 2001;**13**:1444–52.
27. Sakurai T, Amemia A, Ishii M, Matsuzaki I, Chemelli R, Tanaka H, et al. Orexins and orexin receptors: a family of hypothalamic neuropeptides and G coupled-protein receptors that regulate feeding behavior. *Cell* 1998;**4**:573–85.
28. Toshinai K, Date Y, Murakami N, Shimada M, Mondal MS, Shimbara T, et al. Ghrelin-induced food intake is mediated via the orexin pathway. *Endocrinology* 2003;**144**:1506–12.
29. van den Top M, Lee K, Whyment A, Blanks A, Spanswick D. Orexigen-sensitive NPY/AgRP pacemaker neurons in the hypothalamic arcuate nucleus. *Nat Neurosci* 2004;**7**:493–4.
30. Wortley K, Chang G, Davydova Z, Leibowitz S. Orexin gene expression is increased during states of hypertriglceridemia. *Am J Physiol Reg Inter Comp Physiol* 2003;**284**:R1454–65.
31. Yamanaka A, Kunii K, Nambu T, Tsujino N, Sakai A, Matsuzaki I, et al. Orexin-induced food intake involves neuropeptide Y pathway. *Brain Res* 2000;**859**:404–9.
32. Zheng H, Corkern M, Crousillac S, Patterson L, Phifer C, Berthoud HR. Neurochemical phenotype of hypothalamic neurons showing Fos expression 23 h after intracranial AgRP injection. *Am J Physiol Reg Inter Comp Physiol* 2002;**282**:R1773–91.
33. Zheng H, Patterson LM, Berthoud HR. Orexin signaling in the ventral tegmental area is required for high-fat appetite induced by opioid stimulation of the nucleus accumbens. *J Neurosci* 2007;**27**: 11075–82.

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Chapter 157

PYY

Rachel Troke, Tricia M. Tan and Stephen R. Bloom

ABSTRACT

Peptide YY (PYY) is a C-terminally amidated peptide hormone. It is named for the two tyrosine ("Y") residues which occupy both the C- and N-terminals of its 36 amino acid structure. PYY belongs to the "PP fold" family of peptides, which also includes Neuropeptide Y (NPY) and Pancreatic Polypeptide (PP). These peptides have a similar configuration, all with 36 amino acids, a large number of tyrosine residues, and a U-shaped tertiary structure designated the "PP-fold." PYY is secreted from the enteroendocrine L cells of the distal gut in response to a meal, especially protein rich. It exists in two forms, the full length 1-36 form and the processed 3-36 form which results after dipeptidyl-peptidase-4 removes the N-terminal dipeptide. PYY acts via the Y family of receptors (Y1, Y2, Y4, and Y5) to slow gastric emptying (the "ileal brake") and reduce secretion from the pancreas and gallbladder. PYY also acts as a satiety hormone.

DISCOVERY

Peptide YY (PYY) is a 36 amino acid peptide hormone predominantly released from enteroendocrine L cells of the distal gut in response to an oral nutrient load. It was discovered in 1980 by Tatemoto and Mutt at the Karolinska Institute in Stockholm, Sweden.[29] They isolated a novel peptide from porcine small intestine by developing a chemical assay method which identified the C-terminal amide structure, characteristic of a number of biologically active peptides.[29] On analyzing the peptide they had isolated to determine its amino acid sequence (Fig. 1), the investigators noted a large number of tyrosine residues within the amino acid structure of this peptide, including one at both the N- and C-terminals. It was therefore designated Peptide YY, Y being the single letter abbreviation for the amino acid tyrosine.

The amino acid sequence of PYY is related to Pancreatic Polypeptide (PP) and to Neuropeptide Y (NPY). These three peptides are named the "PP fold" family, as they form a common tertiary structure incorporating a hairpin-like U-shaped fold. Please see the relevant chapters on PP and NPY in the Brain and Ingestive Peptides sections of the book for further information.

STRUCTURE OF THE mRNA/GENE

The gene encoding PYY is found on chromosome 17q21.1.[16] The *PYY* gene consists of four exons and three introns,[10] and is in close proximity to the gene encoding PP (*PPY*), being located 10 kilobases away from *PPY* on the same chromosome. By contrast, the gene encoding NPY (*NPY*) is on chromosome 7. These three peptides represent an example of a multiple gene duplication event, giving rise to structurally similar but functionally different products. It is thought that *NPY* was the original gene with first *PYY* and then *PPY* arising as a consequence of gene duplication. The initial gene duplication probably occurred at the same time as a translocation event, explaining the alternative chromosomal location of *PYY* and *PPY*.[10,16]

PYY is synthesized as a prepropeptide which incorporates a signal peptide, the 36 amino acid active PYY sequence, and a carboxy terminal extension peptide.[16] Processing of preproPYY involves amidation of the carboxy terminal tyrosine, which is necessary for biological activity, and proteolytic cleavage by a prohormone convertase to give PYY(1-36), the 36 amino acid form of the peptide which is secreted. After secretion, the cell surface enzyme dipeptidyl-peptidase-4 (DPP-4) cleaves the N-terminal dipeptide from PYY(1-36) to give PYY(3-36), the predominant circulating form of PYY (see Peptide Biosynthesis/Processing section of the book).

Immunohistochemical studies confirm the presence of PYY in enteroendocrine L cells of the gastrointestinal tract. PYY colocalizes in these cells with glucagon-like peptide-1 (GLP-1), although the distribution of GLP-1 in the gut is different from that of PYY.[28] PYY mRNA tissue expression is similar to the distribution of the peptide seen by immunohistochemistry, and in rats, PYY mRNA is expressed at progressively increasing levels in the jejunum, cecum, and then the colon.[35]

RECEPTORS

PYY binds to the seven-trans-membrane-domain G protein-coupled receptors of the "neuropeptide Y receptor family,"

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00157-3
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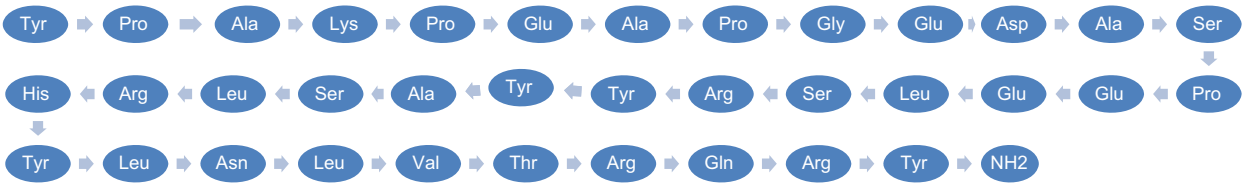

FIGURE 1 The amino acid sequence of peptide tyrosine-tyrosine (PYY). The C- and N-terminal amino acids are both tyrosine residues. In addition, the C-terminal is amidated.

Members of the Y receptor family, like the PP-fold peptides, appear to have arisen from a common ancestor gene through duplication.[19] In mammals, five receptor subtypes have been cloned, Y1, Y2, Y4, Y5, and Y6.[25] The Y3 receptor was initially thought to be a subtype that had a higher affinity for NPY compared with PYY, but has never subsequently been cloned, and no specific agonists or antagonists for this receptor subtype have been identified. As the "Y3" designation had already been allocated to this potential receptor subtype, it was decided not to use it further.[25] The Y6 receptor is functional in mice and rabbits,[19] but the human Y6 gene on chromosome 5 appears to be a pseudogene, inactivated by a frame shift mutation.[1] The Y6 receptor is absent entirely in the rat.

In humans, PYY(1-36) binds to all receptors in the Y family (Y1, Y2, Y4, and Y5). PYY(3-36), the predominant circulating form of PYY immunoreactivity, is more selective for the Y2 and Y5 receptors, and has a lesser affinity for Y1 and Y4 receptors.[18]

Signaling Pathways

Y receptors are coupled to inhibitory, pertussis toxin-sensitive G proteins, and their activation results in a reduction of cyclic-AMP (cAMP) accumulation, modulation of calcium and potassium channels, and the mobilization of intracellular calcium.[25] Y1, Y2, and Y4 are expressed on rabbit gastric smooth muscle cells, and in these cells, activation of Y2 and Y4 but not Y1 receptor types has been shown to cause smooth muscle contraction via inositol 1,4,5-triphosphate (IP_3) dependent calcium release.[26] All three receptors (Y1, Y2, and Y4) are coupled to the inhibition of adenylyl cyclase, the enzyme responsible for formation of cAMP from ATP.[26] Studies on the Y1 receptor in cardiac myocytes show that activation of the receptor by NPY causes a positive inotropic effect in these cells. This effect is the result of the action of phospholipase C (PLC), an element of the downstream signal transduction pathway that leads to IP_3 mediated intracellular calcium release.[15] While Y5 receptors are similar to Y1 receptors in mediating effects on feeding, a study using cloned Y5 receptors stably transfected into a human endometrial cancer cell line suggests that Y5 signal transduction (in response to NPY administration) is limited to inhibition of cAMP accumulation, without the increase in intracellular calcium seen with the other Y receptors, although this may be cell line specific.[6]

Y1 Receptors

Y1 receptors have a wide tissue distribution. By a variety of methods they have been localized in rodents to small arteries and arterioles of many organ systems.[23] Y1 receptors have been identified on enteroendocrine cells of the gut, and in rats Y1 mRNA is found in the proximal colon.[12] The majority of Y1 expression, however, is within blood vessels and nerves. Y1 receptor agonists mediate vasoconstriction in renal and splenic arteries.[22] The Y1 receptor antagonists SR 120107A and BIBP 3226 eliminate the prolonged vasoconstriction mediated by perivascular sympathetic nerves, suggesting an important role for the Y1 receptor in the sympathetic nervous system and blood pressure control. Furthermore, Y1 receptors are detected in the human brain, and throughout the central nervous system (CNS). They are present in the hypothalamic nuclei involved in the regulation of appetite and energy homeostasis.[28] Y1 receptors bind intact PYY (1-36), but PYY(3-36) does not bind well to this receptor.[18]

Y2 Receptors

Y2 receptors were first discovered when it was noted that a C-terminal fragment of PYY, PYY (13-36) had reduced potency in vascular preparations compared with NPY and PYY(1-36), while remaining potent in vas deferens preparations. This was the first suggestion there was more than one NPY receptor subtype, and at that time the two identified subtypes were named Y1 (receptors with poor affinity for C-terminal fragments of NPY) and Y2 (receptors with good affinity for these C-terminal fragments).[24] Y2 receptors have limited homology with Y1 receptors,[25] but a much higher affinity for PYY, particularly PYY(3-36). For Y1 receptor binding, the K_d for PYY(1-36) is 0.42 nM, with a calculated K_i value of 1050 nM for PYY(3-36). At the Y2 receptor, the K_d for PYY(1-36) is 0.03 nM with a K_i value for PYY(3-36) of 0.11 nM.[18] These values show that PYY(3-36) binds poorly to Y1 receptors, as specified above, while PYY(1-36) and PYY(3-36) have roughly equivalent binding affinities for the Y2 receptor. Like Y1 receptors, Y2 receptors are found throughout the peripheral

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nervous system (PNS) as well as the CNS, particularly in the arcuate nucleus of the hypothalamus (ARC). In humans, the Y2 receptor mRNA has been localized in the mucosa of the ileum and ascending colon, as well as in the muscular wall of the ileum and descending colon.[12]

Y4 Receptors

Y4 receptors appear to be the principal receptor for Pancreatic Polypeptide. They have a relatively low affinity for PYY(3-36), and are discussed in further detail in the Pancreatic Polypeptide chapter in the Gastrointestinal Peptides section of the book.

Y5 Receptors

Y5 receptors, like Y1 and Y2, are found with a wide tissue distribution within the brain in murine studies.[27] By Northern Blot analysis, Borowsky et al. have shown that the Y5 receptor also has a wide tissue distribution in the human brain.[8] The potency of PP fold peptides for the Y5 receptor is in the order of NPY ≥ PYY(1-36) ≈ PYY(3-36) > PP.[25]

REGULATION OF PYY SECRETION

PYY is secreted from the enteroendocrine L cells of the distal gut in response to an oral nutrient load, with levels reaching a plateau within 1–2 h after a meal,[28] and remaining elevated for up to 6 h.[2] Plasma concentrations of PYY are lowest in the fasted state, and rise post-prandially. Peak post-prandial concentrations of PYY increase in response to an increasing calorific content of meals.[21] Early studies suggested that PYY release was most strongly stimulated by fat compared with protein or carbohydrate,[28] however Batterham et al. showed in rodents that protein was the most potent stimulus for PYY release, followed by fat and then carbohydrate.[5] PYY levels begin to rise within fifteen minutes of a meal, which suggests that while PYY is released from the distal gut, the mechanisms regulating its secretion may be located more proximally, implying the involvement of other neural or hormonal influences. Indeed, cholecystokinin (CCK), a hormone secreted from the duodenum in response to various fat and protein stimuli promotes PYY release.[28] In CCK receptor null mice, or on administration of a CCK receptor antagonist, PYY secretion is attenuated.[11] In addition to hormonal influences, it seems probable that the neural influence stimulating PYY release is via the vagus nerve.[28]

While nutrients sensed in the proximal gut have an effect on PYY secretion, it has also been shown that the infusion of amino acids,[1] and short chain fatty acids (SCFA) into the colon can stimulate PYY secretion.[1] PYY mRNA expression in the distal gut is inducible by administration of a diet high in resistant starch, which is known to increase the levels of SCFA in the distal gut by fermentation. The SCFA receptors GPR43 and GPR41 have been colocalized to the PYY-releasing enteroendocrine L cells of the distal gut.[17,30] PYY concentrations remain elevated for up to 6 h post prandially, and nutrient sensing in the distal gut, potentially via these SCFA receptors, may be the responsible mechanism for this prolonged elevation of peptide levels.

BIOLOGICAL ACTIONS OF PYY

Gastrointestinal Tract

Peptide YY reduces gastric emptying, and slows intestinal transit time. This effect is termed the "ileal brake" as it represents the ability of the distal small intestine (Ileum) to inhibit gastric emptying.[1] This slowing of intestinal transit is reproduced by infusion of PYY(3-36) to healthy volunteers.[1] In rats, specific PYY binding sites have been identified in the dorsal vagal complex (DVC), an area of the medullary brainstem that incorporates the area postrema (AP), the nucleus tractus solitarius (NTS), and the dorsal motor nucleus of the vagus (DMN). It is probably through these areas that PYY exerts control over the slowing of intestinal transit.[9]

In addition to its effects on gastrointestinal motility, PYY also inhibits gastric acid release when administered peripherally. However, when injected centrally into the DVC, PYY results in an increase in gastric acid secretion.[28] Guo et al.[13] showed that an intravenous infusion of PYY inhibits the cephalic (anticipation and smell/taste of food) and gastric (stimulated by gastric distension and chemical effects of food in the stomach) phases but not the duodenal phase (stimulated by small intestine dilatation and chyme entering the duodenum) of gastric acid secretion. They also noted that PYY does not act to reduce acid secretion via a reduction in gastrin release or binding.

PYY interacts with both GLP-1 and secretin to give an additive reduction of gastric acid output, but also reduces gastric acid secretion via vagal innervation of cells in the fundus of the stomach.[34] Once again, this vagal effect is mediated by the DVC.[34]

After the discovery of PYY in 1980, this peptide was further found to be a potent inhibitor of secretin- and cholecystokinin-mediated pancreatic exocrine secretion.[1] PYY(3-36) inhibition of pancreatic secretion is regulated for the most part via the Y2 receptor—found on vagal afferents. The vagus nerve therefore appears to play multiple important roles in mediating the effects of PYY on the gastrointestinal system.

Inhibition of Appetite and Food Intake

In 2002, Batterham et al. investigated the effects of peripheral administration of PYY(3-36) on feeding. They showed

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in rats that PYY(3-36) had a potent inhibitory effect on food intake both when injected peripherally, and when injected centrally into the ARC, an area of the hypothalamus which is known to have abundant Y2 receptors.[3] They also established that there is no reduction in food intake in response to the administration of PYY(3-36) in Y2 receptor null mice, providing further evidence that PYY(3-36) acts via Y2 receptors to mediate its effects on food intake and satiety.[3] Subsequently, this group further showed a reduction in food intake by more than a third when compared with controls after a 90 min PYY(3-36) infusion in healthy human subjects. Peripheral infusions of 0.8 pmol/kg/min of PYY(3-36) were administered in order to mimic post-prandial concentrations. These healthy subjects had a significant reduction in hunger scores. There was no effect on gastric emptying with the PYY(3-36) infusion, and there was no reported nausea or vomiting.[3] PYY(3-36) infusion was, however, shown to reduce levels of the appetite stimulating hormone ghrelin.[2]

Studies in PYY null mice show that PYY has a physiological role in the control of appetite. These mice were hyperphagic, with a greater cumulative food intake than controls, and an augmented refeeding response after fasting.[5] They were fatter when total body fat was measured by MRI scanning when compared with controls,[5] and treatment with PYY(3-36) as a replacement therapy caused significant weight loss.

After this demonstration of the anorexigenic effects of PYY, many other groups have subsequently replicated these findings to show the effect of PYY on appetite and food intake. The mechanism of this reduction of food intake is mediated by the vagus, as well as areas in the brainstem and hypothalamus.

The hypothalamus is integral to the control of appetite. Neuronal populations within the hypothalamus detect peripheral signals in the circulation such as nutrients, and gut hormones, including PYY. PYY(3-36) administered directly into the ARC has been shown to have an anorexigenic effect.[3] There are two distinct neuronal populations within the ARC that control appetite: firstly, orexigenic (appetite stimulating) neurons expressing NPY and agouti gene-related peptide (AgRP); and secondly anorexigenic (appetite suppressing) neurons expressing Proopiomelanocortin (POMC) and Cocaine and Amphetamine Related Transcript protein (CART). PYY(3-36) inhibits the NPY/AgRP neurons in the hypothalamus in the ARC via presynaptic Y2 receptors, suppressing appetite. Although PYY(3-36) might also work by activating POMC/CART neurons, it still is able to exert anorexigenic effects even in mice bearing knockouts for the POMC gene, suggesting that the principal mechanism here is the presynaptic inhibition of NPY secretion by NPY/AgRP neurons.

In addition, PYY-induced satiety signals are carried to appetite-related areas in the brainstem such as the NTS via the vagus nerve. Such vagal afferent signals are triggered by PYY(3-36) activation of Y2 receptors on the vagus nerve itself.[11]

In contrast to the effects of PYY(3-36) on appetite, mediated via Y2 receptors, administration of PYY(1-36) into the central nervous system of rats causes an increase in food intake, mediated via Y1 receptors.[1] Interestingly, if PYY(1-36) is given peripherally, this causes a reduction in food intake, probably resulting from the peripheral conversion of PYY(1-36) to PYY(3-36) by DPP-4 before the peptide crossing the blood brain barrier.[1] Activation of the Y5 receptor, like Y1, has an orexigenic effect.[1] The effects of PYY(3-36) on food intake are reduced in both Y1 and Y5 null mice, implying that Y2, Y1, and Y5 also play a part in central control of appetite.[28] However, it seems that the major role in this pathway is played by Y2.

Effects on Glucose Metabolism and Energy Expenditure

Van den Hoek et al. in 2004 showed in mice that PYY(3-36) acutely augments insulin-mediated glucose disposal in euglycaemic clamp studies, i.e., it increases insulin sensitivity.[31] They went on to further investigate the effect of PYY on glucose metabolism and energy expenditure.[32] They were unable to show an increase in energy expenditure, heat production, or physical activity in mice treated with PYY(3-36), but they did show that PYY(3-36) treatment reduced the respiratory quotient (RQ), suggesting a shift from carbohydrate metabolism towards fat oxidation. The observed reduction in RQ was not attenuated by chronic administration of PYY(3-36), and the effect was sustained, in comparison to a pair fed group, suggesting that the reduction in RQ is not simply because of the anorectic effect of PYY(3-36).[32]

Further to their previous studies in 2004, they went on to confirm that PYY(3-36) augments chronic as well as acute insulin-mediated glucose disposal. In addition, this group showed that glucose uptake in adipose tissue was significantly increased after treatment with PYY(3-36) compared with controls.[32]

Neuroimaging Studies

Early studies using PYY(3-36) looked at expression of c-*fos*, a marker of neuronal activation, in order to establish which brain areas were involved in PYY action. PYY(3-36) increases c-*fos* expression in the ARC, NTS, AP, amygdale, and thalamus,[7] and studies specifically indicate that PYY(3-36) effects are mediated via Y2 receptors on NPY neurons in the ARC. Functional neuroimaging techniques such a manganese-enhanced magnetic resonance imaging (MEMRI) are now being used to look at the neurophysiology

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underlying these actions. Manganese in particular is able to enter the hypothalamus, and act as an indirect marker of hypothalamic neural activation.[14] PYY(3-36), on subcutaneous administration in rats, was associated with a trend towards a decreased signal intensity in the hypothalamus during MEMRI, although this did not reach significance. A reduction in signal intensity would suggest reduced neuronal activity, in this case of the orexigenic NPY hypothalamic neuronal population. There was no effect of PYY administration on signal intensity in the AP.[14] The effect of PYY on various brain regions has been assessed using whole-brain blood-oxygen-level-dependent (BOLD) functional magnetic resonance imaging of subjects shown pictures of food. This study found that the brain area most modulated by PYY(3-36) administration is the caudolateral orbital frontal cortex (OFC).[4] This is an area which is implicated in reward, and is possibly activated through projections from the hypothalamic nuclei involved in energy regulation and food intake.

PATHOPHYSIOLOGICAL IMPLICATIONS

PYY Levels in Obesity

In obese subjects, PYY secretion in response to meals is blunted. This has been shown in humans with obesity,[21] and also in rodent models of diet induced obesity.[33] In these rodent models, not only is the post prandial secretion of PYY diminished in obesity, but there is also a reduced effect on food intake after administration of exogenous PYY(3-36) in diet-induced obese animals. In human studies, Le Roux et al. showed a significantly lower fasting PYY concentration in obese subjects compared with normal weight controls.[21] Furthermore, they also showed a blunted post-prandial PYY response to meals, with obese people requiring double the amount of calories to achieve post prandial PYY levels equivalent to those of normal weight controls. This study also showed that the lower post-prandial PYY levels achieved in obese subjects are matched by a reduced feeling of satiety. In addition, the results of this research suggest that low fasting PYY levels are a consequence rather than a cause of obesity.[21] Obesity does not appear to confer a peripheral resistance to PYY, unlike leptin.[2] A reduction in PYY secretion in obese individuals suggests an attenuation of satiety signals, and subsequent increased food intake.

PYY LEVELS AFTER GASTRIC BYPASS SURGERY

Obesity has become a major public health issue of epidemic proportions, and there is little to offer in the form of pharmacological therapy. As a result, bariatric surgery procedures are becoming more popular as the only obesity treatment that has been shown to confer long term, sustained weight loss and clear health benefits. The most common bariatric procedure is the Roux-en-Y gastric bypass. Weight loss and reduced appetite are evident very soon after gastric bypass, and for the most part, these are sustained in the long-term. Interestingly, post-prandial levels of PYY and GLP-1 are seen to be elevated as early as two days after gastric bypass surgery, both in humans and in experimental animal models.[20] It has been postulated that these raised post-prandial levels of gut hormones play a large role in the satiety and weight loss that makes bariatric surgery such a successful therapy for obesity. The elevated levels of these gut hormones are also proposed as an explanation for the rapid resolution of diabetes after gastric bypass, although the exact mechanism of this effect remains to be elucidated.

PYY in Disease

Certain disease states are known to be associated with raised levels of PYY. Elevated levels are seen in celiac disease, active Crohn's disease, and in patients with ileal resection.[28] Levels may be raised in these individuals because of malabsorption and therefore increased luminal nutrients in the distal gut triggering increased PYY secretion. Circulating concentrations are also raised in patients with hepatic cirrhosis—a recognized anorectic state.[28] The number of cells expressing PYY in the colon is increased in diabetic gastroparesis, a condition associated with delayed gastric emptying and abnormalities of intestinal transit time.[28]

PYY and PYY Analogs as Potential Obesity Therapies

PYY significantly decreases food intake and increases satiety, as well as slows gastric emptying and intestinal transit. PYY potentially has effects on insulin-mediated glucose disposal and insulin sensitivity. All of these are desired characteristics for an anti-obesity therapy. While natural sequence PYY has a very short halflife when infused (less than 10 min), a long acting analog of PYY that can be administered subcutaneously could have huge potential as a pharmacological therapy for obesity. In combination with other anorectic gut hormones such as oxyntomodulin or GLP-1, the effect of PYY on satiety could be further augmented. Such combination therapies are currently in the early stages of development as obesity and diabetes treatments.

ACKNOWLEDGMENTS

The Section is funded by grants from the MRC, BBSRC, NIHR, an Integrative Mammalian Biology (IMB) Capacity Building Award, an FP7 - HEALTH - 2009 - 241592 EuroCHIP grant and is supported by the NIHR Imperial Biomedical Research Centre Funding Scheme.

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Tricia Tan is funded by Developmental Pathway Funding and Developmental Clinical Schemes from the Medical Research Council. Rachel Troke is the recipient of a Clinical Research Training Fellowship from the MRC.

REFERENCES

1. Ballantyne GH. Peptide YY(1-36) and peptide YY(3-36): Part I. Distribution, release and actions. *Obes Surg* 2006;**16**:651–8.
2. Batterham RL, Cohen MA, Ellis SM, Le Roux CW, Withers DJ, Frost GS, et al. Inhibition of food intake in obese subjects by peptide YY3-36. *N Engl J Med* 2003;**349**:941–8.
3. Batterham RL, Cowley MA, Small CJ, Herzog H, Cohen MA, Dakin CL, et al. Gut hormone PYY(3-36) physiologically inhibits food intake. *Nature* 2002;**418**:650–4.
4. Batterham RL, ffytche DH, Rosenthal JM, Zelaya FO, Barker GJ, Withers DJ, et al. PYY modulation of cortical and hypothalamic brain areas predicts feeding behaviour in humans. *Nature* 2007;**450**:106–9.
5. Batterham RL, Heffron H, Kapoor S, Chivers JE, Chandarana K, Herzog H, et al. Critical role for peptide YY in protein-mediated satiation and body-weight regulation. *Cell Metab* 2006;**4**:223–33.
6. Bischoff A, Puttmann K, Kotting A, Moser C, Buschauer A, Michel MC. Limited signal transduction repertoire of human Y(5) neuropeptide Y receptors expressed in HEC-1B cells. *Peptides* 2001;**22**:387–94.
7. Blevins JE, Chelikani PK, Haver AC, Reidelberger RD. PYY(3-36) induces Fos in the arcuate nucleus and in both catecholaminergic and non-catecholaminergic neurons in the nucleus tractus solitarius of rats. *Peptides* 2008;**29**:112–9.
8. Borowsky B, Walker MW, Bard J, Weinshank RL, Laz TM, Vaysse P, et al. Molecular biology and pharmacology of multiple NPY Y5 receptor species homologs. *Regul Pept* 1998;**75–76**:45–53.
9. Chen CH, Rogers RC. Central inhibitory action of peptide YY on gastric motility in rats. *Am J Physiol* 1995;**269**:R787–92.
10. Conlon JM. The origin and evolution of peptide YY (PYY) and pancreatic polypeptide (PP). *Peptides* 2002;**23**:269–78.
11. Dockray GJ. The versatility of the vagus. *Physiol Behav* 2009;**97**:531–6.
12. Ferrier L, Segain JP, Bonnet C, Cherbut C, Lehur PA, Jarry A, et al. Functional mapping of NPY/PYY receptors in rat and human gastrointestinal tract. *Peptides* 2002;**23**:1765–71.
13. Guo YS, Singh P, Gomez G, Greeley Jr GH, Thompson JC. Effect of peptide YY on cephalic, gastric, and intestinal phases of gastric acid secretion and on the release of gastrointestinal hormones. *Gastroenterology* 1987;**92**:1202–8.
14. Hankir MK, Parkinson JR, Minnion JS, Addison ML, Bloom SR, Bell JD. Peptide YY 3-36 and pancreatic polypeptide differentially regulate hypothalamic neuronal activity in mice in vivo as measured by manganese-enhanced magnetic resonance imaging. *J Neuroendocrinol* 2011;**23**:371–80.
15. Heredia Mdel P, Delgado C, Pereira L, Perrier R, Richard S, Vassort G, et al. Neuropeptide Y rapidly enhances $[Ca^{2+}]i$ transients and Ca^{2+} sparks in adult rat ventricular myocytes through Y1 receptor and PLC activation. *J Mol Cell Cardiol* 2005;**38**:205–12.
16. Hort Y, Baker E, Sutherland GR, Shine J, Herzog H. Gene duplication of the human peptide YY gene (PYY) generated the pancreatic polypeptide gene (PPY) on chromosome 17q21.1. *Genomics* 1995;**26**:77–83.
17. Karaki S, Mitsui R, Hayashi H, Kato I, Sugiya H, Iwanaga T, et al. Short-chain fatty acid receptor, GPR43, is expressed by enteroendocrine cells and mucosal mast cells in rat intestine. *Cell Tissue Res* 2006;**324**:353–60.
18. Keire DA, Mannon P, Kobayashi M, Walsh JH, Solomon TE, Reeve JRJ. Primary structures of PYY, [Pro(34)]PYY and PYY(3-36) confer different conformations and receptor selectivity. *Am J Physiol Gastrointest Liver Physiol* 2000;**279**:G126–31.
19. Larhammar D, Wraith A, Berglund MM, Holmberg SK, Lundell I. Origins of the many NPY-family receptors in mammals. *Peptides* 2001;**22**:295–307.
20. le Roux CW, Aylwin SJ, Batterham RL, Borg CM, Coyle F, Prasad V, et al. Gut hormone profiles following bariatric surgery favor an anorectic state, facilitate weight loss, and improve metabolic parameters. *Ann Surg* 2006;**243**:108–14.
21. le Roux CW, Batterham RL, Aylwin SJ, Patterson M, Borg CM, Wynne KJ, et al. Attenuated peptide YY release in obese subjects is associated with reduced satiety. *Endocrinology* 2006;**147**:3–8.
22. Malmstrom RE, Lundberg JM. Time-dependent effects of ischaemia on neuropeptide Y mechanisms in pig renal vascular control in vivo. *Acta Physiol Scand* 1997;**161**:327–38.
23. Matsuda H, Brumovsky PR, Kopp J, Pedrazzini T, Hokfelt T. Distribution of neuropeptide Y Y1 receptors in rodent peripheral tissues. *J Comp Neurol* 2002;**449**:390–404.
24. Michel MC. Receptors for neuropeptide Y: multiple subtypes and multiple second messengers. *Trends Pharmacol Sci* 1991;**12**:389–94.
25. Michel MC, Beck-Sickinger A, Cox H, Doods HN, Herzog H, Larhammar D, et al. XVI. International Union of Pharmacology recommendations for the nomenclature of neuropeptide Y, peptide YY, and pancreatic polypeptide receptors. *Pharmacol Rev* 1998;**50**: 143–50.
26. Misra S, Murthy KS, Zhou H, Grider JR. Coexpression of Y1, Y2, and Y4 receptors in smooth muscle coupled to distinct signaling pathways. *J Pharmacol Exp Ther* 2004;**311**:1154–62.
27. Nakamura M, Yokoyama M, Watanabe H, Matsumoto T. Molecular cloning, organization and localization of the gene for the mouse neuropeptide Y-Y5 receptor. *Biochim Biophys Acta* 1997;**1328**:83–9.
28. Stanley S, Wynne K, Bloom S. Gastrointestinal satiety signals III. Glucagon-like peptide 1, oxyntomodulin, peptide YY, and pancreatic polypeptide. *Am J Physiol Gastrointest Liver Physiol* 2004;**286**:l G693–7.
29. Tatemoto K, Mutt V. Isolation of two novel candidate hormones using a chemical method for finding naturally occurring polypeptides. *Nature* 1980;**285**:417–8.
30. Tazoe H, Otomo Y, Karaki S, Kato I, Fukami Y, Terasaki M, et al. Expression of short-chain fatty acid receptor GPR41 in the human colon. *Biomed Res* 2009;**30**:149–56.
31. van den Hoek AM, Heijboer AC, Corssmit EP, Voshol PJ, Romijn JA, Havekes LM, et al. PYY3-36 reinforces insulin action on glucose disposal in mice fed a high-fat diet. *Diabetes* 2004;**53**:1949–52.
32. van den Hoek AM, Heijboer AC, Voshol PJ, Havekes LM, Romijn JA, Corssmit EP, et al. Chronic PYY3-36 treatment promotes fat oxidation and ameliorates insulin resistance in C57BL6 mice. *Am J Physiol Endocrinol Metab* 2007;**292**:E238–45.
33. Xu J, McNearney TA, Chen JD. Impaired postprandial releases/syntheses of ghrelin and PYY(3-36) and blunted responses to exogenous ghrelin and PYY(3-36) in a rodent model of diet-induced obesity. *J Gastroenterol Hepatol* 2011;**26**:700–5.
34. Yang H. Central and peripheral regulation of gastric acid secretion by peptide YY. *Peptides* 2002;**23**:349–58.
35. Zhou J, Hegsted M, McCutcheon KL, Keenan MJ, Xi X, Raggio AM, et al. Peptide YY and proglucagon mRNA expression patterns and regulation in the gut. *Obesity (Silver Spring)* 2006;**14**:683–9.

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Chapter 158

Adrenomedullin

Mitchell L. Schubert and Shijian Chu

ABSTRACT

Adrenomedullin, isolated in 1993 from human pheochromocytoma, is a member of the calcitonin family of peptides. It is expressed in gastrointestinal tissues including liver, pancreas, stomach, duodenum, small intestine, colon, and pancreas. The activities of adrenomedullin are mediated via CGRP receptors and specific adrenomedullin receptors, termed AM_1 and AM_2, primarily through the activation of adenylate cyclase. In the stomach, adrenomedullin (1) stimulates somatostatin and thus inhibits histamine and acid secretion; (2) protects against injury by increasing mucosal blood flow, stimulating epithelial cell proliferation, and promoting angiogenesis; and (3) inhibits gastric emptying. In the intestine, adrenomedullin stabilizes gut barrier function. In the colon, it inhibits sodium absorption and ameliorates colitis. Adrenomedullin also inhibits amylase secretion in the pancreas and may contribute to the hemodynamic alterations observed in cirrhosis. More potent and selective adrenomedullin receptor antagonists will be required to define the precise physiological and pathophysiological roles of adrenomedullin in regulating gastrointestinal function.

DISCOVERY

Adrenomedullin, a member of the calcitonin peptide family that also includes calcitonin, calcitonin-gene-related peptide (CGRP), and amylin, was first isolated in 1993 from human pheochromocytoma and termed "adrenomedullin" based on the high concentration in the normal adrenal medulla.[12] In 2004, using sequences from the calcitonin peptide family, a 47-amino-acid peptide termed adrenomedullin-2 (AM-2) or intermedin was identified in mammals. Although AM-2 displays high homology to adrenomedullin and shares similar biological actions, only 18 amino acids are conserved between the peptides, and they are coded by different genes.

Although initially identified as a vasodilator, adrenomedullin is present in the human digestive system and has been implicated in a variety of physiological functions and pathophysiological conditions.

STRUCTURE OF THE mRNA/GENE

Human adrenomedullin is a 52-amino-acid peptide with a ring structure and an amidated C-terminal tyrosine residue.[23] The adrenomedullin gene is located on the short arm of chromosome 11 in humans whereas in mice, it is located on chromosome 7. The adrenomedullin promoter region contains several protein-binding sites including nuclear factor for interleukin-6 expression (NF-IL6) and activator protein 2 (AP-2).

PROCESSING OF THE PRECURSOR

The initial translation product of the gene is preproadrenomedullin, a 185-amino-acid peptide, which is converted to proadrenomedullin. Proadrenomedullin undergoes proteolysis and amidation to yield adrenomedullin and proadrenomedullin N-terminal 20 peptide (PAMP); the latter may possess biologic activity.

Adrenomedullin is characterized by the presence of a six-amino-acid ring formed by a disulfide bridge between residues 16 and 21 at the N-terminus. Its structure is conserved across species, the rat peptide only differing by six substitutions and two deletions compared to the human peptide.

DISTRIBUTION OF THE mRNA/PEPTIDE

Adrenomedullin and its mRNA are expressed not only in the adrenal gland but also in a variety of tissues and cells including lung, heart, kidney, brain, thyroid, bone, skin, nerve, vascular endothelial and smooth muscle cells, submandibular gland, stomach, duodenum, small intestine, colon, pancreas, and liver.[10,27]

In human and rat pancreas, adrenomedullin is expressed in most islet cells, colocalizes with pancreatic polypeptide in islet F cells, and inhibits insulin secretion from β cells. A few adrenomedullin immunoreactive cells are scattered among the exocrine parenchymal cells.

In the hollow digestive system, adrenomedullin is concentrated in mucosal neuroendocrine cells. In the stomach, adrenomedullin has been localized to histamine-containing enterochromaffin-like (ECL) cells and chief cells within the oxyntic mucosa and to gastrin cells within the pyloric mucosa.[5,13,34] In the colon, adrenomedullin immunoreactivity has been localized to serotonin-containing enterochromaffin (EC) cells.[20]

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00158-5
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Adrenomedullin circulates in the plasma in picomolar concentrations. In normal subjects, plasma concentrations range from 2 to 4 pmol/l. Plasma concentrations of adrenomedullin increase with fasting and pregnancy as well as a variety of disease states including acute myocardial infarction, ischemic heart disease, congestive heart failure, essential hypertension, chronic renal failure, hyperparathyroidism, sepsis, and cirrhosis.[15,27] One common denominator may be bacterial infection and/or inflammation as exposure of gastric epithelial cells to various strains of bacteria including *Helicobacter pylori* as well as various cytokines such as interleukin-1, interleukin-6, tumor necrosis factor, interferon, and lipopolysaccharide stimulate adrenomedullin production and secretion.[1] Circulating hormones such as corticosteroids, thyroid hormones, angiotensin II, and norepinephrine also stimulate adrenomedullin secretion. In contrast, interferon-γ, vasoactive intestinal peptide (VIP), and thrombin suppress adrenomedullin production.

In plasma, adrenomedullin complexes with adrenomedullin-binding protein (AMBP). This binding protein was later isolated and found to be identical to complement factor H, a protein that inhibits the alternative complement pathway and modulates neutrophil and monocyte function.[22] It should be noted that only unbound adrenomedullin is measured in plasma by radioimmunoassay. Complement factor H enhances the biological activity of adrenomedullin by protecting the peptide from degradation while unaffecting its ability to bind to its receptor.

RECEPTORS AND SIGNALING CASCADES

Receptors for the calcitonin family of peptides are G-protein-coupled receptors known as calcitonin-receptor-like receptors. The calcitonin-receptor-like receptor (CRLR), a seven-transmembrane domain receptor, can function as either a CGRP receptor or an adrenomedullin receptor, depending on which members of a family of single-transmembrane domain proteins, called receptor-activity-modifying proteins or RAMPs, are expressed.[24] RAMPs function as accessory proteins that traffic CRLR to the plasma membrane and are required for pharmacological specificity. All three RAMPs contain about 160 amino acids and exhibit a common structure that comprises a large extracellular N-terminal domain, a single-transmembrane domain, and a short cytoplasmic C-terminal tail. When CRLR and RAMP1 were cotransfected into Xenopus oocytes, a CGRP pharmacological response was acquired, whereas transfection of CRLR and RAMP2 or RAMP3 resulted in the adrenomedullin receptors AM_1 and AM_2, respectively (Fig. 1).[19,20] Because of the structural similarities among the ligand peptides, there is some crossreactivity among adrenomedullin, amylin, and CGRP. Adrenomedullin can bind to CGRP receptors with an affinity approximately 10-fold less than that of CGRP itself.[6] CGRP binds to the AM_2 receptor with around 50-fold lower affinity than adrenomedullin and has a much lower affinity at AM_1 receptors

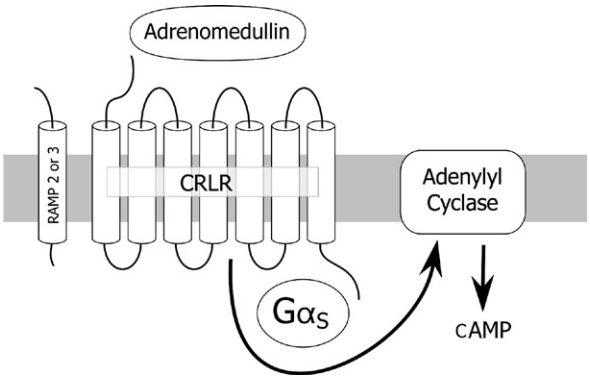

FIGURE 1 Model illustrating the adrenomedullin receptor showing the interactions between the seven-transmembrane domain G-protein-coupled receptor known as the calcitonin-receptor-like receptor (CRLR) and the single domain receptor-associated membrane proteins (RAMPs). Their association forms a high-affinity receptor coupled to $G\alpha_s$ that activates adenylyl cyclase to generate cAMP. The subtype of adrenomedullin receptor formed by the association of CRLR with RAMP2 is termed AM_1, whereas that formed by association with RAMP3 is termed AM_2. Adapted from Ref. 24.

Adrenomedullin22-52 (AM22-52), which lacks the disulfide bond, functions as a low affinity selective adrenomedullin receptor antagonist in some but not all circumstances. It seems to antagonize the effects of adrenomedullin but not CGRP and has significant selectivity for AM_1 receptors over AM_2 receptors.[7] The AM_1 receptor recognizes adrenomedullin over CGRP with a selectivity of two to three orders of magnitude and is more effectively antagonized by AM22-52 than by the CGRP receptor antagonist, CGRP8-37.[16] The AM_2 receptor is sensitive to both CGRP8-37 and AM22-52, although rat and mouse AM_2 receptors show a preference for CGRP8-37 over AM22-52. The amylin receptor antagonist, AC187, can also antagonize specific adrenomedullin receptors but only at high concentrations (>5 μM).

The biologic activities of adrenomedullin are mediated primarily through the activation of adenylyl cyclase although in some cell types adrenomedullin may also release calcium from inositol 1,4,5-trisphosphate-sensitive intracellular stores and stimulate nitric oxide synthase and mitogen-activated protein kinases (MAPKs) (Fig. 1).

BIOLOGICAL ACTIONS WITHIN THE GASTROINTESTINAL TRACT

Gastric Acid Secretion

Adrenomedullin circulates in the plasma and intravenous infusion of the peptide inhibits basal, histamine-stimulated, pentagastrin-stimulated, and 2-deoxy-D-glucose-stimulated

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acid secretion in conscious rats equipped with gastric fistula.[26] The effect is partly, 50–60%, blocked by a selective somatostatin receptor antagonist, suggesting that systemic adrenomedullin inhibits acid secretion via both somatostatin-dependent and -independent pathways. The adrenomedullin receptor antagonist, AM22-52, however failed to significantly antagonize the effect of adrenomedullin. The findings are consistent with those reported in pylorus-ligated rats in which the inhibitory effect on acid secretion of subcutaneous injection of adrenomedullin was more potently blocked by the CGRP receptor antagonist, CGRP8-37, than AM22-52, suggesting that the antisecretory effect is mediated predominantly via CGRP receptors.[28] In the stomach, CGRP is capable of stimulating somatostatin and inhibiting gastrin and acid secretion.[17,29] It should be cautioned, however, that rodent AM_2 receptors display a preference for CGRP8-37.[16]

Studies in isolated mouse stomach and superfused rat fundic segments, preparations that retain intact intramural neural and paracrine pathways but eliminate hormonal influences such as gastrin, indicate that adrenomedullin acts locally, that is, within the oxyntic mucosa, to stimulate somatostatin and inhibit histamine and acid secretion (Fig. 2).[9] The effect is abolished by the axonal blocker, tetrodotoxin, indicating that the effect of adrenomedullin is mediated via the activation of intramural neurons. The identity of the neurotransmitter responsible for adrenomedullin-induced somatostatin secretion is not yet known. In dog mesenteric arteries, adrenomedullin receptors have been identified on adrenergic nerve endings.[3]

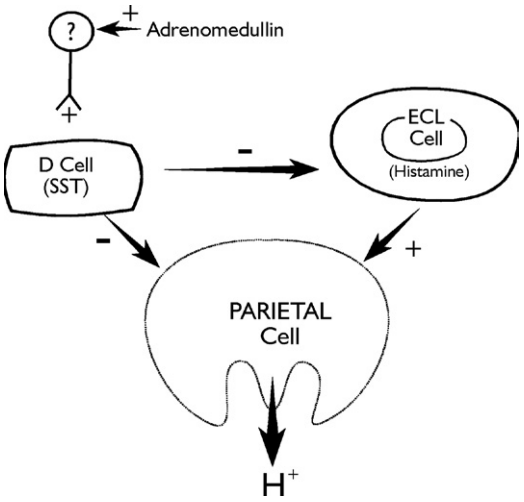

FIGURE 2 Model illustrating the regulation of acid secretion in gastric oxyntic mucosa by adrenomedullin. Adrenomedullin, acting via an as yet unidentified intramural neuron, stimulates somatostatin (SST) and thus inhibits histamine secretion from enterochromaffin-like (ECL) cells and acid secretion from parietal cells. Reproduced by permission from Ref. 9.

Gastrointestinal Protection and Repair

Inhibition of acid secretion and increased mucosal blood flow play important roles in preventing gastric mucosa injury. In rats, central and peripheral administration of adrenomedullin prevents gastric damage induced by 70% ethanol, reserpine, and pylorus ligation. The effect of intracisternal adrenomedullin was blocked by central injection of the CGRP antagonist, CGRP8-37, in one study but not in another.[2,11] The effect was significantly attenuated by subdiaphragmatic vagotomy as well as peripheral injection of atropine, indomethacin, and the nitric oxide synthase inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME). The results suggest that adrenomedullin exerts a gastroprotective effect centrally that is mediated via the vagus nerve with activation of peripheral prostaglandin- and nitric oxide-dependent protective mechanisms.

Peripherally administered adrenomedullin also prevents gastric damage induced by noxious stimuli. The protection is accompanied by inhibition of acid secretion and vasodilation. Studies using CGRP8-37 to block the CGRP receptor and AM22-52 to block the adrenomedullin receptor suggest that the acid effect is mediated predominantly via CGRP receptors, whereas the vasomotor effect is mediated predominantly via adrenomedullin receptors.[28]

Expression of adrenomedullin and its receptor is increased in rat gastric mucosa after direct instillation of ethanol and during gastric ulcer healing,[33] suggesting that adrenomedullin may contribute to gastric mucosal injury repair, perhaps in a paracrine or autocrine manner. In support of this notion, adrenomedullin promotes epithelial restitution, as measured by potential difference, in human oxyntic mucosal sheets mounted in Ussing chamber following injury induced by 0.5 M saline.[5]

The intestinal mucosa constitutes an important barrier between the host and luminal contents containing microbes and noxious substances. Accumulating evidence suggests that adrenomedullin can stabilize gut barrier function. In rat intestinal cells and jejunal epithelium, lipopolysaccharide increases adrenomedullin expression. In a rat model of septic shock, adrenomedullin reduces intestinal permeability as measured by translocation of labeled dextran from the gut into the systemic circulation.[31] Adrenomedullin also preserves gut barrier function after hemorrhagic shock and gut ischemia reperfusion.[35] It reduces the incidence of diarrhea, the severity of colonic damage, and improves survival in trinitrobenzene sulfonic acid (TNBS)-induced colitis in rats.[30]

Gastric Emptying

Intracisternal injection of adrenomedullin dose dependently inhibits gastric emptying in rats.[18] Central injection of PAMP, however, was ineffective in rats[18] but inhibited

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gastric emptying in mice.[21] Intravenous adrenomedullin has been reported to either inhibit[25] or have no significant effect[18] on gastric emptying in rats.

Colonic Transport

Adrenomedullin is present in human and rat colonic mucosa. In rat distal colon mounted in Ussing chamber, adrenomedullin inhibits sodium absorption and stimulates chloride secretion.[4] The effects were inhibited by the axonal blocker, tetrodotoxin, implying that they are mediated via the activation of intramural neurons.

Pancreatic Exocrine Secretion

Adrenomedullin is present in pancreatic islet cells and capable of binding specifically to pancreatic acini.[32] In isolated rat pancreatic acini, adrenomedullin inhibits amylase secretion induced by cholecystokinin and the calcium ionophore, A23187.[32]

Gastrointestinal Malignancy

Adrenomedullin and its receptor are highly expressed in a variety of gastrointestinal cancers where it induces angiogenesis and proliferation, including pancreatic and colorectal cancer.[8] Expression of adrenomedullin is upregulated through a hypoxia-inducible factor-1 (HIF-1)-dependent pathway.

Cirrhotic Portal Hypertension

Peripheral arterial vasodilation leading to decreased systemic vascular resistance is the most outstanding hemodynamic alteration in liver cirrhosis. There is increasing evidence that arterial vasodilation contributes to portal hypertension and the formation of ascites through arterial hypotension, increased cardiac output, and activation of renal sodium retentive mechanisms. The precise cause of the vasodilation remains unknown, but overproduction or reduced degradation of endogenous vasodilators is believed to play a role.

Adrenomedullin causes vasodilation by increasing cAMP in vascular smooth muscle cells and releasing nitric oxide from vascular endothelial cells. Plasma levels of adrenomedullin are increased in patients with cirrhosis and are highest in patients with refractory ascites. Studies in CCL_4-induced cirrhotic rats suggest that overproduction of adrenomedullin may contribute to the observed vascular hyporeactivity to vasoconstrictors.[14]

PATHOLOGICAL IMPLICATIONS

Adrenomedullin is a relatively recently discovered peptide. Although we have made significant progress in understanding its structure, molecular biology, distribution, plasma levels in health and disease, receptors, second messenger systems, and bioactivity in experimental circumstances, questions remain regarding its true physiological role. Recent studies suggest that it may play a role in the pathogenesis of peptic ulcer disease and the hyperdynamic circulation in cirrhosis as well as a growth factor in certain cancers. Endogenous adrenomedullin may also ameliorate damage caused by shock, ischemia reperfusion, and colitis.

Although adrenomedullin circulates in plasma, it is likely that it acts predominantly as an autocrine and paracrine agent. In the gastrointestinal tract, adrenomedullin inhibits gastric acid secretion, protects the stomach as well as intestine and colon from injury, inhibits gastric emptying, inhibits sodium absorption, inhibits amylase secretion, and may contribute to the hemodynamic alterations observed in cirrhotic portal hypertension. More potent and selective adrenomedullin receptor antagonists will be required to define the physiological roles of adrenomedullin in the regulation of gastrointestinal function.

ACKNOWLEDGMENTS

The author thanks Mary E. Beatty for the artwork.

REFERENCES

1. Allaker RP, Kapas S. Adrenomedullin expression by gastric epithelial cells in response to infection. *Clin Diagn Lab Immunol* 2003;**10**:546–51.
2. Clementi G, Caruso A, Cutuli VMC, De Bernardis E, Prato A, Mangano NG, et al. Effects of centrally or peripherally injected adrenomedullin on reserpine-induced gastric lesions. *Eur J Pharmacol* 1998 Oct;**360**(1):51–4.
3. Fujioka H, Okamura T, Toda N. Inhibition by adrenomedullin of amine release from adrenergic nerves in dog mesenteric arteries. *Eur J Pharmacol* 1999 Dec;**385**:155–61.
4. Fukuda K, Tsukada H, Onomura M, Saito T, Kodama M, Nakamura H, et al. Effect of adrenomedullin on ion transport and muscle contraction in rat distal colon. *Peptides* 1998;**19**:1043–7.
5. Fukuda K, Tsukada H, Oya M, Onomura M, Kodama M, Nakamura H, et al. Adrenomedullin promotes epithelial restitution of rat and human gastric mucosa in vitro. *Peptides* 1999;**20**(1):127–32.
6. Hay DL, Conner AC, Howitt SG, Smith DM, Poyner DR. The pharmacology of adrenomedullin receptors and their relationship to CGRP receptors. *J Mol Neurosci* 2003;**22**(1, 2):105–13.
7. Hay DL, Howitt SG, Conner AC, Schindler M, Smith DM, Poyner DR. CL/RAMP2 and CL/RAMP3 produce pharmacologically distinct adrenomedullin receptors: a comparison of effects of adrenomedullin$_{22–52}$, CGRP$_{8–37}$ and BIBN4096BS. *Br J Pharmacol* 2003 Oct;**140**(3):477–86.
8. Hikosaka T, Tsuruda T, Nagata S, Kuwasako K, Tsuchiya K, Hoshiko S, et al. Adrenomedullin production is increased in colorectal adenocarcinomas; its relation to matrix metalloproteinase-9. *Peptides* 2011; http//:dx.doi.org/doi:10.1016/j.peptides.2011.07.012.
9. Hirsch AB, McCuen RW, Arimura A, Schubert ML. Adrenomedullin stimulates somatostatin and thus inhibits histamine and acid secretion in the fundus of the stomach. *Regul Pept* 2003 Feb;**110**(3):189–95.

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10. Ichiki Y, Kitamura K, Kangawa K, Matsuo H, Eto T. Distribution and characterization of immunoreactive adrenomedullin in human tissue and plasma. *FEBS Lett* 1994;**338**(6):10.
11. Kaneko H, Mitsuma T, Nagai H, Mori S, Iyo T, Kusugami K, et al. Central action of adrenomedullin to prevent ethanol-induced gastric injury through vagal pathways in rats. *Am J Physiol Regul Integr Comp Physiol* 1998;**274**:R1783–8.
12. Kitamura K, Kangawa K, Kawamoto M, Ichiki Y, Nakamura S, Matsuo H, et al. Adrenomedullin: a novel hypotensive peptide isolated from human pheochromocytoma. *Biochem Biophys Res Commun* 1993;**192**:553–60.
13. Kitani M, Asada Y, Sakata J, Kitamura K, Sumiyoshi A, Eto T. Cell density of adrenomedullin-immunoreactive cells in the gastric endocrine cells decreases in antral atrophic gastritis. *Histopathology* 1999 Feb;**34**(2):134–9.
14. Kojima H, Sakurai S, Uemura M, Satoh H, Nakashima T, Minamino N, et al. Adrenomedullin contributes to vascular hyporeactivity in cirrhotic rats with ascites via a release of nitric oxide. *Scand J Gastroenterol* 2004 Jul;**39**(7):686–93.
15. Kojima H, Tsujimoto T, Uemura M, Takaya A, Okamoto S, Ueda S, et al. Significance of increased plasma adrenomedullin concentration in patients with cirrhosis. *J Hepatol* 1998;**28**:840–6.
16. Kuwasako K, Cao YN, Nagoshi Y, Kitamura K, Eto T. Adrenomedullin receptors: pharmacological features and possible pathophysiological roles. *Peptides* 2004 Nov;**25**(11):2003–12.
17. Manela FD, Ren J, Gao J, McGuigan JE, Harty RF. Calcitonin gene-related peptide modulates acid-mediated regulation of somatostatin and gastrin release from rat antrum. *Gastroenterology* 1995;**109**:701–6.
18. Martinez V, Cuttitta F, Taché Y. Central action of adrenomedullin to inhibit gastric emptying in rats. *Endocrinology* 1997;**138**:3749–55.
19. McLatchie LM, Fraser NJ, Main MJ, Wise A, Brown J, Thompson N, et al. RAMPs regulate the transport and ligand specificity of the calcitonin-receptor-like receptor. *Nature* 1998;**393**:333–9.
20. Muff R, Born W, Fischer JA. Adrenomedullin and related peptides: receptors and accessory proteins. *Pepitdes* 2001 Nov;**22**(11):1765–72.
21. Ohinata K, Inui A, Asakawa A, Wada K, Wada E, Yoshikawa M. Proadrenomedullin N-terminal 20 peptide (PAMP) inhibits food intake and gastric emptying in mice. *Peptides* 2001;**22**:S89–95.
22. Pio R, Martinez A, Unsworth EJ, Kowalak JA, Bengoechea JA, Zipfel PF, et al. Complement factor H is a serum-binding protein for adrenomedullin, and the resulting complex modulates the bioactivities of both partners. *J Biol Chem* 2001;**276**:12292–300.
23. Poyner DR, Sexton PM, Marshall I, Smith DM, Quirion R, Born W, et al. International Union of Pharmacology. XXXII. The mammalian calcitonin gene-related peptides, adrenomedullin, amylin, and calcitonin receptors. *Pharmacol Rev* 2002 Jun;**54**(2):233–46.
24. Prado MA, Evans-Bain B, Dickerson IM. Receptor component protein (RCP): a member of a multi-protein complex required for G-protein-coupled signal transduction. *Biochem Soc Trans* 2002 Aug;**30**(4):460–4.
25. Reidelberger RD, Kelsey L, Heimann D. Effects of amylin-related peptides on food intake, meal patterns, and gastric emptying in rats. *Am J Physiol Regul Integr Comp Physiol* 2002;**282**:R1395–404.
26. Rossowski WJ, Cheng BL, Jiang NY, Coy DH. Examination of somatostatin involvement in the inhibitory action of GIP, GLP-1, amylin and adrenomedullin on gastric acid release using a new SRIF antagonist analogue. *Br J Pharmacol* 1998 Nov;**125**(5):1081–7.
27. Sakata J, Asada Y, Shimokubo T, Kitani M, Inaatsu H, Kitamura K, et al. Adrenomedullin in the gastrointestinal tract. Distribution and gene expression in rat and augmented gastric adrenomedullin after fasting. *J Gastroenterol* 1998;**33**:828–34.
28. Salomone S, Caruso A, Martinez G, Cutuli VM, Prato A, Bianchi A, et al. Secretory and vascular effects of adrenomedullin in gastric ulcer: role of CGRP- and adrenomedullin-receptors. *Peptides* 2003 Aug;**24**(8):1175–80.
29. Taché Y, Pappas T, Lauffenburger M, Goto Y, Walsh JH, Debas H. Calcitonin gene-related peptide: potent peripheral inhibitor of gastric acid secretion in rat and dog. *Gastroenterology* 1984;**87**:334–49.
30. Talero E, Sanchez-Fidalgo S, Alarcon de la Lastra C, Illanes M, Calvo JR, Motilva V. Acute and chronic responses associated with adrenomedullin administration in experimental colitis. *Peptides* 2008;**29**:2001–12.
31. Temmesfeld-Wollbruck B, Brell B, zu Dohna C, Dorenberg M, Hocke AC, Martens H, et al. Adrenomedullin reduces intestinal epithelial permeability in vivo and in vitro. *Am J Physiol Gastrointest Liver Physiol* 2009;**297**:G43–51.
32. Tsuchida T, Ohnishi H, Tanaka Y, Mine T, Fujita T. Inhibition of stimulated amylase secretion by adrenomedullin in rat pancreatic acini. *Endocrinology* 1999;**140**:865–70.
33. Wang H, Tomikawa M, Jones K, Pai R, Sarfeh IJ, Tarnawski AS. Sequential expression of adrenomedullin and its receptor during gastric ulcer healing. *Dig Dis Sci* 2000;**45**:591–8.
34. Wang H, Tomikawa M, Jones MK, Sarfeh IJ, Tarnawski AS. Ethanol injury triggers activation of adrenomedullin and its receptor genes in gastric mucosa. *Dig Dis Sci* 1999;**44**:1390–400.
35. Zhang F, Wu R, Zhou M, Blau SA, Wang P. Human adrenomedullin combined with human adrenomedullin binding protein-1 is protective in gut ischemia and reperfusion injury in the rat. *Regul Pept* 2009;**152**:82–7.

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Chapter 159

Angiogenic Growth Factors

Sandor Szabo, Xiaoming Deng, Tetyana Khomenko, Ganna Tolstanova, Brankica Paunovic, Longchuan Chen, Ximing Xiong and Zsuzsanna Sandor

ABSTRACT

The etiology and molecular pathogenesis of gastrointestinal (GI) ulcer diseases, for example, duodenal ulcer (DU) and ulcerative colitis (UC), are poorly understood. Our laboratory first postulated that stimulation of angiogenesis alone might be sufficient to accelerate GI ulcer healing. We showed that therapy with genes or peptides of angiogenic growth factors, for example, bFGF, PDGF, and VEGF significantly accelerated healing of experimental DU, whereas blockade of these angiogenic factors delayed DU healing. Because histologically DU and UC look similar, we also predicted that the healing of UC might be also improved by stimulating angiogenesis. However, UC healing apparently involves abnormal "pathologic" angiogenesis, enhanced by VEGF and placental growth factor (PlGF), and anti-VEGF therapy improves experimental UC. Expression of PlGF is apparently decreased in DU but increased in UC. Here, we review similar and differential actions of angiogenic factors in GI ulcer healing and make suggestions for the development of angiogenesis-related drugs.

INTRODUCTION

Gastrointestinal (GI) ulcers are essentially internal wounds that resist normal healing. Because their pathogenesis is poorly understood, and the etiologic (e.g. gastric acid, aspirin-like drugs, stress, innate immunity, environmental exposure) and aggravating factors (e.g. *Helicobacter pylori*) are not well characterized, the remaining therapeutic option is to accelerate healing. Superficial mucosal lesions, that is, erosions usually heal by epithelial regeneration and restitution, but ulcers damage the muscularis propria and smooth muscle cells do not divide/regenerate. These deep lesions are filled by granulation tissue, that is, angiogenesis followed by proliferation of connective tissue fibroblasts that deposit collagen over which adjacent surviving and dividing epithelial cells migrate to complete the healing.

Gastric and duodenal ulcers (GU and DU) are more prevalent than the idiopathic ulcerative colitis (UC) and Crohn's disease (CD), especially because some of the GI ulcers are related to stress, environmental exposure, excessive alcohol and drug use. In most countries of the world, DU is the most prevalent form of "peptic ulcers." At one time during their lives, 8–10% of the US population suffers from DU disease and another 1% from GU.[24,25] Despite advancement in the etiology and the pathogenesis of gastric ulceration, especially after the discovery of the role of *H. pylori*, the pathogenesis of ulcer disease in the GI tract is still poorly understood. Hence, no etiologic and effective therapy is available.

UC and CD that are also known as inflammatory bowel disease (IBD) are associated with substantial inflammation, much more prominently and extensively than gastroduodenal ulcers. In UC, the ulcers are surrounded by acute (e.g. neutrophils) and/or chronic (e.g. lymphocytes, plasma cells, macrophages) inflammatory cells, hence often leading to the mistaken pathogenic target of treating UC by anti-inflammatory drugs to suppress the secondary inflammation response to the previously induced ulcerative lesions. Unlike the more diffuse UC, CD is regional, segmental, leaving uninvolved small or large bowels interspersed by areas of deep lesions, fissures, often fistulas, that are the results of transmucosal involvement. For unknown reasons, nevertheless, UC, unlike CD, often leads to carcinomas.

This chapter focusses on discussing the similar and different expression and actions of angiogenesis and angiogenic factors such as basic fibroblast growth factor (bFGF), platelet growth factor (PDGF), vascular endothelial growth factor (VEGF), placental growth factor (PlGF), and Rac1 in healing of DU and UC. It also provides an overview of the developments of the last 10 years of GI ulcer research, mostly published from our laboratory.

ANGIOGENESIS IN CHRONIC GI ULCER DEVELOPMENT AND HEALING

Angiogenesis is the generation of new blood vessels from an existing vascular bed. The process involves extensive interaction between several cells and molecules.[9,25] The switch to angiogenesis involves a change in the local equilibrium between positive and negative regulators of

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00159-7
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microvessels. The proliferation of endothelial cells and tube formation are crucial elements in granulation tissue production. The formation of granulation tissue, that is, angiogenesis followed by proliferation of fibroblasts and deposition of collagen, on the other hand, is a rate-limiting step in the repair of major damage to tissues that do not regenerate (e.g. after the loss of cardiac or stomach muscle). Only in certain organs such as liver, adrenal cortex, and kidney, does the regeneration involve the proliferation of original parenchymal cells that rapidly replace the lost tissue. The healing process needs the granulation tissue, which forms the basis for proliferation and migration of epithelial cells in the GI tract. The migrating cells cannot grow over necrotic tissue, unless it is gradually replaced by angiogenesis-dependent granulation tissue. Thus, stimulation of only epithelial cell proliferation is counterproductive in the healing of internal and external wounds, unless it is accompanied by the expression of a loose or solid granulation tissue, which provides the basis and physical framework for the migration of epithelial cells to complete the healing process.[29] The healing of deep ulcers that reach or penetrate the muscularis propria almost always requires initial angiogenesis and proliferation of other elements of granulation tissue.[30] Endothelial cell migration, proliferation and microvascular structure formation are initiated and regulated by angiogenic growth factors, such as VEGF which is the most potent and specific stimulator of angiogenesis.[9,30,31]

In normal wound/ulcer healing angiogenesis, known as physiologic angiogenesis, is closely controlled by multiple growth and tissue factors resulting in minimal changes in microvascular permeability, proteolysis, and inflammation. However, abnormal or pathologic angiogenesis, such as that observed in malignant tumors and UC, is characterized by abnormal vasculature, which exhibits defective architecture, increased permeability, and increased inflammatory and thrombogenic potential. Basic and clinical research of the last two decades indicate that angiogenesis plays a crucial role in the healing of both upper[4,10,11,16,17,26,27,31,33] and lower GI tract ulcers.[2,3,12,18,21,32] Development of new vasculature during chronic inflammation may play a negative or "pathologic" role by contributing to increased inflammatory responses resulting from dysfunctional new vessel architecture and increases in the recruitment of inflammatory cell types.[23,32] Indeed, angiogenic inhibition during chronic inflammatory diseases attenuates further inflammation and disease progression.[3,32] However, the microvascular changes that occur during UC have not been closely investigated, and we are only beginning to understand their involvement in the disease process. As such, expanding our knowledge of how pathologic angiogenesis occurs during UC will further our ability to treat patients with these diseases.[2,23,32]

ANGIOGENIC GROWTH FACTORS AND GI ULCER HEALING

Advances in basic sciences such as cell and molecular biology and pharmacology of growth factors provide new methods and new results in the study complex and multifactorial disorders such as ulcer and inflammatory diseases of upper and lower GI tract. Until recently, the pathogenesis of GI ulceration has been investigated mostly from the point of view of aggressive factors and the therapeutic interventions affected the healing process only indirectly. Angiogenic growth factors, for example, bFGF, PDGF, and VEGF are new modulators of ulcer disease, these peptides are relatively large molecules and structurally different from the small molecular mediators and modulators such as glucocorticoids, dopamine, somatostatin, sulfhydryls/antioxidants, endothelins and angiosteroids.[25,29] Furthermore, although the small molecules usually exert multiple effects, the growth factors usually have a single mechanism of actions, that is, stimulating the proliferation of a single or several types of cells.

Similar Therapeutic Effects of bFGF on GU, DU, and UC

bFGF is an 18-kDa polypeptide first isolated under this name from the brain as a fibroblast stimulator. It was later found to be identical to the most potent heparin-binding angiogenic stimulator. Indeed, bFGF is a direct mitogen for vascular endothelial cells, fibroblasts, smooth muscle cells, certain epithelial cells, and neural cells. It has diverse roles in wound healing, tissue regeneration, and embryonic development, and probably in carcinogenesis as well. The high affinity of bFGF for the components of the extracellular matrix, such as heparin and heparan sulfate proteoglycans, might sustain a microenvironment with high availability of bFGF to the target cells.

bFGF Treatment in GU and DU

Our laboratory was the first to show that *per os* (po) treatment with either naturally occurring bFGF or its acid-resistant mutein (bFGF-CS23, in which the second and third cysteines are replaced by serine residues) accelerates the healing of cysteamine-induced chronic DU.[10,27] The results reveal that the ulcer-healing effect of bFGF derivatives is more potent than that of orally administered cimetidine (10 mg/100 g, twice a day for 3 weeks) but that only the acid-resistant mutein bFGF-CS23 significantly decreases both size ($p < 0.01$ vs. control) and prevalence ($p < 0.05$ vs. control) of the remaining chronic ulcers (Fig. 1a). Nevertheless, we also detected an additive ulcer healing effect between bFGF and cimetidine.[10,27,30]

Novel derivatives of recombinant human (rh)bFGF were also tested in the treatment of experimental chronic DU in rats. These included Ser 78,96-rhbFGF (which is bioequivalent to

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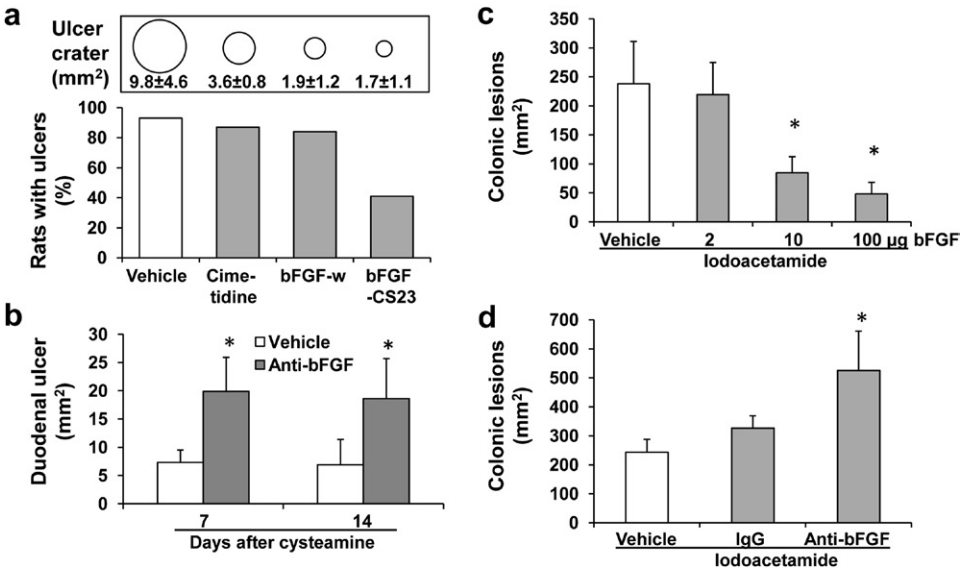

FIGURE 1 Effects of bFGF on DU and UC in rats. (a): Healing of cysteamine-induced DU by cimetidine, wild-type bFGF (bFGF-w) or mutein bFGF (bFGF-CS23). (b): Aggravation of cysteamine-induced DU by bFGF neutralizing antibody. (c), (d): Colonic lesions in iodoacetamide-induced UC after treatment with recombinant rat bFGF or neutralizing anti-bFGF antibody (anti-bFGF). $*p < 0.05$.

rbFGF-CS23), CMC-hrbFGF 9 (a carboxymethyl cysteine derivative of rhbFGF) and PEG-rhbFGF (a polyethylene glycol derivative of hrbFGF). Oral administration of these novel derivatives for 21 days accelerates the healing of cysteamine-induced chronic DU, and PEG-rhbFGF is more active than the other analogs.[28,29]

In a series of experiments, po administration of a mutein of rhbFGF twice a day for 2 weeks significantly accelerates the healing of acetic acid-induced GU in rats: the size of the ulcers decreases, whereas regeneration of the mucosa is enhanced. The same mutein of rhbFGF has no effect on the development of ethanol-induced acute gastric erosions when given to rats before ethanol administration (unpublished results). These results suggest that although bFGF accelerates ulcer healing, it does not exert acute gastroprotection.

Besides the ulcer healing and angiogenic actions, bFGF promotes the reinervation of the newly formed microvessels and regeneration of autonomic nerves in the granulation tissue in the experimental GU induced by acetic acid in rats.[16,17] One human study tested the efficacy and safety of bFGF-CS23 in healing of NSAID-associated GU, which are resistant to or relapse after conventional treatment.[16] Five patients with nine ulcers were treated po and after four weeks, four ulcers had healed, and there was a significant reduction in the size of other ulcers. This is a clinical confirmation of the potent antiulcer effect of bFGF, initially discovered in our animal model.[11] We now know that several forms of bFGF are available, and there is additive antiulcer effect between bFGF and H2 receptor antagonists.[10,27,30]

Our results also support a physiologic role for endogenous bFGF in ulcer healing. Endogenous bFGF was visualized by immunostaining techniques in the normal rat duodenum after administration of the duodenal ulcerogen cysteamine, and bFGF immunoreactivity was time dependently reduced in the mucosa, submucosa, and muscularis propria.[33] The localization of bFGF immunoreactivity was also studied in acetic acid-induced experimental GU in rats. bFGF binding sites, observed in the basal portion of the gastric mucosa in the control rats, are markedly increased in the regenerated tissues around the acetic acid-induced ulcer.[15]

Western blot analysis of rat duodenal mucosa revealed a rapid depletion of mucosal cytoplasmic 18 kDa bFGF and increased synthesis of nuclear 21–25 kDa bFGF in cysteamine-induced ulceration in rats. At 12 h, we found a 2- to 3-fold increase in nuclear forms of bFGF, with a 50% decrease in the cytoplasmic form, whereas 48 hours after the administration of cysteamine, the samples showed a trend toward the pattern seen in control rats. We used ELISAs to actually measure the changes in duodenal mucosal concentration of bFGF during DU development in rats. Endogenous levels of duodenal mucosal bFGF started to increase at 12 h; they reached maximal values at 48 h, when they were significantly higher than the basal value. Despite intragastric administration of cysteamine, bFGF concentrations in the gastric mucosa did not change. Studies with neutralizing anti-bFGF antibodies in cysteamine-induced chronic DU models also support a role for endogenous bFGF in spontaneous ulcer healing: daily injection of these antibodies markedly delays the healing of experimental DU (Fig. 1b).

bFGF Treatment in UC

The effect of bFGF on UC healing was studied in a new chemically induced animal model of UC.[22] We showed that

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intracolonic administration of bFGF improved healing of UC induced by iodoacetamide (Fig. 1c), extending our previous demonstration that intragastric administration of bFGF accelerated healing of chronic DU induced by cysteamine in rats.[18,29] Both pharmacologic and histologic results showed that bFGF treatment significantly improves UC healing which, on the other hand, is impaired by the administration of anti-bFGF antibody (Fig. 1d). The potential molecular mechanisms of the therapeutic actions of bFGF on healing of UC seem to involve increasing angiogenesis and mucosal regeneration, and reducing the inflammatory response in the colon. bFGF seems to accelerate the healing of UC via increasing angiogenesis and cell proliferation in colonic mucosa. Our results showed that angiogenesis and proliferation of other cells (shown by Ki-67-positive staining) are significantly increased around ulcer craters in colonic mucosa treated with bFGF.[18] The finding of increased neovascularization indicates that bFGF facilitates healing via enhancing "normal" angiogenesis which is needed in UC healing. The increased Ki-67-positive cells in bFGF-treated rats with UC indicate that bFGF stimulates cell proliferation in colonic mucosa. Surprisingly, bFGF markedly decreases TNF-α levels and MPO activity in colonic tissues during healing of UC.[18] Matsuura et al.[14] also showed that gene expression of TNF-α is significantly reduced in DSS-induced UC after treatment with bFGF. TNF-α is one of the most important proinflammatory cytokines and directly influences intestinal epithelial cells. TNF-α disrupts the epithelial barrier, and induces apoptosis of epithelial cells and stimulates the secretions of chemokines from intestinal epithelial cells. It also activates the adaptive immune system of the bowel by recruiting and activating neutrophils and macrophages. MPO activity reflects the degree of neutrophilic infiltration and, therefore, is a specific biomarker of inflammation. The concentrations of both TNF-α and MPO in colonic tissues are strongly associated with the intensity of UC inflammation. Thus, reduced TNF-α and MPO levels by bFGF in healing of UC indicate that bFGF may play anti-inflammatory role in UC. These findings suggest that bFGF, unlike VEGF, is a beneficial angiogenic growth factor that provides a promising option for the treatment of UC. bFGF enema may be a clinically safe and useful route that may provide a new therapy way for UC. Moreover, our study showed that the effective doses of bFGF that we used are 4- to 10-fold less than the doses others used to treat UC induced by dextran sulfate sodium (DSS) in rats and mice.

Similar Therapeutic Effects of PDGF on Experimental DU and UC

PDGF was originally described as a product of platelets, but it is also synthesized and secreted by activated macrophages. It consists of two disulfide-linked polypeptides: chain A (14 kDa) and chain B (17 kDa) sharing 60% similarity. Thus, the PDGF dimer has three isoforms: PDGF-AA, -AB, and -BB.[1] Previous studies have shown that various cell types, including macrophages (major source), fibroblasts, capillary endothelial cells, and neutrophils express PDGF-A and/or -B mRNAs and proteins. Macrophage-derived PDGF induces chemotaxis and proliferation of monocytes and fibroblasts during inflammation and wound repair. PDGF plays a central role in the repair process, particularly in fibroblast and endothelial cell proliferation and angiogenesis after acute and chronic tissue injury. PDGF also signals and induces tissue remodeling, cellular differentiation, and morphogenesis. In addition, PDGF directs the migration, differentiation and function of a variety of specialized mesenchymal and migratory cell types during wound healing. Clinical studies showed that PDGF-B (Becaplermin) prevents the formation of diabetic foot ulcers and accelerates the healing of chronic lower extremity diabetic ulcers.[15]

Effect of PDGF on Experimental DU

Soon after recognizing the potent ulcer-healing effect of bFGF, our laboratory tested whether PDGF also accelerates the healing of experimental DU. Macroscopic and microscopic evaluations revealed that treatment with PDGF accelerates the healing of chronic cysteamine-induced ulcers (Fig. 2a), whereas anti-PDGF treatment with a specific neutralizing antibody shows significant aggravation and delayed healing of DU (Fig. 2b). It is important to stress that gastric acid secretion is not influenced by any dose of PDGF.

As with bFGF, ELISA and western blot studies investigated the changes of PDGF concentration in duodenal mucosa during DU development in a rat model.[33] The duodenal mucosal PDGF-AB concentration is elevated at 12 h and reaches its peak at 24 h after cysteamine administration. It decreases to the control value at 48 h after the first dose of cysteamine and decreases further during the healing phase of experimental DU. A high degree of organ specificity for the changes in PDGF-AB in the proximal duodenum is implied by the failure to detect any alteration of PDGF level in the gastric mucosa, despite the intragastric administration of cysteamine.[33] Western blot studies confirm the presence of duodenal mucosal PDGF during the natural history of experimental DU.

As a follow-up to our studies, the effects of bFGF and PDGF were investigated on the migration and proliferation of cultured rabbit gastric epithelial cells. Although any dose of bFGF has virtually no effect on the epithelial cells, PDGF significantly increases the migration of cultured epithelial cells in a dose-dependent manner. The action of PDGF-BB was investigated in addition to the confluent rabbit gastric epithelial cells after wounding.[35] PDGF-BB dose dependently accelerates the migration and proliferation of cultured cells.

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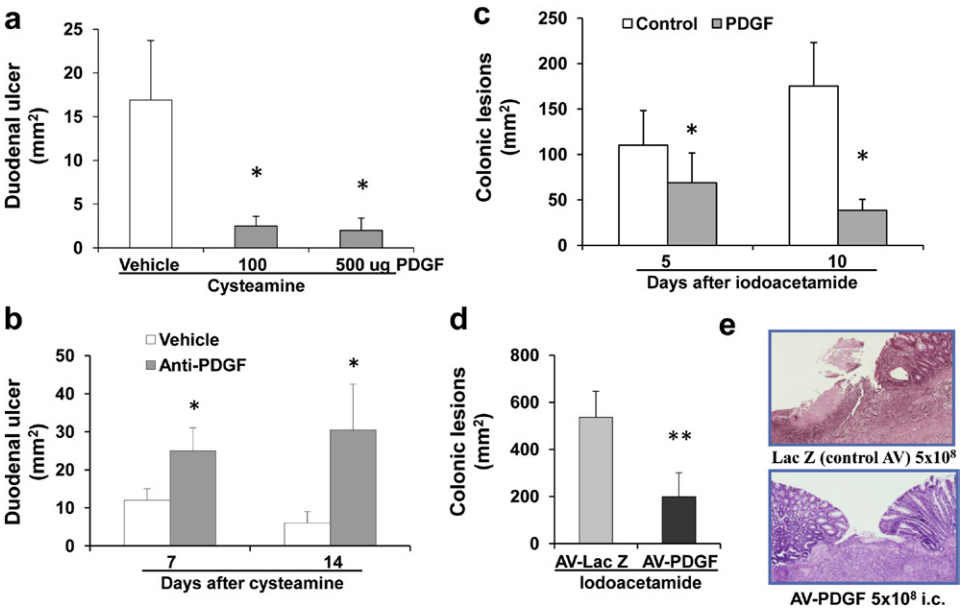

FIGURE 2 Role of PDGF-B in DU and UC in rats. (a), (b): Effect of PDGF-BB and PDGF inhibition by a neutralizing antibody on cysteamine-induced DU in rats. (c): Treatment with PDGF in iodoacetamide-induced UC in rats. (d), (e): Gene therapy using adenoviral vector (AV) encoding PDGF-BB in iodoacetamide-induced UC. $*p < 0.05$; $**p < 0.01$.

Therefore, PDGF-BB may have a role in gastric epithelial cell restoration during the healing of GU.

PDGF Treatment in Experimental UC

We also tested the effects of PDGF-BB on the healing of UC induced by iodoacetamide in rats. PDGF-BB treatment significantly decreases the severity of colitis and extent of adhesions after 5 days of treatment. All parameters, that is, the colonic lesions, severity of colitis, extent of adhesions, and colon wet weight significantly improved 10 days after the treatment (Fig. 2c). Histologically, the ulcer size is smaller, the signs of inflammation are reduced, and in most situations extensive reepithelization is seen. Furthermore, we tested the effects of gene therapy with adenoviral vector (AV) encoding PDGF-BB on the healing of UC. The results showed that treatment with a single dose of AV-PDGF-BB significantly reduces colonic lesions (Fig. 2d). The colonic dilation, colonic thickness, colon wet weight, pericolonic adhesions, and body weight loss are significantly diminished in the group treated with double doses of AV-PDGF-BB. Histology showed advanced reepithelialization of the ulcer after AV-PDGF-BB treatment (Fig. 2e). This is the first demonstration that intracolonic PDGF-BB significantly accelerates the healing of UC in rats.

Different Effects of VEGF on Experimental DU and UC

VEGF is a heparin-binding glycoprotein that occurs in four molecular forms, consisting of 121, 165, 189, and 206 amino acids derived from the same gene by alternative mRNA splicing. In addition to stimulating endothelial cell proliferation, migration, and tube formation (angiogenesis), VEGF, also known as vascular permeability factor (VPF), acts specifically on vascular endothelial cells to increase vascular permeability.[29] Its unique combination of properties largely result from its specific receptors on endothelial cells. VEGF also increases blood flow and apparently prevents endothelial cell apoptosis. Its expression is highly regulated by hypoxia, providing a physiologic feedback mechanism to accommodate insufficient tissue oxygenation by promoting blood vessel formation.[8]

VEGF Treatment in Experimental DU

Because VEGF is the most specific angiogenic peptide, and when this peptide became available for pharmacologic studies in 1997, we again tested the hypothesis that stimulation of angiogenesis alone is sufficient for chronic ulcer healing.[31] The results revealed that treatment with angiogenic factor VEGF significantly reduces the size of DU (Fig. 3a), histologically accompanied by complete healing, prominent angiogenesis, and granulation tissue production. The density of blood vessels is significantly increased in the VEGF-treated rats. Our anti-VEGF study on DU healing showed that inhibition of VEGF by a specific neutralizing antibody significantly delays the healing of DU induced by cysteamine (Fig. 3b).

After we showed that daily intragastric administration of angiogenic growth factors such as bFGF, PDGF, or VEGF accelerates the healing of chronic DU in rats, we hypothesized that a single dose of gene therapy related to these

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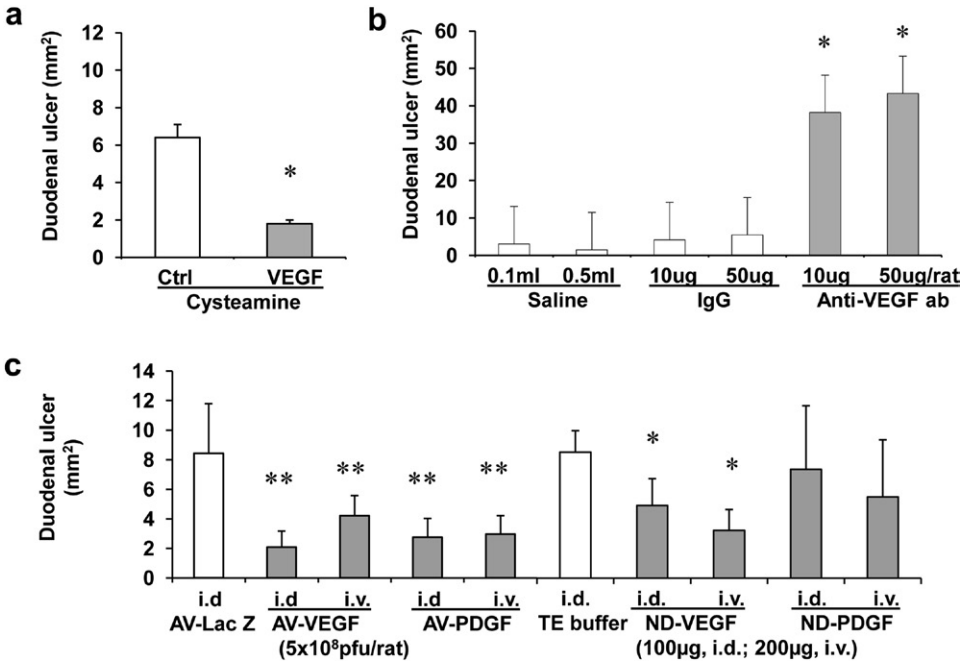

FIGURE 3 Effect of VEGF on DU induced by cysteamine in rats. (a), (b): Healing of DU after recombinant human $VEGF_{165}$ or neutralizing anti-VEGF antibody (anti-VEGF ab). (c): Gene therapy with naked DNA (ND) or adenoviral vector (AV) encoding VEGF in the experimental DU. i.d.: intraduodenal injection; i.v.: intravenous injection; $*p < 0.05$; $**p < 0.01$.

growth factors may be enough to accelerate the healing of DU through enhancement of synthesis of endogenous angiogenic growth factors. Our results showed that a single dose of naked or adenoviral vector DNA of VEGF or PDGF significantly accelerates the healing of DU (Fig. 3c, d). It suggests that gene therapy with either VEGF or PDGF may be a rapid approach to achieve DU healing.[4]

Because VEGF/VPF is highly specific for endothelial cells, we tested the hypothesis that the slightly increased vascular permeability (creating a "histodilutional barrier") might induce gastroprotection. The results showed that pretreatment with the doses (0.04 and 0.2 μg/100 g) of VEGF/VPF significantly reduces the gastric mucosal lesions (Fig. 4a). Histology showed mildly or moderately increased gastric edema in lamina propria or submucosa and only superficial or no gastric erosions in the rats treated with VEGF/VPF (Fig. 4b).

From these results, we concluded that oral treatment of rats with VEGF/VPF accelerates the healing of cysteamine-induced chronic DU and that this healing is accompanied by the stimulation of angiogenesis and granulation tissue production and complete mucosal restoration, VEGF/VPF also provides gastroprotection, which might be related to a histodilutional barrier induced by VEGF/VPF.

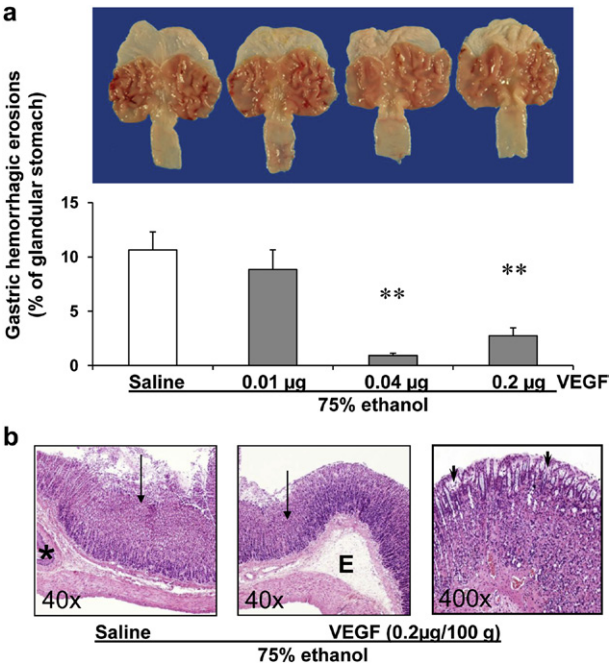

FIGURE 4 Gastroprotection by VEGF in experimental gastric erosions. (a): Gross appearance and quantitative analysis of gastric hemorrhagic erosions induced by 75% ethanol in rats pretreated with recombinant rat $VEGF_{164}$. (b): In control rats, hemorrhagic erosions involve about half of the gastric mucosa (arrow) in the glandular stomach (*). In VEGF-pretreated rats, only mild superficial (arrow) or no mucosal damage is seen, in addition to edema (E) in lamina propria (arrowheads) and submucosa.$**p < 0.01$. See color plate 42.

VEGF Role in Experimental UC

VEGF is a most potent angiogenic factor in the growth factor family. After we showed that therapy with peptide,

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especially, gene of VEGF produces fast healing of experimental DU,[31,25] we predicted a similar beneficial effect of this angiogenic growth factor in experimental UC. We were not only surprised that we did not get the predicted results, but VEGF slightly aggravated experimental UC. Subsequently, we showed a concurrently increased expression of angiogenic VEGF and antiangiogenic endostatin and angiostatin in experimental UC, creating an "angiogenic imbalance" or inappropriate angiogenic response.[21] Furthermore, we realized that the initial name of this peptide was VPF because it increased vascular permeability that represents the first step in inflammation.[5] Thus, the next logical step was to determine the possible mechanistic role of VEGF in the pathogenesis of UC. In the first experiment, we neutralized VEGF in rats with the specific anti-VEGF antibody. Treatment with anti-VEGF antibody has a beneficial effect on the course of iodoacetamide-induced UC, reducing diarrhea and lethargy scores and the macroscopic and microscopic lesions in the colon.[32] Neutralization of VEGF not only reduces VEGF levels but also counteracts the increased vascular permeability in the early stages of experimental UC and decreased the number of infiltrated inflammatory cells in the pathogenesis of UC. Moreover, we found significantly decreased number of leukocytes in the advanced stages of experimental UC after anti-VEGF therapy.[32] However, the widely used antiangiogenic agent bevacizumab (an anti-VEGF antibody) is associated with an increased risk of gastrointestinal perforation and skin ulcers in patients.[20]

Matsuura et al. [14] found that rectal administration of human recombinant bFGF to normal mice significantly increases the expression of VEGF in colonic tissue, but the expression of VEGF in mice with experimental UC after bFGF treatment is lower than that in the untreated mice with colitis in a dose-dependent manner and directly correlates with a beneficial dose of bFGF. We proposed that the beneficial effect of anti-VEGF therapy in experimental UC results from attenuation of VEGF-induced vascular permeability, resulting in reduced vascular leakage and inflammatory cell infiltration. It has been postulated that VEGF produces an abnormal "pathologic" angiogenesis in UC, which plays a critical role in the pathogenesis of UC.[2,32] Scaldaferri et al. [23] showed that VEGF directly links angiogenesis and inflammation in the pathogenesis of both human and experimental UC. Increasing evidence suggests that VEGF is one of the major pro-angiogenic factors involved in pathologic angiogenesis. Blockade of VEGF, that is, antiangiogenesis, may represent a new therapeutic option for IBD.

In addition to bFGF, PDGF, and VEGF, epidermal growth (EGF) has been used to heal experimental gastric, duodenal, and colonic ulcers in animal models.[10,13,26,27,30] Among these, only EGF has an effect on gastroduodenal secretion, whereas the other peptides stimulate virtually all the cellular elements of ulcer healing.[25] Thus, the most consistent common effect of these growth factors in ulcer healing is the stimulation of cell proliferation, especially angiogenesis. The molar potency of bFGF, PDGF, and VEGF is 2–7 million times higher than that of cimetidine in the healing of cysteamine-induced chronic DU in rats.[25] Because VEGF has no major effects other than stimulation of angiogenesis, it is probably safe to conclude that enhancement of angiogenesis, which is accompanied by granulation tissue production, is the most important cellular event leading to ulcer healing. Apparently, spontaneous proliferation and migration of epithelial cells over dense granulation tissue then complete the healing process.

Differential Expression and Pharmacologic Effects of PlGF on Experimental DU and UC

PlGF is a pleiotropic cytokine that stimulates endothelial cell growth, migration, and survival; chemoattracts angiocompetent macrophages and bone marrow progenitors. PlGF selectively binds to VEGFR-1. Besides indirect effects,[19] PlGF signals directly via VEGFR-1, thus, acting independently of VEGF in endothelial cells, macrophages, bone marrow progenitors, and tumor cells, which primarily express VEGFR-1.[19] Gene inactivation studies have revealed that PlGF deficient mice are viable and healthy, indicating that endogenous PlGF is redundant for vascular development. Genetic studies show that PlGF is redundant for vascular development and physiologic vessel maintenance in healthy adults and maintenance but contributes to the angiogenic and inflammatory switch in various diseases, including tumor growth, ischemia, and chronic inflammation. Many cell types express PlGF in pathologic conditions, including endothelial cells, smooth muscle cells, fibroblasts, leukocytes, and bone marrow progenitors.[6,7] Furthermore, PlGF is readily upregulated in pathologic conditions by various stimuli, such as hypoxia, nitric oxide, inflammatory cytokines (e.g. interleukin 1 and TNF-α), and growth factors (e.g. VEGF-A and transforming growth factor-β). Overall, the expression profiling and genetic findings raise the question of whether PlGF is a specific pathologic angiogenic factor.[6,7] If this is shown to be the case, PlGF could be an attractive drug target, because a PlGF inhibitor would be expected to selectively inhibit pathologic angiogenesis without affecting the growth or maintenance of normal vessels and, unlike VEGF/VEGFR inhibitors, would therefore be likely to cause fewer side effects.

Differential Expression of PlGF in Experimental DU and UC in Rats

Because PlGF has been reported as markers of pathologic angiogenesis, we tested the hypothesis that the expression of PlGF might be different between experimental DU and UC. Western blotting showed that the expression of PlGF

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is markedly decreased on the 3rd and 7th days after cysteamine administration but highly increased on the 3rd and 7th days after iodoacetamide administration in rats. This is the first demonstration that PlGF is clearly expressed in the intestines of rats, and the differential expression of PlGF in DU and UC may indicate two distinct angiogenic processes, for example, normal and pathologic angiogenesis occurring during the development and healing of DU and UC, respectively (unpublished results).

Differential Effect of PlGF Inhibition on DU and UC Rats

To determine the pharmacologic role of PlGF in DU and UC, we tested the effect of neutralizing anti-PlGF antibody on cysteamine-induced DU and iodoacetamide-induced UC in rats. The results showed that inhibition of PlGF significantly reduced colonic lesions from 249.1 ± 77.1 (control) to 110.1 ± 73.7 mm^2 ($p < 0.05$), whereas the sizes of DU were not significantly different between controls and anti-PlGF antibody-treated rats. This may further indicate that different angiogenesis are involved in these two GI ulcers. Thus, upregulated PlGF may indicate novel mechanisms of pathologic angiogenesis in UC (unpublished results).

Different Pharmacologic Effects of Rac1 on Experimental DU and UC

Rac1 is a key member of the Rho GTPase family involved in the regulation of cell migration, proliferation, survival, and cell–cell junctions by virtue of its ability to regulate the actin-based cytoskeleton and nuclear gene expression. The activation of Rac1 is mediated by guanine nucleotide exchange factors in response to cytokine stimulation. Rac1 is essential for embryonic angiogenesis and selective deletion of the Rac1 gene in endothelial cells caused defective neovascularization, which was incompatible with life. Endothelial cell culture studies show essential roles for Rac1 in stretch-provoked proliferation, directed migration toward the gradient of thrombin, sphingosine 1-phosphate, VEGF, and Ephrin-A1 and tube formation in a three-dimensional in-gel environment.[34] The genetic deletion of Rac1 causes reduced endothelial cell migration, tube formation, adhesion, and increased vascular permeability. Rac1 has been also suggested to regulate the functions of endothelial progenitor cells, which crucially contribute to neovascularization. Rac1 is also essential for epithelial stem cell function during dermal and oral mucosal wound healing in mice and active Rac1 improves pathologic VEGF neovessel architecture and reduces vascular leak,[34] whereas expression of a dominant inhibitory mutant of Rac1 delays epidermal wound healing. In these studies, excisional wound healing of the skin is also delayed by conditional deletion of the Rac1 gene. The development of the Rac1 specific inhibitor (NSC23766) and agonist (SEW2871) will support the investigation of the roles of Rac1. The technical advance made with these new tools to specifically stimulate or inhibit Rac1 function *in vivo* provides an opportunity for us to reveal the roles of Rac1 in upper and lower GI ulcer diseases such as DU and UC.

Accelerated DU Healing but Impaired UC Healing by Inhibition of Rac1 in Rats

Because Rac1 has been shown to play an important role in epidermal wound healing,[34] we tested the hypothesis whether inhibition of rac1 would affect healing of experimental DU and/or UC. The results show that treatment with a selective inhibitor of Rac1 (NSC23766) accelerates DU healing but delays UC healing. Quantitative data showed that DU craters are significantly reduced from 15.4 ± 3.2 (controls) to 8.7 ± 1.1 mm^2 and UC lesions are increased from 68.3 ± 9.4 (controls) to 102.6 ± 11.7 mm^2. We thus conclude that, in contrast to VEGF, Rac1 seems to play a beneficial role in UC healing but a harmful role in DU healing, indicating that Rac1 may reverse VEGF actions in these two ulcer models (unpublished results).

SUMMARY

Increasing evidence indicates that angiogenesis is a novel and crucial element in the pathogenesis of DU and UC. In this review, we mostly summarized our recent results on ulcer healing with either peptides or genes of angiogenic growth factors, such as bFGF, PDGF, and VEGF without affecting HCl and pepsin secretion. Studies performed in animal models and in humans also show a key role of these endogenous angiogenic peptides in ulcer healing. The molar potency of angiogenic growth factors is 2–7 million times better than the antiulcerogenic effect of antisecretory H2 antagonists. Because histologically gastroduodenal ulcers look similar to the ulcers in the lower GI tract, we also predict that the healing of experimental UC might be also improved by these angiogenic growth factors. Rectal enemas containing bFGF or PDGF indeed accelerate the healing of chemically induced UC in rats. However, the results from our study and those of other studies show opposite effects of angiogenic or antiangiogenic therapies targeting VEGF on the healing of experimental UC. Thus, VEGF induces a "normal" angiogenesis, which accelerates DU healing but a pathologic "abnormal" angiogenesis, which impairs UC healing. It is also important to recognize the inducers and modulators of pathologic angiogenesis because anti-VEGF may also affect angiogenesis needed in normal tissue and organ regeneration. PlGF may be a marker of pathologic angiogenesis and may play critical roles in abnormal angiogenesis, for example, inhibition of PlGF does not affect quiescent vessels in healthy organs.

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In addition, activation of Rac1, a small GTPase, markedly improves VEGF-induced neovessel architecture defect and reduces vascular permeability in an established angiogenic model. Because anti-VEGF therapy causes gastric perforation and skin ulcers in patients, and pathologic angiogenesis is associated with architecturally defective and leaky blood vessels, PlGF and Rac1 may be selected as attractive therapeutic targets for the development of safe antiangiogenic drug. We thus conclude that stimulation of angiogenesis and cell proliferation is the most consistent mechanism of both upper and lower GI ulcers healing by growth factors such as bFGF and PDGF either with peptides or gene transfer. Although VEGF plays a critical role in pathogenesis of lower GI ulcer, for example, UC, enhancement of angiogenesis and granulation tissue production of VEGF is apparently beneficial for the healing of upper GI ulcers. Selective inhibition of pathologic angiogenesis targeting specific markers, for example, PlGF and Rac1 should be considered for GI ulcer healing. Hence, growth factors are potent, endogenously derived agents, which are directly associated with GI ulcer healing in which angiogenesis seems to be the most important process.

REFERENCES

1. Antoniades HN, Hunkapiller MW. Human platelet-derived growth factor (PDGF): amino terminal amino acid sequence. *Science* 1983;**220**:963–5.
2. Chidlow Jr JH, Shukla D, Grisham MB, Kevil CG. Pathogenic angiogenesis in IBD and experimental colitis: new ideas and therapeutic avenues. *Am J Physiol Gastrointest Liver Physiol* 2007;**293**:G5–18.
3. Danese S, Sans M, de la Motte C, Graziani C, West G, Phillips MH, et al. Angiogenesis as a novel component of inflammatory bowel disease pathogenesis. *Gastroenterology* 2006;**130**:2060–73.
4. Deng X, Szabo S, Khomenko T, Jadus MR, Yoshida M. Gene therapy with adenoviral plasmids or naked DNA of vascular endothelial growth factor and platelet-derived growth factor accelerates healing of duodenal ulcer in rats. *J Pharmacol Exp Ther* 2004;**311**:982–8.
5. Dvorak HF. Vascular permeability factor/vascular endothelial growth factor: a critical cytokine in tumor angiogenesis and a potential target for diagnosis and therapy. *J Clin Oncol* 2002;**20**:4368–80.
6. Fischer C, Jonckx B, Mazzone M, Zacchigna S, Loges S, Pattarini L, et al. Anti-PlGF inhibits growth of VEGF(R)-inhibitor-resistant tumors without affecting healthy vessels. *Cell* 2007;**131**:463–75.
7. Fischer C, Mazzone M, Jonckx B, Carmeliet P. FLT1 and its ligands VEGF-B and PlGF: drug targets for anti-angiogenic therapy? *Nat Rev Cancer* 2008;**8**:942–56.
8. Ferrara N, Davis-Smyth T. The biology of vascular endothelial growth factor. *Endocr Rev* 1997;**18**:4–25.
9. Folkman J, Shin Y. Angiogenesis. *J Biol Chem* 1992;**267**:10931–4.
10. Folkman J, Szabo S, Stovroff M, McNeil P, Li W, Shing Y. Duodenal ulcer: discovery of a new mechanism and development of angiogenic therapy which accelerates healing. *Ann Surg* 1991;**214**:414–26.
11. Hull MA, Cullen DJ, Hudson N, Hawkey CJ. Basic fibroblast growth factor treatment for non-steroidal anti-inflammatory drug associated gastric ulceration. *Gut* 1995;**37**:610–2.
12. Kojima T, Watanabe T, Hata K, Nagawa H. Basic fibroblast growth factor enema improves experimental colitis in rats. *Hepatogastroenterology* 2007;**54**:1373–7.
13. Konturek SJ, Brzozowski T, Dembinski A, Warzecha Z, Yamazaki J. Gastric protective and ulcer-healing action of epidermal growth factor. In: Garner A, Whittle BJR, editors. *Advances in drug therapy of gastrointestinal ulceration*. New York: Wiley-Interscience; 1989. p. 261–73.
14. Matsuura M, Okazaki K, Nishio A, Nakase H, Tamaki H, Uchida K, et al. Therapeutic effects of rectal administration of basic fibroblast growth factor on experimental murine colitis. *Gastroenterology* 2005;**128**:975–86.
15. Nagai MK, Embil JM. Becaplermin: recombinant platelet derived growth factor, a new treatment for healing diabetic foot ulcers. *Expert Opin Biol Ther* 2002;**2**:211–8.
16. Nakamura M, Ishii H. *Increased effector site of platelet-derived growth factor-BB and basic fibroblast growth factor during acetic acid-induced gastric ulcer healing. GI Forum-Molecular Biological Approach to Peptic Ulcer Disease* 1995. p. 13–16.
17. Nakamura M, Oda M, Inoue J, Ito T, Akiba Y, Kitajima M, et al. Effect of basic fibroblast growth factor on reinervation of gastric microvessels. Possible relevance to ulcer recurrence. *Dig Dis Sci* 1995;**40**:1451–8.
18. Paunovic B, Deng X, Khomenko T, Ahluwalia A, Tolstanova G, Tarnawski A, et al. Molecular mechanisms of basic fibroblast growth factor effect on healing of ulcerative colitis in rats. *J Pharmacol Exp Ther* 2011;**339**:430–7.
19. Ribatti D. The discovery of the placental growth factor and its role in angiogenesis: a historical review. *Angiogenesis* 2008;**11**:215–21.
20. Saif MW, Elfiky A, Salem RR. Gastrointestinal perforation due to bevacizumab in colorectal cancer. *Ann Surg Oncol* 2007;**14**:1860–9.
21. Sandor Z, Deng XM, Khomenko T, Tarnawski AS, Szabo S. Altered angiogenic balance in ulcerative colitis: a key to impaired healing? *Biochem Biophys Res Commun* 2006;**350**:147–50.
22. Satoh H, Sato F, Takami K, Szabo S. New ulcerative colitis model induced by sulfhydryl blockers in rats and the effects of anti-inflammatory drugs on the colitis. *Jpn J Pharmacol* 1997;**73**:299–309.
23. Scaldaferri F, Vetrano S, Sans M, Arena V, Straface G, Stigliano E, et al. VEGF-A links angiogenesis and inflammation in inflammatory bowel disease pathogenesis. *Gastroenterology* 2009;**136**:585–95.
24. Sobieraj DM, Coleman SM, Coleman CI. US prevalence of upper gastrointestinal symptoms: a systematic literature review. *Am J Manag Care* 2011;**17**:449–58.
25. Szabo S, Deng X, Tolstanova G, Khomenko T, Paunovic B, Chen L, et al. Angiogenic and anti-angiogenic therapy for gastrointestinal ulcers: new challenges for rational therapeutic predictions and drug design. *Curr Pharm Des* 2011;**17**:1633–42.
26. Szabo S, Folkman J, Vattay P, Morales RE, Kato K. Duodenal ulcerogens: effect of FGF on cysteamine-induced duodenal ulcer. In: Halter F, Garner A, Tytgat GNJ, editors. *Mechanisms of peptic ulcer healing*. London: Kluwer Academic Publishers; 1991. p. 139–50.
27. Szabo S, Folkman J, Vattay P, Morales RE, Pinkus GS, Kato K. Accelerated healing of duodenal ulcers by oral administration of a mutein of basic fibroblast growth factor in rats. *Gastroenterology* 1994;**106**:1106–11.
28. Szabo S, Sandor Zs. Basic fibroblast growth factor and PDGF in GI diseases. In: Goodlad RA, Wright NA, editors. *Bailliere's clinical gastroenterology*. London: WB Saunders; 1996. p. 97–112.

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29. Szabo S, Shing Y, Folkman J, Vincze A, Gombos Z, Deng X, et al. Angiogenesis and growth factors in ulcer healing. In: Fan TPD, Kohn EC, editors. *The new angiotherapy*. New Jersey: Humana Press; 2001. p. 199–211.
30. Szabo S, Vincze A. Growth factors in ulcer healing: lessons from recent studies. *J Physiol (Paris)* 2000;**94**:77–81.
31. Szabo S, Vincze A, Sandor Zs, Jadus M, Gombos Z, Pedram A, et al. Vascular approach to gastroduodenal ulceration. New studies with endothelins and VEGF. *Dig Dis Sci* 1998;**43**:S40–5.
32. Tolstanova G, Khomenko T, Deng XM, Chen L, Tarnawski A, Ahluwalia A, et al. Neutralizing anti-VEGF antibody reduces severity of experimental ulcerative colitis in rats. Direct evidence for the pathogenic role of VEGF. *J Pharmacol Exp Ther* 2009;**328**:749–57.
33. Vincze A, Nagata M, Sandor Z, Szabo S. ELISA and Western blot studies with basic fibroblast growth factor (bFGF) and platelet-derived growth factor (PDGF) in experimental duodenal ulceration and healing. *Inflammopharmacology* 1996;**4**:261–5.
34. Wang F, Yamauchi M, Muramatsu M, Osawa T, Tsuchida R, Shibuya M. Rac-1 regulates VEGF/Flt1-mediated cell migration via activation of a PI3-K/Akt pathway. *J Biol Chem* 2011;**286**: 9097–106.
35. Watanabe S, Wang XE, Hirose M, Oide H, Kitamura T, Miwa H, et al. Platelet-derived growth factor accelerates gastric epithelial restoration in a rabbit cultured cell model. *Gastroenterology* 1996;**110**:775–9.

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Chapter 160

Apelin

Song Han, George H. Greeley Jr. and Ella W. Englander

ABSTRACT

Apelin, the endogenous ligand for the APJ receptor was discovered about a decade ago. The aim of this chapter is give an overview of the physiology and pharmacology of apelin in the gastrointestinal tract and pancreas. Signaling cascades activated by apelin and its pathophysiology involvement are also discussed.

DISCOVERY

Apelin is the endogenous ligand for the APJ receptor, a receptor related structurally to the type 1 angiotensin receptor and a member of the seven transmembrane-domain G protein-coupled receptors (GPCRs).[5,30] Since the APJ receptor was characterized before apelin, apelin was identified by means of a "reverse pharmacology" approach in extracts of the cow stomach.[30] APJ is currently the only apelin receptor identified.

STRUCTURE OF THE APELIN mRNA/GENE, PRECURSOR PROCESSING

The apelin cDNA encodes a 77-amino-acid peptide, preproapelin, which is processed to apelin-36 and several smaller variants including apelin-17, -16, -13 and other forms. The human apelin gene is located on chromosome Xq25-26.1. Apelin-36 is most probably the parent peptide.[18,30] With the exception of a single amino acid, the C-terminal apelin-13 sequence [(Q/P) RPRLSHKGPMPF] is fully conserved in all species cloned to date implying that this segment is essential for activation of APJ. Comparison of amino acid sequences across species shows that goldfish preproapelin has a 97% homology with zebrafish preproapelin, a 43% homology with *Xenopus* preproapelin and an about 35% homology with mammalian preproapelins. The promoter regions of rat and human apelin gene have been cloned.[34] Upstream stimulatory factor (USF)-1/-2, a ubiquitous transcription factor that regulates numerous genes involved in metabolism exerts a stimulatory role over breast apelin expression during pregnancy and lactation in the rat.

APELIN DISTRIBUTION

Like many of the peptides expressed in the gastrointestinal tract (GI) tract and pancreas, apelin has a wide distribution in the body. Apelin is synthesized in the brain, heart, lung, kidney, liver, mammary gland, pituitary, white adipose tissue, skeletal muscle, GI tract, and pancreas.[5,7,15,18,23,24,30,32,34] APJ also has a wide distribution in the body. In the GI tract of the adult rat, apelin expression levels (mRNA) are maximal in the stomach and ileum with lower expression levels in the duodenum, jejunum and colon (Fig. 1). Although apelin expression levels are high in the fetal and postnatal rat stomach,[32,33] apelin peptide synthesis is apparently not activated until weaning since apelin immunopositive cells are not detected until weaning. In the adult rat stomach, apelin immunoreactivity is identified in mucous neck, parietal and chief cells (Fig. 2).[17,28] Apelin is also detected in gastric cells immunopositive for chromogranin A (CGA), a marker of enteroendocrine cells.

The ontogeny of enteric apelin and APJ expression have been studied in detail mainly in the mouse and rat.[33] In the mouse stomach, apelin expression levels are maximal at postnatal day 4 (P4), then decreases at the P16 and adult stages (Fig. 3). Apelin expression levels are significantly lower at embryonic day 18.5 (E18.5) when compared with levels at P4. In the duodenum, apelin expression levels are highest at the E18.5 and P4 stages, and then decrease at the P16 and adult stages. In the colon, apelin expression levels are maximal at the E18.5, P4, and P16 stages and much lower in the adult. In the mouse, overall apelin expression levels are of similar magnitudes in the stomach, duodenum, and colon. With regard to ontogeny of APJ expression in mice, expression levels are maximal at the E18.5 stage in the duodenum and colon (Fig. 3). In the mouse stomach, maximal APJ expression is at the E18.5 and P4 stages. Stomach, duodenal and colon APJ expression levels then decrease postnatally and into adulthood.

In the rat, breast apelin expression increases dramatically during pregnancy and lactation, and large amounts of

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00160-3
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apelin occur in milk.[7,32,34] Milk-borne apelin is mentioned since it may have a tropic role via the luminal route on gut development and growth. APJ is expressed in the neonatal rodent GI tract[33] and apelin stimulates gastric and intestinal epithelial cell proliferation.[10,32]

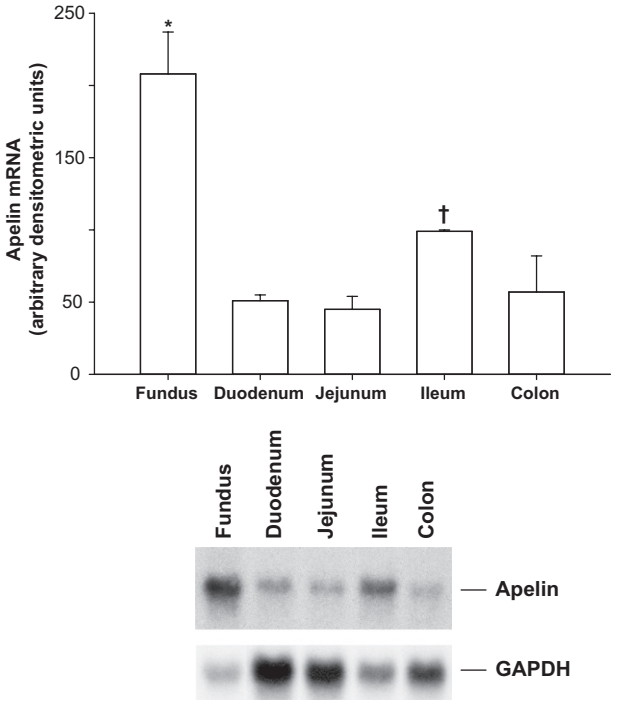

FIGURE 1 Apelin expression in the adult rat gastrointestinal tract. Mean apelin mRNA levels ± SEM of three rats is shown (top panel). Northern hybridization shows apelin mRNA levels in the rat GI tract (bottom panel). Apelin mRNA levels are not shown as a ratio. *, $P < 0.05$ vs. small and large intestine; †, $P < 0.05$ vs. duodenum, jejunum, and colon. (With permission from Wang G, Younes A, Wei W, et al. Apelin, a new enteric peptide: localization in the gastrointestinal tract, ontogeny, and stimulation of gastric cell proliferation and of cholecystokinin secretion. Endocrinology 145:1342–1348, 2004.)

SIGNALING CASCADES

Most of the studies that investigate intracellular signaling cascades activated by apelin use cell lines stably transfected with the APJ receptor, primarily Chinese hamster ovary (CHO) cells. In this experimental model, apelin inhibits forskolin-induced cAMP production.[21] Apelin also causes a sharp elevation in intracellular Ca^{++} levels in human NT2.N neurons.[20] In CHO cells, apelin induces phosphorylation of p70S6 kinase via two transduction cascades, a phosphatidylinositol 3-kinase (PI3 K)/Akt-dependent and an extracellular-regulated kinase (ERK)-dependent pathway.[20] Apelin also activates ERK via a pertussis toxin (PTX)-sensitive G protein in CHO cells. In mouse osteoblastic (MC3T3-E1) cells transfected with APJ, apelin signals by activating JNK and PI3 K/Akt pathways.[29] Apelin is also thought to signal via a nitric oxide (NO)-dependent mechanism to regulate blood pressure.[31] In mice, apelin-activated glucose uptake in skeletal muscle and adipose tissue involves endothelial NO synthase AMP-activated protein kinase and Akt.[3] Apelin's effects on glucose uptake and Akt phosphorylation are mediated partly by Gi and AMPK-dependent pathways.[35]

GI AND PANCREATIC ACTIONS OF APELIN

Apelin has been shown to influence GI secretion, motility, and cell proliferation. In the stomach, apelin secreted by parietal cells exerts a negative feedback action on ECL cells which lowers acid secretion.[17] In mice, central administration of apelin inhibits GI motility.[19] Apelin also stimulates duodenal electrolyte secretion which is apparently mediated by intestinal cholecystokinin (CCK) secretion.[6] Apelin injected intravenously reduced pancreatic juice volume, protein and trypsin outputs in a dose-dependent manner.[14] Apelin given directly into the intestinal lumen increases pancreatic protein and trypsin secretion through a vagal

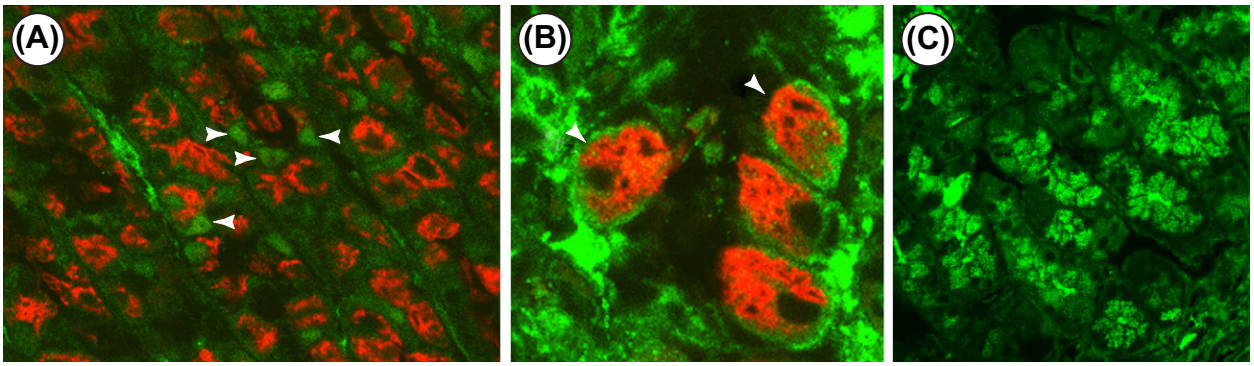

FIGURE 2 Immunofluorescence detection of apelin containing cells in rat stomach. **Panel A**. In the isthmus–neck region of the rat gastric mucosa, mucous neck cells produce apelin (green fluorescence, arrowheads) whereas parietal cells do not produce apelin. Parietal cells are identified by immunostaining for the H^+/K^+ATPase/proton pump (red fluorescence). **Panel B**. In the upper portion of the gastric mucosa parietal cells stain for apelin (arrowheads). Notice that apelin staining (green fluorescence) is stronger at the cell periphery suggesting that cellular localization of the proton pump differs from that of apelin. **Panel C**. Immunofluorescence detection of apelin-containing cells in the base region of the stomach mucosa (green fluorescence). Notice the granular nature of the apelin staining. (With permission from Susaki E, Wang G, Cao G, et al. Apelin cells in the rat stomach. Regul Pept 129:37–41, 2005.). See color plate 43.

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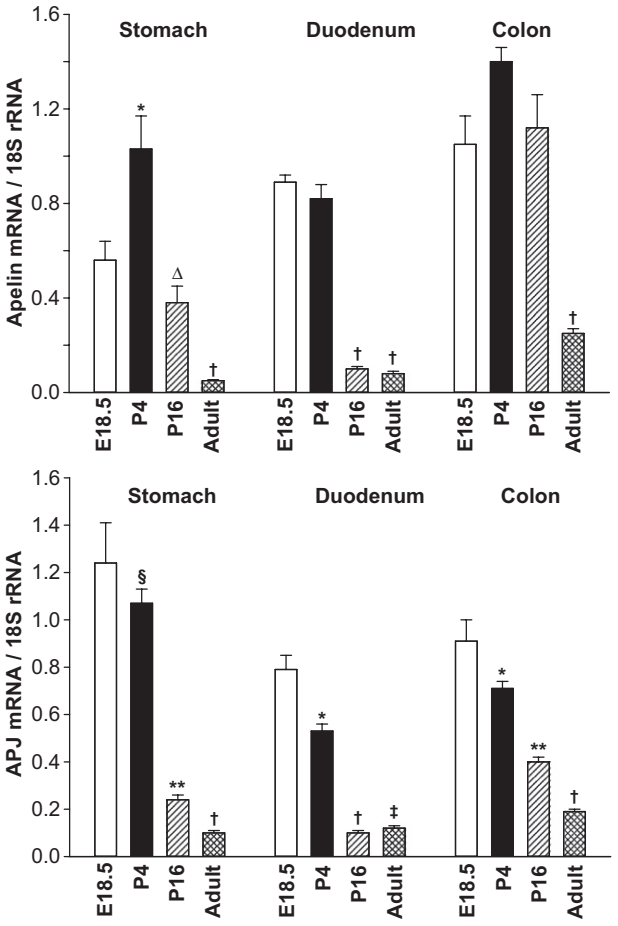

FIGURE 3 Ontogeny of apelin and APJ expression in the mouse stomach, duodenum and colon. $N = 5$ mice/group. $^{*}P < 0.05$ vs. E18.5, P16, adult values; $^{**}P < 0.05$ vs. E18.5, P4, adult values; $^{\Delta}P < 0.05$ vs. P4, adult values; $^{\dagger}P < 0.05$ vs. younger ages; $^{\S}P < 0.05$ vs. P16, adult values. E18.5 = embryonic day 18.5; P4 = postnatal day 4. (With permission from Wang G, Kundu R, Han S, et al. Ontogeny of apelin and its receptor in the rodent gastrointestinal tract. Regul Pept 158:32–39, 2009.)

mechanism. Additionally, apelin-13 can stimulate proliferation of human gastric (SIIA) epithelial cells.[32] Apelin-36 is less potent than apelin-13. Together, these findings indicate that apelin affects a wide array of GI functions.

Apelin and APJ have been localized in pancreatic beta cells of several mammalian species.[25,26] Apelin expression is also detected in human and mouse alpha cells and in a subpopulation of pancreatic polypeptide (PP) cells in human islets. Interestingly, apelin has been shown to inhibit as well as to enhance insulin secretion.[13,25,26] *In vitro*, a high dose (10^{-6} M) of apelin-36 enhances glucose-stimulated insulin secretion by 30% and lower apelin doses (10–100 nM) potently inhibit insulin secretion (50%).[25] In mice, iv administration of apelin-36 (2 nmol/kg) inhibits iv-glucose-induced insulin secretion by 35%.[26] Apelin-36 (10^{-9} M) inhibits glucose-induced insulin secretion from mouse islets. Centrally administered apelin causes hyperinsulinemia, hyperglycemia, glucose intolerance, and insulin-resistance.[4] These findings suggest that CNS apelin has a role in the regulation of glucose and insulin homeostasis.

Apelin exerts a potent glucose-lowering effect that is associated with increased glucose use in skeletal muscle and adipose tissue in mice.[3] Apelin signaling involves endothelial NO synthase, AMP-activated protein kinase, and Akt apelin-provoked glucose uptake. In whole body apelin gene knockout mice (APKO), chronic apelin deficiency results in reduced insulin sensitivity, hyperinsulinemia, and lower circulating adiponectin levels.[35] Soleus muscle lysates have a diminished insulin-induced Akt phosphorylation. *In vivo*, apelin replacement restores insulin sensitivity. In terms of a mechanism underlying the impaired insulin sensitivity in APKO mice, apelin increases glucose uptake and Akt phosphorylation in isolated C2C12 myotubes. Apelin's actions are blocked by pertussis toxin, compound C, and siRNA knockdown of AMPKα1, reduced by phosphoinositide 3-kinases inhibitor, LY-294002, and unaffected by NO synthase inhibitor, L-NAME treatment. Apelin's effects on glucose uptake and Akt phosphorylation are mediated partly by a Gi and AMPK-dependent pathways. In adipocytes, apelin signaling may be mediated by pathways involving Gq, Gi, and AMP-activated protein kinase.[35]

PATHOPHYSIOLOGICAL IMPLICATIONS

Effects of Intestinal Inflammation on Apelin Production

In rodents, induction of experimental colitis with either dextran sodium sulfate (DSS) or trinitrobenzenesulphonic acid (TNBS) results in increased colonic apelin mRNA and peptide levels (Fig. 4).[10] In the DSS model, intestinal elevations in apelin production occur during the inflammatory and repair phases. Increased apelin immunostaining occurs at the surface epithelium, in epithelial cells along the length of the tubular glands of the colon, and in the stem cell region at the gland base. Apelin administration during the recovery phase of DSS-induced colitis increases colonic epithelial cell proliferation, implying that the increased production of endogenous apelin during the recovery stage has a role in epithelial repair. Increased colonic apelin immunostaining is observed in human subjects with ulcerative colitis (UC) and Crohn's disease.[10]

Numerous studies have shown that cellular hypoxia occurs during intestinal inflammation in patients with inflammatory bowel disease (IBD) and in laboratory animals with experimental colitis.[11] Since cellular hypoxia activates production of a hypoxia sensitive transcription factor, hypoxia-inducible factor-1 (HIF-1), the influence of hypoxia on activation of apelin expression has been examined.[9] Exposure of rat pups to acute respiratory hypoxia

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increases hepatic, stomach-duodenal, and colonic apelin mRNA levels 10-, 2-, and 2-fold, respectively. Furthermore, hypoxia increases colonic APJ mRNA levels. *In vitro* studies confirm the *in vivo* findings and show that exposure of cultured cells to hypoxia elevates apelin and APJ mRNA levels. The hypoxia-induced elevation in apelin expression is mediated by HIF, since HIF-activated apelin transcriptional activity is dependent on an intact, putative HIF binding site in the rat apelin promoter (Fig. 5). Site-directed mutagenesis of putative HIF binding sites abrogates HIF-1 induced apelin promoter activity.

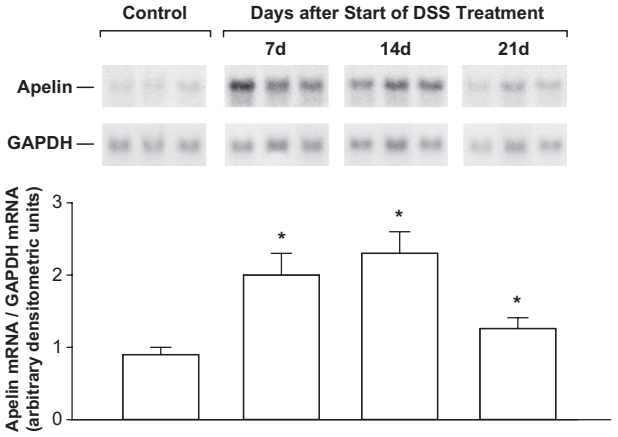

FIGURE 4 Northern analysis of colonic apelin expression in rats with DSS-induced colitis. DSS was given in drinking water ad lib for 5 days and replaced with tap water at the end of day 5. Colonic apelin mRNA levels were measured using poly(A)$^+$ RNA extracts of colon tissue harvested at different times after induction of colitis. Apelin expression was normalized to colon GAPDH mRNA levels. The mean ± SEM of three rats is shown. $^*P < 0.05$ vs. controls. (With permission from Han S, Wang G, Qiu S, et al. Increased colonic apelin production in rodents with experimental colitis and in humans with IBD. Regul Pept 142:131–137, 2007.)

The Jak/Stat signaling pathway is involved in the stimulatory influence of elevated proinflammatory cytokines on apelin promoter activity and expression lebels.[8] *In vivo* and *in vitro* experiments used lipopolysaccharide (LPS) — activated proinflammatory cytokine production and proinflammatory cytokine-treated [interleukin-6, (IL-6) and interferon (IFN)-γ] rodents or enteric cells to activate apelin expression. Pharmacological blockade of Jak/Stat signaling or IL-6 antibody administration inhibits elevations in enteric apelin expression. Furthermore, transient transfection experiments showed that LPS, IL-6 or IFN-γ increase apelin expression by stimulating apelin promoter activity. Blockade of Jak/Stat signaling suppresses IL-6- and IFN-γ-induced apelin promoter activity and elevations in enteric apelin expression in cultured ileal cells exposed to media harvested from LPS-treated macrophages. More importantly, transient transfection experiments in macrophages showed that the LPS-induced elevation in apelin promoter activity is abolished by mutation of a putative Stat3 binding site in the rat apelin core promoter (Fig. 6). Chromatin immunoprecipitation (ChIP) assays showed that phospho-Stat3 is recruited to the apelin promoter. Together, these results indicate that binding of phospho-Stat3 to the apelin promoter is the final step behind proinflammatory cytokine-induced enteric apelin up-regulation during intestinal inflammation.

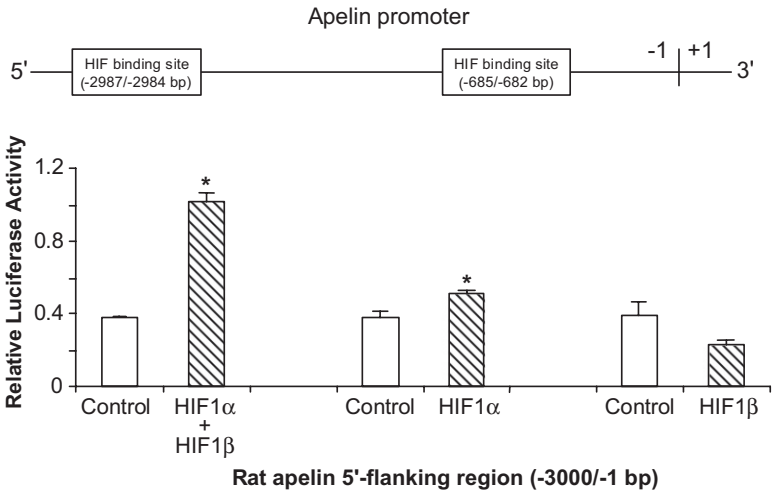

FIGURE 5 Overexpression of HIF-1α- and HIF-1β-stimulated apelin promoter activity. Top: schematic showing that sequence analysis identified 2 putative HIF binding sites in the rat apelin 5′-upstream region (–2987/–2984 and –685/–682 bp). Bottom: transient transfection experiments using an apelin promoter-luciferase reporter construct (–3000/–1 bp) in liver (HepG2) cells showed that overexpression of HIF-1α plus HIF-1β increased apelin promoter activity significantly. Transfection of HIF-1α alone increased promoter activity marginally, whereas transfection with HIF-1β was ineffective. Liver (HepG2) cells were transfected with either an empty expression vector (control) or a HIF expression plasmid. $^*P < 0.05$ vs. control. (With permission from Han S, Wang G, Qi X, et al. A possible role for hypoxia-induced apelin expression in enteric cell proliferation. Am J Physiol Regul Integr Comp Physiol. 294:R1832–R1839, 2008.)

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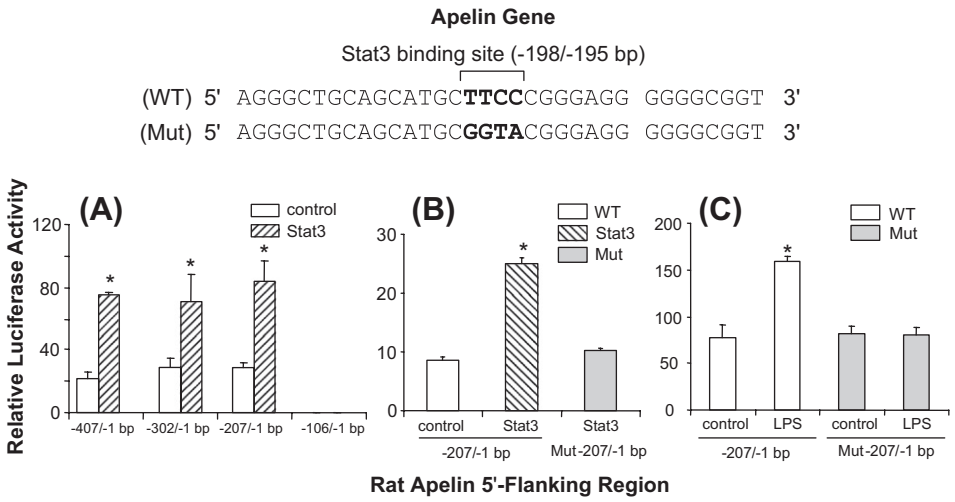

FIGURE 6 A putative Stat3 binding site regulates apelin promoter activity. Top schematic: a putative Stat3 binding site is identified in the wild-type (WT) apelin promoter sequence of the rat (–198/–195 bp); mutated nucleotides are indicated in bold in the mutated (Mut) sequence. Only partial sequences of WT and Mut promoters are shown. A: transient transfection experiments show that Stat3 overexpression increases apelin promoter activity in apelin promoter deletion-luciferase reporter constructs (–407/–1, –302/–1, –207/–1 bp). Promoter activity of the –106/–1 bp apelin promoter reporter construct was not influenced by Stat3 overexpression. B: mutation of a putative Stat3 binding site (–198/–195 bp) in the rat apelin core promoter abolished the elevation in apelin promoter activity induced by Stat3 overexpression. C: mutation of the putative Stat3 binding site inhibited the elevation in apelin promoter activity induced by LPS treatment. $*P < 0.05$ vs. control levels. (With permission from Han S, Wang G, Qi X, et al. Involvement of a Stat3 binding site in inflammation-induced enteric apelin expression. Am J Physiol Gastrointest Liver Physiol 295:G1068–G1078, 2008.)

Apelin and Metabolic Disorders

There is much interest in the physiological role of apelin in obesity and diabetes. Apelin is expressed in adipocytes.[1] Apelin is thought to be secreted by adipose tissue into the systemic circulation and therefore is considered an adipokine. Several human studies show that plasma apelin levels are increased and correlate with body mass index in obese subjects.[1,12] Further reports argue that obesity itself does not increase apelin production but instead speculate that insulin regulates apelin gene expression in adipocytes.[1,26] This idea is based on the finding of large elevations in both adipose tissue apelin expression and plasma apelin plasma levels in mice with hyperinsulinemia-associated obesity. A reduced apelin expression in adipocytes of insulin deficient mice having streptozotocin-induced diabetes was also shown. Additionally, as with pancreatic insulin content, apelin expression in adipose tissue is reduced by fasting and restored by refeeding. Insulin-stimulated apelin expression in fat tissue is associated with stimulation of phosphatidylinositol 3-kinase, protein kinase C and MAPK. Together, these results show that insulin exerts a direct control over apelin gene expression in adipocytes. Treatment of either mice or adipocytes in culture with tumor necrosis factor (TNF)-α, a secretory product of adipose tissue, stimulates apelin expression.[2] The systemic elevation in TNF-α levels may drive the increased blood apelin levels during obesity. Growth hormone (GH) has also been shown to stimulate apelin expression and secretion in mouse 3T3-L1 adipocytes.[15] The stimulatory action of GH is mediated partly by janus kinase 2 and phosphatidylinositol 3-kinase. Lastly, although numerous reports describe obesity-associated changes in circulating apelin levels, a recent paper showed numerous hurdles in measuring authentic apelin in the general circulation.[22]

REFERENCES

1. Boucher J, Masri B, Daviaud D, Gesta S, Guigne C, Mazzucotelli A, et al. Apelin, a newly identified adipokine up-regulated by insulin and obesity. *Endocrinology* 2005;**146**:1764–71.
2. Daviaud D, Boucher J, Gesta S, Dray C, Guigne C, Quilliot D, et al. TNFalpha up-regulates apelin expression in human and mouse adipose tissue. *FASEB J* 2006;**20**:1528–30.
3. Dray C, Knauf C, Daviaud D, Waget A, Boucher J, Buleon M, et al. Apelin stimulates glucose utilization in normal and obese insulin-resistant mice. *Cell Metab* 2008;**8**:437–45.
4. Duparc T, Colom A, Cani PD, Massaly N, Rastrelli S, Drougard A, et al. Central apelin controls glucose homeostasis via a nitric oxide-dependent pathway in mice. *Antioxid Redox Signal* 2011;**15**:1477–96.
5. Edinger AL, Hoffman TL, Sharron M, Lee B, Yi Y, Choe W, et al. An orphan seven-transmembrane domain receptor expressed widely in the brain functions as a coreceptor for human immunodeficiency virus type 1 and simian immunodeficiency virus. *J Virol* 1998;**72**:7934–40.
6. Flemstrom G, Makela K, Purhonen AK, Sjoblom M, Jedstedt G, Walkowiak J, et al. Apelin stimulation of duodenal bicarbonate secretion: feeding-dependent and mediated via apelin-induced release of enteric cholecystokinin. *Acta Physiol (Oxf)* 2011;**201**:141–50.

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7. Habata Y, Fujii R, Hosoya M, Fukusumi S, Kawamata Y, Hinuma S, et al. Apelin, the natural ligand of the orphan receptor APJ, is abundantly secreted in the colostrum. *Biochim Biophys Acta* 1999;**1452**: 25–35.
8. Han S, Wang G, Qi X, Englander EW, Greeley Jr GH. Involvement of a Stat3 binding site in inflammation-induced enteric apelin expression. *Am J Physiol Gastrointest Liver Physiol* 2008;**295**:G1068–78.
9. Han S, Wang G, Qi X, Lee HM, Englander EW, Greeley Jr GH. A possible role for hypoxia-induced apelin expression in enteric cell proliferation. *Am J Physiol Regul Integr Comp Physiol* 2008;**294**:R1832–9.
10. Han S, Wang G, Qiu S, de la Motte C, Wang HQ, Gomez G, et al. Increased colonic apelin production in rodents with experimental colitis and in humans with IBD. *Regul Pept* 2007;**142**:131–7.
11. Harris NR, Carter PR, Yadav AS, Watts MN, Zhang S, Kosloski-Davidson M, et al. Relationship between inflammation and tissue hypoxia in a mouse model of chronic colitis. *Inflamm Bowel Dis* 2011;**17**:742–6.
12. Heinonen MV, Purhonen AK, Miettinen P, Paakkonen M, Pirinen E, Alhava E, et al. Apelin, orexin-A and leptin plasma levels in morbid obesity and effect of gastric banding. *Regul Pept* 2005;**130**:7–13.
13. Higuchi K, Masaki T, Gotoh K, Chiba S, Katsuragi I, Tanaka K, et al. Apelin, an APJ receptor ligand, regulates body adiposity and favors the messenger ribonucleic acid expression of uncoupling proteins in mice. *Endocrinology* 2007;**148**:2690–7.
14. Kapica M, Puzio I, Kato I, Kuwahara A, Zabielski R. Role of feed-regulating peptides on pancreatic exocrine secretion. *J Physiol Pharmacol* 2008;**59**(Suppl. 2):145–59.
15. Kawamata Y, Habata Y, Fukusumi S, Hosoya M, Fujii R, Hinuma S, et al. Molecular properties of apelin: tissue distribution and receptor binding. *Biochim Biophys Acta* 2001;**1538**:162–71.
16. Kralisch S, Lossner U, Bluher M, Paschke R, Stumvoll M, Fasshauer M. Growth hormone induces apelin mRNA expression and secretion in mouse 3T3-L1 adipocytes. *Regul Pept* 2007;**139**:84–9.
17. Lambrecht NW, Yakubov I, Zer C, Sachs G. Transcriptomes of purified gastric ECL and parietal cells: identification of a novel pathway regulating acid secretion. *Physiol Genomics* 2006;**25**:153–65.
18. Lee DK, Cheng R, Nguyen T, Fan T, Kariyawasam AP, Liu Y, et al. Characterization of apelin, the ligand for the APJ receptor. *J Neurochem* 2000;**74**:34–41.
19. Lv SY, Yang YJ, Qin YJ, Xiong W, Chen Q. Effect of centrally administered apelin-13 on gastric emptying and gastrointestinal transit in mice. *Peptides* 2011;**32**:978–82.
20. Masri B, Knibiehler B, Audigier Y. Apelin signalling: a promising pathway from cloning to pharmacology. *Cell Signal* 2005;**17**:415–26.
21. Masri B, Morin N, Pedebernade L, Knibiehler B, Audigier Y. The apelin receptor is coupled to Gi1 or Gi2 protein and is differentially desensitized by apelin fragments. *J Biol Chem.* 2006;**281**:18317–26.
22. Mesmin C, Dubois M, Becher F, Fenaille F, Ezan E. Liquid chromatography/tandem mass spectrometry assay for the absolute quantification of the expected circulating apelin peptides in human plasma. *Rapid Commun Mass Spectrom* 2010;**24**:2875–84.
23. O'Carroll AM, Selby TL, Palkovits M, Lolait SJ. Distribution of mRNA encoding B78/apj, the rat homologue of the human APJ receptor, and its endogenous ligand apelin in brain and peripheral tissues. *Biochim Biophys Acta* 2000;**1492**:72–80.
24. Reaux-Le Goazigo A, Alvear-Perez R, Zizzari P, Epelbaum J, Bluet-Pajot MT, Llorens-Cortes C. Cellular localization of apelin and its receptor in the anterior pituitary: evidence for a direct stimulatory action of apelin on ACTH release. *Am J Physiol Endocrinol Metab* 2007;**292**:E7–15.
25. Ringstrom C, Nitert MD, Bennet H, Fex M, Valet P, Rehfeld JF, et al. Apelin is a novel islet peptide. *Regul Pept* 2010;**162**:44–51.
26. Sorhede Winzell M, Magnusson C, Ahren B. The apj receptor is expressed in pancreatic islets and its ligand, apelin, inhibits insulin secretion in mice. *Regul Pept* 2005;**131**:12–7.
27. Soriguer F, Garrido-Sanchez L, Garcia-Serrano S, Garcia-Almeida JM, Garcia-Arnes J, Tinahones FJ, et al. Apelin levels are increased in morbidly obese subjects with type 2 diabetes mellitus. *Obes Surg* 2009;**19**:1574–80.
28. Susaki E, Wang G, Cao G, Wang HQ, Englander EW, Greeley Jr GH. Apelin cells in the rat stomach. *Regul Pept* 2005;**129**:37–41.
29. Tang SY, Xie H, Yuan LQ, Luo XH, Huang J, Cui RR, et al. Apelin stimulates proliferation and suppresses apoptosis of mouse osteoblastic cell line MC3T3-E1 via JNK and PI3-K/Akt signaling pathways. *Peptides* 2007;**28**:708–18.
30. Tatemoto K, Hosoya M, Habata Y, Fujii R, Kakegawa T, Zou MX, et al. Isolation and characterization of a novel endogenous peptide ligand for the human APJ receptor. *Biochem Biophys Res Commun* 1998;**251**:471–6.
31. Tatemoto K, Takayama K, Zou MX, Kumaki I, Zhang W, Kumano K, et al. The novel peptide apelin lowers blood pressure via a nitric oxide-dependent mechanism. *Regul Pept* 2001;**99**:87–92.
32. Wang G, Anini Y, Wei W, Qi X, AM OC, Mochizuki T, et al. Apelin, a new enteric peptide: localization in the gastrointestinal tract, ontogeny, and stimulation of gastric cell proliferation and of cholecystokinin secretion. *Endocrinology* 2004;**145**:1342–8.
33. Wang G, Kundu R, Han S, Qi X, Englander EW, Quertermous T, et al. Ontogeny of apelin and its receptor in the rodent gastrointestinal tract. *Regul Pept* 2009;**158**:32–9.
34. Wang G, Qi X, Wei W, Englander EW, Greeley Jr GH. Characterization of the 5′-regulatory regions of the rat and human apelin genes and regulation of breast apelin by USF. *FASEB J* 2006;**20**:2639–41.
35. Yue P, Jin H, Aillaud M, Deng AC, Azuma J, Asagami T, et al. Apelin is necessary for the maintenance of insulin sensitivity. *Am J Physiol Endocrinol Metab* 2010;**298**:E59–67.

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Chapter 161

Bombesin-Related Peptides

Robert T. Jensen and Terry W. Moody

ABSTRACT

Bombesin is an amidated tetradecapeptide originally isolated from frog skin that is the prototype of a large number of related peptides, many of which only occur in invertebrates. The mammalian homologs are gastrin-releasing peptide (GRP) and neuromedin B(NMB). Each of these interacts with a distinct G protein-coupled receptor, the GRP-R(BB2) and NMB-R(BB1), respectively. Furthermore, an orphan receptor, with close structural homology to GRP-R an NMB-R has been discovered, bombesin receptor subtype-3(BRS-3)(BB3), whose natural ligand is unknown but is included in the Bn receptor class. Both mammalian Bn peptides (GRP, NMB) as well as their receptors and BRS-3 are widely distributed, but they have been particularly well studied in the gastrointestinal tract and in the CNS. Both the peptides act primarily as neurotransmitter/modulators but have other widespread actions in many tissues, with their CNS effects and their effects on growth of normal and neoplastic tissue receiving the most attention. In this chapter, primarily the mammalian Bn peptides and their receptors will be discussed.

DISCOVERY (BN PEPTIDES AND THEIR RECEPTORS)

The 14-amino acid peptide Bn was isolated from the skin of the European frog *Bombina bombina*,[4] and subsequently a family of related peptides was isolated from other frog skins and named after the genus of the frog from which they were isolated.[13] In general, they could be divided into three families: the Bn group with a carboxyl terminus of Gly-His-Leu-Met-NH_2; the ranatensin–litorin family with a carboxyl terminus of Gly-His-Phe-Met-NH_2, and the phyllolitorin family with a carboxyl terminus of Gly-Ser-Phe/Leu-Met-NH_2.[13] Subsequently, two mammalian members of the Bn-family were isolated: GRP, a 27-amino acid peptide isolated from porcine stomach, which shares seven carboxyl-terminal amino acids with Bn[13] and neuromedin B, from porcine spinal cord[13] a decapeptide sharing seven of its carboxyl amino acids with ranatensin. Studies of GRP and NMB immunoreactivity and mRNA studies demonstrate that these peptides and their mRNAs are widely distributed in mammals in both peripheral tissues, particularly the gastrointestinal tract and in the CNS.[13] In 1990, a mammalian G protein-coupled receptor was described, which interacted with high affinity with GRP (the GRP-R, BB_2) and in 1991 a G protein-coupled receptor with high affinity for NMB was described (NMB-R, BB_1).[13] In 1992/1993, a closely related orphan receptor, BRS-3 (bombesin receptor subtype-3) was described which had 47–51% amino acid identities with the human GRP-R/NMB-R and is thus included in the mammalian Bn-R family, although the native ligand is still unknown.[13] The GRP-R and NMB-R are widely distributed in the CNS and peripheral tissues, whereas the BRS-3 distribution has been less extensively investigated although it appears in many tissues also and particularly in the CNS and gastrointestinal tract.[13,25,27,31] In nonmammalians, other Bn-Rs have been isolated including a bombesin receptor subtype 4(BB4) and a unique Bn-R from chickens termed chBRS-3.5, which at present have no mammalian equivalents.[13]

STRUCTURE OF THE mRNA/GENE

The gene for GRP is located on chromosome 18 and for NMB on human chromosome 15-q11 pter region.[25] The GRP gene has two introns and three exons.[13] Two major transcription start sites for the human GRP-R have been described at location 43 and 36 bp downstream of a TTTAAA motif, which is identified as 407 to 402 bp upstream of the ATG start codon.[13] Similar to the GRP gene, NMB is encoded in the NMB gene in three exons.[25] In humans, NMB is encoded in preproNMB, a 76-amino acid precursor that contains a 24-amino acid signal peptide, NMB-32 and a 17-amino acid carboxyl-terminal extended peptide.[25] The human GRP-R is localized to Xp22 and consists of three exons spanning >27 kb.[13] The human NMB-R is localized to a chromosome 6p21-pter, whereas in the mouse, it is localized to chromosome 10[13] and spans >10 kb. The human BRS-3 receptor is localized to chromosome Xq25 and in the mouse on chromosome XA7.1-7.2.[13]

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00161-5
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PROCESSING OF THE PRECURSOR

Alternative splicing with the GRP gene from a single mRNA produces three isoforms of ProGRP.[13] These three isoforms encode for three peptides of 148, 141, and 138 amino acids, which are identical in the first 122 amino acids. PreproGRP is a 148 prepropeptide, which after removal of the 23-amino acid signal peptide, becomes $ProGRP_{1–125}$ and is subsequently processed to $proGRP_{31–125}$ by a Lys–Lys cleavage at positions 29–30. A carboxypeptidase removes the COOH terminus to produce $GRP_{1–127gly}$ that is subsequently amidated resulting in $GRP_{1–27amide}$, which is finally cleaved at arginine 17 to yield $GRP_{18–27amide}$.[13] In humans, NMB is encoded in preproNMB, a 76-amino acid precursor, which contains a 24-amino acid signal peptide, NMB-32 and a 17-amino acid carboxyl-terminal extended peptide, which is preceded by a Lys–Lys cleavage site.[25] The NMB-32 is subsequently cleaved to yield the decapeptide, NMB. The human preproNMB is similar to that of the rat except that it is 117 amino acids because it contains a 52-amino acid extension peptide rather than the 17 amino acids found in humans.[25]

The three exons of the human GRP-R encode for different parts of the 384-amino acid GRP-R.[13] The first exon encodes for the first three-membrane spanning domains of the GRP-R, and the second exon encodes for transmembrane regions 4 and 5 and most of the third intracellular loop, whereas exon 3 encodes for transmembrane region 5 and the cytoplasmic carboxyl terminus.[13] The three exons of the human NMB-R gene also encode for different parts of the 390-amino acid human NMB-R.[13] The first exon encodes for the amino acids up to a region between the NMB-R transmembrane regions 3 and 4, and the second for a region between transmembrane regions 5 and 6, and exon 3 for the remainder of the NMB-R.[13] Similar to the genes for GRP-R and NMB-R, the gene for the 399-amino acid human BRS-3 receptor has three exons and two introns and spans >5 kB.[13] In human, rat, and mouse, the exon/intron splice sites occurred at Arg^{145} in the second intracellular loop and at Ile^{264} in the third intracellular domain.[13]

DISTRIBUTION OF THE RNA/PEPTIDE

Studies of the distribution of GRP and NMB, as well as their receptor's immunoreactivity (IR) or mRNA studies show that these peptides, their receptors and their mRNAs are widely distributed in both the CNS and peripheral systems, occurring in almost all tissues.[13] The distribution in the gastrointestinal tract will be concentrated on here. In the alimentary tract, GRP-like IR occurs primarily in neurons in the submucosal and/or myenteric plexuses and not in endocrine cells.[13] The highest levels of Bn IR (primarily GRP-10, GRP-27) vary between human and different species,[13] In humans, the amount does not vary between the different areas of the gut, whereas in the rat, the highest amounts are found in the colon followed by the gastric antrum, duodenum, and midileum.[13] A study[20] using PCR of human biopsies from different locations found GRP mRNA in all locations (esophagus, duodenum, ileum, colon, rectum, stomach, pancreas) except for the gallbladder. Northern blot analysis showed the highest amounts of NMB mRNA in the stomach and colon, although it is also detected in the pancreas.[13] In the rat and guinea pig, NMB-IR is found throughout the gastrointestinal tract and in the pancreas.[23] The highest concentrations are found in the esophagus and rectum and immunocytochemistry localized the NMB-IR to nerve fibers in the gastrointestinal tract.[23]

GRP-R-mRNA expression levels are reported in human, mouse, and monkey.[13,31] In the monkey, in which it was studied in detail, the highest levels appear in the pancreas with the next highest (7-fold lower) in the stomach followed by the intestine.[31] A detailed study[7] of GRP-R mRNA distribution in human biopsy species taken from epithelial cells in different locations shows that GRP-R expression is limited to the gastric antrum and not the small intestine or proximal or distal colon. In other studies, GRP-Rs have been described in pancreatic acinar cells, gastric antral G cells, other gastric mucosal cells (D cell, mucus cell, parietal cell), and in epithelial cells lining the normal antrum.[13] NMB-R-mRNA/IR/binding site levels have been reported in human GI tissues as well as in mouse, rat, and monkey.[13,31] In the monkey, the NMB-R-mRNA levels are the highest in the stomach compared with that in the small intestine and pancreas, but much lower than the levels found in the testis or areas of the CNS.[31] In different reports, NMB-R-mRNA/IR/binding sites are distributed in esophageal tissue, stomach, urogenital, and gastrointestinal smooth muscle, human, and rodent adipose tissue and normal colonic mucosal cells.[13] BRS-3 expression has been less well investigated than that of GRP-Rs or NMB-R, but levels in various tissues have been reported in rat, sheep, monkey, human, and guinea pig.[13] In the GI tract, the highest levels in monkeys for BRS-3 mRNA are in the pancreas followed by the stomach and small intestine, and in each of these tissues, it is 4–5 fold lower than in the testis or various CNS regions.[31] BRS-3 IR in the gastrointestinal tract is seen in the tunica muscularis of all regions studied, including the antrum, duodenum, ileum, and colon in nerves and nonneuronal cells but not in muscle cells.[27] It was expressed in both myenteric and submucosal ganglia as well as in nerve fibers and in the cell bodies and process of the c-kit containing interstitial cells of Cayal, lending anatomical support for a potential important role in GI pacemaker function.[27] Animals with targeted knockout of the BRS-3 develop mild obesity, hypertension, impaired glucose metabolism associated with a reduced metabolic rate, as well as increased feeding behavior suggesting

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BRS-3 is important in glucose homeostasis and metabolism.[13,26] BRS-3 receptors are present on pancreatic islets and are involved in glucose regulation.[13] Detailed studies of BRS-3 distribution in all endocrine tissues or adipose tissues that might regulate glucose metabolism have not been done.

GRP-R, NMB-R, and BRS-3 are one of the groups of G protein-coupled receptors that are most frequently ectopically/overexpressed by many common tumors including those of the gastrointestinal tract (colon, pancreatic acinar cells, gastric) as well as other common tumors (breast, prostate, lung, CNS tumors).[13,14,29] In many cases, GRP and/or NMB have an autocrine growth effect on these tumors or can affect their growth.[13,14] Because of this, there is increased interest in the Bn family and their receptors as possible targets for novel tumor treatments, tumor imaging or receptor-direct cytoxicity.[14,29] Each of these areas is specifically dealt with in a separate chapter in the Cancer section of this Volume and therefore will be discussed only briefly in this chapter.

RECEPTORS AND THEIR SIGNAL CASCADES

Receptors for Bn-related Peptides

The actions of GRP are mediated by the GRP-R(BB2), which in humans is a 384-amino acid G protein-coupled receptor; those due to NMB are mediated by the NMB-R (BB1), which is a 390-amino acid G protein-coupled receptor and by an unknown ligand for the orphan receptor BRS-3(BB3), which has a 46–49% homology to GRP-R/NMB-R and is a 399-amino acid G protein-coupled receptor.[13] The human GRP-R has 55% amino acid identities with the human NMB-R and 51% with the human BRS-3.[5,13] The GRP-R, NMB-R, and BRS-3 each has multiple glycosylation sites.[13] There are two consensus potential sites for PKC phosphorylation in the human GRP-R.[13] There is the presence of a putative disulfide bond between cysteines at the end of extracellular domain-1 and middle of extracellular domain-2 (Cys^{113} and Cys^{196} in human GRP-R), which is preserved in all species studied.[13] Detailed studies of the GRP-R show that the mature receptor has a molecular mass of 60 kD in human and 82 kD in mouse with a deglycosylated mass of 43 kD, showing that 35% of the mass of the mature human GRP-R and 47% of the murine GRP-R result from glycosylation.[13] The mouse GRP-R has four potential glycosylation sites, the human two potential sites and detailed studies in the murine GRP-R show that all four potential N-linked glycosylation sites are glycosylated (Asn^5, Asn^{20}, Asn^{24}, Asn^1).[13,16] The amount of glycosylation differs in each site, all of the glycosylation on the mouse GRP-R is N-linked, and the glycosylation moieties are four triantennary and/or tetraantennary complex oligosaccharide chains.[13,16] The presence of the Asn^{24} and Asn^1 glycosylation is especially important for sorting and expression of the murine GRP-R; however, full glycosylation at each site is not needed for receptor expression.[13] The human NMB-R has two consensus sites for potential PKC phosphorylation and three potential N-Linked glycosylation sites.[13] The mature human NMB-R has a molecular mass of 72 kD and a deglycosylated mass of 43 kD. Detailed studies in the rat NMB-R show that there are no O-Linked carbohydrates but that the mature receptor was a sialoprotein,[16] with each of the N-linked sites glycosylated with triantennary and/or tetraantennary complex oligosaccharides.[13,16] The human BRS-3 has a predicted mass of 44 kD, and there are two potential N-linked glycosylation sites and consensus sites for potential PKC phosphorylation in the third cytoplasmic loop and carboxyl terminus.[5,13] There have been no cross-linking studies to establish the extent or type of phosphorylation of the mature BRS-3.

Similar to other G protein-coupled receptors, numerous studies show that GRP-R and NMB-R can undergo numerous regulatory processes when stimulated.[13] These include rapid receptor phosphorylation, receptor internalization when stimulated by agonists, but not antagonists. With prolonged agonist stimulation (>30 min), surface receptors decrease in number and undergo downregulation and the receptors also become desensitized, such that subsequent agonist doses are less potent and efficacious.[13] In various studies, PKC activation has been shown to be important for receptor internalization and receptor interaction with clathrin-coated pits.[13] Both acute (short-term) and long-term desensitization of the GRP-R has been reported, and numerous findings support the conclusion that chronic desensitization and downregulation are coupled processes and occur in a PKC-dependent and independent manner.[13]

The human GRP-R has a very high affinity for Bn and GRP(14–27) (0.07–1.9 nM), the human NMB-R has a very high affinity for NMB (0.052–0.54 nM),[35] and the human BRS-3 has a low affinity (>3000 nM) for Bn, NMB, and GRP, as it does for all naturally occurring Bn-related peptides.[13,35] GRP has >700-fold selectivity for the hGRP-R over the human NMB-R and NMB has a >600-fold selectivity for the human NMB-R over the human GRP-R.[35] Many amphibian peptides (litorin, ranatensin, P-GL, alytesin, Bn) have high affinities (nanomolar range) for both human GRP-R and human NMB-R.[13,35] The natural ligand of the BRS-3 receptor remains unknown. However, two synthetic compounds, which both function as agonists, have been described with high affinity for human BRS-3 which are [D-Phe^6, β-Ala^{11}, Phe^{13}, Nle^{14}) Bn(6–14) (and its analogs)[13,30] and MK-5046 [(2S)-1,1,1-trifluoro-2-[4-(1H-pyrazol-1-yl) phenyl]-3-(4-[[1-(trifluoromethyl) cyclopropyl] methyl]-1H-imidazol-2-yl)propan-2-ol.[11] [D-Phe^6, β-Ala^{11}, Phe^{13},

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Nle[14] Bn(6-14) (and its analogs) have the unique property of interacting as a full agonist with all three Bn-R subtypes with high affinity,[13,30] whereas MK-5046 is selective for the BRS-3 receptor.[11]

Receptor antagonists, which are widely used in various pharmacological and physiological studies, have been described for each classes of human Bn-Rs.[13] Ten different chemical classes of Bn receptor antagonists have been described, of which six classes are GRP-R selective antagonists.[8,13] These GRP-R selective antagonists are all peptide or peptoid analogs, some with high selectivity and affinity for GRP-R in all species.[8,13] Two different classes of antagonists are selective for the NMB-R, with the most selective and potent being the peptoid, PD168368[8] and one class of selective BRS-3 antagonist has been described, Bantag [Boc-Phe-His-4-amino-5-cyclohexyl-2,4,5- trideoxypentonyl-Leu-(3-dimethylamino)benzylamide *N*-methylammonium trifluoroacetate].[10]

Signal Cascades for Bn-Rs

GRP-R, NMB-R, and BRS-3 activation in human cells and all species examined are coupled to activation of phospholipase C(PLC) resulting in the generation of inositol phosphates and diacylglycerol with activation of protein kinase Cs (PKCs) and mobilization of cellular calcium.[13] Each also activates various tyrosine kinase cascades, which in different cells include the activation of focal adhesion kinases (PYK-2, $p125^{FAK}$); MAP kinase (ERK1/2, p38, Jun kinase); Src kinases; various docking proteins including $p130^{Cas}$, paxillin, CRK; and transactivation of EGF receptors.[13] The GRP-R activation in various species has been particularly well studied and also results in the activation of phospholipases A and D. In some, but not most cells, ligand–GRP-R interaction causes the activation of adenylate cyclase; activation of both classical and novel PKCs; activation of PKD in a PKC-dependent and independent manner; activation of p70s6 kinase resulting in an increased expression of immediate early response genes (c-myc, c-jun, c-fos); and activation of various small GTP-binding proteins such as rac or rho.[13]

One of the main signaling cascades of GRP-R, NMB-R, and BRS-3 in both gastrointestinal cancers and in cancers of other sites (lung, prostate) is linked with the transactivation of the epidermal growth factor receptor (EGFR) receptors.[13] This cascade involves the activation of phospholipase C, changes in cytosolic calcium and PKC as well as activation of Src kinases, matrix metalloproteinases with the generation of a EGF-like peptides resulting in EGFR transactivation and the generation of reactive oxygen species.[13,21] This is addressed in detail in the chapter on Bn peptides in the Cancer/Anticancer Peptides section of this book and will not be dealt with further here.

BIOLOGICAL ACTIONS OF BN PEPTIDES WITHIN THE GASTROINTESTINAL (GI) TRACT

General

In older studies, the particular Bn receptor subtype (GRP-R or NMB-R) mediating the various reported responses is frequently not clear because Bn was usually used, and this peptide has high affinity for both GRP-R and NMB-R in both humans and the various nonhuman species studied.[13,35] A second point that can make it difficult to interpret the actions of Bn peptides is that they have the unique ability to not only stimulate tissues directly but also they very frequently stimulate the release of other biologically active peptides, gastrin, cholecystokinin, (CCK), glucagon-like peptide, insulin, motilin, gastric inhibitory polypeptide, enteroglucagon, neurotensin, vasoactive intestinal peptide, peptide YY, which in turn can cause indirect effects.[13] A third point complicating many reported GI actions of various Bn peptides is that it is often unclear as to which are only pharmacological and which are physiological. In the following sections, some of the main actions will be discussed.

Bn stimulating either activation of GRP-R or NMB-R has contractile effects on all gastrointestinal smooth muscle, similar to its effects on smooth muscle in the urogenital tract, and has prominent effects on gastrointestinal motility, especially on stimulating gastric emptying, small intestinal transit and gallbladder emptying.[3,13] Peristalsis is important in both the large and small intestine for the propulsion of intestinal content. In rat colon, activation of GRP-Rs is important in mediating the descending phase of the peristaltic reflex with GRP acting as a modulatory neurotransmitter.[9]

Bn has potent growth effects on various normal gastrointestinal tissues and gastrointestinal tumors through, autocrine and nonautocrine mechanisms.[14] Bn administration in neonatal rats causes hyperplasia of the G cells in the gastric antrum as well as hyperplasia of duodenal and pancreatic acinar glands.[32]

The Action of Bn Peptides in the Esophagus

NMB-Rs were originally described on esophageal muscle, and its activation causes contraction of esophageal smooth muscle.[13] The effect of bombesin infusion on 8 healthy humans was examined.[13] Bn significantly increases lower esophageal sphincter pressure, which is not blocked by atropine or somatostatin and affects esophageal body motility by increasing peristaltic wave amplitude and duration.[13] In cat, lower esophageal sphincter Bn dose dependently increases lower esophageal sphincter tone.[13]

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The Action of Bn Peptides in the Stomach

Activation of GRP-R in gastric smooth muscle results in contraction,[13] which is tetrodotoxin and atropine resistant.[19] Both NMB-R and GRP-Rs are found on isolated gastric smooth muscle cells.[13] In human studies, the use of a GRP-R antagonist inhibits gastric emptying[3] and administration of monoclonal antibombesin antibodies to dogs leads to increased gastric retention.[13,32] GRP-R stimulates both gastrin release from antral G cells and somatostatin release from D cells as well as stimulating gastric acid output.[13] Studies in the isolated vascularly perfused rat stomach show that peptone and various protein nutrients stimulate gastrin release from the stomach antrum by activating stimulatory cholinergic and bombesin/GRP neurons.[13] Activation of GRP-Rs in the stomach stimulates somatostatin secretion in the fundus and antrum by distinct mechanisms. In the fundus, GRP acts indirectly on somatostatin cells by activating stimulatory noncholinergic neurons and to a lesser degree, inhibitory cholinergic neurons. However, in the antrum, GRPs act indirectly on somatostatin cells by stimulating the release of gastrin and to a lesser degree by activation of inhibitory cholinergic neurons.[13]

The Action of Bn Peptides in the Small Intestine

Activation of GRP-R in small intestinal smooth muscle results in the contraction of duodenal circular muscle strips in a atropine- and tetrodotoxin-insensitive manner,[19] as well as contraction of ileal longitudinal muscle strips.[13] *In vivo* infusion of GRP results in a slower propulsive force in the small intestine and inhibition of GRP-R by a GRP-R antagonist *in vivo* accelerates small intestinal transit in humans.[3,13,32] Bn infusion in either an isolated intestinal loop in the dog or in pig produces a 50% reduction in water and electrolyte absorption (Na, Cl) and an increase in bicarbonate secretion.[13] In the isolated mouse jejunum, the effects of Bn on short circuit current and potential difference changes are proposed to be mediated by three levels of action of the peptide: acting on noncholinergic nerves; at preganglionic and postganglionic levels and directly on the enterocyte.[28] Bn has important growth and cytoprotective effects in various small bowel injury models.[13] Bn administration increases absorptive capacity and improves biochemical values in the absence of elemental nutrition in experimental models of short bowel syndrome; it minimizes impairment of interstitial cells of Cajal, the pacemaker cells, induced by FK506 used in small bowel transplantation;[2] its administration can protect the rat small bowel allograft from ischemic reperfusion injury and the use of a selective GRP-R antagonists attenuates the release of proinflammatory cytokines *in vitro* and *in vivo* and improves survival in models of sepsis.[2,13]

The Action of Bn Peptides in the Large Intestine

Activation of NMB-R in colonic smooth muscle results in contraction;[13] however, infusion of GRP increases contractions of colon muscle, which results in slower propulsive force.[32] In various studies, GRP-R/Bn-R are reported to be possibly important in various colonic inflammatory responses. Specifically, a GRP-R antagonist reduces the severity of the colitis in an experimental model of inflammatory bowel disease associated with a reduction in the expression of TNF-alpha,[13] and GRP-R expression is decreased in inflamed and noninflamed colon of Crohn's disease patients but not those with ulcerative colitis. The trefoil factor TFF3 is a peptide produced by mucus-secreting cells in the small and large intestine (see the chapter in this section of the book), which has been implicated in intestinal repair and protection. In the rat colon, Bn induces a dose-dependent increase in the release of TFF3. GRP stimulates colonic ion transport including K and Na secretion across the porcine distal colonic epithelium, and these processes were partially dependent on a bumetanide-sensitive transport pathway in the basolateral membrane.[13,34] GRP can also affect colonic ion transport by modulating the action of prostaglandin E2 on electrolyte transport.

The Action of Bn Peptides Action in the Pancreas

Pancreatic acinar cells from all species examined (rat, man, dog, mouse, guinea pig) possess GRP-Rs, and their activation causes enzyme secretion.[13] Furthermore, *in vivo* Bn stimulates pancreatic secretion in man and in many species (rat, dog, mouse).[32] Because activation of Bn receptors can stimulate CCK release, one of the important questions is whether it stimulates pancreatic secretion indirectly by stimulating the release of CCK. In rats, the CCKAR antagonist L-364,718[1] completely inhibits CCK-stimulated pancreatic enzyme secretion but has no effect on Bn-induced pancreatic secretion, leading to the conclusion that Bn stimulates pancreatic secretion by interacting directly with the acinar cells, which is similar to the findings in humans. Bn can also stimulate pancreatic bicarbonate and fluid secretion, and studies demonstrate that it is mediated by activation of GRP-R.[33] The secretion of the bicarbonate rich fluid stimulated by Bn results from the direct action on interlobular ducts of the pancreas.[33]

Chronic Bn administration increases pancreatic weight and DNA content, and pancreatic enzyme content.[13] Most, but not all studies,[17] especially in the rat, show that these trophic effects of Bn on the pancreas are not mediated by CCK release but are mediated by a direct action.[12]

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Bombesin stimulates insulin secretion from pancreatic islets and recent studies show both GRP-R and BRS-3 receptors are present on pancreatic islets.[13] In normal mice, GRP administration potentiates glucose-stimulated insulin secretion, which is at least partially mediated by the activation of autonomic nerves, and the GRP potentiation effect on glucose-stimulated insulin release is not seen in GRP-R knockout mice.[13] Bn/GRP is reported to have variable effects on the stimulation of other islet hormones pancreatic polypeptide and glucagon with increases in some, but not all, studies reported in the human, dog, and sheep.[13] By contrast, in porcine pancreas, GRP infusion results in the stimulation of insulin and pancreatic polypeptide with inhibition of somatostatin secretion, no effect on glucagon secretion, and no effects are altered by muscarinic cholinergic blockade.[13] Mice with a target disruption of BRS-3 develop hyperphagia, obesity, hypertension, and impaired glucose metabolism.[26] BRS-3 receptors occur in pancreatic islets and BRS-3 knockout mice have elevated insulin levels.[13] Various studies[13] conclude that the BRS-3 receptor activation can contribute to regulating plasma insulin concentrations and/or its secretion and the impaired glucose metabolism in these animals results from impairment of glucose transporter 4 translocation in adipocytes.[13] BRS-3 is highly expressed in human mouse, rhesus monkey, and dog pancreatic islets, (but not rat), and the silencing of BRS-3 with either small interfering RNA or a receptor antagonist reduces glucose-dependent insulin release, showing the widespread importance of this receptor in insulin regulation.[6]

The Action of Bn Peptides Action in the Gallbladder

Activation of Bn receptors in gallbladder muscle results in gallbladder contraction.[13] Studies in the human, dog, guinea pig, and other species show that Bn/GRP can stimulate gallbladder contraction and that it is inhibited by muscarinic cholinergic blockade.[13] Detailed studies in the dog show that bombesin stimulated gallbladder contraction requires involvement of prejunctional cholinergic-, somatostatingeric-, and VIPergic-pathways.[13]

PATHOPHYSIOLOGICAL IMPLICATIONS OF BN PEPTIDES WITHIN THE GASTROINTESTINAL (GI) TRACT

General

At present, no specific diseases have been shown to result from alterations in the genes for Bn peptides or their receptors.[13] Furthermore, at present, no specific therapies for any disease are directed at Bn peptides or their receptors. However, Bn peptides and their receptors are receiving considerable interest in relation to their growth effects on GI and other neoplasms and their overexpression/ectopic expression by many common tumors.[13,14] With regard to the GI tract, numerous experimental studies suggest that they may be particularly important in mucosal protection, regulation of insulin secretion and feeding behavior each of which will be briefly discussed below.

PATHOPHYSIOLOGICAL IMPLICATIONS OF BN PEPTIDES IN GASTROINTESTINAL AND OTHER CANCERS

General: Bn and Cancer

Activation of all three Bn-Rs can have growth effects in various normal tissues and in neoplastic tissues.[13,14] Because Bn-Rs are one of the groups of G protein-coupled receptors that are the most frequently overexpressed/ectopically expressed many common tumors, and these peptides can have a potent effect on their growth and some cases, their differentiation (often by an autocrine mechanism), they are receiving much attention.[13,14] This attention arises both from their role in growth and differentiation of neoplasms and therefore as a target for novel therapies and from their role in imaging tumors related to receptor overexpression for identifying primaries and establishing the extent of the disease. This provides a possible approach for molecular targeting of antitumor therapies (either radioactively labeled Bn analogs or cytotoxic Bn analogs).[13,14,29] This area is specifically dealt with in detail in another chapter on Bn peptides in Cancer in this book and therefore will only be briefly addressed here. Furthermore, only a few key references will be repeated.

POSSIBLE TREATMENTS DIRECTED AT BN PEPTIDES AND/OR THEIR RECEPTORS GROWTH EFFECTS ON TUMORS

Ever since the original description of Bn-like peptide secretion by small-cell lung cancers and the subsequent demonstration that Bn-like peptides have autocrine growth effects on numerous tumors including those in the GI tract, there has been interest in developing novel therapies against this growth cascade.[14] Bn-like peptides have growth stimulatory/differentiating effects in numerous common GI tumors (pancreas, colorectal, gastric, intestinal) and non-GI tumors (lung, prostate, ovarian, CNS, head/neck squamous cell cancers, renal, breast).[14] Promising effects of inhibition of the Bn peptide stimulatory effects by either receptor antagonists or by antibodies have been reported in many experimental

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tumoral models. Furthermore, anti-GRP antibody infusions have been reported in patients with lung cancer with 8% of patients responding.[15] Particularly promising are the reports that inhibition of GRP-R activation may potentiate the inhibitory actions of EGFR inhibitors on various tumors as well as the observation that Bn-R activation often stimulates growth by transactivating the EGFR receptor.[13] These results suggest that inhibition of these cascades could be a novel antitumor treatment approach.

POSSIBLE USE OF BN RECEPTOR OVEREXPRESSION/ECTOPIC EXPRESSION BY TUMORS FOR IMAGING/TUMOR LOCALIZATION

With >70% of many common tumors overexpressing at least one of the three Bn-R subtypes (GRP-R, NMB-R, BRS-3),[14] there is increased interest in using this feature as a means of imaging these tumors for clinical therapies.[29] This possible approach is being considered because of the success of the use of radioactively labeled somatostatin analogs for imaging neuroendocrine tumors, which are now widely used clinically and is the most sensitive imaging modality available.[29] Unfortunately, most common GI tumors, and common tumors in other locations, do not show somatostatin receptor overexpression, so some other G protein receptor, such as the Bn-R family, is being investigated for this purpose instead.[29] Tumor localization is essential in all stages of tumor management including the decision of whether surgery should be done, the extent of surgery, the assessment of the immediate results of surgery or other antitumor treatments, and the follow-up of the patient to assess the effects of treatment or need for a change in treatment approaches. There have been >200 articles published on the use of various labeled Bn analogs to image various tumors, most in the last 5 years and most in experimental studies in animals, and many report promising results.[29] Many COOH terminal Bn/GRP analogs have been used, and they have been labeled with different isotopes depending on whether they were primarily going to be used for imaging, the type of imaging or for Bn receptor-mediated cytotoxicity (next section). Isotopes used including ^{99m}Technetium, ^{111}Indium, ^{64}Copper, ^{125}Iodine, $^{185/187}$Rhenium, ^{18}Fluorine, ^{68}Gallium, ^{90}Yttrium or ^{177}Lutetium.[29] In various *in vitro* and *in vivo* studies, either conventional gamma radiation detection or PET imaging (positron emission tomography) is used to image the tumors.[29] In the original studies, radioactively labeled Bn receptor agonists were used based on analogies to results with somatostatin analogs and numerous studies, which showed that only agonists are internalized and thus believed to give better imaging.[29] Radioactively labeled Bn receptor antagonists may be superior targeting agents to agonists.[13]

POSSIBLE USE OF BN RECEPTOR OVEREXPRESSION/ECTOPIC EXPRESSION BY TUMORS FOR MOLECULAR TARGETING OF CYTOTOXIC AGENTS

Similar to the use of frequent Bn-R overexpression/ectopic expression to image tumors, there is increased interest in using this as a molecular target to deliver cytotoxic agents selectively to the tumor (radioactively labeled or other cytoxic Bn analogs).[29] This occurs, similar to the situation with imaging, because of the success of this approach with radioactively labeled somatostatin analogs in patients with advanced, malignant neuroendocrine tumors[NETs].[29] Using this approach, promising therapeutic effects have been obtained in many studies of patients with advanced NETs and large Phase 3 clinical trials are now being conducted. This has resulted in a similar approach to common GI and other tumors that overexpress/ectopically express Bn-Rs, and there are now >100 articles, primarily published within the last few years using this approach.[29] Similar to the imaging studies discussed above, numerous synthetic agonist COOH terminal analogs of Bn-related peptides are used in these cytotoxic studies.[29] For Bn receptor-mediated radiocytotoxicity, the isotopes primarily used include ^{68}Gallium, ^{90}Yttrium, or ^{177}Lutetium,[29] which are coupled to various Bn analogs with various linkers/chemical groups avidly binding the isotopes.[29] Many of these reports describe radioactively labeled Bn analogs that retain high affinity for the Bn-R bearing tumors *in vivo* and have tumoricidal effects *in vivo*.[29]

Besides coupling Bn analogs to radioactively labeled Bn-related peptides, there is increasing interest in targeting other cytotoxic agents to tumors overexpressing/ectopically expressing Bn receptors.[29] Cytotoxic Bn analogs with Bn-related peptides coupled to chemotherapeutic agents (doxorubicin) (AN-215) inhibit growth of pancreatic, lung, prostate, and gastric cancers[14,29] and GRP analogs coupled to camptothecin inhibit lung cancer growth.[18,22,29] The camptothecin-coupled Bn analogs mediate their cytotoxic effects by interacting with tumoral Bn-Rs.[22] Bn analogs coupled to the marine toxins, hemisasterlin, dolastin, and their conjugates, have cytotoxic effects on various tumors.[13] Conjugation of Bn agonist analogs to magainin, an antimicrobial peptide, markedly enhances (10-fold) its cytotoxicity for tumor cells.[18]

SERUM LEVELS OF BN PEPTIDES AS TUMOR MARKERS

In several studies, plasma levels of GRP precursors (i.e. such as proGRP) in patients with non-small-cell lung cancer or small-cell lung cancer give important prognostic information and are useful in some studies to assess the response to therapy.[13,24]

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OTHER POSSIBLE BN PEPTIDE PATHOPHYSIOLOGICAL IMPLICATIONS IN NONNEOPLASTIC DISEASES

GRP is protective to the small intestine in various injury models[2,13] including by enhancing gut barrier function, preventing atrophy of enteric ganglia caused by FK506 in small intestine and preventing atrophy of Peyer Patches/dysfunction of M cells in rabbits receiving long-term parenteral hyperalimentation, which has led to the suggestion that GRP agonists may have a potential therapeutic role in diseases causing this types of injury.[13]

Studies suggest GRP-R, NMB-R, and BRS-3 are important in regulating metabolism and obesity.[13] This involves both central and peripheral mechanisms including vagal mediated effects.[13] This has led to interest developing antiobesity approaches. Lastly, numerous studies show that BN-R activation (particularly GRP-R and BRS-3) has important roles in regulating insulin release or glucose stimulated insulin release. An increased understanding of the mechanism of their effects may lead to newer approaches to treat diabetes mellitus.

ACKNOWLEDGMENTS

This article was partially supported by intramural funds from NIDDK and NCI, NIH.

REFERENCES

1. Anderson L, Dockray GJ. The cholecystokinin antagonist L-364,718 inhibits the action of cholecystokinin but not bombesin on rat pancreatic secretion in vivo. *Eur J Pharmacol* 1988;**146**:307–11.
2. Dal-Pizzol F, Di Leone LP, Ritter C, Martins MR, Reinke A, Pens GD, et al. Gastrin-releasing peptide receptor antagonist effects on an animal model of sepsis. *Am J Respir Crit Care Med* 2006;**173**:84–90.
3. Degen LP, Peng F, Collet A, Rossi L, Letterer S, Serrano Y, et al. Blockade of GRP receptors inhibits gastric emptying and gallbladder contraction but accelerates small intestinal transit. *Gastroenterology* 2001;**120**:361–8.
4. Erspamer V, Erspamer GF, Inselvini M, Negri L. Occurrence of bombesin and alytesin in extracts of the skin of three European discoglossid frogs and pharmacological actions of bombesin on extravascular smooth muscle. *Br J Pharmacol* 1972;**45**:333–48.
5. Fathi Z, Corjay MH, Shapira H, Wada E, Benya R, Jensen R, et al. BRS-3: novel bombesin receptor subtype selectively expressed in testis and lung carcinoma cells. *J Biol Chem* 1993;**268**(8):5979–84.
6. Feng Y, Guan XM, Li J, Metzger JM, Zhu Y, Juhl K, et al. Bombesin Receptor Subtype-3 (BRS-3) regulates glucose-stimulated insulin secretion in pancreatic islets across multiple species. *Endocrinology* 2011;**152**:4106–15.
7. Ferris HA, Carroll RE, Lorimer DL, Benya RV. Location and characterization of the human GRP receptor expressed by gastrointestinal epithelial cells. *Peptides* 1997;**18**:663–72.
8. Gonzalez N, Mantey SA, Pradhan TK, Sancho V, Moody TW, Coy DH, et al. Characterization of putative GRP- and NMB-receptor antagonist's interaction with human receptors. *Peptides* 2009;**30**:1473–86.
9. Grider JR. Gastrin-releasing peptide is a modulatory neurotransmitter of the descending phase of the peristaltic reflex. *Am J Physiol (Gastrointest Liver Physiol)* 2004;**287**:G1109–15.
10. Guan XM, Chen H, Dobbelaar PH, Dong Y, Fong TM, Gagen K, et al. Regulation of energy homeostasis by Bombesin Receptor Subtype-3: selective receptor agonists for the treatment of obesity. *Cell Metab* 2010;**11**:101–12.
11. Guan XM, Metzger JM, Yang L, Raustad KA, Wang SP, Spann SK, et al. Antiobesity effect of MK-5046, a novel bombesin receptor subtype-3 agonist. *J Pharmacol Exp Ther* 2011;**336**:356–64.
12. Hajri A, Aprahamian M, Damge C. Effect of a new CCK-receptor antagonist, CR 1409, on pancreatic growth induced by caerulein, CCK-8, bombesin and gastrin-releasing peptide in the rat. *Digestion* 1989;**43**:66–72.
13. Jensen RT, Battey JF, Spindel ER, Benya RV. International union of pharmacology. LVIII. Mammalian bombesin receptors: nomenclature, distribution, pharmacology, signaling and functions in normal and disease states. *Pharmacol Rev* 2008;**60**:1–42.
14. Jensen RT, Moody TW. Bombesin-related peptides and neurotensin: effects on cancer growth/proliferation and cellular signaling in cancer. In: Kastin AJ, editor. *Handbook of biologically active peptides*. Amsterdam: Elsevier; 2006. p. 429–34.
15. Kelley MJ, Linnoila RI, Avis IL, Georgiadis MS, Cuttitta F, Mulshine JL, et al. Antitumor activity of a monoclonal antibody directed against gastrin-releasing peptide in patients with small cell lung cancer. *Chest* 1997;**112**:256–61.
16. Kusui T, Benya RV, Battey JF, Jensen RT. Glycosylation of bombesin receptors: characterization, effect on binding and G-protein coupling. *Biochemistry (Mosc)* 1994;**33**:12968–80.
17. Liehr RM, Rosewicz S, Reidelberger RD, Solomon TE. Direct vs. indirect effects of bombesin on pancreatic growth. *Digestion* 1990;**46**(Suppl. 2):202–7.
18. Liu S, Yang H, Wan L, Cai HW, Li SF, Li YP, et al. Enhancement of cytotoxicity of antimicrobial peptide magainin II in tumor cells by bombesin-targeted delivery. *Acta Pharmacol Sin* 2011;**32**:79–88.
19. Milusheva EA, Kortezova NI, Mizhorkova ZN, Papasova M, Coy DH, Balint A, et al. Role of different bombesin receptor subtypes mediating contractile activity in cat upper gastrointestinal tract. *Peptides* 1998;**19**:549–56.
20. Monstein HJ, Grahn N, Truedsson M, Ohlsson B. Progastrin-releasing peptide and gastrin-releasing peptide receptor mRNA expression in non-tumor tissues of the human gastrointestinal tract. *World J* 2006;**12**:2574–8.
21. Moody TW, Berna MJ, Mantey S, Sancho V, Ridnour L, Wink DA, et al. Neuromedin B receptors regulate EGF receptor tyrosine phosphorylation in lung cancer cells. *Eur J Pharmacol* 2010;**637**:38–45.
22. Moody TW, Sun LC, Mantey SA, Pradhan T, Mackey LV, Gonzales N, et al. In vitro and in vivo antitumor effects of cytotoxic camptothecin-bombesin conjugates are mediated by specific interaction with cellular bombesin receptors. *J Pharmacol Exp Ther* 2006;**318**:1265–72.
23. Namba M, Ghatei MA, Bishop AE, Gibson SJ, Mann DJ, Polak JM, et al. Presence of neuromedin B-like immunoreactivity in the brain and gut of rat and guinea-pig. *Peptides* 1985;**6**:257–63.
24. Nisman B, Heching N, Biran H, Barak V, Peretz T. The prognostic significance of circulating neuroendocrine markers chromogranin a, pro-gastrin-releasing peptide and neuron-specific enolase in patients with advanced non-small-cell lung cancer. *Tumour Biol* 2006;**27**:8–16.
25. Ohki-Hamazaki H. *Neuromedin B. Prog Neurobiol* 2000;**62**:297–312.

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26. Ohki-Hamazaki H, Watase K, Yamamoto K, Ogura H, Yamano M, Yamada K, et al. Mice lacking bombesin receptor subtype-3 develop metabolic defects and obesity. *Nature* 1997;**390**(6656):165–9.
27. Porcher C, Juhem A, Peinnequin A, Bonaz B. Bombesin receptor subtype-3 is expressed by the enteric nervous system and by interstitial cells of Cajal in the rat gastrointestinal tract. *Cell Tissue Res* 2005;**320**:21–31.
28. Riviere PJ, Sheldon RJ, Malarchik ME, Burks TF, Porreca F. Effects of bombesin on mucosal ion transport in the mouse isolated jejunum. *J Pharmacol Exp Ther* 1990;**253**:778–83.
29. Sancho V, Di Florio A, Moody TW, Jensen RT. Bombesin receptor-mediated imaging and cytotoxicity: review and current status. *Curr Drug Deliv* 2011;**8**:79–134.
30. Sancho V, Moody TW, Mantey SA, Di Florio A, Uehara H, Coy DH, et al. Pharmacology of putative selective hBRS-3 receptor agonists for human bombesin receptors (BnR): affinities, potencies and selectivity in multiple native and BnR transfected cells. *Peptides* 2010;**31**:1569–78.
31. Sano H, Feighner SD, Hreniuk DL, Iwaasa H, Sailer AW, Pan J, et al. Characterization of the bombesin-like peptide receptor family in primates. *Genomics* 2004;**84**:139–46.
32. Sunday ME, Kaplan LM, Motoyama E, Chin WW, Spindel ER. Gastrin-releasing peptide (mammalian bombesin) gene expression in health and disease. *Lab Invest* 1988;**59**:5–24.
33. Szalmay G, Varga G, Kajiyama F, Yang XS, Lang TF, Case RM, et al. Bicarbonate and fluid secretion evoked by cholecystokinin, bombesin and acetylcholine in isolated guinea-pig pancreatic ducts. *J Physiol* 2001;**535**:795–807.
34. Traynor TR, O'Grady SM. Regulation of colonic ion transport by GRP. I. GRP stimulates transepithelial K and Na secretion. *Am J Physiol* 1996;**270**:C848–58.
35. Uehara H, Gonzalez N, Sancho V, Mantey SA, Nuche-Berenguer B, Pradhan T, et al. Pharmacology and selectivity of various natural and synthetic bombesin related peptide agonists for human and rat bombesin receptors differs. *Peptides* 2011;**32**:1685–99.

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Chapter 162

CCK

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ABSTRACT

Peripheral cholecystokinin (CCK) occurs in intestinal endocrine cells and neurons. It regulates gastric acid secretion, gastric emptying, gallbladder contraction, exocrine pancreatic secretion, and may influence feeding. Characterization of CCK-58 as the most abundant endocrine form and recognition that its physiology differs from that of CCK-8 have important implications for the physiological role of CCK. Circulating levels of CCK may be higher than that previously recognized because of ex vivo degradation of CCK-58 and its lower immunoreactivity. CCK-58 may act in a paracrine manner to activate both CCK_1 and CCK_2 vagal receptors. Higher circulating peptide levels and vagal afferent actions suggest recalibration of "physiological" actions of CCK. Previous conclusions that CCK has minimal therapeutic value in obesity came from CCK-8's reduction of meal size and also the interval between meals. CCK-58 reduces meal size but not intermeal interval. Therefore, the physiology of CCK should be reexamined using the predominant circulating form, CCK-58.

HISTORY

Early classical studies in gut physiology found that extracts of intestinal mucosa caused gallbladder contraction (termed "cholecystokinin") and pancreatic digestive enzyme secretion (pancreozymin).[17] Decades of painstaking chemical and physiological experimentation resulted in the elucidation of the structure of the bioactive peptide now commonly known as cholecystokinin (CCK). Pioneering work by Mutt and Jorpes[22] culminated in reports of full amino acid sequences for peptides containing 33 and 39 residues (CCK-33 and CCK-39). Subsequent studies reported by many investigators indicated that multiple molecular forms of CCK existed in the gut mucosa, brain, and blood.[11] Of particular importance was the discovery of CCK-8.[8] This form and related small analogs proved to be relatively easy to synthesize chemically and demonstrated clear differences in actions from the structurally related gastrin peptide. This resulted in thousands of publications on the physiology and pathophysiology of CCK, based almost exclusively on the use of CCK-8 as a representative probe for bioactivity.

More recent work by Reeve and coworkers has indicated that the major molecular form of CCK in several species is much longer and more structurally complex than the originally characterized form; the peptide has 58 amino acids and is termed CCK-58.[11] Fig. 1 shows the sequence of CCK-58 peptides that have been isolated from dog, cat, and rabbit. The structure of the endogenous peptide that causes cholecystokinin bioactivity and the site of its action are still debated. However, current evidence shows that CCK-58 is the only detectible form in the blood of rats[23] and is the most abundant endocrine form in other species.[10,11] CCK-58 and CCK-8, the most studied peptides, have notable differences in some physiological actions[29] suggesting that the biochemistry, physiology, and pharmacology of cholecystokinin require more investigation.

STRUCTURE OF THE PRECURSOR GENE/ mRNA

The gene for rat cholecystokinin was isolated by Deschenes et al.[7] There are three exons separated by two introns spanning 7 kb. The methionine codon that initiates peptide synthesis is 2 bases into exon 2. A "TATA"-like sequence precedes the transcription initiation site at position 34. The sequence AT-TAAA is found 22 bases 5′ to the site determined to be the polyadenylation addition site. The message-encoding preprocholecystokinin begins with a putative initiator methionine and terminates with a serine followed by a stop codon, TAC. The prepropolypeptide is 115 amino acids long. The cDNA contains 33 nucleotides in the 5′-noncoding region, 199 nucleotides in the 3″-coding region and 345 nucleotides coding for preprocholecystokinin. The amino acid sequence of CCK-58 is flanked by both amino-terminal (45 amino acids) and carboxyl-terminal flanking (12 amino acids) peptides.

PROCESSING OF THE PRECURSOR

Processing of preprocholecystokinin has been suggested by the forms of cholecystokinin observed in tissue extracts. Preprocholecystokinin is processed into procholecystokinin

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00162-7
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Dog     AVQKVDGEPRAHLGALLARYIQQARKAPSGRMSVIKNLQNLDPSHRISDRDY*MGWMDF<
Cat     AVQKVDGEPRAHLGALLARYIQQARKAPSGRMSVIKNLQSLDPSHRISDRDY*MGWMDF<
Rabbit  AAQRTDVESRGHLGALLARYIQQARRAPAGRMSIIKNLQSLDPSHRISDRDY*MGWMDF<
CCK-8                                                   Y*MGWMDF<
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FIGURE 1 The primary sequence of dog, cat, and rabbit CCK-58 and CCK-8. The * indicates the presence of a sulfate group on the tyrosine 7 amino acids from the carboxyl terminus, the Phe< indicates that the carboxyl terminus of all CCK peptides are amidated, the underlining indicates amino acids that differ between cat and rabbit CCK-58 from dog CCK-58. A difference of one amino acid in the amino terminus between dog and cat CCK-58 causes an 18-fold difference in carboxyl-terminal immunoreactivity.

by signal peptidase. The amino terminus of proCCK peptide was determined by its purification and sequence analysis from human intestine.[9] The carboxyl-terminal flanking peptide adjacent to the phenylalanine amide (the carboxyl-terminal amino acid in all cholecystokinin forms) is cleaved from procholecystokinin by PC1 to form CCK-83-Gly-Arg-Arg-COOH. The phenylalanine-amide carboxyl terminus is formed by the actions of two enzyme complexes; first carboxypeptidase D in the duodenum or carboxypeptidase E in the brain[25] removes the two basic groups, then peptidylglycine-α-amidating monooxygenase hydrolyzes the –Phe Gly-COOH to form the carboxyl terminal-Phe-NH_2. This produces CCK-83 with the carboxyl-terminal amide found on all forms of cholecystokinin with accepted bioactivity. Endopeptidase cleavage at single or double basic residues can produce CCK-8, CCK-22, CCK-33, CCK-39, or CCK-58, all with the same phenylalanine-amide carboxyl terminus.

Another distinctive posttranslational modification of cholecystokinin is the sulfation of its carboxyl-terminal tyrosine, seven residues from this end of all cholecystokinin forms. The only gastroenteric peptides with a sulfate group are gastrin and cholecystokinin, which have identical carboxyl-terminal penptapeptide structures with a tyrosine in the sixth and seventh positions from the end of the peptides, respectively. Both gastrin and cholecystokinin exist in sulfated and nonsulfated forms. In dogs, the proportion of sulfated to total gastrin or cholecystokinin is about 66% for both peptides. Importantly, nonsulfated-CCK-58 is as potent, but less efficacious, as the sulfated peptide for stimulation of pancreatic protein and fluid secretion[29] in the intact rat. The fact that nonsulfated-CCK-58 is a full agonist for the CCK_2 receptor but not the CCK_1 (see below), suggests that the *in vivo* pancreatic action may be mediated by the CCK_2 receptor.

However, previous studies lacked an important control for defining the tissue forms of cholecystokinin (and most bioactive peptides), an external indicator of artificial degradation of the endogenous peptide. During extraction and processing of tissue, the peptides are exposed to a plethora of proteolytic enzymes from cellular organelles caused by membrane dissociation during extraction. Some of the enzymes, such as those in the lysozymes, are specifically designed to degrade peptide hormones. Reported cholecystokinin forms (peptides with a carboxyl phenylalanine-amide) include peptides with 4, 5, 8, 22, 33, 39, 58, and 83 amino acids. Other forms have been identified in one species, but this list may well indicate that many of the forms are artifacts of extraction and processing of the tissue being examined. This is especially pertinent for neutral extracts of intestine where active pancreatic enzymes could be responsible for many of the observed forms. Without an external standard that indicates the extent of degradation, there is no validation of observed forms in any studies on intestinal or brain molecular forms, including results reported from our own laboratory.

However, our laboratory has developed a method—"RAPID (Refrigerated, Acidified, Protease inhibitors, Indicator, Dilution)"—for determining the molecular forms of cholecystokinin in blood.[23] In this method, a very important aspect is the use of an indicator to quantify peptide stability and loss. When conditions were developed where the external standard, ^{125}I-CCK-58, was not degraded and all other forms were recovered by >90%, the only detectible molecular form of cholecystokinin in rat blood was CCK-58.[23] In earlier studies in which no indicator was included, CCK-58 was the major form in human and canine blood and tissue.[11] These studies of humans and dogs need to be repeated with indicator before strong conclusions can be drawn. One is tempted to suggest that CCK-58 is the only form in tissue because it is the only detected form in blood. However, this may not be accurate because the liver could remove most or all of CCK-8 before it reaches the general circulation. Others would suggest that CCK-8 is probably present because it is the only molecular form of cholecystokinin observed in the brain. However, this may be premature because CCK-58 is a major form in canine brain,[12] and again, there have been no studies on brain forms of cholecystokinin that have included an indicator of degradation.[23]

Future studies need to include not only indicator, but also recovery studies of most suggested molecular forms including CCK-8, CCK-22, CCK-33, nonsulfated-CCK-58 and sulfated-CCK-58. This quintet of standards reflects the range of size and charge that have been observed in previous studies. At this time, we feel the most probable processing that occurs will produce only CCK-58 or only CCK-58 and CCK-8. The presence of nonsulfated-CCK-58 must be

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evaluated using an indicator such as "heavy CCK-58" (a peptide with five N^{15} atoms) or radioactive sulfated-CCK-58 during extraction and purification of tissue or blood.

PERIPHERAL DISTRIBUTION

The major location of CCK in the periphery is the gastrointestinal tract. CCK has been localized by immunohistochemical methods to a morphologically distinct open-type endocrine cell, the I cell.[5] These cells are found in the proximal small intestine in most species, and immunoassay of tissue extracts also indicates that the highest amounts of CCK are in the proximal intestine but substantial immunoreactivity is also present in the ileum. The peptide has also been detected in secreto-motor nerves in the enteric nervous system in several species.[13] Although no definitive data exist, it is likely—based on the number and density of I cells versus ENS neurons—that most circulating CCK is produced and secreted by mucosal endocrine cells.

RECEPTORS AND SIGNALING CASCADES

It is being increasingly recognized that peripheral cholecystokinin can act at two receptors (CCK_1, CCK preferring, and CCK_2, CCK, and gastrin specific) located on multiple tissues (Fig. 2). There are the classic actions at CCK_1 receptors on the gallbladder and pancreas causing contraction and secretion; in addition, electrophysiological experiments[16] and antagonist studies suggest the CCK_1 receptor is on vagal afferents where it is postulated to regulate pancreatic secretion and at high concentrations meal size (satiation). There is accumulating evidence that CCK_2 receptors are also on the vagus and that both receptors are present on sympathetic nerves.[4]

At this point, it is important to point out a controversial aspect of our work. Many have suggested that only gastrin can activate CCK_2 receptors because of much higher circulating concentrations of gastrin versus cholecystokinin. If CCK is released near receptors containing CCK_2 receptors (as accepted for regulation of meal size by CCK_1 receptors), then CCK could activate the CCK_2 receptor, even if gastrin is circulated at much higher levels. In addition, our group has challenged the concept that CCK circulates at very low levels. We have repeatedly shown that only 20% of endocrine CCK is recovered if plasma is formed and stored before measuring immunoreactive CCK. Many have reported that rats fed a high protein meal (one of the best stimulants of CCK release) have sustained 5–10 pM levels of CCK. If one accounts for the loss of CCK during plasma formation and the diminished immunoreactivity of CCK-58 (Table 2), then the calculated levels would be 25–50 pM, levels that are near the reported gastrin levels in rats (50 pM). Using the above-described method for processing blood and a CCK-58 standard for the immunoreactivity that eluted during HPLC in the region of CCK-58, we observed that protein feeding released 15–25 pM immunoreactive CCK-58 and that no other molecular form was detected (unpublished observation).

The isolated pancreatic acinar cell has been used to study the receptor binding and intracellular signals that respond to CCK.[6] Studies with isolated acinar cells indicate that CCK-evoked specific Ca^{2+} signals are generated through interactions with their respective Ca^{2+}-releasing messengers such as IP_3, cyclic adenosine diphosphoribose (cADPR), and nicotinic acid adenine dinucleotide phosphate (NAADP). In this model, CCK, acting through cADPR generation, would activate the IP_3Rs and RyRs release stimulating the subsequent Ca^{2+} release from the endoplasmic reticulum. At physiological levels (5–50 pM), CCK-8 and CCK-58 cause release of Ca^{2+} spikes near the apical pole (Fig. 3A). Supra physiological levels (5–1000 nM) of both the peptides cause global release of calcium (Fig. 3B).

BIOLOGICAL ACTIONS OF PERIPHERAL CHOLECYSTOKININ

In Fig. 1, the structures of CCK-58 from three species are presented, demonstrating differences in the amino-terminal

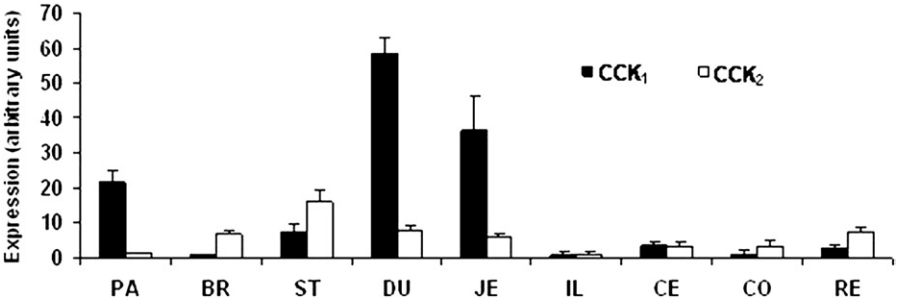

FIGURE 2 Distribution of CCK receptors, CCK_1 and CCK_2, mRNA in whole tissue in the pancreas (PA), brain (BR), stomach (ST), duodenum (DU), jejunum (JE), ileum (IL), cecum (CE), colong (CO), and rectum (RE). Most of CCK_1 receptor mRNA was found in the duodenum, jejunum, and pancreas, whereas CCK_2 receptor mRNA was mostly found in the stomach, duodenum, brain, jejunum, and rectum.

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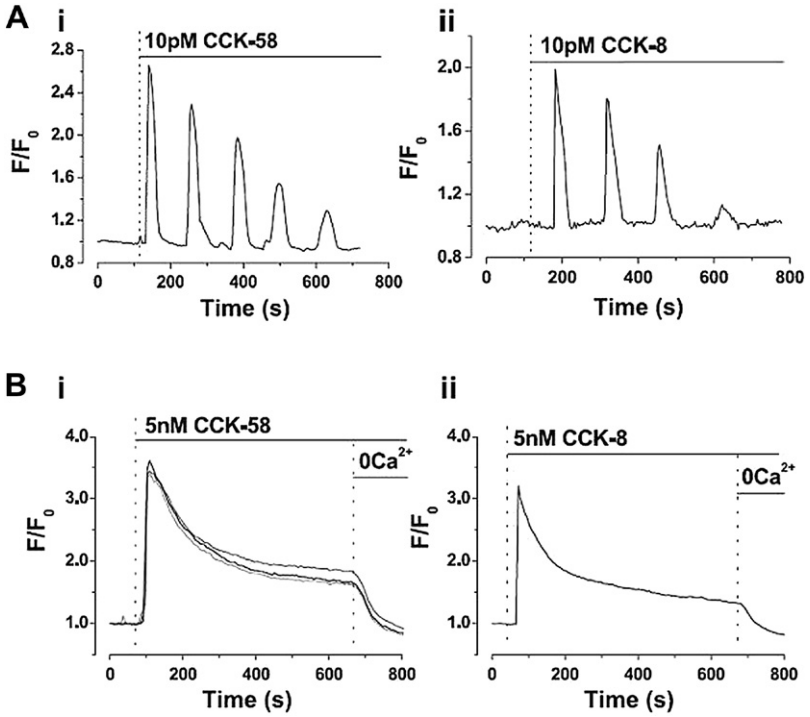

FIGURE 3 Comparative effects of CCK-8 and CCK-58 on the release of cytosolic calcium in the isolated acinar cell at low (A) and high concentrations (B). CCK-8 and CCK-58 cause oscillatory rises of $[Ca^{2+}]_c$ when perfused at 10 pM. Both the peptides cause global release of calcium when they are perfused at 5 nM concentrations. From Criddle DN, Booth DM, Mukherjee R, McLaughlin E, Green GM, Sutton R, Petersen OH, Reeve Jr JR. Cholecystokinin-58 and cholecystokinin-8 exhibit similar actions on calcium signaling, zymogen secretion, and cell fate in murine pancreatic acinar cells. *Am J Physiol Gastrointest Liver Physiol* 2009 Dec;**297**(6):G1085–92. This is an unofficial adaptation or translation of a figure that appeared in a publication of the American Physiological Society. The American Physiological Society has not endorsed the content of this adaptation or translation, or the context of its use.

sequences, and importantly the identity of the recognized biologically active carboxyl-terminal region. In the following discussion, it is important to take into account the observations on the impact of differences in primary structure in the amino-terminal region of CCK-58 on both bioactivity and immunoreactivity. This region, which is separate from the C-terminal portion of CCK that defines bioactivity and immunoreactivity, can nevertheless alter both properties of intact CCK-58. As shown in Tables 1 and 2, CCK-58 forms from different species show altered receptor binding and immunoreactivity, even though they have identical C-terminal bioactive regions. Importantly, canine CCK-58 is more potent for CCK_1 receptor binding than CCK-8 or rabbit CCK-58, suggesting that tertiary structure plays an important role in determining biological activity.

Several experimental criteria must be met to conclude that a circulating factor plays a quantitatively significant role in regulating a specific physiologic response. In the case of gastroenteric peptide hormones and regulation of gastrointestinal responses to ingestion of food, a useful set of such criteria was described by Grossman.[14] These included (1) circulating levels of the hormone should increase after ingesting food; (2) exogenous administration

TABLE 1 Canine and Rabbit CCK-58 Binding to CCK_1 and CCK_2 Receptor Acinar Cell or Brain Membranes

Peptide	IC_{50} CCK_1 receptor binding (pM)	IC_{50} CCK_2 receptor binding (pM)	$\frac{IC_{50}CCK_1}{IC_{50}CCK_2}$
Dog CCK-58	590	280	2.1
Rabbit CCK-58	1990	840	2.4
CCK-8	1780	410	4.3

TABLE 2 Relative Immunoreactivity of CCK-8 to Dog, Rabbit and Cat CCK-58.

Peptide	ID_{50} CCK form/ID_{50} CCK-8
CCK-8	1.0
Dog CCK-58	2.0
Rabbit CCK-58	20
Cat CCK-58	18

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of the hormone should reproduce the physiologic response. In the decades after Grossman's explication of these criteria, many other requirements for accepting a particular hormone as a physiologic regulator have become apparent. CCK provides a useful example of the complex nature of the steps necessary to prove that a peptide acts as a circulating hormone to regulate a specific gastrointestinal response to a meal. (1) As discussed above, the quantitation of circulating peptide levels requires detailed consideration of potential sources of nonphysiologic loss of the peptide during sampling and measurement. (2) The exact molecular forms of peptide that circulate in postprandial blood must be defined. (3) The time course of changes in circulating peptide levels must be known. (4) The cellular localization and density of specific receptors for the peptide should be known. A complicating factor is the possibility that the peptide may act as both a hormone and a local neurotransmitter to cause certain responses, requiring information about the neural distribution and release of the peptide. Thus, (5) the effects of specific receptor antagonists on a response can be a powerful tool, but it must be recognized that such antagonists may be acting to block a peptide delivered by blood or by local release from endocrine cells on nerves acting in a paracrine/neurocrine fashion on a variety of local targets. Furthermore, some specific receptor antagonists may also act centrally, depending on their permeability through the blood–brain barrier, adding a further degree of complication in interpreting experimental results in intact subjects. (6) Inhibitory or facilitative actions of other mediators may be required: exogenous administration of a single mediator in a fasting subject may not reproduce the actual physiological response.

With these criteria in mind, what is the evidence for CCK as a regulator of gastrointestinal motor and secretory responses? In humans, blood levels of CCK increase within minutes of ingesting a meal.[20] Most or all of this response seems to occur in the intestinal phase: circulating CCK concentrations are increased when individual macronutrients (fat, protein, carbohydrate) are infused into the intestine.[2] There is also evidence that bitter taste receptor agonists can induce CCK release.[15] Assuming that these effects occur by the secretion of CCK from mucosal I cells, the cellular mechanisms involved in nutrient sensing by I cells are beginning to be characterized. In all species examined, fat-induced CCK release requires luminal digestion of triglycerides into component fatty acids and monoglycerides, and only fatty acids with chain lengths of ≥12 are effective.[24] More recent findings indicate a role for specific fatty acid transporters in I cell secretion, although there may also be direct effects of fatty acids after absorption by simple diffusion.[16] Evidence suggests that intact (undigested) dietary protein and peptides and free amino acids all increase CCK secretion. Intact protein may act by serving as a luminal protector against proteolytic digestion of specific CCK releasing peptides secreted by mucosal cells and pancreas (LCRF, DBI, monitor peptide), which bind to receptors on I cells.[21] Stimulation of CCK release by peptides and amino acids is in part mediated by the Ca^{2+}-sensing receptor on I cells.[26] The cellular mechanism of the small but reproducible effect of glucose on CCK release is not known. Another releaser of CCK is gastrin releasing peptide (GRP)[18] and conversely, somatostatin[19] inhibits its release. These actions on CCK release could be a function of enteric nervous system (ENS) secreto-motor fibers acting as neurocrine modulators of I cell responses to luminal nutrients. The long held traditional view of CCK as a circulating hormonal regulator of various gastrointestinal functions was based on the assumption that on its release by mucosal I cells, CCK entered the circulation via submucosal capillaries (and subsequently traversed the liver and heart to become an arterial plasma component) and then exerted its effects on distal target cells bearing appropriate receptors. Thus, induction of a particular motor, secretory, or other event during replication of postprandial circulating levels and molecular forms of CCK by exogenous venous administration was thought to be a necessary and sufficient condition for establishing CCK as a regulator of the studied response. Although this is certainly the case, it is now clear that another mechanism—local, paracrine effects of I cell CCK on vagal sensory neurons in gut mucosa—is quite likely to mediate many of the actions of CCK. This pathway, although not yet rigorously proven to exist, seems compatible with a substantial amount of data accumulated in the past two decades showing that CCK often exerts its gastrointestinal actions via a cholinergic mediator rather than or in addition to direct effects on smooth muscle or secretory cells.

Substantial evidence supports an important role for CCK in the inhibition of gastric acid secretion and gastric emptying,[27] stimulation of gallbladder contraction,[20] and stimulation of digestive enzyme secretion by the exocrine pancreas.[1] Small exogenous doses of CCK decrease the gastric acid response to various stimuli, slow down gastric emptying, contract the gallbladder, and increase pancreatic enzyme secretion. All these actions seem to depend on a pathway involving the activation of CCK_1 receptors on afferent vagal sensory neurons. There may also be a nonneural component of CCK activation of CCK_1 receptors on gastric somatostatin cells (inhibition of gastric acid secretion), smooth muscle cells (gastric emptying and gallbladder contraction), and pancreatic acinar cells. Specific CCK_1 receptor antagonists block part or all of the effects of intestinal fatty acids on the inhibition of gastric acid and emptying and stimulation of gallbladder contraction and pancreatic enzyme secretion. There are many other actions of exogenous CCK, including the ability of CCK to decrease the amount and frequency of food intake. This action, because of its extreme importance for the occurrence and treatment of obesity, is a major focus of research.

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PATHOPHYSIOLOGICAL IMPLICATIONS

Potential involvement of CCK in various diseases is suggested by the multiple alterations in gastrointestinal, metabolic, and feeding responses seen in mice and rats with genetic deficiencies in CCK production and CCK_1 receptors. It has not, however, been easy to confirm these findings in humans because of differences in physiological mechanisms, difficulties in accurately measuring CCK release, and limitations on the type and risk of experimental manipulations that can be performed.[3]

FUTURE POSSIBILITIES

CCK-58 is a potential tool for determining the mechanisms that cause pancreatitis. CCK-8 and CCK-58 both stimulate pancreatic digestive enzyme secretion, but only CCK-58 stimulates pancreatic fluid. Even large doses of CCK-58 do not cause pancreatitis, whereas CCK-8 does so in rats.[28] Therefore, defining the differences in acinar intracellular messengers stimulated by CCK-8 and CCK-58 may yield candidate mechanisms resulting in pancreatitis.

Surprisingly, 40 years after a structure of cholecystokinin was reported, there are several aspects of primary structure that require further study. These include the actual forms of CCK in brain and peripheral neurons, the presence or absence of nonsulfated peptides in tissue and blood, the relative contribution of CCK to activation of the CCK_2 receptor, new methods for studying the enteric paracrine actions, and the integrative physiological response of CCK in the intact animal, which may not be the same as in isolated cell systems.

The role of cholecystokinin in feeding needs to be reevaluated using the major endocrine form of cholecystokinin, CCK-58. Our preliminary data are tentative, but there is an indication that this larger form differs from the actions of the most studied form, CCK-8. Several studies have shown that CCK-8 reduces meal size, but its utility as a weapon in the fight against obesity has been challenged by the observation that after a smaller meal there is a concomitant reduction in the interval until the next meal. However, although the most abundant form of CCK reduces meal size, CCK-58 does not decrease the interval until the next meal and even tends to extend the interval. This suggests that endogenous CCK-58 may be effective as a tool against obesity.

ACKNOWLEDGMENTS

This work was supported by NIH grant 1RO1DK083449 (JRR), NIH 1SC1DK094972-01A1 (AIS) and the Birmingham Racing Commission (AIS).

REFERENCES

1. Adler G, Beglinger C, Braun U, Reinshagen M, Koop I, Schafmayer A, et al. Interaction of the cholinergic system and cholecystokinin in the regulation of endogenous and exogenous stimulation of pancreatic secretion in humans. *Gastroenterology* 1991 Feb;**100**(2):537–43.
2. Beglinger C, Degen L. Fat in the intestine as a regulator of appetite–role of CCK. *Physiol Behav* 2004 Dec;**83**(4):617–21.
3. Berna MJ, Jensen RT. Role of CCK/gastrin receptors in gastrointestinal/metabolic diseases and results of human studies using gastrin/CCK receptor agonists/antagonists in these diseases. *Curr Top Med Chem* 2007;**7**(12):1211–31.
4. Brown TA, Washington MC, Metcalf SA, Sayegh AI. The feeding responses evoked by cholecystokinin are mediated by vagus and splanchnic nerves. *Peptides* 2011 Aug;**32**(8):1581–6.
5. Buchan AM, Polak JM, Solcia E, Capella C, Hudson D, Pearse AG. Electron immunohistochemical evidence for the human intestinal I cell as the source of CCK. *Gut* 1978 May;**19**(5):403–7.
6. Criddle DN, Booth DM, Mukherjee R, McLaughlin E, Green GM, Sutton R, et al. Cholecystokinin-58 and cholecystokinin-8 exhibit similar actions on calcium signaling, zymogen secretion, and cell fate in murine pancreatic acinar cells. *Am J Physiol Gastrointest Liver Physiol* 2009 Dec;**297**(6):G1085–92.
7. Deschenes RJ, Haun RS, Funckes CL, Dixon JE. A gene encoding rat cholecystokinin. Isolation, nucleotide sequence, and promoter activity. *J Biol Chem* 1985 Jan;**260**(2):1280–6.
8. Dockray GJ, Gregory RA, Hutchison JB, Harris JI, Runswick MJ. Isolation, structure and biological activity of two cholecystokinin octapeptides from sheep brain. *Nature* 1978 Aug;**274**(5672):711–3.
9. Eberlein GA, Eysselein VE, Davis MT, Lee TD, Shively JE, Grandt D, et al. Patterns of prohormone processing. Order revealed by a new procholecystokinin-derived peptide. *J Biol Chem* 1992 Jan;**267**(3):1517–21.
10. Eberlein GA, Eysselein VE, Hesse WH, Goebell H, Schaefer M, Reeve Jr JR. Detection of cholecystokinin-58 in human blood by inhibition of degradation. *Am J Physiol* 1987 Oct;**253**(4 Pt 1):G477–82.
11. Eysselein VE, Reeve Jr JR, Eberlein G. Cholecystokinin–gene structure, and molecular forms in tissue and blood. *Z Gastroenterol* 1986 Oct;**24**(10):645–59.
12. Eysselein VE, Reeve Jr JR, Shively JE, Miller C, Walsh JH. Isolation of a large cholecystokinin precursor from canine brain. *Proc Natl Acad Sci U S A* 1984 Nov;**81**(21):6565–8.
13. Furness JB, Costa M, Keast JR. Choline acetyltransferase- and peptide immunoreactivity of submucous neurons in the small intestine of the guinea-pig. *Cell Tissue Res* 1984;**237**(2):329–36.
14 Grossman MI. Physiological effects of gastrointestinal hormones. *Fed Proc* 1977 Jun;**36**:1930–2.
15. Hao S, Sternini C, Raybould HE. Role of CCK1 and Y2 receptors in activation of hindbrain neurons induced by intragastric administration of bitter taste receptor ligands. *Am J Physiol Regul Integr Comp Physiol* 2008 Jan;**294**(1):R33–8.
16. Hillsley K, Grundy D. Serotonin and cholecystokinin activate different populations of rat mesenteric vagal afferents. *Neurosci Lett* 1998 Oct;**255**(2):63–6.
17. Jorpes E, Mutt V. Cholecystokinin and pancreozymin, one single hormone? *Acta Physiol Scand* 1966 Jan;**66**(1):196–202.

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18. Ladenheim EE, Wohn A, White WO, Schwartz GJ, Moran TH. Inhibition of gastric emptying by bombesin-like peptides is dependent upon cholecystokinin-A receptor activation. *Regul Pept* 1999 Oct;**84**:101–6.
19. Lembcke B, Creutzfeldt W, Schleser S, Ebert R, Shaw C, Koop I. Effect of the somatostatin analogue sandostatin (SMS 201-995) on gastrointestinal, pancreatic and biliary function and hormone release in normal men. *Digestion* 1987;**36**(2):108–24.
20. Liddle RA, Goldfine ID, Rosen MS, Taplitz RA, Williams JA. Cholecystokinin bioactivity in human plasma. Molecular forms, responses to feeding, and relationship to gallbladder contraction. *J Clin Invest* 1985 Apr;**75**(4):1144–52.
21. McVey DC, Romac JM, Clay WC, Kost TA, Liddle RA, Vigna SR. Monitor peptide binding sites are expressed in the rat liver and small intestine. *Peptides* 1999;**20**(4):457–64.
22. Mutt V. Chemistry, isolation and purification of gastrointestinal hormones. *Biochem Soc Trans* 1980 Feb;**8**(1):11–4.
23. Reeve Jr JR, Green GM, Chew P, Eysselein VE, Keire DA. CCK-58 is the only detectable endocrine form of cholecystokinin in rat. *Am J Physiol Gastrointest Liver Physiol* 2003 Aug;**285**(2):G255–65.
24. Stewart JE, Feinle-Bisset C, Keast RS. Fatty acid detection during food consumption and digestion: associations with ingestive behavior and obesity. *Prog Lipid Res* 2011 Jul;**50**(3):225–33.
25. Wang W, Cain BM, Beinfeld MC. Adult carboxypeptidase E-deficient fat/fat mice have a near-total depletion of brain CCK 8 accompanied by a massive accumulation of glycine and arginine extended CCK: identification of CCK 8 Gly as the immediate precursor of CCK 8 in rodent brain. *Endocrine* 1998 Dec;**9**(3):329–32.
26. Wang Y, Chandra R, Samsa LA, Gooch B, Fee BE, Cook JM, et al. Amino acids stimulate cholecystokinin release through the Ca2+-sensing receptor. *Am J Physiol Gastrointest Liver Physiol* 2011 Apr; **300**(4):G528–37.
27. Whited KL, Thao D, Lloyd KC, Kopin AS, Raybould HE. Targeted disruption of the murine CCK1 receptor gene reduces intestinal lipid-induced feedback inhibition of gastric function. *Am J Physiol Gastrointest Liver Physiol* 2006 Jul;**291**(1):G156–62.
28. Yamamoto M, Reeve Jr JR, Green GM. Supramaximal CCK-58 does not induce pancreatitis in the rat: role of pancreatic water secretion. *Am J Physiol Gastrointest Liver Physiol* 2007 Apr;**292**(4):G964–74.
29. Yamamoto M, Reeve Jr JR, Keire DA, Green GM. Water and enzyme secretion are tightly coupled in pancreatic secretion stimulated by food or CCK-58 but not by CCK-8. *Am J Physiol Gastrointest Liver Physiol* 2005 May;**288**(5):G866–79.

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Chapter 163

CGRP

S. Evangelista

ABSTRACT

Calcitonin gene-related peptide (CGRP), a 37-amino acid peptide produced by the alternative processing of calcitonin gene transcripts, is widely distributed in both the peripheral and central nervous system of mammals. In the gut, CGRP is a marker of afferent fibers in the upper gastrointestinal tract, being almost completely depleted after treatment with the selective neurotoxin capsaicin that targets these fibers via transient receptor potential vanilloid type-1. Its receptor and stable antagonists have implications for migraine. Beside this, several studies have focused on the role of CGRP at the gastrointestinal level. CGRP-containing afferent fibers of the enteric nervous system are involved in the gastrointestinal defense against irritants, intestinal nociception, modulation of gastrointestinal motility and secretion, and experimentally induced models of colitis. Restoration of gastric CGRP levels was found in animals bearing ulcers in the healing state, but its main effects on stomach homeostasis rely on local vasodilator actions during increased acid-back diffusion.

DISCOVERY AND STRUCTURE OF mRNA GENE

Calcitonin gene-related peptide (CGRP) is the first biologically active neuropeptide identified through the molecular biology approach.[28]

In the central and peripheral nervous systems, the RNA transcript from the calcitonin gene yields an mRNA, which encodes the 37-amino acid residue peptide CGRP, proved to be an important physiologic mediator.[32,33] CGRP exists in two forms as CGRP-α, which is generated by transcription and alternative splicing of the calcitonin (CT)/CGRP-α gene, and CGRP-β, which is encoded by the CGRP-β gene. The two forms differ by one and three amino acids in rats and humans, respectively. CGRP-β has been shown to have a pattern of expression that overlaps, but is nonidentical to, that of CGRP-α, with CGRP-α having a higher level of expression in most neurons of the central and peripheral nervous system.[23] One exception is the intrinsic neurons of the enteric nervous systems, which express predominantly CGRP-β mRNA.

CGRP is a member of a family of peptides that share sequence homology and have some overlapping biological activities. The members of the family include amylin (see Ingestive Peptides section of this book), adrenomedullin (see Cancer/Anticancer Peptides, Brain Peptides, Endocrine Peptides, Gastrointestinal Peptides, Cardiovascular Peptides, Renal Peptides, and Respiratory Peptides sections of this book), and CGRP (also see Brain Peptides section).

DISTRIBUTION OF mRNA/PEPTIDE AND RECEPTORS

CGRP is one of the most abundant peptides produced in both peripheral and central neurons. In the spinal cord, the function and expression of CGRP may differ depending on the location of synthesis. CGRP is derived mainly from the cell bodies of motor neurons when synthesized in the anterior horn of the spinal cord and may contribute to the regeneration of nervous tissue after injury. Conversely, CGRP is derived from posterior root ganglion when synthesized in the posterior horn of the spinal cord and may be linked to the transmission of pain. In the trigeminal vascular system, the cell bodies on the trigeminal ganglion are the main source of CGRP. CGRP is believed to play a role in cardiovascular homeostasis and nociception.[32,33]

High levels of CGRP-like immunoreactivity (LI) have been found in the rat[29] and human[31] stomach, and the absence of CGRP-LI in endocrine cells suggests that it is more important as a neurotransmitter than as a circulating hormone. Because no positive CGRP cells are found in the stomach, most gastric CGRP is of extrinsic origin and is contained and released from peripheral endings of capsaicin-sensitive afferents.[18]

The use of capsaicin, a selective neurotoxin for sensory nerves, has led to the exploration of the functional role of these neurons at the gastric level and to the hypothesis of dual "sensory-efferent" functions mediated by capsaicin-sensitive neurons.[15,18] Capsaicin affects the afferent neurons by two distinct phases: an initial excitation followed by a long-lasting refractory state during which the neurons are not responsive to the neurotoxin and other stimuli. The refractory state is usually reversible in terms of weeks, but the systemic administration of high doses of the neurotoxin causes

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00163-9
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permanent neurotoxicity.[15] These neurons are important to transmit sensory information toward the central nervous system and also to release their neurotransmitters toward the periphery, playing a local effector role in several regulatory processes. Recent studies have shown that capsaicin specifically targets these afferent nerves via transient receptor potential vanilloid of type-1 (TRPV-1), a nonselective cation channel with high permeability for Ca^{2+}.[18] TRPV-1 belongs to a family of store operated Ca^{2+} channels and acts as a polymodal detector of potentially harmful stimuli including capsaicin, noxious heat, H^+ ions, anandamide, and several lipoxygenase products.[18]

An example of dual "sensory-efferent" functions is seen with gastric ulcers: animals pretreated with high doses of capsaicin show an aggravation of experimentally induced ulceration because of depletion of protective neurotransmitters. On the other hand, the local application of low doses of capsaicin affords gastric protection through the release of neuropeptides (among them: substance P and CGRP) in the periphery.[7,15,16,30] CGRP is considered to be the likely mediator of sensory neurons in the stomach and their marker in the upper gastrointestinal tract.

In the enteric nervous system, CGRP-β is expressed in a subset of Dogiel type II, intrinsic primary afferent neurons that are not sensitive to capsaicin, whereas CGRP-α is the primary isoform expressed in the sensory afferent neurons of the extrinsic nervous system originating from the dorsal root and vagal ganglia (Fig. 1).

In the past, CGRP receptors have been divided into two classes, CGRP1 and CGRP2. After the cloning of calcitonin receptor-like receptor (CLR) and receptor activity-modifying proteins (RAMPs), it became clear that the CGRP1 receptor was a complex between CLR and RAMP1 (Fig. 2). It is now apparent that the CGRP2 receptor phenotype is the result of CGRP acting at receptors for amylin and adrenomedullin. Accordingly, the term "CGRP2" receptor should no longer be used, and the "CGRP1" receptor should be known as the "CGRP" receptor. The CGRP receptor consists of a seven transmembrane-spanning protein designated RAMP1 and an intracellular component protein (RCP). RAMP1 is involved in receptor trafficking and is required for CGRP binding to CLR, whereas the interaction of CLR with other RAMP proteins forms adrenomedullin receptors.[8]

The human C-terminal fragment of the peptide, $hCGRP_{8–37}$, was the first and widely used tool for determining whether the action of CGRP and that of CT is mediated via specific CGRP receptors or CT receptors. It selectively antagonizes CGRP activity, being also an agonist for CT receptor.[32] So far, two chemically unrelated CGRP antagonists, olcegepant and telcagepant, are in development as a potential treatment for acute migraine attacks. This new class of drugs is designed to block and/or reverse CGRP-mediated dilation of intracranial vessels induced by the activation of the main sensory nerves in the brain.[9]

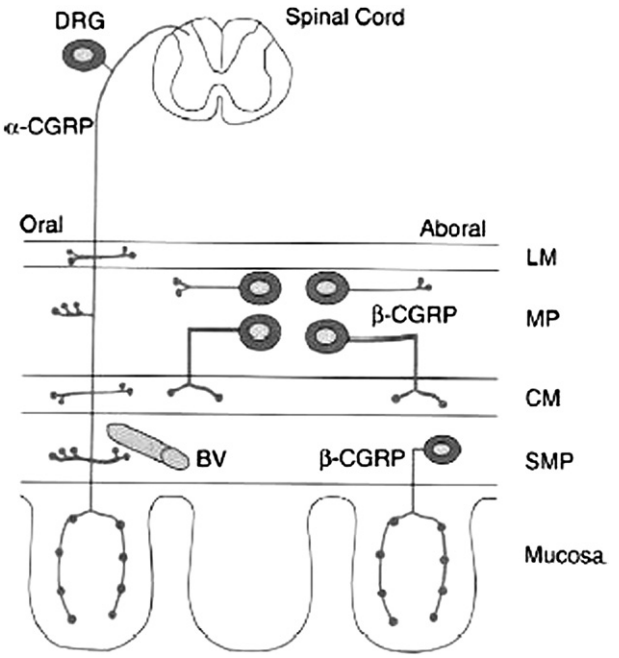

FIGURE 1 Schematic representation of the extrinsic and intrinsic CGRP-containing neurons in the rat intestine. Extrinsic spinal afferents have their cell bodies in the dorsal root ganglia and contain only α-CGRP. CGRP-containing extrinsic nerve terminals innervate the circular and longitudinal smooth muscle layers; the myenteric plexus; the submucosal plexus, where they densely innervate vascular structures; and the mucosa. CGRP-containing intrinsic enteric neurons express only β-CGRP. These neurons are localized in the submucosal and myenteric plexuses. Submucosal neurons innervate the mucosa, whereas myenteric neurons project orally and aborally, both within the myenteric plexus and to the circular muscle layer, and participate in the regulation of motility. BV, blood vessel; CM, circular smooth muscle; DRG, dorsal root ganglion; LM, longitudinal smooth muscle; MP, myenteric plexus; SMP, submucosal plexus.

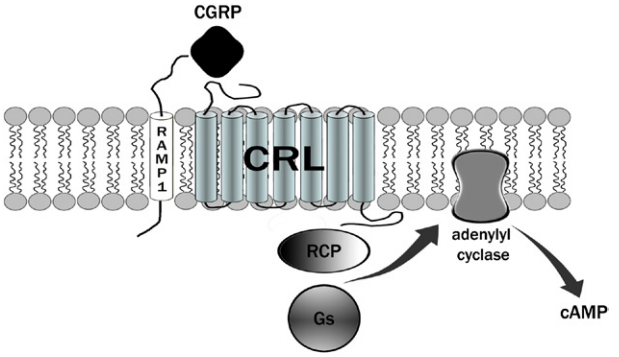

FIGURE 2 Schematic view of the CGRP receptor that consists of calcitonin receptor-like peptide (CLR), receptor activity-modifying protein 1 (RAMP1), and receptor component protein (RCP).

BIOLOGICAL ACTIONS WITHIN GASTROINTESTINAL TRACT

After its discovery, CGRP became the subject of many studies, which have been summarized in several reviews,[32,33] and among them, some have been addressed to study the

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role of CGRP in the gut.[1,7,17,18] CGRP-containing pathways in the enteric nervous system have been shown to be involved in intestinal nociception, modulation of gastrointestinal motility, and secretion and to play a role in experimentally induced models of colitis.

Concerning the motility, two distinct effects seem to be important for CGRP action on intestinal smooth muscle: a direct relaxant effect, observed in the longitudinal and circular muscles of intestinal segments from various species and indirect effects, produced via the stimulation of neuronal receptors leading to the release of other transmitters which, in turn, affect intestinal motility.[1] Similar to other species, the human gut (especially the circular musculature) is relaxed by capsaicin, but this effect seems to be under the control of different neurotransmitter agents, nitric oxide (NO) and VIP, neither of them of intrinsic neuronal origin. The "local efferent" effect of extrinsic afferent neurons can also be demonstrated *in vivo*, in both animals and man. Yet, nearly the normal motility of the small and large intestines is maintained in animals with functionally inhibited capsaicin-sensitive nerves. CGRP is involved also in the peristaltic reflex elicited by muscle stretch and mucosal stimulation.[14] Peristaltic reflexes are subjected to the activity of several neurotransmitters: both the 5-HT_4 receptor antagonists and the CGRP antagonist $hCGRP_{8–37}$ inhibit ascending contraction and descending relaxation. Thus, the reflex in the mouse, like that in rat and human intestine, is initiated by mucosal release of 5-HT and activation of 5-HT_4 receptors on CGRP sensory neurons and is relayed via somatostatin and opioid interneurons to VIP/nitric-oxide synthase inhibitory motor neurons and via cholinergic interneurons to acetylcholine/tachykinin excitatory motor neurons. The importance of this system in regulating GI movements may be exaggerated under pathophysiological conditions, first of all inflammation. The afferent function of capsaicin-sensitive nerves plays a role in sympathetic reflexes, such as the inhibition of GI motility after laparotomy or by peritoneal irritation and diabetic gastroparesis.[1]

CGRP is a potent suppressor of basal and secretagogue-stimulated gastric acid and pepsin secretion in humans, dogs, rabbits, and rats.[7,27] Multiple mechanisms seem to be involved in the release of somatostatin from D cells,[27] where CL ands RAMP1 mRNAs has been found to be involved in the direct inhibition of gastrin- and cholinergic-dependent stimulatory effects and the stimulation of mucus and bicarbonate from gastroduodenal mucosa.[7]

For the ion and fluid intestinal transport, CGRP exhibited concentration-dependent dual actions on rat colonic epithelia acting directly on colonic enterocytes. At low concentrations (i.e. 0.03–10 nM), CGRP produces antisecretory effects that are abolished by pretreating tissues with tetrodotoxin (TTX). At higher concentrations (30–200 nM), secretory responses predominate, and these effects are not abolished by TTX.[5] In guinea-pig colon, CGRP depolarizes myenteric neurons that are synaptically coupled to submucosal neurons. Cholinergic neurons using nicotinic and muscarinic synapses for transmission of information to the epithelium are involved in inducing chloride secretion. CGRP is also present in human epithelia but it is not known as to whether the described secretory effects are of physiological relevance. During inflammation, the activation of CGRP receptors may result in effects on tight junction permeability not observed in normal tissue.[18]

It is known that the vascular system of the gastrointestinal tract is innervated by both sympathetic vasoconstrictor and parasympathetic vasodilator neurons but splanchnic blood vessels receive also a dense supply of fibers containing CGRP.[13,29] These fibers innervate blood vessels of the entire body, and their density is greater around arteries than veins. In the gastrointestinal tract, CGRP-containing nerve fibers are surrounding the mucosa and submucosa blood vessels and species differences are known concerning their presence, being scarce in human and porcine stomach.[13] CGRP exerts also a potent vasodilator and past studies have shown it as a noncholinergic and nonadrenergic transmitter able to dilate mesenteric artery after periarterial stimulation.[17] When released from extrinsic afferent nerve fibers in acid-threatened stomach, CGRP causes a NO-dependent vasodilation that has been shown to be a defensive signal in the face of pending acid injury in the gastric mucosa.[16] The vasodilator effect of CGRP has been widely studied in the upper gastrointestinal tract (see below) and is working under pathological conditions.

PATHOPHYSIOLOGICAL IMPLICATIONS

Role of GCRP in Gastrointestinal Protection

Systemic administration of $hCGRP_{8–37}$ enhances indomethacin- or ethanol-induced mucosal lesions while low dose of capsaicin and/or TRPV-1 agonists have clearly shown to exert gastroprotection via the stimulation of sensory nerves and the local release of CGRP[18] indicating that endogenous CGRP plays a trophic role on mucosal defense of the stomach. Further, gastroprotection by capsaicin was dose dependently antagonized by the CGRP antagonist, $hCGRP_{8–37}$, and by a monoclonal antibody to CGRP.[7] Recent studies using CGRP-knockout mice[24] have strongly confirmed these findings: the protective action of capsaicin against ethanol-induced lesions was completely abolished in CGRP−/− mice (Fig. 3). The authors also showed that the adaptive gastroprotection induced by previous exposure of the stomach to mild irritants was mediated by CGRP being completely absent in CGRP knockout mice.[24]

Furthermore, several studies have reported a depletion of tissue CGRP-LI during the formation of several gastric ulcers.[7] Decreased levels of gastric CGRP-LI were observed after acetic acid-, cysteamine-, concentrated ethanol-, or water-immersion stress ulcers, whereas the ulcerogens did not affect the tissue content of other peptides[7] suggesting

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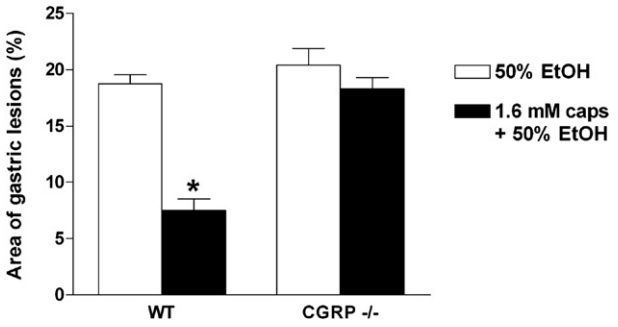

FIGURE 3 Lack of capsaicin (caps)-induced gastric mucosal protection against 50% ethanol-induced lesions in mice knockout to CGRP (CGRP–/–). Mean ± S.E values of 5–6 mice for group. *$P < 0.01$ between wild-type (WT) groups. Modified from Ref. 24.

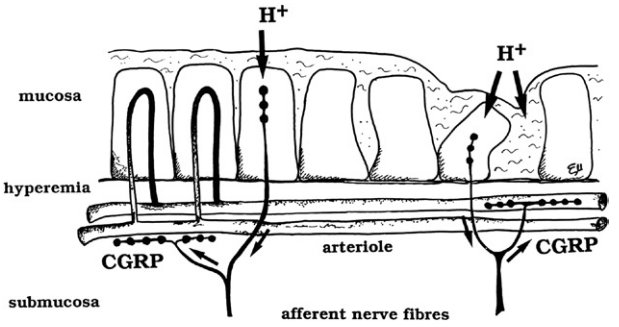

FIGURE 4 CGRP-mediated vasodilation induced by sensory fibers in the stomach. Hydrogen ions diffusing into the gastric wall of disrupted mucosal barrier may activate both mucosal and submucosal branches of afferent nerve endings to release CGRP and cause arteriolar dilation.

that reduction in gastric CGRP-LI cannot be attributed only to damage of the tissue. In humans, the TRPV-1 receptor and CGRP were detected by immunohistochemistry in patients with different gastric diseases[6,31] and TRPV-1 labeling was localized in the parietal cells of stomach from surgical specimens by means of immunohistochemistry, in situ hybridization, and Western blot analysis.[10] Furthermore, in healthy volunteers, low concentrations of capsaicin protect against gastric injuries induced by ethanol or indomethacin,[22] and a suspension of 20 g of chilli was able to protect the human gastroduodenal mucosa from the damage induced by a high dose of acetylsalicylic acid.[34]

Restoration of CGRP-LI was however observed in animals bearing ulcers during healing,[7] and intense CGRP immunostaining was observed in the ulcer margin of patients with gastric ulcers.[31] Healing of ulcer elicited by acetic acid in CGRP knockout mice was markedly delayed as compared with that of wild-type mice as well as the granulation tissue, and neovascularization at the base of these ulcers was poor in CGRP–/– animals.[24] Furthermore, in isolated rat stomachs exposed to hyperosmolar concentrations of sodium chloride, the addition of CGRP enhanced the recovery of mucosal integrity after mild but not severe damage produced by the lesioning agent.[21] These findings and the observation that there is a tonic flow of CGRP toward the peripheral endings of sensory fibers suggest that the peptide is restored in connection with the healing of ulcers after its release produced by the ulcerogen.

Concerning the known CGRP antisecretory effect[7] along with somatostatin releasing activity from gastric D cells,[27] other mechanisms have been explored to explain the CGRP protective role in the stomach. It is known that hyperemia induced by an injury is a physiological response to tissue damage and limits the extent of injury in the stomach.[16,17] Modulation of this response is an important defense mechanism, and it has been reported that CGRP released by capsaicin-sensitive fibers is involved in gastric hyperemic protective effects.[16]

Local application of low doses of capsaicin or TRPV-1 agonists able to stimulate gastric nerve endings[18,30] enhance gastric blood flow through the mucosa, dilate submucosal arterioles, and reduce concomitantly gross mucosal lesions produced by ethanol.[16,30]

Afferent nerve endings originating from dorsal root ganglia and passing through the celiac/superior mesenteric ganglion complex have been reported to modulate these effects, which are abolished by chemical ablation induced by the pretreatment with high doses of capsaicin.[16,30]

It is noteworthy that the gastric hyperemic responses to acid-back diffusion[16] or hypertonic solution[20] depend on sensory innervation of the tissue. On the other hand, it has been shown that exposure of isolated tissues of the rat stomach to low pH solution produces a selective release of CGRP.[12] Hydrogen ions diffusing into the gastric wall of disrupted mucosal barrier may promote protective vasodilation by activating the "efferent" function of capsaicin-sensitive afferent nerves (Fig. 4).

A mediator role of CGRP in this protective hyperemia induced by afferent fibers derives also from the following observations:

1. capsaicin released CGRP from isolated tissues of the rat stomach,[7,16] an effect that was abolished after disruption of capsaicin-sensitive neurons[16] or in CGRP–/– animals,[24]
2. intravascular application of capsaicin induced the release of CGRP into the vascular bed of the rat stomach followed by a marked vasodilator effect,[16,30]
3. low doses of locally applied CGRP dilated submucosal arterioles and enhanced gastric blood flow,[16,18]
4. CGRP-induced vasodilation is in keeping with the distribution of this peptide around blood vessels in the stomach[29] and the large number of vascular CGRP receptors in this region,[11]
5. gastric vasodilating response to topical application of capsaicin was blocked, at least in part, by the antagonist $hCGRP_{8–37}$.[19]

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TABLE 1 Percentage Vanilloid Receptor 1 (TRPV1), Substance P, and CGRP Immunoreactive Areas in Rectal Samples from Patients Characterized by Rectal Hypersensitivity and Controls. Values are Median with a 95% Range. Modified from Ref. 3

	Rectal hypersensitivity	Control	*p*
Mucosa			
TRPV1	0.44 (0.30–0.59)	0.11 (0.00–0.21)	0.0005
Substance P	0.45 (0.29–0.85)	0.39 (0.29–0.61)	0.43
Submucosa			
TRPV1	3.50 (2.20–3.90)	0.80 (0.62–1.30)	0.002
CGRP	24.3 (14.2–42.8)	4.30 (0.10–10.0)	0.0009
Circular muscle			
TRPV1	2.30 (1.20–3.00)	0.78 (0.19–1.62)	0.002
Longitudinal muscle			
TRPV1	1.13 (0.70–2.20)	0.32 (0.14–0.80)	0.01

Taken together, these observations strongly suggest that CGRP is the probable mediator of the protective function of sensory nerves in the stomach.

Similarly, other gastrointestinal disorders involving inflammation, infection or ulceration were reported correlated to change in CGRP innervation.[18] Experimental colitis induced by trinitrobenzensulfonic acid (TNB) and inflammatory bowel diseases were associated with tissue CGRP decrease[26] and upregulation of TRPV1 in nerve fiber of the colon.[35] Silencing of CLR or the use of the CGRP antagonist increased the severity of the TNB lesions.[4,26] This is only an example of how CGRP innervation can be used as a surveillance system that along other ones (immune, endocrine, etc.) can coordinate the defense of all gastrointestinal tracts from injury.

CGRP in Gastrointestinal Motility Disorders and Visceral Sensitivity

Infection, inflammation, intestinal anaphylaxis, trauma, and stress are known to induce change in motility, and all have been reported to be associated with the activity and release of tachykinins and CGRP from extrinsic afferents. Peptides are released from central endings of afferent neurons in the spinal cord and brainstem and mediate transmission to the efferent reflex arc. Likewise, CGRP released within the gut in response to an irritant may facilitate the excitation of afferents and participate in the transmission of nociceptive traffic between afferent neurons and second-order neurons in the spinal cord and brainstem.[2] As an example, the induction of colonic inflammation by acetic acid instillation facilitates the pain reaction to rectal distension, changing the pain threshold, and this hypersensitivity can be blocked by a CGRP antagonist.[2,25] The precise site at which CGRP and also tachykinin receptors mediate colonic hyperalgesia seems to be the spinal cord because CGRP was enhanced there after colonic irritation and its antagonist is more effective if given by intrathecal than intravenous route.[2,25] Because irritation, immune challenge, and inflammation release both tachykinin and CGRP from extrinsic afferents and from intrinsic neurons inside the gut, these peptides may either increase the peripheral sensory gain of extrinsic afferents or contribute to the afferent transmission of algesic signal within the central nervous system. As extrinsic afferents of the gut do not possess receptors for CGRP, it is probable that peptide-induced sensitization or excitation of sensory neurons is an indirect consequence of peptide action on gastrointestinal effectors.[18]

It is noteworthy that rectal biopsy samples taken from patients with well-characterized rectal hypersensitivity showed a marked increase in TRPV1 in muscle, submucosal, and mucosal layers as compared with that of controls[3] (Table 1). Submucosal immunoreactive CGRP expressed in ganglion cells but not SP was increased as well (Table 1). The increase in TRPV1 significantly correlated with the decrease in heat and distension sensory threshold confirming that both afferent and intrinsic fibers take part in the change of signaling pathways of visceral hypersensitivity.

ACKNOWLEDGMENTS

We would like to thank Dr. Stefania Meini for drawing the figures.

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REFERENCES

1. Bartho L, Benko R, Holzer-Petsche U, Holzer P, Undi S, Wolf M. Role of extrinsic afferent neurons in gastrointestinal motility. *Eur Rev Med Pharmacol Sci* 2008;**12**(Suppl. 1):21–31.
2. Bueno L, Fioramonti J, Delvaux M, Frexinos J. Mediators and pharmacology of visceral sensitivity: from basic to clinical investigations. *Gastroenterology* 1997;**112**:1714–43.
3. Chan CLH, Facer P, Davis JB, Smith GD, Egerton J, Williams NS, et al. Sensory fibres expressing capsaicin receptor TRPV1 in patients with rectal hypersensitivity and faecal urgency. *Lancet* 2003; **361**:385–91.
4. Clifton MS, Hoy JJ, Chang J, Idumalla PS, Fakhruddin H, Grady EF, et al. Role of calcitonin receptor-like receptor in colonic motility and inflammation. *Am J Physiol Gastrointest Liver Physiol* 2007;**293**: G36–44.
5. Cox HM, Ferrar JA, Cuthbert AW. Effects of alpha- and beta-calcitonin gene-related peptides upon ion transport in rat descending colon. *Br J Pharmacol* 1989;**97**:996–8.
6. Dömötör A, Peidl Z, Vincze A, Hunyady B, Szolcsányi J, Kereskay L, et al. Immunohistochemical distribution of vanilloid receptor, calcitonin-gene related peptide and substance P in gastrointestinal mucosa of patients with different gastrointestinal disorders. *Inflammopharmacology* 2005;**13**:161–77.
7. Evangelista S. Role of calcitonin-gene related peptide in gastric mucosal defence and healing. *Curr Pharmaceut Des* 2009;**15**:3571–6.
8. Evans BN, Rosenblatt MI, Mnayer LO, Oliver KR, Dickerson IM. CGRP-RCP, a novel protein required for signal transduction at calcitonin gene-related peptide and adrenomedullin receptors. *J Biol Chem* 2000;**275**:31438–43.
9. Farinelli I, Missori S, Martelletti P. Proinflammatory mediators and migraine pathogenesis: moving towards CGRP as a target for a novel therapeutic class. *Expert Rev Neurother* 2008;**8**:1347–54.
10. Faussone-Pellegrini MS, Taddei A, Bizzoco E, Lazzeri M, Vannucchi MG, Bechi P. Distribution of the vanilloid (capsaicin) receptor type 1 in the human stomach. *Histochem Cell Biol* 2005;**124**:61–8.
11. Gates T, Zimmermann RP, Mantyh CR, Vigna SR, Mantyh PW. Calcitonin gene-related peptide-α receptor binding sites in the gastrointestinal tract. *Neuroscience* 1989;**31**:757–70.
12. Geppetti P, Tramontana M, Evangelista S, Renzi D, Maggi CA, Fusco BM, et al. Differential effect of neuropeptide release of different concentrations of hydrogen ions on afferent and intrinsic neurons of the rat stomach. *Gastroenterology* 1991;**101**:1505–11.
13. Green T, Dockray GJ. Characterization of the peptidergic afferent innervation of the stomach in the rat, mouse and guinea-pig. *Neuroscience* 1988;**25**:181–93.
14. Grider JR. CGRP as a transmitter in the sensory pathway mediating peristaltic reflex. *Am J Physiol* 1994;**266**:G1139–45.
15. Holzer P. Capsaicin: cellular targets, mechanisms of action, and selectivity for thin sensory neurons. *Pharmacol Rev* 1991;**43**:143–201.
16. Holzer P. Neural emergency system in the stomach. *Gastroenterology* 1998;**114**:823–9.
17. Holzer P. Peptidergic sensory neurons in the control of vascular functions: mechanisms and significance in the cutaneous and splanchnic vascular beds. *Rev Physiol Biochem Pharmacol* 1992;**121**:49–146.
18. Holzer P. Role of visceral afferent neurons in mucosal inflammation and defense. *Curr Opin Pharmacol* 2007;**7**:563–9.
19. Li D-S, Raybould HE, Quintero E, Guth PH. Role of calcitonin gene-related peptide in gastric hyperemic response to intragastric capsaicin. *Am J Physiol* 1991;**261**:G657–61.
20. Matsumoto J, Ueshima K, Takeuchi K, Okabe S. Capsaicin-sensitive afferent neurons in adaptive responses of the rat stomach induced by a mild irritant. *Japn J Pharmacol* 1991;**55**:181–5.
21. Miller CA, Nakashima M, Gittes G, Debas HT. Calcitonin gene-related peptide (CGRP) upregulates the restitution of rat gastric mucosa in vitro. *J Surg Res* 1995;**58**:421–4.
22. Mozsik G, Szolcsanyi J, Racz I. Gastroprotection induced by capsaicin in healthy human subjects. *World J Gastroenterol* 2005;**11**:5180–4.
23. Mulderry PK, Ghatei MA, Spokes RA, Jones PM, Pierson AM, Hamid QA, et al. Differential expression of alpha-CGRP and beta-CGRP by primary sensory neurons and enteric autonomic neurons of the rat. *Neuroscience* 1988;**25**:195–205.
24. Ohno T, Hattori Y, Komine R, Ae T, Mizuguchi S, Arai K, et al. Roles of calcitonin gene-related peptide in maintenance of gastric mucosal integrity and in enhancement of ulcer healing and angiogenesis. *Gastroenterology* 2008;**134**:215–25.
25. Plourde V, St-Pierre S, Quirion R. Calcitonin gene-related peptide in viscerosensitive response to colorectal distension in rats. *Am J Physiol* 1997;**273**:G191–6.
26. Reinshagen M, Flämig G, Ernst S, Geerling I, Wong H, Walsh JH, et al. Calcitonin gene-related peptide mediates the protective effect of sensory nerves in a model of colonic injury. *J Pharmacol Exp Ther* 1998;**286**:657–61.
27. Ren J, Dunn ST, Tang Y, Wang Y, Gao J, Brewer K, et al. Effects of calcitonin gene-related peptide on somatostatin and gastrin gene expression in rat antrum. *Regul Pept* 1998;**73**:75–82.
28. Rosenfeld MG, Mermod J, Amara SG, Swanson LW, Sawchenko PE, Rivier J, et al. Production of a novel neuropeptide encoded by the calcitonin gene via tissue-specific RNA processing. *Nature* 1983; **304**:129–35.
29. Sternini C, Reeve JR, Brecha N. Distribution and characterization of calcitonin gene-related peptide immunoreactivity in the digestive system of normal and capsaicin-treated rats. *Gastroenterology* 1987;**93**:852–62.
30. Szolcsanyi J, Bartho L. Capsaicin-sensitive afferents and their role in gastroprotection: an update. *J Physiol (Paris)* 2001;**95**:181–8.
31. Tani N, Miyazawa M, Miwa T, Shibata M, Yamaura T. Immunohistochemical localization of calcitonin gene-related peptide in the human gastric mucosa. *Digestion* 1999;**60**:338–43.
32. van Rossum D, Hanisch UK, Quirion R. Neuroanatomical localization, pharmacological characterization and functions of CGRP, related peptides and their receptors. *Neurosci Biobehav Rev* 1997;**21**: 649–78.
33. Wimalawansa SJ. Calcitonin gene-related peptide and its receptors: molecular genetics, physiology, pathophysiology and therapeutic potential. *Endocr Rev* 1996;**17**:533–5.
34. Yeak KG, Kang JY, Yap I, Guan R, Tan CC, Wee A, et al. Chili protects against aspirin-induced gastroduodenal mucosal injury in humans. *Dig Dis Sci* 1995;**40**:580–3.
35. Yiangou Y, Facer P, Dyer NH, Chan CL, Knowles C, Williams NS, et al. Vanilloid receptor 1 immunoreactivity in inflamed human bowel. *Lancet* 2001;**357**:1338–9.

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Chapter 164

Galanin

Duan Chen, Yosuke Kodama, Bård Kulseng, Helene Johannessen and Chun-Mei Zhao

ABSTRACT

Galanin, a 29- to 30-amino acid peptide, was originally discovered in the gut, and the name came from the N-terminal glycine residue and the C-terminal alanine. Galanin nerve fibers are primarily intrinsic to the enteric nervous systems. Galanin receptors, that is, GalR1, GalR2, and GalR3, are coupled to Gβγ and/or $G\alpha_i$, $G\alpha_{q/11}$, and $G\alpha_{i/o}$ signaling pathways, respectively, and located in epithelial cells, smooth muscle, and submucosal and myenteric neurons throughout the gut. Galanin stimulates intestinal chloride secretion and inhibits gastric acid secretion, the release of glucagon-like peptide 1, and the mechanosensitivity of vagal afferents. Galanin modulates the motility and sphincters (both stimulation and inhibition) by acting directly on the smooth muscle and indirectly through the release of other transmitters. The discoveries of gut galanin and its biological actions have potential implications for understanding the pathogeneses of gastrointestinal dysfunction, inflammatory bowel disease, rotavirus- or *Salmonella enterica*-induced diarrhea, esophageal squamous cell carcinoma, and colon cancer.

DISCOVERY

Carboxyamidation is a general characteristic of many biologically active peptides. A technique of fragmentation analysis to reveal the occurrence of peptides with a COOH-terminal α-amide structure in tissue extracts was developed by Kazuhiko Tatemoto and Viktor Mutt at the Department of Biochemistry, Karolinska Institute in Stockholm in 1978. With this method, galanin was discovered in porcine intestinal extract. In brief, a total of 4000 kg of porcine upper intestines was boiled in water, frozen, minced, and extracted. Galanin was determined by the amount of alanine amide released after treatment of the sample with thermolysin. Structural analysis revealed that it consisted of 29 amino acid residues with N-terminal alanine amide (Fig. 1). Thus, this peptide was called galanin because of its N- and C-terminal amino acids (glycine and alanine).[30] Although the isolation work on galanin was already completed in 1980, the structure of galanin was neither determined nor reported until this peptide was found to have effects on plasma glucose levels in dog and rat smooth muscle preparations 3 years later. This natural galanin preparation was used for biological studies until 1986 when a synthetic preparation became available by the use of the trifluoromethanesulfonic acid deprotecting procedure.[13] Galanin isolated from human large intestines consists of 30 amino acid residues that share the sequence of the N-terminal 15 residues with galanin in other mammals but exhibit characteristic differences in the remaining part of the molecules.[3] Unlike the galanin in other mammals, the human galanin is not C-terminally amidated, which is unusual for many biologically active peptides (Fig. 1).

The first galanin receptor, named GalR1, was cloned from the human Bowes melanoma cell line (as a source of mRNAs), and the transcript was identified in human small intestine and fetal brain (by Northern blot analysis) in 1994. Subsequently, subtypes 1 and 2, that is, GalR2 and GalR3, were discovered.[4,12,27] Gene knockout mice were generated by targeted disruption of the endogenous galanin, GalR1 or GalR2, but did not display marked phenotype with respect to the growth rate, relative to their littermates.[7,14,34] Galanin and its receptors have widespread distributions throughout the gastrointestinal tract as well as in the central and peripheral nervous systems of many mammal species. A book entitled "Galanin" covers a variety of the biological and pathobiological functions of galanin in the nervous systems, neuroendocrine, and skin, but little is mentioned in the gastrointestinal tract.[13]

STRUCTURE OF THE GENE AND PROCESSING OF THE PRECURSOR

The structure of the gene encoding galanin is highly conserved among vertebrates. Galanin is mapped to chromosome 19 2.0 cM for mouse, 1q42 for rat, and 11q13.3 for human (http://www.ncbi.nlm.nih.gov/gene/?term=Galanin). The galanin gene is about 6 kB of genomic DNA, depending on the species, and it consists of 6 small exons and 5 introns, which are translated into a 123-(porcine, human) or 124-(murine) amino acid precursor. This preprohormone comprises a 5′ leader sequence followed by a single galanin coding region and a 59–60 amino acid C-terminally flanking

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00164-0
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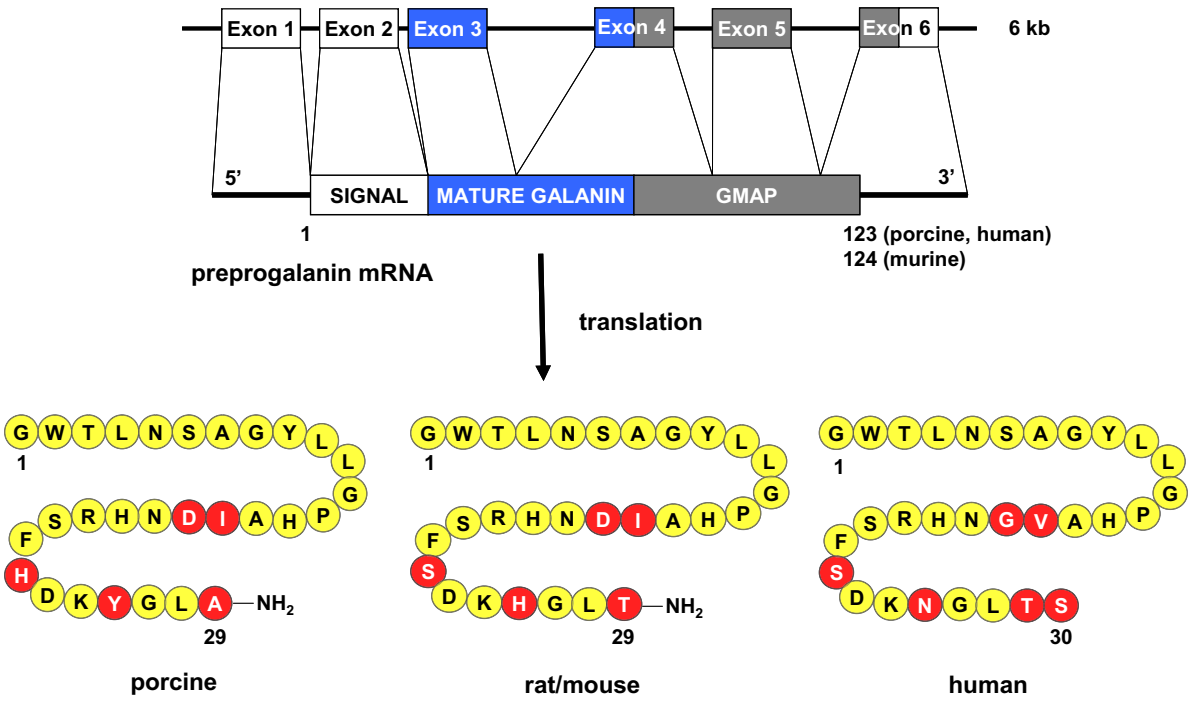

FIGURE 1 Schematic diagrams showing the predicted transcription of galanin gene and the translation of preprogalanin mRNA to produce the mature peptide galanin in porcine, rat, mouse, and human. The first exon encodes the 5′-untranslated region of preprogalanin mRNA. Exon 2 starts with a translation initiation codon of the signal peptide and terminates before the proteolytic site preceding the mature galanin peptide. The first 13 amino acids of galanin are encoded by exon 3; the remaining 16 amino acids and most of galanin message associated peptide (GMAP) by exons 4 and 5. The remaining portion of GMAP and the polyadenylation site are located in exon 6. Note: 29 amino acids with C-terminal amidation in the pig, rat, and mouse, but 30 amino acids without C-terminal amidation in humans, and red circles indicate residues that are different from human. See color plate 44.

peptide, named galanin message–associated peptide (GMAP) (Fig. 1).[13,16] By using radioimmunoassay, chromatographic separations, and tandem mass spectrometry, a series of variant forms of galanin with C-terminal amidation was purified from porcine upper intestine. The modified variants include three β-aspartyl-shifted forms, an oxidized form containing Trp monoxide, and an N-terminally truncated form.[21]

DISTRIBUTIONS OF GALANIN

After it was discovered from porcine intestine tissue extract, the identical galanin was isolated from the porcine gastric corpus tissue.[20] In mice, galanin content in terms of picomoles per gram of wet weight tissue was found in the following ranking order: ileum > duodenum > hypothalamus > stomach > cortex (measured by means of radioimmunoassay).[33] Immunohistochemical analysis has shown that galanin is localized in submucosal and myenteric neurons and in fibers projecting into the mucosa and muscle throughout the gastrointestinal tract in all mammalian species investigated, including humans (Fig. 2). Galanin fibers are primarily intrinsic to the enteric nervous systems and are quite abundant in the muscle layer. They have long descending projections with a dual termination in the myenteric ganglia and in the smooth muscle.[5]

RECEPTORS AND SIGNALING CASCADES

Galanin acts on three receptor subtypes, namely, GalR1, GalR2, and GalR3. All the three receptor subtypes are G protein-coupled receptors (GPCRs) superfamily, with a characteristic core consisting of seven-transmembrane α-helices that weave in and out of the plasma membrane. The chromosomal location of the genes encoding each receptor subtypes are shown in Table 1. A rat GalR1 homolog contains 346 amino acids and shares the same consensus sites with human GalR1 for N-linked glycosylation and intracellular phosphorylation with the exception of two additional phosphorylation sites in the human GalR1 C-terminal domain (Fig. 3A). A rat GalR2 contains 372 amino acids, including 2 consensus sites for extracellular N-linked glycosylation and several intracellular phosphorylation sites distinct from GalR1 (Fig. 3B). A rat GalR3 contains 370 amino acids with a similarity to GalR1 of 36% and to GalR2 of 55% (Fig. 3C).[4]

The mRNAs for these three receptors are expressed throughout the gastrointestinal tract. GalR1 seems to be more abundant in the large intestine than in the stomach and small intestine, GalR2 is present more in the stomach than in the intestine, whereas GalR3 is found only at a detectable level in the gut.[1] Immunohistochemistry shows that

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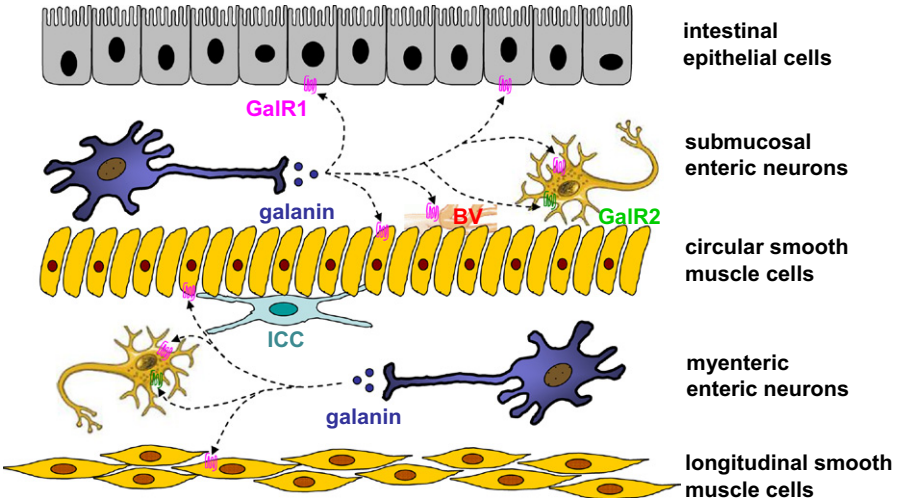

FIGURE 2 Schematic diagrams showing the distributions of galanin-containing nerve cell bodies and nerve fibers, galanin receptors (GalR1, GalR2, not yet GalR3), and acting sites of galanin (indicated by dashed arrows) in the gut. BV, blood vessel; ICC, interstitial cells of Cajal. See color plate 45.

TABLE 1 Galanin Receptor Subtypes GalR1, GalR2, and GalR3*

	GalR1	GalR2	GalR3
Name of gene	GALR1 (human) Galr1 (rat) Galr1 (mouse)	GALR2 (human) Galr2 (rat) Galr2 (mouse)	GALR3 (human) Galr3 (rat) Galr3 (mouse)
Location of chromosome	18q23 (human) 18q12.3 (rat) 18 E3-E4 (mouse)	17q25.3 (human) 10q32.3 (rat) 11 E2 (mouse)	22q13.1 (human) 7q34 (rat) 15 E1 (mouse)
Number of amino acids	349 (human) 346 (rat) 348 (mouse)	387 (human) 372 (rat) 371 (mouse)	368 (human) 370 (rat) 370 (mouse)

IUPHAR database (IUPHAR-DB): Galanin receptors: Last modified on 2008-07-21.

GalR1 is located in the enterochromaffin-like (ECL) cells in the oxyntic mucosa of stomach, the submucosal myenteric neurons, the nerve fibers distributed to the mucosa, plexuses, interconnecting strands, muscle layers, and blood vessels in the gut (Fig. 2). GalR1 immunoreactive fibers form a dense network in the deep muscular plexus in close association with interstitial cells of Cajal (ICC).[1] In the stomach of rats, GalR1 immunoreactive myenteric neurons are both ascending cholinergic/tachikinergic neurons and descending neurons containing nitric oxide or vasoactive intestinal polypeptide (VIP). In the small intestine of rat and guinea pig, GalR1 immunoreactive myenteric neurons are mainly cholinergic. Immunohistochemistry reveals that GalR2 is localized in submucosal and myenteric neurons that are mainly cholinergic in all gastrointestinal segments (Fig. 2).[1,23] There are no reports at present on GalR3 immunoreactivity in the gut, although the antibody is available.

GPCRs interact with heterotrimeric G proteins consisting of α, β, and γ subunits. The α subunits of G proteins are divided into four subfamilies: $G\alpha_s$, $G\alpha_i$, $G\alpha_q$, and $G\alpha_{12}$, and the subfamilies can be further divided into various subtypes. A single GPCR can couple to one or more families of Gα proteins. GalR1 is coupled to a Gβγ and/or $G\alpha_i$ signaling pathway to mediate mitogen-activated protein kinases (MAPK) activation.[15,16,31,32] However, GalR1 is coupled neither to $G\alpha_s$ because GalR1 stimulation does not cause an increase in cAMP accumulation, nor to $G\alpha_{io}$ because GalR1 stimulation is not dependent on protein kinase C (PKC) activity which is $G\alpha_{io}$-mediated MAPK activation. In addition, there is no coupling to $G\alpha_q$ because GalR1 stimulation does not activate inositol phosphate (IP) production.[31,32] Phosphatidylinositol 3-kinases (PI3K) are involved in Gβγ subunit-mediated extracellular-signal-regulated kinases 1 and 2 (ERK1/2) activation. However, the inhibition of PI3K

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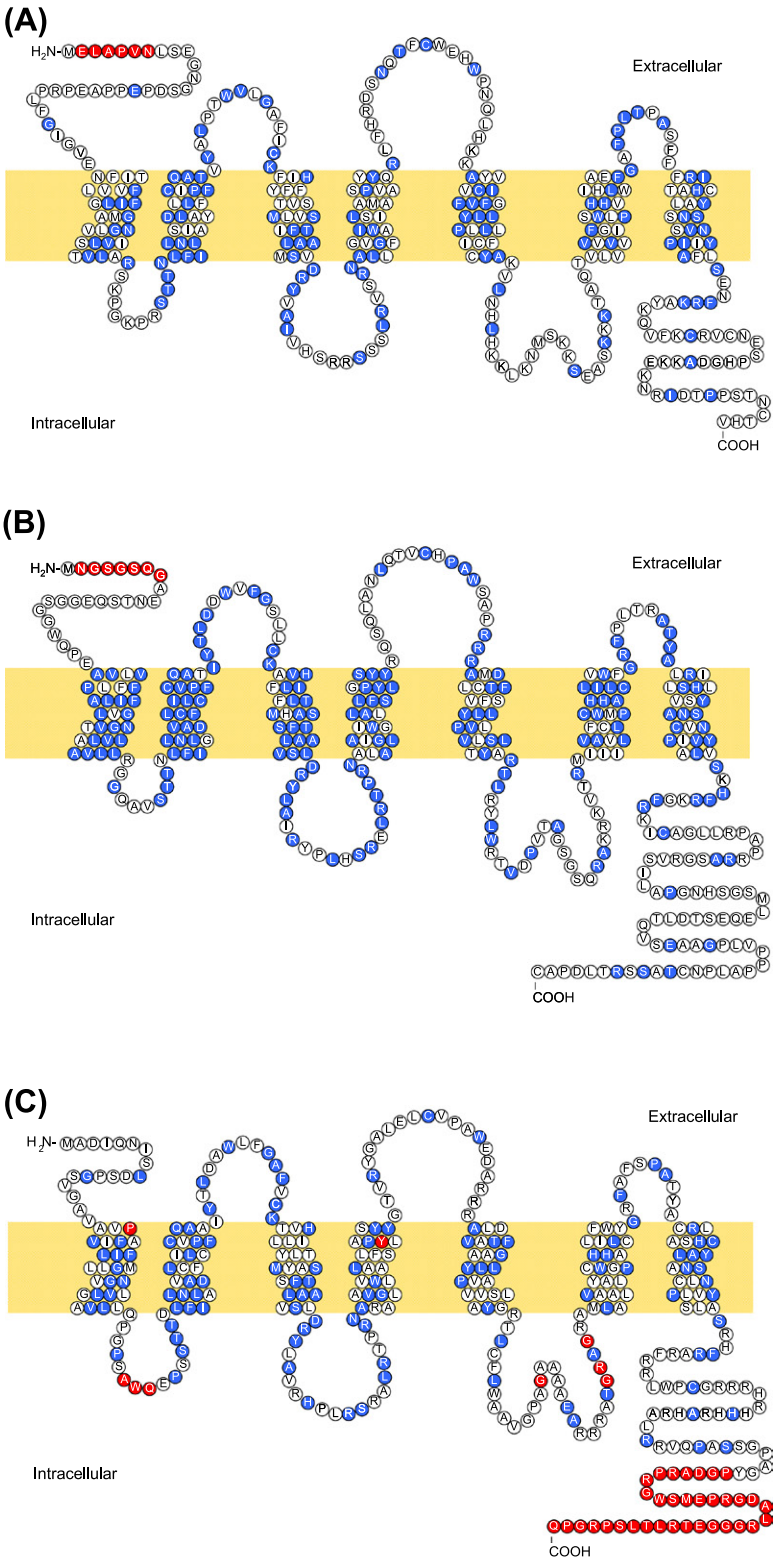

FIGURE 3 Schematic diagrams showing the amino acid sequences of seven-transmembrane domain receptors of GalR1 (A), GalR2 (B) and GalR3 (C) of rat. In A, blue circles indicate residues that are identical with those in B; red circles indicate residues that are absent in B. In B, blue circles indicate residues that are identical with those in C; red circles indicate residues that are absent in C. In C, blue circles indicate residues that are identical with those in A; red circles indicate residues that are absent in A. *(Reprinted from Ref. 4 with permission from Elsevier).* See color plate 46.

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is without any effect on galanin-induced ERK1/2 activation, suggesting a $G\alpha_i$ pathway (Fig. 4A).[15] Both GalR1 and GalR2 mediate pertussis toxin-sensitive MAPK activity. GalR2-mediated MAPK activation can be abolished by the inhibition of PKC activity. Activation of GalR2 is associated with increased IP production, which was not affected by pertussis toxin, suggesting a linkage of GalR2 with $G\alpha_{q/11}$ (Fig. 4B).[31,32] GalR3 mediates the $G\alpha_{i/o}$ pathway. Activation of GalR3 receptors results in inward K^+ currents that are characteristic of $G\alpha_{i/o}$-coupled receptors (Fig. 4C).[28]

BIOLOGICAL ACTIONS WITHIN THE GASTROINTESTINAL TRACT

Regulation of Secretion

The vertebrate stomach performs a variety of functions, serving as a reservoir for food and exposing ingested food to hydrochloric acid and pepsin, which is converted by the acid from pepsinogen. Gastric acid secretion is regulated by endocrine, paracrine, and neurocrine pathways involving at least gastrin, histamine, somatostatin, acetylcholine, pituitary adenylate cyclase-activating polypeptide, gastrin-released peptide, and galanin. Gastrin stimulates histamine release from the ECL cells, which then induces the acid secretion from the parietal cells, whereas somatostatin inhibits the ECL cells and the parietal cells by acting on somatostatin receptor subtype 2 ($SSTR_2$). GalR1 is expressed on the ECL cells, and the expression is upregulated in mice that are deficient in $SSTR_2$. In addition to somatostatin, the ECL cells can be inhibited by galanin via the GalR1, leading to an inhibition of the acid secretion.[24,35]

Intestinal functions include fluid secretion and alterations of intestinal ion flux. Galanin acts on GalR1 in colonic epithelial cells to induce chloride secretion by a mechanism that is $[Ca^{2+}]_i$-dependent, pertussis toxin sensitive, but adenosine 3′,5′-cyclic monophosphate independent.[2]

Gut peptides optimize the process of digestion and nutrient absorption and also play important roles in the regulation of appetite. Galanin can suppress gastrin release evoked by the inhibition of acid secretion and glucagon-like peptide 1 release, which is induced by gastric inhibitory polypeptides.[6,11]

Modulation of Vagal Afferent Mechanosensation

Vagal, splanchnic, and pelvic nerves are three main pathways from the gastrointestinal tract into the central nervous system. Vagal afferent fibers have neuronal cell bodies in the nodose and jugular ganglia and are typically associated with sensations such as satiety and nausea. All three galanin receptors are expressed in the nodose ganglia. Galanin induces potent inhibition of mechnosensitivity of vagal afferents with respect to tension receptors and mucosal receptors, most probably through GalR1.[22] The role of galanin in the pathogenesis of gastrointestinal dysfunction, such as functional dyspepsia and gastroesophageal reflux disease needs further study.

Regulation of Motility

Gastrointestinal tract motility involves the movement of ingested food through the gut for absorbing nutrients, water, and electrolytes and eliminating waste products. The movement is accomplished by coordinating contractions and relaxations of the smooth muscles in the various parts of the gut. The regulation of gut motility is complicated, involving the enteric nervous system, ICC, hormones, paracrine substances, and inflammatory mediators. The enteric nervous system includes intrinsic neural plexuses and autonomic extrinsic neural pathways, which are of fundamental importance for generating major motor patterns and regulating the amplitudes of contractions. The smooth muscle response to external signals ultimately depends on the excitability of the ICC-smooth muscle syncytium. Galanin modulates the motility, either via direct action on the smooth muscle cells or by indirect neuromodulatory mechanisms, such as the release of other transmitters.[16] The effects of galanin on the gastrointestinal motility are both stimulatory and inhibitory, which is consistent with the projection pattern of galanin distribution (Fig. 2). However, it may also depend on the species and the gut segment examined.[16]

Regulation of Sphincters

The function of gastrointestinal sphincters is to serve as one-way valves by tonic contraction and closure to regulate and coordinate the caudal flow of gastrointestinal contents. The sphincters include both skeletal and smooth muscles located in specific sites throughout the gut. The skeletal muscle sphincters, upper esophageal sphincter, and external anal sphincter are under voluntary control. The smooth muscle sphincters are the lower esophageal sphincter, pyloric sphincter, sphincter of Oddi, ileocecal sphincter, and internal anal sphincter. These smooth muscle sphincters are autonomic organs and are controlled by complex interactions between extrinsic nerves from the central nervous system and intrinsic control by the enteric nervous system. Sphincteric relaxation in response to an appropriate reflex is mediated by intramural nonadrenergic noncholinergic (NANC) nerves with neurotransmitters, like VIP and nitric oxide. Most of the actions of galanin on basal smooth muscle sphincteric tone are explained by its actions directly on smooth muscle cells. However, some of the relaxant actions of the peptide may also result from the activation of NANC inhibitory neurons.[25] Galanin colocalizes with nitric oxide

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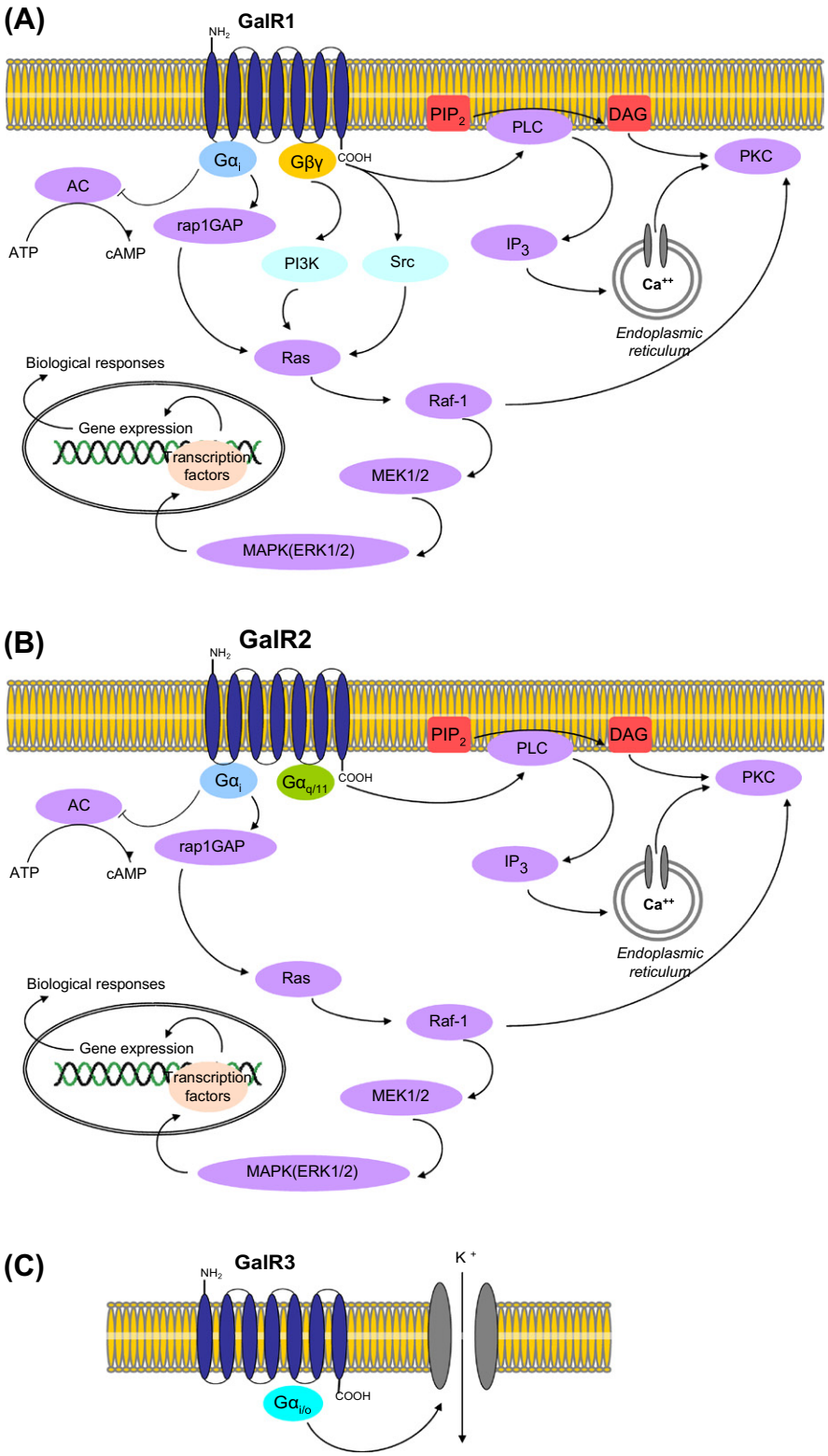

FIGURE 4 Schematic diagrams showing the proposed signaling pathways of GalR1 (A), GalR2 (B), and GalR3 (C). Note: stimulations are indicated by ↓ lines, and inhibitions are indicated by ⊥ lines. Abbreviations: AC, adenylate cyclase; DAG, diacylglycerol; IP_3, inositol triphosphate; MAPK, mitogen-activated protein kinases; MEK, mitogen-induced extracellular kinase; PI3K, phosphatidylinositol 3-kinase; PIP_2, phosphatidylinositol bisphosphate; PKC, protein kinase C; PLC, phospholipase C; rap1GAP, rap1 GTPase-activating protein 1. See color plate 47.

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synthase and VIP-immunoreactive neurons, suggesting the dynamic role of galanin in the regulation and modulation of sphincteric function. For example, in the lower esophageal sphincter, galanin may cause an increase in basal tone and suppression of NANC nerve-mediated relaxation. On the contrary, in the internal anal sphincter, the predominant effect of galanin is to cause smooth muscle relaxation and augmentation of NANC nerve-mediated relaxation.[25]

PATHOPHYSIOLOGICAL IMPLICATIONS

Inflammatory Bowel Disease

Inflammatory bowel disease (IBD) comprises the chronic relapsing inflammatory disorders including Crohn's disease (CD) and ulcerative colitis (UC). Crohn's disease involves any part of the gastrointestinal tract, most commonly the terminal ileum or the perianal region. Ulcerative colitis is limited to the colon. Pathogenesis of IBD involves multiple levels of interaction between the neural, immune, and endocrine systems. Neuropeptides serve as a viable link among all three systems, probably through activation of nuclear factor (NF)-κB, a transcription factor associated with the activation of proinflammatory genes and IBD pathogenesis.[8] There is a robust increased expression of GalR1 in the colonic tissues of patients with CD and UC. Activation of NF-κB induces GalR1 expression in epithelial cells lining the mouse colon. Cloning of the GalR1 gene has revealed multiple recognition sites for the inflammation associated transcription factor NF-κB. In the murine model of IBD, NF-κB activation is associated with increased GalR1 expression as well as excess colonic fluid secretion. Thus, the prominent role of galanin in intestinal fluid secretion and its connection to NF-κB, a critical participant in IBD, links the diarrhea associated with it to an NF-κB-mediated increase in GalR1 expression.[8]

Infectious Diarrhea

Enteric pathogens can alter the balance between absorption and secretion of fluid and electrolytes by the gut, leading to diarrheal disease. The pathogens can either directly modulate epithelial ion transport processes and barrier function or do so indirectly through inflammation, peptides, or loss of absorptive surface.

Rotaviruses are one of the important pathogens causing severe diarrhea in humans and many animal species, mostly the young. Rotavirus infection induces the activation of NF-κB. The NF-κB activation mediates the upregulation of GalR1.[9,17] The promoter region of the GalR1 gene has a recognition site for p50/p65 NF-κB complex.[9] Both constitutive GalR1 expression in the small intestine and rotavirus infection-induced GalR1 expression in the colon are age restricted.[10] Conceivably, the age-restricted GalR1 expression may be mediated by NF-κB signaling that in turn is easier to activate in infants. Rotavirus-infected small intestine could activate the colon remotely by inducing epithelial cells to release biological compounds that can then activate subepithelial nerves and the enteric nerve system with a specific and important role for galanin and GalR1.[10]

Salmonella enterica, including *Typhimurium*, *Enteritidis*, and *Typhi*, are also the important pathogens in causing diseases of the intestines (enteric means pertaining to the intestine). Humans infected with *S. enterica* respond differently according to age: infants develop diarrhea but not bacteremia and survive, whereas the elderly become bacteremic and die. The most common cause of food poisoning by *Salmonella* species is *Salmonella typhimurium* infection. Infection with *S. typhimurium* induces the GalR1 expression in the gut, resulting in increased colonic chloride and fluid secretion.[18] Moreover, galanin and GalR1 expression decline with aging, which is in line with the difference in response to *S. typhimurium* infection with age.[19] However, the infection-induced NF-κB activation is independent of age, probably suggesting that NF-κB activation alone may not regulate galanin and GalR1 expression.[19]

Cancer

Nerves and neuroendocrine peptides play a fundamental role in the function and growth of the normal as well as malignant gastrointestinal epithelium. The neural effects can directly take place on cancer cells or indirectly on the cancer microenvironments.

Esophageal cancers in humans include squamous cell carcinoma (SCC) and adenocarcinoma. Genetic changes that are consistently observed in the esophageal SCC include (i) alterations in tumor suppressor genes, leading to altered DNA replication and repair, cell proliferation and apoptosis; (ii) disruption of the G_1/S cell cycle checkpoint and loss of cell cycle control; and (iii) alterations in oncogene function, for example, *Ras*, leading to deregulation of cell signaling cascades. A study of esophageal SCC cell lines using array-comparative genomic hybridization revealed that the most frequently gained locus was 11q13.1-13.3 and the most commonly lost locus was 18q12.1-23 where GalR1 is mapped.[29] This suggests that GalR1 may function as a tumor suppressor gene. The growth regulation of SCC might be disrupted as a result of galanin overexpression and differential galanin receptors expression. Thus, the cell proliferation could be promoted synergistically by the downregulation of GalR1 and upregulation of galanin signaling via GalR2 or GalR3. Additionally, dysregulation of signaling proteins downstream from the individual receptors may also affect this regulation. Indeed, the activation of GalR2 induces MAPK activation, which is a well-established mitogenic pathway. Hence, the effects of galanin may be

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regulated at the level of the receptor or by differential intracellular signaling mechanisms, and the galanin signaling cascade can be a candidate pathway regulating oncogenesis in the SCC.[29]

Galanin mRNA expression was increased in colon carcinoma tissue in patients. Galanin suppresses chemically induced carcinogenesis in colon cancer in rats. Galanin, together with the octreotide analog of somatostatin and serotonin, inhibited tumor growth in mice that were inoculated with human or rat colon carcinoma cells. The effect of galanin was speculated to be mainly through the inhibition of angiogenesis in the tumor. Whether galanin and its receptors are promising targets for diagnosis and treatment of gastrointestinal cancer needs to be further defined.[26]

REFERENCES

1. Anselmi L, Stella Jr SL, Lakhter A, Hirano A, Tonini M, Sternini C. Galanin receptors in the rat gastrointestinal tract. *Neuropeptides* 2005;**39**:349–52.
2. Benya RV, Marrero JA, Ostrovskiy DA, Koutsouris A, Hecht G. Human colonic epithelial cells express galanin-1 receptors, which when activated cause Cl^- secretion. *Am J Physiol* 1999;**276**:G64–72.
3. Bersani M, Johnsen AH, Højrup P, Dunning BE, Andreasen JJ, Holst JJ. Human galanin: primary structure and identification of two molecular forms. *FEBS Lett* 1991;**283**:189–94.
4. Branchek TA, Smith KE, Gerald C, Walker MW. Galanin receptor subtypes. *Trends Pharmacol Sci* 2000;**21**:109–17.
5. Ekblad E, Rökaeus A, Håkanson R, Sundler F. Galanin nerve fibers in the rat gut: distribution, origin and projections. *Neuroscience* 1985;**16**:355–63.
6. Ericsson P, Håkanson R, Norlén P. Gastrin response to candidate messengers in intact conscious rats monitored by antrum microdialysis. *Regul Pept* 2010;**163**:24–30.
7. Gottsch ML, Zeng H, Hohmann JG, Weinshenker D, Clifton DK, Steiner RA. Phenotypic analysis of mice deficient in the type 2 galanin receptor (GALR2). *Mol Cell Biol* 2005;**25**:4804–11.
8. Gross KJ, Pothoulakis C. Role of neuropeptides in inflammatory bowel disease. *Inflamm Bowel Dis* 2007;**13**:918–32.
9. Hecht G, Marrero JA, Danilkovich A, Matkowskyj KA, Savkovic SD, Koutsouris A, et al. Pathogenic *Escherichia coli* increase Cl^- secretion from intestinal epithelia by upregulating galanin-1 receptor expression. *J Clin Invest* 1999;**104**:253–62.
10. Hempson SJ, Matkowskyj K, Bansal A, Tsao E, Habib I, Benya R, et al. Rotavirus infection of murine small intestine causes colonic secretion via age restricted galanin-1 receptor expression. *Gastroenterology* 2010;**138**:2410–7.
11. Herrmann-Rinke C, Hörsch D, McGregor GP, Göke B. Galanin is a potent inhibitor of glucagon-like peptide-1 secretion from rat ileum. *Peptides* 1996;**17**:571–6.
12. Howard AD, Tan C, Shiao LL, Palyha OC, McKee KK, Weinberg DH, et al. Molecular cloning and characterization of a new receptor for galanin. *FEBS Lett* 1997;**405**:285–90.
13. Hökfelt T, editor. *Galanin, experientia supplementum*, vol. 102. © Springer Basel AG; 2010. http://dx.doi.org/10.1007/978-3-0346-0228-0_1.
14. Jacoby AS, Hort YJ, Constantinescu G, Shine J, Iismaa TP. Critical role for GALR1 galanin receptor in galanin regulation of neuroendocrine function and seizure activity. *Brain Res Mol Brain Res* 2002;**107**:195–200.
15. Kanazawa T, Iwashita T, Kommareddi P, Nair T, Misawa K, Misawa Y, et al. Galanin and galanin receptor type 1 suppress proliferation in squamous carcinoma cells: activation of the extracellular signal regulated kinase pathway and induction of cyclin-dependent kinase inhibitors. *Oncogene* 2007;**26**:5762–71.
16. Lang R, Gundlach AL, Kofler B. The galanin peptide family: receptor pharmacology, pleiotropic biological actions, and implications in health and disease. *Pharmacol Ther* 2007;**115**:177–207.
17. Marrero JA, Matkowskyj KA, Yung K, Hecht G, Benya RV. Dextran sulfate sodium-induced murine colitis activates NF-kappaB and increases galanin-1 receptor expression. *Am J Physiol Gastrointest Liver Physiol* 2000;**278**:G797–804.
18. Matkowskyj KA, Danilkovich A, Marrero J, Savkovic SD, Hecht G, Benya RV. Galanin-1 receptor up-regulation mediates the excess colonic fluid production caused by infection with enteric pathogens. *Nat Med* 2000;**6**:1048–51.
19. Matkowskyj K, Royan SV, Blunier A, Hecht G, Rao M, Benya RV. Age-dependent differences in galanin-dependent colonic fluid secretion after infection with *Salmonella typhimurium*. *Gut* 2009;**58**:1201–6.
20. McDonald TJ, Krantis A, Clarke M, Mutt V, Jörnvall HE. Characterization of porcine gastric galanin. *Peptides* 1992;**13**:589–93.
21. Norberg A, Griffiths WJ, Hjelmqvist L, Jörnvall H, Rökaeus A. Identification of variant forms of the neuroendocrine peptide galanin. *Rapid Commun Mass Spectrom* 2004;**18**:1583–91.
22. Page AJ, Slattery JA, Brierley SM, Jacoby AS, Blackshaw LA. Involvement of galanin receptors 1 and 2 in the modulation of mouse vagal afferent mechanosensitivity. *J Physiol* 2007;**583**:675–84.
23. Pham T, Guerrini S, Wong H, Reeve Jr J, Sternini C. Distribution of galanin receptor 1 immunoreactivity in the rat stomach and small intestine. *J Comp Neurol* 2002;**450**:292–302.
24. Piqueras L, Taché Y, Martinez V. Galanin inhibits gastric acid secretion through a somatostatin-independent mechanism in mice. *Peptides* 2004;**25**:1287–95.
25. Rattan S, Tamura W. Role of galanin in the gastrointestinal sphincters. *Ann N Y Acad Sci* 1998;**863**:143–55.
26. Rauch I, Kofler B. The galanin system in cancer. *EXS* 2010;**102**:223–41.
27. Smith KE, Forray C, Walker MW, Jones KA, Tamm JA, Bard J, et al. Expression cloning of a rat hypothalamic galanin receptor coupled to phosphoinositide turnover. *J Biol Chem* 1997;**272**:24612–6.
28. Smith KE, Walker MW, Artymyshyn R, Bard J, Borowsky B, Tamm JA, et al. Cloned human and rat galanin GALR3 receptors. Pharmacology and activation of G-protein inwardly rectifying K^+ channels. *J Biol Chem* 1998;**273**:23321–6.
29. Sugimoto T, Seki N, Shimizu S, Kikkawa N, Tsukada J, Shimada H, et al. The galanin signaling cascade is a candidate pathway regulating oncogenesis in human squamous cell carcinoma. *Genes Chromosomes Cancer* 2009;**48**:132–42.
30. Tatemoto K, Rökaeus A, Jörnvall H, McDonald TJ, Mutt V. Galanin—a novel biologically active peptide from porcine intestine. *FEBS Lett* 1983;**164**:124–8.
31. Wang S, Hashemi T, Fried S, Clemmons AL, Hawes BE. Differential intracellular signaling of the GalR1 and GalR2 galanin receptor subtypes. *Biochemistry* 1998;**37**:6711–7.

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32. Wang S, He C, Hashemi T, Bayne M. Cloning and expressional characterization of a novel galanin receptor. Identification of different pharmacophores within galanin for the three galanin receptor subtypes. *J Biol Chem* 1997;**272**:31949–52.
33. Wynick D, Small CJ, Bacon A, Holmes FE, Norman M, Ormandy CJ, et al. Galanin regulates prolactin release and lactotroph proliferation. *Proc Natl Acad Sci U S A* 1998;**95**:12671–6.
34. Wynick D, Small CJ, Bloom SR, Pachnis V. Targeted disruption of the murine galanin gene. *Ann N Y Acad Sci* 1998;**863**: 22–47.
35. Zhao CM, Martinez V, Piqueras L, Wang L, Taché Y, Chen D. Control of gastric acid secretion in somatostatin receptor 2 deficient mice: shift from endocrine/paracrine to neurocrine pathways. *Endocrinology* 2008;**149**:498–505.

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Chapter 165

Gastrin

Arthur Shulkes and Graham S. Baldwin

ABSTRACT

Gastrin is synthesized in the gastric antrum as a large precursor, progastrin. After cleavage and processing, progastrin yields a variety of biologically active peptides including glycine-extended gastrin and the final end product gastrin amide. Gastrin amide acting via the cholecystokinin-2 receptor is the major regulator of meal-stimulated gastric acid secretion, and it also has important roles in epithelial cell proliferation in the gastrointestinal tract. Release of gastrin is stimulated by food, predominantly protein, and inhibited by a low gastric pH. The gastrin peptides, particularly the nonamidated forms, may also be autocrine growth factors in the development of colorectal cancers. Overexpression of gastrin as a result of a gastrin-secreting tumor (Zollinger–Ellison syndrome) results in peptic ulcers and thickening of the gastrointestinal mucosa. Gastrin is also overexpressed in some subjects with *Helicobacter pylori* infection. Gastrin immunoneutralization and radiolabeling of cholecystokinin-2 receptor ligands are currently under investigation for treatment and diagnosis of tumors expressing gastrin and/or the cholecystokinin-2 receptor.

DISCOVERY

In 1905, John Edkins provided the first evidence for a blood-borne factor that originated from the distal part of the stomach and stimulated gastric acid secretion. He named the factor gastrin and proposed that its mechanism of action was analogous to that of the pancreatic secretagogue secretin, which had been discovered in 1902 by Bayliss and Starling and shown to originate from the duodenum. However, for many years, the gastrin hypothesis was not accepted, as it was believed that the active agent in Edkin's preparations was histamine. In the 1930s, Simon Komarov, a Russian chemist working at McGill University in Montreal, isolated a gastric acid-stimulating peptide from antral mucosa, separated the peptide from histamine, and thus confirmed the existence of gastrin. The actual purification and sequencing of gastrin were performed by Rod Gregory and his assistant Hilda Tracey in the Department of Physiology at the University of Liverpool, in collaboration with the peptide chemist George Kenner. The arduous extraction of gastrin from hundreds of porcine antra culminated in the report in 1962 of the sequence of two 17 amino acid peptides (Gastrin I and Gastrin II) with Gastrin II having a sulfated tyrosine. Gastrin was the first gastrointestinal peptide to be sequenced and, with the development of a gastrin radioimmunoassay in 1967, it was soon realized that the antral mucosa contained much higher concentrations of gastrin (nanomoles per gram) than the duodenal concentration of the first discovered gastrointestinal hormone secretin (picomoles per gram).

STRUCTURE OF THE GENE/mRNA

The gastrin gene, which is located on chromosome 17 at band q21.2, comprises 3 exons and encodes an mRNA of 435 nucleotides (Fig. 1). Expression of gastrin mRNA may be regulated in response to several factors (see review by Merchant),[22] including mutational activation of either Ras or the Wnt signaling pathway, ligands of the epidermal growth factor (EGF) receptor, cytokines such as interleukin-1 acting via SMADs, or pathogenicity factors such as the *Helicobacter pylori* CagA protein. Moreover, gastrin was recently shown to increase expression of its own gene via an autocrine loop.[20]

Significant progress has been made in defining the regulatory elements that control transcription from the mouse gene *in vitro*. The Brand Laboratory in Boston pioneered work in this area with the identification of a CACC promoter element that activated transcription in rat insulinoma cells. The effects of EGF and related growth factors were later shown to be mediated by the binding of members of either of two families of zinc finger transcription factors (the Sp and ZBP-89 families) to a response element 68 base pairs upstream of the mRNA cap site (reviewed by Merchant[22]).

Less is known about control of expression of the mouse gene *in vivo* or about the corresponding elements in the human gene. The use of bacterial artificial chromosomes as vehicles for the expression of an enhanced green fluorescent protein reporter under the control of the mouse gastrin promoter, or of the human gastrin gene in gastrin-deficient mice, provides new models with which to remedy these deficiencies.

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00165-2
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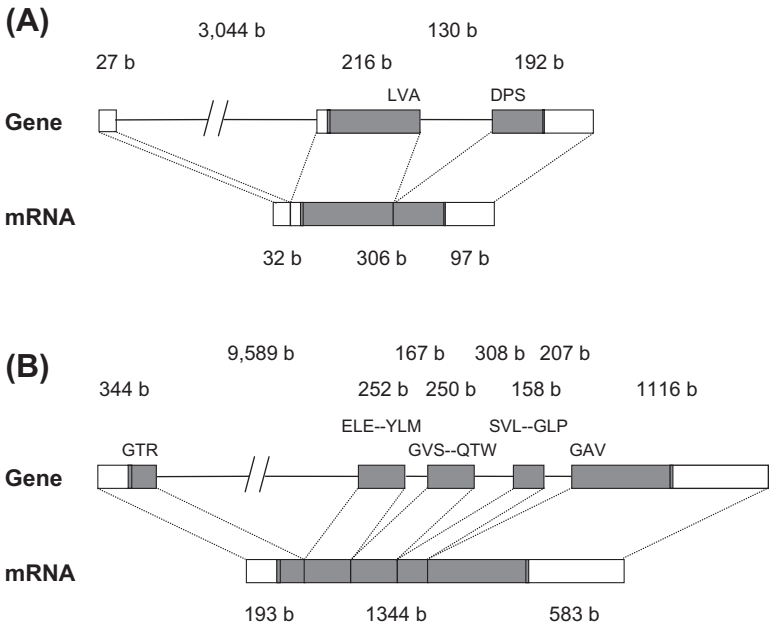

FIGURE 1 Structure of the genes and mRNAs encoding gastrin and the cholecystokinin-2 receptor. The gastrin gene (A) is located on the positive strand of chromosome 17q21.2 between bases 39,868,613 and 39,872,221. The gene contains two introns of 3,044 and 130 bases (b), of which the first occurs in the 5′ untranslated region and the second after the first base of the codon for D71. The mRNA of 435 bases encodes a protein of 101 amino acids. Amino acids at the intron–exon boundaries are shown in the one letter code. The gene encoding the cholecystokinin-2 receptor (B), which is not drawn to the same scale as the gastrin gene, is located on the positive strand of chromosome 11p15.4 between bases 6,280,966 and 6,293,356. The gene contains four introns, which interrupt the coding sequence after the first base of the codon for E51, after the first base of the codon for G135, after the second base of the codon for W218, and after the first base of the codon for G270, respectively. The mRNA of 2120 bases encodes a protein of 447 amino acids. Differential splicing generates several variants, as described in the text.

PROCESSING OF THE PRECURSOR

All forms of gastrin are generated from an 80-amino acid precursor, progastrin, which is cleaved at dibasic sequences by prohormone convertases (PCs) in the secretory vesicle (Fig. 2). Cleavage following Arg^{36}-Arg^{37} by PC1/3 generates the N-terminus of gastrin34 and cleavage following Arg^{73}-Arg^{74} by PC1/3 releases the C-terminal flanking peptide (CTFP). Subsequent removal of Arg^{73} and Arg^{74} by carboxypeptidase E yields glycine-extended gastrin34, and oxidative cleavage by peptidylglycine α-amidating monooxygenase results in C-terminally amidated gastrin34. Finally cleavage following Lys^{53}-Lys^{54} by PC2 exposes the N-terminus of gastrin17, which is then cyclized to a pyroglutamyl residue by glutaminyl cyclase.[7,26] Throughout the remainder of this review, the term gastrin(s) encompasses all progastrin-derived peptides, irrespective of length or amidation status, while gastrin34 and gastrin17 will refer solely to the naturally occurring amidated forms of the hormone, and Gamide and Ggly will refer to synthetic amidated gastrin17 and glycine-extended gastrin17, respectively. Ser^{75}, the residue immediately following Arg^{73}-Arg^{74}, may be phosphorylated *in vivo*, but this modification does not seem to alter the rate of cleavage by PC1 *in vitro*.

The characteristic amidated C-terminal sequence TrpMetAspPhe is also found in the hormone cholecystokinin (CCK), which is responsible for the stimulation of pancreatic enzyme secretion. A second similarity between the two peptides is the presence of a nearby Tyr residue, which is sulfated to varying degrees depending on age and tissue. Isolation and sequencing of both gastrin and CCK from the dogfish *Squalus acanthias*, which is a member of the Chondrichthyes or cartilaginous fishes, established that the gastrin and CCK genes diverged at least 528 ± 56 Myr ago. The evolution of the gastrin and CCK genes has been recently reviewed in detail,[3] and these peptides are also discussed in the Brain Peptides and Ingestive Peptides sections of this book.

A second characteristic of gastrins is an acidic polyGlu sequence. In human gastrins, the central three of the five consecutive glutamates are responsible for the binding of ferric ions.[2] The presence of ferric ions is essential for the biological activity of nonamidated gastrins *in vitro* and *in vivo*[9] but dispensable for amidated gastrins. Related acidic sequences are also found in marsupial gastrins and hence must have been acquired at least 173 ± 12 Myr ago.[3]

DISTRIBUTION OF THE mRNA/PEPTIDE

Progastrin is produced principally by the G cells of the gastric antrum and to a variable extent by the upper small intestine,

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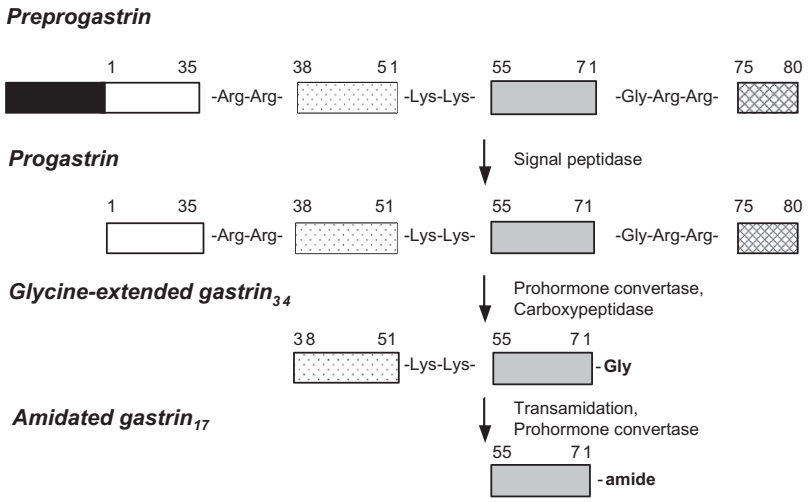

FIGURE 2 Progastrin processing. Preprogastrin (101 amino acids) is converted to progastrin (80 amino acids) by removal of its signal peptide (black box). The sequential action of prohormone convertases and carboxypeptidase E then converts the prohormone to glycine-extended forms such as glycine-extended gastrin$_{34}$ (progastrin$_{38–72}$) and glycine-extended gastrin$_{17}$ (progastrin$_{55–72}$, Ggly). Transamidation of glycine-extended gastrin$_{34}$ to amidated gastrin$_{34}$ by peptidyl α-amidating monooxygenase is followed by cleavage to amidated gastrin$_{17}$ (Gamide) by prohormone convertases, with the extent of cleavage dependent on the tissue. The amino acid sequence of Gamide is ZGPWLEEEEEAYGWMDFamide, where Z represents a pyroglutamate residue. Both nonamidated and amidated forms of gastrin are independently active via different receptors.

with much smaller amounts in the colon and pancreas. As discussed in the previous section, progastrin is processed into multiple forms with the extent of processing dependent on a number of factors, including the site of synthesis, the residence time within secretory vesicles, and the abundance of processing enzymes. In healthy humans, progastrin and the glycine-extended gastrins compose <10% of circulating gastrins. The major stored forms of amidated gastrins are tyrosine-sulfated and nonsulfated gastrin17, in about equal concentrations. Less than 10% of antral amidated gastrin is gastrin34, although the presence of gastrin34 as 50% of duodenal amidated gastrin indicates a decreased cleavage at Lys^{74}-Lys^{75}. The CTFP of progastrin is present in the antrum in at least equimolar concentrations to amidated gastrins (reviewed in Smith et al.[33]).

Human subjects with *Helicobacter pylori* infection have an increased antral concentration of amidated gastrins and decreased concentrations of the nonamidated gastrin forms (progastrin and glycine-extended gastrin). This observation is consistent with an increased conversion of gastrin precursors to amidated gastrins, the forms known to stimulate gastric acidity. By contrast, gastrinoma extracts have a much higher proportion (50–90%) of nonamidated gastrins.[26,33]

Most colorectal polyps and carcinomas synthesize progastrin. The concentrations of the gastrins are much lower than in the antrum, and there is a higher proportion of nonamidated gastrins including progastrin, Ggly, and the CTFP. The diminished processing is consistent with the tumor being the source of gastrin and the low concentrations may be a consequence of the peptides not being stored in secretory granules but secreted constitutively. The observation in a number of studies that gastrin concentrations return to normal after tumor resection confirms that the tumor is the source of the hypergastrinemia.[1] Many colorectal carcinoma (CRC) cell lines synthesize gastrins, again with a predominance of the nonamidated forms.[1]

Gastrin peptides are also found in small amounts in the ovary, pituitary, and in some neurons of the central nervous system. Importantly, gastrin mRNA and peptide are present in the fetal and neonatal pancreas and reappear in patients with Zollinger–Ellison syndrome, in which the gastrin-secreting tumors are predominantly of pancreatic origin.[26] The fact that the hypergastrinemia associated with gastrinoma and antral G cell hyperfunction has a higher proportion of nonamidated gastrins suggests that in the gastrin hypersecretory state the extent of processing is diminished.

Unlike the proportions in the antrum, the circulating concentrations of Gamide and Ggly are similar (around 25 pmol/l). Intact progastrin is present in much lower concentrations at around 1 pmol/l, while the CTFP has been reported to be the major circulating form, although this observation remains to be confirmed.[33] The clearance of gastrin34 is about fivefold slower than that of gastrin17, so although equal concentrations stimulate acid secretion to a similar extent, the effect of gastrin34 is more sustained. Between meals circulating gastrin34 is the predominant amidated form, probably as a result of preferential release of gastrin34 from the duodenum and its slower clearance from the circulation. By contrast, meal-stimulated gastrin is mainly gastrin17.[7]

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RECEPTORS AND SIGNALING CASCADES

Many of the physiological effects of amidated gastrins are mediated by the cholecystokinin-2 receptor (CCK2R). The CCK2R (447 amino acids) shares 46% identity in amino acid sequence with the cholecystokinin1 receptor (CCK1R) (428 amino acids), and both are typical 7-transmembrane domain receptors. The CCK2R gene, which is located on chromosome 11 at band p15.4, comprises 5 exons, and encodes an mRNA of 2120 nucleotides (Fig. 2), whereas the CCK1R, which is located on chromosome 4 at band p15.1-15.2, comprises 5 exons, but encodes an mRNA of 1657 nucleotides.

Several splice variants of the human CCK2R have been described. An N-terminally truncated isoform lacking the first extracellular domain has been cloned from human stomach and was attributed to splicing of an alternative first exon. The alternative receptor had about 10-fold lower affinity for Gamide. Alternative splicing at the 5′ end of exon 4 resulted in a receptor with five extra amino acids in the third intracellular loop, or a receptor with 69 additional amino acids in the third intracellular loop due to the complete retention of intron 4.[14] Some controversy exists over whether the intron 4 variant is expressed in CRC and over whether or not the variant was constitutively active and stimulated cell growth. Multiple forms of the CCK2R, with various deletions in the retained intron 4 and in exon 5, have also been detected in pancreatic adenocarcinomas.

The minimum sequence requirement for high-affinity binding to both the CCK1R and the CCK2R is the amidated C-terminal tetrapeptide TrpMetAspPhe. The two receptors may be distinguished by the fact that the CCK1R binds CCK8 with 14-fold greater affinity than Gamide, whereas the difference for the CCK2R is only 2-fold (Table 1). The CCK1R also binds sulfated CCK8 with 500-fold greater affinity than unsulfated CCK8, whereas the difference for the CCK2R is only 10-fold (Table 1). The CCK2R does not bind either recombinant human progastrin or synthetic Ggly. A variety of potent and selective antagonists have been developed for both receptors (see review by Morton[23]). The Parke-Davis compound PD149168 and the Yamanouchi-Ferring compound YF476 have affinities <0.01nM and selectivity >900-fold for the CCK2R.

In contrast to the abundant structure–function information available for the CCK2R, the identities of the receptors for nonamidated gastrins such as progastrin and Ggly are still controversial. The failure of CCK1R and CCK2R antagonists to inhibit binding of ^{125}I-Ggly to the rat pancreatic cell line AR4-2J clearly differentiated the Ggly binding site from either of the known receptors. However, all attempts to clone the corresponding cDNA have so far been unsuccessful. The identification of annexin II as the progastrin receptor[32] has been disputed.[16]

The signaling pathways downstream of the CCK2R have been thoroughly investigated (reviewed by Rozengurt

TABLE 1 Comparison of Affinities of Agonists and Antagonists for the CCK1 and CCK2 Receptors

	IC_{50} for Binding (nM)		
	CCK1 Receptor	CCK2 Receptor	Company
Agonists			
Gastrin17	845	5.1	
Cholecystokinin8sulfate	0.12	0.27	
Cholecystokinin8	59	2.6	
CCK1 Receptor-Selective Antagonists			
CR 1409	7.1	>1000	Rotta
SR 27897	0.58	489*	Sanofi
L364,718	0.19	32	Merck
CCK2 Receptor-Selective Antagonists			
L365,260	240	5.2	Merck
YF476	502	0.10**	Yamanouchi-Ferring
PD 149164	75	0.083	Parke-Davis

IC_{50} values for the CCK1R and CCK2R were determined by competition for [^{125}I]-CCK8 binding to membranes from rat pancreas (CCK1R) or mouse cerebral cortex (CCK2R).[15]
*Guinea pig.
**Rat.

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and Walsh[30] and by Dufresne et al.[8]). Like many other G protein-coupled receptors, occupation of the CCK2R by agonists activates phospholipase C, which hydrolyzes phosphatidylinositol 4,5-bisphosphate to inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 in turn stimulates the release of Ca^{2+} from intracellular stores, whereas DAG binds to and activates the isoforms of protein kinase C (PKC). The precise mechanisms by which the increased Ca^{2+} and activated PKC lead to an increase in mitogen-activated protein kinase (MAPK) activity, and hence to an increase in proliferation, differ between cell types.

The pathways by which CCK2R activation affects cellular apoptosis also seem to be cell-type dependent. Treatment of the mouse gastric cell line IMGE with Gamide decreases the expression of the proapoptotic protein Bax and increases the expression of the antiapoptotic protein Bcl-xl.[13] Similarly Gamide induces expression of the antiapoptotic protein mcl-1 in AGS-CCK2R human gastric carcinoma cells by a PKC- and MAPK-dependent pathway. By contrast, Gamide induces apoptosis in human CRC cell lines expressing CCK2R by a MAPK-dependent pathway.[24]

Early work indicated that CCK2R activation promoted cell migration via a signaling pathway that involved the GTPase Rho, the Rho-dependent kinase ROCK and focal adhesion kinase (FAK) (reviewed by Rozengurt and Walsh[30] and by Dufresne et al.[8]). A parallel pathway, which involves Gamide-dependent activation of the p21-activated kinase 1 (PAK1) by the GTPases Rac and Cdc42, has been reported recently.[13] The importance of the second pathway in CRC proliferation and metastasis has been clearly demonstrated by the observation in our laboratory that PAK1 knockdown suppresses xenograft growth and liver metastasis of CRC cell lines in animal models. The Rho guanine-nucleotide exchange factor p190RhoGEF, which forms a complex with FAK, has also been implicated in CCK2R-dependent CRC cell motility and invasion. That regulation of β-catenin signaling by Gamide is also dependent on PAK1[12] suggests that PAK1 may occupy a central, crucially important position in Gamide signaling.

Although nonamidated gastrins activate many of the same signaling pathways as amidated gastrins, some significant differences have been observed even in the same cell line. For example, in the mouse gastric cell line IMGE both Ggly and Gamide activate PAK1 with similar time courses.[12] By contrast, although both Ggly and Gamide rapidly activate MAPK in IMGE cells, activation by Ggly is sustained for a much longer period. The different time courses result in different biological effects, as the rapid burst of activation is essential for stimulation of proliferation, while stimulation of migration is dependent on the sustained activation. In the case of apoptosis, the same biological effect can be reached by different pathways, as in IMGE cells both Ggly and Gamide inhibit caspase 3 by activation of Rho, while Gamide can also use an alternative pathway that involves activation of Rac and/or Cdc42.[13]

BIOLOGICAL ACTIONS WITHIN THE GASTROINTESTINAL TRACT

The two major roles of gastrins are stimulation of gastric acid secretion and stimulation of cell proliferation. Recent results strongly suggest that gastrins may also be involved in iron homeostasis. These three areas, and other actions of gastrins, will be considered in turn in this section.

Gastric Acid Secretion

Gastrins are released by food, predominantly by protein. Gastrin gene expression is also increased by these luminal stimuli. Other regulators of gastrins include vagal cholinergic activation (both excitatory and inhibitory) and non-cholinergic excitation with gastrin-releasing peptide being the putative neurotransmitter.[7] Amidated gastrins acting through the CCK2R have a dual stimulatory effect on gastric acid secretion by the gastric parietal cell. The direct effect is via the CCK2R on the parietal cell, while probably the more important pathway involves stimulation by amidated gastrin of histamine synthesis (via an increase in expression of histidine decarboxylase) and of histamine release from fundic enterochromaffin-like (ECL) cells. Histamine in turn activates the parietal cell. The gastrin-acid feedback loop, whereby increased gastric acidity inhibits gastrin secretion and decreased acidity stimulates gastrin release, is central to the regulation of gastric acid secretion. Somatostatin is another important component as it inhibits both gastrin release and gastric acid secretion.[7]

Amidated gastrins are the major hormonal mediators of the gastric phase of acid secretion. Amidated gastrins also contribute to the cephalic and intestinal phases to a variable extent depending on the species, with amidated gastrins being more important in the dog than in the rat. The relative contribution of amidated gastrins to the nongastric phase in humans awaits further studies with CCK2R antagonists.

Studies with knockout mouse models (gastrin$^{-/-}$, CCK2R$^{-/-}$) and transgenic gastrin overexpressing mice have provided additional insight into the role of gastrins (see reviews by Samuelson and Hinkle[31] and by Friis-Hansen[11]). As expected gastrin$^{-/-}$ and CCK2R$^{-/-}$ mice have decreased acid secretion, decreased parietal cell number, and a reduction in functional activity of the histamine-containing ECL cells. Acute infusion of Gamide, cholinergic stimuli, or histamine have no effect, but partial restoration of acid secretion in gastrin$^{-/-}$ mice is achieved by high dose infusion of Gamide for six days. Transgenic mice overexpressing amidated gastrins initially have increased parietal and ECL cell numbers and increased gastric acid secretion, but as the mice age, gastric acid secretion decreases as a result of loss of parietal cells and gastric atrophy, which eventually results in gastric cancer.

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The precise role of nonamidated gastrins in the regulation of gastric acid secretion remains unclear. Ggly seems to contribute to the regulation of gastric acidity at least in the longer term. In gastrin$^{-/-}$ mice administered Gamide, acid secretion increases from very low levels and then gradually decreases over a period of two weeks, whereas in animals receiving Gamide plus Ggly secretion is maintained over many weeks.[4] The potentiation seems to result from a direct effect on the extent and duration of parietal cell activation. In a subsequent study, the same group showed that mice overexpressing Ggly have a normal acid secretion, whereas double transgenic mice overexpressing both Gamide and Ggly have an increased acid secretion compared with transgenic mice overexpressing only Gamide.[5] Taken together, it seems that Ggly increases gastric acidity by maintaining the integrity and activation state of the parietal cell by an unknown mechanism. This effect may be relevant to the sustained hyperchlorhydria seen in patients with either *H. pylori* infection or Zollinger–Ellison syndrome as these subjects tend to have increased ratios of nonamidated gastrins in the circulation.[1] The effect of progastrin on acid secretion has not been reported, whereas the CTFP of progastrin, which is present in high concentrations in the antra of a number of species (reviewed by Smith et al.[33]), does not alter gastric acid secretion after short term (5 min) infusions alone or together with Gamide. The CTFP stimulates histamine release but at 1% of the potency of Gamide. As noted in the previous section, the receptors mediating the effects of Ggly or other nonamidated forms of gastrin have not yet been characterized but are believed to be distinct from the CCK1R and CCK2R that interact with the amidated forms of gastrin and CCK.

Proliferation

Patients with gastrin-secreting tumors have increased thickness of the gastric mucosa and an increased number of parietal and ECL cells. These proliferative effects have been confirmed in cell lines, in transgenic animals overproducing gastrin, and in rodents administered Gamide, as well as in achlorhydric rodents and humans. The proliferative effect is dynamic with increases in gastric epithelial cell proliferation occurring in parallel with the meal-stimulated increase in gastrin secretion. The effects of gastrins are not necessarily direct but may be through a variety of intermediaries such as members of the EGF family. More recently, the nonamidated forms of gastrin including progastrin, Ggly, and the CTFP have been reported to stimulate proliferation in gastric and colorectal cell lines and in the colonic mucosa of mice overexpressing progastrin or Ggly. These mice also support more rapid growth of human CRC xenografts and are more susceptible to the carcinogenic effect of azoxymethane.[1,7,10,33]

Iron Homeostasis

The first indication of a connection between gastrins and iron homeostasis was the report that circulating gastrin concentrations are increased in mice and humans with hemochromatosis (iron overload) resulting from a mutation in the Hfe gene (reviewed in Refs 17, 18). Conversely, iron homeostasis is affected by changes in circulating gastrin concentrations, as seen in juvenile gastrin$^{-/-}$ mice in which saturation of the iron-transporting protein transferrin is reduced, while in juvenile CCK2R$^{-/-}$ mice transferrin saturation is increased.[19] In human patients with hypergastrinemia resulting from Multiple Endocrine Neoplasia Type 1, transferrin saturation positively correlates with circulating gastrin concentration.[19] When placed on a low iron diet gastrin$^{-/-}$ mice developed not only an anemia that was more severe than in their wild-type counterparts but also gross splenomegaly.[17] A possible mechanism based on the known interaction between gastrin and transferrin postulates that gastrin catalyzes the loading of transferrin with iron.[18]

Other Actions

Other actions of gastrins include stimulation of migration and invasion and inhibition of apoptosis. Gamide stimulates a large variety of genes that are involved in gastric acid secretion, proliferation, and organization of the gastrointestinal mucosa. The list includes the genes involved in the generation of parietal and ECL cells, and genes encoding somatostatin, CCK2R, cyclooxygenase-2, interleukin-8, plasminogen activator inhibitor-2, matrix metalloproteinase-7 and -9, trefoil factor 2, sonic hedgehog, transforming growth factor α, and heparin-binding epidermal growth factor.[6,26] Gamide acting via the CCK2R also increases its own expression and indeed may induce the expression of the CCK2R itself.[20] Conversely, in gastrin$^{-/-}$ mice there is a reduction in expression of the genes regulating both parietal and ECL cells. The reported increase in inflammation-related genes in gastrin$^{-/-}$ mice is believed to be the result of the bacterial growth associated with hypochlorhydria.[11]

PATHOPHYSIOLOGICAL IMPLICATIONS

Hypergastrinemia can be the result of a gastrin-secreting tumor, of reduction of gastric acidity as a result of chronic atrophic gastritis or proton pump inhibitors, of reduced clearance in subjects with diminished renal or hepatic function, of *Helicobacter pylori* infection, or of colorectal carcinoma in some patients (see reviews by Murugesan and colleagues[25] and by Rehfeld and colleagues[27]). For a diagnosis of Zollinger–Ellison syndrome, the fasting hypergastrinemia must be associated with a low gastric pH. The diagnostic challenges have been reviewed recently and include the issues of differential processing of the progastrin molecules,

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and the use of radioimmunoassays that are specific for a single form of gastrin.[27] About a third of Zollinger–Ellison patients have a gastrinoma as part of the Multiple Endocrine Neoplasia type-1 syndrome and in these patients the gastrinoma is more probably located in the duodenum, whereas the sporadic type of gastrinoma is more often located in the pancreas.[25]

The hypergastrinemia resulting from *Helicobacter pylori* infection has two distinct mechanisms depending on the location of the infection. If *Helicobacter pylori* infection is predominantly in the antrum, somatostatin is inhibited resulting in an increase in gastrin and gastric acidity, and potentially the formation of a duodenal ulcer. However, if *Helicobacter pylori* infection is associated with chronic atrophic gastirits, the hypergastrinemia is a consequence of the hypochlorhydria.[25]

Sustained hypergastrinemia associated with the continuous administration of proton pump inhibitors to rats results in ECL cell hyperplasia within a few weeks and ECL carcinoids after 12 months. However, the human is less sensitive to chronic hypergastrinemia and even treatment with proton pump inhibitors for years results only in ECL hyperplasia rather than in carcinoids.[1]

The expansion of Barrett's esophagus and the transition to esophageal cancer seem to be related to circulating gastrin concentrations and the local expression of CCK2R. It is not clear whether the clinical advantage of reducing gastroesophageal reflux with proton pump inhibitors is outweighed by the associated increase in gastrins. Studies using specific CCK2R antagonists in patients with Barrett's esophagus have recently been proposed.[21]

Gastrin$^{-/-}$ mice have an increased rate of antral cancer as a result of hypochlorhydria, which permits bacterial growth with consequent inflammatory responses. The loss of trefoil factor (see the chapter in this section of the book) together with *Helicobacter pylori* infection are accelerating factors. Indeed, Gamide prevents the loss of trefoil factor expression and protects against gastric cancer in gastrin$^{-/-}$ mice.[34] Whether moderate hypergastrinemia is protective against the development of gastric cancer in humans is unknown.

Nonamidated gastrins have been implicated in the development and progression of CRC. There have been a number of recent reviews,[1,10] and only the key issues are presented here. Circulating concentrations of nonamidated gastrins are increased in patients with CRC, whereas in normal subjects, a circulating gastrin concentration above normal is associated with a nearly four-fold greater risk of developing CRC. Nonamidated gastrins increase the proliferation of CRC both *in vitro* and *in vivo* and potentiate the development of both chemically and genetically induced CRC. Gastrin expression occurs early in the progression from adenoma to carcinoma with most premalignant adenomatous polyps expressing gastrins, mainly as nonamidated intermediates. That this expression is maintained in CRC is consistent with the suggestion of a paracrine or autocrine role for gastrins. The gastrin gene is a downstream target of the mutated APC protein that is present early in the development of many CRC, and mice with an APC mutation and overexpression of Ggly have an increased number of intestinal polyps. The actions of both amidated and nonamidated forms of gastrin in stimulating proliferation, migration, invasion, and angiogenesis, and in inhibiting apoptosis, are consistent with a role for gastrins in the development of CRC. However in the absence of known receptors for the nonamidated gastrins, the therapeutic potential of blocking the effects of these gastrins remains limited.

The therapeutic options for gastrins include immunoneutralization and receptor antagonists. Gastrimmune is a gastrin–diphtheria conjugate, which elicits antibodies to the amino terminus of gastrin17. Some promising results have been reported in patients with advanced pancreatic and gastric cancers.[35] However, it is not clear whether the antibodies would inhibit all the bioactive forms of gastrin such as progastrin or gastrin34 (either amidated or glycine-extended). CCK2R antagonists have also been used directly for the treatment of ECL cell hyperplasia and gastric carcinoid development and, in combination with gemcitabine, for a phase I/II trial in patients with advanced pancreatic cancer. CCK2R antagonists have in the past been neglected as there are alternate therapies for the regulation of gastric acidity and because the available evidence suggested that CCK2R were not the most relevant target for CRC.[1] The recent surprising observation that the stimulatory effects of progastrin on CRC development in mice are blocked by treatment with CCK2R antagonists or in CCK2R$^{-/-}$ animals[16] suggests that this therapeutic option deserves greater attention.

Considerable effort has been expended in the development of radiopharmaceuticals based on gastrin and CCK for diagnosis and targetting of CCK2R-expressing tumors (reviewed by Roosenburg[29]). Typically chelating groups are attached to the N-terminus of CCK8 to permit radiolabeling with ^{68}Ga or ^{111}In. Further elongation of the CCK/gastrin peptide improves receptor-mediated tumor uptake but may lead to unacceptable levels of retention in the kidney. These ligands provide additional information in patients with neuroendocrine tumors with equivocal or absent somatostatin uptake.[28] As these ligands are internalized by tumor cells, there is potential for tumor therapy and localization.

ACKNOWLEDGMENTS

This work was supported by grants 566555 and 1006245 from the National Health and Medical Research Council of Australia.

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REFERENCES

1. Aly A, Shulkes A, Baldwin GS. Gastrins, cholecystokinins and gastrointestinal cancer. *Biochim Biophys Acta* 2004;**1704**:1–10.
2. Baldwin GS, Curtain CC, Sawyer WH. Selective, high-affinity binding of ferric ions by glycine-extended gastrin(17). *Biochemistry* 2001;**40**:10741–6.
3. Baldwin GS, Patel O, Shulkes A. Evolution of gastrointestinal hormones: the cholecystokinin/gastrin family. *Curr Opin Endocrinol Diabetes Obes* 2010;**17**:77–88.
4. Chen D, Zhao CM, Dockray GJ, Varro A, Van Hoek A, Sinclair NF, et al. Glycine-extended gastrin synergizes with gastrin 17 to stimulate acid secretion in gastrin-deficient mice. *Gastroenterology* 2000;**119**:756–65.
5. Cui G, Koh TJ, Chen D, Zhao CM, Takaishi S, Dockray GJ, et al. Overexpression of glycine-extended gastrin inhibits parietal cell loss and atrophy in the mouse stomach. *Cancer Res* 2004;**64**:8160–6.
6. Dockray G, Dimaline R, Varro A. Gastrin: old hormone, new functions. *Pflugers Arch* 2005;**449**:344–55.
7. Dockray GJ, Varro A, Dimaline R, Wang T. The gastrins: their production and biological activities. *Annu Rev Physiol* 2001;**63**: 119–39.
8. Dufresne M, Seva C, Fourmy D. Cholecystokinin and gastrin receptors. *Physiol Rev* 2006;**86**:805–47.
9. Ferrand A, Lachal S, Bramante G, Kovac S, Shulkes A, Baldwin GS. Stimulation of proliferation in the colorectal mucosa by gastrin precursors is blocked by desferrioxamine. *Am J Physiol Gastrointest Liver Physiol* 2010;**299**:G220–7.
10. Ferrand A, Wang TC. Gastrin and cancer: a review. *Cancer Lett* 2006;**238**:15–29.
11. Friis-Hansen L. Lessons from the gastrin knockout mice. *Regul Pept* 2007;**139**:5–22.
12. He H, Shulkes A, Baldwin GS. PAK1 interacts with beta-catenin and is required for the regulation of the beta-catenin signalling pathway by gastrins. *Biochim Biophys Acta* 2008;**1783**:1943–54.
13. He H, Yim M, Liu KH, Cody SC, Shulkes A, Baldwin GS. Involvement of G proteins of the Rho family in the regulation of Bcl-2-like protein expression and caspase 3 activation by Gastrins. *Cell Signal* 2008;**20**:83–93.
14. Hellmich MR, Rui XL, Hellmich HL, Fleming RY, Evers BM, Townsend Jr CM. Human colorectal cancers express a constitutively active cholecystokinin-B/gastrin receptor that stimulates cell growth. *J Biol Chem* 2000;**275**:32122–8.
15. Hughes J, Boden P, Costall B, Domeney A, Kelly E, Horwell DC, et al. Development of a class of selective cholecystokinin type B receptor antagonists having potent anxiolytic activity. *Proc Natl Acad Sci U S A* 1990;**87**:6728–32.
16. Jin G, Ramanathan V, Quante M, Baik GH, Yang X, Wang SS, et al. Inactivating cholecystokinin-2 receptor inhibits progastrin-dependent colonic crypt fission, proliferation, and colorectal cancer in mice. *J Clin Invest* 2009;**119**:2691–701.
17. Kovac S, Anderson GJ, Alexander WS, Shulkes A, Baldwin GS. Gastrin-deficient mice have disturbed hematopoiesis in response to iron deficiency. *Endocrinology* 2011;**152**:3062–73.
18. Kovac S, Anderson GJ, Baldwin GS. Gastrins, iron homeostasis and colorectal cancer. *Biochim Biophys Acta* 2011;**1813**:889–95.
19. Kovac S, Smith K, Anderson GJ, Burgess JR, Shulkes A, Baldwin GS. Interrelationships between circulating gastrin and iron status in mice and humans. *Am J Physiol Gastrointest Liver Physiol* 2008;**295**:G855–61.
20. Kovac S, Xiao L, Shulkes A, Patel O, Baldwin GS. Gastrin increases its own synthesis in gastrointestinal cancer cells via the CCK2 receptor. *FEBS Lett* 2010;**584**:4413–8.
21. Kuipers EJ. Barrett's oesophagus, proton pump inhibitors and gastrin: the fog is clearing. *Gut* 2010;**59**:148–9.
22. Merchant JL. EGF receptor activation of the human gastrin gene: a tale of two zinc finger transcription factor families. *Keio J Med* 2000;**49**:106–10.
23. Morton M, Prendergast C, Barrett TD. Targeting gastrin for the treatment of gastric acid related disorders and pancreatic cancer. *Trends Pharmacol Sci* 2011;**32**:201–5.
24. Muerkoster S, Isberner A, Arlt A, Witt M, Reimann B, Blaszczuk E, et al. Gastrin suppresses growth of CCK2 receptor expressing colon cancer cells by inducing apoptosis in vitro and in vivo. *Gastroenterology* 2005;**129**:952–68.
25. Murugesan SV, Varro A, Pritchard DM. Review article: strategies to determine whether hypergastrinaemia is due to Zollinger–Ellison syndrome rather than a more common benign cause. *Aliment Pharmacol Ther* 2009;**29**:1055–68.
26. Rehfeld JF, Friis-Hansen L, Goetze JP, Hansen TV. The biology of cholecystokinin and gastrin peptides. *Curr Top Med Chem* 2007;**7**:1154–65.
27. Rehfeld JF, Gingras MH, Bardram L, Hilsted L, Goetze JP, Poitras P. The Zollinger–Ellison syndrome and mismeasurement of gastrin. *Gastroenterology* 2011;**140**:1444–53.
28. Reubi JC, Maecke HR. Peptide-based probes for cancer imaging. *J Nucl Med* 2008;**49**:1735–8.
29. Roosenburg S, Laverman P, van Delft FL, Boerman OC. Radiolabeled CCK/gastrin peptides for imaging and therapy of CCK2 receptor-expressing tumors. *Amino Acids* 2011;**41**:1049–58.
30. Rozengurt E, Walsh JH. Gastrin, CCK, signaling, and cancer. *Annu Rev Physiol* 2001;**63**:49–76.
31. Samuelson LC, Hinkle KL. Insights into the regulation of gastric acid secretion through analysis of genetically engineered mice. *Annu Rev Physiol* 2003;**65**:383–400.
32. Singh P. Role of Annexin-II in GI cancers: interaction with gastrins/progastrins. *Cancer Lett* 2007;**252**:19–35.
33. Smith KA, Patel O, Lachal S, Jennings I, Kemp B, Burgess J, et al. Production, secretion, and biological activity of the C-terminal flanking peptide of human progastrin. *Gastroenterology* 2006;**131**: 1463–74.
34. Tomita H, Takaishi S, Menheniott TR, Yang X, Shibata W, Jin G, et al. Inhibition of gastric carcinogenesis by the hormone gastrin is mediated by suppression of TFF1 epigenetic silencing. *Gastroenterology* 2011;**140**:879–91.
35. Watson SA, Grabowska AM, El-Zaatari M, Takhar A. Gastrin—active participant or bystander in gastric carcinogenesis? *Nat Rev Cancer* 2006;**6**:936–46.

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Chapter 166

GIP

Nigel Irwin and Peter R. Flatt

ABSTRACT

Glucose-dependent insulinotropic polypeptide (GIP) is a 42-amino acid peptide hormone secreted from enteroendocrine K-cells. GIP is recognized as an incretin hormone that potentiates glucose-induced insulin secretion and regulates postprandial glucose homeostasis. The structure of mammalian GIP is well conserved through evolution; however, differential processing may give rise to more than one biologically active form in humans. The GIP receptor is a G protein-coupled receptor and ligand binding leads to stimulation of adenyl cyclase and activation of a number of cAMP-dependent and independent intracellular pathways. In addition to insulinotropic actions, GIP has numerous important effects outside of the pancreas. As such, GIP and GIP receptor mRNA is located throughout the body including adipose, brain, bone, cardiovascular system, and the gastrointestinal tract. Presently, there is optimism that long-acting GIP analogs may offer a new class of drugs for the treatment of diabetes, obesity, neurodegeneration, and bone disorders.

DISCOVERY

GIP is a 42-amino acid peptide hormone released from the proximal small intestine (duodenum and jejunum) in response to feeding. GIP was first isolated from impure preparations of porcine cholecystokinin (CCK; for a detailed account of CCK see the chapter in this section of the book) and shown to be a potent inhibitor of histamine-induced gastric acid secretion. Thus, it was originally named "gastric inhibitory polypeptide."[3] However, in 1973, it was demonstrated that intravenous infusion of a purified preparation of GIP in combination with glucose evoked a greater increase in insulin concentrations than the infusion of glucose alone.[5] This functional link between gut factors and the endocrine pancreas is now termed the "enteroinsular axis."[33] Furthermore, gut factors that stimulate insulin secretion in a glucose-dependent manner at physiological concentrations were later designated as "incretin" hormones.[16] The glucose-dependent nature of the insulinotropic properties of GIP was then later identified in dogs, perfused rat pancreas, and humans, confirming incretin status.[16] Recognition that this was indeed the most important biological property of GIP led to the renaming of the hormone to "glucose-dependent insulinotropic polypeptide" thereby still maintaining its original acronym GIP.[22]

The possible existence of an incretin companion for GIP arose when studies clearly revealed that GIP could not solely account for the full incretin effect in humans.[6] As such, discovery of a coding sequence for a glucagon-related peptide in Anglerfish cDNA contributed to the discovery of glucagon-like peptide-1[20] (see GLP chapters in this section and the Ingestive Peptides section of the book). The insulinotropic effect of GLP-1, as with GIP, is strictly glucose dependent and occurs at physiologically relevant levels corroborating its position as an incretin hormone. Thus, it is now widely accepted that both GIP and GLP-1 act in unison to account for the full incretin effect in humans.[16] Therefore, GIP and GLP-1 possess a unique characteristic in their ability to safeguard against inappropriate stimulation of insulin release, avoiding harmful hypoglycemic episodes and highlighting potential as possible antidiabetic therapy. This chapter will focus primarily on the biology GIP.

STRUCTURE OF THE mRNA/GENE

After purification and amino acid sequence determination of the originally isolated porcine GIP, it was initially believed to contain 43 amino acid residues.[3] However, more sophisticated sequencing techniques using characterization of cDNA clones from a genomic library indicated that an extra glutamine residue was incorporated incorrectly at position 30. This was removed, and the sequence was then corrected to contain 42 amino acid residues.[18] The sequence of bovine, rat, mouse, and numerous other species of GIP was subsequently elucidated and all shown to encode for a highly conserved 42 amino acid peptide.[15] These early studies concentrated on isolation and characterization of the mammalian GIP gene. However, the GIP gene has now been identified in chicken, frogs, and zebrafish with subtle differences between mammalian and nonmammalian species noted.[15] The variation in gene structure might suggest that the GIP gene has undergone rapid sequence modifications

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00166-4
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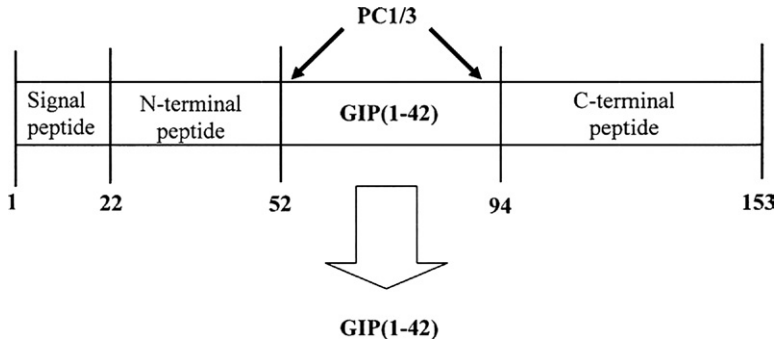

FIGURE 1 Structural organization of the human GIP gene precursor depicting cleavage sites for prohormone convertase (PC) 1/3 enzyme.

early in mammalian evolution, possibly also reflecting differences in biological action of the hormone amongst species.[15] In addition, a recent study suggests that differential enzymatic processing of the proGIP hormone in mammalian gut may yield significant levels of a bioactive shorter version of GIP, GIP(1–30)amide,[21] the full biological significance of which remains to be elucidated. Furthermore, it has been suggested that GIP is expressed and secreted from pancreatic alpha cells, probably promoting insulin secretion.[21] Importantly, however, slight GIP species differences may have an impact on comparisons of studies from separate laboratories using slightly different GIP peptides or antibodies and in the biological activity of GIP within different biological systems.

The GIP hormone is derived from proteolytic processing of a 153-amino acid precursor. The gene coding for the human GIP precursor spans about 10 kilobase pairs and consists of six exons and five introns. Similar to genes encoding other members of the glucagon superfamily, each exon seems to encode a distinct region of the GIP precursor mRNA. For example, the GIP precursor, prepro-GIP is encoded for largely by exons 2 and 5, whereas the GIP(1–42) sequence is encoded by exons 3 and 4.[21] The GIP moiety is flanked on either side by polypeptide segments of 51 and 60 amino acids residues at its amino and carboxyl termini; respectively. The amino terminal flanking region can be additionally subdivided into two distinct regions; a putative signal peptide (21 amino acids) and a hydrophobic N-terminal peptide (30 amino acids) leading to the structural organization of the human GIP precursor. The human GIP gene has been assigned to chromosome 17q21.3→q22 through southern analysis of somatic cell hybrid DNAs and by in situ hybridization using a GIP cDNA.[14]

PROCESSING OF THE PRECURSOR

Processing of the proGIP encoding region occurs via trypsin-like cleavage by prohormone convertase (PC) enzymes at single arginine residues present at either side of the GIP coding sequence at positions 51 and 93. GIP and PC1/3 are colocalized in K-cells and GIP processing is severely impaired in PC1/3 deficient mice,[32] indicating the importance of this enzyme in GIP processing (Fig. 1) (see convertase 1/3 chapter in Peptide Biosynthesis/Processing section of this book). The 5′-upstream region of the human GIP gene contains potential binding sites for a number of transcriptional factors including Sp1, Ap-1 and Ap-2, suggesting regulation by protein kinases A and C.[21] Although the resulting N- and C-termini peptides do not possess biological activity, the C-terminal region of the human GIP precursor does have distinct similarities with amino acid domains within pancreastatin, known to be an inhibitor of insulin secretion in pancreatic beta cells.[31]

In addition, mammalian proGIP contains a highly conserved PC2 cleavage site between residues 52–55[21] (see convertase 2 chapter in Peptide Biosynthesis/Processing section of this book). Interestingly, there seems to be a subpopulation of K-cells that express PC2, representing about 5–15% of the total GIP-immunoreactive cells in mice.[21] This PC2 derived C-terminally truncated form of GIP, GIP(1–31), is probably converted by peptidyl-glycine α-amidating monooxygenase to amidated GIP(1–30). Furthermore, in most mammals GIP(1–42) contains a Gly^{31}-Lys^{32}-Lys^{33} sequence that may undergo cellular processing to also result in the production of GIP(1–30).[21] Therefore, as well as GIP(1–42), GIP(1–30) seems to be a naturally occurring biologically active form of GIP. In this context, GIP(1–30) shares similar insulinotropic properties to the full length molecule, and recently the biological effects of subchronic administration of enzyme-resistant analogs of GIP(1–30) and GIP(1–42) in mice was shown to be identical.[10] Thus, it is possible that biologically active circulating GIP levels have in been underestimated in many early studies owing to antibodies directed toward the C-terminus of GIP(1–42) that would therefore not detect GIP(1–30). Furthermore, GIP is known to exert effects on pancreas, brain, bone, adipose, and possibly cardiac tissue,[16,21] and the possibility of different responses to the two naturally occurring forms of GIP in each tissue necessitates further study.

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DISTRIBUTION OF THE mRNA/PEPTIDE

Immunohistochemical and radioimmunoassay techniques showed that the highest concentrations of GIP were, as expected, found in the jejunum, duodenum, and ileum.[28] In the gastrointestinal tract of dogs and humans, GIP is present in cells primarily in the midduodenal region and, to a lesser extent, in the jejunum.[28] GIP may also be expressed and secreted from pancreatic alpha cells.[21] Other studies would now suggest that GLP-1 and GIP are colocalized throughout the gut in a subpopulation of cells.[24] Indeed Mortensen and colleagues (2003) identified mammalian cells in the upper small intestine that express both GIP and GLP-1 and are capable of coreleasing the two incretin hormones. In agreement with this, the secretion of GIP and GLP-1 to a mixed meal is highly correlated and both incretin hormones have additive, but not potentiating effects on insulin secretion.[25] In terms of the GIP-receptor (GIP-R), Northern blot, RT-PCR, and in situ hybridization studies have shown GIP-R mRNA to be present in the pancreas, adipose tissue, stomach, bone, intestine, pituitary, adrenal cortex, heart, lung, brain, and endothelium of major blood vessels.[34] In addition, there have been several reports of ectopically expressed GIP-Rs on adrenal glands, which are believed to be possibly involved in the onset of food-dependent Cushing's syndrome.[4]

RECEPTORS AND SIGNALING CASCADES

The human GIP-R gene is located on chromosome 19 and contains 14 exons and 12 introns with a coding region of approximately 12.5 kilobase pairs.[21] The GIP-R is a member of the secretin-vasoactive intestinal polypeptide family of G protein-coupled receptors possessing seven transmembrane domains. All mammalian GIP-Rs sequenced to date consist of 392–440 amino acid residues with the exception of the fox (*Felix catus*) whose GIP-R contains 388 residues.[21] The extensive N-terminal extracellular domain of the GIP-R contains numerous glycosylation sites that are important for ligand recognition and binding. As such, it has been demonstrated that the first 132 N-terminal amino acids of the GIP-R are important for high affinity binding and that the first 222 N-terminal residues are important for cAMP production following ligand–receptor coupling.[12] Furthermore, through the use of nuclear magnetic resonance and circular dichroism spectroscopy, the adoption by GIP of an alpha-helical secondary structure was revealed to be an intrinsic requirement for normal biological activity.[1] In addition, the crystal structure of the human GIP-R N-terminal extracellular domain bound to its ligand has also been reported.[27] This study concluded that the C-terminus of the GIP peptide binds to the surface of the extracellular GIP-R domain, with the N-terminal peptide region free to interact with other parts of the receptor. Thus, the N-terminus of GIP is important in the modulation of biological activity and control of agonist/antagonist activity, as demonstrated from numerous chimeric peptide studies.[13]

In addition, it seems the carboxy terminal tail of the GIP-R, although rich in potential phosphorylation sites, is not required for signaling although specific parts may play a role in binding affinity and downregulation of the receptor.[21] Interestingly, a number of studies suggest alternative mRNA splicing of the GIP receptor gene, resulting in GIP receptor variants.[9] For example, genomic analysis identified one exon, designated number 8a, that seems to be alternatively spliced, resulting in a variant mRNA transcript that encodes a truncated receptor with unknown functional properties.[21] Furthermore, a single point mutation of Thr^{340} to Pro^{340} in the sixth transmembrane spanning domain of the GIP-R results in the ligand-independent accumulation of cAMP without masking GIP binding or the GIP-induced cAMP response.[21] However, the biological significance and relevance to humans of these receptor mutants is largely unknown.

Although first identified as an inhibitor of gastric acid secretion, the main biological activity of GIP is associated with the glucose-induced potentiation of insulin secretion. Stimulation of the pancreatic GIP-R has been shown to stimulate adenylyl cyclase and elevate cAMP levels in a number of biological systems.[9] This correlates well with other members of the secretin-VIP family of receptors. Increased GIP-induced cAMP levels are known to increase the activity of protein kinase A (PKA) and the cAMP-specific guanine nucleotide exchange factor (GEF) II/exchange protein directly activated by cAMP (EPAC) 2.[29] The action of both these molecules results in increased insulin exocytosis from the pancreatic beta-cell. In brief, PKA phosphorylates the SUR1 subunit of the K_{ATP} channel, causing channel closure and beta-cell membrane depolarization. This in turns leads to opening of membrane voltage-dependent calcium channels, influx of calcium and exocytosis of insulin. Furthermore, PKA is known to phosphorylate important proteins involved in exocytotic beta-cell machinery.[29] In terms of GEF II activation, one of the main biological effects seems to be interaction with a secretory granule binding protein named Rab3, which is an essential part of the exocytotic system in beta-cells.[29] In addition, GIP is also believed to induce calcium-induced calcium release from intracellular stores, although the mechanism is still not fully understood.[21]

Other distinct signaling pathways for GIP have also been described. For example, GIP has been shown to be involved in the activation of mitogen-activated protein kinase (MAPK) via a wortmannin-sensitive pathway.[19] Indeed, it had been shown that wortmannin, a selective inhibitor of phosphatidylinositol 3-kinase, substantially inhibited

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GIP-stimulated insulin secretion and that VIP and PACAP, two other members of this receptor family, also possess this wortmannin-sensitive signaling pathway.[30] In addition, GIP is thought to promote beta cell survival through cAMP maintained regulation of p38 mitogen-activated protein kinase phosphorylation in the INS-1 pancreatic beta-cell line.[8] Evidence has also emerged that there may be an involvement of phospholipase A_2 in GIP-stimulated insulin secretion mediated via cAMP-dependent and independent pathways.[7] Finally, GIP is known to activate extracellular signal-regulated kinase1/2 (ERK1/2) and has been shown to influence the activity of all known kinases of the ERK1/2 signaling system;[7] this regulation is known to occur via action of PKA and Rap1, a small GTPase.

There are also a number of factors known to control GIP secretion from K-cells of the gastrointestinal tract (Fig. 2). As such, G-protein-coupled receptors (GPRs), believed to mediate fat-induced GIP release, are located on the membrane of K-cells, in particular activation of the G_q-coupled receptors GPR40 and GPR120 augments protein kinase C and inositol (1,4,5)-trisphosphate activity resulting in enhanced secretion.[16] In agreement, genetic knockout of GPR40 in mice diminishes the stimulatory effect of fat on GIP secretion.[16] Furthermore, a small molecular agonist of the G_s-coupled receptor GPR119 was shown to elevate intracellular cyclic AMP levels and stimulate GIP secretion.[16] In addition, K-cells were recently shown to express an active variant of the sodium-coupled glucose transporter-1 (SGLT-1) and α-methyl-glucopyranoside (α-MDG), a transporter substrate, shown to evoke significant GIP secretion.[16] Thus, it is suggested that activation of SGLT-1 results in K-cell depolarization, calcium influx and subsequent secretion of GIP (Fig. 2). In keeping with this α-MDG administration to mice was associated with enhanced GIP secretion, and prolonged treatment for 13 days in *db/db* mice improved oral glucose tolerance.[16] However, the biological processes that trigger GIP secretion are as yet not fully understood.

BIOLOGICAL ACTIONS WITHIN THE GASTROINTESTINAL TRACT

As mentioned above, GIP was first identified as an enterogastrone that inhibited gastric secretions. However, the major recognized biological effects of GIP all occur outside of the gastrointestinal tract. More specifically, GIP has a fundamental role to play in various endocrine tissues including the pancreas, adipose, and bone.[16] However, GIP-Rs are present in several regions of the mammalian gastrointestinal tract, with some putative functions characterized to date. GIP is expressed in rabbit mandibular gland and has a reported inhibitory effect on sodium transport.[21] In addition, GIP can inhibit gastric emptying in rodents, but this effect is not observed in humans.[22] Interestingly, salivary GIP levels are thought to increase with fasting and swallowing in humans; however, the biological relevance of this effect is not known at present.[23] GIP also causes increased intestinal water, electrolyte, and glucose transport and potentiates acetylcholine- and CCK-stimulated pancreatic amylase secretion.[21] Effects on glucose transport seem to be mediated by GIP-induced regulation of the GLUT 2 and the sodium–glucose-transporter systems within the gut. The significance of most of the GIP-mediated effects listed above is uncertain.

Actions on the endocrine pancreas are the most pertinent biological effects of GIP. As detailed previously, GIP is insulinotropic at physiological concentrations and fulfills all the criteria of an incretin hormone.[9] The glucose threshold for GIP insulinotropic activity is about 4.4 mM, with a maximal effect observed at 16.7 mM.[9] Studies with GIP receptor antagonists demonstrate GIP to be the major physiological incretin.[9] Furthermore, genetic knockout of the GIP-R (GIP-$R^{-/-}$) in mice confirm the role of GIP in glucose homeostasis. As such, GIP-$R^{-/-}$ mice display mild glucose intolerance and reduced insulin secretory response to oral glucose.[9] However, isolated islets from GIP-$R^{-/-}$ mice respond normally to glucose, but the insulinotropic response to GLP-1 is augmented, suggesting compensatory upregulatory effects in the islets of GIP-$R^{-/-}$ rodents.[9] Interestingly, mice that have a specific and total destruction of GIP producing intestinal K-cells have remarkably reduced insulin levels in response to oral glucose.[16] Thus, it is possible that GIP producing intestinal K-cells may generate additional compounds that amplify or potentiate the insulin secretory response of incretin hormones. In addition, GIP may be involved in the secretion and action of other gastrointestinal related hormones such as GLP-1, glucagon, pancreatic polypeptide, and somatostatin.[16]

Other Emerging Biological Actions

Regarding effects on the gastrointestinal tract and endocrine pancreas, GIP now has established effects on bone, brain, and adipose tissue.[16] For instance, GIP exerts direct beneficial effects on bone turnover and health.[16] GIP-Rs have been evidenced at the surface of osteoblasts and osteoclasts and daily intravenous injection of native GIP for 6 weeks prevents bone loss in ovariectomized rats.[16] Furthermore, the GIP gene, protein and receptor have also been identified in numerous regions of the human brain.[16] GIP has been shown to induce proliferation of adult-derived hippocampal progenitor cells and potentially protects synapses from the detrimental effects of beta-amyloid accumulation.[16] In terms of adipose tissue, functional GIP-Rs were shown on adipocytes in 1998.[16] Established GIP-mediated effects on lipid metabolism include increased lipoprotein lipase activity, stimulation of lipogenesis, enhancement of fatty acid and

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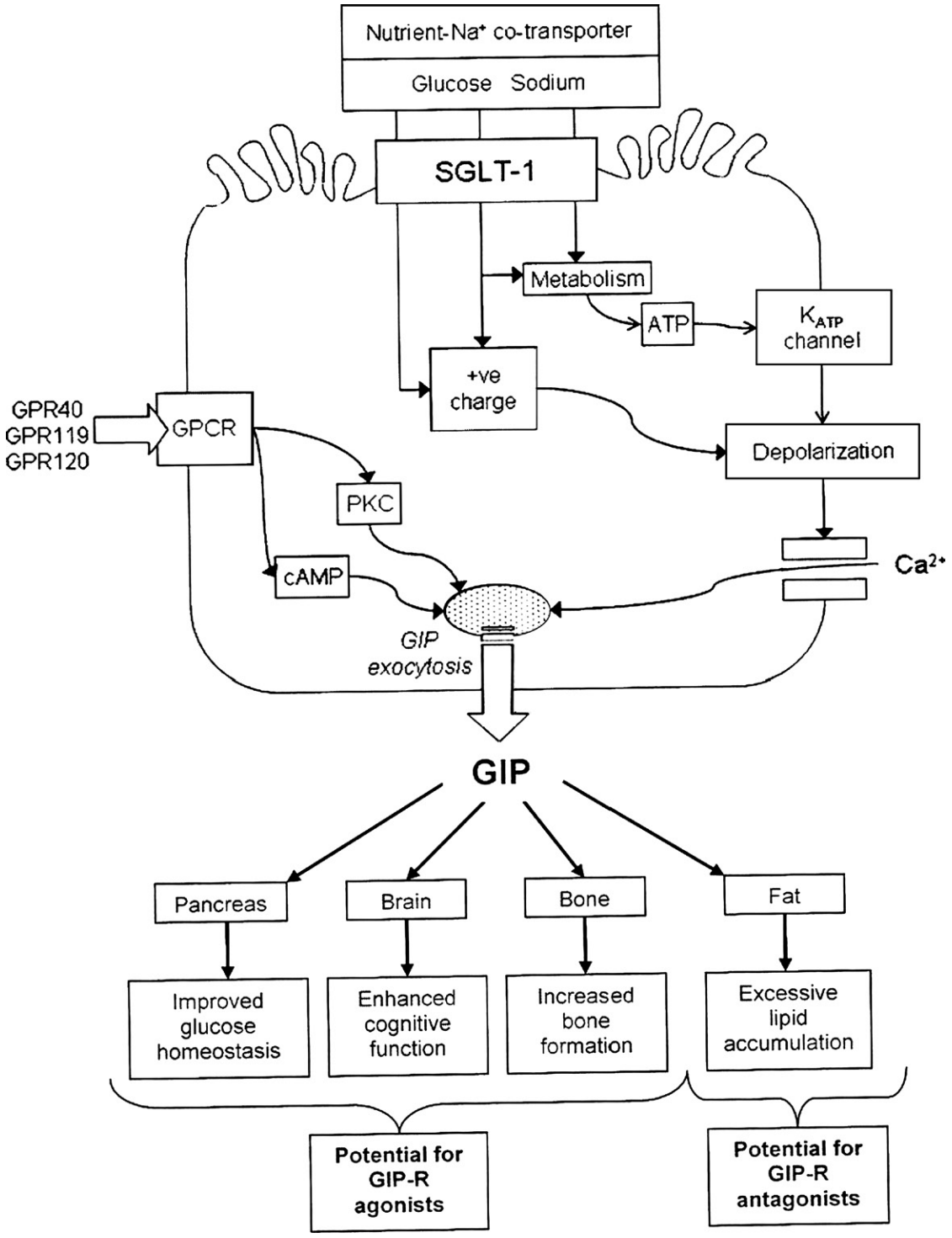

FIGURE 2 Scheme displaying the main signaling pathways involved in GIP secretion from intestinal K-cells together with major physiological functions of GIP and possible involvement in the pathophysiology of disease.

glucose uptake, augmentation of insulin-induced fatty acid incorporation, and inhibition of both glucagon- and adrenergic receptor mediated lipolysis.[16] Interestingly, recently GIP has been suggested to be involved in thermogenesis in brown adipose tissue and the metabolic adaptations to cold acclimation.[17] Putative effects of GIP on the cardiovascular system, pituitary, liver, and muscle have also been considered.[16] Taken together, GIP has several notable important biological

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effects both within and outside the mammalian gastrointestinal tract.

PATHOPHYSIOLOGICAL IMPLICATIONS

There are a number of disease states for which GIP secretion, biological action, or receptor expression are postulated to be involved in the etiology. As discussed earlier, aberrant GIP-R expression is possibly involved in certain forms of food-dependent Cushing's syndrome.[4] However, to date, the major focus of the pathophysiological implications of GIP relate to effects in type 2 diabetes, obesity, bone disorders, and brain function. Much of the evidence for GIP effect in disease comes form preclinical testing, so direct relation to the human setting still needs to be verified. Furthermore, it is uncertain whether disturbances of GIP action are primary factors in disease etiology or secondary consequences of disease progression. Nonetheless, there is evidence for potential therapeutic use of GIP-R agonists and antagonists in a number of disease states (Fig. 2).

GIP and Diabetes

GIP, as an incretin hormone, is secreted rapidly from the gut into the bloodstream after nutrient absorption and makes an inherently important contribution to postprandial blood glucose control.[9] This incretin effect is estimated to account for up to 70% of the total insulin secreted after oral nutrient absorption in normal human subjects.[9] However, the incretin effect is markedly diminished in patients with type 2 diabetes, being less than half that observed in subjects with normal glucose tolerance.[26] Generally, the reduced incretin effect in type 2 diabetes is attributed to defective GIP action and reduced GLP-1 secretion.[16] However, in addition to effects on pancreatic beta-cells, GIP also possesses glucose-lowering extrapancreatic effects. These include inhibition of hepatic glucose production, promotion of glucose uptake in isolated muscle, increase of fatty acid synthesis, stimulation of lipoprotein lipase activity, and reduction of hepatic insulin extraction.[9] The cause of the reported GIP insulin-releasing ineffectiveness in type 2 diabetes is largely unknown but could include reduced GIP-R expression, downregulation of receptor, altered processing of GIP, defective intracellular signaling processes, or mutations of the receptor or protein.[16]

Genetic mutations of the GIP-R are rare in diabetes;[2] one Japanese study did identify two missense mutations in the GIP receptor but showed no relationship between these mutations and type 2 diabetes.[19] Furthermore, a reduced insulin secretion after intravenous glucose infusion was reported in first-degree relatives of patients with type 2 diabetes, but there was no link to decreased insulinotropic action of GIP in these patients.[22] Thus, mutations of the GIP-R as a link to development of type 2 diabetes seem unlikely. To further clarify the role of GIP and its receptor in the pathophysiology of type 2 diabetes numerous studies in rodent models have been conducted. In the hyperglycemic Vancouver diabetic fatty (VDF) rat decreased levels of pancreatic GIP-R mRNA and the GIP-R protein have been observed.[9] Furthermore, chronically elevated glucose levels are associated with downregulation of the GIP-R both *in vivo* and *in vitro*, whereas normalization of glucose levels almost completely restores GIP-R expression levels.[21] In agreement, the acutely diabetic *ob/ob* mouse on the Aston background displays severe hyperglycemia with elevated intestinal and circulating GIP levels, indicative of GIP-R downregulation.[16] However, taken in context downregulation of the GIP-R does not seem to be an inherent defect in the pathophysiology of type 2 diabetes, merely a consequence of hyperglycemia.[16] Furthermore, it is clear that the defect in insulin secretion displayed in type 2 diabetes is not limited to only GIP but it also embraces several factors including glucose and GLP-1.[16] As such, GIP-based pharmaceutics may have promise for the treatment of type 2 diabetes.

However, after release into the circulation, GIP undergoes rapid N-terminal degradation by the ubiquitous enzyme dipeptidyl peptidase IV (DPP IV).[9] DPP IV metabolism effectively eliminates the insulinotropic activity of GIP and thwarts any therapeutic potential. To circumvent this problem, a number of N-terminally modified DPP-IV resistant forms of GIP have been generated.[9,16] Furthermore, speculated abnormalities in GIP-R action can be overcome by administration of pharmacological levels of these GIP analogs in animal models of diabetes.[9,16] In addition, DPP IV inhibitors currently in use to treat human type 2 diabetic patients increase intact levels of both GLP-1 and GIP, and it is unclear to what extent the observed beneficial effects are mediated by GIP.[16] Indeed, recently, it has been shown that combined preparation of enzyme-resistant longer-acting GLP-1 and GIP peptides markedly improved glucose-lowering and insulinotropic properties in mice with type 2 diabetes when compared with either incretin mimetic given individually.[11]

The effects of amino acid modification at position Tyr^1 and Ala^2 in GIP as well as secondary modifications such as fatty acid derivatization or conjugation with polyethylene glycol (PEG) have been extensively reviewed elsewhere.[9,16] In brief, modifications at position at Tyr^1 and Ala^2 were intended to mask the DPP IV binding site of the peptide but not adversely affect biological activity. In general, Tyr^1 were more successful and exhibited complete resistance to DPP IV and enhanced bioactivity compared with the native peptide.[9] Secondary GIP modifications were anticipated to delay renal extraction of GIP by inducing plasma protein binding.[16] Surprisingly, all these secondary modified peptides

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also displayed complete resistance to DPP IV degradation.[16] However, in a head-to-head comparison in *ob/ob* mice, the antidiabetic effectiveness of prolonged once daily injection of a primary or secondary modified GIP analog was almost identical.[16] Preclinical results to date would indicate that further investigation, either alone or in combination with established antidiabetic medications, is required to fully delineate their therapeutic promise.

GIP and Obesity

GIP levels are increased in obesity and consumption of high fat diet induces K-cell hyperplasia and increased GIP protein expression.[16] Indeed, a lipid storage action for GIP is in line with its general anabolic characteristics. There are now numerous studies linking GIP to the development of obesity resulting from chronic overconsumption of energy rich food. In keeping with this, activation of GIP-Rs on adipocytes after feeding results in efficient storage of fat.[16] Thus, when GIP-R signaling is annulled in animal models of obesity diabetes, the blockade of lipid metabolism and fat deposition encourages the use of circulating fat as the primary energy substrate. This results in decreased weight gain and improved insulin sensitivity and considerably outweighs the potential negative impact of loss of the insulin-releasing GIP component of the enteroinsular axis. Moreover, as previously stated, the insulinotropic activity of GIP is already compromised in patients with type-2 diabetes.[26] The beneficial effects of GIP-R antagonism are borne out in a number of preclinical studies using genetic knockout or chemical blockade of GIP-R signaling.[16] Thus, GIP-R$^{-/-}$ mice are resistant to high fat diet induced obesity and subchronic daily administration of the specific GIP-R antagonist, (Pro^{3})GIP, to *ob/ob* mice results in significant reduction in hyperglycemia, alleviation of insulin resistance, and restoration of glucose tolerance.[16] Other similar methodologies to inhibit GIP-R signaling such as active GIP immunization, administration of small molecular weight GIP-R antagonists, specific K-cell destruction, and dietary modification to reduce GIP secretion all result in strikingly similar metabolic effects.[16] No serious adverse effects were observed in any of the animal studies using GIP-R signaling knockdown methodologies to date.

GIP and Bone

GIP stimulates proliferation of the human osteoblastic-like cell lines, consistent with an overall anabolic effect for this hormone.[16] However, GIP-Rs have also been identified on osteoclast cells where GIP was shown to dose dependently inhibit osteoclast resorptive activity.[16] Thus, it seems that GIP may have a dual effect to increase bone formation and decrease bone resorption. In addition, GIP secreting K-cells are ideally located in the upper small intestine to efficiently coordinate the transport of ingested minerals, such as calcium and phosphate, for storage in bone. As such, the activation of GIP-Rs on bone could be extremely beneficial for the treatment of osteoporosis or other similar bone disorders. In agreement, genetic knockout of GIP-R signaling leads to decreased bone formation, increased bone resorption, and significantly lower bone mineral density.[16] Thus there is provisional optimism that longer-acting GIP-R agonists, as detailed above for potential treatment of type-2 diabetes, could also have therapeutic promise for patients with bone disorders.

GIP and Brain

The widespread expression of GIP throughout the brain would suggest an important role for this hormone in the modulation of brain function. As such, in animal models, it has been shown that GIP induces the proliferation of adult-derived hippocampal progenitor cells.[16] In keeping with this, mice that have genetic knockout of the GIP-R or overexpression of GIP peptide display characteristic detrimental and beneficial effects on cognitive function; respectively.[16] Thus, long-acting GIP-R agonists may have additional therapeutic utility for neurodegenerative diseases such as Alzheimer's. Moreover, a long-acting GIP-R agonist has already been shown to possess direct modulating effects on synaptic transmission and enhance the induction of long-term potentiation in mice, a key physiological marker of memory processes.[16] GLP-1 also appears to have similar effects in the hippocampus of animal models.[21] As with effects of GIP analogs on diabetes, obesity and bone, extrapolation of these findings to the human setting is still required.

Summary of Pathological Implications of GIP

GIP-R agonists are suggested as potential therapeutic options for type 2 diabetes, bone disorders and neurodegenerative diseases of the brain. Besides this, research now suggests that GIP-R antagonists may afford an entirely new drug class for alleviation of obesity. Thus, modulation of GIP signaling offers an array of pathophysiological targets that warrant consideration as future therapeutics. However, lack clinical data on the biological effects of GIP analogs, both agonists and antagonists, makes the effectiveness, safety, and tolerability difficult to assess at present.

From the outside it would seem that developing GIP-based drugs that can activate or block the same GIP-R for therapeutic use in disease management may seem a counterintuitive proposition. However, a complete and rational understanding of the physiological and pathophysiological roles of GIP helps explain this paradigm (Fig. 2). Thus, GIP is essentially

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an anabolic hormone, released rapidly in response to feeding to stimulate insulin release and promote energy storage. GIP also possesses numerous insulin-like and insulin-enhancing effects in various tissue including bone and brain.[9] Under pathological conditions, such as overconsumption of energy rich diets in the Westernized World, the anabolic effect of GIP leads to adiposity, lipid deposition in tissue, and development of insulin resistance and subsequent obesity diabetes. Thus, annulment of GIP-R signaling under these circumstances promotes weight loss, improves insulin sensitivity, and counterbalances the hyperinsulinemia and beta-cell exhaust associated with obesity. Therefore, there is a clear rational for the potential use of GIP-R agonists and antagonists as therapeutic options for the treatment and management of a number of degenerative diseases (Fig. 2).

REFERENCES

1. Alaña I, Malthouse JP, O'Harte FP, Hewage CM. The bioactive conformation of glucose-dependent insulinotropic polypeptide by NMR and CD spectroscopy. *Proteins* 2007;**68**:92–9.
2. Almind K, Ambye L, Urhammer SA, Hansen T, Echwald SM, Holst JJ, et al. Discovery of amino acid variants in the human glucose-dependent insulinotropic polypeptide (GIP) receptor: the impact on the pancreatic beta cell responses and functional expression studies in Chinese hamster fibroblast cells. *Diabetologia* 1998;**41**:1194–8.
3. Brown JC, Dryburgh JR. A gastric inhibitory polypeptide. II. The complete amino acid sequence. *Can J Biochem* 1971;**49**:867–72.
4. de Herder WW, Hofland LJ, Usdin TB, de Jong FH, Uitterlinden P, van Koetsveld P, et al. Food-dependent Cushing's syndrome resulting from abundant expression of gastric inhibitory polypeptide receptors in adrenal adenoma cells. *J Clin Endocrinol Metab* 1996;**81**:3168–72.
5. Dupre J, Ross SA, Watson D, Brown JC. Stimulation of insulin secretion by gastric inhibitory polypeptide in man. *J Clin Endocrinol Metab* 1973;**37**:826–8.
6. Ebert R, Unger H, Creutzfeldt W. Preservation of incretin activity after removal of gastric inhibitory polypeptide (GIP) from rat gut extracts by immunoadsorption. *Diabetologia* 1983;**24**:449–54.
7. Ehses JA, Pelech SL, Pederson RA, McIntosh CH. Glucose-dependent insulinotropic polypeptide activates the Raf-Mek1/2-ERK1/2 module via a cyclic AMP/cAMP-dependent protein kinase/Rap1-mediated pathway. *J Biol Chem* 2002;**277**:37088–97.
8. Ehses JA, Casilla VR, Doty T, Pospisilik JA, Winter KD, Demuth HU, et al. Glucose-dependent insulinotropic polypeptide promotes beta-(INS-1) cell survival via cyclic adenosine monophosphate-mediated caspase-3 inhibition and regulation of p38 mitogen-activated protein kinase. *Endocrinology* 2003;**144**:4433–45.
9. Gault VA, O'Harte FP, Flatt PR. Glucose-dependent insulinotropic polypeptide (GIP): anti-diabetic and anti-obesity potential? *Neuropeptides* 2003;**37**:253–63.
10. Gault VA, Porter DW, Irwin N, Flatt PR. Comparison of sub-chronic metabolic effects of stable forms of naturally occurring GIP(1–30) and GIP(1–42) in high-fat fed mice. *J Endocrinol* 2011;**208**:265–71.
11. Gault VA, Kerr BD, Harriott P, Flatt PR. Administration of an acylated GLP-1 and GIP preparation provides added beneficial glucose-lowering and insulinotropic actions over single incretins in mice with type 2 diabetes and obesity. *Clin Sci (Lond)* 2011;**121**:107–17.
12. Gelling RW, Wheeler MB, Xue J, Gyomorey S, Nian C, Pederson RA, et al. Localization of the domains involved in ligand binding and activation of the glucose-dependent insulinotropic polypeptide receptor. *Endocrinology* 1997;**138**:2640–3.
13. Hinke SA, Manhart S, Speck M, Pederson RA, Demuth HU, McIntosh CH. In depth analysis of the N-terminal bioactive domain of gastric inhibitory polypeptide. *Life Sci* 2004;**75**:1857–70.
14. Inagaki N, Seino Y, Takeda J, Yano H, Yamada Y, Bell GI, et al. Gastric inhibitory polypeptide: structure and chromosomal localization of the human gene. *Mol Endocrinol* 1989;**3**:1014–21.
15. Irwin DM, Zhang T. Evolution of the vertebrate glucose-dependent insulinotropic polypeptide (GIP) gene. *Comp Biochem Physiol Part D Genomics Proteomics* 2006;**1**:385–95.
16. Irwin N, Gault V, Flatt PR. Therapeutic potential of the original incretin hormone glucose-dependent insulinotropic polypeptide: diabetes, obesity, osteoporosis and Alzheimer's disease? *Expert Opin Investig Drugs* 2010;**19**:1039–48.
17. Irwin N, Francis JM, Flatt PR. Alterations of glucose-dependent insulinotropic polypeptide (GIP) during cold acclimation. *Regul Pept* 2011;**167**:91–6.
18. Jörnvall H, Carlquist M, Kwauk S, Otte SC, McIntosh CH, Brown JC, et al. Amino acid sequence and heterogeneity of gastric inhibitory polypeptide (GIP). *FEBS Lett* 1981;**123**:205–10.
19. Kubota A, Yamada Y, Yasuda K, Someya Y, Ihara Y, Kagimoto S, et al. Gastric inhibitory polypeptide activates MAP kinase through the wortmannin-sensitive and -insensitive pathways. *Biochem Biophys Res Commun* 1997;**235**:171–5.
20. Lund PK. The discovery of glucagon-like peptide 1. *Regul Pept* 2005;**128**:93–6.
21. McIntosh CH, Widenmaier S, Kim SJ. Glucose-dependent insulinotropic polypeptide (Gastric Inhibitory Polypeptide; GIP). *Vitam Horm* 2009;**80**:409–71.
22. Meier JJ, Nauck MA. GIP as a potential therapeutic agent? *Horm Metab Res* 2004;**36**:859–66.
23. Messenger B, Clifford MN, Morgan LM. Glucose-dependent insulinotropic polypeptide and insulin-like immunoreactivity in saliva following sham-fed and swallowed meals. *J Endocrinol* 2003;**177**:407–12.
24. Mortensen K, Christensen LL, Holst JJ, Orskov C. GLP-1 and GIP are colocalized in a subset of endocrine cells in the small intestine. *Regul Pept* 2003;**114**:189–96.
25. Nauck MA, Bartels E, Orskov C, Ebert R, Creutzfeldt W. Additive insulinotropic effects of exogenous synthetic human gastric inhibitory polypeptide and glucagon-like peptide-1-(7-36) amide infused at near-physiological insulinotropic hormone and glucose concentrations. *J Clin Endocrinol Metab* 1993;**76**:912–7.
26. Nauck MA, Heimesaat MM, Orskov C, Holst JJ, Ebert R, Creutzfeldt W. Preserved incretin activity of glucagon-like peptide 1 [7-36 amide] but not of synthetic human gastric inhibitory polypeptide in patients with type-2 diabetes mellitus. *J Clin Invest* 1993;**91**:301–7.
27. Parthier C, Kleinschmidt M, Neumann P, Rudolph R, Manhart S, Schlenzig D, et al. Crystal structure of the incretin-bound extracellular domain of a G protein-coupled receptor. *Proc Natl Acad Sci U S A* 2007;**104**:13942–7.
28. Polak JA, Bloom SR. Localization of regulatory peptides in the gut. *Brit Med Bull* 1982;**38**:303–7.
29. Seino S, Shibasaki T. PKA-dependent and PKA-independent pathways for cAMP-regulated exocytosis. *Physiol Rev* 2005;**85**:1303–42.

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30. Straub SG, Sharp GWG. A wortmannin-sensitive signal transduction pathway is involved in the stimulation of insulin release by vasoactive intestinal polypeptide and pituitary adenylate cyclase-activating polypeptide. *J Biol Chem* 1996;**271**:1660–8.
31. Takeda J, Seino Y, Tanaka K, Fukumoto H, Kayano T, Takahashi H, et al. Sequence of an intestinal cDNA encoding human gastric inhibitory polypeptide precursor. *Proc Natl Acad Sci USA* 1987;**84**:7005–8.
32. Ugleholdt R, Poulsen ML, Holst PJ, Irminger JC, Orskov C, Pedersen J, et al. Prohormone convertase 1/3 is essential for processing of the glucose-dependent insulinotropic polypeptide precursor. *J Biol Chem* 2006;**28**:11050–7.
33. Unger RH, Eisentraut AM. Entero-insular axis. *Arch Intern Med* 1969;**123**:261–6.
34. Usdin TB, Mezey E, Button DC, Brownstein MJ, Bonner TI. Gastric inhibitory polypeptide receptor, a member of the secretin-vasoactive intestinal peptide receptor family, is widely distributed in peripheral organs and the brain. *Endocrinol* 1993;**133**:2861–70.

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Chapter 167

Ghrelin

Theo L. Peeters

ABSTRACT

Ghrelin is the endogenous ligand for the growth hormone secretagog receptor (GHS-R), but the peptide is most abundant in the stomach, where it is produced in the endocrine cells of the oxyntic mucosa. With motilin, ghrelin forms a new gut peptide family with profound effects on gastrointestinal function. Ghrelin affects gastric acid secretion, has gastroprotective effects, modulates inflammation, and increases gastrointestinal motility by inducing the migrating motor complex and accelerates gastric emptying. These effects result from the activation of central and peripheral ghrelin receptors. Ghrelin agonists have been developed for the treatment of hypomotility syndromes (postoperative ileus, gastroparesis). Some are in phase 2 clinical trials and may soon find clinical application.

DISCOVERY

Ghrelin was discovered in 1999 by Kojima et al.,[13] as the long sought ligand of what was until then known as the Growth Hormone Secretagog receptor (GHS-R) (see his chapter in the Brain Peptides section of the book). The fascinating history of the discovery of ghrelin and of the GHS-R has been reviewed many times. In relation to the effect of ghrelin on the gastrointestinal tract, it is of interest to note that 7 months before the discovery of ghrelin, a peptide with the same sequence was described, and named motilin-related peptide (MTRLP), because the authors noted a similarity with the sequence of motilin. This similarity had escaped the attention of Kojima and collaborators. Moreover, both groups overlooked the fact that their sequence had already been submitted under the name motilin homolog as part of a patent application (for a review of these developments see Ref.[24]). Because the sequences of MTRLP and motilin homolog were derived from cDNA sequences, they did not include the octanoylation of serine 3, which is an important determinant of biological activity of ghrelin. Therefore the true discovery of ghrelin was made by Kojima et al.[13] (also see his chapter of the octanoylation enzyme in the Peptide Biosynthesis/Processing section of the book).

It is now known that the desacylated ghrelin is also secreted and may have biological activities different of those of ghrelin. In more recent literature, ghrelin is therefore sometimes denoted as "acylated ghrelin," but this is a pleonasm. This review mainly concentrates on the acylated peptide, ghrelin. Des-acyl ghrelin and obestatin, another product of the ghrelin gene, are discussed in separate chapters.

STRUCTURE OF THE GENE/mRNA/PEPTIDE

The structure of the ghrelin gene, of the mature mRNA and of the processing of the translated preproghrelin(s) into the active peptide(s) is described in the section on Brain Peptides. As in most peptides, a signal peptide is first split off to produce proghrelin, which is then cleaved by a protein convertase into the active peptide and a fragment, from which obestatin is derived (see the chapter on obestatin in this section of the book).

For every step, there is a large similarity with motilin. The overall gene structure is similar, and the active peptides are encoded by two exons with the boundary between them at a corresponding residue (no. 14). The precursors of both peptides have the same arrangement and overall a large amino acid similarity. For example, to obestatin corresponds an almost identical sequence in the so-called "motilin associated peptide."

The acylation step, usually an octanoylation, is crucial in the synthesis of ghrelin. It precedes the convertase step and the enzyme for this step is named Ghrelin O-acyltransferase (GOAT).[34] In human plasma, a considerable amount of desacylated ghrelin is present, partially secreted as such, partially the result of enzymatic breakdown of ghrelin. The possible role of desacylated ghrelin has been the subject of conflicting reports. However, there is agreement that desacylated ghrelin does not activate the ghrelin receptor so that any role attributed to desacyl ghrelin assumes the existence of another as yet unknown receptor.

The amino acid sequence of ghrelin is well conserved, especially in the N-terminal part. For example, in seven mammalian species (human, monkey, cat, dog, pig, sheep,

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00167-6
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and cow) the first 10 residues are identical. As already noted, the sequence of ghrelin is related to the amino acid sequence of motilin where the N-terminus is also well conserved, although to a lesser extent than in ghrelin. In the same seven species, only the first six residues are identical in motilin. However, there is a paradox here. As already mentioned, a unique and crucially important feature of ghrelin is the octanoylation of serine-3. If the octanoyl group is removed, the potency drops dramatically: >2300-fold. But if the side chains of the other well-conserved amino acids are removed, the effect on potency is quite small, and the peptide can be shortened to an N-terminal fragment of only four residues without appreciable loss of biological activity. It has therefore been suggested that the evolutionary pressure may result from the enzyme responsible for the octanoylation of serine-3 requiring a large recognition site.[29] Available results do not support this hypothesis. Indeed the fact that GOAT is inhibited by a pentapeptide suggests that the recognition site is small. It may be noted that residue number 4 (Phe) of ghrelin, which aligns with residue number 5 of motilin, is identical in all species for which sequences are available.

DISTRIBUTION OF THE mRNA/PEPTIDE

As detailed in the article describing the discovery of ghrelin, the main source of ghrelin is the stomach. This applies to the peptide as well as to the mRNA. The drop in ghrelin plasma concentrations after gastrectomy and small bowel resection suggest that approximately two-thirds of the ghrelin circulating in the plasma is derived from the stomach and the remaining one-third from the small intestine.[4] Ghrelin is expressed along the whole gastrointestinal tract with decreasing concentrations from stomach to colon.[35] The distribution of ghrelin mRNA corresponds with the distribution of GOAT mRNA, suggesting that besides its endocrine effect, locally synthesized ghrelin may have paracrine effects.[17]

RECEPTORS AND SIGNALING CASCADES

The ghrelin receptor has been discussed in the section of Brain Peptides. Here, it may be noted that the ghrelin receptor belongs to a family of receptors including motilin, GPR39, neuromedin U, and neurotensin receptors, but that the motilin and ghrelin receptors are more similar to each other than to any other GPCR. Both are also encoded within two exons, with no untranslated exons, a peculiar feature. The high homology suggests a common ancestry of both receptors consistent with both sharing some GI functions. The interesting species differences in the effects of ghrelin and motolin—most remarkable are the absence of motilin effects in rodents, and the opposite motility effect of ghrelin in dog versus man (see below)—not only suggest different functional constraints acting during evolution but also question the translational value of animal models in drug development.[26]

In the ghrelin receptor, the activation domain involves transmembrane domain 2 (TM2) and TM3, and this domain has been remarkably conserved during >400 million years of evolution. Indeed, it is already present in related receptors in the pufferfish. In particular, glutamic acid residue 124 in TM2 of the GHS-R has a critical interaction with a positively charged nitrogen of ghrelin agonists.[9] In the motilin receptor, the corresponding residue may play a similar role,[32] suggesting the possibility of crossreactivity. However, no noticeable interaction between ghrelin and motilin agonists has been observed, except a weak interaction at high concentration of the motilide EM-574 with the ghrelin receptor.[26]

A peculiar feature of the ghrelin receptor is its high level of constitutional activity. It shares this property with two other members of the ghrelin receptor family, the neurotensin receptor 2 and the orphan receptor GPR39. By contrast, the motilin receptor does not have this property, which seems to be related to an aromatic cluster in the inner face of the extracellular ends of TMs VI and VII.[12] This constitutive activity of the ghrelin receptor depends on the G(q), phospholipase C pathway,[12] although ghrelin itself activates extracellular signal-regulated kinases 1/2 through a calcium-independent novel protein kinase C isoform.[16]

BIOLOGICAL ACTIONS WITHIN GASTROINTESTINAL TRACT

Gastric Acid Secretion

Because ghrelin is present in the oxyntic mucosa, a paracrine or endocrine effect on the cells present in the gastric wall and involved in the regulation of gastric acid secretion, enterochromaffin cells, parietal cells, and gastrin (G) cells, may be considered. However, although ghrelin may increase or inhibit gastric acid secretion, as yet no evidence for any direct effects within the gastrointestinal wall has been found. Instead, ghrelin's effects are vagally or centrally mediated. Indeed, the stimulation of gastric acid secretion after intravenous ghrelin is abolished by vagotomy and the inhibitory effect seen after icv administration is abolished by cysteamine, suggesting the involvement of a central somatostinergic pathway.[24] In contrast, the vagally mediated stimulatory effect involves the release of histamine and may be synergistic with the stimulatory effect of gastrin.[33]

Gastroprotection

The first report of a role of ghrelin in gastroprotection was obtained in a model of ethanol-induced gastric ulcers

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in rats. It was confirmed in the same model and was also found in models of stress-induced ulcers. The protective effect has been observed as well after intraperitoneal, subcutaneous, or icv administration, suggesting that the mechanism can be mediated via peripheral and central ghrelin receptors.[14,24] Ghrelin released in the stomach wall may affect neighboring cells or reach them via the bloodstream. Thus, it has been shown that ghrelin acts directly on enteric neurons to produce nitric oxide (NO) and blockade of NO synthesis blocks the protective effect. The protective effect involves prostaglandin synthesis because it is reduced by indomethacin or the selective COX-2 inhibitor rofecoxib. In agreement with these observations, ghrelin does not offer protection in indomethacin-induced ulcers. Immune cells may also be involved because ghrelin reduces the expression of the proinflammatory cytokine tumor necrosis factor (TNF)-α.[14] Exogenous ghrelin, or ghrelin produced in the stomach, may also reach the central nervous system via the bloodstream (see the chapters on ingestive peptides and the blood–brain barrier later in this book), or the ghrelin signal may be conveyed to the brain via vagal afferents. However, although there seems to be agreement that sensory denervation blocks the effect of ghrelin, vagotomy blocked the effect in one study but was without effect in another. The effect of exogenous ghrelin is blocked by a ghrelin antagonist, but the role of endogenous ghrelin has not been investigated, although it has been noted that mucosal expression of ghrelin and ghrelin plasma concentrations is increased in stress- and ethanol-induced ulcers [data reviewed in Ref. 24].

Intestinal Inflammation

The gastroprotective effects mentioned above and the potent anti-inflammatory effects of ghrelin observed *in vitro* through modulation of cytokine secretion by macrophages[30] suggest a potential beneficial effect in inflammatory bowel diseases. The increased ghrelin plasma concentrations in patients with inflammatory bowel disease and with celiac disease, and the rapid increase of plasma ghrelin in response to endotoxin injection in healthy humans,[8] may also be interpreted as endogenous reactions aimed at countering the inflammatory process. Accordingly, a beneficial effect of a bolus injection of ghrelin was reported in a mouse and rat model of TNBS colitis.[11,15] However, another study found an adverse effect after prolonged administration of ghrelin. Moreover, in the same study,[7] ghrelin KO mice were less susceptible to dextran induced colitis than were normal mice. It was suggested that the treatment schedule is at the basis of the different outcomes[7] or the use of DSS versus TNBS.[5] In view of the long-term administration planned in other therapeutic areas, this should be further investigated.

Gastrointestinal Motility

Ghrelin is structurally related to motilin, the peptide known (and named) for its almost exclusive effects on gastrointestinal motility. Ghrelin is also functionally related to motilin because the two most typical effects of motilin, induction of the migrating motor complex (MMC) and acceleration of gastric emptying have also been shown for ghrelin in humans and in rodents, but not in dogs.

In view of the potential therapeutic application, the effect on gastric emptying has received the most attention and is best documented. Several studies have shown acceleration of gastric emptying by ghrelin in humans[27] and in animal models of delayed gastric emptying [for an extensive review, see Ref. 6). However, this does not necessarily mean that ghrelin plays a physiological role in the regulation of gastric emptying. Indeed in ghrelin KO mice gastric emptying is normal[6] and in humans physiological doses of ghrelin, able to stimulate growth hormone release, have no effect of gastric emptying.[3]

As for the MMC, until the discovery of ghrelin, this phenomenon was almost exclusively studied in humans and in dogs, because in small animal models, such as rats and mice, motilin is without effect. It is now known that this is because rodents are natural KOs for motilin.[25] Ghrelin has opened new perspectives for the study of the MMC, because it has also an effect on the MMC in rats. Elegant studies using various routes of administration of ghrelin and ghrelin antagonists led to the conclusion that in rats the motor effects depend on the activation of central receptors, efferent and afferent vagal pathways.[10,23] In addition, peripheral effects may also be involved. Thus, ghrelin may activate the enteric nervous system. Indeed, studies have localized the ghrelin receptor in the myenteric plexus of guinea pig ileum and in human and rat stomach and colon. *In vitro* studies provide evidence for the activation of cholinergic pathways in rat and mouse preparations.[24]

In those species in which the MMC has been classically studied, humans and dogs, results are conflicting. In humans ghrelin induces MMCs of gastric origin and not related to the release of motilin.[6] However, in dogs, ghrelin does not induce the MMC.[22] By contrast, it has an inhibitory effect via an inhibition of the release of motilin. In physiological conditions, plasma motilin peaks are followed by ghrelin peaks, with the decline of motilin and the decline of contractile activity resulting from the inhibition of motilin by ghrelin.[20] These results offer a fascinating new insight into the regulation of fasting motor activity and require confirmation and further study.

PATHOPHYSIOLOGICAL IMPLICATIONS

The mechanisms controlling the release of ghrelin are poorly understood. The most important factor seems to

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be the nutritional state, but it is unclear as to how fasting increases ghrelin secretion and how a meal reduces it. Blood glucose may play a role and the contact of food components with the stomach wall may as well. Because of the effect of ghrelin on appetite and adiposity, ghrelin may play a role in the development of obesity. The possible role of ghrelin in obesity is discussed in the Brain Peptides and Ingestive Peptides sections.

In gastrointestinal pathophysiology plasma ghrelin has been reported to be increased in IBD, functional dyspepsia, celiac disease, after vagotomy and after Helicobacter eradication, but decreased in gastritis, gastric ulcer, gastrectomy, small bowel resection, and gastric bypass surgery.[8] However, a causal relationship between plasma ghrelin changes and the pathological condition is lacking, except that it seems logical that damage to or removal of the ghrelin producing mucosa may lead to a reduction of ghrelin production. This is what probably happens after *Helicobacter pylori* infection when expression of ghrelin and the number of ghrelin-producing cells decreases, factors that may contribute to the development of ulcers.[24] After eradication however, one study noted a rise of plasma ghrelin, but this was not confirmed in another study based on a larger group of patients. The discrepancy may be explained by the different topography (antrum or fundus, where ghrelin cells are located) and duration of the infection (reversible damage after a short infection, irreversible atrophy after long infection) in both studies. The issue may be important because it has been suggested that eradication of *H. pylori*, via its effect on plasma ghrelin, may contribute to the development of obesity and may also increase the risk of esophageal adenocarcinoma.[19]

Because ghrelin inhibits gastric accommodation[1] a possible role of ghrelin in functional dyspepsia has been explored by measuring plasma levels of ghrelin and desacyl ghrelin in patients in comparison with controls and in relation to gastric emptying, symptom score, and psychological factors. The results, recently summarized by Ogiso et al.,[21] are conflicting and contradictory. It seems safe to conclude that ghrelin does not play a major role in the pathophysiology of functional dyspepsia.

THERAPEUTIC POTENTIAL

The stimulating effects of ghrelin on gastrointestinal motility suggest a role for ghrelin agonists in the treatment of hypomotility syndromes. This potential has been confirmed in animal models of postoperative and septic ileus,[28] and in studies with patients with diabetic or idiopathic gastroparesis.[6]

Ghrelin agonists existed already before the discovery of ghrelin and were then known as Growth Hormone Secretagogues. An extensive review of the many compounds developed since the first discovery of such compounds (in 1977!) by the group of Bowers lists not fewer than 83 drug candidates or lead compounds.[18] Some of the older compounds, such as GHRP-6 and ipamorelin, are now reevaluated as prokinetics. Ipamorelin has been shown to be effective in a model of postoperative ileus and is in phase 2 development (Helsinn Pharmaceuticals). The same company lists a further unspecified compound, ST-1141, in phase 1 development for opioid-induced bowel dysfunction (http://www.helsinn.com). The most advanced seems to be ulimorelin, formerly known as TZP-101 (http://www.tranzyme.com), a molecule specifically developed as a ghrelin agonist. Promising results were obtained in a phase 2 trial with postoperative ileus patients and phase 3 trials have been initiated. Recently ulimorelin was also shown to be useful in patients with severe gastroparesis.[31] Ulimorelin has to be administered intravenously, which limits it therapeutic applications. Its successor, TZP-102 is an orally active compound developed by the same company and granted fast track status by the FDA. Ulimorelin and TZP-102 reduce symptoms, but prokinetic effects were not reported. Their benefit may therefore derive not only from enhanced motility. Therefore, ghrelin may find application in more complex conditions such as dyspepsia.[21]

Considering the high level of constitutive activity of the ghrelin receptor, inverse agonists may also find an application. Because of this phenomenon, not just ghrelin levels but also changes in the expression of the ghrelin receptor should be studied in pathological conditions.

The therapeutic target envisaged for ghrelin antagonists is the treatment of obesity. The fact these compounds delay gastric emptying only at high doses may be asset, as dysmotility side effects are unlikely to occur.[2]

REFERENCES

1. Ang D, Nicolai H, Vos R, Mimidis K, Akyuz F, Kindt S, et al. Influence of ghrelin on the gastric accommodation reflex and on meal-induced satiety in man. *Neurogastroenterol Motil* 2009;**21**:528–33.
2. Camilleri M, Papathanasopoulos A, Odunsi ST. Actions and therapeutic pathways of ghrelin for gastrointestinal disorders. *Nat Rev Gastroenterol Hepatol* 2009;**6**:343–52.
3. Cremonini F, Camilleri M, Vazquez Roque M, Mckinzie S, Burton D, Baxter K. Obesity does not increase effects of synthetic ghrelin on human gastric motor functions. *Gastroenterology* 2006;**131**:1431–9.
4. Cummings DE. Helicobacter pylori and ghrelin: interrelated players in body-weight regulation? *Am J Med* 2004;**117**:436–9.
5. Deboer MD. Use of ghrelin as a treatment for inflammatory bowel disease: mechanistic considerations. *Int J Pept* 2011:art id 189242.
6. De Smet B, Mitselos A, Depoortere I. Motilin and ghrelin as prokinetic drug targets. *Pharmacol Ther* 2009;**123**:207–23.
7. De Smet B, Thijs T, Moechars D, Colsoul B, Polders L, Ver Donck L, et al. Endogenous and exogenous ghrelin enhance the colonic and gastric manifestations of dextran sodium sulphate-induced colitis in mice. *Neurogastroenterol Motil* 2009;**21**:59–70.

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8. El-Salhy M. Ghrelin in gastrointestinal diseases and disorders: a possible role in the pathophysiology and clinical implications (review). *Int J Mol Med* 2009;**24**:727–32.
9. Feighner SD, Howard AD, Prendergast K, Palyha OC, Hreniuk DL, Nargund R, et al. Structural requirements for the activation of the human growth hormone secretagogue receptor by peptide and non-peptide secretagogues. *Mol Endocrinol* 1998;**12**:137–45.
10. Fujino K, Inui A, Asakawa A, Kihara N, Fujimura M, Fujimiya M. Ghrelin induces fasted motor activity of the gastrointestinal tract in conscious fed rats. *J Physiol* 2003;**550**:227–40.
11. Gonzalez-Rey E, Chorny A, Delgado M. Therapeutic action of ghrelin in a mouse model of colitis. *Gastroenterology* 2006;**130**:1707–20.
12. Holst B, Holliday ND, Bach A, Elling CE, Cox HM, Schwartz TW. Common structural basis for constitutive activity of the ghrelin receptor family. *J Biol Chem* 2004;**279**:53806–17.
13. Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H, Kangawa K. Ghrelin is a growth-hormone-releasing acylated peptide from stomach. *Nature* 1999;**402**:656–60.
14. Konturek PC, Brzozowski T, Pajdo R, Nikiforuk A, Kwiecien S, Harsch I, et al. Ghrelin—a new gastroprotective factor in gastric mucosa. *J Physiol Pharmacol* 2004;**55**:325–36.
15. Konturek PC, Brzozowski T, Engel M. Ghrelin ameliorates colonic inflammation. Role of nitric oxide and sensory nerves. *J Physiol Pharmacol* 2009;**60**:41–7.
16. Lau PN, Chow KB, Chan CB, Cheng CH, Wise H. The constitutive activity of the ghrelin receptor attenuates apoptosis via a protein kinase C-dependent pathway. *Mol Cell Endocrinol* 2009;**299**:232–9.
17. Lim CT, Kola B, Grossman A, Korbonits M. The expression of ghrelin O-acyltransferase (GOAT) in human tissues. *Endocr J,* in press. Epub ahead of print. PubMed PMID: 21646729.
18. Moulin A, Ryan J, Martinez J, Fehrentz J- A. Recent developments in ghrelin receptor ligands. *Chem Med Chem* 2007;**2**:1242–59.
19. Nwokolo CU, Freshwater DA, O'Hare P, Randeva HS. Plasma ghrelin following cure of *Helicobacter pylori*. *Gut* 2003;**52**:637–40.
20. Ogawa A, Mochiki E, Yanai M, Morita H, Toyomasu Y, Ogata K, et al. Interdigestive migrating contractions are coregulated by ghrelin and motilin in conscious dogs. *Am J Physiol* 2012;**302**:R233–43.
21. Ogiso K, Asakawa A, Amitani H, Inui A. Ghrelin: a gut hormonal basis of motility regulation and functional dyspepsia. *J Gastroenterol Hepatol* 2011(Suppl. 3):67–72.
22. Ohno T, Kamiyama Y, Aihara R, Nakabayashi T, Mochiki E, Asao T, et al. Ghrelin does not stimulate gastrointestinal motility and gastric emptying: an experimental study of conscious dogs. *Neurogastroenterol Motil* 2006;**18**:129–35.
23. Page AJ, Slattery JA, Milte C, Laker R, O'Donnell T, Dorian C. Ghrelin selectively reduces mechanosensitivity of upper gastrointestinal vagal afferents. *Am J Physiol Gastrointest Liver Physiol* 2007;**292**:G1376–84.
24. Peeters TL. Ghrelin: a new player in the regulation of gastrointestinal function. *Gut* 2005;**54**:1638–49.
25. Peeters TL, Aerssens J, De Smet B, Mitselos A, Thielemans L, Coulie B, et al. The mouse is a natural knock-out for motilin and for the motilin receptor. Functionally they have been replaced by ghrelin. *Neurogastroenterol Motil* 2004;**16**:687.
26. Sanger GJ, Holbrook JD, Andrews PLR. The translational value of rodent gastrointestinal functions: a cautionary tale. *Trends Pharmacol Sci* 2011;**32**:402–9.
27. Tack J, Depoortere I, Bisschops R, Verbeke K, Janssens J, Peeters T. Influence of ghrelin on gastric emptying and meal-related symptoms in gastroparesis. *Aliment Pharmacol Ther* 2005;**22**:847–53.
28. Trudel L, Tomasetto C, Rio MC, Bouin M, Plourde V, Eberling P, et al. Ghrelin/motilin-related peptide is a potent prokinetic to reverse gastric postoperative ileus in rat. *Am J Physiol Gastrointest Liver Physiol* 2002;**282**:G948–52.
29. Van Craenenbroeck M, Gregoire F, De Neef P, Robberecht P, Perret J. Ala-scan of ghrelin (1–14): interaction with the recombinant human ghrelin receptor. *Peptides* 2004;**25**:959–65.
30. Waseem T, Duxbury M, Ito H, Ashley SW, Robinson MK. Exogenous ghrelin modulates release of pro-inflammatory and anti-inflammatory cytokines in LPS-stimulated macrophages through distinct signalling pathway. *Surgery* 2008;**143**:334–42.
31. Wo JM, Ejskjaer N, Hellström PM, Malik RA, Pezzullo JC, Shaughnessy L, et al. Randomised clinical trial: ghrelin agonist TZP-101 relieves gastroparesis associated with severe nausea and vomiting—randomised clinical study subset data. *Aliment Pharmacol Ther* 2011;**33**:679–88.
32. Xu L, Depoortere I, Vertongen P, Waelbroeck M, Robberecht P, Peeters TL. Motilin and erythromycin-A share a common binding site in the third transmembrane segment of the motilin receptor. *Biochem Pharmacol* 2005;**70**:879–87.
33. Yakabi K, Kawashima J, Kato S. Ghrelin and gastric acid secretion. *World J Gastroenterol* 2008;**14**:6334–8.
34. Yang J, Brown MS, Liang G, Grishin NV, Goldstein JL. Identification of the acyltransferase that octanoylates ghrelin, an appetite-stimulating peptide hormone. *Cell* 2008;**132**:387–96.
35. Zhao Z, Sakai T. Characteristic features of ghrelin cells in the gastrointestinal tract and the regulation of stomach ghrelin expression and production. *World J Gastroenterol* 2008;**14**:6306–11.

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Chapter 168

GLP 1/2, Enteroglucagon, Glicentin, and Oxyntomodulin

Jens Juul Holst, Carolyn F. Deacon Bolette Hartmann and Jens Pedersen

ABSTRACT

The glucagon gene is expressed not only in the pancreas but also in endocrine cells of the gut and in neurons of the brain stem. Here, the precursor, proglucagon, gives rise to the peptides glicentin, oxyntomodulin, and glucagon-like peptides 1 and 2 (GLP-1 and 2). Although the physiological role of the former is unclear, the GLPs seem to be important hormones, GLP-1 as an incretin hormone stimulating insulin release and as an enterogastrone, inhibiting appetite and gastrointestinal secretion and motility, and GLP-2 as an intestinotropic factor regulating gut mucosal growth. GLP-1 has shown great promise as a therapeutic for type 2 diabetes, and GLP-1 agonists entered the market in 2005. Inhibitors of the GLP-1-degrading enzyme, dipeptidyl peptidase-4, which increase the levels of endogenous GLP-1, are also available for diabetes therapy. GLP-2 is currently being investigated for treatment of intestinal failure (approved for short bowel syndrome in 2012), whereas oxyntomodulin seems to have appetite-reducing effects.

DISCOVERY, STRUCTURE OF THE GENE, AND PROCESSING OF THE PRECURSOR

It has been known for a long time that the gastrointestinal mucosa contains endocrine cells producing substances with glucagon-like immunoreactivity. The immunoreactivity turned out to result from two peptides containing the entire glucagon sequence of 29 amino acids. One, glicentin, consists of 69 amino acids of which residues no. 33–61 constitutes the glucagon sequence. The other, oxyntomodulin, is a fragment of glicentin, corresponding to amino acids no. 33–69, that is, glucagon + 8 additional amino acids at the C-terminus. Collectively, the two molecules were designated "enteroglucagon" or gut GLI. It was hypothesized that glicentin was part of proglucagon and in agreement with this, the N-terminal fragment corresponding to residues no 1–30 of glicentin, glicentin-related pancreatic polypeptide (GRPP), is secreted in parallel with glucagon. Proglucagon cDNA was first cloned from anglerfish islets and encoded a glucagon-containing glicentin-like peptide plus an additional glucagon-like peptide showing strong sequence homology with (anglerfish) glucagon. On cloning of the mammalian and human proglucagon genes, it was clear that they encode yet another glucagon-like sequence, glucagon-like peptide 2.[1] Further studies revealed that (1) only a single gene encodes proglucagon; (2) identical mRNAs are formed in mammalian species; (3) the gene is expressed in the gut, the pancreas and in the brain; (4) the single, identical precursor, proglucagon, is processed differentially in the different tissues so that different products are secreted from the different organs.[24] Thus, in the pancreas, proglucagon (PG), consisting of 160 amino acids, is cleaved to produce GRPP (PG 1–30), glucagon (PG 33–61) and "Major ProGlucagon Fragment" corresponding to PG 72–158. The three molecules are secreted in parallel and in equimolar amounts.[12] In the gut, the products are glicentin (PG 1–69, part of which is cleaved further to produce GRPP (PG 1–30) and oxyntomodulin (PG 33–69)) and each of the two glucagon-like peptides plus the so-called intervening peptide 2 (corresponding to PG 111–123). Human GLP-1 and 2 correspond to PG 78–107 amide (residue no 108 being lost in the amidation step) and PG 126–158, respectively.[11] In rodents, about half of the GLP-1 appears in the Gly-extended form, GLP-1 7–37. In pigs where proglucagon does not terminate in basic amino acid residues, the fully processed GLP-2 molecule corresponds to PG 126–160. Human GLP-2 corresponds to PG 126–158.[9] Gut processing is catalyzed by the prohormone convertase PC-1/3 (but PC2 in the pancreas) (see the chapters on these enzymes in the Peptide Biosynthesis/Processing section of the book). The fully processed form, now designated GLP-1 (7–36) amide or simply GLP-1, was isolated on the basis of its potent insulinotropic activity.[14] The processing pattern in the brain stem resembles that of the gut. Mutations in PC-1/3 lead to abnormalities in GLP-1 processing and secretion, associated with multiple endocrinopathies (undoubtedly because PC-1/3 is important for processing of many regulatory peptides/hormones), and mice with a

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00168-8
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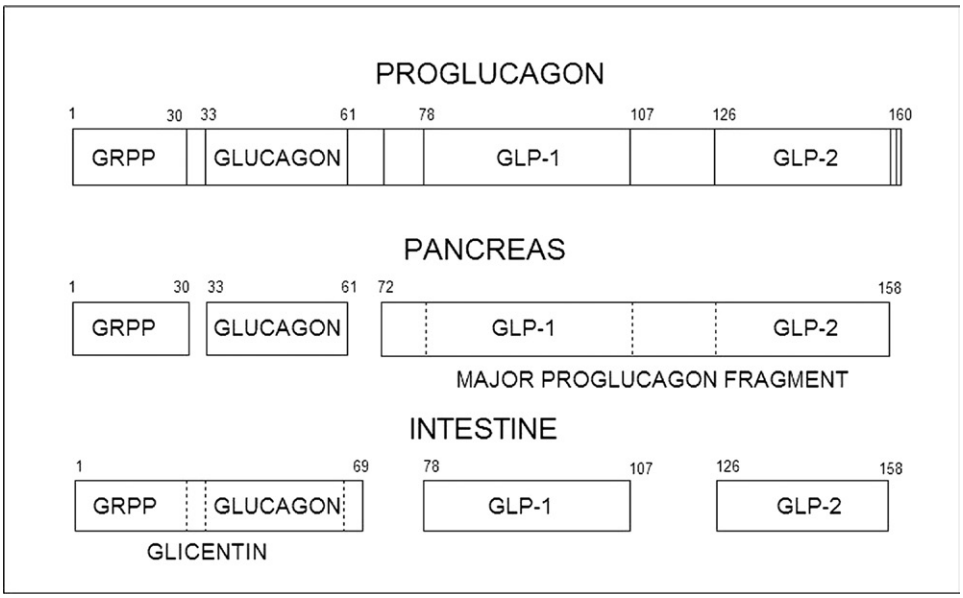

FIGURE 1 Proglucagon processing. Schematic representation of the differential processing of proglucagon as it occurs in the pancreatic islets and in the intestinal mucosa in man. The vertical lines in proglucagon (PG) indicate the positions of the cleavage sites (basic amino acids). GRPP, glicentin-related pancreatic peptide; IP-1, intervening peptide 1; IP-2, intervening peptide 2. Note that in the pancreas, the main products are glucagon itself and GRPP (inactive) and major proglucagon fragment (inactive). Small amounts of N-terminally extended GLP-1 (inactive) may also be formed. In the gut, the main products are glicentin, in which the glucagon sequence is buried (whereby glucagon-like bioactivity is lost); smaller amounts of oxyntomodulin and the same inactive N-terminal fragment as in the pancreas; GLP-1, the majority of which is amidated, whereas the rest is extended by one glycine residue; and GLP-2, which functions as a growth factor for the gut. Thus, although the precursor is the same, the differential processing gives rise to three biologically active, but completely different products in the two tissues, whereas terminal extensions inactivate the "other" active products.

targeted deletion of the PC-1/3 gene are unable to process proglucagon to GLP-2 and GLP-1. The processing is shown diagrammatically in Fig. 1.

EXPRESSION OF THE GENE/DISTRIBUTION OF THE GENE/SECRETION

Regulation of Gene Expression

At least 6 homeodomain proteins are able to interact with the glucagon gene promoter and activate its expression.[16] Thus, the transcription factor, pax6, is expressed in intestinal endocrine cells and activates proglucagon transcription. Mice with a dominant negative pax6 mutation exhibit a total absence of gut endocrine cells expressing GLP-1 or GLP-2. Disruption of the pax6 gene disrupts both islet development and selectively eliminates the endocrine cell population of the small and large bowel, including the proglucagon-producing cells. Beta-catenin, the major effector of the Wnt signaling pathway, activates proglucagon expression in intestinal cells, mediated by the transcription factor TCF-4 (now TCF7L2). Both TCF7L2 and beta-catenin bind to the proglucagon gene promoter, and a dominant negative TCF7L2 repressed proglucagon expression in an intestinal cell line that expresses proglucagon and produces GLP-1. Approximately 1250 nucleotides of the rat proglucagon gene promoter direct the expression to pancreatic islets and neurons of the brain, whereas additional upstream sequences extending to −2,250 are required for the expression of the gene in intestinal endocrine cells. However, the mechanisms regulating tissue-specific proglucagon gene expression in humans seem to differ from those described for the murine gene, and unfortunately, little is known about the regulation of the human gene. A more recent study identified conserved sequences within intron 1 of the human proglucagon gene as being important for the pancreatic expression.

The TCF7L2 mediated regulation of proglucagon expression in the gut is of considerable interest in view of the recent demonstration of a genetic association of a specific single nucleotide polymorphism (SNP), the microsatellite DG10S478 in the intron 3 of the TCF7L2 gene, and the development of type 2 diabetes. This raises the possibility that this SNP influences disease susceptibility through modulation of intestinal proglucagon gene expression and hence possibly plasma concentrations of GLP-1. However, GLP-1 levels seem to be unaffected in the high-risk group, whereas the insulinotropic actions of the incretin hormones are impaired, supporting a role of TCF7L2 for beta cell function.[28] Indeed, decreased incretin receptor levels correlate with beta cell TCF7L2 expression in diabetic models.

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The proglucagon gene in the gut is expressed in the endocrine alpha cells of the pancreatic islets and in the so-called L-cells, open-type endocrine cells of the gastrointestinal mucosa.[4] In some species (dogs, cats, and rats), there are A-like cells in the gastric mucosa, which may produce fully processed glucagon (and this probably explains the finding of glucagon-like bioactivity in stomach extracts in 1948), but such cells are rare in humans (and pigs and rats). In humans, the L-cells are lacking in the duodenum but appear with increasing frequency from the proximal jejunum toward the ileum. A large number of L-cells are also found in the colon, particularly in the cecum and the rectum. Their function in the colon is unknown. The distal L-cells also produce peptide YY, whereas the proximal GLP-1-producing cells also produce glucose-dependent insulinotropic polypeptide (GIP).[21] Most recently, Gribble and Reiman (Minkowsky lecture, EASD 2010) succeeded in isolating primary GLP-1-producing cells from mice expressing a fluorophore under the control of the proglucagon promoter.[26] Their studies clearly show coexpression and even differential coexpression of several bioactive peptides, including neurotensin, somatostatin, and cholecystokin, in the proglucagon-expressing cells. A study using diphtheria toxin-induced knockdown of cells with high expression levels of the glucagon promoter, similarly showed dramatic reductions in the expression of neurotensin, CCK, and PYY in the ileal mucosa, whereas only the expression of CCK and neurotensin are reduced in the proximal jejunum. Therefore, the endocrine cells of the intestinal mucosa show great plasticity and are capable of changing the expression pattern. Local factors may be important, and perhaps maturation and migration along the villous axis. Clearly, speaking of specific cell types, as for instance the L-cells, is no longer tenable.

Secretion

The L-cells have microvilli protruding into the intestinal lumen, and because they secrete on luminal nutrient stimulation, it is assumed that molecules in the villous membranes react to these and activate the secretory machinery.[11] Thus, in an L-cell-like cell line, glucose may activate GLP-1 secretion both in a K_{ATP}-channel-dependent manner by enhancing glucose metabolism but also after intracellular transfer via the sodium dependent glucose transporter-1 (SGLT-1). Thus, a large part of the GLP-1 response to glucose (and therefore most carbohydrates) can be blocked by the inhibitors of SGLT-1. Fructose also stimulates GLP-1 secretion, consistent with the finding that L-cells also express GLUT-5, the fructose transporter. Research using primary proglucagon-expressing cells has confirmed these results.[26] Natural L-cells are now known to express several G protein-coupled receptors that have been associated with lipid ligands including GPR 119, 120, and 131 (aka TRG5, activated by bile acids), and activation of these is associated with GLP-1 secretion both *in vitro* and *in vivo*.[26] The GPR 119 receptor can be activated by 2-monoacylglycerols, which are formed in huge quantities in the gut during normal digestion, both *in vitro* and *in vivo* in humans; this mechanism may explain a large part of the stimulatory actions of ingested lipids on GLP-1 secretion.[7] Although direct studies of L-cell products other than GLP-1 are lacking, it may be assumed that similar patterns of secretion are seen for oxyntomodulin and GLP-2. Thus a picture is emerging where these cells react to luminal factors mainly by the activation of membrane proteins, which then trigger intracellular signaling mechanisms eventually leading to exocysis of processed proglucagon products.

Because of the presence of L-cells in the upper jejunum, secretion is increased rapidly (within 10–15 min on meal ingestion) and, in agreement with the increasing density of L-cells distally, larger meals cause larger responses.[11] Likewise, the responses correlate strongly with the gastric emptying rate. Hormonal mechanisms do not seem to participate at least in pigs, where this has been studied in detail, and although secretion may be influenced by intramural enteric nervous activity, efferent vagal fibers do not stimulate secretion. The sympathetic nerve supply may inhibit secretion, and GLP-1 itself (but not the other proglucagon products) powerfully inhibits L-cell secretion, presumably by the activation of neighboring D-cells releasing somatostatin-28 that acts on the L-cells via sstr 5 receptors.[11]

ACTIVE CONFORMATION AND METABOLISM

Each of the processed products from the pancreas and gut may be found in the circulation where their concentrations reflect their rates of secretion and elimination. Elimination mainly occurs is the kidneys, which extract up to 70 % of GLP-1, greatly exceeding the amount of GLP-1 than can be cleared by glomerular filtration. Thus, the half-lives of oxyntomodulin, glucagon, and GLP-2 are around 6–7 min, whereas that of glicentin is approximately 20 min. No details are known about the metabolism of glicentin and oxyntomodulin in the circulation, but glucagon is subject to some degradation by neutral endopeptidase 24.11. GLP-1 and GLP-2 (but not glucagon) are both substrates for the ubiquitous enzyme, dipeptidyl peptidase-4 (DPP-4), which cleaves off the two N-terminal residues (His-Ala in both peptides) whereby the molecules are rendered inactive.[12] This results in a half-life for GLP-2 of 7 min as mentioned, whereas that of the metabolite, GLP-2 3–33, is around 27 min. GLP-1 is more extensively cleaved by DPP-4, with a plasma half-life of 1.5–2 min and a clearance of 6–10 L of plasma per min, that is, greatly exceeding cardiac output.

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This means that the peptide is degraded rapidly in the circulation and that a steady state is not reached. In fact, the peptide is degraded already as it leaves the endocrine organ, that is, the gut. GLP-1 is stored in the L-cells in intact form, but approximately 75% is already cleaved to GLP-1 9–36 amide as it enters the portal vein draining the gut. Because DPP-4 is localized to the endothelial surface of the capillaries, it is believed that the intact molecule on exocytosis from the L-cells diffuses across the basal lamina and into the lamina propria, where it is taken up by a capillary and degraded.[8] This means that only about 25% reaches the portal circulation in intact form. In the liver, half of the arriving GLP-1 is degraded further so that only 10–15% of that secreted makes it to the systemic circulation and thereby the pancreatic islets. The metabolite, which has a half-life around 4 min, is eliminated mainly in the kidneys. Also, GLP-1 may be degraded by neutral endopeptidase 24.11, although less extensively.

RECEPTOR EXPRESSION

The glucagon receptor is discussed in the glucagon chapter of the Ingestive Peptides section of the book. In agreement with its structure, oxyntomodulin may activate the glucagon receptor, but its potency is only approximately 1/50 of that of glucagon. GLP-1 may also interact with the glucagon receptor but with only about 1/100 of the potency of glucagon. Oxyntomodulin may also interact with the GLP-1 receptor, although less potently than GLP-1.

Glicentin has no known specific receptor and apparently does not interact with any of the other receptors of the family. The GLP-1 receptor was cloned by expression cloning[31] and is related to the glucagon receptor and to receptors for GLP-2, and to the receptors for the other members of the glucagon-secretin family of peptides.[19] It has 7 transmembrane domains, a large N-terminally extracellular domain, and a much smaller C-terminal intracellular domain. It is expressed in kidneys, lung, heart, hypothalamus, hippocampus, and of course in the β-cells in the islet of Langerhans. Interestingly, expression of the receptor has also been found in the nodose ganglion, in specific cells of the immune system, and also in the skin.

In vitro studies have shown that the receptor is desensitized by phosphorylations of serine residues on the cytoplasmatic tail, which also leads to internalization of the receptor. However, in rats, there is no reduction in GLP-1-mediated responses after 1 week of treatment with a GLP-1r agonist, and in humans with type 2 diabetes, the antihyperglycemic effect is unimpaired after 7 days of continuous intravenous infusion or 6 weeks' continuous subcutaneous infusion, indicating that GLP-1 receptor desensitization is of little physiological relevance. Mutagenesis based screening studies reveal that the third intracellular loop of the receptor is involved with coupling of the hetero-trimeric G protein and the coupling with both $G_s\alpha$ and $G_i\alpha$ has later been confirmed with the use of isolated cell membranes. Further *in vitro* studies established a coupling between GLP-1r and $G_q\alpha$. GLP-1r signaling is partly mediated by coupling to β-arrestin-1, and this coupling does not seems to mediate desensitization or internalization of the receptor. Augmentation of glucose-induced insulin secretion subsequent to the activation of the GLP-1r is mediated by cAMP/PKA and cAMP/EPAC2 pathways. Inhibition of apoptosis is mediated by PKA and PI3 K pathways; for instance, tacrolimus-induced cell death in isolated human islets is inhibited by GLP-1 signaling through PI3 K/PKB pathways. Further studies involving cultures of isolated human islets in monolayer have shown that GLP-1-induced cell proliferation is dependent on PKA, PI3 K and mitogen-activated kinases. Song et al. reported that phosphorylation of snapin is an essential step in downstream actions of PKA activation.

The GLP-1 receptor is also expressed in the alpha cells, but "how much" is a highly controversial issue, although the subject is important in view of the powerful and clinically highly relevant inhibitory effect of GLP-1 on glucagon secretion. The receptor is also expressed in the gastric mucosa, perhaps particularly by the parietal cells, and in the lungs where type II pneumocytes have a dense expression. Hepatocytes do not normally express GLP-1 receptors but may do so during development and under pathological conditions, and other cells, perhaps associated with the portal vessels also express the GLP-1 receptor. GLP-1-R (and GLP-2R) mRNA occurs in cells of the nodose ganglion, suggesting that sensory vagal neurons may express the GLP-1 receptors. Receptors have also been localized in the subfornical organ and in the area postrema, areas with leaks of the blood–brain barrier, and GLP-1 injected into the peripheral circulation can access these circumventricular organs suggesting that peripherally secreted GLP-1 may influence the CNS via this pathway. Quantification by the highly sensitive multiple-time regression analysis of blood to brain influx indicates entry into brain parenchyma elsewhere as well.

The GLP-2 receptor (GLP-2R) was cloned in 1999, and is related to the GLP-1 receptor.[22] It is expressed throughout the gastrointestinal tract[22] and in pancreatic alpha cells. The localization in the gut, however, is highly controversial, with some studies showing expression in enteroendocrine cells throughout the gastrointestinal tract, whereas other studies showed immunoreactivity in subepithelial myofibroblasts (SEM) just beneath the basement membrane.[23] In addition, different groups have reported that the receptor is localized to neurons in both Auerbach and Meissner's plexus of the intestine. Expression analysis of isolated epithelium, mucosa, external muscle coat, and enteric ganglia from rat jejunum was negative in the epithelium, but the receptor

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was apparently expressed in enteric ganglia. Expression has also been noted in the nodose ganglion and scattered in the epithelium of a human cervical adenocarcinoma.

Like other class II 7 transmembrane receptors, the GLP-2R undergoes desensitization and internalization on stimulation. The physiological relevance of desensitization and internalization is unclear, however; thus, increased cellularity of the intestine occurs after 7 days of GLP-2 stimulation, and the effect is sustained after another 11 days of stimulation. The GLP-2-mediated effects are reversed 11 days after cessation of GLP-2 stimulation. In people with GLP-2 producing tumors, a growth effect on the small intestine continues until intestinal obstruction develops.

Downstream signaling from the GLP-2R involves accumulation of $[cAMP]_i$ and thereby activation of PKA.[22] However, GLP-2 seems to counteract induced apoptosis by the inhibition of caspases 3, 8, and 9 in a cAMP dependent but PKA and PI3 K independent manner in transfected BHK cells. Phosphorylation of MAPK ERK1/2 is seen after GLP-2R activation in different *in vitro* cell models but not in BHK cells. In primary intestinal cell cultures phosphorylation of ERK1/2 is not induced by GLP-2 stimulation, but jejunum isolated from GLP-2 stimulated mice shows induction of ERK1/2. GLP-2R stimulation was coupled to $G_i\alpha$ in Hela cells. In agreement with this, the cAMP accumulation after GLP-2 stimulation of primary cultures of rat small intestine shows a polynomial appearance, indicating that cAMP induction is inhibited at higher concentration of GLP-2. *In vivo*, parenterally fed piglets infused with GLP-2 dose dependently show activation of protein kinase B (PKB) and inactivation of proapoptotic glycogen-synthase kinase 3 and caspase 3, and upregulation of the prosurvival gene B-cell CLL lymphoma 2 (Bcl2). Stimulation of mice with GLP-2 induces epidermal growth factor receptor family (ErbB) ligands, while effects of GLP-2 are blocked by a pan-ErbB antagonist.[35]

BIOLOGICAL ACTIONS

Glicentin has been reported to influence gastric acid secretion and single reports on growth effects and other effects have been published, but most investigators have found the peptide to be inactive. Thus, in unpublished studies from the authors' laboratory, synthetic glicentin is without effects on gastric acid secretion and insulin secretion in humans. Oxyntomodulin mimics most of the actions of glucagon but is less potent and probably does not act as a glucagon-like agonist physiologically. Like glucagon, it inhibits gastric acid secretion, but by an action that seems to be mediated by GLP-1 receptors (although their exact location is uncertain). However, it is doubtful whether the circulating concentrations of oxyntomodulin under normal circumstances reach sufficient levels to activate the receptors. Intracerebrovascular administration of oxyntomodulin powerfully inhibits food intake in rats by actions that do not seem to be mediated via the GLP-1 receptor. In agreement with the gut-like processing pattern of proglucagon in the proglucagon-producing neurons in the nucleus of the solitary tract,[17] it is possible that oxyntomodulin released from the fibers arising from the proglucagon-producing neurons in the nucleus of the solitary tract may contribute to the inhibitory effect on appetite and food intake apparently resulting from the activation of these neurons, together with GLP-1 and GLP-2. Administration of oxyntomodulin to humans is associated with strong inhibition of appetite and food intake and associated with weight loss. Studies of chimeric proteins with both GLP-1 and glucagon activity show dramatic efficacy in terms of inhibiting appetite and food intake without upsetting blood glucose regulation.[2] Therefore such "dual-agonists" and/or oxyntomodulin-like peptides are currently studied intensely. As dealt with elsewhere, glucagon itself is a surprisingly potent appetite suppressant (see the glucagon chapter: in Ingestive Peptides Section of the book).

ACTIONS OF GLP-1

The most conspicuous effect of GLP-1 is the stimulation of glucose-induced insulin secretion.[11] GLP-1 one of the incretin hormones, the gut hormones that augment insulin secretion on oral as opposed to intravenous administration of glucose. Together, GLP-1 and GIP are responsible for the incretin effect, which may account for as much as 70% of the insulin secreted after carbohydrate intake. Indeed, glucose tolerance and insulin secretion are impaired in mice with the deletions of both the GLP-1 and the GIP receptor and more so in animals with knockout of both receptors. However, GLP-1 has numerous other actions, and its physiologically most important role may be to act as an enterogastrone hormone, inhibiting gastrointestinal motility and secretion, reducing appetite and food intake, and to enhance peripheral deposition of already absorbed nutrients by stimulating insulin secretion.

Effects on the Islets

GLP-1 is strongly insulinotropic and mice with a targeted deletion of the GLP-1 receptor become glucose intolerant and may develop fasting hyperglycaemia.[29] In humans, administration of the GLP-1 receptor antagonist, exendin 9–39, reduces the insulin responses to enteral glucose.[22] The insulinotropic activity of GLP-1, which is strictly glucose-dependent, is exerted via interaction with the GLP-1 receptor located on the cell membrane of the ß-cells.[19] GLP-1 stimulates all steps of insulin biosynthesis and insulin gene transcription, thereby providing continued and augmented supplies of insulin for secretion. Activation of PDX-1, a key

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regulator of islet growth and insulin gene transcription may be involved. In addition, GLP-1 upregulates the genes for the cellular machinery involved in insulin secretion, such as the glucokinase and GLUT-2 genes. GLP-1 also has trophic effects on ß-cells. Not only does it stimulate ß-cell proliferation but it also enhances the differentiation of new ß-cells from progenitor cells in the pancreatic duct epithelium. In addition, GLP-1 is capable of inhibiting apoptosis of beta cells including human beta cells. The complicated intracellular mechanisms whereby GLP-1 may exert these effects on the beta cells are reviewed elsewhere.[5]

GLP-1 also strongly inhibits glucagon secretion from the alpha cells. The mechanism is still unclear but may involve interaction with the alpha cells directly (although in isolated alpha cells secretion has been reported to be stimulated by GLP-1) or a paracrine action by neighboring D-cells whose secretion of somatostatin-14 is potently stimulated by GLP-1. The intraislet hypothesis states that it is GLP-1 stimulated insulin that inhibits glucagon secretion in a paracrine manner. This hypothesis fails to explain the powerful inhibitory actions of GLP-1 in C-peptide negative patients with type 1 diabetes.

Effects on the Gastrointestinal Tract

GLP-1 inhibits gastrointestinal secretion and motility, notably gastric emptying. Physiologically, these effects of GLP-1 may be more important than the insulinotropic activity. When GLP-1 is infused intravenously during ingestion of a meal, the insulin responses are diminished dose dependently, rather than being enhanced. At the same time, gastric emptying is progressively retarded, so that the explanation of the reduced insulin secretion is the reduced gastric emptying of and reduced subsequent absorption of insulinotropic nutrients. Thus, the physiological role of GLP-1 may be to adjust the delivery of chyme to the digestive and absorptive capacity of the gut by retarding propulsion and digestion of the gastric contents. Thus, the GLP-1 receptor antagonist, exendin 9–39 increases fasting antral motility and meal-induced gastric and duodenal motility and decreases pyloric tone. This strongly suggests that endogenous GLP-1 is a physiological regulator of antroduodenal motility. Studies using the same antagonist confirm the physiological nature of the inhibitory effect of postprandial GLP-1 secretion on gastric emptying. The mechanism whereby GLP-1 exerts these actions seems to involve the nervous system. As described above, GLP-1 is degraded extensively in the gut before it reaches the systemic circulation. This has led to the hypothesis that GLP-1 must act locally in the lamina propria before it gets degraded. Thus, on its way to the capillary, while still in the intact form, GLP-1 may interact with afferent sensory nerve fibers arising from the nodose ganglion, causing them to send impulses to the nucleus of the solitary tract. From here, motor nuclei of the vagus or fibers projecting to brain, notably in the hypothalamus, may be activated directly. Exogenous GLP-1 may increase impulse activity in afferent vagus. These impulses may be reflexively transmitted to the pancreas. Studies with ganglionic blockers show that even the insulin response to intraportal administration of GLP-1 and glucose may be reduced to that elicited by glucose alone after ganglionic blockade. Thus, regarding the mechanism of GLP-1 stimulated insulin secretion under physiological circumstances, the neural pathway may be more important than the endocrine route. However, the concentration of intact GLP-1 does increase after meal intake and increases the larger the meal is. Thus, it is possible that the endocrine route becomes more prominent after extensive L-cell stimulation. Also, this inhibition of gastric motility, acid secretion, and emptying seems to involve regulation of efferent vagal activity subsequent to activation of vagal sensory afferents.

Effects on Appetite and Food Intake

Nutrients in the ileum have a satiating effect, curtailing food intake and also cause a release of GLP-1. Infusions of slightly supraphysiological amounts of GLP-1 significantly enhance satiety and reduce food intake in both normal subjects, obese subjects as well as in subjects with type 2 diabetes. Intraperitoneal administration of the GLP-1 antagonist, exendin 9–39 significantly augments food intake, and also strongly reduces the inhibitory effect of a preload of food.[33] A new small molecule antagonist of GLP-1 that can be used more chronically reduces food intake and leads to an increase in weight in a week-long study in rats, indicating that also endogenous GLP-1 regulates food intake. Direct injections of GLP-1 into the cerebral ventricles also inhibit food intake. Here, the peptide presumably interacts with cerebral GLP-1-receptors that are targets for GLP-1 released from nerve fibers ascending from cell bodies in the nucleus of the solitary tract in the brain stem. The question arises whether these neurons are linked to meal-induced satiety. Williams et al.[33] showed with either central (icv) or peripheral (ip) administration of GLP-1 with or without exendin 9–39 that the peripheral effect of GLP-1 is uninfluenced by a central antagonist, while the icv effects of GLP-1 are the same after peripheral blockade. The peripheral and the central GLP-1 systems, therefore, do not seem to require interaction with each other. The mechanism whereby peripheral (intestinal) GLP-1 reduces food intake remains unsettled, but studies indicate that transmission occurs via both vagal sensory afferents and pathways involving central nervous structures, perhaps including the area postrema.[25] Although GLP-1 receptor knockout mice do not become obese,[29] this may reflect the redundancy of the appetite regulating mechanisms rather than ineffectiveness of the signal.

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Other Actions

The cardiovascular effects of GLP-1 were recently reviewed.[30] Intracerebroventricular GLP-1 administration is associated with improved learning in rats and also displays neuroprotective effects. The neuroprotective effects of GLP-1 were recently reviewed.[13] The functions of GLP-1 in the lungs are unclear, although it has been reported to increase the secretion of macromolecules from the neuroendocrine cells.

ACTIONS OF GLP-2

These remained elusive for some time until Drucker et al.[6] observed mucosal hypertrophy in mice carrying proglucagon-producing tumors with an intestinal processing pattern. In subsequent studies of the effects of injected proglucagon products, GLP-2 was found to be capable of producing a similar mucosal growth. GLP-2 presumably activates its receptor on either neuronal cells or subepithelial myofibroblast which in turn produce growth factors that cause mucosal growth. GLP-2 enhances enterocyte proliferation and inhibits enterocyte apoptosis and thereby produces a pronounced growth of the epithelium. In addition, it may increase the expression of nutrient transporters and thereby augment the absorptive capacity of the gut. GLP-2 is currently believed to be an important regulatory factor for an adaptive intestinal growth as illustrated by the blunted intestinal adaptation to refeeding after prolonged fasting in the GLP-2 receptor knockout mouse. Further, modulation of endogenous GLP-2 signaling by immunoneutralization attenuates the adaptive growth of rest ileum after a 75% midsmall intestinal resection and reanastomosis, and immunoneutralization of GLP-2 in streptozotocin treated rats impairs intestinal growth after streptozotocin treatment. GLP-2 also powerfully increases intestinal blood flow. The mechanism is unknown, but it may be related to an increased intestinal metabolic rate. GLP-2 may also act as an enterogastrone hormone. Thus, GLP-2 inhibits sham-feeding induced acid secretion and may inhibit gastric emptying, but it is not as potent as GLP-1. GLP-2 treatment attenuates the inflammatory response in rats after chemically induced colitis by a pathway involving VIP positive enteric neurons, and the anti-inflammatory effects of GLP-2 are reproduced in interleukin 10 knockout mice, a model of chronic colitis. The gastrointestinal effects of GLP-2 have potential in the treatment of patients suffering from intestinal failure, and patients with short bowel syndrome benefit from treatment with GLP-2. Finally, GLP-2 may be one of the possibly several intestinal signals responsible for meal-induced regulation of bone resorption. In studies, where bone resorption was monitored by measurements of cross-linked fragments of collagen-1, the secretion of GLP-1 and 2 was well correlated to meal-induced inhibition of resorption, and upon subcutaneous administration GLP-2 caused a similar reduction of bone resorption.

The GLP-2R knockout (GLP-2R$^{-/-}$) mouse has a blunted intestinal adaptive response to refeeding after fasting for 24 hours. In addition GLP-2R$^{-/-}$ has impaired bone metabolism. The glucose metabolism of GLP-2R$^{-/-}$ and wild-type mice is similar, and weight gains on both high fat and chow diets are also similar. However GLP-2R$^{-/-}$ mice crossed into *ob/ob* mice have, compared with *ob/ob* mice, increased fasting plasma glucose, increased fasting levels of glucagon, and paradoxically improved oral glucose tolerance.

ACTIONS OF OXYNTOMODULIN AND GLICENTIN

Oxyntomodulin is a dual agonist of both the GLP-1r and the glucagon receptor. In humans, oxyntomodulin inhibits both appetite and food intake and induces a significant weight loss in humans.[34] In diet-induced obese mice, treatment with a long-acting oxyntomodulin analog results in weight loss and intraperitoneal glucose tolerance was improved as well. Glicentin probably functions as a precursor of oxyntomodulin, but administration of glicentin to rats was reported to stimulate intestinal growth.[6] A specific receptor or mechanism has not been identified.

PATHOPHYSIOLOGICAL IMPLICATIONS

Disturbances in L-cell Secretion

The secretion of GLP-1 is often decreased in patients with type 2 diabetes, and this may contribute to the severely impaired incretin effect seen in these patients and thereby also to their impaired insulin secretion. The finding is not constant, and the variation probably results from the differences with respect to body mass index, insulin resistance, duration and severity of type 2 diabetes mellitus, gall bladder emptying rates, gastric emptying rates, and importantly antidiabetic therapies, where, for example, metformin increases GLP-1 secretion. In patients with morbid obesity, GLP-1 responses to meal ingestion may be almost eliminated. Because GLP-1 inhibits appetite, the impaired secretion may contribute to or aggravate the development of obesity.

As discussed above, GLP-1 secretion is related to gastric emptying rate, and in patients with accelerated gastric emptying, GLP-1 secretion may be excessive and may be responsible for the occurrence of postprandial reactive hypoglycemia in such patients.[32] Thus, accelerated gastric emptying causes high postprandial glucose concentrations coinciding with high GLP-1 concentrations resulting in exaggerated insulin secretion, which in turn causes hypoglycemia. Inhibition of glucagon secretion may contribute.

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Exaggerated GLP-1 secretion may also be responsible for the cases of hypoglycemia sometimes observed after gastric bypass operations for obesity.[20]

One of the most effective therapies for obesity is gastric bypass operations, which often leads to rapid (within days) remission of diabetes if present. The patients have extremely exaggerated (often around 10-fold) GLP-1 (and GLP-2) responses to meal ingestion, presumably resulting from an accelerated exposure to nutrients in the distal small intestine in which the density of L-cells is much higher than in the bypassed upper small intestine. Note that digestion does not occur until the secretions from the secretory limb are mixed with the contents from the alimentary limb in the distal jejunum, and that the mechanism of nutrient stimulated GLP-1 secretion involves interaction with L-cell membrane proteins, including Na^+/glucose cotransporters, Na^+/H^+-peptone cotransporters and many specific G protein-coupled receptors in the apical membrane.

Tumors producing proglucagon products generally exhibit a pancreatic processing pattern, and the resulting glucagonoma syndrome is caused by the excessive secretion of glucagon. However, in some cases, an intestinal processing pattern is observed, and in these patients, the most dramatic clinical symptoms (intestinal obstruction) are because of intestinal mucosal hyperplasia. This is probably a consequence of increased secretion of GLP-2.

Therapeutic Application of Proglucagon-Derived Peptides

The actions of GLP-1 have attracted great interest, because almost all of them are beneficial in the context of treatment of type 2 diabetes. Infusions of GLP-1 completely normalize fasting blood glucose concentrations in virtually all patients with type 2 diabetes investigated and in clinical studies involving subchronic (6 weeks) continuous subcutaneous infusion, blood glucose levels are greatly reduced, blood lipids improved, body weight reduced, and beta cell secretory capacity and insulin sensitivity increased. Single subcutaneous injections, however, are ineffective because of rapid degradation of the peptide by DPP-IV. To exploit the effects of GLP-1 receptor activation, several pharmaceutical companies have developed resistant analogs of GLP-1 or activators of the receptor. One of these is exendin-4, a peptide of 39 amino acids isolated from the salivary secretions of the lizard, *Heloderma Suspectum*, which is 53% homologous with GLP-1 (with respect to the sequence of the first 30 amino acids) and is a full agonist of the GLP-1 receptor. This peptide is resistant to the actions of DPP-4 and is cleared by the kidneys by glomerular filtration, resulting in a plasma half-life of about 30 min. Single subcutaneous injections, therefore, result in plasma exposure lasting 5–6 h. The peptide that is designated exenatide in its synthetic form has been available for diabetes treatment since 2005 under the name of Byetta. The most conspicuous effects are a sustained reduction of levels of glycated hemoglobinA1c (a measure of the average blood glucose levels) and a weight loss of 7–8 kg that is maintained for >3 years in individuals completing the protocol. A formulation of exenatide with similar clinical effects, which provides continued exposure for several weeks and therefore is useful for once-weekly administration, has been approved in 2012.

An acylated analog of human GLP-1, liraglutid with a half-life of 12–13 h after sc injection has been available since 2010 and has similar or may be even slightly better effects on body weight and HgbA1c. Both exenatide and liraglutid are as effective as insulin therapy but pose very little risk of causing hypoglycemia; their use is therefore recommended in patients with failure of the first oral agent (typically metformin). In addition, both agents function very well in combination with basal insulin therapy, where 70–80% of patients may reach target values of HbA1c (7.0%) without causing weight gain or hypoglycemia, making this combination the most effective therapy for type 2 diabetes to date. The clinical application of GLP-1 agonists for diabetes therapy is reviewed elsewhere.[18]

An alternative approach is inhibition of the enzyme DPP-IV. Unlike the GLP-1 agonists or analogs, DPP-IV inhibitors are small molecules that are orally available. DPP-IV inhibition increases the levels of intact GLP-1 and thereby amplify the actions of the endogenous hormone presumably both with respect to insulin and glucagon secretion. DPP-IV inhibitors have been marketed since 2006, where sitagliptin was launched. The inhibitors elevate the concentrations of both GLP-1 and GIP in their active forms and improve glucose-induced insulin secretion (same secretion elicited at lower plasma glucose concentrations) and lower plasma glucagon concentrations. The fall in HbA1c is about 0.5–1.0 %, but there is no effect on body weight. Unlike the agonists, the inhibitors do not cause gastrointestinal adverse effects (nausea) and are remarkably free from any other adverse effects. They perform well in combination with other oral antidiabetic agents, in particular metformin. One of the ways by which metformin acts is to increase the secretion of GLP-1; with a combined therapy, one therefore obtains stimulation of secretion with one and enhanced survival of GLP-1 with the other, and this may be the explanation for the particular efficacy of this combination. The clinical use of DPP-4 inhibitors is reviewed elsewhere.[3]

Because of its intestinotrophic activities, GLP-2 is currently being investigated as a therapy for intestinal insufficiency. Because of its much slower metabolism compared with that of GLP-1, GLP-2 can be used in its natural form and can enhance intestinal absorption and increase lean body weight in patients with short bowel syndrome in the course of a 5-week study with two daily subcutaneous

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injections. In a subsequent study, patients with short bowel syndrome were treated with native GLP-2 for 1 year, resulting in a reduction in fecal weight of 1000 g/d and enabling patients to maintain their intestinal fluid and electrolyte absorption at lower oral intake. A DPP-IV resistant analog (Teduglutide) may be produced by the substitution of amino acid residue no 2 (with Gly) and has similar, but prolonged effects. Tedoglutide was approved for short bowel syndrome treatment in 2012. Studies in experimental animals show that GLP-2 may also be effective in the therapy of intestinal atrophy after parenteral nutrition, and in intestinal failure after experimental enteritis and after radiation and chemotherapy. GLP-2 is also currently investigated as a therapy for osteoporosis, and is able to strongly reduce the nightly increase in bone resorption in postmenopausal women. In subchronic clinical studies (120 days), GLP-2 administration significantly improves bone mineral density in postmenopausal women.[10] The biology and clinical use of GLP-2 are reviewed elsewhere.[15]

REFERENCES

1. Bell GI, Sanchez-Pescador R, Laybourn PJ, Najarian RC. Exon duplication and divergence in the human preproglucagon gene. *Nature* 1983;**304**(5924):368–71.
2. Day JW, Ottaway N, Patterson JT, Gelfanov V, Smiley D, Gidda J, et al. A new glucagon and GLP-1 co-agonist eliminates obesity in rodents. *Nat Chem Biol* 2009 Oct;**5**(10):749–57.
3. Deacon CF, Carr RD, Holst JJ. DPP-4 inhibitor therapy: new directions in the treatment of type 2 diabetes. *Front Biosci* 2008;**13**:1780–94.
4. Deacon CF, Johnsen AH, Holst JJ. Degradation of glucagon-like peptide-1 by human plasma in vitro yields an N-terminally truncated peptide that is a major endogenous metabolite in vivo. *J Clin Endocrinol Metab* 1995;**80**(3):952–7.
5. Drucker DJ. The biology of incretin hormones. *Cell Metab* 2006 Mar;**3**(3):153–65.
6. Drucker DJ, Erlich P, Asa SL, Brubaker PL. Induction of intestinal epithelial proliferation by glucagon-like peptide 2. *Proc Natl Acad Sci U S A* 1996 Jul;**93**(15):7911–6.
7. Hansen KB, Rosenkilde MM, Knop FK, Wellner N, Diep TA, Rehfeld JF, et al. 2-oleoyl glycerol is a GPR119 agonist and signals GLP-1 release in humans. *J Clin Endocrinol Metab* 2011 Jul..
8. Hansen L, Deacon CF, Orskov C, Holst JJ. Glucagon-like peptide-1-(7-36)amide is transformed to glucagon-like peptide-1-(9-36) amide by dipeptidyl peptidase IV in the capillaries supplying the L cells of the porcine intestine [in process citation]. *Endocrinology* 1999 Nov;**140**(11):5356–63.
9. Hartmann B, Johnsen AH, Orskov C, Adelhorst K, Thim L, Holst JJ. Structure, measurement, and secretion of human glucagon-like peptide-2 [in process citation]. *Peptides* 2000 Jan;**21**(1):73–80.
10. Henriksen DB, Alexandersen P, Hartmann B, Adrian CL, Byrjalsen I, Bone HG, et al. Four-month treatment with GLP-2 significantly increases hip BMD: a randomized, placebo-controlled, dose-ranging study in postmenopausal women with low BMD. *Bone* 2009 Nov;**45**(5):833–42.
11. Holst JJ. The physiology of glucagon-like peptide 1. *Physiol Rev* 2007 Oct;**87**(4):1409–39.
12. Holst JJ, Bersani M, Johnsen AH, Kofod H, Hartmann B, Orskov C. Proglucagon processing in porcine and human pancreas. *J Biol Chem* 1994;**269**(29):18827–33.
13. Holst JJ, Burcelin R, Nathanson E. Neuroprotective properties of GLP-1: theoretical and practical applications. *Curr Med Res Opin* 2011 Mar;**27**(3):547–58.
14. Holst JJ, Orskov C, Nielsen OV, Schwartz TW. Truncated glucagon-like peptide I, an insulin-releasing hormone from the distal gut. *FEBS Lett* 1987;**211**(2):169–74.
15. Hornby PJ, Moore BA. The therapeutic potential of targeting the glucagon-like peptide-2 receptor in gastrointestinal disease. *Expert Opin Ther Targets* 2011 May;**15**(5):637–46.
16. Jin T. Mechanisms underlying proglucagon gene expression. *J Endocrinol* 2008 Jul;**198**(1):17–28.
17. Larsen PJ, Tang-Christensen M, Holst JJ, Orskov C. Distribution of glucagon-like peptide-1 and other preproglucagon-derived peptides in the rat hypothalamus and brainstem. *Neuroscience* 1997 Mar;**77**(1):257–70.
18. Madsbad S, Kielgast U, Asmar M, Deacon C, Torekov SS, Holst JJ. An overview of once-weekly GLP-1 receptor agonists—available efficacy and safety data and perspectives for the future. *Diabetes Obes Metab* 2011 Jan..
19. Mayo KE, Miller LJ, Bataille D, Dalle S, Goke B, Thorens B, et al. International Union of Pharmacology. XXXV. The glucagon receptor family. *Pharmacol Rev* 2003 Mar;**55**(1):167–94.
20. McLaughlin T, Peck M, Holst J, Deacon C. Reversible hyperinsulinemic hypoglycemia after gastric bypass: a consequence of altered nutrient delivery. *J Clin Endocrinol Metab* 2010 Apr;**95**(4):1851–5.
21. Mortensen K, Christensen LL, Holst JJ, Orskov C. GLP-1 and GIP are colocalized in a subset of endocrine cells in the small intestine. *Regul Pept* 2003 Jul;**114**(2–3):189–96.
22. Munroe DG, Gupta AK, Kooshesh F, Vyas TB, Rizkalla G, Wang H, et al. Prototypic G protein-coupled receptor for the intestinotrophic factor glucagon-like peptide 2. *Proc Natl Acad Sci U S A* 1999 Feb;**96**(4):1569–73.
23. Orskov C, Hartmann B, Poulsen SS, Thulesen J, Hare KJ, Holst JJ. GLP-2 stimulates colonic growth via KGF, released by subepithelial myofibroblasts with GLP-2 receptors. *Regul Pept* 2005 Jan;**124**(1-3):105–12.
24. Orskov C, Holst JJ, Knuhtsen S, Baldissera FG, Poulsen SS, Nielsen OV. Glucagon-like peptides GLP-1 and GLP-2, predicted products of the glucagon gene, are secreted separately from pig small intestine but not pancreas. *Endocrinology* 1986 Oct;**119**(4):1467–75.
25. Orskov C, Poulsen SS, Moller M, Holst JJ. Glucagon-like peptide I receptors in the subfornical organ and the area postrema are accessible to circulating glucagon-like peptide I. *Diabetes* 1996 Jun;**45**(6):832–5.
26. Parker HE, Reimann F, Gribble FM. Molecular mechanisms underlying nutrient-stimulated incretin secretion. *Expert Rev Mol Med* 2010;**12**:e1.
27. Salehi M, Aulinger B, Prigeon RL, D'Alessio DA. Effect of endogenous GLP-1 on insulin secretion in type 2 diabetes. *Diabetes* 2010 Jun;**59**(6):1330–7.

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28. Schafer SA, Tschritter O, Machicao F, Thamer C, Stefan N, Gallwitz B, et al. Impaired glucagon-like peptide-1-induced insulin secretion in carriers of transcription factor 7-like 2 (TCF7L2) gene polymorphisms. *Diabetologia* 2007 Dec;**50**(12):2443–50.
29. Scrocchi LA, Brown TJ, MaClusky N, Brubaker PL, Auerbach AB, Joyner AL, et al. Glucose intolerance but normal satiety in mice with a null mutation in the glucagon-like peptide 1 receptor gene. *Nat Med* 1996;**2**(11):1254–8.
30. Sivertsen J, Rosenmeier J, Holst JJ, Vilsboll T. The effect of glucagon-like peptide-1 on cardiovascular risk. *Nat Rev Cardiol* 2011:Forthcoming.
31. Thorens B. Expression cloning of the pancreatic beta cell receptor for the gluco- incretin hormone glucagon-like peptide 1. *Proc Natl Acad Sci U S A* 1992 Sep;**89**(18):8641–5.
32. Toft-Nielsen M, Madsbad S, Holst JJ. Exaggerated secretion of glucagon-like peptide-1 (GLP-1) could cause reactive hypoglycaemia. *Diabetologia* 1998 Oct;**41**(10):1180–6.
33. Williams DL, Baskin DG, Schwartz MW. Evidence that intestinal glucagon-like peptide-1 plays a physiological role in satiety. *Endocrinology* 2009 Apr;**150**(4):1680–7.
34. Wynne K, Park AJ, Small CJ, Patterson M, Ellis SM, Murphy KG, et al. Subcutaneous oxyntomodulin reduces body weight in overweight and obese subjects: a double-blind, randomized, controlled trial. *Diabetes* 2005 Aug;**54**(8):2390–5.
35. Yusta B, Holland D, Koehler JA, Maziarz M, Estall JL, Higgins R, et al. ErbB signaling is required for the proliferative actions of GLP-2 in the murine gut. *Gastroenterology* 2009 Sep;**137**(3):986–96.

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Chapter 169

Leptin

Sandra Guilmeau, Robert Ducroc and André Bado

ABSTRACT

The initial view that leptin, a 16-kDa polypeptide encoded by the ob gene, is an adipocyte-derived signal has been modified by the identification of other producing tissues, such as the stomach. The discovery of stomach-derived leptin, mainly secreted in the lumen, has initiated several investigations leading to the suggested clues: (1) gut leptin may act locally within the gastrointestinal tract to regulate intestinal functions such as nutrient absorption; (2) leptin may integrate meal-related signals from the gut and thus participate in the short-term regulation of energy balance, and (3) leptin secretion may have pathophysiological implications.

INTRODUCTION

Leptin, secreted primarily from white adipose tissue,[35] has been recognized as an adipostatic signal. Thus, leptin administrated peripherally in rodents with genetic or diet-induced obesity decreases body weight and adiposity and improves metabolic control by central nervous system (CNS)-mediated mechanisms.

The leptin receptor Ob-R, encoded by the *db* gene,[32] is a member of the gp130 family of cytokine receptors. It occurs in several isoforms resulting from the alternative splicing of the *db* gene.[19] Only the long isoform, Ob-Rb, can activate the signal transducers and activators of transcription (STAT) pathways, whereas both Ob-Rb and one of the short isoforms (Ob-Ra) can transduce signals through insulin receptor substrates (IRS) and mitogen-activated protein kinase (MAP kinase) pathways.[31]

Leptin is now considered to be involved in a wide range of biological processes. Leptin not only regulates food intake and energy expenditure but it also participates in the regulation of the hypothalamus–pituitary axis, angiogenesis, the immune system and inflammatory response, cell proliferation and apoptosis, and nutrient absorption. This multifunctional role of leptin is consistent with the production of this hormone by various tissues and organs, including the stomach.[2]

This review will focus on stomach-derived leptin and its implication in the physiology of the gastrointestinal tract.

THE STOMACH PRODUCES LEPTIN

The stomach serves as a reservoir in which ingested food accumulates and undergoes chemical and enzymatic primodigestion. The gastric mucosa consists of several types of secretory epithelial cells. Among these cells are endocrine cells secreting hormones, the mucous cells secreting alkaline mucus that protects the epithelium, parietal cells secreting gastric acid, and chief cells secreting pepsinogen, a proteolytic enzyme. The leptin-secreting cells were initially identified as pepsinogen-secreting chief cells in rodents and humans. Subsequent studies have detected leptin in the secretory granules of endocrine P cells in addition to chief cells.[8] Further, ultrastructural studies showed that leptin protein is present along the rough endoplasmic reticulum–Golgi-granule secretory pathways in both chief and endocrine cells.[7]

During development, leptin production in the stomach starts at the onset of suckling in neonatal rats and markedly increases during the transition from liquid to solid food intake.[23,26] This observation underlies the physiological importance of stomach-secreted leptin and the potential role that it could play in the control of gut-derived regulation of food intake in neonates.

Leptin secretion in the stomach is regulated by the nutritional status, acetylcholine-released by the vagus nerve, and intestinal hormones, that is, cholecystokinin and secretin.[14] In addition, gastric leptin shows diurnal variations influenced by food intake rhythms, and the changes occurring just before the beginning of the feeding period are opposite to those of the appetite stimulating peptide ghrelin.[27] Gut-derived leptin can be distinguished from fat-derived leptin by its rapidly increased secretion induced by a meal and its main secretion into the gastric lumen suggesting different functions for these two pools of leptin.

GASTRIC LEPTIN IS A NEUROENDOCRINE MOLECULE FOR SATIETY

One of the first questions raised by gastric leptin is whether it can act locally in the stomach to generate signals

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00169-X
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contributing to meal-induced termination of food intake, namely, on the vagus nerve conveying those signals to the CNS. This implies, however, the expression of leptin receptors on the vagus nerve. Interestingly, mRNA and protein for leptin receptors have been localized in the nodose ganglion.[5] The vagal afferent neurons possess functionally active leptin receptors proteins and leptin may activate intracellular STAT3 in these neurons.[5] Moreover, leptin receptors have been also described on the intestinal vagal mechanoreceptors. These vagal receptors are responsive to leptin directly delivered in the stomach, as evidenced by the increase of the tonic activity of gastric-related neurons in the NTS.[33] These results correlate well with those initially obtained from *in vitro* gastric vagus rat stomach preparation in which electrophysiological studies identified two types (1 and 2) of leptin-responsive gastric vagal afferent fibers.[34] Pretreatment of cholecystokinin-8 (CCK-8) fibers is required to increase the sensitivity for leptin of the type 2 fibers.[34] This clearly provides a basis for the reported leptin-CCK potentiating effects to increase the firing frequency of gastric vagal terminals,[34] neuronal activity in the nucleus tractus solitarius (NTS), and to reduce food intake[3] and body weight.[20]

Altogether, the vagus nerve seems to be the primary target of leptin produced by and secreted into the stomach. This leptin acts from the luminal side via a neurocrine mode of action through the vagal leptin receptors. The molecular basis of this neurocrine action of leptin remains to be elucidated.

GASTRIC LEPTIN ENTERS THE INTESTINE

Some of the stomach-derived leptin is not fully degraded by proteolysis, reaching the intestine in an active form to initiate biological functions. Thus, high amounts of free leptin and leptin bound to high molecular weight proteins are present in duodenal lumen in rats.[15] Such leptin-binding proteins were reported as the soluble leptin receptor in the blood and also identified as clusterin, also termed apolipoprotein J, a protein whose function has not yet been defined. Interestingly, in the rat colonic lumen, leptin can only be detected as free leptin.[15] Although the significance and the precise nature of these high molecular weight leptin-binding proteins in the intestinal juices needs further investigation, they may have physiological implications in the bioavailability of leptin in the gut lumen. Notably, the amount of leptin in the duodenum is greater than that expected from the stomach, suggesting contribution from other leptin-producing sites. In addition to gastric secretions, the duodenum receives hepatobiliary and exocrine pancreatic secretions and the liver also produces leptin.[17] Thus, the liver can contribute to the levels of leptin trafficking in the intestinal lumen by direct hepatic synthesis of leptin and/or by increasing the release of free leptin from the hepatic stores of the complexes of leptin bound to OB-Re.[17] More precise quantification of leptin and additional molecular studies are needed to ascertain the respective contribution of the different parts of the digestive system to leptin in the intestinal lumen. In addition to the presence of leptin in the gut lumen, leptin receptors occur at both the apical and basolateral sides of the enterocytes all along the small and large intestine, supporting the contention that leptin, acting from the luminal side, affects biological functions of the intestinal epithelium.[10]

LEPTIN REGULATES INTESTINAL ENDOCRINE SECRETIONS

CCK-1 receptor antagonists can both prevent peripheral leptin-induced inhibition of food intake[3] and the stimulation of pancreatic exocrine secretions.[14,22] In addition, intraduodenal leptin also stimulates pancreatic enzyme secretion in a CCK-1 receptor-dependent manner, probably through the activation of duodeno-pancreatic reflexes.[22] These results suggest that endogenously released CCK may be involved in those leptin actions. Circulating leptin (mainly reflecting adipocyte leptin) and, more importantly duodenal leptin, increases plasma CCK at levels comparable with those induced by feeding.[15] This action of duodenal leptin is probably relayed via a direct activation on the CCK-producing endocrine I cells. Finally, the leptin-induced release of CCK determines a positive feedback loop, because CCK can stimulate the release of gastric leptin.[2] CCK is one of the meal-generated molecules inducing satiety signals that are conveyed by the viscerosensitive vagal afferent neurons to the NTS, and then to the hypothalamus. Thus, leptin stimulation of duodenal CCK secretion suggests that under physiological conditions both peptides may potentiate their own actions by cross-stimulating their secretions. This is in accordance with the reported dampening of CCK or leptin inhibitory action on food intake when either peptide is absent or their receptors functionally inactive.[3]

Leptin can also stimulate the secretion of glucagon-like peptide-1 (GLP-1), another satiety signal, *in vitro* in enteroendocrine L cells and *in vivo* in rodents[1] providing evidence for the existence of an adipoenteroendocrine axis involved in the regulation of nutrient homeostasis.

GUT LEPTIN AND INTESTINAL ABSORPTION OF NUTRIENTS

The small intestinal lining contains functionally and morphologically polarized enterocytes that play a central role in the absorption of nutrients after digestion. In the intestine, the chyme undergoes hydrolysis by proteolytic enzymes from pancreatic, bile, and the intestinal juices, pursuing

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the primodigestion started in the stomach. Nutrients thus degraded in smaller molecules cross the intestinal brush-border by active transport, passive diffusion, or facilitated processes. The arrival of the meal in the intestine stimulates the release of gastrointestinal hormones that control the absorption of nutrients and are also signals for induction of postprandial satiety. Thus, it is conceivable that gut leptin rapidly secreted in the lumen after a meal (in contrast to adipocyte leptin) is a key molecule controlling intestinal absorption of nutriments.

Jejunal Absorption of Proteins

Under physiological conditions, dietary proteins are degraded in a series of steps by hydrolytic enzymes originating from the stomach, pancreas, and small intestine. This results in a mixture of free amino acids and small peptides that are efficiently absorbed by enterocytes. These small peptides are cleared from the intestinal lumen by the brush-border transporter PepT-1, which cotransports dipeptides and tripeptides with protons. Luminal but not basolateral leptin increases the absorption of dipeptides through PepT-1 in the enterocyte-like Caco2 cells *in vitro*.[4] These results were confirmed *in vivo* in the rat in which the direct administration of leptin in the jejunum (mimicking gastric leptin) rapidly increases the absorption of dipeptides.[4] The mechanism of this short-term action involves increased recruitment of membrane PepT-1 molecules from an intracellular preformed pool to the apical membrane. From a physiological point of view, the facilitation of protein absorption through PepT-l activation by gut leptin is consistent with the reports of a satiety effect of dietary proteins and is in line with the aminostatic hypothesis.

Jejunal Absorption of Fats

A major function of intestinal cells is to absorb a large amount of dietary lipids. After a digestive phase, the free fatty acids (FAs) lipolytic products are absorbed by the enterocytes, in which sequential events result in their packaging as chylomicrons. The formation and secretion of these intestinal lipoproteins are key steps in the transport of dietary fats. The assembly of triglyceride (TG) rich lipoproteins within the enterocytes, involves multiple pathways including (1) the uptake of FA by several specific carriers, such as Fatty Acid Transporter FAT/ and its human homolog CD36; (2) their translocation from the brush-border membrane to the endoplasmic reticulum by intestinal and liver Fatty Acid Binding Proteins (I- and L-FABPs); (3) their esterification in TG and subsequent assembly with apolipoprotein (apoB, apoA-IV) to form lipoprotein particles. Leptin seems to play a role in regulating the synthesis of apolipoproteins. Leptin administrated to fat-loaded *ob/ob* mice induces STAT5 DNA binding, and reduces apolipoprotein transcript levels in the mice jejunum.[21] Leptin is also involved in the regulation of circulating apo-AIV by suppressing apo-AIV synthesis in the small intestine.[9] Moreover, leptin can regulate the expression of microsomial triglyceride transfer protein (MTP) in mouse enterocytes through intestinal melanocortins and MC4-receptors.[18] Such an enterocyte-specific regulatory mechanism may provide additional peripheral control of fat mobilization independently of that determined by central nervous leptin signaling. It remains unknown however whether these leptin functions are assumed by leptin trafficking in the lumen of the intestine.

In the enterocyte-like Caco2 cells, leptin reduces the output of *de novo*-synthesized apolipoprotein ApoB-100 and ApoB-48 as well as that of newly formed chylomicrons and of low-density lipoproteins, supporting a role for leptin in the reduction of intestinal TG secretion into the circulation. Moreover, I-FABP expression is decreased by leptin in Caco2 cells. These effects of leptin on FA uptake and assembly in the enterocyte are probably involved in the regulation of energy homeostasis.

Jejunal Absorption of Sugars

Dietary carbohydrates are digested in the intestine through the action of amylase and intestinal brush-border membrane disaccharides into monosaccharides, D-glucose, D-galactose, and D-fructose. Sodium-dependent glucose cotransporter-1 (SGLT-1) is the specific transporter for D-glucose and D-galactose, whereas D-fructose is transported into the enterocytes by the pentose transporter GLUT-5. After accumulation in the enterocytes, the monosaccharides exit the cell across the basolateral membrane by the Na+-independent transporter GLUT-2. In the jejunum, leptin can inhibit the active absorption of galactose mediated by SGLT-1 without affecting the passive component of the absorption.[10] This leptin inhibition of galactose absorption *in vitro* probably involves leptin–receptor coupled activation of protein kinase C and A. In contrast, systemic leptin administration to rats after massive small bowel resection (MSBR) leads to an increase in the amount of GLUT-5 protein with no change in the levels of SGLT-1.[24]

Gut leptin also retains the ability to modulate the absorption of glucose-galactose in the rat jejunum. Indeed, luminal addition of leptin on rat jejunum, isolated in Ussing chambers, rapidly and dramatically decreases active glucose transport.[10] This rapid inhibition of glucose entry into the enterocyte by luminal leptin involves a reduced recruitment of SGLT-1 from an intracellular preformed pool to the apical membrane. The inhibition of active glucose transport by serosal leptin is however slower and probably involves the release of CCK as a relay molecule. This leptin regulation

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of active glucose absorption has been extended to GLUT-2 mediating the passive component of glucose absorption and to GLUT-5 mediated fructose transport. These studies clearly show that introduction of leptin into intestinal lumen results in the upregulation of both GLUT-2 and GLUT-5 transport activities through mechanisms critically dependent on the catalytic α_2 subunit of 5′ adenosine-monophosphate-activated protein kinase.[29] Because the small intestine is now recognized as an insulin-sensitive organ, a better understanding of the mechanisms by which leptin affects the intestinal absorption of monosaccharides may have physiological relevance in the management of diabetes, in particular non-insulin-dependent diabetes mellitus.

Importantly, the increased activity of the fructose GLUT-5 transporter is associated with a concurrent increase of blood glucose and liver fructose. This latter effect is accompanied by hypertriglyceridemia, suppression of intestinal and hepatocyte Fasting-induced adipocyte factor (Fiaf) expression, and by the induction of hepatic sterol regulatory element-binding protein transcription factor, targeting genes in the fatty acid biosynthesis pathway. Finally, a positive regulatory control loop between gut leptin and fructose has been identified in which fructose triggers the release of gastric leptin which in turn upregulates GLUT-2/GLUT-5 leading to increased fructose absorption and changes in some metabolic functions in the liver.[29]

Jejunal Absorption of Amino Acids

L-Glutamine is the primary metabolic fuel for enterocytes. Glutamine from the diet is transported into the absorptive cells by two sodium-dependent neutral amino acid transporters present at the apical membrane: ASCT2/SLC1A5 and B^0AT1/SLC6A19. The capacity of luminal leptin to control Na^+-dependent transport of glucose led to the question of leptin action on Na(+)-dependent glutamine absorption. Thus, mucosal leptin inhibits the uptake of glutamine and phenylalanine (substrate of B^0AT1) in everted intestinal rings.[15] In Ussing chambers, glutamine and phenylalanine absorption follows as Na+-induced short-circuit current is inhibited by leptin in a dose-dependent manner. The mechanisms of leptin regulation of the active entry of neutral amino acids involve a reduction of both B^0AT1 and ASCT2 proteins traffic to the apical plasma membrane and modulation of their gene expression.[11]

Colonic Absorption of Short Chain Fatty Acid

Butyrate, a short chain fatty acid (SCFA), is one of the products of the microbial digestion of carbohydrates and dietary fibers in the large bowel, which represents a dominant energy source for the colonocytes. Gut leptin is involved in the regulation of butyrate uptake by the intestinal epithelial cells through the proton-linked monocarboxylate transporter type 1 (MCT-1).[6] Indeed, in the human intestinal Caco2 cells, luminal leptin increases butyrate uptake by increasing MCT-1 mRNA levels and the amounts of MCT-1 protein on the apical membrane.[6] Such a control of MCT-1 activity by leptin which affects the availability of SCFA in the mucosa probably modulates the intracellular events regulating normal differentiation and proliferation in the colonic mucosa. Along this line, it has been reported that *in vitro* leptin promotes colon cancer HT-29 cell survival on butyrate challenge by counteracting the apoptotic programs initiated by this short chain fatty acid,[25] suggesting potential implications for the diseased colon (see the leptin chapter in Cancer/Anticancer section of the book). More investigations are needed to establish the relevance of these current results in the pathophysiology of colon cancer.

LEPTIN AND GUT PATHOLOGIES

Leptin is involved in maintaining gastric epithelial cell integrity and gastroprotection. In rats, systemic leptin is effective in attenuating both ethanol- and aspirin-induced damage to the gastric mucosa. This is correlated with an increase of leptin production by the stomach during experimentally-induced gastric damage in rats and during H pylori infection in humans. This gastric cytoprotective effect of leptin involves an increase in blood flow, local production of nitric oxide and prostaglandin E_2, and vagus nerve-dependent mechanisms.

One of the important functions of leptin is its role in the regulation of inflammatory processes. There is indeed a large body of evidence that leptin-mediated signal pathways play an active role in innate and adaptive immunity through alteration of various target genes transcription.[13] Interestingly, leptin-deficient *ob/ob* mice are resistant to colonic inflammation induced by oral dextran sulfate sodium or trinitrobenzene sulfonic acid and replacement of leptin converts their resistance to disease into susceptibility.[28] The mechanisms responsible for such an action largely involve T cell activation capacity of leptin.[13] Human inflamed colonic cells exhibit a strong leptin-immunoreactivity that is concentrated at subapical part of the colonic cells, whereas normal colonic epithelial cells do not show this.[30] This supports the idea that fat- and gut-derived leptin may be a key component in the control of intestinal inflammation processes. Whether the aberrant expression of leptin is a cause or consequence of inflammation in colon mucosa or whether leptin acting from the luminal side contributes to the progression and maintenance of the chronicity of the inflammation, remains to be further investigated.

Beside this proinflammatory action of leptin acting in the intestinal mucosa, leptin signaling can be important in the mucosal defense against enteric infection. Thus, a mutation

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of leptin receptor is associated with increased susceptibility to *Entamoeba histolytica* infection with greater levels of mucosal destruction in children.[12] In mice with deleted intestinal leptin receptors, leptin signaling in the intestinal epithelium mediates resistance to enteric infection by *Entamoeba histolytica* through the STAT3 and protein–tyrosine phosphatase SHP2/ERK pathways.[16]

CONCLUSIONS

The gastrointestinal epithelial cells are in contact with submucosal immune cells that are tightly and finely controlled by the enteric nervous system. There is increasing evidence pointing out the importance of leptin at the interface between the immune, endocrine, and enteric nervous systems, and the epithelial barrier within the gut. These results make leptin, a hormone and a cytokine, a good candidate to link neuroendocrine and immune systems to metabolic status. A better understanding of the role of gut leptin in the physiology of gastrointestinal functions will provide a basis for the determination of its relevance in several disease states such as obesity, diabetes, and IBD.

ACKNOWLEDGMENTS

The authors' work was supported by the Institut National de la Santé et de la Recherche Médicale (Inserm), grant APEX99 n°4X006E to A. Bado, by The French Society of Nutrition (SFN). S. Guilmean has been supported by a grant from Danone Institute.

REFERENCES

1. Anini Y, Brubaker PL. Role of leptin in the regulation of glucagon-like peptide-1 secretion. *Diabetes* 2003;**52**:252–9.
2. Bado A, Levasseur S, Attoub S, Kermorgant S, Laigneau JP, Bortoluzzi MN, et al. The stomach is a source of leptin. *Nature* 1998;**394**:790–3.
3. Barrachina MD, Martinez V, Wang L, Wei JY, Tache Y. Synergistic interaction between leptin and cholecystokinin to reduce short-term food intake in lean mice. *Proc Natl Acad Sci USA* 1997;**94**:10455–60.
4. Buyse M, Berlioz F, Guilmeau S, Tsocas A, Voisin T, Peranzi G, et al. PepT1-mediated epithelial transport of dipeptides and cephalexin is enhanced by luminal leptin in the small intestine. *J Clin Invest* 2001;**108**:1483–94.
5. Buyse M, Ovesjö ML, Goiot H, Guilmeau S, Péranzi G, Moizo L, et al. Expression and regulation of leptin receptor proteins in afferent and efferent neurons of the vagus nerve. *Eur J Neurosci* 2001;**14**:64–72.
6. Buyse M, Sitaraman SV, Liu X, Bado A, Merlin D. Luminal leptin enhances CD147/MCT-1-mediated uptake of butyrate in the human intestinal cell line Caco2-BBE. *J Biol Chem* 2002;**277**:28182–90.
7. Cammisotto PG, Renaud C, Gingras D, Delvin E, Levy E, Bendayan M. Endocrine and exocrine secretion of leptin by the gastric mucosa. *J Histochem Cytochem* 2005;**53**:851–60.
8. Cinti S, Matteis RD, Pico C, Ceresi E, Obrador A, et al. Secretory granules of endocrine and chief cells of human stomach mucosa contain leptin. *Int J Obes Relat Metab Disord* 2000;**24**:789–93.
9. Doi T, Liu M, Seeley RJ, Woods SC, Tso P. Effect of leptin on intestinal apolipoprotein AIV in response to lipid feeding. *Am J Physiol Regul Integr Comp Physiol* 2001;**281**:R753–9.
10. Ducroc R, Guilmeau S, Akasbi K, Devaud H, Buyse M, Bado A. Luminal leptin induces rapid inhibition of active intestinal absorption of glucose mediated by sodium-glucose cotransporter 1. *Diabetes* 2005;**54**:348–54.
11. Ducroc R, Sakar Y, Fanjul C, Barber A, Bado A, Lostao MP. Luminal leptin inhibits L-glutamine transport in rat small intestine: involvement of ASCT2 and B0AT1. *Am J Physiol (Gastrointest and Liver Physiol)* 2010;**299**:G179–85.
12. Duggal P, Guo X, Haque R, Peterson KM, Rickfels S, Mondal D, et al. A mutation in the leptin receptor is associated with *Entamoeba histolytica* infection in children. *J Clin Invest* 2011;**121**(3):1191–8.
13. Fantuzzi G, Faggioni R. Leptin in the regulation of immunity, inflammation, and hematopoiesis. *J Leukoc Biol* 2000;**68**:437–46.
14. Guilmeau S, Buyse M, Bado A. Gastric leptin: a new manager of gastrointestinal function. *Curr Opin Pharmacol* 2004;**4**:561–6.
15. Guilmeau S, Buyse M, Tsocas A, Laigneau JP, Bado A. Duodenal leptin stimulates cholecystokinin secretion: evidence of a positive leptin-cholecystokinin feedback loop. *Diabetes* 2003;**52**:1664–72.
16. Guo X, Roberts MR, Becker SM, Podd B, Zhang Y, et al. Leptin signaling in intestinal epithelium mediates resistance to enteric infection by *Entamoeba histolytica*. *Nat Immunol* 2011;**4**(3):294–303.
17. Ikejima K, Takei Y, Honda H, Hirose M, Yoshikawa M, et al. Leptin receptor-mediated signaling regulates hepatic fibrogenesis and remodeling of extracellular matrix in the rat. *Gastroenterology* 2002;**122**:1399–410.
18. Iqbal J, Li X, Chang BH-J, Chan L, Schwartz G, Chua SG Jr, et al. An intrinsic gut leptin–melanocortin pathway modulates intestinal microsomal triglyceride transfer protein and lipid absorption. *J Lipid Res* 2010;**51**:1929–42.
19. Lee GH, Proenca R, Montez JM, Carroll KM, Darvishzadeh JG, Lee JI, et al. Abnormal splicing of the leptin receptor in diabetic mice. *Nature* 1996;**379**:632–5.
20. Matson CA, Reid DF, Cannon TA, Ritter RC. Cholecystokinin and leptin act synergistically to reduce body weight. *Am J Physiol Regul Integr Comp Physiol* 2000;**278**:R882–90.
21. Morton NM, Emilsson V, Liu YL, Cawthorne MA. Leptin action in intestinal cells. *J Biol Chem* 1998;**273**:26194–201.
22. Nawrot-Porabka K, Jaworek J, Leja-Szpak A, Palonek M, Szklarczyk J, et al. Leptin is able to stimulate pancreatic enzyme secretion via activation of duodeno–pancreatic reflex and CCK release. *J Physiol Pharmacol* 2004;**55**(Suppl. 2):47–57.
23. Oliver P, Pico C, De Matteis R, Cinti S, Palou A. Perinatal expression of leptin in rat stomach. *Dev Dyn* 2002;**223**:148–54.
24. Pearson PY, O'Connor DM, Schwartz MZ. Novel effect of leptin on small intestine adaptation. *J Surg Res* 2001;**97**:192–5.
25. Rouet-Benzineb P, Aparicio T, Guilmeau S, Pouzet C, Descatoire V, et al. Leptin Counteracts Sodium Butyrate-induced Apoptosis in Human Colon Cancer HT-29 Cells via NF-{kappa}B Signaling. *J Biol Chem* 2004;**279**:16495–502.
26. Sanchez J, Oliver P, Miralles O, Ceresi E, Pico C, Palou A. Leptin orally supplied to neonate rats is directly uptaken by the immature stomach and may regulate short-term feeding. *Endocrinology* 2005;**146**:2575–82.

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27. Sanchez J, Oliver P, Pico C, Palou A. Diurnal rhythms of leptin and ghrelin in the systemic circulation and in the gastric mucosa are related to food intake in rats. *Pflugers Arch* 2004;**448**:500–6.
28. Siegmund B, Lehr HA, Fantuzzi G. Leptin: a pivotal mediator of intestinal inflammation in mice. *Gastroenterology* 2002;**122**: 2011–25.
29. Sakar Y, Nazaret C, Lettéron P, Ait Omar A, Avenati M, et al. Positive regulatory control loop between gut leptin and intestinal GLUT2/GLUT5 transporters links to hepatic metabolic functions in rodents. *PLoS ONE* 2009;**4**(11):e7935doi: 10.1371.
30. Sitaraman S, Liu X, Charrier L, Gu LH, Ziegler TR, Gewirtz A, et al. Colonic leptin: source of a novel proinflammatory cytokine involved in IBD. *Faseb J* 2004;**18**:696–8.
31. Tartaglia LA. The leptin receptor. *J Biol Chem* 1997;**272**:6093–6.
32. Tartaglia LA, Dembski M, Weng X, Deng N, Culpepper J, Devos R, et al. Identification and expression cloning of a leptin receptor, OB-R. *Cell* 1995;**83**:1263–71.
33. Wang L, Barachina MD, Martinez V, Wei JY, Tache Y. Synergistic interaction between CCK and leptin to regulate food intake. *Regul Pept* 2000;**92**:79–85.
34. Wang YH, Tache Y, Sheibel AB, Go VL, Wei JY. Two types of leptin-responsive gastric vagal afferent terminals: an in vitro single-unit study in rats. *Am J Physiol* 1997;**273**:R833–7.
35. Zhang Y, Proenca R, Maffei M, Barone M, Leopold L, Friedman JM. Positional cloning of the mouse obese gene and its human homologue [published erratum appears in Nature 1995 Mar 30;374(6521):479. *Nature* 1994;**372**:425–32.

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Chapter 170

Motilin

Pierre Poitras

ABSTRACT

Motilin is released from the M cell of the duodenal mucosa into the blood circulation to help regulate the interdigestive motility of the stomach and small intestine. Motilin, as an interdigestive hormone, induces the phase III contraction of the migrating motor complex (MMC) during the fasting period. Motilin receptor stimulation is a potent pharmacological stimulus of gastric motor activity and can be used to accelerate the gastric emptying of a meal. Motilin is part of the motilin/ghrelin peptide family.

DISCOVERY OF MOTILIN

The sequence of motilin was first published in 1973 by Brown et al.[2] Inspired by observations made at the beginning of the 20th century that gastric motility was inhibited by perfusion of an acid solution into the duodenum, Brown discovered that instillation of pH 9 Tris buffer into the duodenum increased motor activity in a transplanted gastric pouch in dogs. This experiment, confirming the regulation of gastric motor activity by an endocrine factor released from the duodenum, led to the purification of this hormone. As starter material, he used a side fraction produced during the purification of porcine intestinal secretin by Victor Mutt at the Karolinska Institute. Tracing the purification steps with the *in vivo* model of a canine transplanted gastric pouch, Brown, through successive chromatographic steps on CM cellulose, Sephadex G25, and DEAE cellulose, isolated a 22-amino acid peptide with a molecular weight of 2700. He chose the name motilin "because of the original observation that it caused an increase in motor activity in pouches of gastric fundus gland area of the stomach." The structure of porcine motilin is shown in Table 1.

STRUCTURE OF MOTILIN AND RELATED PEPTIDES

Structural Heterogeneity and Species

Species heterogeneity in the structure of motilin soon became a major issue in the physiological and pharmacological exploration of the peptide; indeed, it had an impact on the detection of motilin by radioimmunoassay (RIA) and on the bioactivity of motilin on various experimental models. Biochemical characterization of motilin was, therefore, undertaken in numerous animal species. In the late 1970s, using successive chromatographies (gel, ion, and HPLC) of small intestinal extracts, and detection of motilin-like-immunoreactivity by RIA with an antiserum against porcine motilin, we purified canine motilin and found differences in 5 of its 22 amino acids (Table 1). From the mid-1980s, molecular biology techniques allowed the isolation and characterization of cDNA clones encoding motilin precursors in numerous species. Human motilin was found to be similar to the pig peptide, but differences in the amino acid sequence were subsequently identified in many species as shown in Table 1.

Mouse and Rat Motilin

Confusion certainly arose from studies in rats and mice; the 2 common laboratory animals could not be used for motilin research because motilin could not be detected in these species and was present without any biological activity. The efforts of many research teams to characterize motilin and motilin receptors in these rodents were in vain. An important step in the comprehension of this unusual situation in the field of peptide research was taken by T.L. Peeters using an "in silico" approach to identify motilin and the motilin receptor in the now elucidated rat genome. Peptide-conserved sequences were discovered, but the putative translation products contained stop codons that disable the formation of full prepromotilin peptides. A partial 1–13 fragment, which has amino acid substitutions in positions 2, 3, and 7, might eventually be formed. This fragment showed contractile activity similar to porcine motilin in the rabbit duodenum *in vitro*, confirming its motilin nature.[17] RTPCR analysis with a probe made from mouse motilin of mouse duodenum, stomach, or brain failed to reveal motilin in these organs, suggesting that motilin is not expressed in mice. Moreover, "in silico" analysis of the motilin receptor also suggested the presence of homologous regions but with many stop codons and a frame-shift making

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00170-6
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TABLE 1 Sequence of Motilin in Various Species

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
Pig (1972)	**PHE**	**VAL**	**PRO**	**ILE**	**PHE**	**THR**	**TYR**	**GLY**	**GLU**	**LEU**	**GLN**	**ARG**	**MET**	**GLN**	**GLU**	**LYS**	**GLU**	**ARG**	**ASN**	**LYS**	**GLY**	**GLN**
Dog (1979)	-	-	-	-	-	-	His	Ser	-	-	-	Lys	Ile	Arg	-	-	-	-	-	-	-	-
Man (1987)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Rabbit (1991)	-	-	-	-	-	-	-	-	Ser	-	-	-	-	-	-	Arg	-	-	-	Arg	-	His
Cat (1993)	-	-	-	-	-	-	His	Ser	-	-	-	Arg	Ile	Arg	-	-	-	-	-	-	-	-
Chicken (1995)	-	-	-	Phe	-	-	Gln	Ser	Asp	Ile	-	Lys	-	-	-	-	-	-	-	-	-	-
Sheep (1997)	-	-	-	-	-	-	-	-	-	Val	-	-	-	-	-	-	-	-	Tyr	-	-	-
Monkey (1998)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Ser	-	-	-
Horse (1999)	-	-	-	-	-	-	-	Ser	-	-	-	-	-	-	-	-	-	-	-	Arg	-	-
Cow (1999)	-	-	-	-	-	-	-	-	-	Val	Arg	-	-	-	-	-	-	-	Tyr	-	-	-
Guineapig (2001)	-	-	-	-	-	-	-	Ser	-	-	Arg	-	Thr	-	-	Arg	-	Gln	-	-	Arg	Leu
Mouse (2004)	-	Ile	Thr	-	-	-	His	-	-	-	-	-	-	x	x	x	x	x	x	x	x	x
Rat (2004)	-	Ile	Thr	-	-	Val	His	-	-	-	-	-	Leu	-	x	x	x	x	x	x	x	x
Suncus (2009)	-	Met	-	-	-	-	-	-	-	-	-	Lys	-	-	-	-	-	Gln	-	-	-	-

( ): year of discovery; -: identical AA; x: absent AA

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the synthesis of the mature protein impossible. Mouse motilin, indeed, was inactive in the mouse duodenum *in vitro* or in the mouse stomach *in vivo*, suggesting that motilin receptors were absent in the mouse digestive tract. A recent study[7] confirmed that the genomes of mice and rats only contain remnants of the motilin and motilin receptor genes. Heterogeneity in the structure of motilin among species is unusual in the field of peptides.

Motilin/Ghrelin Family

Motilin was in a peculiar position because it was not identified as belonging to a family of peptides, as, for example, peptide families such as gastrin/CCK, secretin/VIP. However, ghrelin, a newly discovered peptide of the mammalian stomach, shares sufficient structural similarity with motilin to have been called motilin-related peptide.[23] Motilin and ghrelin (see the chapters on ghrelin in this Gastrointestinal Peptides Section of the book, as well as in the Brain, Endocrine, Ingestive, and Cardiovascular Peptide Sections) have a number of biological actions in common. The motor effects of ghrelin on the GI tract are indeed comparable with those observed for motilin, that is, induction of phase III of the migrating motor complex (MMC) in the fasting period and stimulation of gastric emptying during the postprandial period. In the central nervous system, motilin and ghrelin show comparable activities on the release of growth hormone (GH) or on appetite stimulation.

Not only do motilin and ghrelin show structural similarity, but their also receptors have sequence homology. Resemblance between motilin and ghrelin in the structure of the ligands and in the structure of their receptors suggests of course crossreactivity between ghrelin and motilin analogs on their receptors. However, recent studies have failed to show a significant interaction between motilin ligands and the ghrelin receptor and, vice versa, between ghrelin ligands and the motilin receptor. The significance and implication of the motilin/ghrelin peptide family have still to be identified.

GENE STRUCTURE AND POSTTRANSLATIONAL PROCESSING

The human motilin gene was mapped to the P 21.2→P 21.3 region of chromosome 6. It consists of 5 exons separated by 4 introns; the bioactive peptide structure is encoded in 2 distinct exons (exon 2 and exon 3). Nucleotide sequence analysis predicts that motilin is first synthesized as a precursor of 114–119 amino acids. A 25-amino acid signal peptide precedes the 22-amino acid sequence of the active peptide, followed by 2 lysine residues that precedes a 65-amino acid sequence that has been called motilin-associated peptide (MAP) whose biological importance remains unknown.

Northern blot analysis of human duodenal mRNA revealed a single band and suggested that the multiple forms of motilin-like immunoreactive material found by gel permeation chromatographies were produced by post-translational processing of a single motilin precursor. The importance of these promotilins, however, seems marginal; in tissues and in blood, the 22-amino acid peptide is by far the dominant molecular form encountered.

DISTRIBUTION IN THE GI TRACT

The presence of motilin in the gut of numerous animal species has been verified by RIA measurement of motilin-like-immunoreactivity (MLI) in tissue extracts, by immunostaining of motilin-containing cells or structures, and by Northern blots to detect motilin mRNA in various tissue regions of the GI tract. By far, the most prevalent region for motilin expression is the proximal gut mucosa, mainly the duodenum, and, to a minor extent, the jejunum.

Motilin is produced in a specific endocrine cell of the intestinal mucosa. The M cell is well characterized in electron microscopy by its relatively small, solid secretory granules, often of irregular shape and density. The presence of motilin in a subpopulation of 5-hydroxytryptamine-enterochromaffin cells has been initially described but remains controversial.

The presence of motilin in extramucosal structures of the GI tract is also debated. MLI was detected in extracts from gut muscle, but this could be explained by mucosal contamination during tissue preparation. Some studies, most often unconfirmed, suggested that motilin was present in the vagus nerve and in neurons of the intestinal wall. Motilin appeared as one of the rare peptides not considered as a brain–gut peptide. Motilin concentrations measured in the brain were always much lower than those found in the gut and much lower than other brain peptides such as CCK or VIP. However, motilin mRNA has been detected in specific brain regions of various animal species, including humans. Motilin receptors also seem to be present in the brain, and we must now face the concept that motilin could, indeed, like most other peptides, play a role as a neural transmitter.

RECEPTOR AND STRUCTURE ACTIVITY

Structure Activity

Nuclear magnetic resonance analysis of the 3-dimensional structure of motilin revealed that the 7 first amino acids of the N-terminal portion of the molecule are positioned in a curved fashion, whereas the C-terminal segment of the peptide displays an alpha-helix appearance (Fig. 1).

The N-terminal portion of the molecule is responsible for receptor binding and *in vitro* bioactivity. Peptide fragments containing the first 12 amino acids of the molecule

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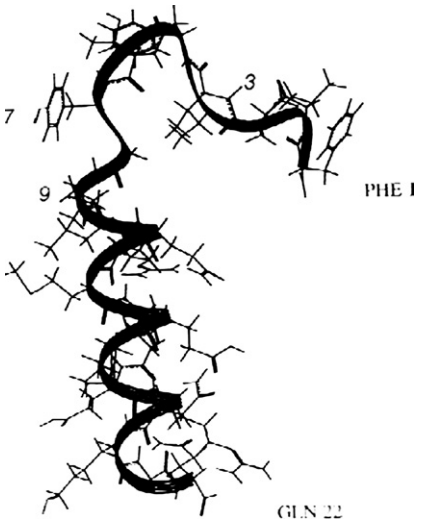

FIGURE 1 Structure of the 22-amino acid motilin peptide revealed by nuclear magnetic resonance. The N-terminal curved sequence binds to the receptor, whereas the α-helix C-terminal structure protects the molecule for *in vivo* bioactivity.

demonstrate full binding capacity and full contractile activity *in vitro*. Phe1, ILe4, and Tyr7 are key amino acids for the pharmacophore of motilin.

Interestingly, the C-terminal structure, not required for *in vitro* bioactivity, was found to be important for *in vivo* bioactivity. N-terminal fragments of 15 amino acids, fully active *in vitro*, were indeed inactive *in vivo*, whereas longer fragments with 19, 20, or 21 amino acids could mimic the motor activity of the 22-amino acid native peptide when injected into conscious dogs.[19] This is an interesting concept in the field of peptide pharmacology where the C-terminal structure seems to be important in protecting the whole molecule probably against circulating degrading enzymes. A similar observation on the protective role of the C-terminal segment *in vivo* has been made for ghrelin,[22] the other member of this peptide family.

Receptor

The motilin receptor was cloned in 1999. For years before that, all attempts to sequence the intestinal motilin receptor failed while using a goal-directed approach with a motilin probe for its recognition (of course, we now realize that strategies with mouse or rat as starting material were destined to fail due to the now documented absence of motilin receptors in these species). Researchers in the Merck Laboratories[5] followed a different strategy by first cloning and sequencing a large variety of G protein-coupled receptors from human genomic DNA libraries and then looking at the nature of the ligands for these receptors. Starting from an orphan receptor with a structure close to that of the receptor for the growth hormone secretagogue (GHS), mass screening of >500 peptide and nonpeptide molecules was performed to obtain positive results for motilin. Starting tissue for this work came from the thyroid, and the significance of this receptor localization remains an enigma.

The motilin receptor gene was mapped to human chromosome 13 q14–q21. It consists of 412 amino acids with 7 transmembrane domains and has 52% homology with the ghrelin receptor. Cloning of the motilin receptor of the rabbit and dog[14] confirmed the structural heterogeneity of motilin receptors among species, as it was suspected in earlier pharmacological studies looking at the interaction of various fragments and analogs of motilin on various tissue membranes or organs *in vitro* and *in vivo*.

In *in situ* hybridization studies,[5] motilin receptor RNA was expressed in a subset of interstitial cells mostly apparent in human duodenum, jejunum, and colon. These cells also demonstrated immunoreactivity for neuron-specific enolase and, to some extent, for nitric oxide synthase (NOS) and choline acetyltransferase. In the duodenum, motilin receptor expression was also found in a separate population of cells having an elongated appearance consistent with smooth muscle cells. In another study, motilin receptor immunoreactivity was present in muscle cells and the myenteric plexus of human GI tract.[21] In canine stomach, motilin receptor immunoreactivity was localized in neuronal cell bodies and fibers.[14]

Functional experiments in humans and in animals suggested that motilin receptors indeed exist on nerves as well as on muscles of the gut. Receptors on neurons, sensitive to antimuscarinic blockade, are activated in humans by low doses of exogenous motilin to induce phase III contraction of the MMC during the fasting period, whereas muscle receptors, insensitive to atropine, seem to be activated by higher doses of ligands to accelerate gastric emptying of a meal.[1,3] In receptor binding studies with solutions specifically enriched in muscle or in neural elements of the intestinal wall,[12] specific and distinct responses to various motilin synthetic analogs and fragments suggested the existence of structurally heterogeneous motilin receptor subtypes specific to muscle or neural elements (that we have called M and N receptor subtypes).

Motilin receptors in the brain remain an enigma. Biological responses (such as an increase in GH secretion, increase in food intake, or decrease in anxiety) to intracerebral injection of motilin were indeed observed, but, most of the time, in rats or mice, animals with a questionable contribution to motilin research. Motilin binding sites were, however, identified by autoradiographic studies of rabbit brain tissues, and receptor binding studies allowed the identification of high and low affinity binding sites for motilin on these tissues. The identification of motilin receptors in the rabbit brain and the documentation of motilin RNA in the brain of most species in which it has been investigated

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until now suggest a role for motilin as a central neurotransmitter, a role that has still to be clarified.

BIOLOGICAL ACTIONS

Difficulties in Experimental Conditions

Motilin was discovered >30 years ago, but many questions still seem unanswered. Exploration of its biological action was difficult for various reasons: (a) Experimental subjects: rat and mouse are convenient laboratory animals for biological studies, but, as discussed previously, were of no help in motilin research. We therefore had to rely on more difficult experimental animals, including the dog that was very important in the elucidation of motilin physiology. (b) Biological actions: as discussed later, the main biological action of motilin is the induction of phase III of the MMC. Recording of this gastrointestinal contractile activity is, therefore, essential for physiological studies on motilin, but this is a complicated task; it requires, in most cases, an invasive technology (surgical implantation of recording electrodes, fluoroscopic positioning of nasally or orally introduced recording probes, etc.) that demands technical expertise from researchers as well as physical and even emotional tolerance from test subjects. Furthermore, the basal cyclical nature of the studied biological phenomenon requires prolonged and tedious experimental protocols. (c) Species heterogeneity: as discussed earlier in this chapter, species heterogeneity in the structure of motilin and of the motilin receptor, as well as in the regulatory mechanisms of the MMC, limits the possibility of extrapolating data from species to species and forces extreme caution in the transfer of information from one species to another.

Pharmacological Action

As its name implies, motilin acts on digestive motility. When administered in pharmacological doses, motilin, or motilin derivatives such as erythromycin (see later), can increase contraction of the lower esophageal sphincter, of the fundic and antral stomach, of the duodenum, of the gallbladder, and, to a lesser extent, of the sigmoid colon (this poor action of motilin on the colon is surprising considering the high number of motilin receptors in this organ). Secretions from the pancreas and from the small intestine can also be stimulated by the exogenous administration of motilin.

Stimulation of antroduodenal motility remains, however, the dominant action of the peptide. Initial *in vitro* studies with intestinal tissues from rabbits and humans revealed that motilin can stimulate smooth muscle contraction by a direct effect on muscle cells; the peptide action was seen despite the addition of all neural blockers, including tetrodotoxin. Experiments with isolated muscle cells have confirmed the presence of motilin receptors on muscle cell membranes. However, *in vivo* studies in dogs and humans clearly showed that the motor action of motilin is mediated by muscarinic transmitters.

Physiological Role

The characteristic action of motilin is the induction of phase III contraction of the migrating motor complex (MMC). The MMC is the basic organization of motor activity of the gut during the fasting interdigestive period. It lasts 80–120 minutes and consists of 3 successive phases: phase I, no signification contractions are seen for 20–60 minutes; during phase II, intermittent and irregular contractions start to occur 20–60 minutes before phase III during which strong peristaltic contractions, lasting 3–10 minutes, start from the stomach and lower esophagus to migrate distally to the duodenum, jejunum and ileum until the colon. This phase III peristaltic wave has been proposed to clean the digestive tract from bacteria or nutrients that could accumulate during the digestive period and could generate deleterious effects on the gut (e.g., bacterial overgrowth). Feeding interrupts the MMC and induces more constant motor activity of moderate amplitude to allow optimal absorption of ingested nutrients, because accelerated transit during phase III would indeed impair nutrient absorption by the small intestine.

Regulatory peptides can exert their influence via different pathways: endocrine (hormonal), neurocrine, paracrine, or autocrine. At present, we believe that the main action of motilin is to regulate MMC activity through an endocrine mechanism, that is, through variation in its plasma concentrations. Motilin is in a unique position, as a GI regulatory peptide, of being active during the interdigestive period, and being therefore not a digestive but an interdigestive hormone. Its role as an interdigestive hormone has been well identified in the dog, in which all of the Morton Grossman's criteria[4] establishing the endocrine contribution of peptides have been fulfilled:[18] (1) Regulation of the cyclical pattern of the MMC is indeed under the control of circulating factors, as shown in various experiments where MMC persists in animals when their stomach had been completed denervated. (2) Plasma motilin levels are variable during the fasting period, and there is a perfect correlation between circulating peak levels of endogenous motilin in the blood and the initiation of phase III contractions from the stomach or proximal duodenum. (3) Administration of exogenous motilin induces phase III contraction of the MMC, and this can be obtained even when motilin is given in small doses reproducing physiological plasma variations. (4) Inhibition of circulating plasma motilin by the administration of specific motilin antisera blocks phase III contractions from the upper gut. Although the situation could not be explored under such perfect experimental conditions in humans, most evidence suggests that circulating motilin also plays a key role in the regulation of phase IIIs initiated from the antrum in humans.

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Release Mechanisms

Most gastrointestinal hormones are released after a meal to allow or facilitate the digestion and absorption of nutrients. Motilin is a unique hormone; it is released periodically during the interdigestive fasting period, and its cyclical release is abolished after a meal (as shown schematically in Fig. 2). Therefore, a "biological clock," still of unknown nature, somewhere in the organism periodically signals motilin cells to release the peptide into the circulation. *In vitro* preparations of intestinal mucosal cells enriched in motilin cells showed that muscarinic receptors are present on the motilin cell membrane and that protein kinase C activators are the most potent second messengers eliciting motilin release. In the *ex vivo* perfused canine intestine, bombesin has been identified as a direct stimulant of motilin release, whereas the stimulatory effect of opiates was mediated by acetylcholine. However, phenylephrine and somatostatin seem to act directly on M cell membrane receptors to block the release of the peptide. Interactions with luminal serotonin[13] as well as plasma ghrelin[25] on the release of plasma motilin have been suspected recently. Vagus nerves seem to have some influence on the release of plasma motilin because acute vagal cooling inhibited the cyclical peak increases in plasma motilin; however, vagotomized dogs displayed a normal cyclical profile of motilin plasma release.

As the stimulation of motilin cyclical peak release during the interdigestive period is incompletely understood, inhibition of this cyclical release by a meal (Fig. 2) is also incompletely understood. The situation is still more complicated by the fact that meal ingestion is followed in humans by a very early and brief increase in plasma motilin before the interdigestive release cycle is interrupted, as in the dog. This early release in humans can be mimicked experimentally by central stimulation with modified sham feeding and by distension of the gastric fundus with an air-filled balloon. The contribution of this postprandial motilin release, not present in the dog, in the process of nutrient digestion in humans remains to be characterized.

PATHOPHYSIOLOGICAL IMPLICATION

Disorder of Motilin Secretion

Some GI hormones, such as gastrin and vasointestinal polypeptide (VIP), are important to clinicians because of the disease symptoms they can generate in response to tumoral hypersecretion by gastrinomas or VIPomas. One patient with a motilinoma has been reported;[6] suffering from liver metastases from a rectal neuroendocrine tumor, the patient had an isolated increase in plasma motilin, but was without any specific clinical manifestation. Up to now, there has been no clinical phenotype attributed to motilin hypersecretion. High levels of circulating motilin have been documented, as cosecreted factors, in some patients with pancreatic tumors and Zollinger–Ellison syndrome and in patients with carcinoid tumors of the gut. Although it could be tempting to speculate on the role of motilin in the diarrhea found in these patients, its contribution to the biological alterations remains unknown.

Because motilin hypersecretion could be expected to generate GI hypermotility and hypersecretion with probable diarrhea, it is logical to expect that hypomotilinemia

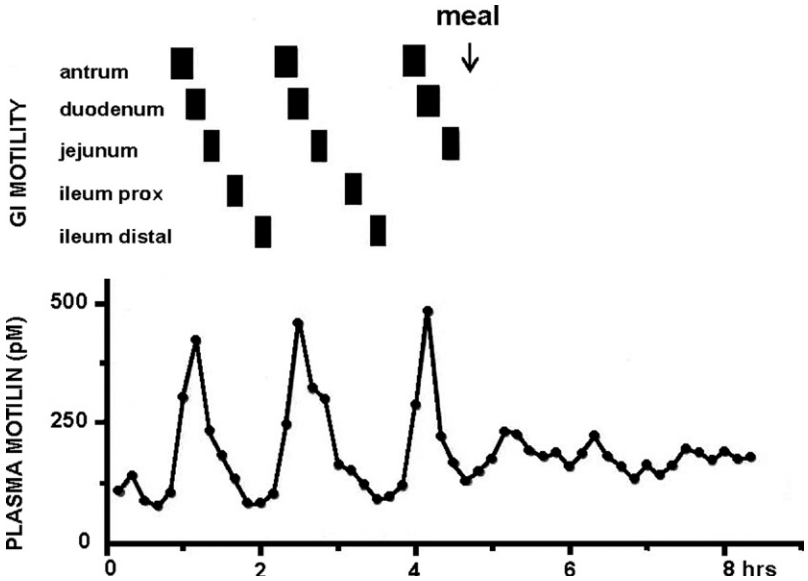

FIGURE 2 Schematic representation of canine plasma motilin variations. During the fasting interdigestive period, motilin is released cyclically every 80–120 min (lower panel) to induce the phase III contraction of the migrating motor complex (MMC) from the stomach to the ileum (indicated by the dark boxes in the upper panel). After eating, motilin cyclical peak increases are abolished for 2–8 h (depending on the content and nature of the meal) while the MMC is interrupted and the fed pattern motility profile is taking place.

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would induce GI hypomotility. Some investigators have indeed reported altered levels of plasma motilin in patients with idiopathic intestinal pseudoobstruction, idiopathic or postoperative gastroparesis, or irritable bowel syndrome (IBS). To this point, however, plasma motilin measurement has not been shown to be useful for inclusion in the workup diagnoses of any clinical situations.

Motilin Receptor Agonists and Antagonists

Motilin was recently of major interest to medical clinicians because of the capacity of motilin receptor agonists to act as powerful stimulants of GI motor activity in patients with hypokinetic disorders. Zen Itoh was the first to observe that erythromycin, a macrolide antibiotic, could mimic the motor effect of motilin when injected into dogs.[8] It was soon established that erythromycin was in fact acting on motilin receptors, and Janssens et al.[9] made the capital observation that erythromycin was the most potent gastrokinetic ever tested to stimulate gastric emptying in diabetic patients with gastroparesis. Since then, erythromycin, whether administered intravenously (iv) or by mouth (po), is used by many clinicians for the treatment of patients with gastroparesis or intestinal pseudoobstruction. Motilides or motilin receptor agonist substances derived from the erythromycin macrolide and with improved gastrokinetic activity but devoid of antibiotic properties have been developed by many pharmaceutical companies.[16] At least 3 motilides have been tested in humans, but for various reasons (e.g., rapid tachyphylaxis, no significant clinical benefit, potential side effects), clinical trials with these newly derived molecules have failed to substantiate the impressive pharmacological potential seen with iv erythromycin. The motilide mitemcinal (GM-611) accelerated gastric emptying in gastroparesis patients and provided relief of symptoms attributed to gastroparesis.[11] Atilmotin (a 14-amino acid motilin analog IV administered) accelerated gastric emptying of solids and liquids in humans.[10] The small molecule compound GSK 962040 also seems to be a motilin receptor agonist with a potential therapeutic role in humans.[20]

Motilin receptor antagonists were developed recently [MA-2029,[15] TZP-201[24]]. Based on our knowledge of the contractile capacity of motilin, we could wonder if these antagonists could have a therapeutic role in relaxing the small intestine or colon in patients with IBS or the gastric fundus in patients with functional dyspepsia.

REFERENCES

1. Boivin M, Pinelo LR, St-Pierre S, Poitras P. Neural mediation of the motilin motor effect on the human antrum. *Am J Physiol* 1997;**272** (1 Pt 1):G71–6.
2. Brown JC, Cook MA, Dryburgh JH. Motilin, a gastric motor activity stimulating polypeptide: the complete amino acid sequence. *Can J Biochem* 1973;**51**:533–7.
3. Coulie B, Tack J, Peeters T, Janssens J. Involvement of two different pathways in the motor effects of erythromycin on the gastric antrum in humans. *Gut* 1998;**43**(3):395–400.
4. Drossman MI. Physiological effects of gastrointestinal hormones. *Fed Proc* 1977;**36**:1930–1.
5. Feighner SD, Tan CP, McKee KK, Palyha OC, Hreniuk DL, Pong SS, et al. Receptor for motilin identified in the human gastrointestinal system. *Science* 1999;**284**(5423):2184–8.
6. Fiasse R, Deprez P, Weynand B, De Clercq P, Wibin E, Pauwels S, et al. An unusual metastatic motilin-secreting neuroendocrine tumour with a 20-year survival. Pathological, biochemical and motility features. *Digestion* 2001;**64**(4):255–60.
7. He J, Irwin DW, Chen R, Zhang YP. Stepwise loss of motilin and its specific receptor genes in rodents. *J Mol endocrinol* 2010;**44**(1):37–44.
8. Itoh Z, Nakaya M, Suzuki T, Arai H, Wakabayashi K. Erythromycin mimics exogenous motilin in gastrointestinal contractile activity in the dog. *Am J Physiol* 1984;**247**(6 Pt 1):G688–94.
9. Janssens J, Peeters TL, Vantrappen G, Tack J, Urbain JL, De Roo M, et al. Improvement of gastric emptying in diabetic gastroparesis by erythromycin. *N Engl J Med* 1990;**322**(15):1028–31.
10. Korimilli A, Parkman HP. Effect of atilmotin, a motilin receptor agonist, on esophageal, lower esophageal sphincter, and gastric pressures. *Dig Dis Sci* 2010;**55**(2):300–6.
11. McCallum RW, Cynshi O. US investigative Team. Efficacy of mitemcinal, a motilin agonist, on gastrointestinal symptoms in patients with symptoms suggesting diabetic gastropathy: a randomized, multi-center, placebo-controlled trial. *Aliment Pharmacol Ther* 2007;**26**(1):107–16.
12. Miller P, Roy A, St-Pierre S, Dagenais M, Lapointe R, Poitras P. Motilin receptors in the human antrum. *Am J Physiol Gastrointest Liver Physiol* 2000;**278**(1):G18–23.
13. Nakajima H, Mochiki E, Zietlow A, Ludwig K, Takahashi T. Mechanism of interdigestive migrating motor complex in conscious dogs. *J Gastroenterol* 2010;**45**(5):506–14.
14. Ohshiro H, Nonaka M, Ichikawa K. Molecular identification and characterization of the dog motilin receptor. *Regul Pept* 2008;**146** (1–3):80–7.
15. Ozaki K, Onoma M, Muramatsu H, Sudo H, Yoshida S, Shiokawa R, et al. An orally active motilin receptor antagonist, MA-2029, inhibits motilin-induced gastrointestinal motility, increase in fundic tone, and diarrhea in conscious dogs without affecting gastric emptying. *Eur J Pharmacol* 2009;**615**(1–3):185–92.
16. Peeters TL. Erythromycin and other macrolides as prokinetic agents. *Gastroenterology* 1993;**105**:1886–99.
17. Peeters TL, Aerssens J, DeSmet B, Mitselos A, Thielemans L, Coulie B, et al. The mouse is a natural knock-out for motilin and for the motilin receptor. *Neurogastro Mot* 2004;**16**:687:(abstract).
18. Poitras P. Motilin is a digestive hormone in the dog. *Gastroenterology* 1984;**87**:909–13.
19. Raymond MC, Boivin M, St-Pierre S, Gagnon D, Poitras P. Studies on the structure–activity of motilin in vivo. Effect of motilin synthetic analogues in conscious dog. *Regul Pept* 1994;**50**(2):121–6.
20. Sanger GJ, Westaway SM, Barnes AA, Macpherson DT, Muir AI, Jarvie EM, et al. GSK962040: a small molecule, selective motilin receptor agonist, effective as a stimulant of human and rabbit gastrointestinal motility. *Neurogastroenterol Motil* 2009;**21**(6):657–64:e30-1.
21. Takeshita E, Matsuura B, Dong M, Miller LJ, Matsui H, Onji M. Molecular characterization and distribution of motilin family receptors in the human gastrointestinal tract. *J Gastroenterol* 2006;**41**(3):223–30.

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22. Tolle V, Zizzari P, Tomasetto C, Rio MC, Epelbaum J, Bluet-Pajot MT. In vivo and in vitro effects of ghrelin/motilin-related peptide on growth hormone secretion in the rat. *Neuroendocrinology* 2001;**73**(1):54–61.
23. Tomasetto C, Karam SM, Ribieras S, Masson R, Lefebvre O, Staub A, et al. Identification and characterization of a novel gastric peptide hormone: the motilin-related peptide. *Gastroenterology* 2000;**119**(2):395–405.
24. Venkova K, Thomas H, Fraser GL, Greenwood-Van Meerveld B. Effect of TZP-201, a novel motilin receptor antagonist, in the colon of the musk shrew (*Suncus murinus*). *J Pharm Pharmacol* 2009;**61**(3):367–73.
25. Zietlow A, Nakajima H, Taniguchi H, Ludwig K, Takahashi T. Association between plasma ghrelin and motilin levels during MMC cycle in conscious dogs. Regulatory peptides. *Regul Pept* 2010;**164**(2–3):78–82.

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Chapter 171

Neurotensin

James M. Bugni and Charalabos Pothoulakis

ABSTRACT

Neurotensin (NT) is an enteric and brain peptide that elicits cellular signals in the gastrointestinal (GI) tract mainly on binding the 7-transmembrane G protein-coupled neurotensin receptor 1 (NTR-1). This receptor is expressed in a variety of cell types in the gut and regulates several enteric functions. NT is secreted in large quantities in the distal region of the small intestine in response to bacterial products and ingested fats and aids in the regulation of digestion, gastrointestinal secretions, and gut motility. Pathophysiologically, both NT and NTR-1 are significantly increased in expression during colitis, and signaling through this axis is important for modifying the degree of inflammation and also promoting the ensuing mucosal healing through proliferative and antiapoptotic effects. The ability of NT/NTR-1 to stimulate cell growth is usurped by neoplastic cells during cancer development, as the expression of NTR-1 is increased in colon cancer and colon cancer cell lines. This review summarizes the diverse roles of this peptide hormone in normal and diseased states in the GI tract.

DISCOVERY

The 13-amino acid peptide NT was first isolated by Carraway and Leeman from acid acetone extracts of bovine hypothalmi by column chromatography and paper electrophoresis.[7] Subsequently, high levels of the identical peptide were isolated using similar methods from calf small intestine extracts.[22] In the original report of its isolation, NT was discovered to stimulate contraction of the guinea pig ileum and contraction of the duodenum, indicating an important role for this peptide in gut motility and showing that NT is another biologically active peptide produced in both the brain and the gut.

STRUCTURE OF THE mRNA/GENE

The gene for NT (*NTS*) is located on Ch12 in humans and is about 9 kb in length. The primary transcript is processed from 4 exons to form a major mRNA species of about 1200 bp with the coding sequence for the precursor peptide spanning all 4 exons (Fig. 1A). On isolation of polyA+ RNA from canine mucosa and hybridization with probe specific to the coding region, the presence of multiple smaller mRNA species ranging in size from 500 to 980 bases are revealed.[11] These probably represent differentially spliced products. Indeed, 2 alternative splice products are predicted from the genomic sequence, one of which omits the second and another which omits the third exon. Proteins produced by these putative open reading frames (ORFs) would code for the two biologically active motifs (see below). However, the verified existence, differential expression, and functional significance of these variants have not been investigated.

PROCESSING OF THE PRECURSOR

NT is synthesized as a part of a 169- to 170-amino acid precursor polypeptide (proneurotensin/neuromedin N) that is processed to form NT and the 6-amino acid peptide neuromedin N, both located near the carboxy-terminal region of the propeptide (Fig. 1B). The amino terminal portion of the protein contains the signal peptide that targets the propeptide to the endoplasmic reticulum for secretion. The NT and neuromedin N peptides are separated by and each flanked by Lys-Arg amino acids that are recognition sequences for proteolytic processing. A homologous sequence is also present in the middle of the precursor protein, but it is not processed to a mature peptide. This internal sequence shares homology with an amphibian peptide, and thus, it probably represents and ancestral peptide. The propeptide sequence probably arose from tandem sequence duplication events and the mammalian structure is similar to the avian structure showing that the duplications occurred before the evolutionary divergence of these classes.[11]

Although NT and neuromedin N are synthesized from the same precursor peptide, they are often produced in distinct cell populations within the brain, gut, and the adrenal glands. The distinct cell type- and tissue-specific expression of either NT or neuromedin N is attributed to differential expression of prohormone convertases (PC), endopeptidases that recognize the dibasic Lys-Arg sequences that

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00171-8
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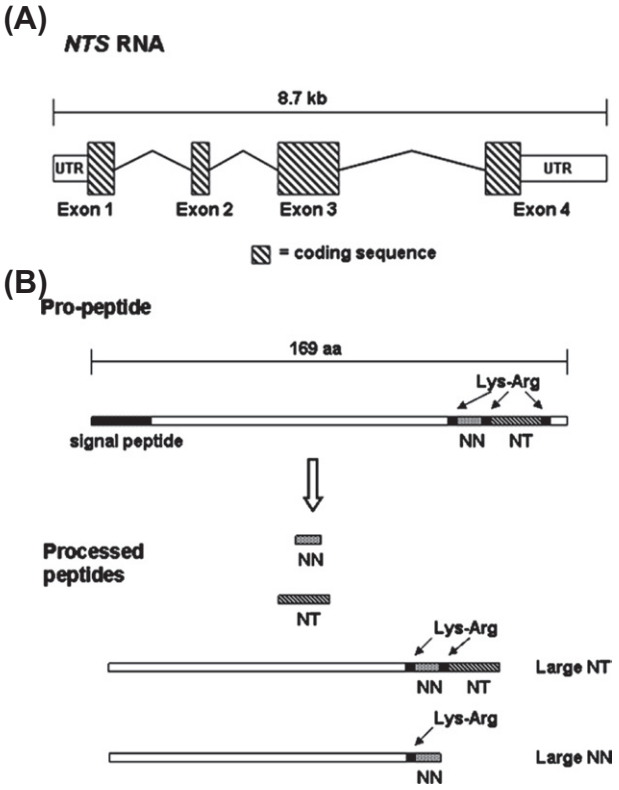

FIGURE 1 Neurotensin exon–intron and pro-peptide organization.

flank the NT and neuromedin N peptides.[21] These enzymes are discussed in detail in the Peptide Biosynthesis/Processing section of this book. Processing by the endopeptidases PC-1, PC-2, and PC-5A gives rise mainly to NT and neuromedin N in the brain, NT, and a large peptide with a C-terminal neuromedin N moiety (large NN) in the gut, and NT, large NN, and a large peptide with a C-terminal NT moiety (large NT) in the adrenals (Fig. 1B). Large NT seems to have activity as an agonist and is expressed normally in the intestine and in colon cancer cell lines. However, the full biological significance of large NT remains to be elucidated.

DISTRIBUTION OF THE PEPTIDE

Early studies showed that the GI tract is a major source of NT and that most NT in rats is localized in the intestine (~90%), whereas a much smaller percentage (~10%) is present in the brain.[22] Among the different areas of the intestine, the jejuno-ileal section expresses the highest NT concentration, although other parts of the GI tract such as esophagus, stomach, duodenum, and large intestine also express this peptide.[19] In mammals, NT-containing cells are scattered among enteroendocrine cells of the jejunal and and ileal mucosa, whereas in chicken, NT cells are more evident in the antrum.[19] NT-containing cells are abundant in the cat, dog, and human intestine and in most mammals are localized predominately on the villi, although in the canine intestine they are more numerous in the crypts. Ultrastructural analysis indicates that NT-producing neuroendocrine cells, also called N cells, contain cytoplasmic granules, predominantly in the basal portion of the cell that represent the storage site for this peptide.[19] This cellular localization facilitates rapid release of NT following specific stimuli and enables this peptide via receptor binding to target intestinal cells to modulate a variety of intestinal functions.

RECEPTORS AND SIGNALING CASCADES

Three NT receptors have been identified. The high affinity NTR1 (expressed from the NTSR1 gene) mediates most of the GI functions of NT as determined by use of specific, nonpeptide antagonists. Moreover, most NTR-2 expression is evident in the brain and other extragastrointestinal sites. The NTR3 is not of the GPCR class of receptors but belongs to the sortilin class. Expression of this receptor has been detected in colonic adenocarcinoma HT-29 cells, but the roles of NTR2 and 3 in gut physiology or pathophysiology remain uncharacterized. By contrast, NTR1 is highly conserved across mammals, and mediates several GI functions including chloride secretion, intestinal cell growth, and intestinal inflammatory and stress responses as described below.

A variety of intracellular signaling pathways are activated by neurotensin in colon cancer cell lines and in the nontransformed colonic epithelial cell line NCM460. Of the small G proteins that couple to GPCRs, NTR-1 is Gq/11 preferring, but this preference seems to be dependent on the cell type.[30] G protein activation stimulates and increases intracellular calcium and causes activation of the Pi3-kinase pathway, which mediates the NT/NTR-1-stimulated phosphorylation of the prosurvival molecule AKT. NT signaling through NTR-1 in NCM460 cells also elicits phosphorylation of insulin growth factor 1 receptor (IGF1R), which leads to NT-dependent activation of AKT and MAPK pathways.[32] Moreover, NT binding to NTR-1 causes the activation of the epidermal growth factor receptor (EGFR) and the downstream MAPK pathway by direct intracellular activation of EGFR and additionally through a mechanism that involves metalloproteinase-dependent cleavage of proTGFα in colonocytes.[34] NT-mediated activation of MAPK signaling and of the downstream transcription factor AP1 also occurs rapidly in human colorectal cancer cell lines and the pancreatic cancer PaCa-2 cells.[12] Moreover, NT stimulates activation of the small Rho-like GTPases RhoA, Rac1, and Cdc42, as well as activation of PKCδ that are, at least partially, responsible for NT-stimulated activation of the transcription factor NF-κB.[35]

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BIOLOGICAL ACTIONS WITHIN THE GASTROINTESTINAL TRACT

Stimulation of NT Production and Role in Fat Absorption

Certain bacterial products and food intake particularly in the form of dietary fat are major stimuli for NT release from the GI tract. For example, consumption of a meal or administration of oleic acid dramatically increases plasma concentrations of NT.[33] Presumably, signals generated at the proximal gut after fat or meal ingestion stimulate NT production in the distal small intestine, the main source of NT.[33] In humans, infusion of emulsions of fatty acids and several other lipids into the intestine inhibits acid secretion and increases NT levels, suggesting that NT plays a causal role in inhibiting gastric secretion.[27]

NT has been also implicated as a critical mediator of bile acid cycling, both release and uptake. In support for an important role for NT in fat digestion, NT infusion into the bloodstream causes increased oleic acid translocation across the intestinal lumen into the lymph of rats.[3] Intestinal absorption of taurocholate, an important conjugated bile acid, is potently enhanced by NT administration to fasted rats.[17] NT levels seem to be responsive to bile acid concentrations suggesting a dynamic relationship between the two. Administration of intestinal bile acid during feeding increases intestinal NT mRNA expression and circulating levels of NT,[18] and treatment of rats with the NTR1 antagonist SR-48692 inhibits intestinal taurocholate TC absorption.[18] These results suggest a role for endogenous NT in enterohepatic bile acid cycling. Although the mechanism of this NT response is not entirely clear, pharmacologic evidence indicates that NT-stimulated taurocholate uptake in rat jejunum may be dependent on mast cells.[17]

NT Inhibits Motility in the Intestine While Stimulating Motility in the Colon

Tests of isolated stomach preparations showed that administration of NT inhibits spontaneous motor activity.[2] NT infusion in humans also inhibits gastric and duodenal motility and shifts the motility pattern of the duodenum and proximal jejunum from a fasting- to a fed-type.[33] These studies support the role of NT in regulating postprandial motility in the upper intestine. Apart from peripheral NT, Studies by Buéno et al. indicate that central NT might also exert inhibitory effects in gastric motility.[33] Administration of NT to normal volunteers reduces pressure of lower esophageal sphincter, suggesting that this peptide can affect esophageal function.

In contrast to its inhibitory effect in the upper GI tract, peripheral NT administration increases motility in dog and cat colon.[29] In humans, intravenous NT administration increases the duration of contractions in both the ascending colon and the rectosigmoid area and stimulates defecation. The mechanism(s) of these NT effects may be mediated by release of endogenous acetylcholine, although direct NT effects on smooth muscle cells have also been proposed. Recent results indicate that NT can induce both excitatory and inhibitory responses in motor activity of the rat or human colon, indicating the complexity of this response.[31]

NT Inhibits Gastric Acid Secretion but Stimulates Chloride Secretion in the Colon

Several studies have shown that NT functions to inhibit gastric acid secretion in mammals. Gastric acid secretion is induced in response to a variety of stimuli in humans and dogs, including exposure to pentagastrin, a test meal, or insulin hypoglycemia, and several reports have shown that administration of NT reduces this response.

NT also seems to mediate secretory responses in small intestine and colon. In an early study, Eklund et al. showed that NT stimulates fluid secretion in the cat ileum.[13] This initial report was followed by several other studies, which showed that serosal administration of NT stimulates chloride secretion in guinea pig ileum,[20] porcine jejunum, isolated rabbit colonocytes, and human colonic epithelial HT29 cell monolayers.[26] Also, NT might play a role in exacerbating several colonic diarrheal disorders. Using chamber studies show that NT induces chloride secretion in human colon and that this effect is mediated by nerve cells, adenosine, and prostaglandins.[28] Pharmacologic and immunohistochemical studies also provide evidence that NT-stimulated chloride secretion is mediated by NTR1 expressed on nerve cells of the colonic mucosa.[28] Several mechanisms have been proposed to mediate the effects of NT in gastric and small intestinal secretion and motility, including release of somatostatin, norepinephrine, and/or in the case of motility direct effects on myocytes and cells of the myenteric plexus.[1] Although a direct molecular link for participation of NT in gastric acid output and gastric and upper small intestinal motility has not been firmly established, it does not seem to involve modulation of gastrin levels.[25]

PATHOPHYSIOLOGICAL IMPLICATIONS

Role of NT and NTR1 in Intestinal Inflammation

Early investigation of NT and intestinal inflammation used an *in vivo* model of acute colitis with *Clostridium difficile* toxin A, an enterotoxin that mediates antibiotic-associated colitis in animals and humans.[16] Later chemical injury models of colitis involving intracolonic administration of

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trinitrobenzene sulfonic acid (TNBS) or oral administration of the detergent dextran sulfate sodium (DSS) also showed an important role for NT in modulating inflammation. Examination of the causal role of NT in intestinal inflammation has been investigated mainly by the use of specific nonpeptide NTR1 antagonists and more recently with NT and NTR1 genetically deficient mice.

Injection of toxin A elicits a dramatic increase in NT and NTR1 expression in rat colonic mucosa within 4 h.[9] After exposure of ileal loops to luminal toxin A, pretreatment of rats with the NTR1 antagonist SR-48,692 significantly inhibits colonic fluid secretion, intestinal permeability, mucosal neutrophil infiltration, colonocyte damage, and mast cell activation.[9] These results strongly indicate that NT is a proinflammatory peptide in acute colonic secretion and inflammation mediated by a bacterial enterotoxin. The mechanism of the proinflammatory responses to NT may involve mast cell activation,[9] because mast cells contain NTR1 receptors that mediate histamine release in response to NT. NT signaling directly in leukocytes including neutrophils, macrophages, and lymphocytes may enhance inflammation, as NT stimulation has been shown to affect phagocytic, chemotactic, and adherence activity in these cell types.[16] NT may also affect inflammation by modulating vascular permeability through direct signaling through the NTR1 receptor expressed on endothelial cells.[16]

NT most probably also enhances inflammation indirectly by stimulating the expression of proinflammatory cytokines in human colonocytes and mesenteric preadipocytes. NTR1 is expressed in colonic epithelial cells in rat, mouse, and human colon, in the nontransformed human colonic epithelial cell line, NCM460, and in several human colonic epithelial cancer cell lines. NT-stimulated activation of NTR-1 elicits secretion of the proinflammatory cytokine interleukin-8 (IL-8), and the full response is dependent on an increase in intracellular calcium and activation of MAP kinase, NF-κB, PKC delta, and the small GTPases RhoA, Rac1, and Cdc42.[16] Mesenteric fat is another important tissue involved in colitis, particularly Crohn's disease in humans and experimental TNBS-induced colitis in animals. Levels of NT and NTR-1 increase dramatically in mouse preadipocytes in response to TNBS-induced colitis.[23] Studies using the NTR-1 antagonist SR-48629 showed that NT/NTR-1 signaling in preadipocytes causes a PKC-delta- and NF-κB-dependent increase in secretion of the proinflammatory cytokine IL-6, and causes a reduction in macrophage migration to inflamed foci in the colon. Treatment with SR-48629 also significantly ameliorates the degree of colitis in this model.[23] In the DSS model of colitis, mice deficient in NTR-1 have significantly lower total colonic expression of IL-6 and are resistant to lethality induced by DSS, although the degree of inflammation and epithelial damage is not significantly different from wild type mice.[6]

NT Promotes Cell Growth, Regeneration, and Healing of the GI Mucosa

NT is a trophic peptide able to stimulate cell growth and proliferation of epithelial cells in the gastric, small intestinal, and colonic mucosa in pathophysiologic situations where there is damage to the mucosa.[8] NT also increases cellularity and reverses mucosal hypoplasia in the intestine of rats fed an elementary diet. In rats, administration of NT accelerates the restoration of gut mucosal integrity after radiation-induced mucosal injury and small and large intestinal mucosal adaptation after colon resection.[24] NT stimulation of a proliferative response is also observed in cultured cells as NT activates growth in several colonic cancer cell lines. The positive effects of NT on cell growth seem to be usurped by cancer cells during tumor development as discussed below.

There is evidence that NT/NTR1 signaling participates in the mucosal healing phase of intestinal inflammation. Brun et al. showed that NT promotes mucosal healing in the DSS model of chronic colitis.[5] After oral administration of DSS, treatment with the NTR-1-specific antagonist SR 48692 exacerbates, whereas treatment with NT ameliorates mucosal damage, body weight loss, and neutrophil infiltration.[5] With DSS exposure, expression of NTR-1 shifts from mainly expression in the lamina propria to predominantly the epithelial layer, especially near epithelial erosions.[5] Moreover, NTR-1-mediated signals are probably important for ulcerative colitis (UC), as NTR-1 is expressed at higher levels in colonic biopsies from patients with active UC than noninvolved areas.[5] Exposure of human colonocytes to NT transactivates the EGFR, a critical receptor in colon tissue repair. Stimulation of proliferation and inhibition of apoptosis seem to be critical for epithelial expansion over eroded regions of the colon in the healing phase of colitis and NT/NTR-1 stimulation of the EGFR pathway and AKT are likely major contributors to this response.

NT Deregulation in GI Cancers

High levels of NTR-1 expression are found in human cancers from several anatomic sites including pancreatic, lung, breast, and prostate cancers as well as UC-associated colon cancer, and sporadic colorectal cancers.[14] Receptor overexpression in colon cancers can be explained, at least in part, by increased transcription of the NTR-1 gene via T cell transcription factor (TCF)/ lymphoid enhancer-binding factor (Lef)- and nuclear β-catenin-dependent pathways, as there are consensus TCF/Lef-binding sites in the NTR-1 promoter that are active in luciferase/reporter assays. Similarly, NT is also found at higher levels in colon cancers than in normal colon,[15] and *in vivo* evidence shows that neurotensin/NTR-1 signaling is causal for tumor growth. Xenograft

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transplants of human colon cancer cell lines become larger after NT administration and smaller when treated with the NTR-1 specific antagonist SR 48692.[14] NF-κB is an important regulator of inflammation-associated colorectal cancer and functions in both cell autonomous and non-autonomous mechanisms to enhance tumor development. At least part of the mechanism for the increased growth of subcutaneous colon cancer xenografts in nude mice seems to be through NT-stimulated expression of micro-RNAs, specifically miR-21 and miR-155, in an NF-κB dependent manner.[4] These miRNAs have been implicated in cancer development by inhibiting expression of PTEN and SOCS1, which negatively regulate the prosurvival kinase AKT.

In chemical carcinogenesis models, administration of NT in depot form to rats injected with the carcinogen azoxymethane increases tumor size and invasion into the muscularis.[14] Interestingly, this pathway is important for colon tumor formation in mice genetically deficient for NTR-1. NTR-1 deficiency reduces the number of tumors that develop but does not affect tumor size in a model of sporadic cancer.[6] Although NTR-1 plays a role in modifying expression of inflammatory cytokines in the DSS model of colitis, this pathway does not affect tumor development in the azoxymethane-DSS model of inflammation-associated cancer.[6] Moreover, the positive effect of NTR-1 in promoting colon tumor development in mice does not involve NTR-1 overexpression, unlike colon cancer development in humans where NTR-1 is often expressed at higher levels in cancer than in normal colon.[6] It is still unclear whether the effect of NT is directly on the neoplastically transformed epithelial cells, for example, through stimulation of proliferation and inhibition of apoptosis, or whether indirect mechanisms such as maintenance of tumor associated macrophages are important.

NT and Colonic Stress Responses

Irritable bowel syndrome (IBS) is a common debilitating functional gastrointestinal disorder with a wide variety of symptoms known to be affected by psychological stress. Thirty minutes of immobilization stress in rats increases plasma NT concentrations and stimulates colonic mucin release, goblet cell depletion, prostaglandin E2 secretion, and mast cell activation.[10] Administration of the NTR1 antagonist SR-48692 before restraint of rats attenuates stress-mediated secretion of colonic mucin and prostaglandin E2 and activates colonic mucosa mast cells. Although the mechanism of the NT-mediated colonic effects is not entirely clear, direct NT effects on colonic epithelial cells, and mast cells, leading to mucin and mast cell mediators release, respectively, might represent a likely mechanism.[10] These results indicate that NT may mediate some of the colonic responses and symptoms in patients with IBS.

SUMMARY

The findings discussed in this review show that NT and its high affinity receptor NTR-1 play an important role in the physiology and pathophysiology of several GI functions. Important normal intestinal functions associated with this peptide include modulation of motility, enterohepatic circulation of bile acids, and GI secretions. NT also plays a role in disease or other abnormal states include colonic cancer, mucosal regeneration and healing after damage, secretory diarrhea, IBS, and inflammation induced by chemicals or a bacterial enterotoxin. Evidence indicates that NT can directly affect many intestinal cell types such as myocytes, enteric nerves, mast cells, adipocytes, and epithelial cells of the small intestine and colon via specific high affinity receptors expressed in these cells. Thus identification of the particular cells of the GI mucosa expressing NT receptors in humans and studies on the signaling cascades that are activated after exposure of the different intestinal cells to NT would facilitate an understanding of the pathways activated by this peptide. Elucidation of these NT-related pathways is critical for designing effective therapeutic strategies for possible treatment of various GI diseases using NT receptor antagonists.

REFERENCES

1. Al-Saffar A. Analysis of the control of intestinal motility in fasted rats, with special reference to neurotensin. *Scand J Gastroenterol* 1984;**19**:422–8.
2. Andersson S, Rosell S, Hjelmquist U, Chang D, Folkers K. Inhibition of gastric and intestinal motor activity in dogs by (Gln4) neurotensin. *Acta Physiol Scand* 1977;**100**:231–5.
3. Armstrong MJ, Parker MC, Ferris CF, Leeman SE. Neurotensin stimulates [3H]oleic acid translocation across rat small intestine. *Am J Physiol* 1986;**251**:G823–9.
4. Bakirtzi K, Hatziapostolou M, Karagiannides I, Polytarchou C, Jaeger S, Iliopoulos D, et al. Neurotensin signaling activates microRNAs -21 and -155 and Akt, promotes tumor growth in mice, and is increased in human colon tumors. *Gastroenterology* 2011;**141**:1749–61.
5. Brun P, Mastrotto C, Beggiao E, Stefani A, Barzon L, Sturniolo GC, et al. Neuropeptide neurotensin stimulates intestinal wound healing following chronic intestinal inflammation. *Am J Physiol Gastrointest Liver Physiol* 2005;**288**:G621–9.
6. Bugni JM, Al-Rabadi L, Jubbal K, Karagiannides I, Lawson G, Pothoulakis C. The neurotensin receptor-1 promotes tumor development in a sporadic but not an inflammation-associated mouse model of colon cancer. *Int J Cancer* May 2011.
7. Carraway R, Leeman SE. The isolation of a new hypotensive peptide, neurotensin, from bovine hypothalami. *J Biol Chem* 1973;**248**:6854–61.
8. Carraway RE, Plona AM. Involvement of neurotensin in cancer growth: evidence, mechanisms and development of diagnostic tools. *Peptides* 2006;**27**:2445–60.
9. Castagliuolo I, Wang CC, Valenick L, Pasha A, Nikulasson S, Carraway RE, et al. Neurotensin is a proinflammatory neuropeptide in colonic inflammation. *J Clin Invest* 1999;**103**:843–9.

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10. Castagliuolo I, Wershil BK, Karalis K, Pasha A, Nikulasson ST, Pothoulakis C. Colonic mucin release in response to immobilization stress is mast cell dependent. *Am J Physiol* 1998;**274**:G1094–100.
11. Dobner PR, Barber DL, Villa-Komaroff L, McKiernan C. Cloning and sequence analysis of cDNA for the canine neurotensin/neuromedin N precursor. *Proc Natl Acad Sci U S A* 1987;**84**:3516–20.
12. Ehlers RA, Zhang Y, Hellmich MR, Evers BM. Neurotensin-mediated activation of MAPK pathways and AP-1 binding in the human pancreatic cancer cell line, MIA PaCa-2. *Biochem Biophys Res Commun* 2000;**269**:704–8.
13. Eklund S, Fahrenkrug J, Jodal M, Lundgren O, Sjoqvist A. Mechanisms of neurotensin-induced fluid secretion in the cat ileum in vivo. *Acta Physiol Scand* 1987;**129**:203–10.
14. Evers BM. Neurotensin and growth of normal and neoplastic tissues. *Peptides* 2006;**27**:2424–33.
15. Evers BM, Ishizuka J, Chung DH, Townsend Jr CM, Thompson JC. Neurotensin expression and release in human colon cancers. *Ann Surg* 1992;**216**:423–30:[discussion 30–1].
16. Gross KJ, Pothoulakis C. Role of neuropeptides in inflammatory bowel disease. *Inflamm Bowel Dis* 2007;**13**:918–32.
17. Gui X, Carraway RE. Involvement of mast cells in basal and neurotensin-induced intestinal absorption of taurocholate in rats. *Am J Physiol Gastrointest Liver Physiol* 2004;**287**:G408–16.
18. Gui X, Dobner PR, Carraway RE. Endogenous neurotensin facilitates enterohepatic bile acid circulation by enhancing intestinal uptake in rats. *Am J Physiol Gastrointest Liver Physiol* 2001;**281**: G1413–22.
19. Holzer P, Bucsics A, Saria A, Lembeck F. A study of the concentrations of substance P and neurotensin in the gastrointestinal tract of various mammals. *Neuroscience* 1982;**7**:2919–24.
20. Kachur JF, Miller RJ, Field M, Rivier J. Neurohumoral control of ileal electrolyte transport. II. Neurotensin and substance P. *J Pharmacol Exp Ther* 1982;**220**:456–63.
21. Kitabgi P. Neurotensin and neuromedin N are differentially processed from a common precursor by prohormone convertases in tissues and cell lines. *Results Probl Cell Differ* 2010;**50**:85–96.
22. Kitabgi P, Carraway R, Leeman SE. Isolation of a tridecapeptide from bovine intestinal tissue and its partial characterization as neurotensin. *J Biol Chem* 1976;**251**:7053–8.
23. Koon HW, Kim YS, Xu H, Kumar A, Zhao D, Karagiannides I, et al. Neurotensin induces IL-6 secretion in mouse preadipocytes and adipose tissues during 2,4,6,-trinitrobenzensulphonic acid-induced colitis. *Proc Natl Acad Sci U S A* 2009;**106**:8766–71.
24. Mata A, Gomez de Segura IA, Largo C, Codesal J, De Miguel E. Neurotensin increases intestinal adaptation and reduces enteroglucagon-like immunoreactivity after large bowel resection in rats. *Eur J Surg* 1997;**163**:387–93.
25. Mogard MH, Maxwell V, Sytnik B, Walsh JH. Regulation of gastric acid secretion by neurotensin in man. Evidence against a hormonal role. *J Clin Invest* 1987;**80**:1064–7.
26. Morris AP, Cunningham SA, Benos DJ, Frizzell RA. Cellular differentiation is required for cAMP but not Ca(2+)-dependent Cl-secretion in colonic epithelial cells expressing high levels of cystic fibrosis transmembrane conductance regulator. *J Biol Chem* 1992;**267**:5575–83.
27. Read NW, McFarlane A, Kinsman RI, Bates TE, Blackhall NW, Farrar GB, et al. Effect of infusion of nutrient solutions into the ileum on gastrointestinal transit and plasma levels of neurotensin and enteroglucagon. *Gastroenterology* 1984;**86**:274–80.
28. Riegler M, Castagliuolo I, Wang C, Wlk M, Sogukoglu T, Wenzl E, et al. Neurotensin stimulates Cl(-) secretion in human colonic mucosa in vitro: role of adenosine. *Gastroenterology* 2000;**119**:348–57.
29. Rothstein RD, Ouyang A. Mechanism of action of neurotensin at the ileocecal sphincter region. *Life Sci* 1989;**45**:1475–82.
30. Skrzydelski D, Lhiaubet AM, Lebeau A, Forgez P, Yamada M, Hermans E, et al. Differential involvement of intracellular domains of the rat NTS1 neurotensin receptor in coupling to G proteins: a molecular basis for agonist-directed trafficking of receptor stimulus. *Mol Pharmacol* 2003;**64**:421–9.
31. van der Veek PP, Schots ED, Masclee AA. Effect of neurotensin on colorectal motor and sensory function in humans. *Dis Colon Rectum* 2004;**47**:210–8.
32. Zhao D, Bakirtzi K, Zhan Y, Zeng H, Koon HW, Pothoulakis C. Insulin-like growth factor-1 receptor transactivation modulates the inflammatory and proliferative responses of neurotensin in human colonic epithelial cells. *J Biol Chem* 2011;**286**:6092–99.
33. Zhao D, Pothoulakis C. Effects of NT on gastrointestinal motility and secretion, and role in intestinal inflammation. *Peptides* 2006;**27**:2434–44.
34. Zhao D, Zhan Y, Koon HW, Zeng H, Keates S, Moyer MP, et al. Metalloproteinase-dependent transforming growth factor-alpha release mediates neurotensin-stimulated MAP kinase activation in human colonic epithelial cells. *J Biol Chem* 2004;**279**:43547–54.
35. Zhao D, Zhan Y, Zeng H, Koon HW, Moyer MP, Pothoulakis C. Neurotensin stimulates interleukin-8 expression through modulation of I{kappa}B{alpha} phosphorylation and p65 transcriptional activity: involvement of protein kinase C{alpha}. *Mol Pharmacol* 2005; **67**:2025–31.

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Chapter 172

Nesfatin-1

Shinsuke Oh-I, Hiroyuki Shimizu and Masatomo Mori

ABSTRACT

Nesfatin-1, an amino-terminal fragment derived from its precursor, nucleobindin-2 (NUCB2), has been identified as a novel anorexigenic molecule that is localized in adipose tissues and hypothalamic nuclei, which are involved in the feeding regulation and integrally connected with other brain areas. Nesfatin-1 is also expressed in gastrointestinal (GI) tract tissues such as the gastric mucosa, showing colocalization with ghrelin-positive granules, the duodenal mucosa, and the pancreatic β-cell cells, showing colocalization with insulin-positive granules. Peripheral injection of nesfatin-1 apparently produces anorexia in rodents, suggesting that the brain–gut axis of nesfatin-1 influences feeding behavior. Nesfatin-1 is also speculated to influence certain diseases of the central nervous system that are associated with digestive symptoms, as has been observed in some patients with a restricted type of anorexia nervosa and digestive organ diseases. However, a receptor specific to nesfatin-1 and the main tissue secreting nesfatin-1 into the peripheral blood remain to be clarified.

DISCOVERY, STRUCTURE, AND PROCESSING

Nesfatin/NEFA/nucleobindin-2 (NUCB2) was identified as a new appetite-regulating molecule by a subtraction-cloning assay used to determine genes activated by a peroxisome proliferator-activated receptor-γ activator (troglitazone) in SQ-5 cells. A computer-based program determined the signal peptides in the coding region of each of the 596 genes cloned in the assay. We performed further searches for genes expressed in both adipocytes and neuronal cells. NUCB2 is composed of a signal peptide of 24 amino acids in the N-terminal region and a protein structure containing 396 amino acids. NUCB2 is expressed in adipose and neuronal cells, and belongs to a gene family homologous to that of nucleobindin-1 (NUCB1). The functions of NUCB1 and NUCB2 as secreted proteins *in vivo* remain unknown, although both have been suggested to contribute to bone mineralization and antibody production. Nesfatin/NUCB2 may be processed by a prohormone convertase (PC) to yield nesfatin-1, nesfatin-2, and nesfatin-3. Among these fragments, only nesfatin-1, an 82-amino acid peptide located in the N-terminal region of nesfatin, is strongly anorexigenic in rodents (Fig. 1A). Nesfatin-1 is precisely present as a processing fragment in the cerebrospinal fluids of rats,[8] but our preliminary *in vitro* observations have shown that CHO cells overexpressing both NUCB2 and murine PC-1/3 or PC-2 fail to produce a fragment corresponding to nesfatin-1, suggesting the involvement of other PCs in the cleavage of NUCB2. Nesfatin-1 is distributed throughout the central nervous system and peripheral tissues, including white adipose tissues and those of the stomach, duodenum, and pancreas.[8,11] This chapter discusses the pathophysiological function of nesfatin-1 in these tissues.

CENTRAL AND PERIPHERAL SATIETY ACTION OF NESFATIN-1

Acute intracerebroventricular injection of nesfatin-1 reproducibly reduces food intake in rodents during the dark phase. In particular, acute or chronic injection of nesfatin-1 into the third ventricle of the rat brain produces decreases in body weight gain with concomitant reduction of body fat mass. It is especially interesting that the anorexigenic effect induced by nesfatin-1 is apparently independent of leptin signaling in the hypothalamus, because nesfatin-1-induced anorexia occurs in rats with a leptin receptor mutation. By contrast, previous injection of SHU9119, a melanocortin3/4 receptor (MC3/4R) antagonist, can block central nesfatin-1-induced reduction in food intake.[1] More recent studies support the notion that anorexia induced by nesfatin-1 is mediated, at least in part, by the oxytocin signaling pathway in the hypothalamic paraventricular nucleus (PVN), where urocortin and CRH_2 receptors are expressed.[4,10] The mechanisms by which nesfatin-1 act either in series or in parallel to recruit CRH_2, MC3/4R receptors, and oxytocin signal are still yet to be determined. However, these findings are interpreted as supporting the notion that a nesfatin-1-related signaling pathway is involved in the complex and integrated neurotransmission networks in the brain hypothalamus that produce anorexia.

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00172-X
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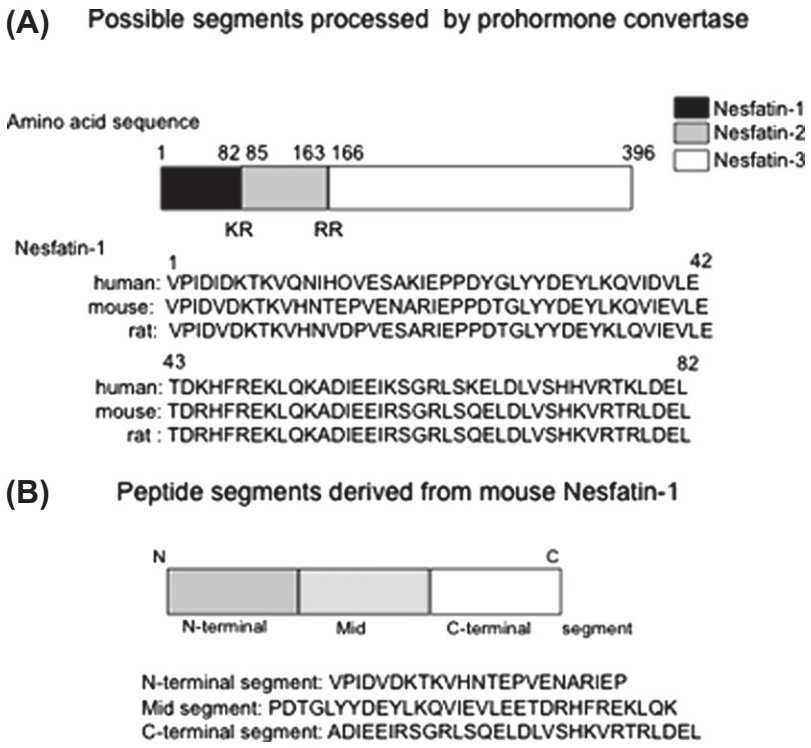

FIGURE 1 A. Structure of the precursor of nesfatin-1, NUCB2. B. Amino acid sequence of each segment derived from nesfatin-1.

Intraperitoneal injection of nesfatin-1 also produces a dose-dependent reduction in food intake in mice fed *ad libitum*. Because nesfatin-1 possesses a characteristic beta-sheet structure, three peptide segments based on the structure of the beta-sheet were artificially produced as follows: an N-terminal segment (23 amino acid residues), a midsegment (30 residues), and a C-terminal segment (29 residues). These segments correspond to the nesfatin-1 alignment of 1–23, 24–53, and 54–82 amino acid residues (Fig. 1B). Among these segments, only the midsegment (24–53 residues) showed the ability to reduce food intake. Moreover, the midsegment had an interesting structure similar to that of an activation site of agouti-related protein (AgRP), and α-melanocyte stimulating hormone (α-MSH). Intraperitoneal injection of the midsegment produces anorexigenic effects in the leptin-resistant animal models of obesity, such as genetically diabetic (*db*/*db*) mice and mice fed a high-fat diet, suggesting again that nesfatin-1-induced anorexia is independent of leptin signaling. Taken together, these findings indicate that nesfatin-1 is a new bioactive substance associated with antiobesity and that nesfatin-1 may suppress hyperphagia in obese patients with leptin resistance.

DISTRIBUTION AND BIOLOGICAL ACTION OF NESFATIN-1 IN THE GI TRACT

The fact that nesfatin-1-immuno-positive cells are selectively localized in the stomach, duodenum, and pancreas suggests that nesfatin-1 may play important roles in gastrointestinal functions similar to those observed with other satiety-regulating neuropeptides. Signal transmission from peripheral vagal afferent nerves to the neurons in the nucleus tractus solitarius (NTS) in the brain stem involves muscarinic and nicotinic cholinergic receptors. We characterized the possible role of cholinergic signals transmitted to the NTS to produce anorexia. Although atropine methyl nitrate, an antagonist for the muscarinic receptor, does not affect food intake suppression induced by peripheral injection of the midsegment of nesfatin-1, hexamethonium, a nicotinic cholinergic blocker, abolishes this suppression.[9] These results indicate that part of the anorexigenic effect of peripheral nesfatin-1 is mediated by cholinergic neurons with the nicotinic receptor.

Nesfatin-1 is expressed in the X/A-like cells of the gastric mucosa, which consists of 20–25% of the endocrine cells and contains vesicles with bioactive

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substances. X/A-like cells have key roles in the regulation of gastrointestinal mobility and ghrelin secretion. Ghrelin is a stimulatory factor of growth hormone and a potent orexigenic factor (see ghrelin chapters in this and other sections of the book). Immunopositive vesicles for nesfatin-1 and ghrelin are differently recognized in X/A-like cells of the gastric oxyntic mucosa. These observations suggest that nesfatin-1 and ghrelin are released separately under different metabolic conditions. However, whether these factors coordinate food intake within the X/A-like cells remain to be clarified. Peripheral injection of some anorexigenic molecules, such as peptide YY, produces aversive effects, resulting in reduced appetite. By contrast, nesfatin-1 has no such an effect in rodents.[9] Intracerebroventricular injection of nesfatin-1 decreases food intake and inhibits gastroduodenal motility in a dose-dependent manner,[1,10] by a pathway independent of CRH receptor-2 signaling. Nesfatin-1 expressed in the gastric mucosa decreases on starvation,[11] suggesting that peripheral nesfatin-1 has significant effects on gastrointestinal function.

In rodents, nesfatin-1 is also expressed in Brenner's gland in the duodenum. Brenner's gland is located in the submucosa of the duodenum and pancreas,[15] and constitutes the hepatopancreatic sphincter (Sphincter of Oddi). This gland plays an important role in maintaining appropriate pH conditions in the duodenum by activating digestive alkalizing enzymes, thus protecting the duodenal mucosa from tissue-damaging factors such as gastric acid. Although the physiological function of nesfatin-1 in Brunner's gland *in vivo* is not clear, there is a possibility that nesfatin-1 influences exocrine function in the pancreas by stimulation of propancreatic external secretion and internal secretion.

Some of the brain–gut peptides, such as pituitary adenylate cyclase-activating polypeptide (PACAP) and ghrelin, are expressed and secreted in both the brain and pancreatic islets.[6,13] Interestingly, nesfain-1 is also synthesized and secreted by islet cells and potentiates glucose-induced insulin release in an autocrine manner by promoting Ca^{2+} influx through L-type Ca^{2+} channels in β-cells,[5] suggesting that it has insulinotropic effects.[3] The possibility of nesfatin-1 regulating glucose metabolism requires detailed clarification under diabetic conditions.

PATHOPHYSIOLOGICAL IMPLICATION

To investigate the clinical significance of nesfatin-1 in humans, we had established a sandwich-typed enzyme-linked immunosorbent assay (ELISA) system to detect human nesfatin-1 in the peripheral blood.[12] This specific and sensitive ELISA does not detect whole nesfatin/NUCB2. Exogenous administration of nesfatin-1 may be effective in improving hyperphagia in obese subjects with low blood levels of nesfatin-1. Blood levels of nesfatin-1 in patients with restricted type of anorexia nervosa are significantly higher than those of nesfatin-1 in normal controls, suggesting that nesfatin-1 is regulated by certain nutrition status and contributes to anorexia in such patients.[7] Circulating concentrations of nesfatin-1 are lower in obese subjects than in nonobese subjects, showing an inversed correlation with increased body weights.[12] Patients with newly diagnosed primary generalized epilepsy have about 120- and 160-fold higher circulating and salivary NUCB2/nesfatin-1 levels, respectively, and these are normalized after treatment.[2] These findings suggest that monitoring of nesfatin-1 in blood is useful as a biomarker not only for diagnosing illness but also for estimating responsiveness to treatment among patients with anorexia nervosa or epilepsy.

Nesfatin-1 also influences cardiac function by increasing blood pressure via its activation of the sympathetic nervous system through the MC3/4R signaling pathway in the brain,[14] suggesting that nesfatin-1 neurons are connected with MC3/4R signal and regulate the cardiovascular system.

PERSPECTIVE

Nesfatin/NUCB2 is possibly processed to nesfatin-1, which is present in a number of peripheral tissues including white adipocytes and those of the gastrointestinal tract (stomach, duodenum, and pancreas), in addition to the brain regions. In response to changes in nutrient conditions, nesfatin-1 is released to the peripheral blood. Nesfatin-1 has an important function in the brain, especially in the hypothalamic PVN and NTS, which receives directly and/or indirectly satiety signals from peripheral tissues. However, how nesfatin-1 functions to regulate the systemic energy balance remains to be elucidated (Fig. 2). One of the key means of addressing this function will be to identify a receptor specific to nesfatin-1. We have shown that nesfatin-1 activates the MC3/4R-dependent anorexigenic pathway being independent of leptin signaling in the central nervous system and that it does not directly stimulate intracellular concentrations of cyclic AMP in cells expressing MC3/4R. Although the exact intracellular signaling system remains unclear, nesfatin-1-induced suppression of food intake may be associated with a type of G protein-coupled receptor that evokes Ca^{2+} influx.

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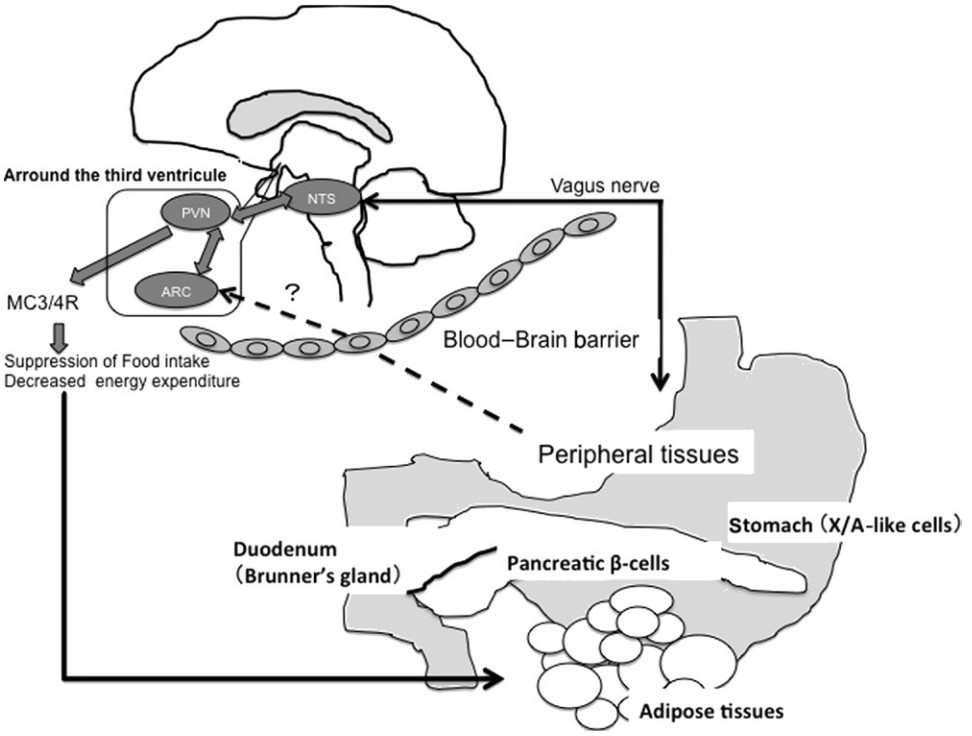

FIGURE 2 Possible interactions among tissues/organs expressing nesfatin-1. PVN, paraventricular nucleus; NTS, nucleus tractus solitarius; ARC, arcuate nucleus.

REFERENCES

1. Atsuchi K, Asakawa A, Ushikai M, Ataka K, Tsai M, Koyama K, et al. Centrally administered nesfatin-1 inhibits feeding behaviour and gastroduodenal motility in mice. *Neuroreport* 2010;**21**:1008–11.
2. Aydin S, Dag E, Ozkan Y, Erman F, Dagli AF, Kilic N, et al. Nesfatin-1 and ghrelin levels in serum and saliva of epileptic patients: hormonal changes can have a major effect on seizure disorders. *Mol Cell Biochem* 2009;**328**:49–56.
3. Gonzalez R, Perry RL, Gao X, Gaidhu MP, Tshushima RG, Ceddia RB, et al. Nutrient responsive nesfatin-1 regulates energy balance and induced glucose-stimulated insulin secretion in rats. *Endocrinology* 2011;**152**:3628–37.
4. Maejima Y, Sedbazar U, Suyama S, Kohno D, Onaka T, Takano E, et al. Nesfatin-1-regulated oxytocinergic signaling in the paraventricular nucleus causes anorexia through a leptin-independent melanocortin pathway. *Cell Metab* 2009;**10**:355–65.
5. Nakata M, Manaka K, Yamamoto S, Mori M, Yada T. Nesfatin-1 enhances glucose-induced insulin secretion by promoting Ca (2+) influx through L-type channels in mouse islet β-cells. *Endocr J* 2011;**58**:305–13.
6. Nakata M, Yada T. PACAP in the glucose and energy homeostasis: physiological role and therapeutic potential. *Curr Pharm Des* 2007;**13**:1105–12.
7. Ogiso K, Asakawa A, Amitani H, Nakahara T, Ushikai M, Haruta I, et al. Plasma nesfatin-1 concentrations in restricting-type anorexia nervosa. *Peptides* 2011;**32**:150–3.
8. Oh-I S, Shimizu H, Satoh T, Okada S, Adachi S, Inoue K, et al. Identification of nesfatin-1 as a satiety molecule in the hypothalamus. *Nature* 2006;**443**:709–12.
9. Shimizu H, Oh-I S, Hashimoto K, Nakata M, Yamamoto S, Yoshida N, et al. Peripheral administration of nesfatin-1 reduces food intake in mice: the leptin-independent mechanism. *Endocrinology* 2009;**150**:662–71.
10. Stengel A, Goebel M, Wang L, Rivier J, Kobelt P, Mönnikes H, et al. Central nesfatin-1 reduces dark-phase food intake and gastric emptying in rats: differential role of corticotrophin-releasing factor2 receptor. *Endocrinology* 2009;**150**:4911–9.
11. Stengel A, Goebel M, Yakubov I, Wang L, Witcher D, Coskun T, et al. Identification and characterization of nesfatin-1 immunoreactivity in endocrine cell types of the rat gastric oxyntic mucosa. *Endocrinology* 2009;**150**:232–8.
12. Tsuchiya T, Shimizu H, Yamada M, Osaki A, Oh-I S, Ariyama Y, et al. Fasting concentrations of nesfatin-1 are negatively correlated with body mass index in non-obese males. *Clin Endocrinol* 2010;**73**:484–90.
13. Yada T, Dezaki T, Sone H, Koizumi M, Damdindorj B, Nakata M, et al. Ghrelin regulates insulin release and glycemia: physiological role and therapeutic potential. *Curr Diabetes Rev* 2008;**4**:18–23.
14. Yosten GL, Samson WK. Nesfatin-1 exerts cardiovascular actions in brain: possible interaction with the central melanocortin system. *Am J Physiol Regul Integr Comp Physiol* 2009;**297**:R330–6.
15. Zhang AQ, Li XL, Jiang CY, Lin L, Shi RH, Chen JD, et al. Expression of nesfatin-1/NUCB2 in rodent digestive system. *World J Gastroenterol* 2010;**16**:1735–41.

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Chapter 173

Obestatin

Guillaume Gourcerol

ABSTRACT

Obestatin is a novel 23-amino acid peptide derived from the processing of the ghrelin gene. The peptide name was in keeping with its initially reported actions to inhibit food intake and gastrointestinal motility thereby opposing the stimulatory effect of ghrelin. However, subsequent reports challenged these findings because several groups hardly reproduced the original obestatin actions on satiation and gut motility and questioned the synthesis of obestatin in the stomach to further act as a gastrointestinal hormone via the bloodstream. In addition, although GPR39 has been initially considered to be the cognate receptor of obestatin, this has been recently disclaimed leaving GPR39 as an orphan receptor again. Moreover, plasma obestatin has been found to decrease postprandially, and to be negatively correlated with the body mass index, paralleling the concentration of plasma ghrelin. Hence, further investigations are warranted to ascertain the real role of obestatin as a bioactive peptide rather than as a ghrelin-gene product.

OBESTATIN DISCOVERY

In 2005, Zhang et al. from the Stanford University School of Medicine discovered a novel peptide derived from the ghrelin peptide precursor.[33] The 23-amino acid peptide was identified by a bioinformatic approach, which compared the preproghrelin sequences among 11 mammalian species and spotted a conserved region flanked by a potential cleavage site. Based on the initial reported biological actions, these authors named the newly discovered peptide "obestatin" as a contraction from the Latin verb "obedere," meaning to devour, and "statin," implying suppression. Contrary to the appetite and gastrointestinal (GI) motility-stimulating effects of ghrelin, the authors reported in their initial study that obestatin decreased food intake, body weight gain, and gastric transit. Moreover, the authors found that obestatin inhibited the ghrelin stimulatory effects on feeding and gastrointestinal contractile activity. These "*Yin and Yang* personalities of ghrelin and obestatin" were reinforced by selective binding of obestatin to the orphan receptor, G protein-coupled receptor (GPR) 39, which belongs, as well as the ghrelin receptor (GHS-R1a) and motilin receptor (GPR38), to the class A family of rhodopsin receptors. Although it was shown that one gene could encode more than one peptide, the fact that two biologically active peptides issued from the same prepropeptide antagonizes each other's actions remains a unique finding. This major breakthrough brought a novel exciting insight into the gut peptide field and triggered a flurry of investigations that, however, reported discrepant results that still fuel the debate on the real impact of obestatin on food intake and gastric motor function.

PROCESSING FROM THE GHRELIN GENE TO OBESTATIN

Obestatin is a putative 23-amino acid peptide, which derives from the ghrelin gene (GHRL),[33] located in humans in the short arm of chromosome 3 (3p25–26). The ghrelin gene consists of 4 exons that give rise to a 117-amino acid preprohormone named preproghrelin[7] (Fig. 1). Preproghrelin harbors the preproghrelin signal peptide encoded by exon 1, the 28-amino acid ghrelin peptide hormone encoded by parts of exons 1 and 2, and a 66-amino acid C-terminal peptide named C-ghrelin, encoded by the remainder of exon 2 and exons 3 and 4.[7] Although this C-terminal peptide has been detected in the human circulation, its physiological relevance remains unknown and may rather reflect changes in the transcription or the processing of preproghrelin. Finally, the ghrelin gene also encodes for an exon 3-deleted truncated C-ghrelin which further codes for ghrelin but not for obestatin.

The obestatin$_{1–23}$ sequence is identical to the residues 76–98 of the C-ghrelin, but whether obestatin is posttranslationally cleaved from C-ghrelin or is a product of alternative gene splicing remains unknown. Obestatin requires a further C-terminal amidation to be biologically active, which is a common posttranslational feature of biologically active peptides. To date, an additional isoform of obestatin, Obestatin$_{11–23}$ has also been identified in stomach extracts.[33] However, the biological role of obestatin$_{11–23}$ has not yet been established. A recent nuclear magnetic resonance study detailed the structural properties of both obestatin isoforms and identified a conserved sequence QYQQHGR

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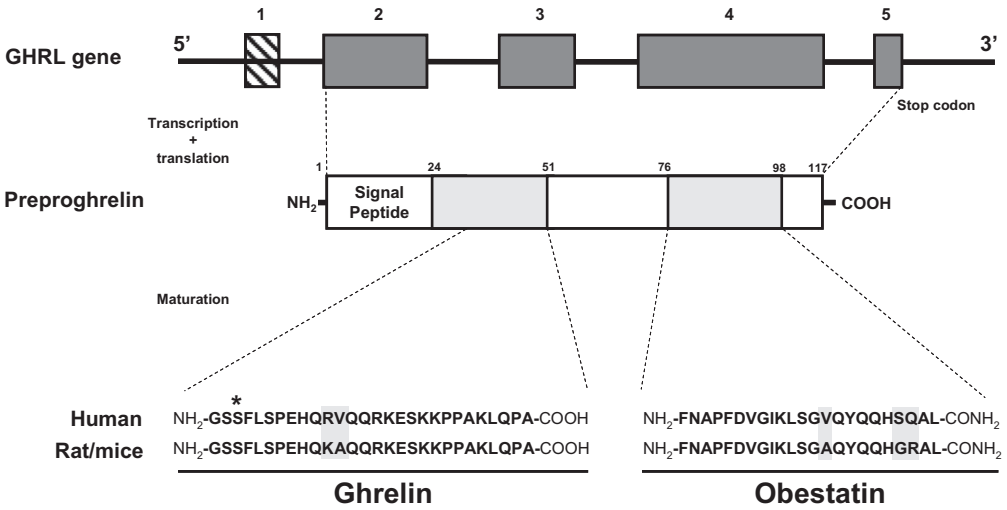

FIGURE 1 Schematic representation of the ghrelin gene and of transcriptional, translational, and posttranslational processes leading to the synthesis of ghrelin and obestatin. Note that the first exon is noncoding. The acylation of the hydroxyl group of Ser^3 indicated by asterisk (*) is required for the biological activity of ghrelin. Nonconserved amino acid are represented as shaded letters. Adapted from Gourcerol G and Taché Y. Neurogastroenterol Motil 2007;19:161–5.

with a 3-D conformation sharing similarities with neurotensin, which might indicate the ability of obestatin to bind a GPR.[26]

OBESTATIN DISTRIBUTION

Tissue Distribution

In contrast with ghrelin, which is known to be primarily synthesized in the stomach and then released into the blood circulation to act as a hormone,[7] limited studies have attempted to detect C-ghrelin-derived peptides, including obestatin, in GI tissues. In the initial report from Zhang et al., these authors used combined gel permeation chromatography and mass spectrometric analyses, and found that endogenous $obestatin_{1–23}$ and $obstestatin_{11–23}$ isoforms could be detected from rat stomach tissues.[33] To date, this has not been further investigated by the same approach, whereas subsequent reports using immunochemistry or radioimmunoassay (RIA) on stomach tissues drew divergent conclusions. For instance, obestatin immunoreactivity was found in cells of rat and human gastric mucosa,[10,16] as well as in gastric myenteric cholinergic neurons.[10] On the other hand, Chanoine et al. detected very little obestatin in rat stomach tissues.[5] Similarly, Bang et al. using combined high-performance liquid chromatography and RIA failed to detect obestatin immunoreactivity in the gastric extract from fasted rats.[3] Therefore, the biochemical identification of obestatin as a distinct and endogenous peptide in stomach remains to be confirmed. In addition to the stomach, obestatin immunoreactivity has also been found in the pancreas,[16] whereas discrepant immunoreactivity has been reported in the testis.[10,16]

Plasma Concentrations

The initial report from Zhang et al. unexpectedly pointed out that plasma obestatin, unlike ghrelin and other GI hormones involved in food intake, did not vary after a meal.[33] Although further studies in rodents led to divergent observations, with a fast inducing increased or decreased plasma obestatin, results in humans have been more consistent. Most of the reports found that plasma obestatin parallels plasma ghrelin concentration. For instance, circulating obestatin decreases after a meal in most of the studies in healthy volunteers.[7] Likewise, fasting plasma obestatin is negatively correlated with the body mass index (BMI) and plasma insulin. In parallel to ghrelin, obestatin is decreased after sham feeding or after atropine administration in healthy subjects.[7] Plasma obestatin levels are lower in patients with chronic atrophic gastritis or during *Helicobacter pylori* infection, and after gastrectomy, which suggests that circulating obestatin originates from the stomach.[7] Recently, obestatin plasma concentrations have been reinvestigated with reversed-phase high-performance liquid chromatography coupled with RIA.[22] These authors found very small amounts of obestatin that were 50- and 20-fold lower in the stomach and plasma, respectively, by comparison with ghrelin. Similarly, Bang et al. failed to detect obestatin immunoreactivity in rat blood samples.[3] This contrasts with the measured fasting plasma concentrations of obestatin in normal-weight volunteers, which range from 35 to 95 pg/mL, whereas total fasting circulating ghrelin varies from 100 to 200 pg/mL.[34] In the study by Bang et al., both ghrelin and proghrelin immunoreactivity were found in high concentrations and further decreased after feeding and an oral glucose test.[3] Moreover, specific immunoassays directed to the

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N-terminus, C-terminus, and midregion of proghrelin all detected the same molecular species of 7000 kDa, suggesting that the only forms detected were close to the full length 66-amino acid C-ghrelin peptide.[3] Therefore, in addition to the crossreactivity with C-terminal ghrelin gene products, the release of obestatin and obestatin precursors accompanying preprandial ghrelin release might explain the parallel obestatin and ghrelin levels detected in blood samples. In addition, the variability of measured plasma obestatin concentrations might relate to the short half life of the peptide (<15 min), which suggests that obestatin may act through paracrine/autocrine pathway rather than as a GI hormone.[24]

OBESTATIN AND GPR39

GPR39 was originally cloned in 1997 and characterized to belong to the class A of the 7-transmembrane domain GPR as part of the ghrelin subfamily that also includes GHS-R1a, GPR38, neurotensin receptor 1, neurotensin receptor 2, neuromedin U receptor 1 (GPR66), and neuromedin U receptor 2.[7,20] GPR39 signals with high constitutive activity mainly through G_q and $G_{12/13}$ signaling pathways resulting in increased inositol phosphate turnover, as well as activation of the cAMP response element and serum response element transcriptional activity. The GPR39 receptor was first reported by Northern-blot analysis to be widely expressed in the human brain, while its peripheral distribution was restricted to the stomach and small intestine[20] supporting the idea that cognate ligand would exert an action within the GI tract. Because GHS-R1a and GPR38 are important receptors regulating GI motility, the influence of GPR39 on GI transit was recently shown in transgenic mice lacking GPR39. GPR39−− mice display accelerated gastric emptying and increased fecal pellet output compared with their wild-type littermates.[21] However, GPR39 knockout mice have similar body weight, adiposity, and food intake compared with their age-matched littermate controls, suggesting that GPR39 is not a major regulator of body weight at least in lean mice.[21,29] GPR39 is expressed in the endocrine pancreas, and it decreases postprandial plasma insulin concentrations in mice.[29] Zinc ions activate GPR39 signaling at micromolar concentrations.[6,17] It remains to be determined whether Zn++ is the only natural ligand for GPR39, and whether Zn++ functions as an agonist by itself or as an enhancer of GPR39 activation by another endogenous ligand, which still remains to be identified.

In 2005, Zhang et al. reported that obestatin was the cognate ligand for the orphan GPR39 receptor.[33] They observed, in a crude plasma membrane preparation of rat jejunum, that obestatin bound with high affinity to GPR39 although other members of the ghrelin subfamily did not.[33] Furthermore, obestatin displayed a high affinity to GPR39 receptors overexpressed in CHO or HEK293—T cells and also stimulated cAMP release.[33] In addition, Zhang et al., using real-time quantitative polymerase chain reaction (RT-PCR) analyses reported that the highest levels of GPR39 transcripts were expressed in the small intestine, stomach, liver, and hypothalamus, which was consistent with the results of ^{125}I-obestatin binding studies. However, later these findings were challenged by several independent groups. For instance, subsequent studies using in situ hybridization in mice and RT-PCR analysis in rats failed to detect the expression of GPR39 mRNA in the hypothalamus,[17,23] a region primarily involved in food intake regulation. In addition, Holst et al., who previously established and characterized the constitutive activity of GPR39, were unable to confirm that obestatin has agonist properties on the GPR39 receptor.[17] This was supported by the absence of obestatin signaling in GPR39 expressing cells as monitored by inositol phosphate turnover, cAMP, arrestin mobilization, as well as cAMP response element-dependent and serum response element-dependent transcriptional activity.[17] In addition, radiolabeled obestatin did not bind GPR39 in different types of GPR39 expressing cells.[17] Similarly, Lauwers et al. [19] were unable to show that obestatin elicits GPR39 signaling in transfected cells as monitored by cAMP under similar conditions of incubation as reported previously.[33] Lastly, Chartrel et al. reported in 2007 that ^{125}I-obestatin did not bind GPR39 and observed no effects of obestatin on GPR39-transfected cells using similar functional assays (cAMP production, Ca++ mobilization, and GPR39 internalization). This led the obestatin discoverers to acknowledge inconsistencies in the binding of iodinated obestatin to GPR39 resulting from impurities of iodinated obestatin. Thus, the authors disclaimed the ability of obestatin to bind GPR39 in a partial retraction.[32] More recently, however, the same research group using purified iodinated obestatin reported that obestatin was capable of binding to GPR39 in human embryonic kidney 293—T-transfected cells and observed obestatin binding to the jejunum, stomach, ileum, pituitary, and adipose tissue.[31] In addition to GPR39, Granata et al. reported that obestatin could also bind the glucagon-like peptide-1 receptor on HIT-T15 and INS-1E β-cells with low affinity.[14] However, this was later questioned by Unniappan et al., who did not observe binding of obestatin to INS-1 β-cells or HEK cells overexpressing GLP-1 receptor.[30] Altogether, although the involvement of GPR39 in GI motility has been shown by several groups, there is to date no clear evidence that GPR39 is activated by obestatin.

Biological Actions of Obestatin

Food Intake and Body Weight

Zhang et al.'s main findings in their initial report indicated that obestatin suppressed food intake for 5 h after a single intraperitoneal (ip) injection at doses ranging from 320 μg/kg

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to 2.5 mg/kg (125–1000 nmol/kg) in fasted mice.[33] This was associated with a reduction in the body weight on repeated daily ip administration in lean mice. In addition, obestatin injected into the mouse lateral brain ventricle (icv) at 20 μg/kg inhibited food intake almost completely for the first hour and significantly reduced cumulative food intake for the following 5-h experimental period. The initial promise of a new regulator of appetite and body weight was curtailed by convergent studies negating these findings under a variety of conditions. To date, out of 22 studies, only 5 reproduced the anorexigenic action of obestatin whereas 17 failed to observe any effect (for a review, see Ref. 13).

Such a large discrepancy between the initial and subsequent reports is hardly explained by the different experimental protocols or species used.[13] Site and modalities of obestatin injection, either ip, subcutaneous (sc), intravenous (iv), or intracerebroventricular (icv), on acute or chronic administration, were equally ineffective in influencing food intake or decreasing body weight.[13] This was observed under conditions in which anorexigenic control substances such as cholecystokinin (CCK)-8, melanocortin-receptor agonist, MT-II, exendin, or sibutramine resulted in the expected satiety effect or body weight reduction.[13] In addition, doses of obestatin injected centrally or peripherally were in a similar or even higher range than those reported by Zhang et al. because doses up to 2.5 mg/kg, ip and 200 μg/kg, icv in mice, and doses up to 3 mg/kg, ip in rats remained ineffective in decreasing food intake.[11] Since then, Zhang et al. specified that anorexigenic effects of obestatin could only be observed under a precise experimental protocol, for example, with peptide injection performed 15 min before the chow is given to fasted animals.[32] However, negative findings were also obtained under testing conditions that mimicked those used in the initial report.[11,17,23] In addition, these discrepancies cannot be explained by the species used (rat versus mouse), nutritional status (fed versus fasted, lean versus obese animals), animal background (ghrelin-deficient versus wild-type mice) or obestatin form (humans versus rodents).[13]

Molecular approaches also resulted in contradictory findings as observed in the different functional assays. For instance, acute iv injection of obestatin induced c-fos protein expression within the nucleus of the solitary tract and the paraventricular nucleus of the hypothalamus, two key nuclei involved in the control of food intake and GI motility.[2] However, further studies failed to observe any increase in c-fos expression in these nuclei[18] or activation of gastric vagal afferent discharge[12] after peripheral injection of obestatin. In addition, the icv injection of obestatin (20 μg/kg/day for 7 days) did not influence the expression of key hypothalamic peptides regulating food intake, namely, neuropeptide Y, proopiomelanocortin, agouti-related peptide, or cocaine and amphetamine-regulated transcript.[23] Finally, the short half life of obestatin in the blood circulation and the absence of a specific transport system by endothelial cells composing the blood–brain barrier do not support the role of obestatin as a stable hormone involved in food intake and energy balance regulation.[24]

One explanation for these discrepancies might rely on the interaction between obestatin and other GI hormones influencing the satiety signal at the peripheral level. It has become increasingly recognized that different signals governing meal initiation, meal-end satiation, and intermeal satiety could modulate each other. Interaction between these signals results in a more potent satiety effect than each signal alone. Alternatively, one signal can reduce the effects of that induced by another. Therefore, obestatin and ghrelin, which both arise from posttranslational cleavage of the preproghrelin peptide, are well positioned to interact. This was highlighted in the original Zhang et al. report.[33] Both ghrelin and obestatin were coinjected ip at the same molar ratio resulting in the normalization of food intake compared with the inhibitory effect of obestatin administered alone. Conversely, repeated daily injections of obestatin prevented the body weight gain induced by ip ghrelin in lean mice over a 1-week period.[33] Although it seems unlikely that obestatin acts as an antagonist to ghrelin, interplay between ghrelin and obestatin was also reported by Zizzari et al.[35] This group observed that obestatin antagonized GH secretion and food intake induced by ghrelin only when ghrelin and obestatin were administered with equimolar quantities, whereas obestatin alone was unable to decrease GH release in different assays.[35] Subsequent studies, using central or peripheral injections of obestatin together with ghrelin, however, failed to confirm such an interaction.[23] Under conditions of chronic obestatin and ghrelin administration at the same molecular ratio, obestatin did not yield to a reduction of ghrelin-induced increased food intake and body weight gain over a 7-day treatment period in mice.[23] Furthermore, the lack of an obestatin effect to influence food intake in fasted rodents, a condition associated with the highest endogenous circulating levels of ghrelin, suggests that obestatin also does not alter the orexigenic action of endogenous ghrelin. Furthermore, another study showed that there was no interaction between obestatin and CCK-8 on food intake, gastric emptying, or gastric vagal afferent activity, while CCK-8 is known to interact synergistically with other satiety signaling hormones including leptin or bombesin.[12] Hence, these discrepancies emphasize that the originally given name of obestatin was premature because the effects of obestatin on food intake and body weight remain hardly consistent despite a growing literature on the subject.

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Gastrointestinal Motility

Most of the gut peptides that display significant effects on food intake also exert biological actions on digestive motor function, especially on the upper GI. Such effects were also tested in the original report describing the anorexigenic properties of obestatin.[33] Obestatin diminished the contractile activity of jejunal muscular strips *in vitro*, whereas ghrelin displayed the opposite effects, suggesting a role for obestatin to slow down upper GI transit.[33] This was expended by *in vivo* assay where obestatin injected ip in doses ranging from 320 μg/kg to 2.5 mg/kg delayed gastric emptying of a caloric meal in mice for the 1- to 2 h period postinjection. In addition, one report detected obestatin in cholinergic myenteric plexi,[10] but the latter further questions the role of obestatin as an inhibitor of gastric emptying since these neurons enhance propulsive activity of the stomach.

Subsequently, the ability of obestatin to delay gastric emptying or gastric motor activity in the fasted state has so far not been replicated. Indeed, all five subsequent reports failed to observe an inhibition of gastric emptying in fasted rats or mice after peripheral administration of obestatin at doses ranging from 0.15 to 3 mg/kg.[4,8,9,11,12] Again, it is unlikely that divergent results between the initial and subsequent reports arise from experimental procedures. Gastric emptying was tested by different techniques, with noncaloric and caloric test meals, in both rats and mice, and with established positive controls.[13] Likewise, *in vitro* gastrointestinal contractions explored with rat and mouse jejunal, duodenal, and fundic strips were not influenced by obestatin in the presence or absence of electrical field stimulation.[4,8] Results on *in vivo* gastric and intestinal contractions are more intriguing because one study found an inhibition of antral and duodenal motor activity 30 min after iv obestatin injection in fed rats, probably recruiting a vagal and central CRF_{1-2} pathway.[2] However, two studies failed to find an inhibitory effect of iv or intracisternal obestatin on antral and intestinal motility in fasted rats at doses up to 3 mg/kg, ip.[4,11] Another study did not observe gastric relaxation after iv obestatin administration.[12] Finally, all reports failed to antagonize the ability of ghrelin with obestatin to stimulate either GI motility or gastric transit.[2,4,8,11] Hence, it seems unlikely that obestatin, by contrast with GPR39, acts as an inhibitor of gastric emptying and motility, at least in the fasted state, when plasma ghrelin peaks.

Glucose Homeostasis

As ghrelin increases blood glucose through the inhibition of insulin release by the pancreatic β-cells, much attention has been paid to the possible involvement of obestatin in glucose homeostasis and insulin secretion (for review, see Refs. 7, 14, 15, 27). This was reinforced by the detection of obestatin in the pancreas where it colocalizes with ghrelin.[16] In addition, an inverse relationship has been found between obestatin and insulin plasma levels in humans. However, discrepant results on insulin secretion and blood glucose were observed after obestatin administration in both *in vitro* and *in vivo* assays. Several reports found either little or no effect of obestatin on these parameters, although others reported a decrease and an increase in insulin release after obestatin administration.[7,14,15,27] It is currently difficult to speculate on the pathways recruited in these divergent effects of obestatin on pancreatic β-cells. Nevertheless, it was suggested that obestatin could stimulate pancreatic/duodenal homeobox-1 gene and antiapoptotic BCL2 protein expression, and promote survival of pancreatic β-cells.[15] In addition, obestatin has been shown to increase β-cell cAMP and activate extracellular signal-related kinase 1/2 and phosphatidylinositol 3-kinase/Ak[14] in pancreatic β-cells. Consequently, and comparable with food intake and GI motility, research on the effects of obestatin on glucose homeostasis and insulin secretion has generated numerous results with variable and occasionally contradictory conclusions.

Other Biological Actions

Since the original report by Zhang et al. which focused on obestatin as a regulator of food intake and GI motility, numerous and sometimes unexpected effects have been reported (for a review, see Ref. 27). As ghrelin stimulates GH release in the pituitary, obestatin was therefore investigated for its ability to inhibit GH release. Although the initial reports by Zhang et al. and Nogueiras et al. stated that obestatin did not influence GH release,[23,33] three subsequent studies have indicated that obestatin could either increase GH release or reduce the ghrelin-induced increase of GHRH release.[27] At the gut level, obestatin has been shown to increase the secretion of pancreatic-biliary juice and trypsin. In adipose tissue, obestatin could play a role in cholesterol transport, promote adipogenesis, and regulate adipocyte differentiation. Obestatin has also been suggested to increase the force of heart contractions probably through a β-adrenergic pathway, and to stimulate cell proliferation in retinal pigment epithelial cells. In the brain, obestatin has been proposed to promote sleep, improve memory, and alter thirst through a decrease in central vasopressin.[27] Most of these findings have not led so far to further investigations or confirmation and therefore require further evidence in light of the preceding controversies.

PATHOPHYSIOLOGICAL IMPLICATIONS

In the absence of pharmacological agonists and antagonist of the still unknown receptor of obestatin, limited results are available on the possible pathophysiological involvement of obestatin in diseases and rely on plasma measurement in

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several conditions related to disturbance of food intake and glucose homeostasis.

Eating Disorders

Anorexia nervosa (AN) is a major cause of malnutrition and death in young women. In all studies to date, plasma ghrelin levels are elevated in restrictive AN. In most studies, plasma obestatin parallels ghrelin concentrations and is elevated in restrictive AN patients compared with normal-weight or BMI-matched healthy subjects (for review, see Ref. 35). However, ghrelin/obestatin ratio has been shown to be either elevated or decreased in AN.[35] Conversely, convergent studies all found decreased circulating obestatin concentrations in obese patients compared with normal-weight subjects, while the ghrelin/obestatin ratio remains unchanged.[34] In patients with bulimia nervosa, plasma obestatin has also been found to be lower than in control subjects, whereas the ghrelin/obestatin ratio is decreased.[35] As expected in this nutritional condition, plasma concentrations of ghrelin and acylated ghrelin also decrease in obese patients. Although elevated plasma ghrelin in AN patients may be interpreted as an adaptive mechanism to stimulate food intake and maintain normal blood glucose and body weight in starving situations, the elevated circulating obestatin in these patients is to date not fully understood. The same conclusion could be drawn for the decreased concentrations of circulating ghrelin and obestatin in obese patients. One explanation might rely, as mentioned above in physiological conditions, on the parallel release of ghrelin and ghrelin gene products, including obestatin. This is further supported by the elevated level of circulating obestatin observed in Prader–Willi syndrome, characterized by obesity associated with hyperghrelinemia.[7] Whether obestatin rather than ghrelin plasma level or ghrelin/obestatin ratio might represent a reliable diagnostic or prognostic marker of eating disorders remains to be further evaluated.

Insulin Resistance and Diabetes

As obestatin has been shown to be expressed in the pancreas with ghrelin to possibly regulate insulin secretion and blood glucose, a few studies have investigated obestatin plasma concentrations in patients with insulin resistance and diabetes mellitus. To date, two studies have shown that fasting concentrations of plasma obestatin, but not ghrelin, are reduced in nondiabetic subjects with insulin resistance.[1] Obestatin has even been proposed as a marker of insulin resistance in a multiple linear regression model that however did not take in account plasma ghrelin in the analysis.[25] Conversely, fasting circulating obestatin has been found either decreased or unchanged in type-2 diabetes mellitus.[25,28] Postprandial obestatin concentrations remain stable in patients with insulin resistance or in type-2 diabetes mellitus, contrasting with the decrease usually observed in control subjects.[1,28] The real significance, if confirmed, of this finding however remains not fully understood. To date, plasma obestatin levels have not been systematically measured in type-1 diabetes patients.

Other Pathophysiological Conditions

Based on the tissue expression, and preliminary reports of some organ-specific action of obestatin, several authors have suggested that obestatin could be part of the ontogeny of several disorders, and might even represent a future therapeutic target (for a review, see Ref. 27). For instance, obestatin concentrations are reduced in patients with mild to moderate high blood pressure, and are elevated in cachectic patients with congestive heart failure. In addition, obestatin has been suggested to play a role in myocardial protection after reperfusion. Although results indicating obestatin function in the renal system are lacking, obestatin concentrations are elevated in the plasma of patients with chronic kidney disease or with renal failure and normalized after hemodialysis. Obestatin has also been suggested to play a role in cancer progression. In support of this hypothesis, plasma obestatin levels have been shown to be higher in patients with ovarian cancer compared with in healthy controls, but not in prostate cancer or uterine leiomyoma. In addition, obestatin stimulates the proliferation of the KATO-III gastric cancer cell, whereas it inhibits proliferation of TT-human medullary thyroid carcinoma and BON-1 endocrine pancreatic tumor cell line. Lastly, obestatin immunoreactivity is decreased in thyroid and pancreatic tumor tissue. Hence, these preliminary and promising results on the role of obestatin as a marker or even as a therapeutic approach in heart and kidney disease, and in cancer physiopathology, require further attention and confirmation.

CONCLUSIONS

Obestatin was initially reported to reduce food intake, body weight gain, and gastric emptying and to suppress intestinal motility through an interaction with the orphan receptor GPR39. However, a number of subsequent studies undertaken in different laboratories suggest that these initial results should be interpreted with caution. To date, the obestatin receptor is yet to be discovered, and the presence of obestatin in various tissues, including the stomach still remains to be confirmed. In addition, the parallel variation of obestatin with ghrelin in the blood circulation adds another clue to the physiological functions of obestatin in regulating appetite and metabolism. The future identification of the obestatin receptor and development of more reliable assays for obestatin detection, as well as pharmacological tools, are therefore warranted to confirm and better understand the real role of obestatin as

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a potential bioactive peptide and GI hormone rather than a ghrelin-gene product.

ACKNOWLEDGMENT

The author is grateful to Richard Medeiros, Rouen University Hospital Medical Editor, for editing the manuscript.

REFERENCES

1. Anderwald-Stadler M, Krebs M, Promintzer M, Mandl M, Bischof MG, Nowotny P, et al. Plasma obestatin is lower at fasting and not suppressed by insulin in insulin-resistant humans. *Am J Physiol Endocrinol Metab* 2007;**293**(5):E1393–8.
2. Ataka K, Inui A, Asakawa A, Kato I, Fujimiya M. Obestatin inhibits motor activity in the antrum and duodenum in the fed state of conscious rats. *Am J Physiol Gastrointest Liver Physiol* 2008;**294**(5):G1210–8.
3. Bang AS, Soule SG, Yandle TG, Richards AM, Pemberton CJ. Characterisation of proghrelin peptides in mammalian tissue and plasma. *J Endocrinol* 2007;**192**(2):313–23.
4. Bassil AK, Haglund Y, Brown J, Rudholm T, Hellstrom PM, Naslund E, et al. Little or no ability of obestatin to interact with ghrelin or modify motility in the rat gastrointestinal tract. *Br J Pharmacol* 2007;**150**(1):58–64.
5. Chanoine JP, Wong AC, Barrios V. Obestatin, acylated and total ghrelin concentrations in the perinatal rat pancreas. *Horm Res* 2006;**66**(2):81–8.
6. Chartrel N, Alvear-Perez R, Leprince J, Iturrioz X, Reaux-Le Goazigo A, Audinot V, et al. Comment on "Obestatin, a peptide encoded by the ghrelin gene, opposes ghrelin's effects on food intake." *Science* 2007;**315**(5813):766:[author reply 766].
7. Chen CY, Asakawa A, Fujimiya M, Lee SD, Inui A. Ghrelin gene products and the regulation of food intake and gut motility. *Pharmacol Rev* 2009;**61**(4):430–81.
8. De Smet B, Thijs T, Peeters TL, Depoortere I. Effect of peripheral obestatin on gastric emptying and intestinal contractility in rodents. *Neurogastroenterol Motil* 2007;**19**(3):211–7.
9. Depoortere I, Thijs T, Moechars D, De Smet B, Ver Donck L, Peeters TL. Effect of peripheral obestatin on food intake and gastric emptying in ghrelin-knockout mice. *Br J Pharmacol* 2008;**153**(7):1550–7.
10. Dun SL, Brailoiu GC, Brailoiu E, Yang J, Chang JK, Dun NJ. Distribution and biological activity of obestatin in the rat. *J Endocrinol* 2006;**191**(2):481–9.
11. Gourcerol G, Coskun T, Craft LS, Mayer JP, Heiman ML, Wang L, et al. Preproghrelin-derived peptide, obestatin, fails to influence food intake in lean or obese rodents. *Obesity (Silver Spring)* 2007;**15**(11):2643–52.
12. Gourcerol G, Million M, Adelson DW, Wang Y, Wang L, Rivier J, et al. Lack of interaction between peripheral injection of CCK and obestatin in the regulation of gastric satiety signaling in rodents. *Peptides* 2006;**27**(11):2811–9.
13. Gourcerol G, St-Pierre DH, Tache Y. Lack of obestatin effects on food intake: should obestatin be renamed ghrelin-associated peptide (GAP)? *Regul Pept* 2007;**141**(1–3):1–7.
14. Granata R, Settanni F, Gallo D, Trovato L, Biancone L, Cantaluppi V, et al. Obestatin promotes survival of pancreatic beta-cells and human islets and induces expression of genes involved in the regulation of beta-cell mass and function. *Diabetes* 2008;**57**(4):967–79.
15. Granata R, Volante M, Settanni F, Gauna C, Ghe C, Annunziata M, et al. Unacylated ghrelin and obestatin increase islet cell mass and prevent diabetes in streptozotocin-treated newborn rats. *J Mol Endocrinol* 2010;**45**(1):9–17.
16. Gronberg M, Tsolakis AV, Magnusson L, Janson ET, Saras J. Distribution of obestatin and ghrelin in human tissues: immunoreactive cells in the gastrointestinal tract, pancreas, and mammary glands. *J Histochem Cytochem* 2008;**56**(9):793–801.
17. Holst B, Egerod KL, Schild E, Vickers SP, Cheetham S, Gerlach LO, et al. GPR39 signaling is stimulated by zinc ions but not by obestatin. *Endocrinology* 2007;**148**(1):13–20.
18. Kobelt P, Wisser AS, Stengel A, Goebel M, Bannert N, Gourcerol G, et al. Peripheral obestatin has no effect on feeding behavior and brain Fos expression in rodents. *Peptides* 2008;**29**(6):1018–27.
19. Lauwers E, Landuyt B, Arckens L, Schoofs L, Luyten W. Obestatin does not activate orphan G protein-coupled receptor GPR39. *Biochem Biophys Res Commun* 2006;**351**(1):21–5.
20. McKee KK, Tan CP, Palyha OC, Liu J, Feighner SD, Hreniuk DL, et al. Cloning and characterization of two human G protein-coupled receptor genes (GPR38 and GPR39) related to the growth hormone secretagogue and neurotensin receptors. *Genomics* 1997;**46**(3):426–34.
21. Moechars D, Depoortere I, Moreaux B, de Smet B, Goris I, Hoskens L, et al. Altered gastrointestinal and metabolic function in the GPR39-obestatin receptor-knockout mouse. *Gastroenterology* 2006;**131**(4):1131–41.
22. Mondal MS, Toshinai K, Ueno H, Koshinaka K, Nakazato M. Characterization of obestatin in rat and human stomach and plasma, and its lack of acute effect on feeding behavior in rodents. *J Endocrinol* 2008;**198**(2):339–46.
23. Nogueiras R, Pfluger P, Tovar S, Arnold M, Mitchell S, Morris A, et al. Effects of obestatin on energy balance and growth hormone secretion in rodents. *Endocrinology* 2007;**148**(1):21–6.
24. Pan W, Tu H, Kastin AJ. Differential BBB interactions of three ingestive peptides: obestatin, ghrelin, and adiponectin. *Peptides* 2006.
25. Qi X, Li L, Yang G, Liu J, Li K, Tang Y, et al. Circulating obestatin levels in normal subjects and in patients with impaired glucose regulation and type 2 diabetes mellitus. *Clin Endocrinol (Oxf)* 2007;**66**(4):593–7.
26. Scrima M, Campiglia P, Esposito C, Gomez-Monterrey I, Novellino E, D'Ursi AM. Obestatin conformational features: a strategy to unveil obestatin's biological role? *Biochem Biophys Res Commun* 2007;**363**(3):500–5.
27. Seim I, Walpole C, Amorim L, Josh P, Herington A, Chopin L. The expanding roles of the ghrelin-gene derived peptide obestatin in health and disease. *Mol Cell Endocrinol* 2011;**340**(1):111–7.
28. St-Pierre DH, Settanni F, Olivetti I, Gramaglia E, Tomelini M, Granata R, et al. Circulating obestatin levels in normal and type 2 diabetic subjects. *J Endocrinol Invest* 2010;**33**(4):211–4.
29. Tremblay F, Richard AM, Will S, Syed J, Stedman N, Perreault M, et al. Disruption of G protein-coupled receptor 39 impairs insulin secretion in vivo. *Endocrinology* 2009;**150**(6):2586–95.
30. Unniappan S, Speck M, Kieffer TJ. Metabolic effects of chronic obestatin infusion in rats. *Peptides* 2008;**29**(8):1354–61.
31. Zhang JV, Jahr H, Luo CW, Klein C, Van Kolen K, Ver Donck L, et al. Obestatin induction of early-response gene expression in gastrointestinal and adipose tissues and the mediatory role of G protein-coupled receptor, GPR39. *Mol Endocrinol* 2008;**22**(6):1464–75.

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32. Zhang JV, Klein C, Ren PG, Kass S, Ver Donck L, Moechars D, et al. Response to comment on "Obestatin, a peptide encoded by the ghrelin gene, opposes ghrelin's effects on food intake." *Science* 2007;**315**(5813):766.
33. Zhang JV, Ren PG, Avsian-Kretchmer O, Luo CW, Rauch R, Klein C, et al. Obestatin, a peptide encoded by the ghrelin gene, opposes ghrelin's effects on food intake. *Science* 2005;**310**(5750):996–9.
34. Zhang N, Yuan C, Li Z, Li J, Li X, Li C, et al. Meta-analysis of the relationship between obestatin and ghrelin levels and the ghrelin/obestatin ratio with respect to obesity. *Am J Med Sci* 2011;**341**(1):48–55.
35. Zizzari P, Hassouna R, Grouselle D, Epelbaum J, Tolle V. Physiological roles of preproghrelin-derived peptides in GH secretion and feeding. *Peptides* 2011.

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Chapter 174

Opioids

Catia Sternini, Celine Duraffourd and Laura Anselmi

ABSTRACT

In the gastrointestinal tract, the endogenous opioid peptides enkephalin, dynorphin, and endorphin, and their receptors, the ∂, μ, and k opioid receptors (∂OR, μOR, kOR) are localized to neurons, and endocrine and immune cells. Enkephalin and dynorphin are primarily localized to enteric neurons, whereas endorphin for the most part is found in enteroendocrine cells. Opioid receptors are predominantly localized to enteric neurons, with the ∂OR and μOR often colocalizing in subsets of neurons; μORs is also found in gut mucosal immune cells. Both ∂ORs and μORs undergo ligand-mediated endocytosis in enteric neurons, whereas μORs recycle to the cell surface, ∂ORs do not. The differential trafficking might reflect differential functions of distinct opioid receptors in the gut. μOR is the main target of opiates, potent analgesics, and mediates the opioid-bowel syndrome, a major complication of chronic opiate treatment. μOR also exerts a protective effect in intestinal inflammation.

DISCOVERY OF OPIOID PEPTIDES AND RECEPTORS

The powerful biological effects attributed to the opioid system, including analgesia, motor activity, stress response, immunity, autonomic and digestive functions, are induced by endogenous opioid peptides and opiate drugs through the activation of distinct opioid receptors,[12,28] which were originally identified on the basis of pharmacological studies with synthetic opiates. The evidence for different receptor types was originally provided by Martin et al. in 1976,[15] who named two distinct opioid receptors, the receptor μ (μ for morphine) and k (k for ketocyclazocine) after the drugs used in their studies. In 1977, the third principal type of opioid receptor, ∂ receptor (∂OR), was discovered by pharmacological analysis of opioid peptide effects on mouse vas deferens.[14] In 1992, the groups of Evans and Kieffer, using expression cloning, isolated and cloned the first opioid receptor gene, Oprd1, which encodes the ∂OR.[11] The Oprm1 and Oprk1, encoding μOR and kOR receptor, respectively, were cloned in the following 2 years.[11] The natural ligands for the opioid receptors were also discovered in the 1970s, the first being the pentapeptides, leucine-enkephalin and methionine-enkephalin, natural ligands for the ∂OR, followed by β endorphins, potent ligands for μOR and ∂OR and then dynorphins, natural ligands for kORs.[1] The cloning of cDNAs for the endogenous opioids followed shortly their isolation.[1] It is only in the 1990s that two opioid tetrapeptides, named endomorphin 1 and endomorphin 2, highly selective for μOR were isolated from the brain;[34] however, the genes for these peptides have not been identified. Opiate drugs or alkaloids, including morphine and fentanyl—the most potent analgesics widely used in humans for pain control[20]—are structurally distinct, nonpeptide ligands that activate the same receptors as native opioids.

STRUCTURE OF THE mRNA/GENE AND PROCESSING OF THE PRECURSOR

Endogenous opioids are generated by three distinct genes, the proopiomelanocortin, proenkephalin, and prodynorphin genes, which generate several biologically active peptides, including beta-endorphin, enkephalin, and dynorphin, via the cleavage of precursor proteins. In addition to processing, posttranslational events also influence the final products, pharmacological profiles and selectivity that might vary in different regions of the brain or periphery.[1] The diverse effects of opioids and opiates are mediated by the activation of multiple receptors, which are classified as members of the superfamily of seven transmembrane G protein-coupled receptors.[4,11] The three major classes of receptors, ∂OR, kOR, and μOR, share about 60% similarity in their sequences. The transmembrane regions share the highest identity (~73–76%), followed by the intracellular regions with 63–66% homologies, whereas they are much more divergent in their extracellular regions with only 34–40% identity. These extracellular domains confer selectivity to the opioid receptors as to their ability to bind agonists and antagonists. Given the complex pharmacological diversity of opioid receptors based on binding sites and ligand affinities, one would have expected the identification of more

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00174-3
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than three major ORs. Alternative splicing of the three opioid receptors has been reported, thus providing evidence for the existence of multiple opioid receptors subtypes, classified as μOR1-3, kOR1-3, and ∂OR1-2.[4] In addition, rearrangement of positioning of transmembrane domains and modulation of cytoplasmic domains in response to different agonists, as well as posttranscriptional modifications (phosphorylation, palmitoylation, or glycosylation), receptor dimerization and interactions with intracellular proteins may also account for the diversity of the opioid receptors family as suggested by earlier pharmacological studies.[4]

Opioid receptors partly overlap in their ligand affinity but maintain a certain degree of selectivity for the three families of endogenous opioids and differ in their pharmacological profiles.[4,11] Enkephalins have the highest affinity for the ∂OR, but they also have high affinity for the μOR and very low affinity for kOR. Dynorphins preferentially bind kOR and can also bind μOR and ∂OR. Endorphin binds both μOR and ∂OR with the same affinity, but it has only a low affinity for kOR. By contrast, endomorphins have a preference for μOR that is several thousand-fold that of ∂OR and kOR.[34] Similarly, opiates used clinically preferentially activate the μOR even though their ligand selectivity profiles are impaired when they accumulate at high concentrations, in which conditions they can interact with other opioid receptors.[7]

DISTRIBUTION OF THE mRNA/PEPTIDE

In the gastrointestinal tract, endogenous opioid peptides are localized to the enteric nervous system and to endocrine cells.[12,31] Specifically, in the enteric nervous system, the derivatives of proenkephalins are confined mainly to myenteric neurons projecting to the circular muscle and submucosal plexus,[6] prodynorphin-derived peptides are localized to submucosal and myenteric neurons and to fibers originating from the celiac ganglion,[30] and β endorphins are localized to myenteric ganglion cells and fibers distributed to both plexuses and the muscle.[13] Enkephalins and dynorphins colocalize with classical transmitters (for example, acetylcholine) or peptides, including substance P, vasoactive intestinal polypeptide, gastrin-releasing peptides, galanin, or nitric oxide (NO), the major transmitter from descending neuronal pathways.[6,30] Endogenous opioids are also localized to enteroendocrine and enterochromaffin cells of the stomach, intestine, and pancreas.[31] Opioid receptor binding sites have been reported throughout the mammalian gastrointestinal tract in association with different structures, whereas OR immunoreactivities with specific antibodies for the cloned opioid receptors are predominantly distributed to the enteric nervous system with different sites of expression in different species.[2,9,26,31] Most of the results on opioid receptor localization are concerned with the μOR and ∂OR, whereas results on kOR distribution are sparse. In rats, μOR neurons are more abundant in the submucosal than myenteric plexus, whereas kOR are more numerous in the myenteric than submucosal plexus.[2] μOR and kOR myenteric neurons are more abundant in the stomach and proximal colon compared with those in other regions of the GI tract, and μOR and kOR fibers are distributed to the muscle and mucosal layers. Finally, both μOR and kOR have been reported in interstitial cells of Cajal in the rat myenteric plexus,[2,8] but not in other species.[9] In the guinea pig, an animal model that has been widely used for functional studies of opioid receptors in the GI tract, μOR immunoreactivity is confined mainly to the myenteric plexus.[9,31] Myenteric μOR neuron terminals are most numerous in the small intestine, followed by the stomach and the proximal colon. μOR receptor immunoreactive fibers are dense in the muscle layer and the deep muscular plexus in close association with interstitial cells of Cajal. This distribution closely matches the pattern of enkephalin. μOR enteric neurons comprise functionally distinct populations of neurons of the ascending and descending pathways of the peristaltic reflex.[9] In human gut, μOR immunoreactivity is localized to myenteric and submucosal neurons and to immune cells of the lamina propria.[31] ∂OR immunoreactivity is located in both plexuses where it is predominant in varicose fibers, muscle, and mucosa. kOR immunoreactivity seems to be confined to the myenteric plexus and to bundles of fibers in the muscle. Gray et al. identified the cellular expression patterns of ∂, μ, and k-ORs in the rat ileum[8] and showed μ/∂OR coexpression in interstitial cells adjacent to myenteric plexus and in the submucous plexus. Punctate kOR immunoreactivity was seen broadly in nerve fibers in both plexuses, suggesting localization in varicosities. Although kOR immunoreactivity was often apposed to μOR immunoreactivity, μ/kOR colocalization was not apparent in these neurons but was detected in interstitial cells in submucous plexus ileum rat preparations.[8] Poole et al.[25] used mice in which the enhanced green fluorescent protein (eGFP) was inserted into the ∂OR gene and showed that ∂OR fluorescence is predominantly localized to enteric neurons of both plexuses with higher abundance in the small intestine compared with that in the large intestine and to fibers distributed to the muscle layer. ∂OR-eGFP is localized to secretomotor and vasomotor neurons of the submucosal plexus and to excitatory and inhibitory neurons in the myenteric plexus of the small intestine but mostly in inhibitory neurons of the colon myenteric plexus. Frequent colocalization between ∂OR-eGFP and μOR immunoreactivity is observed.[25]

RECEPTORS AND SIGNALING CASCADES

Activation of opioid receptors is translated into physiological responses via coupling to inhibitory Gi/Go proteins through a pertussis toxin-sensitive pathway resulting in activation of

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several effectors. opioid receptors act via G proteins to inhibit adenylyl cyclase and the formation of cyclic AMP, increase potassium currents, inhibit calcium channel activity, modulate inositol triphosphate turnover, and activate mitogen-activated protein kinase.[11,28] Coupling of the opioid receptors to the effector system results in attenuation of cellular activity. Opioid receptors are G protein coupled receptors (GPCRs); thus, they follow the scheme of most GPCRs after ligand activation. Interaction of ligand with its GPCR initiates a cascade of events including phosphorylation by G protein receptor kinases (GRKs) that promote interaction with β arrestins (β-arrestins 1 and 2), which prevent further stimulation and downstream signaling pathway by uncoupling receptors from G proteins; therefore, inducing rapid acute desensitization.[3] The phosphorylated opioid receptor–ligand complex then translocates from the cell surface into the cytoplasm through a dynamin-dependent and clathrin-mediated process, undergoes intracellular sorting into endosomes, dephosphorylation and either degradation into lysosomes or recycling to the surface.[3] Phosphorylation, endocytosis, intracellular sorting, and recycling are important regulatory processes that mediate desensitization, downregulation, and resensitization, events that regulate cellular responsiveness to stimulation.

Most of the information available on opioid receptor activation, trafficking, and signaling in the gastrointestinal tract derives from studies on μOR. The presence of μOR receptor at the cell surface is essential for cellular activation, and agonist-induced receptor internalization is an important event in the regulation of cellular responsiveness because it depletes the cell surface of receptors and initiates the intracellular trafficking, thus contributing to late desensitization and to resensitization, respectively. In enteric neurons, in organotypic cultures *in vitro* and *in vivo*, ligand-induced μOR endocytosis occurs in both the soma and neuronal processes and is prevented by naloxone, an opioid receptor antagonist and by CTOP (d-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2), a selective μOR antagonist. μOR internalization is rapid, reaches a maximum at 30 min and persists for 4–6 h. Internalized μORs recycle to the cells surface, without requiring new receptor synthesis, and internalize again.[18] Ligand-induced μOR internalization in enteric neurons is concentration dependent and ligand-selective, because it is induced by endogenous opioids with high affinity for μOR, such as enkephalins and endomorphins, by enkephalin analogs such as DAMGO ([D-Ala2,N-Me-Phe4,Gly-ol5] enkephalin) (Fig. 1) and by most opiates, including etorphine and fentanyl.[17,18,32] By contrast, morphine, the prototype of opiate drugs, which activates μOR through the same signaling pathways as other opiates and opioids, fails to induce receptor internalization under the same experimental conditions (Fig. 1), even at concentrations far in excess of those inducing maximal inhibition of electrically induced muscle contraction and inhibition of cAMP formation. Moreover, morphine partially inhibits μOR endocytosis induced by etorphine, indicating activation of the receptor.[32] Opiates with high efficiency in inducing μOR internalization (e.g. etorphine or fentanyl) have less propensity to induce tolerance than morphine, which seems to be resistant to internalization and has a higher propensity to induce tolerance.[5] This suggests that perhaps receptor internalization could serve as a mechanism to protect from tolerance, a major side effect of opiate chronic treatment that implies the need of increasing opiate doses to achieve the same analgesic effect. This ligand-selective internalization is generalized to other neurons throughout the brain. The resistance of morphine-activated μORs to undergo internalization is of interest because morphine is a drug of high clinical relevance for its widespread use for pain control and after surgery. The recruitment or upregulation of proteins mediating endocytosis, such as β-arrestin, dynamin, and the G protein-coupled receptors kinases proteins (GRKs) might be an important factor in determining agonist efficiency in inducing receptor internalization. This is supported by the observation that overexpression of GRKs or β arrestins in heterologous

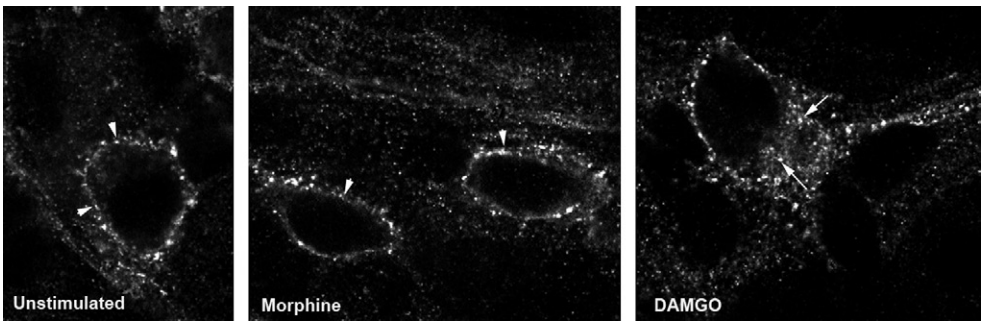

FIGURE 1 μOR immunoreactivity in enteric neurons of guinea pig ileum. μOR immunoreactivity is predominantly found at the cell surface (arrowheads) in unstimulated neurons and in neurons stimulated with morphine, a poor internalizing μOR agonist, whereas it is present in endosomes in the cytoplasm (arrows) in neurons stimulated with DAMGO, a μOR selective agonist with high endocytic efficacy. Organotypic cultures of the small intestine were incubated in medium alone (unstimulated) or containing saturating concentrations of morphine (100 μmol/L) or DAMGO (10 μmol/L) for 1 h at 40 °C to allow ligand–receptor binding, transferred to ligand-free medium at 37 °C for 30 min to allow receptor internalization, fixed, and processed for immunohistochemistry as described.[21]

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cells increase the efficiency of morphine to induce receptor internalization.[33,35] However, it is not known whether this occurs with native receptors in highly differentiated cells as neurons. Our group has shown that morphine acquires the ability to induce pronounced μOR internalization in enteric neurons from animals rendered tolerant by chronic administration of morphine (Fig. 2). This process is accompanied by increased expression and translocation of dynamin from the intracellular pool to the membrane, whereas β-arrestin levels were not changed.[21] Morphine-induced internalization in enteric tolerant neurons is inhibited by a dynamin inhibitor that blocks coated vesicle formation, further supporting the hypothesis that the increased level and cellular location of dynamin plays a role in inducing internalization of morphine-activated μORs. DAMGO, a selective μOR agonist with high internalizing efficiency retains its ability to induce μOR internalization in tolerant neurons (Fig. 2), indicating that chronic activation of μOR does not impair receptor trafficking in enteric neurons. It is probable that different mechanisms at the receptor level and downstream of the receptor contribute to ligand-induced μOR internalization and to the regulation of opiate drug actions in the gut. These findings are of clinical relevance because μOR enteric neurons are part of the neuronal circuits controlling gastrointestinal functions including motility, which is severely impaired by chronic opiate treatment. μOR internalization also occurs *in vivo* in response to endogenously released opioids, as it occurs in stress conditions such as abdominal surgery.[22]

Like the μORs, the ∂ORs undergo ligand-mediated internalization in enteric neurons, in both the soma and neurites, which is selectively blocked by a ∂OR antagonist and is dynamin dependent. However, unlike μORs, ∂ORs do not recycle to the cell surface and are degraded in lysosomes. Replenishment of ∂ORs at the cell membrane occurs 6–16 h later and requires synthesis of new receptors. The sustained receptor downregulation might play a role in long-lasting tolerance to ∂OR agonists.[25]

BIOLOGICAL ACTIONS WITHIN THE GASTROINTESTINAL TRACT

Opioid effects are complex because they are mediated by different classes of cell-surface receptors, and they depend on different sites of action and species. Opioids and opiates affect a variety of functions within the digestive system, including motility, secretion, and electrolyte and fluid transport.[12] This is consistent with the distribution of opioid receptors in functionally distinct neuronal populations in the enteric nervous.[9] Because opioid receptors are expressed in both excitatory and inhibitory pathways within the neuronal circuits regulating gastrointestinal motility, their activation can result in the inhibition of acetylcholine or other excitatory transmitter release with consequent inhibition of peristaltic contraction; at the same time, opioid receptor activation can result in the suppression of nitric oxide or other inhibitory transmitters thus resulting in the inhibition of peristaltic distension. Thus, activation of these pathways might impair intestinal transit by changing the coordinated reflex motor activity into a segmenting and nonpropulsive motility pattern.[31] By contrast, in the guinea pig, which has been extensively used as a model for functional studies to characterize the physiological and pathophysiological effects of opioids, activation of opioid receptors on myenteric neurons inhibits nerve-mediated contraction of circular and longitudinal muscle and opiod-induced blockade of propulsion and transit is not associated with muscular spasm. Opioid neurons might also exert a modulatory influence on motor neurons subserving peristalsis and might function as a restraining mechanism in conditions of fatigues, that is, when the intestine empties against high resistance. Furthermore, the observation that μOR fibers surround interstitial cells of Cajal (ICC), which are pacemakers coordinating intestinal motor activity and are likely to be involved in neurotransmission from neurons to muscles, suggests an additional pathway through which

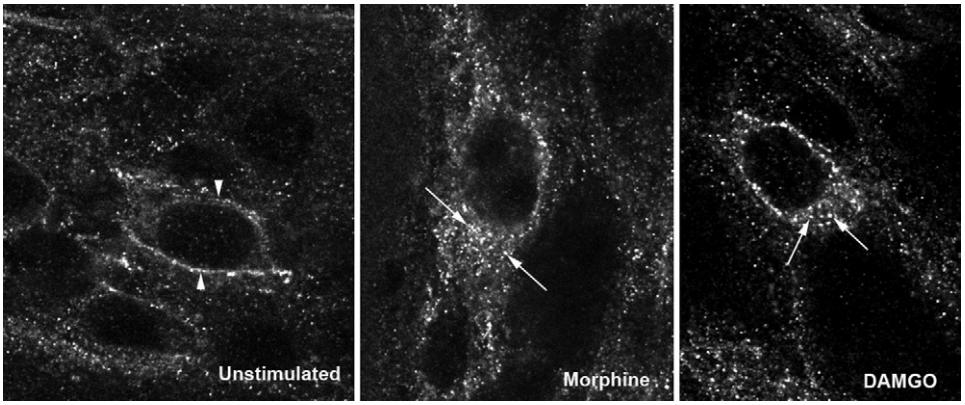

FIGURE 2 μOR immunoreactivity in enteric neurons of guinea pig ileum rendered tolerant by chronic treatment with increasing concentration of morphine for 7 days as described.[21] μOR immunoreactivity is predominantly found at the cell surface (arrowheads) in unstimulated neurons, but it translocates into endosomes in the cytoplasm (arrows) in neurons stimulated with morphine or DAMGO, as described in Fig. 1.

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OR activation might affect gastrointestinal motility.[9] opioid receptor activation might result in the inhibition of ICC activity via the reduction of transmitter release by excitatory and inhibitory neurons, which innervate ICCs. Opioids also attenuate electrolyte and water secretion and facilitate net absorption of fluids. Together, these inhibitory effects on motility and secretion induce constipation.

Even though the central administration of opiates can delay gastrointestinal transit, there is overwhelming evidence that the effects of opioids on gastrointestinal functions are mostly peripheral resulting from the direct activation of opioid receptors in the gut, which is supported by morphological and functional evidence and by the observation that opioid receptor antagonists with peripherally restricted sites of action prevent opiate-induced delay of transit and that antagonists, which do not cross the blood–brain barrier block opiate effects in the gut.

PATHOPHYSIOLOGICAL IMPLICATIONS

Opiates are sill the mainstay therapeutic approach for chronic pain. However, the prolonged use of these drugs induce a variety of side effects, including tolerance, which requires increasing doses of opiates to obtain the desired analgesic effects, which in turn aggravate tolerance thus creating a vicious cycle. Furthermore, the chronic use of opiates induces serious side effects at the level of the gastrointestinal tract resulting in the development of opioid-bowel dysfunction (OBD).[19] OBD is mediated by μOR, which is the main target of opiates and is characterized by a variety of symptoms, including decreased gastric emptying, abdominal cramping and bloating, delayed gastrointestinal transit, and constipation, which is the main and most severe symptom that can be so intolerable to induce patients to stop opiate treatment. OBD is a clinical problem affecting as many as 60–90% of patients who receive opioids for chronic metastatic malignancy or chronic noncancer pain. Currently, the best treatment available is the use of peripheral μOR antagonists, such as Alvimopan and Methylnaltrexolone to counteract the peripheral effect of opiates without affecting the central, analgesic effect.

μORs are also contribute to the worsening of postoperative ileus (POI), a transient condition that develops after abdominal or pelvic surgery.[10] POI is characterized by the delay of gastrointestinal transit with bowel distension, bloating, nausea, and vomiting. POI is caused by a combination of inflammatory, immunological, and neurogenic mechanisms, and it is aggravated by the use of opiates as postsurgery analgesics. The best results for POI treatment have been reported with the use of the selective, peripherally acting μOR antagonist, Alvimopam.

Increasing evidence supports a role of the opioid system in intestinal inflammation.[23,24] Indeed, immune cells express endogenous opioids and their receptors, μOR, kOR, and dOR, and opioid peptides are released by immune cells. *In vivo* treatment with opioids and opioid antagonists has been reported to modulate release of proinflammatory cytokines, including tumor necrosis factor (TNF) α, and opioids can sensitize T-lmphocytes and macrophages to proinflammatory stimuli. In addition, opioid antagonists have been shown to improve the inflammatory scores and quality of life of patients with Crohn's disease and to reduce inflammation in moderate experimental colitis, but not in severe colitis, probably through the activation of ∂OR, supporting a proinflammatory role of ∂OR.[16,29] By contrast, μOR seems to exert immunosuppressive effects. μOR agonists have been shown to ameliorate inflammation in animal models of acute intestinal inflammation, and μOR deficient mice have been shown to be more susceptible to colon inflammation than their littermate controls suggesting that μOR agonists might represent a novel therapeutic approach for inflammatory bowel diseases (IBD). In addition, the upregulation of μOR mRNA has been reported in intestinal inflammation and in patients with IBD. μOR has been localized on both enteric neurons and immunocytes in patients with IBD, but it is overexpressed in inflammatory cells in patients with active IBD.[23] In addition, μOR is increased by cytokines and ex vivo treatment with a selective μOR agonist reduces the expression of TNF-α in the mucosa of patient with active IBD. These results support a protective effect of μOR in intestinal inflammation and suggest that μOR agonists could be potential new therapeutic molecules for the treatment of IBD. We have shown that treatment with a selective μOR agonist, DAMGO, at concentrations that do not pass the blood–brain barrier and do not affect gastrointestinal transit, reduces the acute inflammatory processes and epithelial damage induced by intestinal ischemia/reperfusion.[27] Intestinal ischemia is a life-threatening emergency with high mortality rate that ranges from 30% to 90% depending on the etiology. It can occur in several clinical–surgical conditions, including abdominal surgery for aortic aneurysm, small bowel transplantation, cardiopulmonary bypass, strangulated hernias, and neonatal necrotizing enterocolitis. Intestinal ischemia, a consequence of reduction of tissue oxygenation, induces progressive tissue damage and necrosis, which is paradoxically aggravated by restoration of the blood flow or reperfusion. DAMGO reduction of inflammation in intestinal ischemia is accompanied by a reduction in myeloperoxidase, an index of neutrophil infiltration, and of the proinflammatory cytokines. These findings provide evidence for a protective role of μORs in intestinal inflammation induced by ischemia/reperfusion. This effect probably predominantly results from the activation of μOR in inflammatory cells and is mediated by the proinflammatory cytokine, TNF-α. These findings support the concept that peripheral μORs could be explored as possible targets for novel therapeutic approaches to ameliorate inflammation, the first acute event in intestinal injury induced by ischemia/reperfusion.

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REFERENCES

1. Akil H, Watson SJ, Young E, Lewis ME, Khachaturian H, Walker JM. Endogenous opioids: biology and function. *Annu Rev Neurosci* 1984;**7**:223–55.
2. Bagnol D, Mansour A, Akil H, Watson SJ. Cellular localization and distribution of the cloned mu and kappa opioid receptors in rat gastrointestinal tract. *Neuroscience* 1997;**81**:579–91.
3. Bohm S, Grady EF, Bunnett NW. Mechanisms attenuating signaling by G protein-coupled receptors. *Biochem J* 1997;**322**:1–8.
4. Dietis N, Rowbotham DJ, Lambert DG. Opioid receptor subtypes: fact or artifact? *Br J Anesth* 2011;**107**:8–18.
5. Duttaroy A, Yoburn BC. The effect of intrinsic efficacy on opioid tolerance. *Anesthesiology* 1995;**82**:1226–36.
6. Furness JB, Costa M, Miller RJ. Distribution and projections of nerves with enkephalin-like immunoreactivity in the guinea-pig small intestine. *Neuroscience* 1983;**8**:644–53.
7. Goldstein A, Naidu A. Multiple opioid receptors: ligands selectivity profiles and binding site signature. *Mol Pharmacol* 1989;**36**:265–72.
8. Gray AC, Coupar IM, White PJ. Comparison of opioid receptor distributions in the rat ileum. *Life Sci* 2006;**78**:1610–6.
9. Ho A, Lievore A, Patierno S, Kohlmeier SE, Tonini M, Sternini C. Neurochemically distinct classes of myenteric neurons express the μ-opioid receptor in the guinea pig ileum. *J Comp Neurol* 2003;**458**:404–11.
10. Holzer P. Opioid antagonists for prevention and treatment of opioid-induced gastrointestinal effects. *Curr Opin Anaesthesiol* 2010;**23**: 616–22.
11. Kieffer BL, Evans CJ. Opioid receptors: from binding sites to visible molecules in vivo. *Neuropharmacology* 2009;**56**(Suppl. 1):205–12.
12. Kromer W. Endogenous and exogenous opioids in the control of gastrointestinal motility and secretion. *Pharmacol Rev* 1988;**40**:121–62.
13. Leander S, Ekman R, Uddman R, Sundler F, Hakanson R. Neuronal cholecystokinin, gastrin-releasing peptide, neurotensin, and beta-endorphin in the intestine of the guinea pig. Distribution and possible motor functions. *Cell Tissue Res* 1984;**235**:521–31.
14. Lord JAH, Waterfield AA, Hughes J, Kosterlitz HW. Endogenous opioid peptides: multiple agonists and receptors. *Nature* 1977;**267**:495–9.
15. Martin WR, Eades CC, Thompson JA, Gilbert PE, Huppler RE. The effect of morphine and nalorphine-like drugs in the nondependent and morphine dependent chronic spinal dog. *J Pharmacol Exp Ther* 1976;**197**:517–32.
16. Matters GL, Harms JF, McGovern C, Fitzpatrick L, Parikh A, Nilo N, et al. The opioid antagonist naltrexone improves murine inflammatory bowel disease. *J Immunotoxicol* 2008;**5**:179–87.
17. McConalogue K, Grady EF, Minnis J, Balestra B, Tonini M, Brecha NC, et al. Activation and internalization of the μ opioid receptor by the newly described endogenous agonists, endomorphin-1 and endomorphin-2. *Neuroscience* 1999;**90**:1051–9.
18. Minnis J, Patierno S, Kohlmeier SE, Brecha N, Tonini M, Sternini C. Ligand-induced μ opioid receptor endocytosis and recycling in enteric neurons. *Neuroscience* 2003;**119**:33–42.
19. Panchal SJ, Muller-Schwefe P, Wurzelmann JI. Opioid-induced bowel dysfunction: prevalence, pathophysiology and burden. *Int J Clin Pract* 2007.
20. Pasternak GW. Pharmacological mechanisms of opioid analgesics. *Clin Neuropharmacol* 1993;**16**:1–8.
21. Patierno S, Anselmi L, Jaramillo I, Scott D, Garcia R, Sternini C. Morphine induces mu opioid receptor endocytosis in guinea pig enteric neurons following prolonged receptor activation. *Gastroenterology* 2011;**140**:618–26.
22. Patierno S, Raybould HE, Sternini C. Abdominal surgery induces μ opioid receptor endocytosis in enteric neurons of the guinea pig ileum. *Neuroscience* 2004;**123**:101–9.
23. Philippe D, Chakass D, Thuru X, Zerbib P, Tsicopoulos A, Geboes K, et al. Mu opioid receptor expression is increased in inflammatory bowel diseases: implications for homeostatic intestinal inflammation. *Gut* 2006;**55**:815–23.
24. Philippe D, Dubuquoy L, Groux H, Brun V, Chuoi-Mariot MT, Gaveriaux-Ruff C, et al. Anti-inflammatory properties of the mu opioid receptor support its use in the treatment of colon inflammation. *J Clin Invest* 2003;**111**:1329–38.
25. Poole DP, Pelayo JC, Scherrer G, Evans CJ, Kieffer BL, Bunnett NW. Localization and regulation of fluorescently labeled delta opioid receptor, expressed in enteric neurons of mice. *Gastroenterology* 2011;**141**: 982–91:e8.
26. Poonyachoti S, Kulkarni-Narla A, Brown DR. Chemical coding of neurons expressing ∂- and k-opioid receptor and type I vanilloind receptor immunoreactivities in the porcine ileum. *Cell Tissue Res* 2002;**307**:23–33.
27. Saccani F, Anselmi L, Jaramillo I, Bertoni S, Barocelli E, Sternini C. Protective role of μ opioid receptor activation in intestinal inflammation induced by mesenteric ischemia/reperfusion in mice. *J Neurosci Res*. 2012; doi10.1002.jnr23108 (Epub ahead of print).
28. Simon EJ. Opioid receptors and endogenous opioid peptides. *Med Res Review* 1991;**11**:357–74.
29. Smith JP, Stock H, Bingaman S, Mauger D, Rogosnitzky M, Zagon IS. Low-dose naltrexone therapy improves active Crohn's disease. *Am J Gastroenterol* 2007;**102**:820–8.
30. Steele PA, Costa M. Opioid-like immunoreactive neurons in secretomotor pathways of the guinea-pig ileum. *Neuroscience* 1990;**38**: 771–86.
31. Sternini C, Patierno S, Selmer IS, Kirchgessner A. The opioid system in the gastrointestinal tract. *Neurogastroenterol Motil* 2004;**16**(Suppl. 2):3–16.
32. Sternini C, Spann M, Anton B, Keith Jr DE, Bunnett NW, von Zastrow M, et al. Agonist-selective endocytosis of μ opioid receptor by neurons *in vivo*. *Proc Natl Acad Sci U S A* 1996;**93**: 9241–6.
33. Whistler JL, von Zastrow M. Morphine-activated opioid receptors elude desensitization by β-arrestin. *Proc Natl Acad Sci U S A* 1998;**95**:9914–9.
34. Zadina JE, Hackler L, Ge L-J, Kastin A. A potent and selective endogenous agonist for the μ-opiate receptor. *Nature* 1997;**386**: 499–502.
35. Zhang J, Ferguson SSG, Barak LS, Bodduluri SB, Laporte SA, Law P-Y, et al. Role of G protein-coupled receptor kinase in agonist-specific regulation of μ-opioid receptor responsiveness. *Proc Natl Acad Sci U S A* 1998;**95**:7157–62.

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Chapter 175

PACAP

John P. Vu, Patrizia M. Germano and Joseph R. Pisegna

ABSTRACT

Pituitary adenylate cyclase polypeptide (PACAP) is a member of the superfamily of peptide hormones that includes vasoactive intestinal polypeptide (VIP) and secretin. PAC1 is a specific receptor with a 1000-fold greater affinity for PACAP compared with VIP or secretin. PAC1 is expressed in the gastrointestinal tract where PACAP functions to regulate gastrointestinal secretion and motility. PACAP seems to play a key role in the central nervous system regulation of appetite, although its potential peripheral mechanism remains unknown. It is involved in the regulation of innate and adaptive immune responses and may have a role in inflammatory bowel diseases. In neuroendocrine tumors, both PAC1 and PACAP are expressed and seem to regulate the tumor growth and the release of other neuropeptides and biogenic amines.

INTRODUCTION

PACAP was isolated by Arimura and coworkers in 1989 by examining fractions of ovine hypothalamus capable of stimulating adenylyl cyclase (AC). PACAP is derived from a 170-amino-acid long precursor, which gives rise to two biological forms, PACAP 38 and PACAP 27, in which the first 27 N-terminal amino acids share complete identity. PACAP activity is mediated through binding to three G protein-coupled receptors:[14] the high affinity PAC1 and VPAC1/VPAC2, which bind with similar affinity to PACAP and VIP, inducing an intracellular cyclic adenosine monophosphate (cAMP) increase, and a Ca^{2+} response (only PAC1).[25] Although all the biologically active forms of PACAP can be identified in tissues, the predominant form is PACAP 38, whose structural similarity places it in the superfamily of hormones including also secretin, glucagon, glucagon-like peptides, and gastric inhibitory peptide (GIP).[14] These peptides are covered in this and other sections of the book, and specific chapters on PACAP are in the Brain, Cancer, Endocrine, and Respiratory Peptide sections.

The amino acid sequence of PACAP hormone shows interspecies mammalian similarity. Disruption of the PACAP gene has resulted in a greater understanding of the role of PACAP on lipid and carbohydrate metabolism, and thermogenesis.[12] In this murine model, the lack of PACAP expression results in inadequate heat production resulting from insufficient norepinephrine stimulation of brown adipose tissue. PACAP may play a role in the central nervous system (CNS) in the regulation of appetite/satiety by modulating leptin signaling mechanisms;[16] after blockade of endogenous central PACAP signaling with the partial PACAP antagonist, PACAP (6-38), leptin-induced hypophagia and hyperthermia are attenuated *in vivo*. In the gastrointestinal tract, PACAP expression has been identified in the esophagus, stomach, duodenum, and intestine, where its primary function is to regulate secretion and motility.[4] In intestinal warm ischemia in PACAP null mice,[7] PACAP-38 seems to have a protective role.

In intestinal smooth muscle cells, PACAP, released from the enteric nerves acts as a potent nonadrenergic, noncholinergic (NANC) transmitter, supposedly in concert with other mediators to regulate gastrointestinal smooth muscle contraction.[21] NANC inhibition of gastrointestinal smooth muscle involves several intracellular mechanisms, including increase of cyclic guanosine monophosphate (cGMP), increase of cAMP, and hyperpolarization of the cell membrane via direct or indirect activation of potassium ion (K^+) channels. PACAP-induced responses in the intestine are believed to involve intracellular mechanisms of hyperpolarization of the cell membrane, by direct or indirect activation of the apamin-sensitive potassium ion (K^+) channels, by cGMP or cAMP increase, or activation of nitric oxide.

PACAP is also a potent stimulator of cellular growth and differentiation as seen in AR-42 J cells, in which PACAP stimulates an increase of ornithine decarboxylase activity and cell proliferation,[1] and in PC-12, a pheochromocytoma cell line, in which PACAP stimulates neurite outgrowth.[6] After small bowel autotransplantation, PACAP-38 reduces small bowel oxidative stress and injury induced by cold ischemia and reperfusion, suggesting that PACAP-38 may be useful in small-bowel transplantation.[8]

PACAP has been specifically linked to some human motility disorders such as Hirschsprung's disease, in which a reduction in PACAP-immunoreactive nerve fibers is found. PACAP is expressed at high levels in neuroendocrine tumor cells where it induces intracellular signaling, release

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00175-5
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of intracellular preformed mediators such as 5-hydroxytryptamine, and cellular proliferation.[11]

CLONING AND CHARACTERIZATION OF PAC1

Radioligand binding studies with a rat pancreatic acinar carcinoma cell line, AR42 J, as well as hypothalamic and brain cell membranes, suggested that a specific receptor for PACAP exists.[25] The AR42 J cDNA library was used for the cloning and characterization of PAC1 in 1993.[25] The receptor cDNA encodes a putative protein containing 495 amino acids with a molecular weight of approximately 50 kDa.[25] The deduced amino acid sequence of the PAC1 shows that it belongs to the family of VIP, secretin, and glucagon receptors.[14,25] Pharmacologically, PAC1 has >1000-fold greater affinity for either PACAP-27 or PACAP-38, whereas there is little to no affinity for secretin and glucagon.[25] PACAP-27, PACAP-38, and VIP have equal binding affinity for VPAC1 or VPAC2 receptors. Studies of the PAC1 gene identified that the receptor could exist in four major splice variants, which differ in the length of the third intracellular domain, each with a differential tissue expression and different signal transduction properties regarding their ability to differentially activate AC and phospholipase C (PLC).[25] A fourth splice variant occurring in the transmembrane region of PAC1 does not couple to either cyclic AMP or PLC but couples to an L-type Ca^{2+} channel.[14] A similar observation was made after the discovery of the human PAC1, wherein two exons could be differentially spliced and expressed.[25] The differential activation of PLC by the splice variants may have important consequences in the signal transduction cascades observed in the gastrointestinal tract and expressed on tumors.[10] The human PAC1 gene is localized on chromosome 7, while in the rat, it is localized on chromosome 4. Maxadilan, a peptide isolated from a sand fly salivary gland, appears to selectively bind to PAC1 but it does not share peptide homology with either PACAP or VIP.[29] Leukocyte accumulation and plasma leakage induced by maxadilan administration involves a mechanism mediated through the activation of PAC1-receptors expressed on leukocytes and endothelial cells.[29]

RECEPTOR BINDING AND INTRACELLULAR SIGNALING CHARACTERISTICS OF PAC1

Structure–function studies indicate that the N-terminal first extracellular (EC1) domain of PAC1 is the dominant region of the receptor for PACAP hormone recognition and binding.[5,32] Several domains of PACAP hormone have been recognized to be involved in the interaction with PAC1 receptor, and the presence of the helical conformation is not a sufficient feature for receptor activation.[5] PAC1 uses a similar conserved fold of Class B G-protein-coupled receptor extracellular domain for PACAP binding, indicating that there is a conserved consensus for this family of receptors.[17] Subsequent to the discovery of PACAP, Deutsch and Sun, working with the pheochromocytoma cell line PC-12 showed that PACAP is coupled to both AC and PLC.[6] These observations were confirmed in the anterior pituitary-derived somatotrophs and gonadotrophs, as well as of adrenal chromaffin cell cultures.[2] In somatotrophs, PACAP stimulates cAMP-dependent Ca^{2+} influx, whereas in gonadotrophs PACAP induced PLC-stimulated Ca^{2+} release.[2] An examination of the carboxyl-terminal region of PAC1 indicated that there are two critical amino acids (Ser and Arg) responsible for signal transduction coupling.[19] It has been hypothesized that the ability of PAC1 to signal differentially, through either the Gαs or Gαq signal transduction pathway in a tissue-specific manner, suggests a possible mechanism by which PACAP can modulate the proliferation of some tumoral cells such as the pheochromocytoma cell line, PC-12, and neuroblasts.[6,10]

PAC1 is expressed also on many tumor cell lines, including the human colon cancer HCT8 and HCT-116 cell lines[10] and BON neuroendocrine cells of pancreatic carcinoid origin as shown by immunofluorescent staining by confocal laser imaging microscopy in Fig. 1.[11] Differential coupling of PAC1 SV1 splice variant onto HCT8 and HCT-116 human colonic tumor cells induces tumor growth only when both cAMP and Ca^{2+} responses are simultaneously activated. cAMP elevation without Ca^{2+} increase does not seem to activate cell proliferation.[10]

The expression of PACAP in the suprachiasmatic nuclei of the hypothalamus, and the development of transgenic animal models, suggest that PACAP and PAC1 are involved in the regulation of circadian rhythms.[3,13] PACAP has potent neuroprotective effects not only *in vitro* on cultured astrocytes, but also in *in vivo*.[28] The expression of PAC1 in the amygdala and the demonstration of a reduced anxiety behavior suggest that PACAP may be involved in the regulation of stress responses.[15]

ROLE OF PACAP IN THE GASTROINTESTINAL TRACT

In peripheral tissues, the maximum density of PAC1 occurs in the adrenal gland[14] and also the human prostate gland and testis express high levels of PAC1.[14] In the gastrointestinal tract, PACAP is expressed in the esophagus, stomach, duodenum, as well as the small and large intestines.[4] PAC1 has been observed in the rat and mouse enterochromaffin-like cells of the stomach[24] and in gastric and colonic myenteric cells and neurons. In colonic enteric nerve fibers, PACAP and PAC1 are colocalized.[22]

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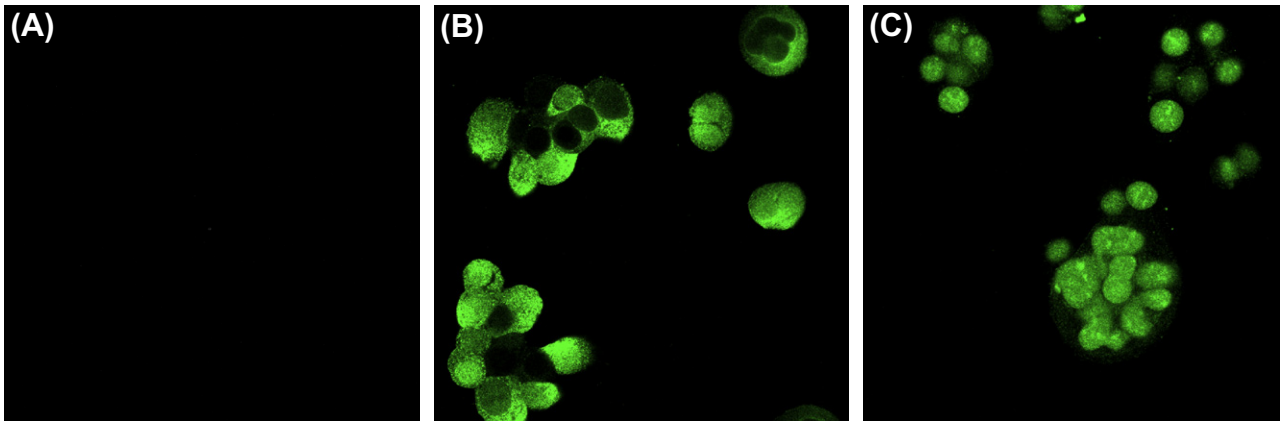

FIGURE 1 Confocal MIcrograph of BON cells stained with (B) anti-PAC1 antibody or (C) anti-PACAP antibody. See color plate 48.

In these studies, whole mount sections were stained with specific anti-PACAP and anti-PAC1 antibodies to show PACAP and PAC1 colocalization in enteric neurons and myenteric cells.[22] Other PACAP receptors as VPAC1 are expressed in the gastrointestinal tract: in the stomach VPAC1 (classical VIP receptor) are expressed on gastric D cells and on gastric chief cells.[33] These different gastric cell types are primarily involved in the secretory digestive functions of the stomach and seem to play a key role in the regulation of gastric acid secretion. In addition to being expressed on secretory cells, both VPAC1 and PAC1 seem to be expressed on gastrointestinal smooth muscle cells.

Other studies indicate that gastrointestinal lymphocytes and antigen presenting cells express VPACs and PAC1,[9] which may play a role in the regulation of gastrointestinal inflammatory responses. In the rat pancreas, PACAP is a potent stimulant of amylase release and presumably the neuropeptide is localized within nerve fibers innervating the pancreas.[26] The existence of PACAP immunoreactivity in the pancreas may implicate a role for PACAP in exocrine pancreatic function.[26] On gastrointestinal smooth muscles PACAP is a potent NANC inhibitory neurotransmitter, which is thought to act via apamin-sensitive potassium channels.[27] PACAP-containing nerve fibers are present in the small and large intestines where they are involved in motility functions. In rats, PACAP stimulates apamin sensitive K^+ channels[27] and both VIP and PACAP stimulate nitric oxide synthase (NOS), as in the porcine jejunum. Other studies show that PACAP modulates colon-inferior mesenteric ganglion (IMG) reflexes. The role of VIP and PACAP on the relaxation of the gastrointestinal smooth muscle is not clear; some results indicate that this effect would be mediated via the release of nitric oxide. In dispersed rabbit gastric muscle cells, crosscompetition by either VIP or PACAP suggests a potential interaction of both peptides with the same receptors via differential signal transduction pathways. In studies on gastric smooth muscle isolated from rabbit or guinea pig, it seems that VPAC2 is the principally expressed receptor.[31] In a guinea pig model, there is specific expression of a subclass of VPAC receptors with specificity for VIP that may represent a novel splice variant. However, the role of PAC1 has been supported by a study in a mutant mouse model showing that the effects of PACAP in mediating NANC relaxation of longitudinal muscles are mediated specifically by PAC1. Currently, it is unclear whether the discrepancy observed in these studies results from species-related differences, or whether there are multiple classes of VPAC and PAC receptors, which are intimately involved in regulating gastrointestinal motor functions. In humans, PACAP has been linked to motility disorders such as Hirschsprung's disease, in which a decrease in the number of PACAP- immunoreactive nerve fibers has been detected.

In gastric PACAP-containing enteric nerve fibers, the neuropeptide has been observed to colocalize with PAC1 receptors.[22] The physiological significance of PACAP expression in the gastric epithelium seems to be related to the gastric ECL cell stimulation of histamine release and thereby of gastric acid secretion.[18] However, PACAP activation of VPAC1 receptors, expressed on the somatostatin-containing D cells, plays an opposite effect by inhibiting gastric acid secretion.[33] The effects of PACAP stimulation of ECL cells during the nocturnal hours may account for the observed increase in gastric acid secretion that occurs in humans at approximately 0200–0400 and, therefore, may have clinical implication in the development of nocturnal gastroesophageal reflux symptoms.[33]

The isolation of pure cell populations of rat gastric ECL cells[24] has enhanced the understanding of the PAC1 receptor expression and the PACAP physiological function in the stomach. PACAP stimulation of pure populations of ECL cells results in intracellular Ca^{2+} release, cAMP stimulation, and histamine release. The use of microarray analysis has confirmed the expression profile of genes predominantly involved in the ECL secretory pathways.[24] Similarly, in

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whole rabbit gastric glandular preparations PACAP activates intracellular Ca^{2+} release in ECL cells, and through the release of histamine stimulates parietal cell acid secretion.[33]

ROLE OF PACAP IN THERMOGENESIS, OBESITY, AND APPETITE

PACAP and PAC1 seem to play an important role in the regulation of appetite/satiety and, more recently, the development of PACAP or PAC1 deletion mouse models has allowed to better study their role in the regulation of feeding behavior. Obesity and particularly abdominal fat accumulation are associated with numerous metabolic abnormalities, including insulin resistance and type II diabetes in a significant proportion of patients. PACAP is a strong secretagog of insulin from pancreatic islets, contributing to the homeostasis of glycemia and modulation of the pathogenesis of diabetes.[26] *In vivo* and *in vitro* studies[26] suggest that PACAP has trophic effects on pancreatic beta cells and regulates both their proliferation and viability, thereby protecting against diabetes. PACAP reduces food intake and regulates energy homeostasis;[20] mRNA expression of PACAP decreases in the VMN during fasting but is increased during high fat diet. PACAP injections into the VMN inhibit food intake under normal and fasting conditions via PAC1 signaling, which may contribute to the thermogenic response of PACAP through activation of α-MSH (melanocortin system), a mediator of PACAP-induced autonomic nerve activity.[16,23] In animal models, intracerebroventricular (icv) injection of PACAP decreases food intake in vertebrates, including rodents, chick, and goldfish,[20] indicating an anorexigenic role for PACAP at the CNS level in the arcuate nucleus (ARC). In chick, PACAP and VIP icv inhibit food intake, but the coinjection of PAC1 receptor antagonist, PACAP (6–38), attenuates only the anorexigenic effect of PACAP, but not that of VIP; whereas, icv coinjection of VPACs receptor antagonist, [D-p-Cl-Phe6, Leu17]-VIP, does not alter the effects of PACAP and VIP, suggesting that PAC1 and not VPACs receptors mediate PACAP anorexigenic mechanisms in the chicken brain. PACAP, released on leptin binding to PACAP containing neurons in the nucleus arcuate, plays an anorexigenic effect through the activation of α-MSH.[23] In contrast, PACAP also stimulates the orexigenic NPY neurons in the ARC, indicating a dual and opposite central activity.

ROLE OF PACAP IN THE IMMUNE SYSTEM

PAC1 is expressed on macrophages and lymphocytes, where it seems to regulate innate and adaptive immune responses, promoting a Th1 cellular phenotype.[9] The PACAP knockout mouse model shows a hyperinflammatory effect in an autoimmune encephalomyelitis (EAE) model of multiple sclerosis, with an enhanced Th1/Th2 cytokine profile.[30] PAC1 expression has been identified on intestinal and peripheral circulating lymphocytes, where it seems to play an important role in the regulation of inflammatory cytokine gene expression.

To investigate the role of PAC1 in colonic mucosal inflammation, the DSS-colitis model was used in PAC1 -/- mice. After four days of DSS treatment, elevated inflammatory parameters are increased in PAC1-deficient mice compared with that of wild-type mice. These results suggest that the PAC1 receptor may be involved in the modulation of the pathogenetic mechanisms that underlie colonic mucosal inflammation and repair mechanisms, and that a deficiency state might increase the tissue damages, that lead to the evolution of inflammatory bowel disease (IBD).

CONCLUSIONS

The discovery of PACAP and its receptor PAC1 has resulted in a new era of understanding the role of neuropeptides in the regulation of physiological processes. PACAP and its receptors are expressed throughout the body, and especially in the central nervous system, where they play an important role in the embryological development of the brain, induce a neurotrophic effect, are involved in the regulation of circadian rhythms, act as anxiolytic agents, and control feeding behavior. In the gastrointestinal tract, the neuronal and mucosal cell expression of this peptide suggests that PACAP is one of the key regulators of the neuroenteric nervous system. PACAP seems to stimulate secretion from the stomach through its actions on the PAC1 receptor,[18] whereas it clearly has a regulatory function in inhibiting gastric acid secretion through the VPAC1 receptor expressed on the gastric D cells.[33] In the intestine, PACAP acting at either via PAC1 or VPAC1 seems to be a regulator of gut motility and responsible for smooth muscle relaxation. In the pancreas, PACAP acts as a trophic factor and is involved in the stimulation of insulin secretion at the exocrine pancreas level.[26] PACAP and PAC1 are potential therapeutic targets for future clinical development.

Supported by US VA Merit Review, AGA IRSA, and VA Career Development Award.

REFERENCES

1. Buscail L, Cambillau C, Seva C, Scemama JL, De Neef P, Robberecht P, et al. Stimulation of rat pancreatic tumoral AR4-2J cell proliferation by pituitary adenylate cyclase-activating peptide. *Gastroenterology* 1992;**103**:1002–8.
2. Canny BJ, Rawlings SR, Leong DA. Pituitary adenylate cyclase-activating polypeptide specifically increases cytosolic calcium ion concentration in rat gonadotropes and somatotropes. *Endocrinology* 1992;**130**:211–5.

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3. Colwell CS, Michel S, Itri J, Rodriguez W, Tam J, Lelièvre V, et al. Selective deficits in the circadian light response in mice lacking PACAP. *Am J Physiol Regul Integr Comp Physiol* 2004;**287**: R1194–201.
4. Cox HM. Pituitary adenylate cyclase activating polypeptides, PACAP-27 and PACAP-38: stimulators of electrogenic ion secretion in the rat small intestine. *Br J Pharmacol* 1992;**106**:498–502.
5. Dejda A, Bourgault S, Doan ND, Létourneau M, Couvineau A, Vaudry H, et al. Identification by photoaffinity labeling of the extracellular N-terminal domain of PAC1 receptor as the major binding site for PACAP. *Biochimie* 2011;**93**(4):669–77.
6. Deutsch PJ, Sun Y. The 38-amino acid form of pituitary adenylate cyclase-activating polypeptide stimulates dual signaling cascades in PC12 cells and promotes neurite outgrowth. *J Biol Chem* 1992;**267**(8):5108–13.
7. Ferencz A, Kiss P, Weber G, Helyes Z, Shintani N, Baba A, et al. Comparison of intestinal warm ischemic injury in PACAP knockout and wild-type mice. *J Mol Neurosci* 2010;**42**(3):435–42.
8. Ferencz A, Racz B, Tamas A, Reglodi D, Lubics A, Nemeth J, et al. Influence of PACAP on oxidative stress and tissue injury following small-bowel autotransplantation. *J Mol Neurosci* 2009;**37**(2):168–76.
9. Ganea D, Rodriguez R, Delgado M. Vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide: players in innate and adaptive immunity. *Cell Mol Biol* 2003;**49**(2):127–42.
10. Germano PM, Le SV, Oh DS, Fan R, Lieu S, Siu A, et al. Differential coupling of the PAC1 SV1 splice variant on human colonic tumors to the activation of intracellular cAMP but not intracellular Ca^{2+} does not activate tumor proliferation. *J Mol Neurosci* 2004;**22**(1–2):83–92.
11. Germano PM, Lieu SN, Xue J, Cooke HJ, Christofi FL, Lu Y, et al. PACAP induces signaling and stimulation of 5-hydroxytryptamine release and growth in neuroendocrine tumor cells. *J Mol Neurosci* 2009;**39**(3):391–401.
12. Gray SL, Yamaguchi N, Vencová P, Sherwood NM. Temperature-sensitive phenotype in mice lacking pituitary adenylate cyclase-activating polypeptide. *Endocrinology* 2002;**143**(10):3946–54.
13. Hannibal J, Brabet P, Fahrenkrug J. Mice lacking the PACAP type I receptor have impaired photic entrainment and negative masking. *Am J Physiol Regul Integr Comp Physiol* 2008;**295**(6):R2050–8.
14. Harmar AJ, Arimura A, Gozes I, Journot L, Laburthe M, Pisegna JR, et al. International Union of Pharmacology. XVIII. Nomenclature of receptors for vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide. *Pharmacol Rev* 1998;**50**(2):265–70.
15. Hashimoto H, Shintani N, Tanida M, Hayata A, Hashimoto R, Baba A. PACAP is implicated in the stress axes. *Curr Pharm Des* 2011;**17**(10):985–9.
16. Hawke Z, Ivanov TR, Bechtold DA, Dhillon H, Lowell BB, Luckman SM. PACAP neurons in the hypothalamic ventromedial nucleus are targets of central leptin signaling. *J Neurosci* 2009;**29**(47):14828–35.
17. Kumar S, Pioszak A, Zhang C, Swaminathan K, Xu HE. Crystal structure of the PAC1R extracellular domain unifies a consensus fold for hormone recognition by class B G-protein coupled receptors. *PLoS One* 2011;**6**(5):e19682.
18. Lu Y, Germano PM, Ohning GV, Vu JP, Pisegna JR. PAC1 deficiency in a murine model induces gastric mucosa hypertrophy and higher basal gastric acid output. *J Mol Neurosci* 2011;**43**(1):76–84.
19. Lyu RM, Germano PM, Choi JK, Le S, Pisegna J. Identification of an essential amino acid motif within the C terminus of the pituitary adenylate cyclase-activating polypeptide type I receptor that is critical for signal transduction but not for receptor internalization. *J Biol Chem* 2000;**275**(46):36134–42.
20. Matsuda K, Maruyama K, Nakamachi T, Miura T, Uchiyama M, Shioda S. Inhibitory effects of pituitary adenylate cyclase-activating polypeptide (PACAP) and vasoactive intestinal peptide (VIP) on food intake in the goldfish, *Carassius auratus*. *Peptides* 2005;**26**(9):1611–6.
21. Matsuda NM, Miller SM. Non-adrenergic non-cholinergic inhibition of gastrointestinal smooth muscle and its intracellular mechanism(s). *Fundam Clin Pharmacol* 2010 Jun;**24**(3):261–8. [Epub 2009 Aug 6].
22. Miampamba M, Germano PM, Arli S, Wong HH, Scott D, Tache Y, et al. Expression of pituitary adenylate cyclase-activating polypeptide and PACAP type 1 receptor in the rat gastric and colonic myenteric neurons. *Regul Pept* 2002;**105**(3):145–54.
23. Mounien L, Do Rego JC, Bizet P, Boutelet I, Gourcerol G, Fournier A, et al. Pituitary adenylate cyclase-activating polypeptide inhibits food intake in mice through activation of the hypothalamic melanocortin system. *Neuropsychopharmacology* 2009;**34**(2):424–35.
24. Oh DS, Lieu SN, Yamaguchi DJ, Tachiki K, Lambrecht N, Ohning GV, et al. PACAP regulation of secretion and proliferation of pure populations of gastric ECL cells. *J Mol Neurosci* 2005;**26**(1):85–98.
25. Pisegna JR, Wank SA. Cloning and characterization of the signal transduction of four splice variants of the human pituitary adenylate cyclase activating polypeptide receptor. Evidence for dual coupling to adenylate cyclase and phospholipase C. *J Biol Chem* 1996;**271**(29):17267–74.
26. Sakurai Y, Shintani N, Hayata A, Hashimoto H, Baba A. Trophic effects of PACAP on pancreatic islets: a mini-review. *J Mol Neurosci* 2011;**43**(1):3–7.
27. Schworer H, Katsoulis S, Creutzfeldt W, Schmidt WE. Pituitary adenylate cyclase activating peptide, a novel VIP-like gut–brain peptide, relaxes the guinea-pig taenia caeci via apamin-sensitive potassium channels. *Naunyn Schmiedebergs Arch Pharmacol* 1992; **346**(5):511–4.
28. Seaborn T, Masmoudi-Kouli O, Fournier A, Vaudry H, Vaudry D. Protective effects of pituitary adenylate cyclase-activating polypeptide (PACAP) against apoptosis. *Curr Pharm Des* 2011;**17**(3):204–14: Review.
29. Svensjö E, Saraiva EM, Amendola RS, Barja-Fidalgo C, Bozza MT, Lerner EA, et al. Maxadilan, the lutzomyia longipalpis vasodilator, drives plasma leakage via PAC1-CXCR1/2-pathway. *Microvasc Res* 2012;**83**(2):185–93.
30. Tan YV, Abad C, Lopez R, Dong H, Liu S, Lee A, et al. Pituitary adenylyl cyclase-activating polypeptide is an intrinsic regulator of Treg abundance and protects against experimental autoimmune encephalomyelitis. *Proc Natl Acad Sci U S A* 2009;**106**(6):2012–7.
31. Vanneste G, Robberecht P, Lefebvre RA. Inhibitory pathways in the circular muscle of rat jejunum. *Br J Pharmacol* 2004;**143**(1):107–18.
32. Yu R, Li J, Wang J, Liu X, Huang L, Ding Y, et al. The functional recombinant first extracellular (EC1) domain of PACAP receptor PAC1 normal form (PAC1-EC1(N)) recognizes selective ligands and stimulates the proliferation of PAC1-CHO cells. *Neurosci Lett* 2010;**480**(1):73–7.
33. Zeng N, Athmann C, Kang T, Lyu RM, Walsh JH, Ohning GV, et al. PACAP type I receptor activation regulates ECL cells and gastric acid secretion. *J Clin Invest* 1999;**104**(10):1383–91.

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Chapter 176

Pancreatic Polypeptide

Tricia M. Tan and Stephen R. Bloom

ABSTRACT

Pancreatic polypeptide (PP) is a 36-amino acid, C-terminally amidated peptide, and is a member of the PP-fold peptide family. It is produced and secreted by the PP cells of the islets of Langerhans. PP secretion is stimulated by food intake proportional to calorie intake under the control of the vagus nerve. Its physiological functions include actions as a satiety hormone, suppression of exocrine pancreatic secretion and possibly in slowing gastric emptying. The PP cognate receptor is the neuropeptide Y4 receptor, and key anatomical structures expressing this receptor are the brainstem (area postrema, dorsal vagal complex), hypothalamus and the small and large intestines. There is evidence of a distinct PP receptor in the liver, but this is not as well characterized as yet.

DISCOVERY

PP was originally discovered as a contaminant of insulin preparations from chicken pancreas by Kimmel et al.,[19] and bovine pancreas by Lin and Chance.[24]

STRUCTURE OF THE GENE AND PROTEIN

The *Homo sapiens* gene encoding the precursor preproPP (HGNC name: *PPY*) is located on chromosome 17, adjacent to the gene encoding the precursor of peptide YY (HGNC name: *PYY*). Evolutionarily, *PPY* seems to have arisen as a tandem duplication of the *PYY* gene with or just before the emergence of tetrapods. In a second evolutionary event, the *PYY-PPY* gene cluster has been duplicated in the primate and ungulate lineages, after the divergence of the rodents, to create a *PYY2-PPY2* cluster on chromosome 17. The *PPY2* gene seems to be a pseudogene as there is a point mutation introducing a premature stop codon in the signal peptide region, and the gene does not encode a functional protein.[13]

The cDNA for *PPY*, first isolated and sequenced by Boel et al.,[10] consists of 4 exons, all constitutively spliced together. Exon 1 contains the sequence of the 5′ untranslated region (45 nt long). Exons 2–4 contain the coding sequences. Notably, exon 2 contains the signal peptide and most of the functional domain of PP with the exception of the C-terminal Tyr^{36}. Exon 3 encodes the Tyr^{36} residue and the C-terminal PP "icosapeptide." Exon 4 encodes the C-terminal heptapeptide and the 3′ end of exon 4 contains a 3′ untranslated region of 124 nt.

PP is a 36-amino acid, C-terminally amidated peptide (Fig. 1). It is a member of the so-called PP-fold peptide family. This family encompasses neuropeptide Y (NPY), peptide YY (PYY), and PP. These peptides contain a common structural motif, the PP-fold, which consists of two antiparallel helices, a type 2 proline helix and an alpha helix, connected by a type 2 beta turn. The two helices interact via hydrophobic residues, stabilizing the fold.[35]

The sequences of human and bovine PP are closely related and differ only by two residues.[10] A sequence comparison between the PP orthologs isolated from mammals, avians, and reptiles identifies 8 conserved (and presumably key) residues, namely, Pro^5, Pro^8, Gly^9, Ala^{12}, Leu^{24}, Tyr^{27}, Arg^{33}, and Arg^{35}.[14]

PROCESSING OF PREPROPP AND PP

The precursor preproPP (Fig. 2) is a 95-amino acid peptide, which is processed to PP (residues 30–65 of preproPP). The N-terminal signal peptide is cleaved in the endoplasmic reticulum (preproPP residues 1–29) to release proPP (residues 30–95). Residues 66–68 (Gly-Lys-Arg—"GKR") represent the cleavage and amidation signal.[11] Residues 69–87 encode the PP icosapeptide, which is followed by the C-terminal heptapeptide (residues 88–95).

ProPP is believed to be first cleaved at the "dibasic cleavage site" Lys-Arg (residues 67,68) to release PP and the proPP C-terminal peptide. The proPP C-terminal peptide is subsequently processed at the "monobasic cleavage site" to release the PP icosapeptide and the C-terminal heptapeptide.

Although multiple forms of PP immmunoreactivity separable by gel filtration chromatography are found in circulation, normal pancreatic tissue only produces one form of PP that is indistinguishable from the pure PP peptide.[2] Therefore, the circulating forms probably represent incompletely cleaved or breakdown products.

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00176-7
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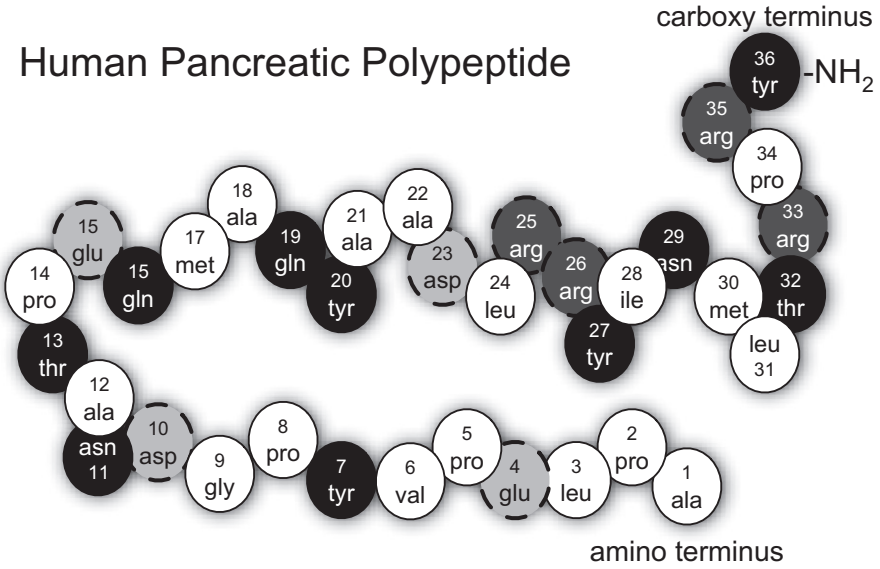

FIGURE 1 Primary sequence of human pancreatic polypeptide. Amino acid residues are abbreviated as follows: Ala = alanine, Pro = proline, Leu = leucine, Glu = glutamic acid, Val = valine, Tyr = tyrosine, Gly = glycine, Asp = aspartic acid, Asn = asparagine, Thr = threonine, Gln = glutamine, Met = methionine, Arg = arginine, Ile = isoleucine. NH_2 = carboxy terminal amide group. Dashed line, dark gray = positively charged side chains; Dashed line, light gray = negatively charged side chains, Black = polar uncharged side chains; Solid line, white = hydrophobic side chains. Numbers denote the residue numbers from the amino terminus.

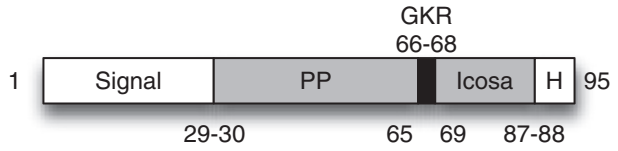

FIGURE 2 Structure of preproPP peptide. Key: Signal = signal peptide; GKR = Gly-Lys-Arg cleavage and amidation signal; Icosa = Icosapeptide; H = C-terminal heptapeptide.

Pharmacokinetic studies with bovine PP infusions into human volunteers reveal that PP is rapidly broken down, with an apparent terminal elimination half life of 6.9 ± 0.3 min, a metabolic clearance rate of 5.1 ± 0.2 ml/kg/min and an apparent volume of distribution of 51 ± 3 ml/kg.[4] This rapid breakdown is mediated by endogenous peptidases such as neprilysin (neutral endopeptidase 24.11) and dipeptidyl peptidase IV.

DISTRIBUTION AND SECRETION OF PP

PP is secreted by PP cells of the islets of Langerhans. The PP cells are localized more in the periphery of the islets. PP cells tend to be found in greater quantity in the islets in the head of the pancreas rather than the tail. In primates, 93% is found in the pancreas, but PP is also found in smaller quantities in the distal gut. The mean fasting plasma level of PP in normal volunteers is 31.2 ± 6.2 pmol/L.[2]

Food intake stimulates PP secretion, for example, a "normal" hospital lunch caused a postprandial increase in PP levels to 164 ± 46.2 pmol/L. The postprandial secretion seems to be biphasic, incorporating an acute peak of secretion at 0–60 min and a more sustained peak from 60 to at least 360 min.[1] Postprandial PP secretion is proportional to the size of the meal eaten. Postprandial PP secretion seems to be under the control of the vagus nerve, such that vagotomy or atropine treatment markedly reduce secretion.[32]

PP release is not stimulated by parenteral infusions of glucose, amino acids, or fat. Insulin-induced hypoglycemia induces a sharp increase in PP secretion from the pancreas.[1]

Other influences on PP secretion include the following:

- *Cholecystokinin* (*CCK*): Adrian et al. showed that an infusion of caerulein, a CCK analog, in humans stimulates PP release,[1] and this is also true of the CCK peptides found in humans.
- *Secretin*, *Gastric Inhibitory Peptide* (*GIP*): Secretin and GIP are gut hormones that are related in sequence. Secretin stimulates PP release in human volunteers.[1] Similarly, a bolus injection of GIP has been shown to stimulate PP release by 2- to 3-fold in volunteers.[6]
- *Somatostatin*: Studies with an isolated perfused human pancreas preparation show that there seems to be no direct effect of somatostatin-14 infusion on PP secretion. There was also no effect of immunoneutralization of intraislet somatostatin on PP secretion.[20] However, infusion of somatostatin into human volunteers does suppress PP secretion, suggesting that the effects of somatostatin may be indirectly mediated.[27]
- *Ghrelin and related peptides*: The octanoylated form of ghrelin and obestatin (an alternative product of the processing of the proghrelin precursor) seem to inhibit the secretion of PP by isolated rodent islets.[30]

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RECEPTORS AND SIGNALING CASCADES

PP-fold peptides bind to the neuropeptide Y family of seven-transmembrane-domain receptors, Y1, Y2, Y4, and Y5, all of which are expressed in the hypothalamus and in the periphery.[9]

PP binds with highest affinity to the Y4 receptor with a K_D of 13.8 pM, and also to Y1 and Y5 with lesser affinity. The Y4 receptor is distributed widely, with expression in the small and large intestines and testis (in the rat).[9] It is distributed widely in the CNS, particularly in the hypothalamus, area postrema, the dorsal vagal complex and a subregion of the nucleus tractus solitarus called the subnucleus gelatinosus.[34] Y4 receptor exists in the membrane as homodimers, but these dissociate on binding PP. The activated Y4 receptor inhibits adenylyl cyclase via the G_i G-protein, therefore reducing intracellular cyclic AMP levels.[25] Y4 also activates G_q, inositol triphosphate release, and intracellular Ca^{2+} release.[28]

The key residues of PP involved in binding of PP to Y4 appear to be Pro^2, Pro^3, Arg^{33}, Arg^{35}, Tyr^{36}. Deletion of the C-terminal amide substantially reduces binding, indicating its key importance.[16]

A specific PP-binding receptor activity has been found in the liver that binds PP with a higher K_D of 3.4–4.6 nM.[29] Y4 receptor is not known to be expressed in the liver, implying that the hepatic PP receptor is a distinct receptor type, the exact nature of which is as yet unknown.

BIOLOGICAL ACTIONS

Effects of PP on Appetite, Energy Expenditure, and Energy Balance

A major physiological action of PP seems to be as a postprandial satiety hormone. PP was originally shown to reduce food intake when administered peripherally to mice in 1977.[26] Batterham et al. showed that an iv infusion of PP at a dose of 10 pmol/kg/min for 90 min reduced food intake in lean human volunteers by 21.8 ± 5.7%, as assessed by consumption of a buffet lunch. There was a significant inhibition of cumulative food intake during 24 h of 25.3 ± 5.8%. During the infusion, PP levels increased from a baseline of 15.5 ± 4.4 to 258.5 ± 21.4 pmol/L, a supraphysiological concentration. Although PP is known to slow gastric emptying in other studies,[31] at this dose, there was no detectable effect on gastric emptying.[8]

Schmidt et al. also infused PP at lower doses of up to 2.25 pmol/kg/min in lean human volunteers.[31] Interestingly, this lower-dose infusion achieved peak levels of 299 ± 23.7 pmol/L, higher than those reported by Batterham et al., and three times normal postprandial levels. The study did not focus on food intake as an endpoint, but it was noted that PP infusion was not associated with significant reductions in hunger and appetite as assessed by visual analog scales.

The exact site of action of PP's anorexigenic (appetite-reducing) action is not completely known. Possible sites of action include the area postrema as Y4 receptors are particularly rich in this area, and also visceral vagal nerve afferents to the brainstem as vagotomy abolishes the anorectic effect of PP.[21] Indeed, intraperitoneal PP activates the area postrema and nucleus tractus solitarius as assessed by staining for the neuronal activation marker c-*fos* (Fig. 3).[23]

The hypothalamus is also another possible site of action, circulating PP perhaps diffusing to the hypothalamus through the incomplete blood–brain barrier at the median eminence. Intraperitoneal PP also activates c-*fos* expression in the arcuate nucleus of the hypothalamus and, more specifically, the anorexigenic neuronal group that expresses alpha-MSH (Fig. 3, POMC/CART). The importance of alpha-MSH signaling in mediating the anorexigenic action of PP is underlined by the observation that knockout of the alpha-MSH receptor, MC4, abolishes the anorexigenic effect of PP. Moreover, a conditional knockout of the Y4 receptor in the hypothalamus abolishes the activation of the alpha-MSH neurons by intraperitoneal PP, suggesting that the principal site of action is indeed in the hypothalamus.[23] In addition to its action on food intake, repeated intraperitoneal injections of PP also stimulate sympathetic activity and oxygen consumption, perhaps causing an increased energy expenditure that would tend to augment weight loss.[7]

Effects of PP on Pancreatic Secretion and Gall Bladder Function

PP inhibits the exocrine secretion from the pancreas and the motor function of the biliary tree and gall bladder. This was assessed by measurement of trypsin and bilirubin output, respectively, from duodenal juice samples in volunteers undergoing infusion of secretin and caerulein to stimulate pancreatic secretion and gall bladder contraction: infusion of bovine PP to achieve typical postprandial PP levels inhibited trypsin output four-fold and suppressed bilirubin secretion almost completely.[17] Extrinsic denervation of the pancreas does not affect PP inhibitory activity, which suggests that intrinsic mechanisms are involved.[15]

Gastric Effects of PP

Bovine PP does not seem to have any significant effects on gastric emptying nor on gastric secretion in humans when given at doses to achieve circulating PP levels equivalent to the postprandial state.[5] By contrast, Schmidt et al. infused human PP (as opposed to bovine PP) into volunteers eating a radioactively labeled meal, and they showed PP is able to slow gastric emptying of solid food but not water. However, they used a dose that was sufficient to increase circulating levels to approximately 3-fold normal postprandial levels,

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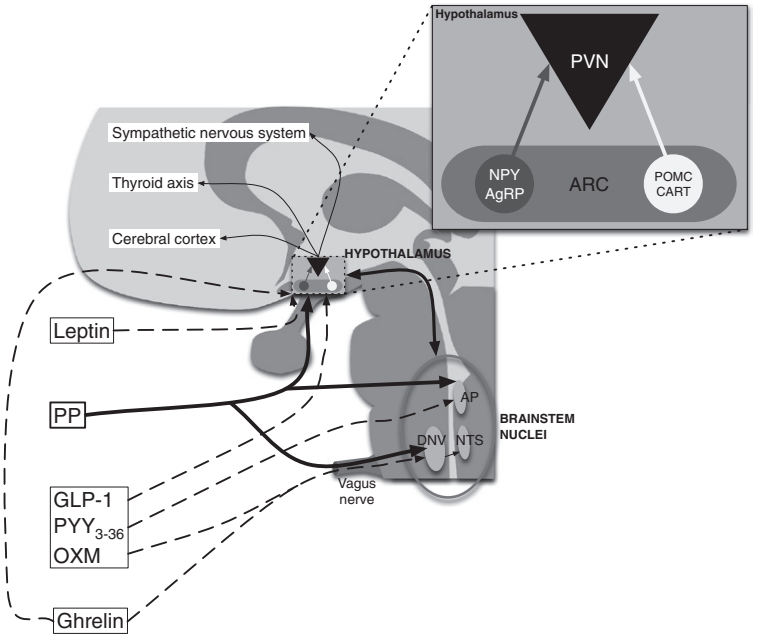

FIGURE 3 Appetite regulatory centers of the CNS. The two main centers in the CNS that regulate appetite, the brainstem nuclei (DNV—Dorsal Nucleus of the Vagus; NTS—Nucleus Tractus Solitarius; AP—Area Postrema) and the hypothalamus (ARC—arcuate nucleus; PVN—paraventricular nucleus) are indicated. These two centers enjoy extensive reciprocal connections with each other. Within the ARC, there exist two antagonistic sets of neurons projecting to the PVN: orexigenic neurons expressing neuropeptide Y (NPY) and Agouti gene Related Peptide (AgRP), and anorexigenic neurons expressing pro-opiomelanocortin (POMC) and Cocaine-Amphetamine Regulated Transcript protein (CART). POMC is processed to alpha-MSH (top right hand of the figure). The regulation of food intake and weight is determined by the balance of activities between these two sets of neurons, and their influence on the PVN. The PVN in turn influences higher centers in the cerebral cortex (to regulate food-seeking behavior), the thyroid axis and sympathetic nervous system (to regulate basal metabolism). Some peripheral influences on the brainstem nuclei and the hypothalamus are indicated (leptin, GLP-1, $PYY_{3–36}$, oxyntomodulin (OXM), Ghrelin—the dashed arrows indicate likely mechanisms of action). In particular, PP is believed to exert its effects by (1) diffusion through areas where the blood–brain barrier is incomplete with activation of Y4 NPY receptors at the AP and perhaps the median eminence of the hypothalamus and (2) activating visceral vagal nerve afferents.

and so the physiological relevance of this observation may be questioned.[31]

Metabolic Effects of PP

The infusion of bovine PP does not affect the basal nor the postprandial secretion of insulin.[3] Similarly, an infusion of human PP does not affect the basal secretion of insulin.[8] Both studies show no significant effects of PP infusion on the secretion of glucagon, gastrin, GIP, secretin, oxyntomodulin (enteroglucagon), peptide YY, glucagon-like peptide-1 (GLP-1), ghrelin, or leptin.[3,8] Schmidt et al. did show a slight delay to the peak of postprandial insulin secretion by about 16 min at the highest dose of human PP used, together with a sustained postprandial increase in glucose. This may be explained by the delay in gastric emptying of solid food that they observed and therefore may not represent a direct effect of PP.[31]

Can PP influence insulin sensitivity? This question was studied in patients with chronic pancreatitis, which exhibit PP deficiency and a blunted response to insulin in terms of suppression of hepatic glucose output during euglycemic–hyperinsulinemic clamp studies, that is, evidence of insulin resistance. Brunicardi et al. showed that an 8-h bovine PP infusion restores insulin sensitivity. PP reduced patients' mean glucose levels in response to an oral glucose challenge in two of the five patients studied, indicating that this change in insulin sensitivity has some influence on glucose tolerance in these patients.[12] It is unclear, however, if PP significantly influences insulin sensitivity in healthy humans.

PATHOPHYSIOLOGICAL IMPLICATIONS

High concentrations of plasma PP are frequently observed in patients with pancreatic neuroendocrine tumors (PNET). Some cases of this phenomenon result from PP cell hyperplasia in the normal islets surrounding a PNET that may be secreting other hormones (insulin, VIP, glucagon), and some result from incorporation of PP-secreting cells within the tumor itself. More rarely, some cases are because of a primary PP-secreting PNET, that is, a "PPoma."

PPomas are generally diagnosed during the investigation of abdominal pain, hepatomegaly, liver metastases, obstructive jaundice, pancreatitis, or gastrointestinal hemorrhage (either resulting from direct erosion of blood vessels or resulting from portal hypertension as a consequence

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of portal vein thrombosis). Although watery diarrhea was initially believed to be part of the tumor syndrome, it is now evident that this occurs only in one-third of patients. Weight loss can occur as a presenting symptom, consistent with PP's satiety-inducing action. In contrast to other varieties of PNETs, metastatic/malignant PPomas are relatively uncommon, but not unknown.[22]

Given its postprandial release and postulated role in reducing appetite after food intake, it may be conjectured that obesity may result, at least partially, from a reduction in PP secretion, therefore decreasing the feedback inhibition of food intake. Some studies have shown that the fasting and postprandial levels of PP are lower in obese subjects compared with that in lean subjects. Conversely, other studies have found that there are no significant differences in PP levels (fasting or postprandial) between lean and obese subjects. Thus, there remains some controversy as to the exact relationship between circulating PP levels and obesity. This may perhaps be because there are marked variations in PP secretion, influenced by diurnal variation, by age (where increasing age is associated with increased PP levels), and by large interindividual variations.[18]

Finally, given the actions of PP as a satiety hormone, it represents an attractive therapeutic target for an antiobesity drug. Longer-lasting analogs of PP and other Y4 agonists are currently in trial for this purpose.[33]

REFERENCES

1. Adrian TE, Besterman HS, Cooke TJ, Bloom SR, Barnes AJ, Russell RC. Mechanism of pancreatic polypeptide release in man. *Lancet* 1977;**1**:161–3.
2. Adrian TE, Bloom SR, Bryant MG, Polak JM, Heitz PH, Barnes AJ. Distribution and release of human pancreatic polypeptide. *Gut* 1976;**17**:940–4.
3. Adrian TE, Greenberg GR, Barnes AJ, Christofides ND, Alberti KG, Bloom SR. Effects of pancreatic polypeptide on motilin and circulating metabolites in man. *Eur J Clin Invest* 1980;**10**:235–40.
4. Adrian TE, Greenberg GR, Besterman HS, Bloom SR. Pharmacokinetics of pancreatic polypeptide in man. *Gut* 1978;**19**:907–9.
5. Adrian TE, Greenberg GR, Fitzpatrick ML, Bloom SR. Lack of effect of pancreatic polypeptide in the rate of gastric emptying and gut hormone release during breakfast. *Digestion* 1981;**21**:214–8.
6. Ahren B, Pettersson M, Uvnas-Moberg K, Gutniak M, Efendic S. Effects of cholecystokinin (CCK)-8, CCK-33, and gastric inhibitory polypeptide (GIP) on basal and meal-stimulated pancreatic hormone secretion in man. *Diabetes Res Clin Pract* 1991;**13**:153–61.
7. Asakawa A, Inui A, Yuzuriha H, Ueno N, Katsuura G, Fujimiya M, et al. Characterization of the effects of pancreatic polypeptide in the regulation of energy balance. *Gastroenterology* 2003;**124**:1325–36.
8. Batterham RL, Le Roux CW, Cohen MA, Park AJ, Ellis SM, Patterson M, et al. Pancreatic polypeptide reduces appetite and food intake in humans. *J Clin Endocrinol Metab* 2003;**88**:3989–92.
9. Berglund MM, Hipskind PA, Gehlert DR. Recent developments in our understanding of the physiological role of PP-fold peptide receptor subtypes. *Exp Biol Med* 2003;**228**:217–44.
10. Boel E, Schwartz TW, Norris KE, Fiil NP. A cDNA encoding a small common precursor for human pancreatic polypeptide and pancreatic icosapeptide. *EMBO J* 1984;**3**:909–12.
11. Bradbury AF, Finnie MD, Smyth DG. Mechanism of C-terminal amide formation by pituitary enzymes. *Nature* 1982;**298**:686–8.
12. Brunicardi FC, Chaiken RL, Ryan AS, Seymour NE, Hoffmann JA, Lebovitz HE, et al. Pancreatic polypeptide administration improves abnormal glucose metabolism in patients with chronic pancreatitis. *J Clin Endocrinol Metab* 1996;**81**:3566–72.
13. Conlon JM. The origin and evolution of peptide YY (PYY) and pancreatic polypeptide (PP). *Peptides* 2002;**23**:269–78.
14. Conlon JM, Platz JE, Chartrel N, Vaudry H, Nielsen PF. Amino acid sequence diversity of pancreatic polypeptide among the amphibia. *Gen Comp Endocrinol* 1998;**112**:146–52.
15. DeMar AR, Taylor IL, Fink AS. Pancreatic polypeptide and peptide YY inhibit the denervated canine pancreas. *Pancreas* 1991;**6**: 419–26.
16. Gehlert DR, Schober DA, Beavers L, Gadski R, Hoffman JA, Smiley DL, et al. Characterization of the peptide binding requirements for the cloned human pancreatic polypeptide-preferring receptor. *Mol Pharmacol* 1996;**50**:112–8.
17. Greenberg GR, McCloy RF, Adrian TE, Chadwick VS, Baron JH, Bloom SR. Inhibition of pancreas and gallbladder by pancreatic polypeptide. *Lancet* 1978;**2**:1280–2.
18. Johns CE, Newton JL, Westley BR, May FE. Human pancreatic polypeptide has a marked diurnal rhythm that is affected by ageing and is associated with the gastric TFF2 circadian rhythm. *Peptides* 2006;**27**:1341–8.
19. Kimmel JR, Pollock HG, Hazelwood RL. Isolation and characterization of chicken insulin. *Endocrinology* 1968;**83**:1323–30.
20. Kleinman R, Gingerich R, Ohning G, Wong H, Olthoff K, Walsh J, et al. The influence of somatostatin on glucagon and pancreatic polypeptide secretion in the isolated perfused human pancreas. *Int J Pancreatol* 1995;**18**:51–57.
21. Kojima S, Ueno N, Asakawa A, Sagiyama K, Naruo T, Mizuno S, et al. A role for pancreatic polypeptide in feeding and body weight regulation. *Peptides* 2007;**28**:459–63.
22. Kuo SC, Gananadha S, Scarlett CJ, Gill A, Smith RC. Sporadic pancreatic polypeptide secreting tumors (PPomas) of the pancreas. *World J Surg* 2008;**32**:1815–22.
23. Lin S, Shi YC, Yulyaningsih E, Aljanova A, Zhang L, Macia L, et al. Critical role of arcuate Y4 receptors and the melanocortin system in pancreatic polypeptide-induced reduction in food intake in mice. *PLoS One* 2009;**4**:e8488.
24. Lin TM, Chance RE. Candidate hormones of the gut. VI. Bovine pancreatic polypeptide (BPP) and avian pancreatic polypeptide (APP). *Gastroenterology* 1974;**67**:737–8.
25. Lundell I, Statnick MA, Johnson D, Schober DA, Starback P, Gehlert DR, et al. The cloned rat pancreatic polypeptide receptor exhibits profound differences to the orthologous receptor. *Proc Natl Acad Sci U S A* 1996;**93**:5111–5.
26. Malaisse-Lagae F, Carpentier JL, Patel YC, Malaisse WJ, Orci L. Pancreatic polypeptide: a possible role in the regulation of food intake in the mouse. Hypothesis. *Experientia* 1977;**33**:915–7.
27. Marco J, Hedo JA, Villanueva ML. Inhibitory effect of somatostatin on human pancreatic polypeptide secretion. *Life Sci* 1977;**21**:789–92.
28. Misra S, Murthy KS, Zhou H, Grider JR. Coexpression of Y1, Y2, and Y4 receptors in smooth muscle coupled to distinct signaling pathways. *J Pharmacol Exp Ther* 2004;**311**:1154–62.

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29. Nguyen TD, Wolfe MS, Heintz GG, Whitcomb DC, Taylor IL. High affinity binding proteins for pancreatic polypeptide on rat liver membranes. *J Biol Chem* 1992;**267**:9416–21.
30. Qader SS, Hakanson R, Rehfeld JF, Lundquist I, Salehi A. Proghrelin-derived peptides influence the secretion of insulin, glucagon, pancreatic polypeptide and somatostatin: a study on isolated islets from mouse and rat pancreas. *Regul Pept* 2008;**146**:230–7.
31. Schmidt PT, Naslund E, Gryback P, Jacobsson H, Holst JJ, Hilsted L, et al. A role for pancreatic polypeptide in the regulation of gastric emptying and short-term metabolic control. *J Clin Endocrinol Metab* 2005;**90**:5241–6.
32. Schwartz TW, Holst JJ, Fahrenkrug J, Jensen SL, Nielsen OV, Rehfeld JF, et al. Vagal, cholinergic regulation of pancreatic polypeptide secretion. *J Clin Invest* 1978;**61**:781–9.
33. Tan TM, Field BC, Minnion JS, Cuenco-Shillito J, Chambers ES, Zac-Varghese S, et al. Pharmacokinetics, adverse effects and tolerability of a novel analogue of human pancreatic polypeptide, PP 1420. *Br J Clin Pharmacol* 2011. doi:10.1111/j.1365-2125.2011.04082.x.
34. Whitcomb DC, Puccio AM, Vigna SR, Taylor IL, Hoffman GE. Distribution of pancreatic polypeptide receptors in the rat brain. *Brain Res* 1997;**760**:137–49.
35. Wood SP, Pitts JE, Blundell TL, Tickle IJ, Jenkins JA. Purification, crystallisation and preliminary X-ray studies on avian pancreatic polypeptide. *Eur J Biochem* 1977;**78**:119–26.

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Chapter 177

Paneth Cell α-Defensins

André J. Ouellette and Michael E. Selsted

ABSTRACT

Enteric α-defensins are constituents of dense core granules that Paneth cells secrete from small intestinal crypts into the lumen in response to cholinergic or bacterial stimuli. The molecular signature of the ~4.5 kDa α-defensins consists of six Cys residues which form an invariant pattern of disulfide pairings. The tridisulfide array establishes a conserved backbone topology and protects against proteolysis, enabling the peptides to function in the environment of the intestinal lumen. α-Defensins are broad-spectrum microbicides that constitute the majority of bactericidal peptide activity released by Paneth cells. Enteric α-defensins modulate the composition of the small intestinal microbiota, and they confer immunity to experimental infection by orally-administered *Salmonella enterica* serovar Typhimurium. Mutations that disrupt Paneth cell homeostasis may cause defective α-defensin secretion or reduce the solubility of secreted granules, diminishing luminal Paneth cell α-defensin levels, impairing innate immunity, and contributing to immunopathologies.

DISCOVERY OF PANETH CELL α-DEFENSINS

The defensins are one of two major antimicrobial peptide (AMP) families in mammals and were among the first AMPs to be identified and characterized.[26] They comprise three cationic, Cys-rich subfamilies, termed α-, β-, and θ-defensins, all of which have broad-spectrum antimicrobial activities.[28] The α-defensins are ~4.5 kDa and contain six Cys residues positioned in a pattern that facilitates the formation of invariant Cys^{I}–Cys^{VI}, Cys^{II}–Cys^{IV}, Cys^{III}–Cys^{V} disulfide bond pairings that define the α-defensins (Fig. 1).

Paneth cell α-defensins were found as abundant small intestinal mRNAs expressed in adults but not in fetal mouse small bowel, which coded for α-defensin precursors as inferred from the spacing of six Cys residues in the C-terminal region of the deduced mRNA products. Prepro-α-defensin mRNAs were abundant only in small intestine and localized by in situ hybridization to Paneth cells at the base of the crypts of Lieberkühn. The mouse α-defensin peptides were termed "cryptdins" (Crps) for "crypt defensin."[16] α-Defensin mRNAs were soon identified in human Paneth cells with oligonucleotide probes based on sequences conserved in known α-defensin cDNAs and genes.[10] Cloning of mouse crypt α-defensin cDNAs showed that mouse Paneth cells express numerous α-defensin variants, many with very similar primary structures, and genomic studies confirmed that the peptide isoforms were coded by individual, closely linked genes. By immunohistochemical analysis, Crp1 was localized exclusively to the cytoplasm of small bowel Paneth cells and subsequently to their dense core secretory granules (Fig. 2). The bactericidal activities predicted for Paneth cell α-defensins were confirmed with the purification mouse enteric α-defensin peptides and the demonstration of their potent microbicidal activities.[27]

DEFENSIN GENE AND PRECURSOR STRUCTURES

The determinants regulating α-defensin gene transcription and peptide abundance are poorly understood. Paneth cell defensin genes are closely linked at chromosomal loci along with the myeloid α-defensin genes. Genes that encode HD5 and HD6, the two human Paneth cell α-defensins, map to 8p21-8pter and are the syntenic with mouse genes located in the proximal region of chromosome 8.[1,17,30] α-Defensin gene expression is tissue-restricted, with major sites of expression in promyelocytes of the bone marrow and Paneth cells of the small intestine. The fidelity and Paneth cell specificity of HD5 transgene expression of *DEFA5*-transgenic (+/+) mice suggests that *cis*-acting β-catenin/TCF-4 sites in the human Paneth cell HD5 gene are conserved and sufficient to direct transgene gene transcription with accuracy.[21]

Most myeloid α-defensin genes consist of three exons, and genes expressed in Paneth cells have a two-exon structure.[28] Most myeloid α-defensin genes, as well as the related θ-defensin genes in primates, have an additional intron ranging from 1 to 6.5 kb that interrupts the 5′-untranslated region (Fig. 3). Whether α-defensin genes consist of

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00177-9
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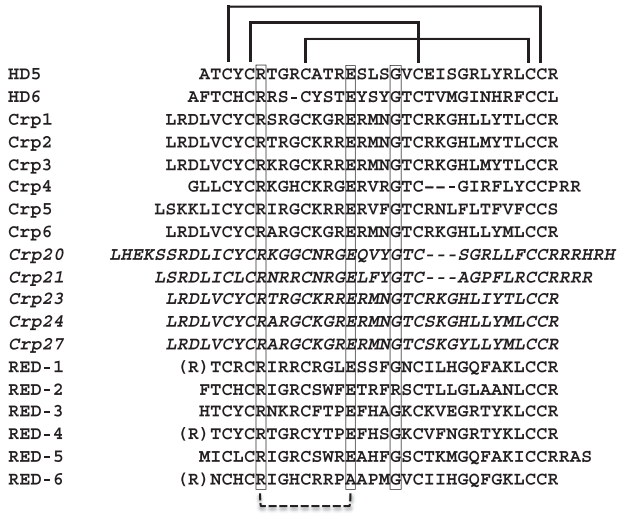

FIGURE 1 Primary structures and canonical features of representative Paneth cell α-defensins. The single letter notation amino acid sequences of human Paneth cell α-defensins HD-5 and HD-6, mouse Crps 1-6, 20, 21, 23, 24, and 27, and rhesus macaque enteric α-defensins REDs 1-6, respectively, were positioned manually to align canonical Cys residue positions. Crps 20, 21, 23, 24, and 27, identified only in the C57BL/6 strain to date [Ref. 29], are shown in italicized font. The canonical Arg, Glu, and Gly residue positions are identified within boxes, and the conserved Arg-Glu salt bridge is depicted by the dotted bracket below the RED-6 sequence. Dash characters denote spaces introduced to maintain the alignment of conserved Cys residues and the disulfide array. The tridisulfide array that defines the α-defensin peptide family is shown above the HD5 sequence.

two- or three-exons, the distal 3′-exon codes for the mature α-defensin peptide. To date, no single α-defensin gene has been reported to be expressed in both bone marrow and in Paneth cells, irrespective of gene organization. In macaques, however, the RED-1 and RED-6 Paneth cell α-defensin genes, which have characteristic 3-exon myeloid gene structures and are closely related to rhesus myeloid α-defensin 4 (RMAD-4), are not transcribed in bone marrow (R.A. Llenado and A.J. Ouellette, unpublished).

The α-defensin gene loci appear to be inherently unstable, as judged by extensive genomic and proteomic differences between species and even between inbred strains of mice.[1,29] Detailed annotations of defensin gene loci are not available for most mammalian species, but rhesus macaques, mice and horses express numerous, diverse enteric α-defensin genes based on cloning studies. In the mouse, comparisons of the α-defensin gene loci of the NIH C57BL/6 and Celera mixed strain genome assemblies revealed extensive genomic and proteomic differences between inbred strains.[1,29] For example, the C57BL/6 genome contains five strain-specific α-defensins (Crps 20, 21, 23, 24, 27 in Fig. 1), a repeated multigene cassette found only in that assembly, and several non-defensin coding genes within the locus that are oriented differently in the two assemblies.[29] In addition, the five most abundant C57BL/6 strain α-defensins have not been reported in other inbred strains of mice and differ from those identified previously in mice. Instability of the gene locus in mice is illustrated further by the existence of α-defensin-related subfamilies of cysteine-rich sequence (CRS) peptides found only in mice. CRS1C and CRS4C are coded by linked two-exon genes whose second exons diverge markedly from the α-defensin genes, coding for peptides with 11 or 9 Cys residues that occur in a series of [C]-[X]-[Y] triplet repeats. Phylogenetically, CRS1C and CRS4C genes are more closely related to the rat enteric α-defensin genes than to any known mouse defensin gene, showing that they are evolutionarily distinct from mouse α-defensin genes.[29] Also, although mice lack neutrophil α-defensins,[5] both mouse genome assemblies contain over twenty α-defensin ancestral rat myeloid α-defensin pseudogenes.

α-Defensin Precursors

The bactericidal activity of Paneth cell α-defensins requires proteolytic removal of inhibitory proregion acidic amino acids from the precursor to enable membrane disruptive activity of the mature peptide. Paneth cell α-defensins derive from ~10 kDa prepropeptides that contain canonical signal sequences, usually acidic proregions, and a ~4.5 kDa mature α-defensin peptide in the C-terminal portion of the precursor (Fig. 3). In nearly all pro-α-defensins, the electropositive charge of the α-defensin component, essential for bactericidal peptide activity, is balanced or neutralized by anionic amino acid residues in the prosegment. Pro-α-defensins lack bactericidal activity and have reduced ability to bind to and permeabilize model membrane systems until activated proteolytically by specific converting proteinases.

The details of posttranslational Paneth cell α-defensin precursor processing differ in humans and mice. For example, human Paneth cells store and release unprocessed α-defensin precursors, e.g., proHD5, which are processed by trypsin isoforms during or after secretion to generate the mature, activated HD5 molecules.[8] By contrast, mouse Paneth cell pro-α-defensin processing is mediated intracellularly by matrix metalloproteinase-7 (MMP7), which cleaves at conserved sites in the proregion of the precursor, including at the junction of the propeptide and the α-defensin moiety of the proform.[6] In studies of mouse Paneth cell pro-α-defensin proCrp4$^{20–92}$ activation *in vitro*, the cleavage step at Ser43↓Ile44 removes nine acidic amino acids that collectively block the membrane disruptive behavior of the Crp4 moiety of the precursor.[6,33] Further mutagenesis studies showed that the four acidic residues near the proCrp4 amino terminus, at residue positions D20, D26, E27, E28, are primarily responsible for inhibition of Crp4-mediated membrane disruption in the precursor. Also, analyses of proCrp4 variants with proregion truncations showed that, as measured along the polypeptide backbone, decreasing the

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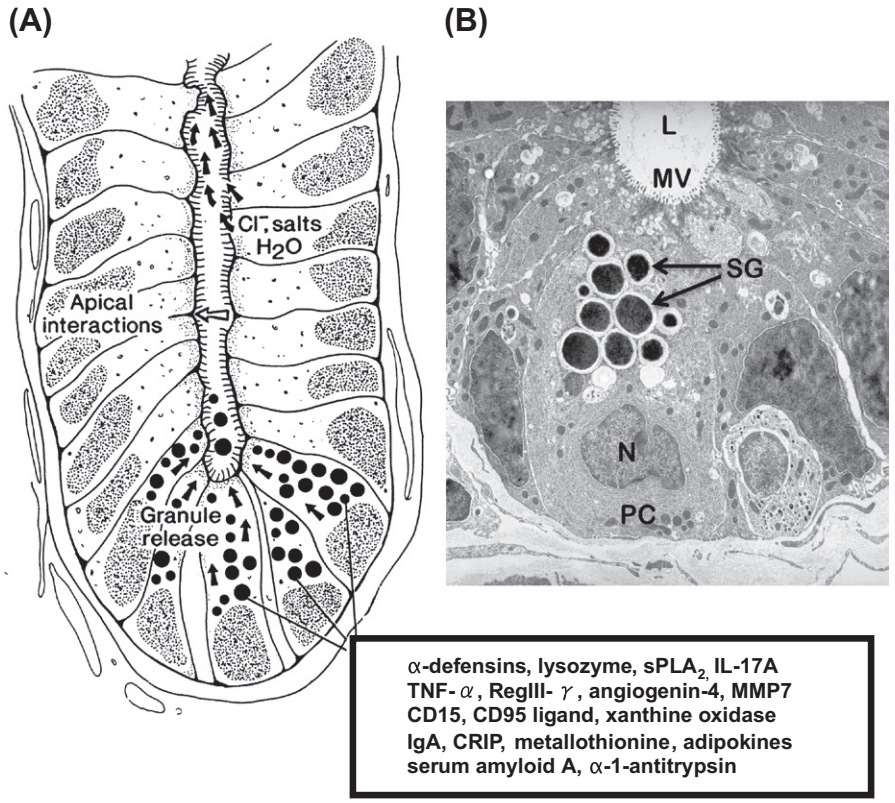

FIGURE 2 Paneth cell release of α-defensins in dense core secretory granules. In A, the apical release of α-defensin-containing dense core granules and additional constituents into the crypt lumen is illustrated. Solubilized α-defensins inhibit microbial colonization and shape the composition of the microbiota. Because mouse Paneth cell α-defensin cryptdin-3 stimulates the release of chloride ion from T84 cells [Ref. 11], certain secretory components may provide a luminal clearance mechanism by drawing water into the crypt lumen. In B, large, electron-dense secretory granules (SG) are shown for a single Paneth cell. SGs are clustered at the apical cell surface with its stubby microvilli (MV) in proximity of the crypt lumen (L). The basolaterally-oriented nucleus (N), characteristic of this lineage also is indicated. The electron micrograph was provided by Dr. Susan J. Hagen, Beth Israel Deaconess Medical Center, Boston, MA.

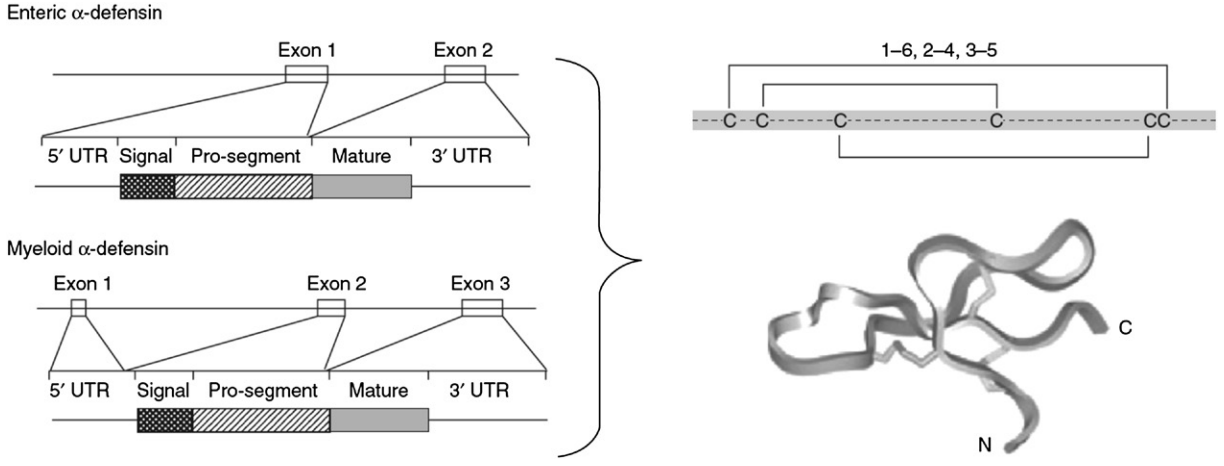

FIGURE 3 α-Defensin gene and precursor structures. Schematics of the myeloid and Paneth cell α-defensin genes and precursors are aligned at left. The signal peptide and proregions are crosshatched differentially, and the gray C-terminal region of the precursors denote the residues that constitute the mature α-defensin peptide. At right, the conserved spacing and disulfide connectivities of the α-defensin peptide family is illustrated at top, and the three-dimensional structure shown below is that of rabbit α-defensin RK-1. Adapted from Reference 28.

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length of the linear proregion is not structurally important for maintaining proCrp4 in an inhibited state. MMP7 gene disruption ablates proCrp processing, creating a deficiency of activated Crp peptides. The resulting MMP7-null mice have impaired innate immunity to oral bacterial infection and their small bowel microbiota differ from wild-type littermate controls.[22,34]

Distribution of α-defensins in the GI Tract

The major site of enteric α-defensin biosynthesis is in Paneth cells, which normally are restricted to the crypts of Lieberkühn of the small intestine and secrete dense core granules apically into the lumen.[18] As components of a regulated secretory pathway, the granules dissolve rapidly to deliver a complex mixture of host defense peptides and proteins to the crypt lumen (Fig. 2). In addition to α-defensin, Paneth cell granules contain secretory phospholipase A2 (Pla2g2a), lysozyme, lipopolysaccharide-binding protein, RegIII-γ, xanthine oxidase, matrix metalloproteinase 7 (MMP7), CD95 ligand, IgA, and CD1d as granule constituents.[18] Paneth cells also produce diverse proinflammatory mediators that include IL-17A, TNF-α, IL-1β, and adipokines.[4]

Mechanisms that regulate Paneth cell differentiation and maturation provide understanding into the distribution of enteric α-defensins. The epithelial cell lineages of the small intestine are specified by transcription factors that are controlled by the Wnt and Notch signaling pathways.[32] Paneth cell α-defensin genes are Wnt targets, and their transcription is mediated in part by β-catenin/TCF-4 (TCF7L2) along with additional tissue specific transcription factors, including Cdx2.[32] In addition to their distinctive role in enteric innate immunity, Paneth cells also provide key signals for maintenance of the crypt stem cell niche, Lgr5+ stem cell survival, and crypt homeostasis.[23]

In health, small intestinal Paneth cells are the sole source of enteric α-defensins, yet microbicidal α-defensins of Paneth cell origin are abundant in mouse distal colonic lumen.[13] The colonic epithelium does not express α-defensins, but the peptides accumulate in colonic luminal contents as a result of their proximal secretion in the small intestine and inherent resistance to proteolysis. The peptides recovered from the colonic lumen have bactericidal peptide activities equivalent to native α-defensins extracted from ileum.[13] Because the colonic microbial burden is several orders of magnitude greater than that of the small bowel, α-defensins may affect the colonic microbiota by means other than direct peptide-mediated cell killing. For example, at low peptide concentrations, certain defensins inhibit bacterial peptidoglycan synthesis by lipid II binding,[25] and we speculate that α-defensins may use a lipid II binding mechanism to exert selective antimicrobial effects on the microbiota of the colon. Although it remains speculative, α-defensin inhibition of bacterial peptidoglycan biosynthesis by lipid II binding could provide a mechanism for low α-defensin peptide levels to influence the colonic microbiota.

Structures of Paneth Cell α-defensins

Several α-defensin structures have been determined by NMR and X-ray crystallographic techniques.[28] The three-dimensional structures contain a canonical triple-stranded antiparallel β-sheet motif (Figs. 3 and 4) constrained by the C^{I}–C^{VI}, C^{II}–C^{IV}, C^{III}–C^{V} α-defensin tridisulfide array. The crystal structure of the human neutrophil α-defensin HNP-3 is a non-covalent, amphipathic dimer[9] as are the crystal structures of all human α-defensin peptides.[31] On the other hand, α-defensins from rabbit neutrophils, mouse Paneth cells, and rabbit kidney are monomeric in solution.[15,28] (Fig. 4).

Four structural features are conserved in the α-defensin peptide family (Figs. 1 and 4). Numbering residue positions from the N-terminus of the mouse Crp4 mature peptide, the conserved features are: (1) the disulfide array, (2) a salt bridge formed by Arg^{7}-Glu^{15} hydrogen bonds, (3) a conserved Gly at residue position 19, and (4) a high Arg:Lys molar ratio. In most instances, site-directed mutagenesis at these conserved positions does not alter *in vitro* bactericidal peptide activities.[15] For example, disulfide-null α-defensins are as bactericidal as the native molecules; however, the disulfide array is essential in conferring protection against proteolysis. Peptide mutants of the Arg^{7}-Glu^{15} salt bridge fold inefficiently and are sensitive to proteolysis, but, once folded and oxidized, their bactericidal activities are equivalent to those of the native molecules.[19,20] Perhaps, rather than having been selected to optimize bactericidal activity, these conserved features evolved to enable or facilitate efficient peptide folding, interactions with chaperone proteins, trafficking in the ER, and thereby mimimize unfolded protein responses. Effects of complete Lys-for-Arg replacements on bactericidal peptide activity vary with peptide primary structure, in that they attenuated HNP-1 and Crp4 activity but improved the activity of RMAD-4.[12,35] In both Lys substituted Crp4 and RMAD-4, complete Lys-for-Arg substitutions increased the sensitivity of the peptides to inhibition by NaCl, suggesting that Arg may be under selection as the electropositive α-defensin amino acid to confer improved bactericidal peptide activity under conditions of higher ionic strength.

Receptor Expression and Regulation

α-Defensins are microbicidal against gram-positive and gram-negative bacteria, certain fungi, spirochetes, protozoa, and certain viruses,[28] but those activities are not mediated by receptor-based mechanisms. In phagocytes, diverse AMPs are stored in azurophilic granules and mediate non-oxidative microbial cell killing following phagolysosomal fusion by permeabilizing the outer and inner membranes of *E. coli*, inducing the formation of ion channels in lipid

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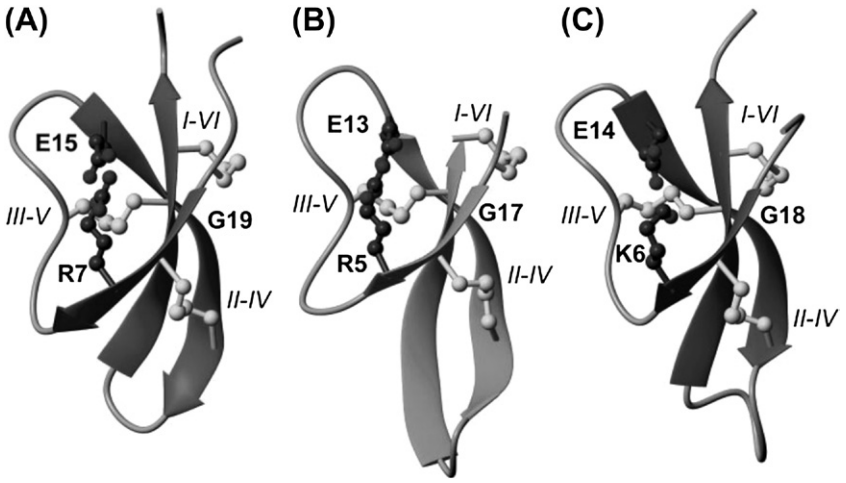

FIGURE 4 Ribbon diagrams of the NMR solution structure of Crp4 (A), the crystal structure of the HNP3 monomer (B) (Protein Data Bank code 1DFN), and the NMR solution structure of rabbit kidney α-defensin RK-1 [Ref. 22] (C) (Protein Data Bank code 1EWS) are displayed. Disulfide bonds are shown in *ball-and-stick* representation, illustrating the cross-braced central β-sheet. The main structural differences occur in the hairpin loop formed between Cys^{IV} and Cys^{V} resulting from the number of residues between the disulfide pairs, 6, 8, and 9 residues in Crp4, RK-1, and HNP3, respectively. The side chains of the conserved Arg and Glu residue positions form a tight electrostatic interaction in all three structures. Note that the rare R6K substitution in the natural RK-1 peptide retains the capability of forming a salt bridge with Glu^{35} in that peptide. The N and C termini are flexible in all peptides as evident from their disorder in the Crp4 and RK-1 structures and from the lack of density for the terminal residues in the X-ray analysis of HNP3. Reprinted from Rosengren, K.J., Daly, N.L., Fornander, L.M., Jönsson, L.M.H., Shirafuji, Y., Qu, X., Vogel, H.J., Ouellette, A.J., and Craik, D.J.: Structural and functional characterization of the conserved salt-bridge in mammalian Paneth cell α-defensins: Solution structures of mouse cryptdin-4 and (E15D)-cryptdin-4. J. Biol. Chem. 281: 28068–28078, 2006, with permission.

bilayers. Both peptide-elicited effects are influenced by membrane energetics and are selective for membranes that mimic the composition of electronegative bacterial cell membranes. Certain α-defensins have distinct membrane disruptive mechanisms even though their overall topologies are very similar. For example, human neutrophil α-defensin HNP-2 dimers form stable, 20 Å multimeric pores after insertion into model membranes, but the monomeric rabbit NP-1 peptide permeabilizes the membrane by creating large, short-lived defects in model phospholipid bilayers. High-resolution synchrotron small-angle X-ray scattering studies of defensin interactions with heterogeneous membranes show that α-defensins selectively generate saddle-splay, or "negative Gaussian," membrane curvature in model membranes of bacterial phospholipid composition, consistent with vesicle leakage assays.[24]

Biological Actions within Gastrointestinal Tract

Paneth cells in mouse small intestinal crypts secrete granules rich in microbicidal peptides when exposed to bacteria or bacterial lipopolysaccharide, lipoteichoic acid, lipid A, and muramyl dipeptide, or when stimulated pharmacologically with cholinergic agonists.[2] Paneth cell secretion is modulated by the Ca^{2+}-activated intermediate conductance K^+ channel KCa3.1 (also known as mIKCa1 and KCNN4), and highly selective blockers of KCa3.1 block Paneth cell secretion by 50–60%.[3] In *ex vivo* crypt preparations, mouse Paneth cells respond equivalently to purified bacterial cell envelope glycolipids, regardless of acyl chain modifications associated with attenuation or virulence in human infections. Total secreted protein, secreted lysozyme, and the bactericidal activities of collected secretions are independent of glycolipid acyl chain modifications of the glycolipid agonists associated with virulence. In an oral challenge mouse model of *Toxoplasma gondii* infection, Tlr9 mRNA levels were induced, as was type I IFN mRNA, and Crp3 and Crp5 α-defensin gene expression was elevated.[7] The *T. ondii* effects are mediated by Tlr9-dependent production of type I IFNs, because IFN-β replicated the responses in Tlr9 -/- mice, and responses were abrogated in IFNAR -/- mice.

The contribution of Paneth cell α-defensins to enteric mucosal immunity is evident from the phenotype of mice that are transgenic for the human Paneth cell α-defensin, HD5 (*DEFA5*-transgenic (+/+)).[21] *DEFA5*-transgenic (+/+) mice express a 3 kb HD5 minigene encompassing the peptide-coding exons only in Paneth cells and at levels that are similar to those encoded by endogenous mouse Paneth cell α-defensin genes. When orally challenged with *Salmonella enterica* serovar Typhimurium, *DEFA5*-transgenic (+/+) mice are highly resistant to infection.[21] Thus, addition of a single new α-defensin to Paneth cell secretions of *DEFA5*-transgenic (+/+) mice is sufficient to confer immunity to the lethal effects of this virulent pathogenic bacterium.

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As the *DEFA5*-transgenic (+/+) mouse phenotype illustrates, differential sensitivities of bacterial species to particular α-defensins suggests that peptide diversity may confer selective advantage against certain enteric pathogens.

α-Defensins secreted by Paneth cells shape the composition of the mouse small intestinal microbiota, apparently by selecting for peptide-tolerant resident microbial species *in vivo*.[22] For example, in the distal ileum of individual *DEFA5*-transgenic (+/+) mice, Firmicutes constituted 25% of bacterial species compared to 59% in FvB mice, the reference strain for the *DEFA5* (+/+) transgenic animals. Also, Bacteroidetes made up 69% of bacterial species in *DEFA5* (+/+) transgenic ileum, but in FvB controls only 35% of species were in the Bacteroidetes group.[22] Thus, the ileal microflora of mice secreting one additional exogenous α-defensin was markedly different and genotype-dependent. Also in the same study, the distinct repertoire of C57BL/6 mouse Paneth cell α-defensins (Fig. 1) appears to influence the ileal microbiota, because significant differences were found in the percent distribution of the Firmicutes and Tenericutes groups in C57BL/6 and FVB mice.[22]

Pathophysiological Implications

Paneth cell α-defensins contribute to enteric innate immunity to infection and also determine the composition of the small intestinal microbiota.[21,22] As noted above, *DEFA5*-transgenic (+/+) mice are immune to oral challenge with wildtype *S. enterica* serovar Typhimurium, and the composition of their resident ileal microbiota differs profoundly from that of controls.[21,22] Because a resident microflora is essential in IBD pathogenesis, the quantity and/or the repertoire of Paneth cell α-defensins and other gene products may influence gastrointestinal inflammation by shaping a healthy microbiota or one that promotes disease in genetically predisposed individuals. Thus, a reduction in luminal α-defensins resulting from disrupted Paneth cell homeostasis or Paneth cell deficiency could skew the resident microbiota toward more a proinflammatory composition and thereby contribute to disease.

Necrotizing enterocolitis (NEC) is a leading cause of morbidity and mortality in neonatal intensive care units, and many predisposing factors have been associated with NEC pathogenesis, including deficits or changes in Paneth cell function. By *in situ* hybridization, HD5 and HD6 mRNA levels appear to be 2-3 fold greater in NEC patients than in atresia or meconium ileus controls, yet immunohistochemical analyses showed that levels of HD5 peptide were equivalent in NEC and the control groups. These findings have led to the proposal that Paneth cells of NEC patients may be hypersecretory and thus accumulate reduced levels of mature α-defensin. We speculate that this may be the result of disrupted Paneth cell homeostasis resulting from induced autophagy or ER stress responses in NEC. The finding of reduced lysozyme immunostaining in Paneth cells of NEC patients is consistent with a diminished level of host defense protein production in the Paneth cell lineage in NEC individuals.

Mucosal immunity could be compromised by deficient α-defensin levels, defects in crypt homeostasis that impair Paneth cell delivery of secretory granules, or impaired solubility of α-defensins and secreted proteins following release into the crypt microenvironment.[14] Because Paneth cell α-defensins modulate the composition of the mouse small intestinal microbiota,[22] a reduction in luminal α-defensins could contribute to disease by skewing the composition of resident microorganisms toward more a proinflammatory microbial population. Disruptions to Paneth cell homeostasis induced by varied genetic defects may lead to induced autophagy, ER stress, unfolded protein responses, and apoptosis leading to ileitis and enhanced sensitivity to colitis.[14]

ACKNOWLEDGMENTS

This work is supported by NIH grants R01DK044632 and R01AI059346 (A.J. Ouellette) and NIH grants R37AI022931 and RO1DE15517 (M.E. Selsted) and by the USC Norris Cancer Center Support Grant P30 CA014089.

REFERENCES

1. Amid C, Rehaume LM, Brown KL, Gilbert JG, Dougan G, Hancock RE, et al. Manual annotation and analysis of the defensin gene cluster in the C57BL/6J mouse reference genome. *BMC Genomics* 2009;**10**:606.
2. Ayabe T, Satchell DP, Wilson CL, Parks WC, Selsted ME, Ouellette AJ. Secretion of microbicidal alpha-defensins by intestinal Paneth cells in response to bacteria. *Nat Immunol* 2000;**1**:113–8.
3. Ayabe T, Wulff H, Darmoul D, Cahalan MD, Chandy KG, Ouellette AJ. Modulation of mouse Paneth cell alpha-defensin secretion by mIKCa1, a Ca^{2+}-activated, intermediate conductance potassium channel. *J Biol Chem* 2002;**277**:3793–800.
4. Cadwell K, Liu JY, Brown SL, Miyoshi H, Loh J, Lennerz JK, et al. A key role for autophagy and the autophagy gene Atg16l1 in mouse and human intestinal Paneth cells. *Nature* 2008;**456**:259–63.
5. Eisenhauer PB, Lehrer RI. Mouse neutrophils lack defensins. *Infect Immun* 1992;**60**:3446–7.
6. Figueredo SM, Weeks CS, Young SK, Ouellette AJ. Anionic amino acids near the pro-alpha-defensin N terminus mediate inhibition of bactericidal activity in mouse pro-cryptdin-4. *J Biol Chem* 2009;**284**:6826–31.
7. Foureau DM, Mielcarz DW, Menard LC, Schulthess J, Werts C, Vasseur V, et al. TLR9-dependent induction of intestinal alpha-defensins by Toxoplasma gondii. *J Immunol* 2010;**184**:7022–9.
8. Ghosh D, Porter E, Shen B, Lee SK, Wilk D, Drazba J, et al. Paneth cell trypsin is the processing enzyme for human defensin-5. *Nat Immunol* 2002;**3**:583–90.
9. Hill CP, Yee J, Selsted ME, Eisenberg D. Crystal structure of defensin HNP-3, an amphiphilic dimer: mechanisms of membrane permeabilization. *Science* 1991;**251**:1481–5.

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10. Jones DE, Bevins CL. Paneth cells of the human small intestine express an antimicrobial peptide gene. *J Biol Chem* 1992;**267**:23216–25.
11. Lencer WI, Cheung G, Strohmeier GR, Currie MG, Ouellette AJ, Selsted ME, et al. Induction of epithelial chloride secretion by channel-forming cryptdins 2 and 3. *Proc Natl Acad Sci USA* 1997;**94**:8585–9.
12. Llenado RA, Weeks CS, Cocco MJ, Ouellette AJ. Electropositive charge in alpha-defensin bactericidal activity: functional effects of Lys-for-Arg substitutions vary with the peptide primary structure. *Infect Immun* 2009;**77**:5035–43.
13 Mastroianni JR, Ouellette AJ. Alpha-defensins in enteric innate immunity: functional Paneth cell alpha-defensins in mouse colonic lumen. *J Biol Chem* 2009;**284**:27848–56.
14 McGuckin MA, Eri RD, Das I, Lourie R, Florin TH. ER stress and the unfolded protein response in intestinal inflammation. *Am J Physiol Gastrointest Liver Physiol* 2010;**298**:G820–32.
15 Ouellette AJ. Paneth cell alpha-defensins in enteric innate immunity. *Cell Mol Life Sci* 2011;**68**:2215–29.
16 Ouellette AJ, Greco RM, James M, Frederick D, Naftilan J, Fallon JT. Developmental regulation of cryptdin, a corticostatin/defensin precursor mRNA in mouse small intestinal crypt epithelium. *J Cell Biol* 1989;**108**:1687–95.
17 Ouellette AJ, Pravtcheva D, Ruddle FH, James M. Localization of the cryptdin locus on mouse chromosome 8. *Genomics* 1989;**5**:233–9.
18 Porter EM, Bevins CL, Ghosh D, Ganz T. The multifaceted Paneth cell. *Cell Mol Life Sci* 2002;**59**:156–70.
19 Rajabi M, de Leeuw E, Pazgier M, Li J, Lubkowski J, Lu W. The conserved salt bridge in human alpha-defensin 5 is required for its precursor processing and proteolytic stability. *J Biol Chem* 2008;**283**:21509–18.
20 Rosengren KJ, Daly NL, Fornander LM, Jonsson LM, Shirafuji Y, Qu X, et al. Structural and functional characterization of the conserved salt bridge in mammalian Paneth cell alpha-defensins: solution structures of mouse cryptdin-4 and (E15D)-cryptdin-4. *J Biol Chem* 2006;**281**:28068–78.
21 Salzman NH, Ghosh D, Huttner KM, Paterson Y, Bevins CL. Protection against enteric salmonellosis in transgenic mice expressing a human intestinal defensin. *Nature* 2003;**422**:522–6.
22 Salzman NH, Hung K, Haribhai D, Chu H, Karlsson-Sjoberg J, Amir E, et al. Enteric defensins are essential regulators of intestinal microbial ecology. *Nat Immunol* 2010;**11**:76–83.
23 Sato T, van Es JH, Snippert HJ, Stange DE, Vries RG, van den Born M, et al. Paneth cells constitute the niche for Lgr5 stem cells in intestinal crypts. *Nature* 2010;**469**:415–8.
24 Schmidt NW, Mishra A, Lai GH, Davis M, Sanders LK, Tran D, et al. Criterion for amino acid composition of defensins and antimicrobial peptides based on geometry of membrane destabilization. *J Am Chem Soc* 2011;**133**:6720–7.
25 Schneider T, Sahl HG. Lipid II and other bactoprenol-bound cell wall precursors as drug targets. *Curr Opin Investig Drugs* 2010;**11**:157–64.
26 Selsted ME, Brown DM, DeLange RJ, Lehrer RI. Primary structures of MCP-1 and MCP-2, natural peptide antibiotics of rabbit lung macrophages. *J Biol Chem* 1983;**258**:14485–9.
27 Selsted ME, Miller SI, Henschen AH, Ouellette AJ. Enteric defensins: antibiotic peptide components of intestinal host defense. *J Cell Biol* 1992;**118**:929–36.
28 Selsted ME, Ouellette AJ. Mammalian defensins in the antimicrobial immune response. *Nat Immunol* 2005;**6**:551–7.
29 Shanahan MT, Tanabe H, Ouellette AJ. Strain-specific polymorphisms in Paneth cell alpha-defensins of C57BL/6 mice and evidence of vestigial myeloid alpha-defensin pseudogenes. *Infect Immun* 2011;**79**:459–73.
30 Sparkes RS, Kronenberg M, Heinzmann C, Daher KA, Klisak I, Ganz T, et al. Assignment of defensin gene(s) to human chromosome 8p23. *Genomics* 1989;**5**:240–4.
31 Szyk A, Wu Z, Tucker K, Yang D, Lu W, Lubkowski J. Crystal structures of human alpha-defensins HNP4, HD5, and HD6. *Protein Sci* 2006;**15**:2749–60.
32 van der Flier LG, Clevers H. Stem cells, self-renewal, and differentiation in the intestinal epithelium. *Annu Rev Physiol* 2009;**71**:241–60.
33 Weeks CS, Tanabe H, Cummings JE, Crampton SP, Sheynis T, Jelinek R, et al. Matrix metalloproteinase-7 activation of mouse Paneth cell pro-alpha-defensins: Ser43-Ile44 proteolysis enables membrane-disruptive activity. *J Biol Chem* 2006;**281**:28932–42.
34 Wilson CL, Ouellette AJ, Satchell DP, Ayabe T, Lopez-Boado YS, Stratman JL, et al. Regulation of intestinal alpha-defensin activation by the metalloproteinase matrilysin in innate host defense. *Science* 1999;**286**:113–7.
35. Zou G, de Leeuw E, Li C, Pazgier M, Li C, Zeng P, et al. Toward understanding the cationicity of defensins: Arg and Lys versus their noncoded analogs. *J Biol Chem* 2007;**282**:19653–65.

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Chapter 178

PYY

Louise Purtell and Herbert Herzog

ABSTRACT

Peptide YY (PYY) is produced as a 36 amino acid peptide hormone within the distal gastrointestinal tract and the pancreas where it acts in a para- and endocrine fashion. It circulates in two biologically active forms, which act upon Y receptors to exert digestive, absorptive, antisecretory and satiety effects. PYY is implicated in a number of gastrointestinal diseases and has been proposed as a putative anti-obesity therapy.

DISCOVERY

PYY is a 36 amino acid, straight-chain polypeptide. It shares structural similarity with pancreatic polypeptide (PP) and neuropeptide Y (NPY); all three peptides have tyrosine residues at the C-terminal which is also amidated. PYY has also a tyrosine at the N-terminus, designated in peptide nomenclature as Y, which gives PYY its name.[1] PYY was first isolated from porcine small intestine by Takemoto et al in 1980, and in 1988 its protein sequence was determined in humans.[28,29]

STRUCTURE OF GENE

The PYY gene has been cloned and sequenced from a large number of species. It comprises four exons and three introns, with each of the exons encoding a different functional domain of the mRNA[7,15] (Fig. 1). The human PYY gene is located in a cluster with PP on chromosome 17q.21.1 and spans approximately 1.2 kbp. There is significant conservation of gene, sequence and protein structure between PYY, NPY, and PP, indicating that these genes arose from a common ancestor by gene duplication. In higher mammals additional gene duplication events lead to the generation of other PYY-like genes which in at least one species, bovine, lead to the production of the active peptide, seminalplasmin.[8]

PROCESSING OF THE PRECURSOR

Transcription and translation of the PYY gene produces a 98 amino acid prepropeptide. A signal peptide is followed by the 36 amino acid PYY sequence and the cleavage and amidation sequence Gly-Lys-Arg, with a 31 amino acid flanking peptide at the carboxy terminal[7] (Fig. 1).

There are two biologically active forms of endogenous PYY: $PYY_{1\text{-}36}$ and $PYY_{3\text{-}36}$, which have different affinities for the Y receptors. Dipeptidyl peptidase IV (DPP-IV) cleaves the tyrosine and proline residues from the N-terminal end of the full-length peptide ($PYY_{1\text{-}36}$) to form the truncated peptide ($PYY_{3\text{-}36}$).[21,22] DPP-IV, a short-acting, abundantly-expressed cell surface enzyme, also acts to metabolize GLP-1 to its inactive form. Approximately 40% of total PYY released is converted to $PYY_{3\text{-}36}$[10] (Fig. 1). The half-live time of the peptides in the serum is approximately 8 min[17] and proteases such as kallikreins specifically remove amino acids 36 and 35 and thereby render the peptide inactive.

DISTRIBUTION IN THE GASTROINTESTINAL TRACT

PYY is produced by the endocrine L-cells of the epithelial mucosa (Fig. 2). PYY is abundantly expressed in the colon, terminal ileum, and rectum, and produced to a lesser extent in the esophagus, duodenum, proximal jejunum, and stomach.[18] Furthermore, in the pancreas specific PYY expressing cells surround the insulin producing cells in the islets of Langerhans (Fig. 3). PYY is colocalized in the gastrointestinal tract with proglucagon, glicentin, glucagon-like peptide 1 (GLP-1), and GLP-2 and, upon arrival of food in the duodenum, co-released from the same secretory granules.[2] The level of PYY expression increases from the proximal end to the distal end of the gastrointestinal tract. PYY-expressing neurons can be found in the central nervous system and in specific gastric neurons in some species.

REGULATION OF SECRETION

PYY release from the endocrine L-cells in the gut can be triggered by a number of mechanisms. Circulating levels rise within 15 min of ingestion of a meal, reaching a peak at approximately 60–120 min postprandially (Fig. 4). PYY

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00178-0
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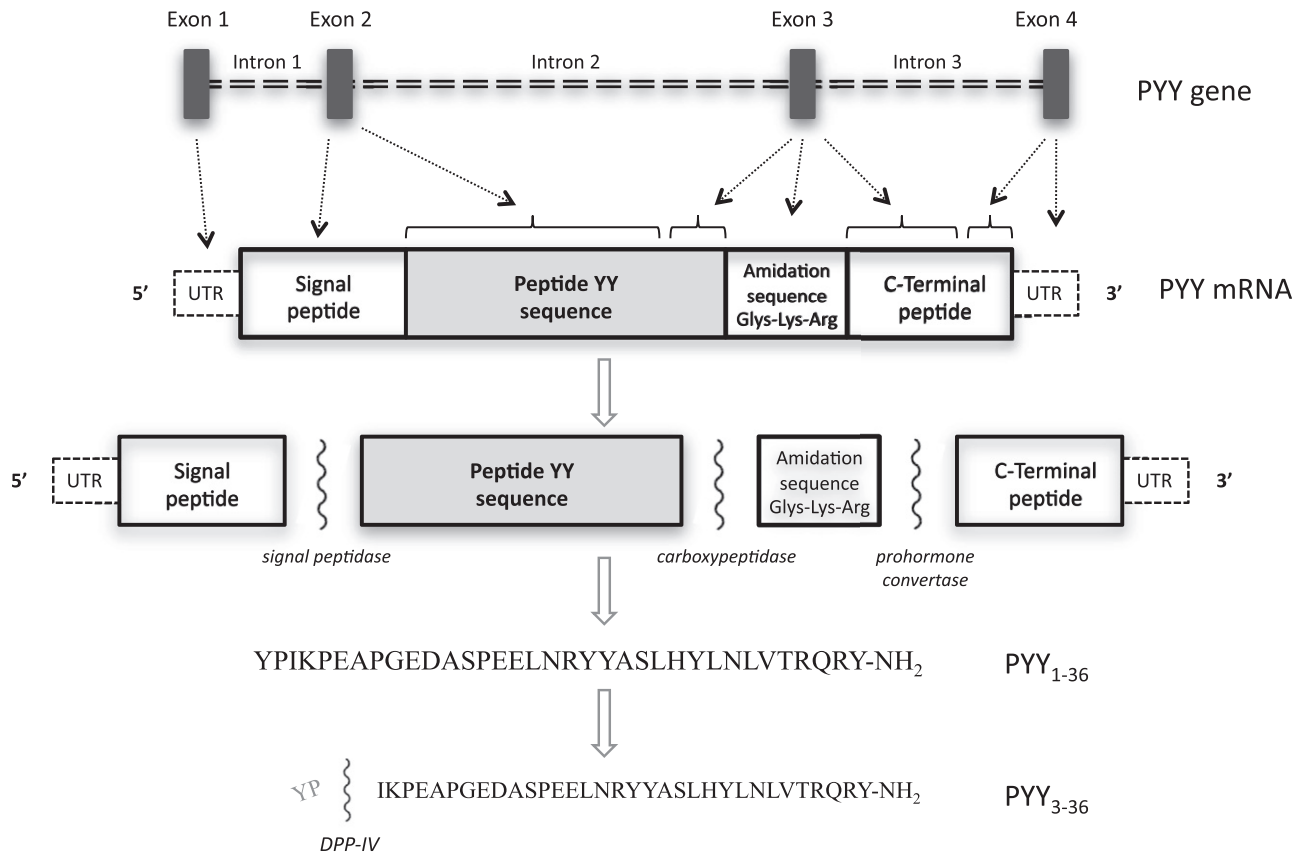

FIGURE 1 Transcription and translation of the PYY gene and processing of the PYY precursor protein to yield PYY_{1-36} and PYY_{3-36}. UTR—untranslated region.

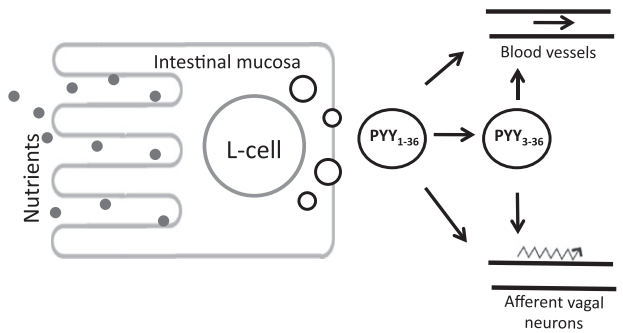

FIGURE 2 Nutrient-stimulated secretion of PYY from the intestinal L-cells and release through humoral and vagal channels.

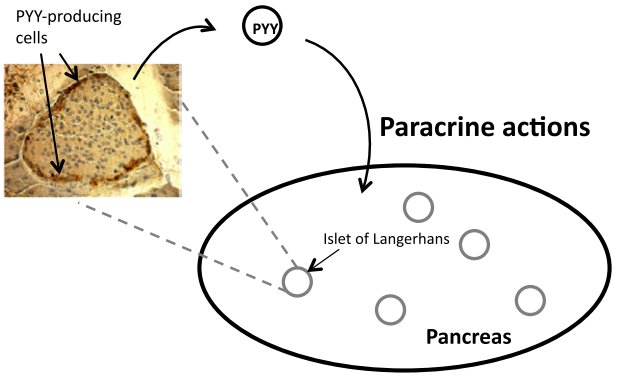

FIGURE 3 Pancreatic secretion of PYY from the islets of Langerhans to exert paracrine effects. See color plate 49.

plasma concentrations can remain elevated for as long as 6 h, depending on the calorie load of the meal ingested. Meal components shown to stimulate PYY secretion include glucose, bile salts, lipids, short-chain fatty acids, and amino acids. Of these, dietary lipids are the most potent stimulant of secretion.[11] The sustained postprandial production of PYY is consistent with the hypothesis that the L-cells of the distal bowel and colon detect levels of these macronutrients and release PYY in response. Upon meal ingestion, plasma PYY concentrations become elevated before nutrients reach the distal gastrointestinal tract. This suggests that the initial postprandial PYY release may be modulated by indirect neural or humoral pathways, such as the vagus nerve.[2] The presence of regulatory peptides such as cholecystokinin (CCK) and vasoactive intestinal polypeptide (VIP) may also stimulate PYY release in the absence of a meal cue.[2] PYY secretion has been further shown to be stimulated by exercise, both long-term and after only a single session of high intensity exercise.[16]

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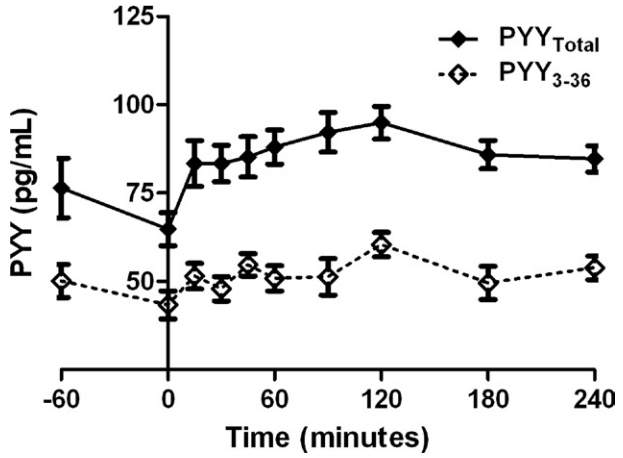

FIGURE 4 Plasma levels of PYY_{Total} and PYY_{3-36} in response to an oral mixed meal in lean adults ($n = 10$). Circulating PYY is elevated postprandially with a peak at 120 minutes. (Adapted from Purtell L, Sze L, Loughnan G, Smith E, Herzog H, Sainsbury A, et al. In adults with Prader–Willi syndrome, elevated ghrelin levels are more consistemt with hyperphagia than high PYY and GLP-1 levels. *Neuropeptides* 2011;**45**:301–7.)

RECEPTORS AND SIGNALING CASCADES

PYY, NPY and PP exert their physiological actions through at least five G-protein coupled receptors: Y1, Y2, Y4, Y5, and y6. These belong to the guanine nucleotide binding regulatory protein (G-protein) coupled receptors superfamily. Upon activation of the receptor, the G_{ialpha}-subunit is released from the receptor/G-protein complex, inhibiting the production of second messenger cyclic AMP through the inhibition of adenylyl cyclase.[35] In addition, the Y-receptors alter neuronal excitability and neurotransmitter release through inhibition of voltage-gated K^+ channels and stimulation of Ca^{2+} channels.[35] PYY and NPY have indistinguishable affinities for all Y-receptors with a rank order of ligand-binding affinities of Y2 > Y1 > Y5 > Y6 >> Y4; this is reversed for PP, which binds with greatest affinity to Y4 > Y6 >> Y2 > Y5 > Y1. Despite some structural and functional similarities, the Y-receptors have differential distribution profiles. In humans, the Y1 receptor is localized in the proliferative zone of the colonic epithelium and the mucosal nerves, as well as centrally where it is involved in appetite regulation and anxiety.[9] The Y1 receptor has been found to have a role in regulating the proliferation of gut epithelial cells.[20] The Y2 receptor has been found in the human small intestine, as well as in rat jejunum and colon mucosa. In the brain, it has been shown to be expressed in mouse hippocampus, hypothalamus and amygdale. Like the Y1 receptor, it binds PYY and NPY in preference to PP; however unlike Y1 it also shows considerable affinity for C-terminal PYY and NPY fragments such as PYY_{3-36}. Postprandial suppression of gastric motility, and the subsequent satiety, is mediated in part through PYY's action on the Y2 receptor. Y4 receptors are localized in both small and large intestine in humans, as well as the pancreas; they are also expressed in the brain, with the hypothalamus being the region of highest expression. Upon binding of PP to the Y4 receptor, increases in colonic muscle contraction and fecal output is triggered in mice.[24] The Y5 receptor shows overlapping expression with Y1 receptors in the brain where it is also involved in appetite and energy homeostasis regulation.[14] PP is thought to also act as a ligand for the y6 receptor. While functional in mouse and rabbit, this Y-receptor is truncated in the human genome.[6] The truncated y6 receptor is present in a number of human tissues, including the small and large intestine; however, a specific physiological role has not yet been found for it. In mice, the expression of y6 in the suprachiasmatic nuclei of the hypothalamus suggests involvement in the circadian regulation of energy homeostasis.

TABLE 1 Physiological Actions of Peptide YY

Organ	Physiological action
Pancreas	• Inhibits pancreatic exocrine secretion • Inhibits insulin secretion (mouse, dog) • Inhibits insulinotropic effects of GRP and GIP (dogs)
Stomach	• Slows gastric motility • Inhibits gastric acid secretion
Small intestine & colon	• Inhibits Cl^- secretion • Promotes electrolyte absorption • Lengthens mouth to cecum transit time
Kidney	• Suppresses glomerular filtration rate, renin and aldosterone • Promotes sodium secretion
Appetite & food intake	• Increases appetite and promotes weight gain (PYY1-36) • Inhibits appetite and promotes weight loss (PYY3-36)
Vascular	• Promotes vasoconstriction • Increases systolic and diastolic blood pressure
Growth	May facilitate GI growth and development
Bone	Inhibits osteoblast activity

BIOLOGICAL ACTIONS WITHIN GASTROINTESTINAL TRACT

PYY is a key player in a number of physiological processes acting in an autocrine, paracrine, and endocrine fashion. Its main action in the gastrointestinal tract is to enhance nutrient absorption by suppressing gastric motility and inhibiting electrolyte secretion in the small intestine and colon. It also has an important role in the regulation of appetite and food intake (Table 1).

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Motor Effects

When nutrients, particularly lipids, are present in the lumen of the ileum, the "ileal brake" mechanism takes effect. This acts on the upper gut to slow gastric emptying and is probably mediated by PYY. The ileal brake has been found in humans, where infusion of lipids into the ileum exerted inhibitory feedback on gastric motility.[27] Studies in dogs show that ileal lipid infusions increase the interval between jejunal migrating motor complexes.[33] PYY's effects on gastrointestinal motility may act vasovagally. Witte et al compared the effects on gastric motility of PYY_{1-36} and PYY_{3-36} in humans and found the latter to be significantly more potent, both in half emptying time and gastric emptying rate.[34]

Gastric Secretion

PYY acts as a physiological enterogastrone in a number of species, including human. Gastric acid secretion in the stomach is stimulated by the parasympathetic nervous system via the vagal nerve, as well as by hormones, primarily gastrin. The presence of lipids in the lumen of the ileum causes PYY release, which in turn inhibits gastric acid secretion. In studies of dogs, PYY to inhibits pentagastrin- and histamine-stimulated, but not bethanecol-stimulated gastric acid secretion.[12] Further, it can abolish cephalic stage secretion, which is mediated by the vagus. This may be because of an inhibition of acetylcholine release from vagal nerve fibers.

Pancreatic Exocrine Secretion

PYY inhibits pancreatic secretion. Exogenous and endogenous CCK, secretin, intraduodenal amino acids and neurotensin stimulate secretion of pancreatic juice, bicarbonate ions and pancreatic enzymes (Fig. 3). PYY acts to counter these stimulatory effects through the Y2 receptor in rats. PYY also inhibits postprandial nutrient-stimulated pancreatic secretion. Further, treatment with anti-PYY serum causes enhanced pancreatic secretion, demonstrating PYY's importance in this feedback mechanism. As yet there is no consensus as to the mechanism by which PYY regulates pancreatic secretion. Dog studies suggest that the mechanism is independent of the vagus, and is instead mediated by adrenergic pathways. Others suggest that the feedback effects are modulated by the CCK pathway.

Pancreatic Endocrine Secretion

Animal studies strongly support a role for PYY in the inhibition of pancreatic endocrine secretion, but this evidence is not borne out by clinical studies. While no effect of intravenous PYY on plasma insulin was seen in mice, it significantly reduced circulating insulin levels in dogs (Fig. 3). Both mouse and dog studies show a glucose-dependent inhibition of insulin by PYY in the postprandial state. These effects were mirrored in *in vitro* studies: PYY dose-dependently inhibit insulin production in the presence of glucose, but not under basal conditions. Knockout mouse models also implicate PYY in pancreatic endocrine regulation and suggest that its actions are mediated by the Y1 receptors. PYY may also have indirect effects on the enteroinsular axis, as it inhibited the insuliotropic effects of gastrin releasing peptide (GRP) and gastric inhibitory peptide (GIP) in dogs. However, these insulin-inhibitory effects of PYY have not been replicated in clinical studies. Intravenous administration of PYY has not been found to influence insulin secretion in humans, either basally or under glucose-dependent conditions.

Small Intestinal and Colonic Secretion

PYY has an antisecretory action in the small intestine and colon, acting to increase fluid and electrolyte absorption. VIP modulates secretion of water and chloride ions in the small intestine through stimulation of intracellular cAMP production. PYY reduces this VIP-mediated secretion through mucosal Y1, Y2 and Y4 receptors.[5,9] PYY can also exert this effect on basal or prostaglandin-stimulated cAMP production. High postprandial PYY levels ensure that electrolytes are optimally absorbed at the intestinal mucosa; in the fasted state the relative absence of this feedback allows secretory hormones to maintain fluid homeostasis.

Appetite and Food Intake

PYY is an important modulator of appetite, feeding behavior, and body weight homeostasis. Importantly, the two circulating forms of PYY, PYY_{1-36} and PYY_{3-36}, can both act through the Y2 receptor to induce satiety. Thus, the extent and rate that DPP-IV converts PYY_{1-36} to PYY_{3-36} is not critical for the satiety action of PYY. However, conversion of PYY_{1-36} to PYY_{3-36} reduces the action on the Y1 receptor, which in general has inhibitory functions in many peripheral tissues such as fat and bone.

Renal Function

PYY's influence on renal function has been studied in humans. PYY, when infused at concentrations mimicking normal postprandial levels, decreases glomerular filtration rate by 10%. Sodium secretion increased by 30%, while rennin and aldosterone is significantly suppressed. Infusion with a high dose mimicking levels in pathological conditions, shows, in addition to these changes, reduced renal plasma flow. The mechanism behind these changes is not yet clear, but, whether directly or indirectly, PYY appears to have a major role in modulating postprandial natriuresis.

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Vascular Effects

PYY plays a role in vasoconstriction, reducing blood flow to, in particular, the intestine through predominantly Y1 mediated actions.[19] In humans, PYY infusion is associated with increased systolic and diastolic blood pressure.

Growth and Development

PYY is one of the earliest gut peptides to be expressed in the fetal development of mice, suggesting a role in gastrointestinal growth and differentiation. *In vitro* studies indicate that these effects are mediated by the Y1 receptor. High levels of PYY or NPY during embryonic development is lethal because of the failure of midline formation.

PATHOPHYSIOLOGICAL IMPLICATIONS

The secretion pattern of PYY is altered in, and may contribute to, a number of gastrointestinal and systemic disorders.

Anorexia Nervosa

Anorexia nervosa (AN) is a condition in which patients deliberately suppress their food intake in order to reduce body weight. Whether this decreased food intake is modulated by endocrine cues has long been debated. Adolescent girls with AN have higher fasting PYY concentrations than healthy controls.[23] Interestingly, another study observed that PYY levels in women with AN are strongly correlated with bone mineral density (BMD), suggesting that PYY may contribute to the bone loss often seen in this condition via over-stimulating the inhibitory Y1 receptor on bone forming cells.[30]

Prediabetes

Low circulating PYY may not only be a function of type 2 diabetes (T2D), but also a predictor. A 2008 study investigated postprandial PYY response in healthy individuals with and without a family history of T2D. The authors found that the family history positive group have a blunted PYY secretory response, a defect that could precede insulin resistance and adiposity on the path to T2D in genetically susceptible individuals.[31]

Gastrointestinal Diseases

Many diseases involving the gastrointestinal tract are characterized by altered patterns of PYY secretion. Malabsorptive disorders are often accompanied by elevated PYY concentrations. Celiac disease, or sprue, is an autoimmune disorder involving severe small intestinal malabsorption of nutrients because of villous atrophy. It is caused by an allergic response to the gluten protein gliadin. Tropical sprue causes a similar malabsorptive state, but has an unknown infectious origin. In both types of sprue, PYY concentrations are greatly elevated in both basal and postprandial states. Circulating PYY is also high in patients with other forms of gastric malabsorption, such as chronic pancreatitis, infective diarrhea, and the inflammatory bowel diseases Crohn's disease and ulcerative colitis.[3]

PYY's involvement in malabsorptive gastrointestinal disorders is consistent with its ileal brake role. Ingested nutrients are primarily absorbed in the proximal small intestine in healthy individuals. When there is a defect in intestinal absorption, such as in these conditions, unabsorbed nutrients reach the distal ileum and colon, triggering PYY release in an adaptive attempt to slow gastric emptying and thus increase mucosal contact time.

Gastrointestinal Surgery

PYY concentrations increase after significant bowel resection.[3] This is an adaptive response to the shortening of the gastrointestinal tract and some subsequent loss of absorptive capability. Thus PYY secretion is not elevated after resection of small bowel segments, as in this case absorptive power is not greatly altered. Significant colon resection, however, can produce the opposite effect—decreased PYY levels.[3] This reflects the fact that the colonic mucosa is where a large proportion of PYY-expressing cells are localized. Removal of colon segments reduces the ability to produce and release PYY.

Bariatric Surgery

The purpose of bariatric surgery is to promote weight loss through restrictive or maladaptive means. Candidates for these types of surgery are generally morbidly obese, with the low fasting and postprandial PYY levels associated with obesity. In general, bariatric surgery tends to increase circulating PYY, reflecting both decreased nutrient intake (after restrictive surgery such as vertical banded gastroplasty) and reduced nutrient absorption (malabsorption operations such as jejuno-ileal bypass).[13] Surgery which combines both restrictive and malabsorption elements, such as Roux-en-Y gastric bypass, undoubtedly reaps the benefit of both mechanisms. Despite bariatric surgery's stimulatory effects on PYY, post-surgery patients rarely attain levels equivalent to those of healthy individuals. This may be because many postoperative patients are still overweight, if not obese.

Pancreatitis

In addition to neuroendocrine actions, a role for PYY has been found in immune function. A 2007 study found that therapeutic PYY confers significant survival benefits on mice with ethionine-induced necrotizing pancreatitis.[32] The

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same group observed *in vitro* that these effects are mediated by a PYY-dependent suppression of amylase and cytokine secretion.

Obesity

There has been much research into PYY's role in obesity, and particular interest has been given to potential therapeutic uses. Basal PYY concentrations are lower in obese individuals than in lean, in both adults and children.[25,26] An inverse linear relationship between PYY and BMI had been suggested; however, this does not hold true in the case of morbid obesity. PYY release is similarly blunted in obese individuals after mixed meal ingestion. This difference disappears, however, when the mixed meal is replaced with an oral glucose load. It may be that obese patients respond appropriately to glucose but develop resistance to fat-stimulated PYY secretion. PYY has been widely proposed as a possible anti-obesity drug because of its inhibitory effects on appetite. Batterham et al conducted a placebo-controlled study in which healthy individuals were peripherally infused with $PYY_{3\text{-}36}$ to achieve postprandial concentrations. Participants reduced their *ad libitum* calorie intake by 36% in a meal 2 h after the infusion, and 24 h calorie intake was reduced by 33%.[4] There was a comparable reduction in caloric intake when the experiment was repeated with obese participants, showing that obese and lean individuals have a similar sensitivity to peripheral PYY.

REFERENCES

1. Adrian TE, Ferri GL, Bacarese-Hamilton AJ. Human distribution and release of a putative new gut hormone, peptide YY. *Gastroenterology* 1985;**89**:1070–7.
2. Ballantyne G. Peptide YY(1-36) and Peptide YY(3-36): part I. Distribution, release and actions. *Obes Surg* 2006;**16**:651–8.
3. Ballantyne G. Peptide YY(1-36) and Peptide YY(3-36): part II. Changes after Gastrointestinal surgery and Bariatric surgery: part I. Distribution, release and actions appeared in the last issue. *Obes Surg* May 2006;**2006**(16):795–803.
4. Batterham RL, Bloom SR. The gut hormone peptide YY regulates appetite. *Ann NY Acad Sci* 2003;**994**:162–8.
5. Bilchik AJ, Hines OJ, Adrian TE, McFadden DW, Berger JJ, Zinner MJ, et al. Peptide YY is a physiological regulator of water and electrolyte absorption in the canine small bowel in vivo. *Gastroenterology* 1993;**105**:1441–8.
6. Burkhoff AM, Linemeyer DL, Salon JA. Distribution of a novel hypothalamic neuropeptide Y receptor gene and its absence in rat. *Mol Brain Res* 1998;**53**:311–6.
7. Conlon JM. The origin and evolution of peptide YY (PYY) and pancreatic polypeptide (PP). *Peptides* 2002;**23**:269–78.
8. Couzens M, Liu M, Tüchler C, Kofler B, Nessler-Menardi C, Parker RMC, et al. Peptide YY-2 (PYY2) and Pancreatic Polypeptide-2 (PPY2): species-specific evolution of novel members of the Neuropeptide Y gene family. *Genomics* 2000;**64**:318–23.
9. Cox HM. Endogenous PYY and NPY mediate tonic Y1- and Y2-mediated absorption in human and mouse colon. *Nutrition* 2008;**24**:900–6.
10. Cox HM. Peptide YY: a neuroendocrine neighbor of note. *Peptides* 2007;**28**:345–51.
11. Essah PA, Levy JR, Sistrun SN, Kelly SM, Nestler JE. Effect of macronutrient composition on postprandial peptide YY levels. *J Clin Endocr Metab* 2007;**92**:4052–5.
12. Guo YS, Fujimura M, Lluis F, Tsong Y, Greeley Jr GH, Thompson JC. Inhibitory action of peptide YY on gastric acid secretion. *Am J Physiol* 1987;**253**:G298–302.
13. Hanusch-Enserer U, Roden M. News in gut-brain communication: a role of peptide YY (PYY) in human obesity and following Bariatric surgery? *Eur J Clin Invest* 2005;**35**:425–30.
14. Iyengar S, Li DL, Simmons RM. Characterization of neuropeptide Y-induced feeding in mice: do Y1-Y6 receptor subtypes mediate feeding? *J Pharmacol Exp Ther* 1999;**289**:1031–40.
15. Kohri K, Nata K, Yonekura H, Nagai A, Konno K, Okamoto H. Cloning and structural determination of human peptide YY cDNA and gene. *Biochim Biophys Acta* 1993;**1173**:345–9.
16. Li JB, Asakawa A, Li Y, Cheng K, Inui A. Effects of exercise on the levels of peptide YY and Ghrelin. *Exp Clin Endocr Diab* 2011;**119**(163):6.
17. Lluis F, Fujimura M, Gómez G, Salvá J, Greeley GJ, Thompson J. Cellular localization, half-life, and secretion of peptide YY. *Rev Esp Fisiol* 1989;**45**:377–84.
18. Lundberg J, Tatemoto K, Terenius L, Hellström P, Mutt V, Hökfelt T, et al. Localization of peptide YY (PYY) in gastrointestinal endocrine cells and effects on intestinal blood flow and motility. *Proc Natl Acad Sci USA* 1982;**79**:4471–5.
19. Lundberg JM, Tatemoto K. Vascular effects of the peptides PYY and PHI: comparison with APP and VIP. *Eur J Pharmacol* 1982;**83**:143–6.
20. Mannon PJ, Mele JM. Peptide YY Y1 receptor activates mitogen-activated protein kinase and proliferation in gut epithelial cells via the epidermal growth factor receptor. *Biochem J* 2000;**350**:655–61.
21. Medeiros MS, Turner AJ. Post-secretory processing of regulatory peptides: The pancreatic polypeptide family as a model example. *Biochimie* 1994;**76**:283–7.
22. Mentlein R, Dahms P, Grandt D, Krüger R. Proteolytic processing of neuropeptide Y and peptide YY by dipeptidyl peptidase IV. *Regul Peptides* 1993;**49**:133–44.
23. Misra M, Miller KK, Tsai P, Gallagher K, Lin A, Lee N, et al. Elevated peptide YY levels in adolescent girls with anorexia nervosa. *J Clin Endocr Metab* 2006;**91**:1027–33.
24. Moriya R, Fujikawa T, Ito J, Shirakura T, Hirose H, Suzuki J, et al. Pancreatic polypeptide enhances colonic muscle contraction and fecal output through neuropeptide Y Y4 receptor in mice. *Eur J Pharmacol* 2010;**627**:258–64.
25. Pfluger PT, Kampe J, Castaneda TR, Vahl T, D'Alessio DA, Kruthaupt T, et al. Effect of human body weight changes on circulating levels of peptide YY and peptide YY3–36. *J Clin Endocr Metab* 2007;**92**:583–8.
26. Renshaw D, Batterham RL. Peptide YY: a potential therapy for obesity. *Curr Drug Targets* 2005;**6**:171–9.
27. Spiller RC, Trotman IF, Higgins BE, Ghatei MA, Grimble GK, Lee YC, et al. The ileal brake–inhibition of jejunal motility after ileal fat perfusion in man. *Gut* 1984;**25**:365–74.

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28. Tatemoto K. Isolation and characterization of peptide YY (PYY), a candidate gut hormone that inhibits pancreatic exocrine secretion. *Proc Natl Acad Sci USA* 1982;**79**:2514–8.
29. Tatemoto K, Nakano I, Makk G, Angwin P, Mann M, Schilling J, et al. Isolation and primary structure of human peptide YY. *Biochem Biophys Res Commun* 1988;**157**:713–7.
30. Utz AL, Lawson EA, Misra M, Mickley D, Gleysteen S, Herzog DB, et al. Peptide YY (PYY) levels and bone mineral density (BMD) in women with anorexia nervosa. *Bone* 2008;**43**:135–9.
31. Viardot A, Heilbronn LK, Herzog H, Gregersen S, Campbell LV. Abnormal postprandial PYY response in insulin sensitive nondiabetic subjects with a strong family history of type 2 diabetes. *Int J Obes* 2008;**32**:943–8.
32. Vona-Davis L, McFadden DW. PYY and the pancreas: inhibition of tumor growth and inflammation. *Peptides* 2007;**28**:334–8.
33. Wen J, Leon EL-D, Kost LJ, Sarr MG, Phillips SF. Duodenal motility in fasting dogs: humoral and neural pathways mediating the colonic brake. *Am J Physiol-Gastr L* 1998;**274**:G192–G1G5.
34. Witte AB, Grybäck P, Holst JJ, Hilsted L, Hellström PM, Jacobsson H, et al. Differential effect of PYY1-36 and PYY3-36 on gastric emptying in man. *Regul Peptides* 2009;**158**:57–62.
35. Yulyaningsih E, Zhang L, Herzog H, Sainsbury A. NPY receptors as potential targets for anti-obesity drug development. *Br J Pharmacol* 2011;**163**:1170–202.

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Chapter 179

Secretin

Laurence J. Miller

ABSTRACT

Secretin is a linear 27-residue amidated peptide that is prototypic of natural peptide ligands that bind to and activate family B G protein-coupled receptors. All these peptides have diffuse pharmacophoric domains, with important determinants for high affinity binding spread throughout their length and with key determinants for biological activity at their amino terminus. Recent insights provide an evolving understanding of the structural basis of secretin binding to its receptor and stimulation of receptor signaling. The structure of the gene encoding secretin, its primary transcripts, and the processing of products of translation are well described. Physiological functions of this hormone include stimulation of secretion of biliary and pancreatic bicarbonate-rich fluid and inhibition of gastric secretion and gastric emptying. There are clinical pathological states of both elevation and deficiency in the levels of secretin, as well as clinical diagnostic utility for secretin infusion in testing pancreatic exocrine function and provocative testing for gastrinoma.

DISCOVERY

Secretin and its receptor hold unique and important roles in history, with the peptide representing the first hormone and giving birth to the field of endocrinology,[1] and the receptor representing the first member of the B family of G protein-coupled receptors.[17] Secretin was identified in 1902 by Bayliss and Starling[1] when they observed that the infusion of acid into a denervated loop of canine jejunum resulted in the stimulation of pancreatic exocrine secretion. The response was believed to be mediated by a blood-borne transmitter that they called a hormone, based on the Greek word meaning "I arose to activity." Indeed, secretin is one of the many gastrointestinal peptide hormones that have subsequently been isolated from the intestine, purified, structurally characterized, and ultimately synthesized and used to reproduce their physiological effects by Jorpes and Mutt.[23] Because the source of secretin and other gastrointestinal hormones are isolated enteroendocrine cells scattered throughout the intestinal mucosa, making them much more difficult to study than the classical solid endocrine organs, the field of endocrinology moved ahead quite quickly working on the classical endocrine organs, although it took many years to assemble the cast of mediators comprising the gastrointestinal endocrine system and to develop tools and reagents to characterize them.

The secretin receptor cDNA was cloned using the expression cloning technique in which a pool of genes from the NG108-15 neuroblastoma-glioma hybrid cell line in a mammalian expression vector was used to transfect COS cells that were then screened for secretin radioligand binding.[17] The primary sequence of the secretin receptor included seven hydrophobic segments likely to represent the seven transmembrane helical segments typical of the G protein-coupled receptors (GPCRs) known at that time, yet the classical signature sequences known to be present in family A GPCRs were absent in this receptor. Within a short time, the calcitonin and parathyroid hormone receptor sequences were also reported, and it became clear that a new and unique family (family B GPCRs) had been identified. Although this family has similar predicted heptahelical transmembrane topology and the ability to couple with heterotrimeric G proteins to that of family A GPCRs, clear structural differences from family A and coherence in themes have defined this as a unique family within the GPCR superfamily.[30]

STRUCTURE OF THE mRNA/GENE

The porcine secretin gene was first cloned in 1990[18] and found to include four exons and three introns spanning 813 bp.[19] Similar organization is present in genes subsequently cloned from rats, pigs, and humans (Fig. 1). All these include an untranslated region and coding regions for a signal peptide, an amino-terminal peptide that can be part of a biologically active form of secretin, and a long carboxyl-terminal extension peptide of unknown biological significance. The organization of the secretin gene is similar to that of several other peptides in this family, including glucagon, pituitary adenylate cyclase–activating polypeptide, and vasoactive intestinal polypeptide, with all having the mature hormonal peptides encoded by a single exon. Other members of

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00179-2
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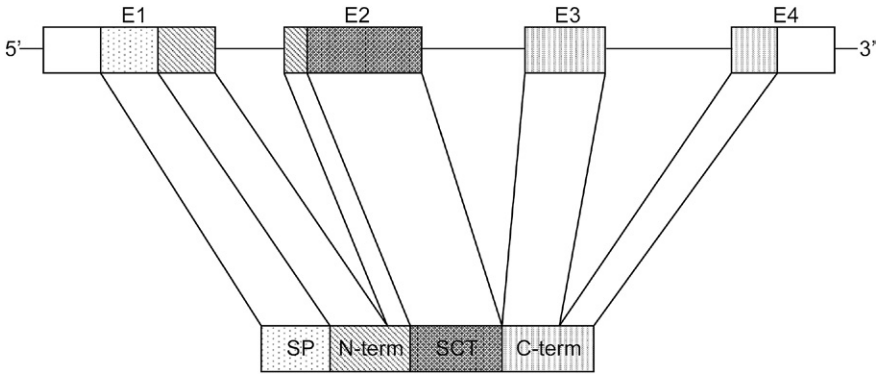

FIGURE 1 Genomic organization of the porcine secretin gene. Shown is the general organization of the genomic sequence encoding secretin, including four exons (E1–E4) and intervening introns. The mature transcript for this hormone includes a signal peptide (SP) and amino terminal-coding region (N-term) that comes from the first exon and the 5′-end of the second exon. The classical mature peptide hormone (SCT) is encoded by the second exon. A carboxyl-terminal extension (C-term) is encoded by portions of the third and fourth exons.

	1	6	11	16	21	26
Pig, cow, goat, sheep	HSDGT	FTSEL	SRLRD	SARLQ	RLLQG	LV*
Dog	-----	-----	----E	-----	-----	--
Rat	-----	-----	---Q-	-----	-----	--
Human	-----	-----	----E	G----	-----	--
Mouse	----M	-----	-----	-----	-----	--
Rabbit	-----	L----	-----	R----	-----	-L
Guinea pig	-----	----K	-----	-----	-----	--
Chicken	----L	----Y	-KM-G	N-OV-	KFI-N	-M

FIGURE 2 Amino acid sequence of secretin from various animal species. Shown are the amino acid sequences of secretin from multiple species with the one-letter code. These sequences in multiple mammalian species are highly conserved, and the biologically critical amino-terminal sequence is also most conserved in an avian species as well. Identical amino acids are shown as (-), whereas amino acid replacements are directly identified.

this family, such as glucose-dependent insulinotropic peptide and growth hormone-releasing hormone, have the hormone-coding region spanning two exons. Although there is only a single copy of the secretin sequence within its gene in these species, glucagon and vasoactive intestinal polypeptide genes that have been cloned to date do include duplications such as glucagon-like peptides 1 and 2 and peptide histidine-methionine (PHM).

PROCESSING OF THE PRECURSOR

Mature secretin peptides are highly conserved in multiple mammalian species (Fig. 2). To date, one avian species, from *Gallus gallus* (chicken) has been reported (Fig. 2) and found to retain most of the amino-terminal sequence that is so important for the biological activity of this hormone. In addition to the mature, carboxyl-terminally-amidated form of secretin, various proforms of this hormone have been isolated. These are analogous to those of several other family members. These include a nine amino acid amino-terminally-extended form (N-prosecretin), glycine-extended forms believed to be in various stages of processing (secretin-Gly and secretin-Gly-Lys-Arg), and carboxyl-terminally-extended precursor forms of unknown significance.[2,9] The carboxyl-terminal peptides predicted from mRNA molecules isolated from rats and pigs are 72 residues in length, although these peptides are minimally conserved and far more divergent than the sequences of the coding regions of the mature peptides. Of interest, the first precursor form characterized to include portions of this sequence represented secretin plus 41 residues at its carboxyl terminus, although it did not include the first 32 residues of the predicted carboxyl-terminal peptide that had been replaced with an Arg residue.[10] Other precursors were ultimately characterized, including one with the full length predicted carboxyl-terminal peptide and one with a shortened form of this peptide.[19] The Gly-Lys-Arg sequence that is present immediately after the carboxyl-terminal Val residue in the mature porcine and human secretin peptides provides the classical site for dipeptidyl-peptidase cleavage, as well as the amino group that is donated during α-amidation of peptides that occurs as an important part of the posttranslational processing of the hormone precursor.

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DISTRIBUTION OF THE mRNA/PEPTIDE

Secretin-secreting S cells are scattered in the small intestinal mucosa, particularly in the villus region, throughout the length of the small intestine of most species, with a gradient decreasing in frequency from proximal to distal. Consistent with this, secretin-like immunoreactivity is the highest in the duodenum where secretin-containing cells can represent six per one thousand cells,[16] and the frequency of these cells is found to progressively decrease to very low levels of immunoreactivity in the ileum. There are some differences among species, with rats having less of a proximal-to-distal gradient and pigs extending the presence of secretin into the colon, but these species seem to represent exceptions. The presence of secretin-like immunoreactivity in tissues outside of the intestine has been inconsistent and somewhat confusing, with some studies reporting its presence and others failing to find it in the same tissue. Tissues following this theme include the gastric antrum, pancreatic islets, and various regions of the central nervous system.

Although the identification of the cDNA encoding secretin and the ability to determine the presence of secretin mRNA could help clarify the distribution of this hormone, the apparent lack of correlation of the amount of mRNA with the amount of secretin peptide provides challenges. Clearly, the predominant site of secretin mRNA is in the intestine, but, of interest, it seems to be present in the highest concentration in the distal small intestine and is also present in the colon, sites that typically contain little mature peptide. Similarly, Northern blotting has failed to detect secretin mRNA in the stomach, pancreas, and many brain regions, probably because of levels that are too low to detect by this technique, whereas the very sensitive polymerase chain reaction amplification shows the presence of this mRNA in these tissues. It is of interest to note that polymerase chain reaction has provided positive results for the amplification of secretin transcripts present in first-strand cDNA prepared from the heart, lung, kidney, and testis.[25] The significance of the latter observations is unclear, particularly with no immunoreactive secretin peptide being identified in these tissues.

Secretin has been identified during the late stages of fetal development in the intestine and pancreas, remaining at high levels in the early postnatal period and then falling to adult levels.[19] This pattern of temporal expression is quite distinct from that of other gastrointestinal peptides such as cholecystokinin, which increase after feeding begins. It has been suggested that the prenatal expression of secretin could relate to the trophic action of this hormone in stimulating pancreatic growth and development.

RECEPTORS AND SIGNALING CASCADES

The secretin receptor is a family B guanine nucleotide-binding protein (G protein)-coupled receptor (GPCR). It was the first member of this family to be cloned[17] and has turned out to be prototypic of all peptide-binding members of this family[30] (Fig. 3). Although these receptors have the seven transmembrane topology and G protein coupling typical of all other GPCRs, the family B GPCRs are quite distinct in relation to their signature sequences, missing the key conserved amino acids present in the much larger and more extensively studied family A group. All the crystal structures for GPCRs that have been published to date are family A receptors,[28] and even the helical bundle region of those receptors that has been most conserved among them is predicted to be distinct in family B.[6] The most characteristic feature of the family B GPCRs is the relatively long extracellular amino-terminal tail region (>125 residues) that includes six conserved cysteine residues that contribute to three intradomain disulfide bonds.[12] Recently, a series of members of this family have had the structures of their amino-terminal tail regions solved using either NMR or X-ray crystallography. This has resulted in the characterization of a highly conserved structure that includes a highly conserved core with two antiparallel β-sheet regions, three disulfide bonds, and multiple loop regions with variable presence of an amino-terminal α-helix.[27] This structure also includes a conserved hydrophobic peptide-binding cleft above the stable core and between the helix and loop region.

The carboxyl-terminal portion of secretin, like other natural ligands for receptors in this family, assumes an alpha-helical conformation and occupies this peptide-binding cleft in the receptor amino terminus (Fig. 3). At the end of this helical segment, there is a helix N-capping motif that is believed to stabilize the important helical conformation and to direct the biologically critical peptide amino-terminal segment toward the core of the receptor[24] (Fig. 3). Although the details of the docking of the peptide amino terminus are not clear, this interaction is key for the biological activity of the natural hormonal ligand. Because the amino-terminal tail domain of the receptor is only covalently linked with the helical bundle core domain through the peptide backbone (carboxyl-terminal end of the amino-terminal tail region to the top of transmembrane segment one), the tethering process mediated by the bound natural agonist ligand is believed to be very important to induce the conformational change in the helical bundle that exposes the G protein-coupling site at the cytosolic face of the receptor. It is probable that these processes are conserved in this family, based on the similarity of peptide ligand and receptor structures and structure–activity considerations for both molecules.

In addition to its binding and activation of the secretin receptor, secretin is also capable of binding and activating another family B GPCR, the VPAC1 receptor.[13] This receptor binds secretin with low affinity, yet high concentrations

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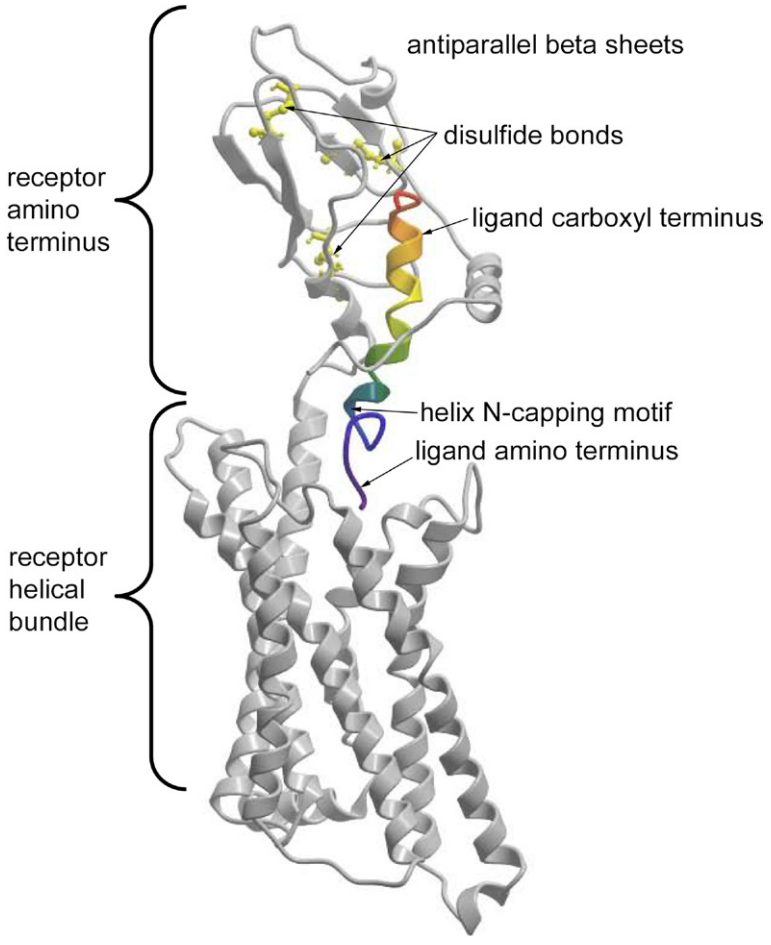

FIGURE 3 Illustration of the structure of the secretin receptor. Shown is a prototypic family B GPCR in gray, with the extracellular amino-terminal domain above the intramembranous helical bundle domain. Within the amino terminus are three highly conserved disulfide bonds (yellow), two antiparallel beta sheet regions, and several loop and helical structures. The peptide ligand is shown colored from purple-to-red, representing amino-to-carboxyl-terminal regions. The carboxyl terminus is illustrated in the typical alpha helix conformation ending in the conserved helix N-capping motif and the biologically important amino terminus directed toward the helical bundle. See color plate 50.

of secretin are capable of stimulating a full biological response. This is fully consistent with the common themes for structures and mode of binding and activation of these receptors. The carboxyl-terminal ends of the peptide ligands provide considerable binding energy but without a high degree of specificity. The amino-terminal ends of the peptide ligands provide the biological activity with a higher degree of specificity, while also adding binding energy to form the productive ligand–receptor complex.

Another interesting feature of secretin activation of its receptor is an evolving theme of receptor dimerization. There is a relatively recent appreciation that GPCRs can associate with themselves and with each other to form oligomers, although the presence of such higher-order complexes and their functional implications vary substantially across the superfamily.[7] For family C GPCRs, dimerization is quite common and even necessary to achieve some types of responsiveness to particular ligands. For family A GPCRs, oligomeric complexes can form for select members, with the implications varying substantially, from disrupting preformed complexes to stabilizing and stimulating the formation of such complexes, and ranging from no functional effects to substantial effects on ligand affinity, specificity, and signaling. For family B GPCRs, the secretin receptor has been studied more extensively than any other member[11] and seems to be prototypic of the typical behavior of members of this receptor family. Secretin receptors form structurally specific homodimeric complexes along the lipid-facing surface of TM4. This complex is key for the high affinity G protein-coupled state of this receptor. This complex is also critical for the negative cooperativity of binding observed for this receptor and other members of this receptor family.[11]

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Like other GPCRs, the secretin receptor is regulated by both biochemical and cellular mechanisms. The major biochemical regulatory mechanism involves receptor phosphorylation.[26] This occurs via signaling kinases such as protein kinase A and protein kinase C, and via G protein-coupled receptor kinases.[29] Phosphorylation is believed to mediate arrestin association with the receptor and uncoupling of the receptor from its G protein. The major cellular mechanism for regulation involves receptor internalization to move the peptide-binding site off the cell surface to a protected environment. This can be reversed by receptor recycling back to the cell surface or by new receptor biosynthesis.

BIOLOGICAL ACTIONS WITHIN THE GASTROINTESTINAL TRACT

The major physiological actions of secretin in the gastrointestinal tract provide a critical mechanism that contributes to nutritional homeostasis.[3] This relates to the ability of secretin to stimulate alkaline secretions from the pancreatic and biliary ductular systems. It is noteworthy that secretin is secreted from its intestinal S cells in response to acidic chyme in the lumen, with this requiring neutralization by the alkaline secretions to achieve an optimal pH environment for the actions of many digestive enzymes required for the breakdown of complex nutrients into absorbable forms. The components of intestinal chyme described to stimulate secretion of secretin include gastric acid and products of fat digestion (long chain fatty acids) and protein digestion (oligopeptides and amino acids), bile acids, and certain herbal extracts (curcuma longa and licorice root). The gastric acid delivered to the duodenum seems to be the most quantitatively important stimulant, with the pH threshold for secretin secretion of about 4.5 (release of hormone at pH levels below this threshold). Of interest, a secretin-releasing peptide has been postulated to exist,[22] mediating this effect of the luminal acid and providing still another level of potential feedback inhibition.

Secretin is also known to stimulate gastric secretion of mucus and pepsinogen, duodenal Brunner's gland secretion, pancreatic islet cell secretion of insulin, glucagon, pancreatic polypeptide, and somatostatin, and pancreatic growth. It can also inhibit the secretion of gastric acid and inhibit motility and transit in the esophagus, stomach, small intestine, and colon.

Another coherent set of responses to secretin have become clear in studies with knockout animal models.[5] These relate to body fluid homeostasis, where central nervous system effects of secretin can stimulate drinking and vasopressin release into the circulation; renal effects can result in water retention in the kidney particularly through effects on aquaporins, and effects of cardiovascular osmoregulatory neurons to regulate blood volume.[4] Indeed, insights into these effects may provide therapeutic opportunities for secretin receptor–active drugs in the future.

The actions of secretin are known to be synergistic with other gastrointestinal hormones such as cholecystokinin, with both acting together to stimulate the release and delivery of enzyme-rich pancreatic secretions into the duodenum. Additional interactions have been described for neurotensin and insulin with secretin. How this might affect normal human physiology is not yet clear. Interactions with nerves and neuronal actions are also prominent for secretin, being particularly affected by the integrity of the vagus nerve and with cholinergic potentiation of some effects of this hormone.

PATHOPHYSIOLOGICAL IMPLICATIONS

Clinical situations have been described in which levels of circulating secretin are elevated. These include gastric hypersecretory syndromes such as peptic ulcer and Zollinger–Ellison (gastrinoma) syndrome, renal failure with inadequate clearance of secreted peptide, and even a rare secretin-secreting islet cell tumor. Reduced levels of secretin have been observed in patients with achlorhydria and celiac disease, probably reflecting the absence of physiological stimulation or reduced S cell mass.

Secretin is also administered in diagnostic testing as a means of stimulating secretion in the pancreatic and biliary ductular systems, particularly together with imaging of these ducts, and as a provocative test for Zollinger–Ellison (gastrinoma) syndrome.[8] Most islet cell tumors responsible for this syndrome have excess secretin receptors on their cell surface and respond with a substantial elevation of serum gastrin during this provocative test, although rare tumors with excessive misspliced forms of this receptor do not respond.[20]

To date, there have not been any reports of abnormal or variant secretin molecules with recognized clinical presentations. There had been some excitement about an early report suggesting a role for secretin as a therapeutic for autism,[15] but further studies have failed to confirm the utility of this approach.[21]

Misspliced forms of the secretin receptor have been described in the setting of neoplasia.[20] This includes both islet cell tumors, such as the unresponsive gastrinoma described above, and pancreatic ductal adenocarcinomas. The dominant negative action of the variant splice form can actually have an inhibitory effect on the growth inhibitory role of this hormone, thereby stimulating the growth of such tumors.[14]

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ACKNOWLEDGMENTS

This work was supported by a grant from the National Institutes of Health, DK46577.

REFERENCES

1. Bayliss WM, Starling EH. The mechanism of pancreatic secretion. *J Physiol* 1902;**28**:325–53.
2. Bonetto V, Jornvall H, Mutt V, Sillard R. Two alternative processing pathways for a preprohormone: a bioactive form of secretin. *Proc Natl Acad Sci U S A* 1995;**92**:11985–9.
3. Chey WY, Chang TM. Secretin, 100 years later. *J Gastroenterol* 2003;**38**:1025–35.
4. Chu JY, Cheng CY, Lee VH, Chan YS, Chow BK. Secretin and body fluid homeostasis. *Kidney Int* 2011;**79**:280–7.
5. Chu JY, Chung SC, Lam AK, Tam S, Chung SK, Chow BK. Phenotypes developed in secretin receptor-null mice indicated a role for secretin in regulating renal water reabsorption. *Mol Cell Biol* 2007;**27**:2499–511.
6. Donnelly D. The arrangement of the transmembrane helices in the secretin receptor family of G-protein-coupled receptors. *FEBS Lett* 1997;**409**:431–6.
7. Filizola M, Weinstein H. The study of G protein-coupled receptor oligomerization with computational modeling and bioinformatics. *FEBS J* 2005;**272**:2926–38.
8. Frucht H, Howard JM, Slaff JI, Wank SA, McCarthy DM, Maton PN, et al. Secretin and calcium provocative tests in the Zollinger–Ellison syndrome. A prospective study. *Ann Intern Med* 1989;**111**:713–22.
9. Gafvelin G, Carlquist M, Mutt V. A proform of secretin with high secretin-like bioactivity. *FEBS Lett* 1985;**184**:347–52.
10. Gafvelin G, Jornvall H, Mutt V. Processing of prosecretin: isolation of a secretin precursor from porcine intestine. *Proc Natl Acad Sci U S A* 1990;**87**:6781–5.
11. Gao F, Harikumar KG, Dong M, Lam PC, Sexton PM, Christopoulos A, et al. Functional importance of a structurally distinct homodimeric complex of the family B G protein-coupled secretin receptor. *Mol Pharmacol* 2009;**76**:264–74.
12. Grace CR, Perrin MH, DiGruccio MR, Miller CL, Rivier JE, Vale WW, et al. NMR structure and peptide hormone binding site of the first extracellular domain of a type B1 G protein-coupled receptor. *Proc Natl Acad Sci U S A* 2004;**101**:12836–41.
13. Harmar AJ, Arimura A, Gozes I, Journot L, Laburthe M, Pisegna JR, et al. International union of pharmacology. XVIII. Nomenclature of receptors for vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide. *Pharmacol Rev* 1998;**50**:265–70.
14. Hayes GM, Carrigan PE, Dong M, Reubi JC, Miller LJ. A novel secretin receptor splice variant potentially useful for early diagnosis of pancreatic carcinoma. *Gastroenterology* 2007;**133**:853–61.
15. Horvath K. Secretin treatment for autism. *N Engl J Med* 2000;**342**:1216:author reply 8.
16. Inokuchi H, Fujimoto S, Hattori T, Kawai K. Tritiated thymidine radioautographic study on the origin and renewal of secretin cells in the rat duodenum. *Gastroenterology* 1985;**89**:1014–20.
17. Ishihara T, Nakamura S, Kaziro Y, Takahashi T, Takahashi K, Nagata S. Molecular cloning and expression of a cDNA encoding the secretin receptor. *EMBO J* 1991;**10**:1635–41.
18. Kopin AS, Wheeler MB, Leiter AB. Secretin: structure of the precursor and tissue distribution of the mRNA. *Proc Natl Acad Sci U S A* 1990;**87**:2299–303.
19. Kopin AS, Wheeler MB, Nishitani J, McBride EW, Chang TM, Chey WY, et al. The secretin gene: evolutionary history, alternative splicing, and developmental regulation. *Proc Natl Acad Sci U S A* 1991;**88**:5335–9.
20. Korner M, Miller LJ. Alternative splicing of pre-mRNA in cancer: focus on G protein-coupled peptide hormone receptors. *Am J Pathol* 2009;**175**:461–72.
21. Krishnaswami S, McPheeters ML, Veenstra-Vanderweele J. A systematic review of secretin for children with autism spectrum disorders. *Pediatrics* 2011;**127**:e1322–5.
22. Li P, Lee KY, Chang TM, Chey WY. Mechanism of acid-induced release of secretin in rats. Presence of a secretin-releasing peptide. *J Clin Invest* 1990;**86**:1474–9.
23. Mutt V, Jorpes JE, Magnusson S. Structure of porcine secretin. The amino acid sequence. *Eur J Biochem* 1970;**15**:513–9.
24. Neumann JM, Couvineau A, Murail S, Lacapere JJ, Jamin N, Laburthe M. Class-B GPCR activation: is ligand helix-capping the key? *Trends Biochem Sci* 2008;**33**:314–9.
25. Ohta M, Funakoshi S, Kawasaki T, Itoh N. Tissue-specific expression of the rat secretin precursor gene. *Biochem Biophys Res Commun* 1992;**183**:390–5.
26. Ozcelebi F, Holtmann MH, Rentsch RU, Rao R, Miller LJ. Agonist-stimulated phosphorylation of the carboxyl-terminal tail of the secretin receptor. *Mol Pharmacol* 1995;**48**:818–24.
27. Parthier C, Reedtz-Runge S, Rudolph R, Stubbs MT. Passing the baton in class B GPCRs: peptide hormone activation via helix induction? *Trends Biochem Sci* 2009;**34**:303–10.
28. Rasmussen SG, Choi HJ, Rosenbaum DM, Kobilka TS, Thian FS, Edwards PC, et al. Crystal structure of the human beta2 adrenergic G-protein-coupled receptor. *Nature* 2007;**450**:383–7.
29. Shetzline MA, Premont RT, Walker JK, Vigna SR, Caron MG. A role for receptor kinases in the regulation of class II G protein-coupled receptors. Phosphorylation and desensitization of the secretin receptor. *J Biol Chem* 1998;**273**:6756–62.
30. Ulrich CD, Holtmann M, Miller LJ. Secretin and vasoactive intestinal peptide receptors: members of a unique family of G protein-coupled receptors. *Gastroenterology* 1998;**114**:382–97.

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Chapter 180

Somatostatin

Vicente Martinez

ABSTRACT

Somatostatin is a tetradecapeptide isolated in the early 1970s from sheep hypothalami. It has been found in virtually every organ of the body, and it is particularly abundant in the gastrointestinal tract. Somatostatin exerts an inhibitory action on numerous physiological functions, acting as a classical endocrine hormone, a local (paracrine) regulator, or a neurotransmitter. Somatostatin actions are mediated through five distinct receptor subtypes (sst_{1-5}). sst_2 and sst_5 are the main mediators of the gastrointestinal effects of the peptide. Within the gastrointestinal tract, somatostatin exerts a broad range of physiological and pharmacological effects, mostly inhibitory, of which suppression of gastric acid secretion is a landmark response. Several diseases of the gastrointestinal tract imply disturbances in the somatostatin production or an altered expression of somatostatin receptors. The development of selective somatostatin agonists and antagonists has been critical in understanding the physiological and pathophysiological functions of somatostatin within the gastrointestinal tract.

Somatostatin was discovered in 1973 in sheep hypothalamic extracts and named originally hypothalamic growth hormone inhibiting factor.[2] Since then, the peptide has been found throughout virtually every organ of the body. Apart from its numerous endocrine and central nervous actions, it was quite clear that somatostatin has extensive gastrointestinal actions, acting mostly as an inhibitory mediator. During the last four decades, continued scientific interest resulted in the cloning and characterization of a family of five somatostatin receptors, the identification of three related neuropeptides, namely, coststatin, thrittene, and neuronostatin, and the development of numerous synthetic somatostatin analogs, some of them with clinical applications.

DISCOVERY

On searching for soluble factors involved in the inhibition of growth hormone secretion, a hypothalamic peptide consisting of 14 amino acids was isolated, identified as an inhibitor of growth hormone release, and named hypothalamic growth hormone inhibiting factor or somatostatin.[2] A few years later, a second biologically active, amino-terminally extended form of 28 residues, called somatostatin-28, was isolated from the porcine gut.[24] Both forms of somatostatin exhibit a disulfide bridge that allows a cyclic structure. Somatostatin peptides of 25 and 20 amino acids have also been purified from sheep hypothalamus and porcine intestine, respectively. However, the possibility that these peptides result from the proteolytic cleavage of somatostatin-28 during the isolation process has not been excluded.[3]

Both, somatostatin-14 and somatostatin-28 have a cyclic structure and derive from a single precursor, preprosomatostatin, a product of the somatostatin 1 gene, through tissue-specific posttranslational processing.[12,30] Somatostatin is a phylogenetically ancient peptide that is widely distributed throughout the body. The somatostatin-14 sequence is totally conserved across species, whereas there is only a 40–66% homology between somatostatin-28 and its counterparts in fish. In mammals, somatostatin-28 contains the somatostatin-14 moiety, responsible for the biological activity, at its carboxyl-terminal end. The processing machinery required for its synthesis is present in almost all tissues, with different relative activities. This leads to different tissue-specific types of synthesis and release of somatostatin-14 and somatostatin-28. Somatostatin-14 is the predominant form in the CNS and in most peripheral organs, including the gastrointestinal tract. By contrast, somatostatin-28 is mainly produced by intestinal enteroendocrine cells.[3,20]

During the previous years, the somatostatin family of regulatory peptides has expanded with the identification in mammals of three additional somatostatin-related peptides, namely, cortistatin, thrittene, and neuronostatin.

Cortistatin was identified in rats as a neuropeptide with strong homology to somatostatin. Like somatostatin, cortistatin has a short (cortistatin-17 in humans and cortistatin-14 in rats) and a long form (cortistatin-29 in rats and in humans). Rat cortistatin-14 shares 11 of its 14 amino acids with somatostatin-14, including the FWKT tetramer believed to be responsible for somatostatin's receptor interactions and the pair of cysteine residues that probably render the peptides cyclic. Unlike the broad tissue expression of the somatostatin-1 gene, the cortistatin

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00180-9
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gene has a more restricted expression pattern, mainly in the cortex and hippocampus.[7,8,28]

Recent studies using unique polyclonal antisera and a strain of somatostatin-deficient mice showed the existence of an additional somatostatin-like gastrointestinal peptide that has been named thrittene. Thrittene is homologous with the 13 residues of the proximal region of somatostatin-28 (somatostatin-$28_{(1–13)}$) but is not derived from the somatostatin-1 gene or the cortistatin gene.[10]

Bioinformatic analyses of evolutionarily conserved sequences predicted the existence of a novel somatostatin-related peptide within the prosomatostatin molecule. After this, a 13-residue amidated product of the prosomatostatin, named neuronostatin, was purified from porcine pancreas. Unlike somatostatin-14, somatostatin-28 and the cortistatins, neuronostatin is not a cyclic polypeptide.[25] Although derived from the same preproprotein as somatostin, the processing of neuronostatin and somatostatin could be differentially regulated. Moreover, because of the need for the amidation of neuronostatin, but not somatostatin, expression of amidation enzymes in cells expressing somatostatin transcripts could determine neuronostatin levels.

The physiological role of these somatostatin-related peptides is still unclear.

STRUCTURE OF THE mRNA/GENE

The abundant expression of somatostatin in anglefish pancreatic islets was vital for the initial cloning of a somatostatin cDNA encoding the full amino acid sequence of preprosomatostatin in 1980.[13,14] Subsequently, cDNAs encoding preprosomatostatin of numerous species have been isolated and shown to have virtually complete homology in the region encoding somatostatin-28 and somatostatin-14.[30]

Somatostatin is encoded by a single gene, the somatostatin 1 gene, which, in humans, maps to the long arm of the chromosome 3. In the rat, the gene spans 1.2kb and is interrupted within the coding sequence of preprosomatostatin, the 116-amino-acid precursor of somatostatin-14 and somatostatin-28, by a single intron of 630 bases. The somatostatin 1 gene is present in all vertebrate classes, showing a virtually complete homology in the region encoding both forms of the peptide.[12,30] A single promoter directs transcription of the gene in all tissues, and there are no known alternative mRNA splicing events.

Cortistatin is the product of a second somatostatin gene present in most mammals (somatostatin 2/costistatin gene).[12,30] A revised evolutionary concept of the somatostatin gene family suggests that a primordial gene underwent duplication during or before the advent of chordates and that the two resulting genes underwent differing rates of mutation to produce the distinct preprosomatostatin (somatostatin 1) and preprocortistatin (somatostatin 2/cortistatin) genes in mammals. A second gene duplication event from the ancestral somatostatin gene occurred in teleosts to generate the so-called somatostatin 3 and somatostatin 4 genes.[12,30]

Therefore, so far, there is evidence for the existence of at least four distinct somatostatin genes in vertebrates: (i) the somatostatin 1 gene, encoding for somatostatin-14 and somatostatin-28 and representing the ancestral gene of the family; (ii) the somatostatin 2/cortistatin gene, encoding for cortistatin; (iii) the somatostatin 3 gene, encoding for several somatostatin-like peptides in teleosts; and (iv) the somatostatin 4 gene, encoding for somatostatin-like peptides in ostariophysi.

The gene encoding for thrittene, which is not derived from either the somatostatin-1 or the somatostatin 2/cortistatin genes, remains elusive.

PROCESSING OF THE PRECURSOR

As mentioned, both forms of mammalian somatostatin (14 and 28) are derived post-translationally from a common preprohormone (116-amino-acid) by the action of specific proprotein convertases (PCs). For instance, genetic studies indicate a primary role for PC2 in the generation of somatostatin-14[17] (see the PC2 chapter in Peptides Biosynthesis/Processing section of this book).

The processing machinery required for the synthesis of somatostatins is present in almost all tissues, including some that ordinarily do not synthesize the peptide, with different relative activities.[3] This leads to different tissue-specific types of synthesis and release of somatostatin-14 and somatostatin-28. Depending on the tissue considered, the somatostatin precursor is processed to somatostatin-14 or to both somatostatin-14 and somatostatin-28, with no processing intermediates and with no evidence of conversion of somatostatin-28 to somatostatin-14. However, the detection of fragments somatostatin-$28_{1–12}$ (the amino terminal fragment after somatostatin-28 cleavage) in tissues producing somatostatin-14 suggests the possible production of this form of the peptide from somatostatin-28.[3]

Somatostatin-14 is the predominant form in the CNS and in most peripheral organs, including the gastrointestinal tract, where it can be found in the stomach, duodenum, colon, and pancreas. By contrast, somatostatin-28 is produced mainly by intestinal enteroendocrine cells and represents >50% of the somatostatin content in the small intestine.[20]

DISTRIBUTION AND RELEASE

The central nervous system, gastrointestinal tract, and endocrine glands are the major sites of production and action of somatostatin. In the rat, the gastrointestinal tract accounts

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for approximately 65% of the total body somatostatin, the pancreas for approximately 5%, the brain for approximately 25%, and the remaining organs for approximately 5%.[20] Somatostatin mRNA is distributed throughout the gastrointestinal tract, being present in both enteroendocrine cells and neurons. The gastrointestinal tract is the main source for the picomolar concentration of somatostatin measured in the circulation. Somatostatin, however, degrades rapidly in the circulation, with a half-life of <3min. Although the shorter somatostatin-14 form is mainly synthesized in the stomach, colon, and pancreas, the longer somatostatin-28 form is mainly found in the small intestine, in particular in its proximal part.[3,20]

Distribution of Somatostatin and Somatostatin-like Peptides in the Gastrointestinal Tract

In mammals, somatostatin is found mainly in the mucosal layer of the gastrointestinal tract. For instance, >90% of immunoreactive somatostatin in extracts of human gastrointestinal tissue is measured in the mucosa, in particular in the stomach, the duodenum, and the jejunum; <10% occurs in the muscle layers. Within the mucosa, somatostatin is localized in epithelial endocrine cells termed D cells, found preferentially in the upper gastrointestinal tract. In the stomach, D cells are localized both in the antral and the fundic mucosa. They have a characteristic morphology, with cytoplasmatic processes that terminate in close proximity to the target cells. Such morphology supports the concept of a paracrine/local mode of action of somatostatin in the gastric mucosa. In the fundic mucosa, D cells are of a "closed type," without luminal contact, whereas in the antrum, they are of an "open type," with their apical membrane exposed to the gastric lumen. This organization correlates with functional responses to different stimuli.[3,20]

Endocrine D cells are also found in the intestinal mucosa and pancreas. Intestinal D cells are "flask-shaped" cells, with their apical membranes open to the lumen, and, therefore, respond to luminal stimuli. In the pancreas, endocrine D cells are a functional component of the pancreatic islets, participating in the local endocrine control.[3,20]

Although gastric somatostatin is mainly of endocrine origin, intestinal somatostatin is mostly present in neurons of the submucosal and myenteric plexus, throughout the whole gastrointestinal tract, with the lowest levels observed in the colon. Most neuronal somatostatin-like immunoreactivity is present in the intrinsic innervation of the gut. For instance, 20% of the cell bodies in the submucous plexus of the small intestine of guinea pigs contain somatostatin-like immunoreactivity, corresponding to cholinergic secretomotor neurons. Pancreatic ganglia and nerves also contain significant amounts of somatostatin.[3,20]

Cortistatin mRNA is expressed in the same gastrointestinal tissues as somatostatin mRNA but at levels significantly lower than those of somatostatin. Besides regions of the central nervous system (cortex and hippocampus), selective expression of cortistatin is only found in cells of the immune system, where it was proposed to function as the main endogenous somatostatin receptor ligand.[7,8]

Thrittene has been localized in enteroendocrine cells of the stomach and the distal small intestine, and in the enteric nervous system.[10] Neuronostatin has been localized in parietal cells of the gastric mucosa and in pancreatic islets, probably acting as a local regulator of gastric acid secretion and pancreatic endocrine function, although this needs to be confirmed.[25]

Regulation of Somatostatin Release in the Gastrointestinal Tract

Somatostatin is secreted mostly in a paracrine/neurocrine fashion, and, therefore, circulating levels are relatively low and might not reflect release at local sites. Moreover, to avoid spreading of actions, somatostatin is rapidly degraded by plasma enzymes, having a relatively short half-live in circulation, about 3min. These factors complicate the study of somatostin release and regulation *in vivo*. Somatostatin release in the gastroenteropancreatic system is regulated by intraluminal, neural, and endocrine factors in a complex network.

Release by Intraluminal Stimuli

Nutritional factors and acid (low pH) are the main intraluminal stimulants of somatostatin release. These factors, and in particular luminal acid, are key regulators of somatostatin release from open-type endocrine D cells in the antral region of the stomach. Similarly, the presence of nutrients and acid in the duodenum stimulates the release of gastric somatostatin, an effect probably mediated through the combined release of intestinal enterogastrone(s) and the activation of neural reflexes targeting antral D cells.[3,18,20] Because somatostatin is a potent inhibitor of gastric acid secretion, stimulation of its release by intraluminal acid represents a physiologically significant negative feedback regulatory loop. Physiological postprandial changes of somatostatin levels in the peripheral or portal circulation derive from gastric and intestinal somatostatin release. Other sources of somatostatin do not contribute significantly to the peripheral plasma levels of the peptide.[3]

Release by Neural Mechanism

The sympathetic and parasympathetic nervous systems have opposite effects on somatostatin release. Adrenergic agonists, through β-adrenergic receptors, stimulate gastric somatostatin release. On the other hand, muscarinic cholinergic agents inhibit somatostatin release in *in vitro* conditions. However,

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in vivo, there is evidence for vagal-dependent enhancement and inhibition of somatostatin release, an effect that has been explained by the existence of vagal nonadrenergic stimulatory pathways. Cholinergic agonists directly inhibit fundic D cells. Therefore, cholinergic effects *in vivo* may lead to a stimulation of acid secretion by direct action on parietal cells and also by inhibition of fundic somatostatin release. Moreover, the contribution of intrinsic peptidergic nerves targeting antral and fundic D cells needs to be taken into consideration.[3,20,22,35]

Release by Gut Regulatory Peptides

Several regulatory peptides, acting as classical hormones or as neuropeptides, modulate gastrointestinal somatostatin release. These can be categorized as stimulatory and inhibitory agents. The most important stimulants of somatostatin release are gastrin and cholecystokinin (CCK). Peptides of the secretin family, bombesin/gastrin-releasing peptide (GRP), oxyntomodulin, glucagon-like peptide-1 (GLP-1), and calcitonin gene-related peptide (CGRP), which consistently inhibit gastric acid secretion, may function by stimulating somatostatin release. On the contrary, substance P, insulin, glucagon, pancreatic polypeptide, and opioids are potent inhibitors of somatostatin release. The exact signaling pathways by which these stimuli trigger somatostatin release from D cells are complex and not completely understood. They involve mucosal cells and afferent and efferent vagal nerves.[3,20]

RECEPTORS AND SIGNALING CASCADES

Somatostatin Receptors

Somatostatin acts on its target cells through a family of rhodopsin-like G protein-coupled receptors (GPCR) with seven transmembrane domains, of which five subtypes (sst_1–sst_5) have been cloned and characterized from various species.[15] In addition, two splice variants of the sst_2, sst_{2A}, and sst_{2B}, which differ in length and composition of their intracellular carboxy termini, have been cloned in the mouse and in the rat. Sequence homology is 39–57% among the five subtypes, being highly conserved across species. For instance, there is an 80–99% homology among the human, rat, and mouse isoforms. The nearest relatives of the somatostatin receptors are the opioid receptors, sharing about 37% sequence similarity to that of the mouse sst_1. According to structural and functional characteristics, the somatostatin receptors are divided into two groups: group 1, including sst_2, sst_3, and sst_5 and group 2 that comprises sst_1 and sst_4.

In humans, somatostatin receptors are encoded by five nonallelic genes, mapped to chromosomes 14, 17, 22, 20, and 16, respectively. Genes coding for sst_1, sst_3, sst_4, and sst_5 are intronless; however, the gene for sst_2 contains three introns, which result in the generations of the unspliced sst_{2A} and the spliced sst_{2B} variants mentioned above. Recently, the existence of truncated sst_5 variants in humans, pigs, mice, and rats that lack one or more transmembrane domains has been reported. These truncated forms are formed by noncanonical splicing and show characteristic molecular and functional properties. They show a broad distribution in normal and tumoral tissues. However, their functional involvement, if any, in somatostatin (or cortistatin) actions remains to be elucidated.[4]

An increasing body of evidence suggests that in many instances somatostatin receptors do not function as monomers but that they associate with other receptors to form homodimers and heterodimers whose binding, pharmacological and signal properties can diverge significantly from those of the corresponding single receptor. However, these potential interactions have not been shown in native cells, and their relevance for receptor physiology is not clear.[9]

Signaling of Somatostatin Receptors

The binding of somatostatin to its receptors triggers a complex set of signaling events, initiated by the interaction of the activated receptors with a large number of different protein partners (Table 1). The interactions occurring between the activated receptor and its effectors determine the subsequent intracellular signaling events and, ultimately, the associated biological responses. For review on the subject see Refs. 16, 27.

Signaling of somatostatin receptors involves the activation of heterotrimeric G proteins. All somatostatin receptors decrease adenylyl cyclase activity, reducing the concentration of intracellular cAMP, via the pertussis-sensitive G protein family G_i/G_o, leading, further down, to the stimulation of a vanadate-sensitive phosphotyrosine phosphatase (PTP), the modulation of mitogen-activated protein kinases (MAPKs), as well as the stimulation of phospholipase C. Further, some somatostatin receptors activate inwardly rectifying K^+ channels and/or inhibit voltage-dependent Ca^{2+} channels. The net effect of coupling to these membrane channels is the hyperpolarization of the membrane potential leading to an inhibitory effect on secretion. Moreover, somatostatin receptors can act via pertussis toxin-independent mechanisms, via the activation of G proteins such as G_{12} or G_{14}. In some instances, a direct interaction between somatostatin receptors and phosphatases or kinases might happen.

The lowering of intracellular cAMP and Ca^{2+} are the most relevant mechanisms for the inhibition of hormone secretion from endocrine cells, whereas activation of PTP and MAPK are postulated to play a role in the antiproliferative effect of on tumor cells.

In addition, a different series of agonist-triggered events lead to somatostatin receptor desensitization and internalization. Somatostatin binding to sst_1, sst_{2A}, and sst_3 results in the activation of kinases and the subsequent receptor

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TABLE 1 Characteristics of the Human Cloned Somatostatin Receptor Subtypes

Characteristics	sst_1	sst_{2A}	sst_3	sst_4	sst_5
Chromosomal localization	14q13	17q24	22q13.1	20p11.2	16p13.3
Amino acids	391	369 356 (sst_{2B})	418	388	364
G protein-coupling	G_i/G_o	G_i/G_o	G_i/G_o	G_i/G_o	G_i/G_o
Transduction pathways					
Adenylyl cyclase	↓	↓	↓	↓	↓
Tyrosine phosphatase	↑	↑	↑	↑	↑
MAP kinases	↑↓	↑↓	↑	↑	↑↓
K^+ channels	↓	↑	↑	↑	↑
Ca^{2+} channels	↓	↓	–	–	↓
Na+/H+ exchanger	↓	–	↓	↑↓	–
Phospholipase C/IP_3	↑	↑	↑	↑	↑↓
Phospholipase A2	↓	↓	–	↑	–
Nitric oxide synthases	–	↑	↓	–	–
Selective agonist	CH-275 L797,591	BIM23027 L-054,22 MK-678	L-796,778	NNC269100 L-803,087	BIM23268 BIM23052 L-817,818
Selective antagonists	NVP-SRA880	Cyanamid-154806 PRL-2903	NVP-ACQ090	–	BIM23056
Tissue distribution	Brain, pituitary, GI tract, kidney, liver	Brain, pituitary, GI tract, kidney, adrenals	Brain, pituitary, GI tract	Brain, pituitary, GI tract, placenta, lungs	Brain, pituitary, GI tract, adrenals

phosphorylation. This results in a receptor subtype-specific recruitment of arrestins, thus stopping all G protein-mediated signaling and starting the receptor internalization process.

Somatostatin Receptor Localization in the Gastrointestinal Tract

All somatostatin receptor subtypes are present in the gastrointestinal tract; however, their exact localization is still a matter of debate. This results from the complex physiology of somatostatin (and cortistatin), the existence of numerous species-specific differences in receptor distribution, and discrepancies depending on whether the receptors are estimated at the mRNA or protein level (Table 1).

At the transcriptional level, mRNAs for all somatostatin receptor subtypes, have been detected throughout the whole rat gastrointestinal tract in the epithelium and the lamina propria of the mucosa, the submucosa, the smooth muscle layers, and the submucosal and myenteric nerve plexus. In humans, mRNAs for the various somatostatin receptor subtypes were found in the stomach, in the small intestine, and in the epithelium and lamina propria of the colon.

In the stomach, sst_2 is the predominant receptor subtype, with both splice variants (sst_{2A} and sst_{2B}) being present, as shown by *in situ* hybridization studies and RT-PCR techniques. This was further confirmed at the protein level with immunohistochemical studies using antipeptide antibodies specific for the sst_{2A} and sst_{2B} isoforms. These studies showed that sst_{2B} is mainly present in parietal cells and, in high concentration, in enterochromaffin-like (ECL) cells. On the other hand, sst_{2A} seems to be restricted to a small population of ECL cells. So far, no somatostatin receptors have been found in other cellular types of the gastric mucosa. Sst_{2A} has also been localized in nerve fibers arising from the enteric nervous system and innervating the mucosa, with fibers running parallel to the epithelia. Many of these fibers are in close proximity to D cells of the gastric mucosa. This distribution supports the view that sst_{2A}

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receptors might mediate somatostatin effects in the gastrointestinal tract via neuronal and paracrine pathways.[18,20,31]

In situ hybridization studies have identified all five receptor subtypes in the epithelium, submucosal plexus, and muscle layers of the rat small intestine and colon. However, only sst_1, sst_2, and sst_3 are found in the myenteric plexus. The main receptor subtype found in the intestine is the sst_2 form. Imunohistochemical studies showed the presence of sst_{2A} receptors in the myenteric and submucosal plexuses and in nerve fibers innervating the muscle, mucosa, and vasculature, thus suggesting an important role for this receptor subtype in the regulation of intestinal motor and secretory functions. Sst_{2A} receptors have been described also in interstitial cells of Cajal, indicative of other target cells for somatostatin to regulate smooth muscle activity.[20,31]

Within the pancreas, early autoradiographic studies showed the presence of somatostatin binding sites in alpha and beta cells. Subsequent molecular studies showed that rat islets contain mRNA for all five receptor subtypes, although this does not imply the presence of functional receptors. In humans, immunohistochemical studies showed a predominant expression of sst_2 and sst_5, with a cell-specific distribution pattern: beta and delta cells contain sst_5, while alpha cells contain mostly sst_2. In the exocrine pancreas, sst_2 has been detected in rodent, but not human, acinar cells.[23]

In addition to normal tissues, a vast majority of human tumors, either benign or malignant, express somatostatin receptors in high concentrations, frequently featuring more than one isotype.[6,29] In particular, somatostatin receptors are present in almost all neuroendocrine enteropancreatic tumors. The expression patterns seem to be tumor specific, with a general profile of $sst_2 > sst_1 > sst_3 > sst_4 > sst_5$. For instance, sst_2 predominance is found in about 90% of carcinoid tumors and 80% of endocrine pancreatic tumors.[20]

Cortistatin Receptors

It has been suggested that human MrgX2 and the GH secretagog receptor 1a (GHS-R1a) might function as cortistatin receptors. However, there is no convincing evidence supporting this view. First, cortistatin has up to 1000-fold higher affinities for the classical somatostatin receptors than it has for MrgX2 or GHS-R1a. Moreover, there is a major discrepancy between receptors and peptide distribution; with MrgX2 (actually the complete Mrgx family) being absent in rodents, where cortistatin was discovered. Therefore, as of today, cortistatin and somatostatin share the same receptors.[28] Functional differences reported to distinguish cortistatin and somatostatin may relate to ligand-driven transductional selectivity and/or other factors such as receptor dimerization and/or the influence of accessory proteins.

BIOLOGICAL ACTIONS WITHIN THE GASTROINTESTINAL TRACT

Somatostatin, acting in a paracrine fashion, as a classical hormone or as a neurotransmitter, mediates a broad range of physiological actions, mostly inhibitory, in the gastrointestinal tract (Table 2). Biological actions of somatostatin-related peptides are not yet fully investigated.

TABLE 2 Receptor Selectivity of Somatostatin Actions in the Gastrointestinal Tract

Somatostatin action	Receptor subtype	Localization
Inhibition of acid secretion	sst_2	Parietal cells
Inhibition of histamine release	sst_2	Enterochromaffin-like cells
Inhibition of gastric emptying	sst_2/other undetermined	Myenteric plexus
Modulation of intestinal motility	sst_2	Interstitial cells of Cajal
Modulation of the peristaltic reflex	sst_2/other undetermined	Myenteric plexus
Modulation of epithelial function and blood flow	sst_2/other undetermined	Submucous plexus
Stimulation of ion transport	sst_2	Colonic epithelial cells
Inhibition of chloride secretion	sst_2	Colonic epithelial cells
Inhibition of pancreatic amylase release	sst_5	Pancreatic acinar cells
Inhibition of insulin secretion	sst_5/sst_1 (sst_2 in humans)	Pancreatic beta cells
Inhibition of glucagon secretion	sst_2	Pancreatic alpha cells
Inhibition of cell proliferation	sst_1/sst_2/sst_3/sst_4/sst_5	Tumor cells

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Somatostatin Actions in the Stomach

Gastric acid secretion reflects a complex balance between stimulatory and inhibitory factors. Somatostatin is a potent inhibitor of gastric acid secretion and probably the main inhibitory regulator during the cephalic, gastric, and intestinal phases of secretion.

The somatostatin-dependent regulation of gastric acid secretion involves gastric somatostatin produced by fundic antral endocrine D cells, acting locally through paracrine, rather than endocrine, mechanisms. Fundic D cells are stimulated by gastrin and acetylcholine, as well as by other neuropeptides; they are closely associated with parietal cells and ECL cells through cytoplasmic extensions. Antral D cells respond to changes in gastric luminal acidity and are closely associated with their target cells, that is, G cells, resulting in the local release of somatostatin, which in turn, inhibits gastrin synthesis and release from G cells. During the cephalic and gastric phases of a meal, gastric acid secretion is stimulated through the release of gastrin. Attenuation of excessive acid secretion can be attributed to the local release of somatostatin, regulating (inhibiting) the activity of G cells, parietal cells and ECL cells. In addition, *in vivo* and *in vitro* observations suggest that somatostatin also controls basal gastric acid secretion during the interdigestive phases, acting as a tonic restraint on acid secretion.[3,18,20]

Several *in vivo* and *in vitro* studies in rats, mice, dogs, and humans using different relatively selective agonists and antagonists of somatostatin receptors established that sst_2 mediates the inhibitory effects of somatostatin on gastric acid secretion.[18–22] The most conclusive evidence for the involvement of sst_2 receptors in the control of gastric acid secretion comes from functional studies in mice with targeted disruption of the sst_{2A} receptor gene (sst_2–/–).[18–22,34] Because the sst_{2B} variant is a spliced product of the sst_{2A}, these animals lack both forms of the receptor; therefore, none of the splice variants is expressed, and no information about the isoform mediating somatostatin action on acid secretion can be derived.

Several gut regulatory peptides [i.e., amylin, adrenomedullin, CGRP, bombesin, glucose-dependent insulinotropic polypeptide, GLP-1, and pituitary adenylate cyclase-activating polypeptide (PACAP)] inhibit gastric acid secretion through the release of somatostatin or have somatostatin-dependent mechanisms of action. Moreover, receptors for several of these neuropeptides have been localized in gastric D cells. These observations suggest that gastric D cells function as an integrator of inhibitory signals controlling gastric acid secretion. Activation of D cells translates into the release of somatostatin, the activation of sst_2 receptors in parietal and ECL cells and the subsequent inhibition of acid output (Fig. 1).[18,20–22,35]

Against that expected, sst_2–/– mice have normal levels of circulating gastrin and unchanged basal acid output,[19,35] thus suggesting the existence of alternate compensatory mechanisms that can replace the integrative role of D cells and somatostatin in the inhibitory control of acid secretion. Indeed, it has been shown that the genetic ablation of the paracrine somatostatin/sst_2-dependent inhibitory control of acid secretion in sst_2–/– mice leads to (i) a shift toward a galanin-neuroendocrine-dependent control of ECL activity and (ii) an enhancement of the direct action of gastrin and vagal cholinergic pathways on parietal cells.[35] Overall, this plasticity of the gastric neuroendocrine control is able to maintain similar basal acid secretion and postprandial stimulated secretion in sst_2–/– and wild-type mice.

As it relates to gastric motor functions, somatostatin inhibits the normal reflex response to volume increase, slows down the emptying of a meal, and decreases the perception of fullness.[5,11,31] The receptors mediating these effects are not fully identified although the expression of sst_2 receptors in the enteric nervous system points toward this receptor subtype. Nonetheless, gastric emptying was normal in sst_2–/– mice.

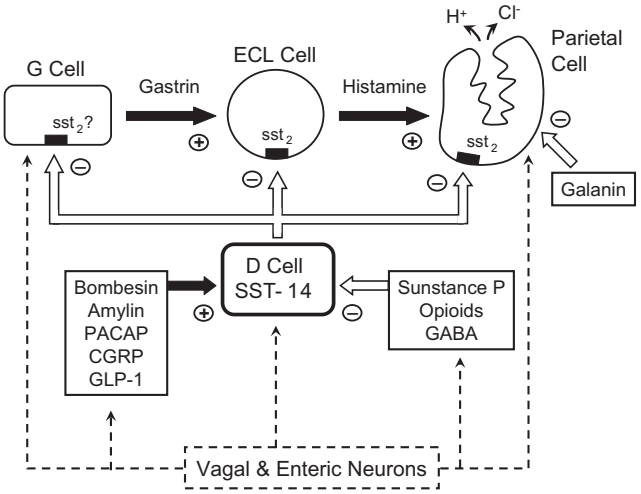

FIGURE 1 Basic mechanisms controlling gastric acid secretion. Somatostatin secreted from gastric D cells modulates the gastrin-enterochromaffin-like (ECL) cell–parietal cell axis. Gastrin, secreted from G cells, stimulates the release of histamine from ECL cells, which is the major stimulant of acid from gastric parietal cells. Somatostatin, acting through sst_2, inhibits secretion from each of these cell types, although the predominant actions seem to be on ECL and parietal cells. The activity of these cells is also modulated by neural signals from the vagus and the enteric nervous system. In fact, D cells might function as a common integrator center for the action of neuropeptides that either stimulate or inhibit somatostatin release and, through this mechanism, inhibit or stimulate gastric acid secretion, respectively. Some neuropeptides, such as galanin, might function as alternative somatostin-independent pathways controlling acid production and might be upregulated when the somatostin-sst_2-dependent control is altered, as during the targeted disruption of the sst_2 in mice. The pathways illustrated are not all-inclusive but represent many of the key regulatory steps.

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Somatostatin Actions in the Small Intestine

Within the small intestine, somatostatin acts as an important neurotransmitter, acting through somatostatin receptors located in the submucosal and myenteric nervous plexus of the enteric nervous system. The physiological implications of these actions are complex because of the broad spectrum of functions modulated by the enteric nervous system (i.e., motility, exocrine and endocrine secretions, absorption of nutrients, microcirculation, or the local immune system). In the small intestine, neuronal somatostatin is released locally by intestinal distension, and it participates, as a neuromodulator, in the coordination of the peristaltic reflex. Pharmacological studies and the use of sst_2−/− mice support the notion that sst_2 in enteric ganglia is involved in the regulation of peristalsis; however the implication of other receptor subtypes cannot be ruled out.[1,5,31,34] The presence of sst_2 receptors in interstitial cells of Cajal further supports a role for somatostatin modulating intestinal motility.

Expression of sst_2 receptors in submucosal ganglia suggests an involvement in the regulation of mucosal function and blood flow. Indeed, somatostatin decreases intestinal fluid secretion, stimulates the absorption of water and electrolytes, and reduces collateral splanchnic blood flow up to 30%, without causing adverse systemic effects. Comparable effects can also be detected in humans on the application of somatostatin analogs such as octreotide.

Somatostatin Actions in the Colon

In humans, somatostatin has been reported to modulate colonic motor and sensory functions. As in the small intestine, somatostatin participates in colonic peristaltic activity, decreases postprandial colonic tone, increases the threshold for visceral sensory perception, prolongs transit, and modulates epithelial function. Motor and sensory effects of somatostatin are probably mediated through sst_1 and sst_2 receptors, with sst_1 located in the longitudinal colonic smooth muscle and sst_2 in the circular.[5]

Somatostatin Actions in the Pancreas

Somatostatin affects both endocrine and exocrine pancreatic secretion, acting through paracrine and endocrine mechanisms. Although the pancreas contains mRNA for all somatostatin receptor subtypes, pharmacological studies in normal and sst_2−/− mice have shown that pancreatic actions of somatostatin are mediated through sst_1, sst_2, and sst_5 receptors.[20,32–34]

In mice, intraislet somatostatin, acting locally through sst_1 and sst_5 receptors, exerts a tonic inhibitory effect on insulin secretion. Similar effects, although exerted through sst_2 receptors, are observed on glucagon-secretory alpha cells. In this sense, somatostatin not only exerts a tonic inhibitory effect on stimulus-induced glucagon secretion but also regulates the suppression of glucagon secretion by glucose. Conversely, a recent study with human pancreatic islets indicated that sst_2 mainly mediates the somatostatin inhibition of insulin release, suggesting the existence of potential species differences.

In the rat, somatostatin acts directly on acinar cells to inhibit pancreatic enzyme and bicarbonate secretion. Pharmacological studies identified sst_5 as the main receptor subtype inhibiting pancreatic amylase release in rats. In humans, circulating somatostatin-14 and somatostatin-28 are equally potent inhibiting basal and CCK-stimulated exocrine enzyme secretion. However, no somatostatin receptors have been identified in the human exocrine pancreas so far.

PATHOPHYSIOLOGICAL IMPLICATIONS

Disturbances of the somatostatin system have been observed in pathologic human gastrointestinal tissues, both neoplastic and nonneoplastic. They include disturbances of somatostatin production and of somatostatin receptor expression. There have been no reported cases of heritable genetic syndromes caused by reduced or absent expression of either somatostatin or any of its receptors. A number of polymorphisms have been identified in the somatostatin 1, sst_2, and sst_5 genes, but no linkage has been found between particular alleles and specific disease states.

A clear pathophysiological role of somatostatin has been shown only in cases with somatostatin-producing tumors (somatostatinomas). Somatostatinomas are rare neuroendocrine tumors that arise typically from transformed D cells in the pancreatic islets or duodenum. The clinical syndrome of somatostatin hypersecretion is characterized by malabsorption, steatorrhea, cholelithiasis, diabetes, and pancreatic insufficiency.

Alterations in the responsiveness and number of gastric D cells might be related to the hypersecretory states observed in patients with nonatrophic gastritis associated with *Helicobacter pylori* infection and in patients with duodenal or gastric ulcers. Because of its medical interest, the relationship between chronic *H. pylori* infection and reduced gastric somatostatin has been actively investigated.[5] *H. pylori* infection induces a gastric inflammation with release of several cytokines that induce a decrease in both D cell mass and somatostatin secretion, leading to elevated gastrin levels and increased gastric acid output. The eradication of *H. pylori* infection is followed by structural and functional restoration of endocrine D cells. Animal models of human *H. pylori* infection suggest that somatostatin may play a role in the disease not only by interacting with acid regulation but also by influencing the inflammatory status.

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Somatostatin Receptor Expression in Neoplastic Disease

Of much greater importance and clinical impact than the production of somatostatin is the expression of somatostatin receptors in neoplasms. A very high incidence, and often a high density, of somatostatin receptors have been found in the gastroenteropancreatic endocrine tumors with receptor autoradiography and by *in situ* hybridization methods. They include endocrine neoplasms of the pancreas and the intestinal tract that express predominantly sst_2, with or without other subtypes.[6,29]

Somatostatin Analogs and their Clinical Applications in Gastrointestinal Disease

The natural somatostatins are very potent molecules, produced locally and degraded very rapidly, thus limiting their clinical use. Therefore, extensive pharmaceutical discovery programs have been ongoing to synthesize somatostatin analogs with receptor subtype selectivity (or pan-activity, as the natural ligands), improved pharmacokinetics and oral bioavailability. As a result, stable somatostatin agonist analogs, such as octreotide, have been developed not only for diagnostic and therapeutic applications but also to elucidate the physiological role and mechanism of actions of the native hormones. In addition, more recently, nonpeptidergic, receptor subtype selective, agonists, and antagonists have been synthesized (Table 1).

Diagnostic Applications with Radioactively Labeled Somatostatin

Somatostatin receptors were the first peptide receptors to exemplify the principle of *in vivo* targeting of human cancers expressing a high receptor density. Radioactively labeled synthetic somatostatin analogs are now used diagnostically for *in vivo* scintigraphy of primary neoplasms overexpressing somatostatin receptors and their metastases, in particular gastroenteropancreatic cancers.[6]

Therapeutic Applications

Targeting of the overexpressed somatostatin receptors by synthetic somatostatin analogs in diseased tissues can be used therapeutically in several ways: (i) as radioactive and nonradioactive cytotoxic analogs for tumor radiotherapy, and (ii) as nonradioactive noncytotoxic analogs for symptomatic tumor therapy or therapy of nonneoplastic diseases.

i. *Tumor therapy with somatostatin analogs*—Because sst_2 seems to be the main receptor subtype in many human tumors, several somatostatin analogs with a particularly high sst_2 affinity have been developed for tumor therapy.[6] Some potent chemotherapeutic agents, such as camptothecin, methotrexate, paclitaxel, and doxorubicin, have been coupled to sst_2-preferential somatostatin analogs to generate selective cytotoxic agents.[26] In these conjugates, the ability of the peptide portion to bind the receptor specifically is preserved and the cytotoxicity of the attached radical is retained, thus displaying significant selective antitumor abilities in many different types of tumors. The clinical efficacy of these compounds has not been assessed. (see the Somatostatin chapter in the Cancer/Anticancer Peptides section of this book).

ii. *Nonradioactive noncytotoxic somatostatin analogs*—Stable somatostatin analogs can be used for the symptomatic treatment of a variety of neoplastic and nonneoplastic diseases of the gastrointestinal tract. Somatostatin and its stable analog octreotide have been consistently used in the treatment of esophageal variceal bleeding, pathological states of the exocrine pancreas, and neuroendocrine enteropancreatic tumors.

Long-acting somatostatin analogs can be administered to treat symptoms caused by the oversecretion of hormones from gastroenteropancreatic endocrine tumors. Octreotide can, for example, alleviate hypokalemia, dehydration, and diarrhea in patients with vasoactive intestinal polypeptide-secreting tumors (VIPomas) and can reduce peptic ulcers and necrolytic skin lesions in patients with gastrin- or glucagon-producing tumors, respectively. Although somatostatin seems to have an inhibitory effect on cell growth and proliferation, a true antiproliferative effect of somatostatin analogs in these tumors is, however, modest.

Somatostatin and octreotide reduce intestinal secretion and have, therefore, been used to treat diarrhea. Somatostatin was originally shown to be effective in treating diarrhea caused by VIPomas. It has since been recognized that somatostatin analogs are also effective in treating other diarrheal conditions. Octreotide improves secretory diarrhea, prolonging gastrointestinal transit time, decreasing endogenous fluid secretion, and stimulating the absorption of water and electrolytes. Moreover, octreotide has been shown to improve diarrhea associated with irritable bowel syndrome by slowing intestinal transit time and to increase the rectal perception threshold for discomfort, an effects probably mediated via the inhibition of exaggerated release of serotonin from enteroendocrine cells.

ACKNOWLEDGMENTS

The author is supported by grants BFU2009-08229 from the Ministerio de Ciencia e Innvovacion and 2009SGR708 from the Generalitat de Catalunya.

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REFERENCES

1. Abdu F, Hicks GA, Hennig G, Allen JP, Grundy D. Somatostatin sst(2) receptors inhibit peristalsis in the rat and mouse jejunum. *Am J Physiol Gastrointest Liver Physiol* 2002;**282**:G624–33.
2. Brazeau P, Vale W, Burgus R, Ling N, Butcher M, Rivier J, et al. Hypothalamic polypeptide that inhibits the secretion of immunoreactive pituitary growth hormone. *Science* 1973;**179**:77–9.
3. Chiba T, Yamada T. Gut somatostatin. In: Walsh JH, Dockray GJ, editors. *Gut peptides*. New York: Raven Press; 1994. p. 123–45.
4. Córdoba-Chacón J, Gahete MD, Durán-Prado M, Luque RM, Castaño JP. Truncated somatostatin receptors as new players in somatostatin–cortistatin pathophysiology. *Ann N Y Acad Sci* 2011; **1220**:6–15.
5. Corleto VD. Somatostatin and the gastrointestinal tract. *Curr Opin Endocrinol Diabetes Obes* 2010;**17**:63–8.
6. Culler MD, Oberg K, Arnold R, Krenning EP, Sevilla I, Díaz JA. Somatostatin analogs for the treatment of neuroendocrine tumors. *Cancer Metastasis Rev* 2011;**30**(Suppl. 1):9–17.
7. Dalm VA, Van Hagen PM, de Krijger RR, Kros JM, Van Koetsveld PM, Van Der Lely AJ, et al. Distribution pattern of somatostatin and cortistatin mRNA in human central and peripheral tissues. *Clin Endocrinol (Oxf)* 2004;**60**. 625–9. Erratum in: *Clin Endocrinol (Oxf)* 2005;**62**:387.
8. de Lecea L, Criado JR, Prospero-Garcia O, Gautvik KM, Schweitzer P, Danielson PE, et al. A cortical neuropeptide with neuronal depressant and sleep-modulating properties. *Nature* 1996;**381**:242–5.
9. Durán-Prado M, Malagón MM, Gracia-Navarro F, Castaño JP. Dimerization of G protein-coupled receptors: new avenues for somatostatin receptor signalling, control and functioning. *Mol Cell Endocrinol* 2008;**286**:63–8.
10. Ensinck JW, Baskin DG, Vahl TP, Vogel RE, Laschansky EC, Francis BH, et al. Thrittene, homologous with somatostatin-28((1-13)), is a novel peptide in mammalian gut and circulation. *Endocrinology* 2002;**143**:2599–609.
11. Foxx-Orenstein A, Camilleri M, Stephens D, Burton D. Effect of a somatostatin analogue on gastric motor and sensory functions in healthy humans. *Gut* 2003;**52**:1555–61.
12. Gahete MD, Cordoba-Chacón J, Duran-Prado M, Malagón MM, Martinez-Fuentes AJ, Gracia-Navarro F, et al. Somatostatin and its receptors from fish to mammals. *Ann N Y Acad Sci* 2010;**1200**:43–52.
13. Goodman RH, Jacobs JW, Chin WW, Lund PK, Dee PC, Habener JF. Nucleotide sequence of a cloned structural gene coding for a precursor of pancreatic somatostatin. *Proc Natl Acad Sci U S A* 1980;**77**:5869–73.
14. Hobart P, Crawford R, Shen L, Pictet R, Rutter WJ. Cloning and sequence analysis of cDNAs encoding two distinct somatostatin precursors found in the endocrine pancreas of anglerfish. *Nature* 1980;**288**:137–41.
15. Hoyer D, Epelbaum J, Feniuk W, Humphrey PP, Meyerhof W, Patel Y, et al. Somatostatin receptors. In: Girdleston D, editor. *The IUPHAR compendium of receptor characterization and classification*. Royston: Nightingale Press; 2000. p. 354–64.
16. Lahlou H, Guillermet J, Hortala M, Vernejoul F, Pyronnet S, Bousquet C, et al. Molecular signaling of somatostatin receptors. *Ann N Y Acad Sci* 2004;**1014**:121–31.
17. Low MJ. The somatostatin neuroendocrine system: physiology and clinical relevance in gastrointestinal and pancreatic disorders. *Best Pract Res Clin Endocrinol Metab* 2004;**18**:607–22.
18. Martinez V. Somatostatin receptors and the control of gastric acid secretion. In: Taché Y, Goto Y, Ohning G, Yamada T, editors. *Gut–brain peptides in the new millennium*. Los Angeles: CURE Foundation; 2002. p. 167–78.
19. Martinez V, Curi AP, Torkian B, Schaeffer JM, Wilkinson HA, Walsh JH, et al. High basal gastric acid secretion in somatostatin receptor subtype 2 knockout mice. *Gastroenterology* 1998;**114**: 1125–32.
20. Martinez V, Taché Y. Somatostatin. In: Johnson LR, editor. *Encyclopedia of gastroenterology*. New York: Elsevier; 2004. p. 426–33.
21. Piqueras L, Martínez V. Role of somatostatin receptors on gastric acid secretion in wild-type and somatostatin receptor type 2 knockout mice. *Naunyn Schmiedebergs Arch Pharmacol* 2004;**370**: 510–20.
22. Piqueras L, Taché Y, Martínez V. Somatostatin receptor type 2 mediates bombesin-induced inhibition of gastric acid secretion in mice. *J Physiol (London)* 2003;**549**:889–901.
23. Portela-Gomes GM, Stridsberg M, Grimelius L, Oberg K, Janson ET. Expression of the five different somatostatin receptor subtypes in endocrine cells of the pancreas. *Appl Immunohistochem Mol Morphol* 2000;**8**:126–32.
24. Pradayrol L, Jörnvall H, Mutt V, Ribet A. N-terminally extended somatostatin: the primary structure of somatostatin-28. *FEBS Lett* 1980;**109**:55–8.
25. Samson WK, Zhang JV, Avsian-Kretchmer O, Cui K, Yosten GL, Klein C, et al. Neuronostatin encoded by the somatostatin gene regulates neuronal, cardiovascular, and metabolic functions. *J Biol Chem* 2008;**283**:31949–59.
26. Schally AV, Engel JB, Emons G, Block NL, Pinski J. Use of analogs of peptide hormones conjugated to cytotoxic radicals for chemotherapy targeted to receptors on tumors. *Curr Drug Deliv* 2011;**8**:11–25.
27. Schonbrunn A. Selective agonism in somatostatin receptor signaling and regulation. *Mol Cell Endocrinol* 2008;**286**:35–9.
28. Siehler S, Nunn C, Hannon J, Feuerbach D, Hoyer D. Pharmacological profile of somatostatin and cortistatin receptors. *Mol Cell Endocrinol* 2008;**286**:26–34.
29. Sun LC, Coy DH. Somatostatin receptor-targeted anti-cancer therapy. *Curr Drug Deliv* 2011;**8**:2–10.
30. Tostivint H, Lihrmann I, Vaudry H. New insight into the molecular evolution of the somatostatin family. *Mol Cell Endocrinol* 2008;**286**:5–17.
31. Van Op den Bosch J, Adriaensen D, Van Nassauw L, Timmermans JP. The role(s) of somatostatin, structurally related peptides and somatostatin receptors in the gastrointestinal tract: a review. *Regul Pept* 2009;**156**:1–8.
32. Wang XP, Norman M, Yang J, Magnusson J, Kreienkamp HJ, Richter D, et al. Alterations in glucose homeostasis in SSTR1 gene-ablated mice. *Mol Cell Endocrinol* 2006;**247**:82–90.
33. Wang XP, Yang J, Norman MA, Magnusson J, DeMayo FJ, Brunicardi FC. SSTR5 ablation in islet results in alterations in glucose homeostasis in mice. *FEBS Lett* 2005;**579**:3107–14.
34. Zeyda T, Hochgeschwender U. Null mutant mouse models of somatostatin and cortistatin, and their receptors. *Mol Cell Endocrinol* 2008;**286**:18–25.
35. Zhao CM, Martinez V, Piqueras L, Wang L, Taché Y, Chen D. Control of gastric acid secretion in somatostatin receptor 2 deficient mice: shift from endocrine/paracrine to neurocrine pathways. *Endocrinology* 2008;**149**:498–505.

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Chapter 181

Tachykinins

Peter Holzer

ABSTRACT

Within the gastrointestinal tract, the tachykinins substance P and neurokinin A occur in neurons of the enteric nervous system, in fibers of extrinsic afferent neurons, and in enterochromaffin and immune cells of the mucosa. Their biological actions are mediated by three different types of G protein-coupled receptors, termed NK1, NK2, and NK3 and are involved in the regulation of gastrointestinal motility, secretion, vascular permeability, and pain sensitivity. Pathophysiologically, tachykinins seem to be involved in inflammation-induced disturbances of gut function such as dysmotility, secretory diarrhea, inflammation, and hyperalgesia. Expressed in a cell-specific manner, tachykinin receptors thus represent an attractive target for novel therapeutic strategies in gastroenterology. Indeed, NK1 receptor antagonists have proved beneficial in vagus nerve-mediated nausea and vomiting, and a combined NK1, NK2, and NK3 receptor antagonist shows promise in the treatment of irritable bowel syndrome.

DISCOVERY OF TACHYKININS IN THE GUT

The biological activity of substance P (SP) was discovered in 1931 when Ulf S. von Euler and John H. Gaddum reported that both the intestine and the brain contained a substance that caused contraction of gastrointestinal (GI) smooth muscle, the substance being different from any of the endogenous compounds known at that time. In the early 1950s, Bengt Pernow realized the wide distribution of SP in the GI tract, and in the 1960s, Fred Lembeck and others found that SP causes copious salivary secretion in rats and dogs. This biological effect was the lead bioassay whereby Susan E. Leeman and the workers in her laboratory identified SP as an undekapeptide in 1970. SP turned out to be the mammalian counterpart of a family of amphibian and nonvertebrate peptides named tachykinins by Vittorio Erspamer, because they caused fast contraction of GI smooth muscle (see the chapters on tachykinins in the Invertebrate, Amphibian/Skin, and Brain Peptides sections of this book). This breakthrough was followed by the discovery of another tachykinin, neurokinin A (NKA), in the mammalian gut, the identification of three tachykinin receptors labeled as NK1, NK2, and NK3, and the development of highly selective nonpeptide antagonists for all three tachykinin receptors. As a result, the distribution, biological actions, and pathophysiological implications of tachykinins in the mammalian gut have been thoroughly investigated, and tachykinin receptor antagonists have been explored for their utility in the treatment of inflammatory bowel disease (IBD), irritable bowel syndrome (IBS) and other GI disorders.[8,9,10,16,31] NK1 receptor antagonists have proved to be effective in preventing nausea and emesis caused by GI and other emetic stimuli.[6]

DISTRIBUTION OF TACHYKININ mRNAs AND PEPTIDES IN THE GI TRACT

The structures of the 3 known tachykinin genes (TAC1, TAC3, TAC4) and their protein products are discussed in the chapter of N.M. Page in the Brain Peptide section. SP and NKA, the major tachykinins in the GI tract, are derived from the TAC1 gene (previously called PPT-A or PPT-I for preprotachykinin A or I, respectively). The primary RNA transcript of TAC1 is alternatively spliced to produce 4 different types of mRNA (α-TAC1, β-TAC1, γ-TAC1, δ-TAC1), which all encode SP, whereas β-TAC1 and γ-TAC1 also encode NKA and some N-terminally extended forms of NKA such as neuropeptide K (derived from β-TAC1) and neuropeptide γ (derived from γ-TAC1).[27] The TAC3 (PPT-B or PPT-II) gene can be transcribed to 3 mRNA isoforms (α-TAC3, β-TAC3, γ-TAC3), of which only the α-TAC3 and β-TAC3 mRNA are translated into NKB.[8,16,27] The tachykinins and tachykinin gene-related peptides encoded by TAC4 vary considerably among mammalian species.[27] The human TAC4 (PPT-C) gene can be transcribed to 4 mRNA forms to yield endokinin A (α-TAC4v1), endokinin B (α-TAC4v2, β-TAC4, γ-TAC4, δ-TAC4), endokinin C (α-TAC4v1, α-TAC4v2), or endokinin D (β-TAC4), while the mouse and rat TAC4 gene encodes hemokinin 1.[27]

In the human colon, the expression of TAC1 mRNA is particularly abundant in the muscle layer, whereas the expression of TAC3 mRNA is low or negligible.[18] Because

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00181-0
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TAC1 mRNA prevails in the gut, SP and NKA are the predominant tachykinins in this organ system, whereas NKB has proved difficult to be detected in the bowel.[8] Expression of TAC4 has been shown to occur in the human stomach, small intestine, and colon.[18,27] The levels of TAC4 mRNA are similar in the muscle and mucosa layers of the human colon,[18] but the precise cellular distribution of TAC4 gene products in the GI tract has not yet been mapped in detail.

Although some SP is contained in enterochromaffin and immune cells of the GI mucosa, the major source of tachykinins in the gut is the enteric nervous system (ENS).[8,9,31] This predominantly neural expression of SP and NKA is conserved across mammalian species, yet there are both regional and species differences in the quantitative distribution of tachykinins to different classes of enteric neurons.[8,9,25,31] In the guinea pig intestine, SP is typically present in intrinsic primary afferent neurons (IPANs) of the myenteric and submucosal plexus and, within the myenteric plexus, in ascending interneurons, as well as in excitatory motoneurons to the longitudinal and circular muscle (Fig. 1). Most enteric neurons containing SP are cholinergic as they coexpress choline acetyltransferase. The tachykininergic enteric neurons can be further subgrouped by their content of calbindin/calretinin, neurofilament protein triplet, and dynorphin/enkephalin-like immunoreactivity (Fig. 1). Primary afferent neurons that originate from dorsal root ganglia and reach the gut via sympathetic and sacral parasympathetic nerves also contribute to the tachykinin content of the gut. The peripheral fibers of these extrinsic sensory neurons project primarily to submucosal arterioles and also supply the mucosa and enteric nerve plexuses (Fig. 1).

TACHYKININ RECEPTOR SUBTYPES, SIGNALING, AND DISTRIBUTION IN THE GI TRACT

The biological actions of tachykinins in the GI tract are mediated by 3 tachykinin receptors,[8,16,31] termed NK1, NK2, and NK3, and encoded by 3 different genes, TACR1, TACR2, and TACR3. Although SP, NKA, and NKB are full agonists at all 3 tachykinin receptors, the potency order of tachykinins is SP > NKA > NKB for NK1 receptors, NKA > NKB > SP for NK2 receptors, and NKB > NKA > SP for NK3 receptors.[31] Rat and mouse hemokinin 1 is a preferential NK1 receptor agonist.[27] As outlined in the chapter by N.M. Page, the membrane topology of tachykinin receptors is typical of metabotropic receptors with seven transmembrane domains. Being associated with the pertussis toxin-insensitive G proteins Gq/G11, tachykinin receptors use the phospholipase C/inositol trisphosphate/diacylglycerol/protein kinase C system as major transduction pathway.[28,31] In addition, tachykinin receptors can couple to the G proteins Gs and Gi, resulting in adenylyl cyclase activation and inhibition, respectively.[28,31]

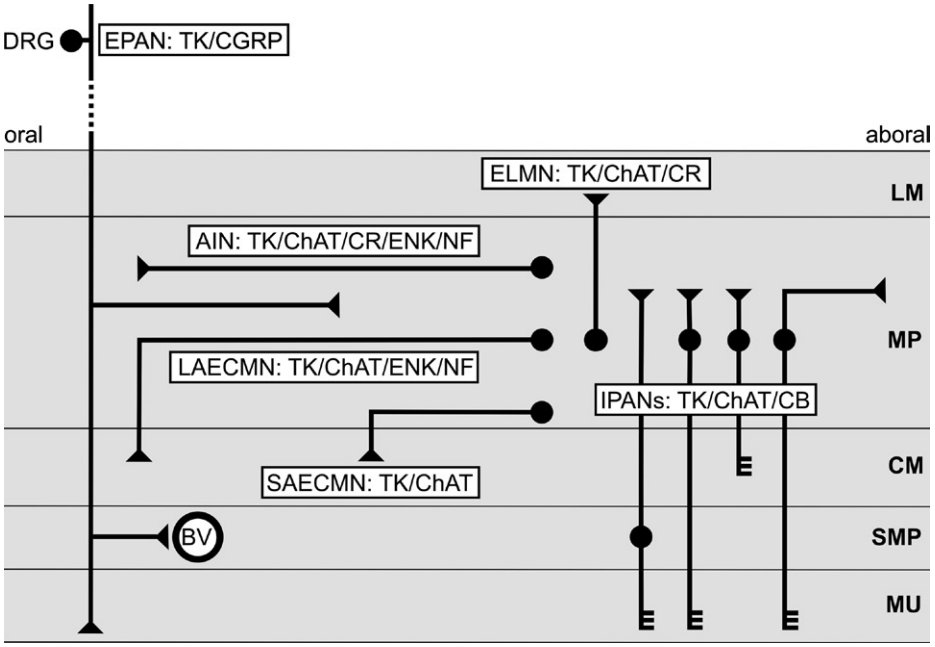

FIGURE 1 Chemical coding and projections of the most important classes of tachykinin (TK)-immunoreactive neurons in the mammalian gut. AIN, ascending interneuron; BV, blood vessel; CB, calbindin; CGRP, calcitonin gene-related peptide; ChAT, choline acetyltransferase; CM, circular muscle; CR, calretinin; DRG, dorsal root ganglion; ELMN, excitatory longitudinal muscle motoneuron; ENK, enkephalin; EPAN, extrinsic primary afferent neuron; IPANs, intrinsic primary afferent neurons; LAECMN, long ascending excitatory circular muscle motoneuron; LM, longitudinal muscle; MP, myenteric plexus; MU, mucosa; NF, neurofilament protein; SAECMN, short ascending excitatory circular muscle motoneuron; SMP, submucosal plexus.

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The pharmacology of tachykinins is one of the most advanced among neuropeptides. By modification of the amino acid sequence of endogenous tachykinins, selective agonists for all three tachykinin receptor types have been obtained, and this advance has been complemented by the design of potent and receptor-selective nonpeptide antagonists for NK1, NK2, and NK3 receptors.[8,31] It is important to note, however, that the potency of nonpeptide tachykinin receptor antagonists can greatly vary among different species, one typical example being that a number NK1 receptor antagonists with high affinity for the human NK1 receptor has only moderate affinity for the rat NK1 receptor.[31]

The distribution of tachykinin NK1, NK2, and NK3 receptors to GI neurons and effector cells (Fig. 2) enables tachykinins to modify GI motility, secretory activity, vascular diameter, vascular permeability, immune function, gut sensitivity, and nociception.[8,9,10,22] Although all 3 receptor types are expressed in the human colon, mRNA for TACR2-α, the gene encoding the functional NK2 receptor, is more abundant than TACR1 and TACR3 mRNA.[12,18] NK1 receptors have been localized to the muscularis mucose, the media of submucosal blood vessels (Fig. 2), and some immune cells of the human GI tract.[8] In the rodent gut, NK1 receptors occur on longitudinal and circular muscle cells, interstitial cells of Cajal (ICCs), IPANs, excitatory and inhibitory motoneurons, secretomotor neurons, epithelial cells, and granulocytes.[8,31] NK2 receptors are typically expressed by the longitudinal muscle, circular muscle, and muscularis mucose of the rodent GI tract and, in addition, are present on epithelial cells and enteric nerve endings (Fig. 2). NK2 receptors also abound in the smooth muscle of the human intestine[16] but in addition are expressed by a majority of myenteric neurons in the human colon, these neurons sending varicose fibers to the longitudinal and circular muscle.[13] NK3 receptors are largely confined to enteric neurons; in the rodent intestine, these NK3 receptor-bearing neurons comprise IPANs, ascending and descending interneurons, excitatory and inhibitory motoneurons, as well as secretomotor neurons (Fig. 2).

BIOLOGICAL ACTIONS OF TACHYKININS IN THE GUT

Transmitter Function in the Enteric Nervous System

SP and NKA are cotransmitters of intrinsic enteric and extrinsic afferent neurons, which is consistent with their vesicular localization and calcium-dependent release upon nerve stimulation.[8,9,10,16,22,31] Within the ENS, tachykinins mediate slow postsynaptic excitation, a process in which both NK1 and NK3 receptors are involved. Slow postsynaptic excitation contributes to the communication among IPANs (mediated primarily by NK3 receptors), between IPANs and ascending as well as descending interneurons (mediated by NK3 receptors), between ascending interneurons and excitatory motoneurons (mediated by NK3 receptors), as well as between IPANs and inhibitory motoneurons

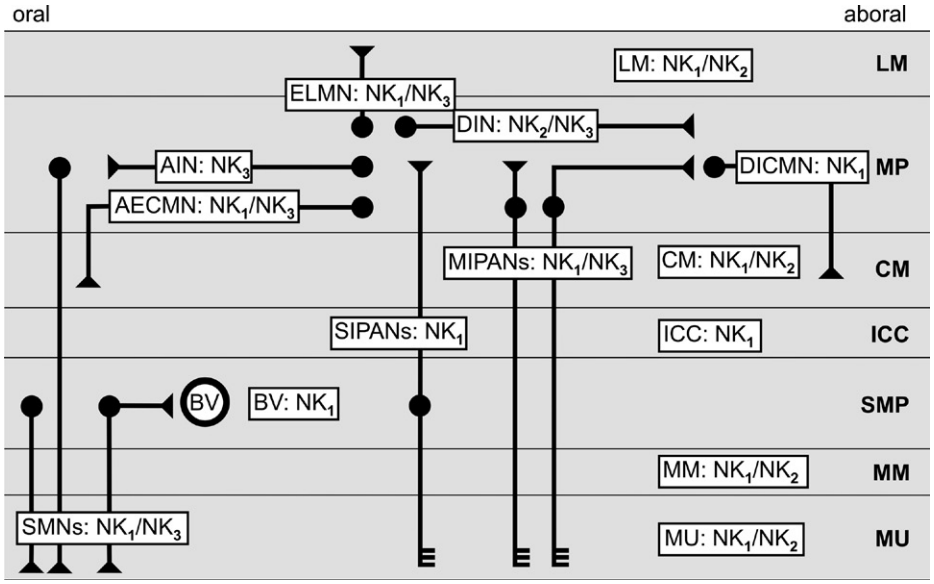

FIGURE 2 Cellular expression of tachykinin NK1, NK2, and NK3 receptors in the mammalian gut. Filled circles depict neuronal somata, filled triangles nerve endings. AECMN, ascending excitatory circular muscle motoneuron; AIN, ascending interneuron; BV, blood vessel; CM, circular muscle; DICMN, descending inhibitory circular muscle motoneuron; DIN, descending interneuron; ELMN, excitatory longitudinal muscle motoneuron; ICC, interstitial cell of Cajal; LM, longitudinal muscle; MIPANs, myenteric intrinsic primary afferent neurons; MM, muscularis mucose; MP, myenteric plexus; MU, mucosa; SIPANs, submucosal intrinsic primary afferent neurons; SMNs, secretomotor neurons; SMP, submucosal plexus.

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(mediated by NK1 receptors).[8,9,31] The activation of after hyperpolarization-type myenteric neurons by electrical stimulation of the guinea pig small intestinal mucosa is primarily mediated by NK1 receptors.[5] Apart from neuro-neuronal transmission, tachykinins also participate in excitatory neuromuscular transmission (mediated by NK2 and to some extent NK1 receptors), in which they share this role with the principal transmitter acetylcholine.[8,22,31]

Motor Activity

Tachykinins can both stimulate and inhibit GI motility, the net response depending on the type and site of tachykinin receptors that are activated.[8,9,16,22,31] Facilitation of GI motor activity is typically brought about by the activation of NK1 receptors on IPANs, ICCs, and muscle cells, NK2 receptors on muscle cells, and NK3 receptors on IPANs, cholinergic interneurons, and cholinergic motoneurons (Fig. 2). The stimulation of NK1 receptors on ICCs enforces motility by prolonging the duration of the slow waves generated by these cells.[8] Tachykinin-induced muscle contraction in the human gut *in vitro* is prominently mediated by muscular NK2 receptors,[16] and in the circular muscle of the isolated human sigmoids, it seems as if tachykinins acting via NK2 receptors are the main excitatory neurotransmitters released by nerve stimulation.[16] NK2 receptors also make an important contribution to the effects of SP and NKA to stimulate motility in the human small intestine *in vivo* and to replace the regular pattern of interdigestive motor activity by a pattern of irregular activity.[16] The contractile response to NK3 receptor stimulation in the guinea pig intestine is predominantly mediated by cholinergic neurons.[8]

NK1 and NK3 receptors on inhibitory motor pathways within the ENS enable tachykinins to depress motor activity via release of nitric oxide and adenosine triphosphate.[8]

Despite the high pharmacological potency of tachykinins to modify GI motility, NK1, NK2, and NK3 tachykinin receptor antagonists have, overall, little effect on GI motor performance in the guinea pig and human gut under physiological conditions both *in vitro* and *in vivo*. NK1 receptor antagonists cause a minor stimulation of peristaltic motility in the guinea pig isolated small intestine.[8] This activity profile is consistent with the failure of the NK1 receptor antagonist aprepitant to change gastric retention, gastric emptying, as well as small and large intestinal transit in humans,[21] although cases of mild diarrhea have been reported.[8] Likewise, NK2 receptor antagonists do not alter small intestinal motility in humans and in the long-term fail to modify bowel habits,[16] but lead to a minute inhibition of peristalsis in the guinea pig small bowel.[8] Only when the overwhelming cholinergic component in the neural activation of smooth muscle has been compromised, does blockade of tachykinin receptors impair peristalsis in the guinea pig small intestine. SP and NKA thus function as a backup system in the cholinergic activation of GI muscle during small intestinal peristalsis, a role that in the guinea pig small intestine is brought about by NK1 and NK2 receptors but in the human intestine is primarily mediated by NK2 receptors.[8,16]

In the colon of the guinea pig and mouse, however, tachykinins seem to play a more prominent role in the regulation of peristalsis than in the small intestine. Thus, when all 3 tachykinin receptors are blocked simultaneously, peristalsis in the guinea pig distal colon is significantly depressed even without concomitant blockade of cholinergic transmission.[8] Similarly, propulsion of fecal pellets in the mouse isolated colon is inhibited by both NK1 and NK2 receptor antagonists in the absence of acetylcholine receptor antagonists.[2] From these findings it seems as if tachykinins regulate motility primarily in the colon. Furthermore, multi- or pan-tachykinin receptor antagonists would seem to be more efficacious than mono-receptor antagonists in modifying GI motor activity and, eventually, GI motor disorders.[8]

Secretory Activity

Tachykinins modify endocrine and exocrine secretory processes in the GI tract including the stomach and pancreas.[8,10] Electrolyte and fluid output in the rodent intestine can be stimulated through activation of tachykinin NK1 and NK3 receptors on cholinergic and noncholinergic secretomotor neurons in the submucosal plexus as well as NK1 and NK2 receptors on epithelial cells. Mucosal ion transport in the isolated human colon is enhanced by both NK1 and NK2 receptor activation and subsequent stimulation of enteric neurons.

The neurogenic actions of tachykinins to elicit electrolyte and fluid secretion in the intestine are consistent with a role of SP and NKA as transmitters of enteric secretory reflexes. The implication of tachykinins in these reflexes can be threefold.[8] First, tachykinins released from IPANs or interneurons can activate cholinergic and noncholinergic secretomotor neurons. Second, SP can be released from axon collaterals of IPANs close to the epithelial effector cells and elicit chloride secretion via a mechanism resembling an axon reflex. Third, tachykinins released from extrinsic sensory nerve endings in response to capsaicin, *Clostridium difficile* toxin A, or distention can stimulate enteric secretomotor neurons through activation of NK1 and NK3 receptors.[8]

Proinflammatory Function

Tachykinins, particularly SP, are vasoactive peptides that can induce dilation or constriction of blood vessels in the digestive tract, the type of action depending on the vascular bed and species under study.[8,10] The tachykinin-evoked

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vasodilation in the intestines of cat, dog, and guinea pig is mediated by NK1 receptors. Both SP and NKA enhance blood flow in the proximal small intestine of humans, probably through activation of NK1 receptors which have been localized to the media of submucosal blood vessels. Conversely, blood flow in the rat gastric mucosa is diminished by tachykinins via constriction of collecting venules, a mechanism which may depend on the release of proteases from mast cells.[8]

Another effect of substance P acting via endothelial NK1 receptors is to increase venular permeability in the intestine and, thereby, to facilitate the extravasation of plasma proteins and leukocytes.[8] In addition, tachykinins can influence the activity of various immune cells in the gut. Thus, NK1 and NK2 receptors have been localized to monocytes/macrophages, granulocytes, lymphoid cells, and eosinophils, and stimulation of tachykinin receptors can lead to recruitment and activation of granulocytes as well as mast cells in the GI tract.[8,10]

PATHOPHYSIOLOGICAL IMPLICATIONS OF TACHYKININS IN THE GI TRACT

Implications of Tachykinins in GI Motor Disturbances

In view of the motor effects of SP and NKA and their apparent involvement in the regulation of GI motor activity, particularly in the colon, there is accumulating evidence that pathological alterations of GI motility are related to a disturbed function of the tachykinin system.[8,9,16,31] Experimental support for this hypothesis comes from the findings that certain GI diseases are associated with (i) changes in the expression of tachykinins and/or tachykinin receptors, (ii) changes in the functionality of tachykinin receptors and, consequently, in the motor effects of tachykinins, and/or (iii) a beneficial effect of tachykinin receptor antagonists.[8] For instance, slow-transit constipation in some pediatric patients is accompanied by a decrease in the density of nerve fibers containing substance P in the colon.[15] In line with this finding, neuromuscular transmission to the circular muscle via NK2 receptors is impaired in pediatric patients with slow-transit constipation.[33] In adult patients with slow-transit constipation, the responsiveness of the colonic circular muscle to NK1 or NK2 receptor agonists is either enhanced or decreased,[19,23] whereas in ulcerative colitis, Crohn's disease, and diverticular disease the contractile response of the colonic circular muscle to an NK2 receptor agonist is decreased.[23] Similarly, the responsiveness of the colonic musculature to tachykinin receptor agonists is depressed in rats and rabbits with trinitrobenzene sulfonic acid (TNBSA)-induced colitis, whereas in ricin-evoked ileitis of the rabbit tachykinin-mediated neurogenic contractions are amplified.[8]

Tachykinin receptor antagonists are able to correct various types of experimentally disturbed motility in the gut.[8,9,16,31] Thus, NK1 receptor antagonists inhibit stress-induced defecation and correct the hypomotility and muscular hyporesponsiveness caused by anaphylaxis, inflammation, and pain. Postoperative and peritonitis-induced ileus is ameliorated by NK1 and NK2 receptor antagonists, while the giant colonic contractions associated with inflammatory diarrhea are effectively suppressed by NK2 receptor antagonists.[8] Although the usefulness of NK3 receptor antagonists remains to be explored, it seems as if NK1 receptor antagonists are particularly efficacious in alleviating GI motor inhibition. NK2 receptor antagonists seem to be beneficial in attenuating pathological hypermotility without causing constipation.[8]

Apart from acting on mast cells, enteric neurons and muscle cells, tachykinin receptor antagonists may correct GI dysmotility by interfering with the function of extrinsic afferents which can contribute to GI motor dysregulation in two ways.[8] First, they participate in autonomic intestino-intestinal reflexes in which SP and NKA, released from the central endings of sensory neurons in the spinal cord, mediate transmission to the efferent reflex arc. Second, tachykinins released from sensory nerve endings in the gut can disturb GI motility, an instance that may be reflected by the ability of NK1 receptor antagonists to ameliorate dysmotility caused by esophageal acidification, anaphylaxis, and inflammation.[8]

Implications of Tachykinins in GI Inflammation, Hypersecretion, and Cancer

Many studies have shown that GI infection, inflammation, and mucosal injury are associated with time-related changes in the expression and release of tachykinins as well as with alterations in the expression and function of tachykinin receptors in the gut. However, the alterations in SP and NKA expression are variable, given that the GI tachykinin levels in patients with gastritis, IBD or diverticular disease have been reported to be decreased, increased, or unchanged.[8,10,18] The expression of TAC4 mRNA is enhanced in IBD, particularly in the mucosa of patients with ulcerative colitis.[18] Whole-mount analysis of the myenteric plexus has revealed that the chemical coding of enteric neurons in ulcerative colitis is shifted in as much as the ratio of SP-positive versus SP-negative cholinergic neurons is significantly enhanced. Animal studies have shown that experimental infection and inflammation in the alimentary canal also cause changes in the tachykinin levels, which mirror those seen in human diseases to a variable degree.[4,8,10]

By contrast, there is more consistent evidence that IBD and experimentally induced infection or inflammation in the gut are accompanied by an increased expression of NK1 and NK2 receptors in the inflamed and noninflamed regions

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of the small and large intestines.[4,8,24] The upregulation and ectopic occurrence of SP binding sites in pseudomembranous colitis resulting from *C. difficile* infection has been reproduced by treatment of rats with *C. difficile* toxin A.[8]

There is ample evidence that, in addition to alterations in the expression of SP, NKA, and their receptors, disturbances of the GI tachykinin system contribute to the manifestations of IBD and functional GI disorders.[8] For example, the secretory response to SP is blunted in mucosal tissues isolated from patients with Crohn's disease or ulcerative colitis, and tachykinin receptor antagonists display beneficial effects in various models of experimental GI infection, GI inflammation, GI injury, diarrhea, and pancreatitis.[8] A search through the contemporary literature shows that, in rodents, esophagitis evoked by chronic acid reflux, colitis induced by TNBSA, dinitrobenzene sulfonic acid, dinitrofluorobenzene, dextran sulfate sodium, zymosan, or injection of T cells in SCID mice, and pancreatitis elicited by cerulein are blunted by an NK1 receptor antagonist.

Tachykinins play a particular role in the inflammation (granulocyte, mast cell, and macrophage activation) and hypersecretion evoked by *C. difficile* toxin A, which involves activation of capsaicin-sensitive extrinsic afferent neurons, release of SP, and activation of NK1 receptors on enteric neurons. Many other types of GI hypersecretion involve tachykinin receptors, which is exemplified by the observations that the intestinal hypersecretion evoked by *Cholera* toxin and the diarrhea caused by castor oil in rats are inhibited by NK1 and NK2 receptor antagonists.[8,10]

Of further relevance is the question whether tachykinins play a role in the initiation and/or maintenance of GI hypersecretion and inflammation. Experimental results indicate that SP and NKA participate primarily in the initial stage of TNBSA- and acetic acid-induced colitis.[8] If so, tachykinin receptor antagonists may be more beneficial in the initiation or reactivation of inflammation than in the suppression of an ongoing inflammatory process. The effect of SP to increase vascular permeability in the GI tract is amplified in the inflamed tissue because inflammation leads to downregulation of neutral endopeptidase which in the normal tissue maintains low levels of SP in the extracellular fluid and, thus, limits its proinflammatory effects.[8]

Preclinical studies suggest that various immune cells in the gut are under the influence of SP and NKA. The tachykinin-responsive cells include mast cells, lymphocytes, and granulocytes.[8] Furthermore, immune cells are not only targets of tachykinin actions but under pathological conditions can themselves be induced to synthesize and release tachykinins. This is true for macrophages in the mucosa of the rat ileum exposed to *C. difficile* toxin A and for eosinophils from the mucosa of IBD patients.[8]

Tachykinins may play a role in GI tumor biology. Tachykinin-like immunoreactivity is synthesized in and released from carcinoid tumor cells, and circulating levels of tachykinin-like immunoreactivity are significantly correlated with symptoms of flush and diarrhea in patients with metastasizing ileocecal serotonin-producing carcinoid tumors.[1] Substance P promotes colitis-associated cancer and acts as a mitogen on cell lines derived from human gastric and colonic adenocarcinomas, these effects being prevented by NK1 receptor antagonists.[26,29]

Implications of Tachykinins in GI Hyperalgesia, Pain, and Irritable Bowel Syndrome

The majority of spinal afferents supplying the rodent GI tract contain SP and NKA; and NK1, NK2, as well as NK3 receptors are expressed at many levels of the gut–brain axis. It is in keeping with these circumstances that a plethora of preclinical studies attests to a role of tachykinin receptors in visceral pain and hyperalgesia.[8,30] For instance, genetic deletion of NK1 receptors in mice prevents intracolonic acetic acid and capsaicin from eliciting pseudoaffective pain responses, from inducing primary mechanical hyperalgesia in the colon, and from causing referred mechanical hyperalgesia in abdominal skin.[8] Experimental studies with selective tachykinin receptor antagonists indicate that all 3 tachykinin receptors play a role in visceral nociception and inflammation-induced hyperalgesia, although to a different degree.[8] Thus, the visceromotor pain response to acute gastric and colorectal distention in the rat is inhibited by NK2 and NK3 receptor antagonists but left unaltered by NK1 receptor antagonists.[8] The infection-, inflammation-, or stress-induced hypersensitivity to distention in the rabbit, rat, gerbil, and guinea pig jejunum or colon is likewise reduced by either NK1 or NK2 receptor antagonists.[8,14] Results in the contemporary literature indicate that pancreatitis-evoked abdominal hyperalgesia in rodents is likewise blunted by NK1 and NK3 receptor antagonists.[20]

Tachykinin receptor antagonists may target multiple relays in the nociceptive pathways from the periphery to the brain. One site of action is within the spinal cord where tachykininergic transmission from primary afferents is interrupted. This seems to be true for the antihyperalgesic effect of NK1 receptor antagonists in the rabbit, guinea pig, and rat. Intrathecal administration of NK1 and NK3 receptor antagonists abrogates the abdominal hyperalgesia associated with experimental pancreatitis,[20] inhibits the visceromotor pain response to colorectal distention, and counteracts the GI hypersensitivity caused by repeated distention or experimental GI inflammation.[8] Further consistent with a central site of the antinociceptive action of NK1 receptor antagonists is the finding that experimental colitis or cystitis in the rat leads to an upregulation and de novo expression of NK1 receptors in the dorsal horn of the spinal cord.[8]

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Apart from blocking tachykininergic transmission in the spinal cord, NK1, NK2, and NK3 receptor antagonists may be antihyperalgesic by a peripheral site of action.[8,30] Although the expression of tachykinin receptors by primary afferent nerve fibers remains to be clarified, the NK2 receptor antagonist nepadutant has been observed to inhibit the enhanced firing that lumbosacral afferent neurons exhibit after distention of the experimentally inflamed rat colon.[8] Because the activity in pelvic and somatic afferent neurons is not affected, nepadutant has been proposed to be antihyperalgesic by a peripheral action on hypersensitive afferents supplying the colon. A similar conclusion has been reached for the brain-impermeable NK3 receptor antagonist SB-235375 which is able to inhibit the visceromotor reaction to painful colorectal distention in rats.[30] Because colonic motility and compliance are little affected and mechanonociception in the skin is not altered, it has been inferred that NK3 receptor antagonists exhibit intestine-specific antinociceptive activity. Part of the antinociceptive action of tachykinin receptor antagonists in the periphery may be indirect and result from their ability to blunt hyperexcitability in the enteric nervous system, to normalize aberrant GI motility and secretion, and to dampen inflammatory processes.[8,30]

Taking all preclinical studies together, it would seem that tachykinin receptor antagonists have considerable potential for the treatment of visceral pain and functional GI disorders.[8] By correcting hyper- or hypomotility, hypersecretion, and inflammation, tachykinin receptor antagonists may reduce the sensory gain of extrinsic afferents in the GI tract and, in addition, block tachykininergic transmission in the spinal cord. Furthermore, the effects of brain-penetrant NK1 receptor antagonists at the level of the gut and afferent system may favorably combine with their inhibitory actions on emesis, anxiety, depression, and stress reactions in the brain.[8] In clinical studies, however, this therapeutic promise of tachykinin receptor antagonists has thus far been borne out only to a limited extent.

For instance, when esophageal pain was studied in healthy volunteers, a NK1 receptor antagonist proved ineffective in altering pain thresholds to electrical stimulation both at baseline and following acid-induced esophageal allodynia.[34] The NK3 receptor antagonist talnetant was likewise without effect on sensory function and compliance in the colon of healthy volunteers.[11] In addition, talnetant was unable to provide any benefit to IBS patients as revealed in a phase II trial.[3] Tested in a small group of patients with IBS, the NK1 receptor antagonist ezlozipant also failed to modify the sensory response to rectosigmoid distention, but attenuated the associated emotional response.[17] It thus seems as if selective mono-tachykinin receptor antagonists are unable to alter GI pain and hyperalgesia in IBS patients.

The situation seems to be different, however, when nonselective multi-receptor antagonists with activity at 2 or all 3 tachykinin receptors are used. DNK333, an antagonist with affinity for NK1, NK2, and NK3 receptors has been reported to increase the rate of satisfactory relief from abdominal discomfort and pain and to reduce global IBS symptoms in women with diarrhea-predominant IBS.[35] It follows that pan-tachykinin receptor antagonists may hold therapeutic potential, although their efficacy and safety remain to be thoroughly tested.

Nausea and Vomiting

Preclinical studies have revealed that NK1 receptor antagonists inhibit vomiting caused by a variety of factors including the anticancer agent cisplatin, irradiation, copper sulfate, morphine, apomorphine, and motion.[6] This broad-spectrum profile of activity against peripherally and centrally acting emetogenic stimuli and the requirement for brain penetration show that NK1 receptor antagonists interrupt the emetic reflex at a central site of action within the brainstem, close to the nucleus of the solitary tract and the Bötzinger complex. The experimental observation that NK1 receptor antagonists block both the acute and delayed phase of cisplatin-induced emesis has been confirmed in clinical trials. The NK1 receptor antagonists aprepitant and casopitant add significantly to the antiemetic efficacy of 5-HT3 receptor antagonists and dexamethasone in humans and, in this combination, provide significant control over both the acute and delayed phase of chemotherapy-induced nausea and emesis.[6,7] Both aprepitant and casopitant have also been shown to enhance the antiemetic effect of 5-HT3 receptor antagonists in preventing postoperative nausea and vomiting.[6,32] Like casopitant mesylate, fosaprepitant (a prodrug bioequivalent to aprepitant) can be administered by intravenous injection, which is of benefit to patients who do not tolerate oral medication.

ACKNOWLEDGMENTS

The artistry of Evelin Painsipp in preparing the figures is greatly appreciated. Work in the author's laboratory is supported by the Austrian Science Funds, the Jubilee Funds of the Austrian National Bank, the Austrian Federal Ministry of Science and Research, and the Zukunftsfonds Steiermark.

REFERENCES

1. Cunningham JL, Janson ET, Agarwal S, Grimelius L, Stridsberg M. Tachykinins in endocrine tumors and the carcinoid syndrome. *Eur J Endocrinol* 2008;**159**:275–82.
2. Deiteren A, De Winter BY, Nullens S, Pelckmans PA, De Man JG. Role of tachykinin receptors in the modulation of colonic peristaltic activity in mice. *Eur J Pharmacol* 2011;**667**:339–47.
3. Dukes GE, Dewit OE, Sanger GJ, Ameen VZ, Northcutt AR, Perschy YL, et al. Lack of effect of the NK3 receptor antagonist, talnetant SB223242 on symptoms of IBS: results of 2 randomized, double-blind, placebo-controlled dose ranging trials. *Gastroenterology* 2007;**132**:A60.

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4. Evangelista S, Tramontana M, Maggi CA. Spatial and temporal expression of tachykinins and NK1- and NK2-receptor gene during TNB induced colitis in rats. *Neuropeptides* 2008;**42**:663–70.
5. Gwynne RM, Bornstein JC. Electrical stimulation of the mucosa evokes slow EPSPs mediated by NK1 tachykinin receptors and by P2Y1 purinoceptors in different myenteric neurons. *Am J Physiol* 2009;**297**:G179–86.
6. Hargreaves R, Ferreira JC, Hughes D, Brands J, Hale J, Mattson B, et al. Development of aprepitant, the first neurokinin-1 receptor antagonist for the prevention of chemotherapy-induced nausea and vomiting. *Ann N Y Acad Sci* 2011;**1222**:40–8.
7. Herrstedt J, Apornwirat W, Shaharyar A, Aziz Z, Roila F, Van Belle S, et al. Phase III trial of casopitant, a novel neurokinin-1 receptor antagonist, for the prevention of nausea and vomiting in patients receiving moderately emetogenic chemotherapy. *J Clin Oncol* 2009;**27**:5363–9.
8. Holzer P. Role of tachykinins in the gastrointestinal tract. In: Holzer P, editor. *Tachykinins. Handbook of experimental pharmacology*, vol. 164. Berlin: Springer; 2004. p. 511–58.
9. Holzer P, Holzer-Petsche U. Tachykinins in the gut. Part I. Expression, release and motor function. *Pharmacol Ther* 1997;**73**:173–217.
10. Holzer P, Holzer-Petsche U. Tachykinins in the gut. Part II. Roles in neural excitation, secretion and inflammation. *Pharmacol Ther* 1997;**73**:219–63.
11. Houghton LA, Cremonini F, Camilleri M, Busciglio I, Fell C, Cox V, et al. Effect of the NK3 receptor antagonist, talnetant, on rectal sensory function and compliance in healthy humans. *Neurogastroent Motil* 2007;**19**:732–43.
12. Jaafari N, Hua G, Adélaïde J, Julé Y, Imbert J. Expression of the tachykinin receptor mRNAs in healthy human colon. *Eur J Pharmacol* 2008;**599**:121–5.
13. Jaafari N, Khomitch-Baud A, Gilhodes JC, Hua G, Julé Y. Qualitative and quantitative analysis of tachykinin NK2 receptors in chemically defined human colonic neuronal pathways. *J Comp Neurol* 2008;**507**:1542–58.
14. Kakol-Palm D, Brusberg M, Sand E, Larsson H, Martinez V, Johansson A, et al. Role of tachykinin NK1 and NK2 receptors in colonic sensitivity and stress-induced defecation in gerbils. *Eur J Pharmacol* 2008;**582**:123–31.
15. King SK, Sutcliffe JR, Ong SY, Lee M, Koh TL, Wong SQ, et al. Substance P and vasoactive intestinal peptide are reduced in right transverse colon in pediatric slow-transit constipation. *Neurogastroent Motil* 2010;**22**:883–92.
16. Lecci A, Capriati A, Altamura M, Maggi CA. Tachykinins and tachykinin receptors in the gut, with special reference to NK2 receptors in human. *Auton Neurosci* 2006;**126/127**:232–49.
17. Lee OY, Munakata J, Naliboff BD, Chang L, Mayer EA. A double blind parallel group pilot study of the effects of CJ-11,974 and placebo on perceptual and emotional responses to rectosigmoid distension in IBS patients. *Gastroenterology* 2000;**118**:A846.
18. Liu L, Markus I, Saghire HE, Perera DS, King DW, Burcher E. Distinct differences in tachykinin gene expression in ulcerative colitis, Crohn's disease and diverticular disease: a role for hemokinin-1? *Neurogastroen Motil* 2011;**23**:475–83.
19. Liu L, Shang F, Morgan MJ, King DW, Lubowski DZ, Burcher E. Cyclooxygenase-dependent alterations in substance P-mediated contractility and tachykinin NK1 receptor expression in the colonic circular muscle of patients with slow transit constipation. *J Pharmacol Exp Ther* 2009;**329**:282–9.
20. Liu L, Shenoy M, Pasricha PJ. Substance P and calcitonin gene related peptide mediate pain in chronic pancreatitis and their expression is driven by nerve growth factor. *JOP* 2011;**12**:389–94.
21. Madsen JL, Fuglsang S. A randomized, placebo-controlled, crossover, double-blind trial of the NK1 receptor antagonist aprepitant on gastrointestinal motor function in healthy humans. *Aliment Pharmacol Ther* 2008;**27**:609–15.
22. Maggi CA. Principles of tachykininergic co-transmission in the peripheral and enteric nervous system. *Regul Pept* 2000;**93**:53–64.
23. Menzies JR, McKee R, Corbett AD. Differential alterations in tachykinin NK2 receptors in isolated colonic circular smooth muscle in inflammatory bowel disease and idiopathic chronic constipation. *Regul Pept* 2001;**99**:151–6.
24. Michalski CW, Autschbach F, Selvaggi F, Shi X, Di Mola FF, Roggo A, et al. Increase in substance P precursor mRNA in noninflamed small-bowel sections in patients with Crohn's disease. *Am J Surg* 2007;**193**:476–81.
25. Mitsui R. Immunohistochemical analysis of substance P-containing neurons in rat small intestine. *Cell Tissue Res* 2011;**343**:331–41.
26. Pagán B, Isidro AA, Coppola D, Chen Z, Ren Y, Wu J, et al. Effect of a neurokinin-1 receptor antagonist in a rat model of colitis-associated colon cancer. *Anticancer Res* 2010;**30**:3345–53.
27. Page NM. Characterization of the gene structures, precursor processing and pharmacology of the endokinin peptides. *Vascul Pharmacol* 2006;**45**:200–8.
28. Poole DP, Amadesi S, Rozengurt E, Thacker M, Bunnett NW, Furness JB. Stimulation of the neurokinin 3 receptor activates protein kinase C epsilon and protein kinase D in enteric neurons. *Am J Physiol* 2008;**294**:G1245–56.
29. Rosso M, Robles-Frías MJ, Coveñas R, Salinas-Martín MV, Muñoz M. The NK-1 receptor is expressed in human primary gastric and colon adenocarcinomas and is involved in the antitumor action of L-733,060 and the mitogenic action of substance P on human gastrointestinal cancer cell lines. *Tumour Biol* 2008;**29**:245–54.
30. Sanger GJ. Neurokinin NK1 and NK3 receptors as targets for drugs to treat gastrointestinal motility disorders and pain. *Br J Pharmacol* 2004;**141**:1303–12.
31. Shimizu Y, Matsuyama H, Shiina T, Takewaki T, Furness JB. Tachykinins and their functions in the gastrointestinal tract. *Cell Mol Life Sci* 2008;**65**:295–311.
32. Singla NK, Singla SK, Chung F, Kutsogiannis DJ, Blackburn L, Lane SR, et al. Phase II study to evaluate the safety and efficacy of the oral neurokinin-1 receptor antagonist casopitant (GW679769) administered with ondansetron for the prevention of postoperative and postdischarge nausea and vomiting in high-risk patients. *Anesthesiology* 2010;**113**:74–82.
33. Stanton MP, Hengel PT, Southwell BR, Chow CW, Keck J, Hutson JM, et al. Cholinergic transmission to colonic circular muscle of children with slow-transit constipation is unimpaired, but transmission via NK2 receptors is lacking. *Neurogastroen Motil* 2003;**15**:669–78.
34. Willert RP, Hobson AR, Delaney C, Hicks KJ, Dewit OE, Aziz Q. Neurokinin-1 receptor antagonism in a human model of visceral hypersensitivity. *Aliment Pharmacol Ther* 2007;**25**:309–16.
35. Zakko S, Barton G, Weber E, Dunger-Baldauf C, Rühl A. Randomised clinical trial: the clinical effects of a novel neurokinin receptor antagonist, DNK333, in women with diarrhea-predominant irritable bowel syndrome. *Aliment Pharmacol Ther* 2011;**33**:1311–21.

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Chapter 182

TFF Peptides

Werner Hoffmann

ABSTRACT

The trefoil factor family (TFF) domain is a cysteine-rich shuffled module forming the basic unit for the TFF peptides (in short, TFFs). TFFs are typically secreted by mucin-producing cells of the gastrointestinal tract and other epithelia. Furthermore, they are synthesized in the brain and some other organs. The three mammalian TFFs consist of one (TFF1, TFF3) or two TFF domains (TFF2). In amphibia, the pattern differs remarkably. The biosynthesis of mammalian TFFs is rather complex. TFF1 and TFF3 form disulfide-linked heteromers with gastrokine-2 and IgG Fc binding protein, respectively. TFFs play a key role in the maintenance of the surface integrity of mucous epithelia in health and disease. Their multiple molecular functions include (1) formation of the mucus barrier, (2) enhancement of mucosal repair (restitution) on different levels, (3) modulation of mucosal differentiation processes, and (4) modulation of the immune response. Of special note, TFFs are also connected with oncogenic pathways.

DISCOVERY

Secretory trefoil factor family peptides (in short, TFFs; for reviews, see Refs. 4, 10, 13, 28, 29, 31, 34) share a common structural motif: the TFF domain (formerly: P-domain or trefoil-domain). This unique ancient shuffled module arose well before amphibian evolution and contains six conserved cysteine residues. TFF domains are also present in mosaic proteins associated with mucous surfaces (for review, see Ref. 10). The actual nomenclature of TFF peptides is based on an agreement reached in 1997 at the *Conférence Philippe Laudat* to avoid the term "trefoil" because of its multiple meanings.

Historically, the discovery of the three mammalian and various amphibian TFFs was the result of four groups working in different fields (for review, see Ref. 10). Mammalian TFFs consist of one (TFF1, TFF3) or two TFF domains (TFF2). The amino acid sequence of human TFF1 (formerly: pS2/BCEI/pNR-2/pNR-105/Md2) was deduced in 1984 and the primary structure of porcine TFF2 (formerly: [pancreatic] spasmolytic polypeptide/[P] SP) was reported in 1985. However, the high similarity between TFF1 and TFF2 was first recognized in 1988 by the analysis of TFF domains from the frog integumentary mucin FIM-A.1 (formerly: spasmolysin). Human TFF2 was first described in 1990. TFF3 (formerly: intestinal trefoil factor/ITF/P1.B) was detected in 1991 in rats and in 1993 in humans.

The first amphibian TFFs have been characterized in 1991 (for review, see Ref. 10). Typical of the *Xenopus laevis* gastrointestinal (GI) tract are xP1 (single TFF domain), xP4.1, and xP4.2 (four TFF domains).

STRUCTURE OF THE mRNA/GENE

The three human and the three mouse TFF genes as well as part of the amphibian TFF genes have been characterized (for review, see Ref. 10). All three human TFF genes are clustered on chromosome 21q22.3 in a head-to-tail arrangement within 54.5 kb in the order tel-TFF1-TFF2-TFF3-cen. Transcription of all three genes is directed toward the centromere (Fig. 1). A syntenic 40kb region was characterized in the mouse genome on chromosome 17q. This locus also encodes an antisense TFF1-related transcript.

All TFF genes analyzed thus far share common structural features. Most notably, TFF domains are typical shuffled modules always encoded by a single exon belonging to the class 1-1. Consequently, the TFF1 and the TFF3 genes consist of three exons; whereas the TFF2 gene contains four exons (Fig. 1).

The situation in amphibia is rather complex (for review, see Ref. 10) because additional TFF genes exist for which no mammalian ortholog is known (e.g., xP2 expressed in the skin and xP1-L in the tadpole stomach). Whether these genes form a cluster as in mammals is not yet known.

The expression of the clustered mammalian TFF genes is coordinatedly regulated (for reviews, see Refs. 4, 10, 13). This has been observed also in various TFF-deficient mice. There are numerous studies characterizing the promoter regions of the mammalian and amphibian TFF genes and their complex transcriptional regulation by various stimuli such as estrogen, cytokines, gastrin, transforming growth factor-α, prostaglandins, peroxisome

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00182-2
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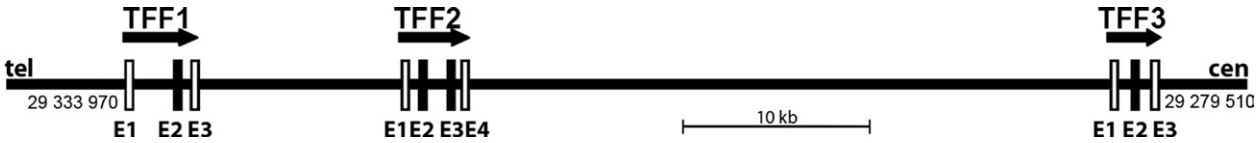

FIGURE 1 Chromosomal localization of the human TFF genes. Illustrated is the TFF cluster on chromosome 21q22.3. Indicated also are the orientation of the telomere and the centromere as well as the nucleotide positions. The exons (E) encoding the TFF domains are shown in black. Each of the first exons (E1) encodes the signal sequences typical of precursors of secretory proteins.

proliferator-activated receptors (PPARs), osmotic stress, and hypoxia (for reviews, see Refs. 2, 4, 10, 13, 28). Pathologically relevant are the induction of TFF expression by hypoxia and its modulation by nitric oxide[20] as well as the regulation of TFF1 by Ras/ERK signaling and TFF3 by STAT1/3 (for reviews, see Refs. 2, 13). Furthermore, TFF1 and TFF2 expression is regulated by gastrin,[2,32] whereas TFF3 expression is inducible by Toll-like receptor activation.[21]

PROCESSING OF THE PRECURSOR

All TFF peptides are synthesized via precursors containing a cleavable N-terminal signal sequence typical of secretory proteins. A hallmark of the unique TFF modules (comprising about 42 amino acid residues; for compilation, see Refs. 10, 29) are six conserved cysteine residues which form intramolecular disulfide bridges in the order C^1–C^5, C^2–C^4, C^3–C^6 (for review, see Ref. 29), creating three characteristic loop structures (L1–L3). A planar projection of this structure resembles a trefoil (Fig. 2). The complex pattern of disulfide bridges is probably one reason for the remarkable resistance of TFFs against proteolytic degradation particularly in the stomach.[13,29] However, shortened forms of TFF3 are found in the human gastric juice[16] and intestine.[1]

In the human GI tract, all TFFs are capable of forming heteromers with different partner proteins (Fig. 2). Gastric TFF1 forms a disulfide-linked heterodimer with gastrokine-2 (GKN2; formerly: GDDR, TFIZ1) via C^7,[15,18] and intestinal TFF3 is disulfide-linked to IgG Fc binding protein (FCGBP) via C^7.[1] Furthermore, gastric TFF2 mainly exists as a high molecular weight complex associated with an unknown partner protein.[15] By contrast, porcine pancreatic TFF2 has been reported to exist as a monomer that is stabilized by an additional intramolecular disulfide bridge between cysteine residues outside the TFF domains (Fig. 2).[29] Taken together, the differences concerning their partner proteins point to highly specialized functions for TFF1, TFF2, and TFF3.

Besides the heteromeric forms, also monomeric and homodimeric forms of TFF1 and TFF3 were detectable *in vivo*.[1,15,16] For example, about 15–20% of colonic TFF3 exist mainly in a monomeric form, which could be released from the TFF3–FCGBP heteromer *in vivo* by reduction with exogenous hydrogen sulfide (H_2S) because of high H_2S concentrations particularly in the lumen of the colon.[1] Of special note, TFF3 liberated in this manner could be posttranslationally modified by H_2S, that is, by S-sulfhydration.[1] Alternatively, TFF3 could also be released from the TFF3–FCGBP heteromer by a disulfide isomerase activity, may be as TFF3 homodimer,[1] which is detectable, for example, in the gastric juice.[16]

The N-terminal glutamic acid residues of human TFF1 and human TFF3 as well as recombinant human TFF2 produced in yeast are not cyclized to form a pyroglutamic acid residue.[1,29] By contrast, porcine pancreatic TFF2 contains an N-terminal pyroglutamic acid residue formed by cyclization of a glutamine residue in the precursor.

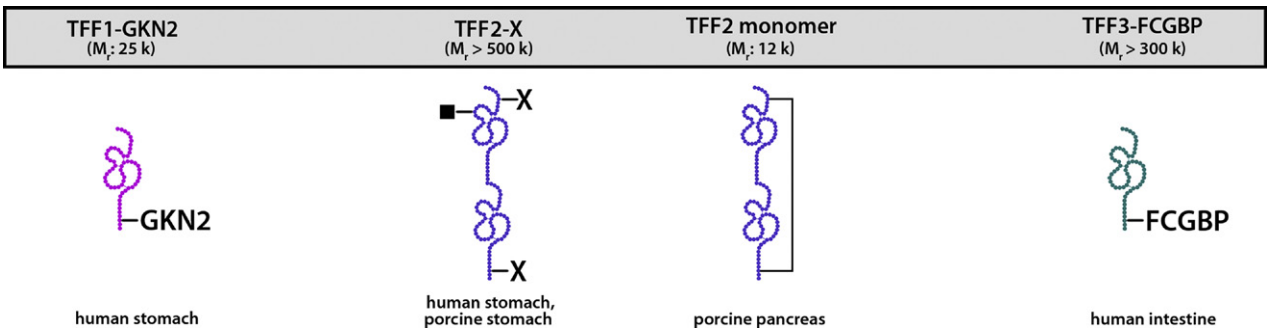

FIGURE 2 TFF forms in the mammalian GI tract. Schematically represented are the TFF1–GKN2 heteromer, the monomeric form of TFF2 (including the additional disulfide bridge), the high molecular weight complex of TFF2 (partner X is not known thus far), and the TFF3–FCGBP heteromer. The TFF1 and TFF3 heteromers are disulfide-linked. Also indicated is the N-glycosylation site of human gastric TFF2 (rectangular knob). See color plate 51.

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Additionally, most of human gastric TFF2 (in contrast to porcine and murine TFF2) is posttranslationally modified by N-glycosylation. Here the unusual fucosylated N,N′-diacetyllactosediamine (LacdiNac) oligosaccharide was identified (Hanisch et al., *Glycobiology*, in press). Interestingly, the ratio of glycosylated to nonglycosylated TFF2 in the gastric juice showed diurnal variations and is modulated by food intake.[27]

The three-dimensional structures of porcine pancreatic TFF2 and recombinant human TFF1 (monomer and homodimer) and TFF3 (homodimer) were determined (for reviews, see Refs. 10, 29). A hydrophobic cleft between loops L2 and L3 is a characteristic feature of all TFF domains, and all the conserved amino acid residues are located here. Particularly, the tryptophan residue just before C^6 is probably of great importance.

DISTRIBUTION OF THE mRNA/PEPTIDE

TFF peptides are characteristic exocrine products of mucous epithelia and in particular of the GI tract (for reviews, see Refs. 10, 13, 34). Here, they are often synthesized together with secretory mucins (Table 1). However, this does not imply that expression of TFFs and mucins is regulated together; for example, expression of TFF3 and MUC2 shows a marked divergence along the rat intestine. Furthermore, TFFs are found in the human serum, and they are also typical neuropeptides (for review, see Ref. 10).

There are remarkable species differences concerning TFF expression in the GI tract. The focus will be put here on the human GI tract. TFF expression on the protein level often does not parallel the corresponding mRNA levels.[14,16] Similar results were reported for expression of mucins. The reason for this may be mRNA instability.[16] Furthermore, particularly TFF2 mRNA and protein expression is not congruent in the murine and human stomach corpus because of differentiation and migration processes of precursor cells.[14,23]

Expression in the Human Alimentary Tract

TFFs are secreted from all parts of the normal human alimentary tract but in variable amounts (Table 1). TFF3 seems to represent the "standard" TFF peptide, which is found in all GI organs (and in other regions of the human body), whereas TFF1 and TFF2 are predominantly expressed in the stomach.

The synthesis of TFFs starts in the oral cavity where TFF1, TFF2, and TFF3 are detectable in the submandibular and labial salivary glands. TFF3 is the major TFF secreted (submandibular glands), and it represents an important constituent of the saliva.

The normal esophagus is also a site of TFF peptide synthesis. Here, TFF3 is a typical secretory product of the submucosal glands.

The most complex TFF expression pattern of the whole body is present in the stomach. Only here, are all three TFF

TABLE 1 TFF Peptides Synthesized in the Normal Human Alimentary Tract. Scarce TFF Peptide Levels are Indicated by Parentheses. Also Shown are the Secretory Mucins Coexpressed

Organ	Cell	TFF peptide	Secretory mucin
Salivary glands	Submandibular glands	(TFF1, TFF2), TFF3	MUC5B, MUC7, MUC19
	Labial glands (mucous acini)	(TFF1, TFF2), TFF3	MUC5B, MUC7, MUC19
Esophagus	Submucosal glands	TFF3	MUC5B
Stomach	Cardiac and antral SMCs	TFF1, TFF3	MUC5AC
	Corpus SMCs	TFF1	MUC5AC
	Mucous neck cells	TFF2	MUC6
	Antral glands	TFF2	MUC6
Intestine	Brunner's glands	TFF2, TFF3	MUC6
	Goblet cells	TFF3	MUC2
Vater's ampulla	Goblet cells	TFF3	MUC5AC, MUC5B
Pancreas	(Duct epithelium), islets	(TFF1), TFF3	(MUC6)
Liver	LBDs, SBDs, PBGs	TFF1, (TFF2), TFF3	MUC6
Gall bladder	Epithelial cells	(TFF1), (TFF2), TFF3	MUC5B, MUC6

SMC, surface mucous cell; LBD, large biliary duct; SBD, small biliary duct; PBG, peribiliary gland.

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peptides synthesized in relatively large amounts.[14] TFF1 peptide predominates in the surface mucous cells (SMCs), whereas TFF2 peptide is detectable in mucous neck cells (MNCs) and antral gland cells. Of special note, TFF2 mRNA and protein expression is not congruent as shown for the murine and human stomach corpus.[14,23] TFF2 mRNA is typically observed in precursor cells, whereas TFF2 protein appears in mature MNCs only. Gastric TFF3 expression has been neglected for a long time. However, there is significant synthesis at the gastric border zones, that is, at the cardia and the antrum[14,16] where TFF3 peptide is detectable in early SMCs located close to the isthmus (by contrast, TFF1 peptide occurs primarily in superficial, late SMCs).

All three TFFs are also present in the gastric juice with great individual variations. Here, a shortened form of TFF3 is found that is probably the result of a peptic cleavage at the N-terminal region.[16] Remarkably, the concentration of TFF2 in the gastric juice is subject to diurnal variations.[27]

There is an abrupt change of the TFF expression pattern at the antroduodenal transitional zone. TFF1 is absent at the protein level in the intestine, whereas TFF2 is secreted from duodenal Brunner's glands. TFF3 is found in Brunner's glands and in goblet cells of the small and large intestine. Furthermore, significant amounts of TFF3 can be detected in the meconium of human infants as early as 12 weeks of gestation.

TFF2 expression in the pancreas is rather scarce, and there is little TFF1 synthesis in ductal cells. Only recently has abundant TFF3 immunoreactivity been reported in the pancreatic islets colocalized with insulin indicating endocrine secretion (for reference, see Ref. 5).

Synthesis of TFFs also occurs in the biliary system (for review, see Ref. 26). Here, mainly TFF1 and TFF3 are occasionally detectable in large and rarely in small bile ducts, and TFFs are also secreted from the gall bladder. Furthermore, TFF3 was identified in goblet cells of the hepatopancreatic ampulla, also known as the ampulla of Vater, where it is localized together with MUC5AC, MUC5B, and MUC6.

Expression in the Alimentary Tract of Other Mammals

Remarkable species differences have been observed in the TFF expression patterns. These variations also affect the respiratory tract.[8] However, currently, information from other mammals is mainly restricted to the porcine, murine, and rat.

Considerable differences exist for TFF2 expression in the pancreas. In contrast to the situation in humans, the pancreas is the major source for porcine TFF2 (acinar cells). Relatively high TFF2 expression levels are also found in the mouse and rat pancreas. Furthermore, in the murine liver (and kidney) an antisense TFF1-related transcript has been identified, which is not detectable in humans.

Expression in the Amphibian Alimentary Tract

The situation in amphibia is quite different from that in mammals (for review, see Ref. 10). There are differences in the histology of the GI organs and in the structure of the TFFs. Limited information is available only for the South African toad *Xenopus laevis* (GI TFFs: xP1, xP4.1, xP4.2; xP1-L).

xP1, the amphibian ortholog of TFF1, is detectable in gastric SMCs with a decreasing gradient from the corpus toward the antrum, whereas the pronounced amphibian gastric MNCs are completely devoid of xP1. Of note, expression of the homologous xP1-L gene was detected in larval SMCs only.

xP4.1 is N-glycosylated and comprises four TFF domains. It is localized together with mucins in the characteristic MNCs and less in SMCs in all parts of the stomach. Based on its expression pattern, xP4.1 is considered as a functional equivalent of TFF2. xP4.2 is very similar to xP4.1 (89.7% identity), but it lacks the N-glycosylation site. This peptide is mainly expressed in a distinct population of esophageal goblet cells and in MNCs of the gastric fundus and corpus but not the antrum.

RECEPTORS AND SIGNALING CASCADES

Many of the multiple biological functions of TFFs are expected to be triggered by receptor ligation. However, there are no molecular data published unambiguously describing TFF binding molecules with the classical characteristics of high affinity receptors in spite of circumstantial evidence for their existence on the basolateral side of mucous epithelia (for reviews, see Refs. 4, 9, 13). For example, injected TFF1 and TFF3 have been shown to bind predominantly to TFF2-positive MNCs of the stomach (for references, see Ref. 13). Furthermore, the β-subunit of the fibronectin receptor (i.e., a β-integrin) and a transmembrane protein with similarity to CRP-ductin/DMBT1 were identified from porcine stomach because of their TFF2-binding capacity (for review, see Ref. 13). Only lately has the chemokine receptor CXCR4 been described as a low affinity receptor for TFF2 (for review, see Ref. 9). This receptor, together with its predominant physiological ligand CXCL12 plays a key role for various chemotactic processes. However, there are considerable differences *in vitro* between TFF2 and CXCL12 concerning signaling cascades involved, and the biological role of a TFF2-CXCR4 ligation is not clear thus far (for review, see Ref. 9).

There are only limited studies identifying the signaling cascades triggered by TFFs (for reviews, see Refs. 4, 7) including ERK1/2, JNK, Akt, and NF-κB.[6,12] Generally, the recombinant TFFs and porcine TFF2 used for these studies showed only relatively weak effects. Of note, TFF1 and TFF3 differ in their ability to induce activation of STAT3. Reports on TFF-triggered gene expression are also rare.

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Possible target genes include decay-accelerating factor, NOS2, COX2, MMP1, 3, 13, and TFFs themselves[25] (for review, see Ref. 7). TFFs have occasionally been claimed to trigger phosphorylation of the EGF receptor in certain cell lines (for review, see Refs. 4, 7). However, TFFs do not bind directly to the EGF receptor,[28] but they show synergistic effects with EGF (for review, see Ref. 7). For example, TFF2 and EGF synergistically enhance haptotactic migration but trigger different signaling cascades.[7]

BIOLOGICAL ACTIONS WITHIN THE GASTROINTESTINAL TRACT

Protective and Healing Effects in Animal Models

There are numerous *in vivo* studies indicating protective and healing effects of all three recombinant TFFs after various damages to the rodent GI tract[22,33] (for reviews, see Refs. 10, 13). Recombinant TFFs were applied via different routes, that is, luminally or systemically, or transgenic mice overexpressing TFFs were investigated. GI damage was induced chemically, by restraint, hypoxia, or radiation. Generally, only TFF2 and the dimeric forms of TFF1 and TFF3 were active but not the monomeric forms. The beneficial effect of TFFs was correlated with reductions in VCAM-1, IL-6, and TNF-α expression. Also, a synergistic protective effect with EGF was reported.

Detailed comparative studies with recombinant TFF2 and TFF3 clearly documented that luminal application is superior over systemic delivery.[22] Surprisingly, systemic TFFs (in particular the TFF3/monomer) even aggravated colitis scores.[22] Furthermore, induction of colitis was effectively prevented after intragastric administration of TFFs by genetically modified *Lactococcus lactis*.[33] This also points to biological activity after luminal application.

In agreement with the observed protective role of TFFs, all transgenic TFF-deficient mice show abnormalities in the GI tract. TFF1-deficient mice fail to develop a functional gastric mucosa and develop obligatory antroplyloric adenomas, 30% of which progressed to carcinomas (for review, see Ref. 31). TFF2-deficient animals show a modulation of crucial genes involved in innate and adaptive immunity,[3] and they have an altered gastric surface pH regulation after damage.[35] TFF3-deficient mice show a decreased resistance to colonic injury, they are particularly sensitive to chemotherapy and radiation-induced mucosal injury (for review, see Ref. 28) and have a reduced body mass when compared with the wild-type animals.

There is evidence now that TFFs support a variety of different mucosal defense and repair mechanisms to various extents. These mechanisms synergistically enhance the surface integrity of the GI mucosa. The multiple molecular functions include (1) formation of the mucus barrier, (2) enhancement of mucosal repair (restitution) on different levels, (3) modulation of mucosal differentiation processes, and (4) modulation of the immune response.

TFFs are Integral Constituents of the Mucus Barrier

All three TFF peptides appear as constituents of laminated mucous gels when localized by immunohistochemistry[16] (for review, see Ref. 10). The hydrated mucus also contains, for example, different oligomeric mucins, FCGBP, soluble forms of DMBT1, and immunoglobulins. Remarkably, the mode of association differs for each TFF peptide. In this context, it is also interesting to note that TFF domains are integral constituents of the frog integumentary mucins FIM-A.1 and FIM-C.1 and other mosaic proteins associated with mucous surfaces (for review, see Ref. 10). Furthermore, a pH-dependent, lectin-like activity has been reported for both TFF1 and TFF3.[24]

Gastric TFF1 is cosecreted with the mucin MUC5AC and has been reported also to interact with MUC5AC (for review, see Ref. 10). However, the majority of the TFF1-GKN2 heterodimer does not associate with the mucin fraction after size exclusion chromatography; only a small proportion (<0.1%) appeared together with mucins.[15] The TFF1–GKN2 heteromer may have a role in the complex oligomerization and/or packaging of MUC5A during the secretory pathway in gastric surface mucous cells. This view is supported by the observation that TFF1-deficient mice have an altered gastric mucus layer and show activation of the "unfolded protein response."[31]

In contrast, gastric TFF2 mainly exists as a high molecular weight complex associated with the mucin fraction even after size exclusion chromatography.[15] Thus, TFF2 could act as "link peptide" crosslinking an unknown partner protein (Fig. 2). One candidate would be the mucin MUC6, which is cosecreted with TFF2. Furthermore, porcine pancreatic TFF2 monomer has been shown to interact noncovalently with a gastric DMBT1 isoform (porcine CRP-ductin; for review, see Ref. 13) and recombinant TFF2 changed specifically the viscoelastic properties of mucous gels.[30] Of note, this effect is characteristic of TFF2 and is not observed for TFF1 or TFF3.[13,30]

Recent results showed that TFF3 also is mucus-associated by forming a disulfide-linked heteromer with FCGBP in the intestine.[1] The cysteine-rich repetitive glycoprotein FCGBP (Mr > 300 k) is a typical constituent of mucus in various organs. It is autocatalytically cleaved, and in the intestine parts are covalently attached to the mucin MUC2 (for references, see Ref. 1). Thus, it is tempting to speculate that the mucus-associated TFF3-FCGBP heteromer might serve as a reservoir for

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biologically active, modified TFF3 monomers (and probably also dimers) upon release.

TFFs Enhance Mucosal Restitution and Modulate Apoptosis and Angiogenesis

Migration of epithelial cells is particularly observed in the gastric and intestinal mucosa after superficial injury. This rapid repair is called "restitution" and starts within minutes of damage and well before extensive inflammatory processes and proliferation occur. TFFs have been shown in a number of *in vitro* models to influence different stages of this multistep process (for reviews, see Refs. 7, 10). Generally, TFFs are believed to act from the luminal side reaching their basolateral receptors only when the epithelial integrity is lost, that is, after local mucosal damage. This view is strongly supported by systematic protection studies *in vivo*.[22]

The first step in mucosal restitution is the reduction of cell–cell contacts directly at the borders of the injured area, followed by acquisition of these cells into a migratory phenotype. There are reports that TFF3 modulates adherens and tight junctions.[19] This would be in line with the observation that recombinant TFF3 changes the migration pattern of epithelial cells *in vitro*.

Furthermore, TFFs have been shown to speed up migration of epithelial cells and also immune cells in different *in vitro* model systems (motogenic effect; for reviews, see Refs. 7, 10). This effect is critically dependent on activation of ERK1/2.[6]

Restitution is also supported by the antiapoptotic effect of TFFs [12] (for reviews, see Refs. 4, 7, 28, 31). This effect is in line with the motogenic effect of TFFs because both cell migration and apoptosis are linked by common signaling mechanisms, for example, Rac1/XIAP. Of special note, under inflammatory conditions recombinant TFF3 has been reported to have a proapoptotic effect and has also increased the expression of matrix metalloproteinases.[25]

Restitution *in vivo* is also dependent on uninterrupted mucosal blood flow. Consequently, the pro-angiogenic activity of TFFs (for reviews, see Refs. 4, 7) supports particularly the final period of restitution, that is, remodeling, which is overlooked often and can take months.

TFF1 and TFF3 Modulate Mucosal Differentiation Processes

Continuous regeneration of the GI mucosa via proliferation and differentiation from stem cells followed by directed migration is an essential mechanism for maintaining its surface integrity.[14] A role of TFFs for differentiation processes of gastric and respiratory epithelial cells has been demonstrated (for reviews, see Refs. 8, 31). For example, TFF1-deficient mice show an increased number of SMCs, whereas the number of parietal cell is decreased.[31] This effect is explained by altered differentiation from prepit to preparietal cells. Furthermore, the amplification of mucous progenitor cells is even more changed in the pyloric antrum of TFF1-deficient mice leading gradually to dysregulated differentiation and the development of adenomas and carcinomas.[11]

Furthermore, SMCs in the human gastric corpus and the antrum are generated via different differentiation programs.[14,16] There are indications that TFF3 plays a role for the stepwise maturation of antral SMCs and that this process is also responsible for the higher turnover rate of the antral mucosa when compared with the body mucosa.

However, cell proliferation is not enhanced by TFFs.[13,28] Only in the presence of EGF has TFF3 been reported to stimulate β-cell replication.[5] There are rather reports on an antiproliferative effect of TFFs (for reviews, see Refs. 4, 13, 28, 31).

TFF2 Modulates the Immune Response

There is increasing evidence that particularly TFF2 is involved in immune responses and inflammatory processes (for review, see Ref. 3). For example, TFF2 and TFF3 are expressed in lymphoid tissues such as spleen, thymus, lymph nodes, and bone marrow, and they stimulate migration of monocytes. Thus, TFF2 expression is not only limited to the gastric mucosa but is also present in macrophages and lymphocytes.[17] TFF2-deficient mice show altered expression particularly of genes involved in innate and adaptive immunity[3] and these animals have also an increased susceptibility to *Helicobacter felis*-induced gastritis.[17] Furthermore, $TFF2^{-/-}$ cells were hyperresponsive to interleukin 1β stimulation suggesting TFF2 as a negative regulator of gastrointestinal inflammation and immune cell cytokine response.[17] In addition, the tumor necrosis factor-α-induced secretion of IL-6 and IL-8 is enhanced by TFF2 and TFF3 *in vitro* pointing to a possible function in the recruitment of leukocytes during mucosal inflammation.[6]

TFFs Probably Differ in Their Biological Actions

Based on the observation that TFFs are capable of forming heteromers or high molecular weight complexes with different partner proteins (Fig. 2), one might expect different biological functions also. It is still an open question whether the various TFF heteromers represent the functional forms or they are storage forms of TFFs or TFFs have just a structural function in these heteromers. Generally, TFF monomers can be released from the heteromers by reduction of disulfide linkages. During this process also

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posttranslationally modified monomers could be generated. These modifications could be essential for TFF ligation to specific high affinity receptors and thus could alter dramatically the biological activity of TFFs. Unfortunately, the functional results are limited thus far because mainly recombinant TFF monomers and homodimers have been tested, which showed only weak biological activities. Taken together, the following picture appears concerning the multiple biological actions of TFFs in the GI tract.

TFF1 forms a heterodimer with GKN2, which could act as a motogen by triggering a specific receptor. Here, GKN2 could synergistically support the motogenic activity of TFF1. This hypothesis is strengthened by the report that the closely related peptide GKN1 has both mitogenic and motogenic activities. Furthermore, TFF1 could also have a function for the complex oligomerization and/or packaging of MUC5AC.

TFF2 could act as "link peptide" crosslinking a yet unknown high molecular weight component of the gastric mucus, for example, MUC6. By acting as a structural component of mucus, it could influence the rheological properties of this barrier dramatically. Furthermore, monomeric TFF2 could also activate a specific receptor, such as CXCR4.

TFF3 could have a motogenic effect by triggering a specific receptor after its release as a modified monomer or a homodimer from the TFF3-FCGBP heteromer.

However, the precise molecular mechanisms of TFFs still await their full elucidation.

PATHOPHYSIOLOGICAL IMPLICATIONS

Generally, TFF expression is dysregulated in inflammatory diseases and many neoplastic conditions (for reviews, see Refs. 4, 13, 31, 34). Furthermore, the unusual TFF plasma levels are indicative for the progression of certain cancers and can improve, for example, gastric cancer screening. TFFs are also important factors affecting the bacterial microbiota and progression of diseases of the alimentary/GI tract.

For example, *Helicobacter pylori* infection induces TFF expression and TFF1 (and to a lesser extent TFF3) enhances the efficient colonization of the gastric mucosa with *H. pylori* because these peptides interact in a lectin-like fashion specifically with *H. pylori* core oligosaccharide portion of lipopolysaccharide.[24] This TFF-sugar interaction is pH-dependent. It would be interesting to extend such studies to the gastric and intestinal microbiota.

There is a vast literature on the ectopic expression of TFFs during inflammatory diseases of the GI tract (such as gastroesophageal reflux disease, gastric ulcerations, ulcerative duodenitis and colitis, Crohn's disease, hepatolithiasis, cholecystitis, and pancreatitis; (for reviews, see Refs. 10, 13, 34)). Under these conditions, a unique glandular structure known as the ulcer-associated cell lineage (UACL) is a prominent site of TFF synthesis. The UACL is thought to represent a natural repair kit activated after mucosal damage.[34] Interestingly, a selective reduction in TFF3 expression has been reported in untreated celiac disease.

Inflammation is a critical component of tumor progression. Pathological TFF expression is also observed in premalignant conditions, such as Barrett's metaplasia and metaplastic polyps and TFF3 is a promising biomarker for Barrett's esophagus nowadays. Biosynthesis of TFFs is also dysregulated in many epithelial tumors (e.g., of the esophagus, stomach, biliary tract, pancreas, and intestine), suggesting a role for TFFs in cancer progression (for reviews, see Refs. 4, 10, 31). Particularly, TFF3 is a marker for bad prognosis of both stomach and colon carcinomas. Of particular interest, coexpression of TFF1 and GKN2, which normally form a heterodimer, is deregulated in gastric cancer.[18] Furthermore, there are also indications that formation of the TFF3–FCGBP heteromer is deregulated during colon carcinogenesis.

TFF1 and TFF3 are believed to represent key players in the different responses of the stomach and the intestine to inflammation. TFF1 and TFF3 expression is differentially regulated by SHP2/ERK and STAT3 signaling, respectively. Cancer develops in the stomach, and inflammatory bowel disease hits the intestine when this critical gp130-regulated balance is disrupted (for reviews, see Refs. 2, 13). Consequently, reduced TFF1 expression and increased TFF3 synthesis have been observed in many gastric cancers indicating a clear shift toward STAT3 signaling in gastric neoplasia.

Interestingly, TFF2 arises also in a metaplastic lineage of the gastric fundus and corpus called "spasmolytic polypeptide-expressing metaplasia." This cell type probably arises in part through trans-differentiation of chief cells and is at least as strongly associated with the development of cancer as intestinal metaplasia.[23]

Deregulation of TFFs is also observed after incisional wounds of GI tissue, in various induced gastric ulcerations, and after irradiation and chemotherapy. Particularly, the late and sustained TFF1 and TFF3 responses are indicative for a role in late-stage repair processes (for review, see Ref. 10).

Taken together, TFFs are expected to play important roles in physiological wound healing and in inflammatory processes, carcinogenesis, and metastasis.

REFERENCES

1. Albert TK, Laubinger W, Müller S, Hanisch FG, Kalinski T, Meyer F, et al. Human intestinal TFF3 forms disulfide-linked heteromers with the mucus-associated FCGBP protein and is released by hydrogen sulfide. *J Prot Res* 2010;**9**:3108–17.
2. Baus-Loncar M, Giraud AS. Multiple regulatory pathways for trefoil factor (TFF) genes. *Cell Mol Life Sci* 2005;**62**:2921–31.
3. Baus-Loncar M, Kayademir T, Takaishi S, Wang T. Trefoil factor family 2 deficiency and immune response. *Cell Mol Life Sci* 2005;**62**:2947–55.

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4. Emami S, Rodrigues S, Rodrigue CM, Le Floch N, Rivat C, Attoub S, et al. Trefoil factor family (TFF) peptides and cancer progression. *Peptides* 2004;**25**:885–98.
5. Fueger PT, Schisler JC, Lu D, Babu DA, Mirmira RG, Newgard CB, et al. Trefoil factor 3 stimulates human and rodent pancreatic islet β-cell replication with retention of function. *Mol Endocrinol* 2008;**22**: 1251–9.
6. Graness A, Chwieralski CE, Reinhold D, Thim L, Hoffmann W. Protein kinase C and ERK activation are required for TFF-peptide-stimulated bronchial epithelial cell migration and tumor necrosis factor-α-induced interleukin-6 (IL-6) and IL-8 secretion. *J Biol Chem* 2002;**277**:18440–6.
7. Hoffmann W. TFF (trefoil factor family)-triggered signals promoting mucosal restitution. *Cell Mol Life Sci* 2005;**62**:2932–8.
8. Hoffmann W. TFF (trefoil factor family) peptides and their potential roles for differentiation processes during airway remodeling. *Curr Med Chem* 2007;**14**:2716–9.
9. Hoffmann W. Trefoil factor family (TFF) peptides and chemokine receptors: a promising relationship. *J Med Chem* 2009;**52**:6505–10.
10. Hoffmann W, Jagla W. Cell type specific expression of secretory TFF peptides: colocalization with mucins and synthesis in the brain. *Int Rev Cytol* 2002;**213**:147–81.
11. Karam SM, Tomasetto C, Rio M-C. Amplification and invasiveness of epithelial progenitors during gastric carcinogenesis in trefoil factor 1 knockout mice. *Cell Prolif* 2008;**41**:923–35.
12. Kinoshita K, Taupin DR, Itoh H, Podolsky DK. Distinct pathways of cell migration and antiapoptotic response to epithelial injury: structure–function analysis of human intestinal trefoil factor. *Mol Cell Biol* 2000;**20**:4680–90.
13. Kjellev S. The trefoil factor family—small peptides with multiple functionalities. *Cell Mol Life Sci* 2009;**66**:1350–69.
14. Kouznetsova I, Kalinski T, Meyer F, Hoffmann W. Self-renewal of the human gastric epithelium: new insights from expression profiling using laser microdissection. *Mol BioSyst* 2011;**7**:1105–12.
15. Kouznetsova I, Laubinger W, Kalbacher H, Kalinski T, Meyer F, Roessner A, et al. Biosynthesis of gastrokine-2 in the human gastric mucosa: restricted spatial expression along the antral gland axis and differential interaction with TFF1, TFF2 and mucins. *Cell Physiol Biochem* 2007;**20**:899–908.
16. Kouznetsova I, Peitz U, Vieth M, Meyer F, Vestergaard EM, Malfertheiner P, et al. A gradient of TFF3 (trefoil factor family 3) peptide synthesis within the normal human gastric mucosa. *Cell Tissue Res* 2004;**316**:155–65.
17. Kurt-Jones EA, Cao LC, Sandor F, Rogers AB, Whary MT, Nambiar PR, et al. Trefoil family factor 2 is expressed in murine gastric and immune cells and controls both gastrointestinal inflammation and systemic immune responses. *Infect Immun* 2007;**75**:471–80.
18. May FEB, Griffin SM, Westley BR. The trefoil factor interacting protein TFIZ1 binds the trefoil protein TFF1 preferentially in normal gastric mucosal cells but the co-expression of these proteins is deregulated in gastric cancer. *Int J Biochem Cell Biol* 2009;**41**:632–40.
19. Meyer zum Büschenfelde D, Tauber R, Huber O. TFF3-peptide increases transepithelial resistance in epithelial cells by modulating claudin-1 and -2 expression. *Peptides* 2006;**27**:3383–90.
20. Ortiz-Masiá D, Hernández C, Quintana E, Velázquez M, Cebrián S, Riano A, et al. iNOS-derived nitric oxide mediates the increase in TFF2 expression associated with gastric damage: role of HIF-1. *FASEB J* 2010;**24**:136–45.
21. Podolsky DK, Gerken G, Eyking A, Cario E. Colitis-associated variant of TLR2 causes impaired mucosal repair because of TFF3 deficiency. *Gastroenterology* 2009;**137**:209–20.
22. Poulsen SS, Kissow H, Hare K, Hartmann B, Thim L. Luminal and parenteral TFF2 and TFF3 dimer and monomer in two models of experimental colitis in the rat. *Regul Peptides* 2005;**126**:163–71.
23. Quante M, Marrache F, Goldenring JR, Wang TC. TFF2 mRNA transcript expression marks a gland progenitor cell of the gastric oxyntic mucosa. *Gastroenterology* 2010;**139**:2018–27.
24. Reeves EP, Ali T, Leonard P, Hearty S, O'Kennedy R, May FEB, et al. *Helicobacter pylori* lipopolysaccharide interacts with TFF1 in a pH-dependent manner. *Gastroenterology* 2008;**135**:2043–54.
25. Rösler S, Haase T, Claassen H, Schulze U, Schicht M, Riemann D, et al. Trefoil factor 3 is induced during degenerative and inflammatory joint disease, activates matrix metalloproteinases, and enhances apoptosis of articular cartilage chondrocytes. *Arthritis Rheum* 2010;**62**:815–25.
26. Sasaki M, Ikeda H, Nakanuma Y. Expression profiles of MUC mucins and trefoil factor family (TFF) peptides in the intrahepatic biliary system: physiological distribution and pathological significance. *Prog Histochem Cytochem* 2007;**42**:61–110.
27. Semple JI, Newton JL, Westley BR, May FEB. Dramatic diurnal variation in the concentration of the human trefoil peptide TFF2 in gastric juice. *Gut* 2001;**48**:648–55.
28. Taupin D, Podolsky DK. Trefoil factors: initiators of mucosal healing. *Nat Rev Mol Cell Biol* 2003;**4**:721–32.
29. Thim L, May FEB. Structure of mammalian trefoil factors and functional insights. *Cell Mol Life Sci* 2005;**62**:2956–73.
30. Thim L, Madsen F, Poulsen SS. Effect of trefoil factors on the viscoelastic properties of mucus gels. *Eur J Clin Invest* 2002;**32**:519–27.
31. Tomasetto C, Rio M-C. Pleiotropic effects of trefoil factor 1 deficiency. *Cell Mol Life Sci* 2005;**62**:2916–20.
32. Tu S, Chi AL, Lim SH, Cui G, Dubeykovskaya Z, Ai W, et al. Gastrin regulates the TFF2 promoter trough gastrin-responsive *cis*-acting elements and multiple signaling pathways. *Am J Physiol* 2007;**292**: G1726–37.
33. Vandenbroucke K, Hans W, van Huysse J, Neirynck S, Demetter P, Remaut E, et al. Active delivery of trefoil factors by genetically modified *Lactococcus lactis* prevents and heals acute colitis in mice. *Gastroenterology* 2004;**127**:502–13.
34. Wong WM, Poulsom R, Wright NA. Trefoil peptides. *Gut* 1999; **44**:890–5.
35. Xue L, Aihara E, Podolsky DK, Wang TC. In vivo action of trefoil factor 2 (TFF2) to speed gastric repair is independent of cyclooxygenase. *Gut* 2010;**59**:1184–91.

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Chapter 183

Urocortins

Vincent Wu, Pu-Qing Yuan, Muriel Larauche, Lixin Wang and Mulugeta Million

ABSTRACT

Urocortins (Ucn 1, Ucn 2, and Ucn 3) are members of the corticotropin-releasing hormone (CRH) family of peptides with ancient evolutionary lineage. In mammals, Ucns and CRH make up the four known CRH paralogous peptides. The mature Ucn peptides have comparable amino acid length, 38–40 amino acids, and share significant sequence homology. Ucns are distributed throughout the neuraxis and peripheral tissues including the epithelial, neuronal, and immune cells of the gastrointestinal tract. They exert biological actions by activating two related class B G protein–coupled CRH receptors, CRH_1 and CRH_2. Ucn 1 binds to both CRH_1 and CRH_2, whereas Ucn 2 and Ucn 3 bind selectively to CRH_2. In mammals, Ucns administered peripherally exert profound effects on food intake and on the gastrointestinal secretomotor, mucosal barrier, inflammation, and visceral pain responses. The identification of Ucns-like peptides throughout evolution, their distribution, and their biological actions support their critical role in gastrointestinal tract homeostasis during metabolic demands and/or in stress-related gut pathophysiologies.

DISCOVERY OF UROCORTINS

The identification of urocortins followed the earlier discoveries of corticotropin-releasing hormone (CRH) and the nonmammalian CRH-related peptides; urotensins, and sauvagine (see the CRH/Urocortins chapter in the Brain Peptides section of the book). CRH was isolated from the ovine hypothalamus, urotensins from the caudal spinal cord/neurosecretory organ (urophysis) of teleost fishes, and sauvagine from the skin of frog *Phyllomedusa sauvageii*. The differential responses of tissues to CRH and the nonmammalian peptides and their differential affinities to CRH receptors provided impetus to search for other CRH-related peptides in mammals. This resulted in the discovery of the first of the three urocortins from rat brain named urocortin, now renamed urocortin 1 (Ucn 1).[1,11,33] The discovery of Ucn 1, using antisera against the suckerfish urotensin-I, and the phylogeny of the then known CRH-related peptides led Lovejoy and Balment[21] to further suggest the existence of at least 4 CRH-like peptides in the vertebrate genome (see Lovejoy's chapter in the Brain Peptides section of the book). This and the lack of a pervasive Ucn 1 projection to CRH receptor type 2 (CRH_2)-expressing cells guided studies and increased the plausibility for the existence of other CRH peptides that are endogenous ligands for CRH_2. Consistent with this contention, the use of the public genome database, bioinformatic analysis, and molecular cloning, resulted in the identification of two new peptides named urocortin 2 (Ucn 2)/stresscopin-related peptide (SRP), and urocortin 3 (Ucn 3)/stresscopin (SCP) in humans and mice by two independent groups.[12,18,28] The two CRH/Ucns-related peptides share significant sequence homology with the mammalian CRH and Ucn 1. Furthermore, the sequence homology of the human Ucn 2 and Ucn 3 and that of the mouse mature Ucn 2 and Ucn 3 peptides is 76% and 90%, respectively.[18,28] Phylogenetically, the mammalian Ucns are part of the superfamily of neuropeptides that includes CRH, urotensin-I, sauvagine, and the insects diuretic peptides I and II (see the Diuretic/Antidiuretic chapter in the Invertebrate Peptides section of the book). The orthologous peptides are believed to have evolved through species divergence whereas the mammalian paralogs have evolved as a result of two gene duplications.[21] The discovery of Ucns and their functions have thus strengthened the long held thoughts and predictions that the CRH peptides are one of the oldest peptide lineages that are conserved throughout the species and confer biological actions that are critical for survival.

UROCORTIN GENES AND PRECURSOR STRUCTURES

The CRH/UCN gene family members share similar genomic organization and sequence homology. Like CRH, all the three UCN genes contain two exons and a single intron varying greatly in size from 200 to 8000 bp in human UCNs and their rodent orthologs (Table 1). Unspliced and alternatively spliced transcripts have been found in Ucn 1 and Ucn 3. However, the presence of the intron or alternative splicing would not affect the coding sequence of preproUcns since the preferred start codon is located downstream on the second exon.

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00183-4
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TABLE 1 Chromosomal Location and Physical Size of Intron and Exons of UCN genes. The Annotations are Based on the Latest Assemblies of Human GRCh37, Rat RGSC3.4, and Mouse NCBIM37 Genome

		DNA (bp)			Protein (aa)	
	Chromosome	Exon 1	Intron	Exon 2	Precursor	Mature peptide
Human						
UCN1	2p23-p21	286	509	251	124	40
UCN2	3p21.3	71	1410	566	112	38, 43
UCN3	10p15.1	222	492	8484	161	38, 40
Rat						
UCN1	6q14	35	535	261	122	40
UCN2	8q32	71	651	655	109	38
UCN3	17q12.3	511	922	3119	164	38, 41
Mouse						
UCN1	5B1	33	471	263	122	40
UCN2	9F2	74	1002	715	113	38
UCN3	13A1	509	969	3184	164	38, 41

The retained intron may contain regulatory sequences or form secondary structure to affect cell-specific mRNA processing (e.g. RNA splicing, antisense RNA) and protein translation.

Ucn 1

The human urocortin 1 (hUcn 1) is a 40-amino acid (aa) peptide. It has been identified in other mammalian species including in rats and mice. The human, mouse, and rat Ucn 1 share a highly conserved sequence (83–95%).[5,33] In addition, human and rat Ucn 1 display 44–63% sequence identity with the urotensin-I identified in various fish species, 45% identity with rat/human (r/h)CRH, and only 33% and 35% identity, respectively, with frog sauvagine.[5,33] The human Ucn 1 precursor is a 124 polypeptide encoded by the UCN1 gene (on chromosome 2p23-p21). The precursor is processed by a cleavage of paired dibasic arginine (R) by proprotein convertase subtilisin/kexin type enzyme and removal of carboxy-terminal lysine (K) by carboxypeptidase E, followed by amidation of the eventual valine (V) residue by peptidylglycine alpha-amidating monooxygenase (PAM) resulting in the generation of the intermediate Ucn-57 amide (with N-term 17 aa extension) and the mature Ucn1-40 amide (see the Peptide Biosynthesis/Processing section of the book). Outside the brain, Ucn 1 mRNA is expressed in diverse visceral organs including the heart and GI organs, more prominently in the stomach, colon, pancreas, and intestinal barrier/lining, and secretory epithelium.

Ucn 2

Human Ucn 2, also known as stresscopin-related peptide, is a 38–43 aa peptide processed from a 112 aa polypeptide precursor encoded by human gene UCN2 (on chromosome 3p21.3). In rodents, Ucn 2 has a conserved carboxy-terminal tripeptide GRR sequence and, therefore, can be amidated at the proximal Val after dibasic residue removal by carboxypeptidase E and α-amidation from Gly donor by the PAM enzyme complex. Posttranslational processing of human prohUcn 2 is not yet fully characterized. Human Ucn 2, with its unique C-terminal tripeptide GHC sequence, may require a different strategy to use carboxypeptidases or prohormone-converting enzymes to generate a mature amidated peptide. However, amidated hUcn 2 peptides with predicted 38–43 residues possess full agonist activity. In rodents, Ucn 2 is expressed in the brain, esophagus, heart, skin, and many other tissues.[12,28]

Ucn 3

Human Ucn 3, originally named stresscopin to implicate its stress-coping activity, is a 38 or 40 aa carboxy-terminal amidated peptide processed from the 161 aa polypeptide precursor encoded by UCN3 gene on chromosome 10p15.1. In rodents, posttranslational processing of 164 aa proUcn 3 yields mature peptides with predicted 38 or 41 residues (Ucn3-41 amide and Ucn3-38 amide) with full agonist activity. Ucn 3 is expressed in the brain, pancreas, cardiovascular, endocrine, intestinal epithelium barrier/lining, and several other tissues.[12,18]

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RECEPTORS AND SIGNALING CASCADES

Previous studies showed that binding and biological actions of urocortins are mediated by two class B G protein-coupled receptors (GPCR), referred to as CRH_1 and CRH_2. Ucn 1 binds to both receptor subtypes with equally high affinity and thus is considered as a nonselective agonist. Ucn 2 and Ucn 3, however, bind selectively with high affinity to CRH_2 receptors while showing little or no affinity or activity toward CRH_1. Thus, they have been implicated as the cognate ligands of ubiquitously expressed CRH_2 in the periphery. The molecular basis for ligand selectivity involves a single amino acid difference in the N-terminal extracellular domains (ECDs) of the receptors (CRH_1 Glu104/ $CRH_2\alpha$ Pro-100) at a site proximate to peptide residue 35 (Arg in CRH/Ucn 1, Ala in Ucn 2/3).[27] As members of the class B1 GPCR, CRH receptors are primarily coupled to Gαs, the hallmark feature known to this GPCR subfamily. On ligand stimulation, GTP binding and dissociation of Gαsβγ heterotrimer complex activates adenylyl cyclase, which in turn elevates second messenger cAMP production. Increased intracellular cAMP concentrations would lead to several cAMP-dependent signaling cascades, including PKA and EPAC, which have multiple effects on cytoplasmic and nuclear events, in particular, CREB-regulated gene activation.[9]

Cell Signaling Events Mediated by Ucn 1-Activated CRH_1 Receptor

More detailed studies of G protein coupling to transfected CRH_1[9] show that the type 1 receptors are capable of interacting with other Gq, Gi/o subunits to activate additional cell signaling pathways such as PKC, MAPK (ERK1/2), and AKT relevant to the CRH/Ucn actions. Furthermore, identification of multiple CRH_1 isoforms expressed in human and rodents may expand the role of CRH_1 in coordinating ligand and cell type-specific cell signaling.[35]

Emerging Cell Signaling Events Mediated by Ucns-Activated CRH_2 Receptor

More recent studies on CRH_2-mediated cell signaling show more subdued responses to CRH and Ucn 1. Lack of a robust response to CRH is not surprising because CRH has a much lower affinity to CRH_2. Given that Ucns are considered as endogenous ligands of CRH_2 and that CRH_2 has been considered to primarily couple with Gαs and activate a cAMP-dependent pathway, the subdued response to Ucns was surprising. However, although Ucns elicit relatively weaker cell response in cAMP production and ERK activation,[35] they trigger elevated inhibitory response in nitric oxide (NO) and cell apoptosis. Ucn 2 has also been implicated in calcium channel activation via CRH_2,[9] although the underlying mechanism is not yet clear. As shown in the case of CRH_1, current[35] and future identification of additional CRH_2 isoforms that are coupled to multiple G-proteins other than Gαs shall provide a better understanding of Ucns-activated CRH_2 signaling.

DISTRIBUTION OF UROCORTINS IN THE GI TRACT

Morphological and molecular studies show that Ucns are distributed throughout the neuraxis and the peripheral tissues.[7] Although, abundant data established the expression of Ucns in specific and distinct brain regions that control the stress response and energy balance,[2] convergent evidence show that Ucns are expressed in the gastrointestinal tract. The distribution of Ucn 1, Ucn 2, and Ucn 3 mRNA in the GI tract was first detected by in situ hybridization and reverse transcription polymerase chain reaction (RT-PCR).[4,12] Thereafter, immunohistochemical techniques provided an insight into their cellular localizations.[2,16] Most of the reports on Ucn distribution are related to Ucn 1 and less on Ucn 2 and Ucn 3, primarily because of the lack of specific Ucn 2 and Ucn 3 antibodies. Differences in sensitivity between the techniques used or the specificity of the molecular and immunological reagents used also account for some of the discrepancies in the reports on the distribution of Ucns. Nonetheless, the expression of Ucns and their receptors in the GI tract suggests a local autocrine and/or paracrine action of Ucns in the modulation of GI functions.

Urocortin 1

Stomach

After the initial report of Ucn 1 immunoreactivity (IR) in rat duodenal extracts,[33] Ucn 1 IR was later detected throughout the gastrointestinal tract of rats[14] and in the human gastric tissue.[4] Specifically, Ucn 1 IR is present in the gastric mucosa parietal cells of both human and rats.[2] Similarly Ucn 1 IR is detected in fibers of the gastric submucosal and myenteric plexi colocalized with tyrosine hydroxylase (TH) and in serotonin (5-HT)-immunoreactive (IR) enterochromaffin cells of the rat stomach.[2]

Small intestine

Ucn 1 mRNA-expressing cells are exclusively localized to the submucosal and myenteric plexi in the rat small intestine.[7] Ucn 1 immunoreactivity, on the other hand, is shown in TH positive fibers of the enteric nervous system primarily in fibers near the Lieberkühn crypts but not in enteric neuronal cell bodies.[2,14,16] Whether Ucn 1 protein is present in the small intestinal enteric neuron cell bodies that are not

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TH positive is yet to be determined. These results suggest that Ucn 1 may play an important role in the regulation of intestinal secretomotor function.

Colon

Ucn 1 in the rat colon is expressed in different cell types. The majority (70–80%) of rat colonic submucosal and myenteric neurons express Ucn 1.[29] The Ucn 1 positive colonic myenteric neurons are mainly cholinergic and serotonergic neurons. Ucn 1 is expressed in very few cells of colonic mucosa.[2,16] By contrast in the human colon, Ucn1 mRNA and peptide are primarily expressed in mucosal immune cells, specifically in the lamina propria macrophages and isolated lamina propria mononuclear cells. Human studies further show that Ucn 1 peptide expression is detected as early as three months of age but not before birth or in neonates. These data suggest that Ucn 1 synthesis may be subject to diet and luminal factors and that Ucn 1 may play a role in modulating local inflammation through an autocrine/paracrine effect on mucosal immune cells.[2,16] The presence of the local effects of neuronal Ucn 1 in the human colon is yet to be established, as results in support of the expression of Ucn 1 in rodents[30] but lack of expression in human are reported.[26]

Urocortin 2

Stomach

Ucn 2 expression in the stomach has only been described at the mRNA level, but it is present in the mouse, rat, and human.[2] A comparison with other peripheral tissues in the mouse reveals that gastric expression of Ucn 2 is low.[2]

Small intestine

Ucn 2 mRNA is detected in both the rat and mouse small intestine.[2,16] Throughout the rat small intestine, Ucn 2 mRNA is detected in the epithelium, lamina propria immune cells, and the enteric plexuses with the highest expression in the jejunum.[3] In human small intestine, Ucn 2 mRNA is absent. The cellular location of Ucn 2 peptide in the stomach and small intestine is still unknown.

Colon

Ucn 2 mRNA is not detected in the human colon, expressed at a very low level in the murine colon, but is abundantly expressed in the rat colon.[3,12] The location of Ucn 2 IR in the small and large intestines of rats include epithelial cells, the lamina propria immune cells, and enteric neurons.[3] The protein levels of Ucn2 in the rat colon are however lower than that in the jejunum. Ucn 2 IR in Peyer's patches, although faint, is reported.[3] The Ucn 2 mRNA distribution on the other hand shows antergorade gradient in that the highest level is found in the distal colon, followed by the proximal colon and the terminal ileum.[3]

Urocortin 3

In rats, the GI tract contains a relatively high level of Ucn 3 mRNA compared with other peripheral organs/tissues.[12] Significant levels of Ucn 3 mRNA are found in the colon, the small intestine and stomach, but not in the ileocecal region, cecum, and esophagus.[12] Immunohistochemical studies detected specific Ucn 3 signals in the muscularis mucosae of the small intestine of the mouse.[12] In humans, Ucn 3 mRNA is in the stomach and all regions of the small intestine, except for the ileocecal area.[12] Both immunohistochemistry and in situ hybridization techniques show Ucn 3 expression in the myenteric and submucosal plexus, in vascular endothelial cells, and vascular smooth muscle cells of blood vessels in subserosa and in smooth muscle layers. Immunohistochemical analysis reveals the expression of Ucn 3 in serotonergic enterochromaffin cells and the epithelium, albeit at a rather low level. Immunohistochemical results further show Ucn 3 localized in both enteric neurons and S-100-ir enteric glial cells. The signal is the strongest in the myenteric plexus. In contrast to Ucn 1, Ucn 3 is hardly detected in the lamina propria inflammatory cells in colonic mucosa. Ucn 3 peptide is also detected in the large intestine by RIA, with high concentrations in the rectum and sigmoid colon.[2] In addition, Ucn 3 is expressed in the pancreatic islets.[15] All these findings suggest that Ucn 3 has the potential to play physiological or pathological roles in the modulation of gastrointestinal functions during stressful conditions. Overall, the distribution of Ucns and their receptors in the gut is compatible with a local role in the GI response to stress and metabolic demand.

BIOLOGICAL ACTIONS WITHIN THE GASTROINTESTINAL TRACT

The biological actions of urocortins on the gastrointestinal functions are primarily derived from experimental *in vivo* injections or *in vitro* applications of physiological or pharmacological doses of urocortins. These studies show that Ucns exert a profound effect on gut tissue contractility, secretion, mucosal barrier function, transit, visceral pain, inflammatory responses, and food intake.[7,23]

Gastrointestinal Motility and Transit

Stomach

Intraperitoneal (ip) or intravenous (iv) injection of Ucn 1 and Ucn 2 exert an inhibitory action on gastric motility and emptying. In rats and mice, the peripheral actions of Ucn 1

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and Ucn 2 are prevented by either nonselective CRH_1 and CRH_2 antagonist, a selective CRH_2 antagonist, astressin$_2$-B, but not by a selective CRH_1 antagonist suggesting the primary role of CRH_2 in the responses.[23] Overall, peripherally injected CRH and Ucns-induced inhibition of gastric motility and emptying are fairly consistent across the species and involve inhibition of gastric contractility and/or gastroduodenal coordination.

Colon

In rodents, peripherally injected Ucn 1 (iv or ip) mimics the central effects of Ucn 1 in that it stimulates colonic transit and contractility.[22,23] Direct application of Ucn 1 to enteric neurons of rats and guinea-pigs activates neurons through CRH_1 and not CRH_2.[20] By contrast, peripherally injected Ucn 2 reduces stress-induced colonic motor activity and fecal pellet output that are known to be mediated through CRH_1.[8] These results suggest that the CRH_1 and CRH_2 receptors may play different roles in the colonic response to CRH peptides and stress.

Gastrointestinal Secretion

Gastrointestinal secretory function is an active process that plays an important role in digestion as well as in the protection of the mucosal surface. The regulation of GI secretions is effected through humoral and neural factors. Studies on peripheral Ucn effect on the gastric and small intestinal secretory responses are scant. However, earlier studies show that iv injected CRH decreases pentagastrin-stimulated gastric acid secretion in dogs and rats[32] and duodenal bicarbonate secretion in rats.[17] It is, thus, likely that peripheral Ucns modulate gastric and intestinal secretions.

In the lower gut, restraint stress or iv CRH-induced colonic mucin secretion is prevented by CRH receptor antagonists suggesting the role of CRH signaling in stress-related colonic mucin secretion. Intraperitoneal administration of CRH, Ucn 1, or sauvagine mimic stress-induced watery diarrhea, reminiscent of intestinal secretion, through a CRH_1-dependent pathway.[22] The intestinal capacity for high ion and water secretion is a defense mechanism against pathogens, which is commonly observed as the phenomenon of diarrhea. The passive movement of water at the epithelial level is assured by an active ion transport across the tissue, whose changes can be measured in Ussing chambers by modifications of the short circuit current (I_{sc}). Chronic peripheral administration of Ucn 3 increases basal colonic I_{sc}, suggesting a role for CRH_2 receptors in ion secretion.[16] Although direct evidence on the role of Ucns on intestinal secretion proper is scarce, given that Ucns and CRH act on the same receptors, it is plausible that results with CRH also apply to Ucns. For instance, in rats, treated with antagonists and selective agonists, CRH_1 receptors are involved in the increased basal colonic Isc and diarrhea occurring with rapid onset after peripheral and central CRH administration.[16] Although both CRH receptors are involved in ion secretion, the maximal epithelial I_{sc} responses in rat colonic tissue obtained with sauvagine administered *in vitro*, at doses 200–1000 fold lower than CRH, suggest a predominant CRH_2 effect in the modulation of secretory epithelial functions in the rat colon.[16]

Emerging evidence show that Ucn 1 stimulates pancreatic exocrine secretion in the rat through a CRH_1 receptor–mediated mechanism.[10] These actions are exerted indirectly and seem to involve cholinergic intrapancreatic neurons. Exogenous Ucn 3 also increases glucagon and insulin levels through CRH_2.[19] The results suggest that both CRH_1 and CRH_2 receptors may play a role in the Ucn-induced pancreatic secretions.

Intestinal Permeability

The gastrointestinal mucosal barrier function is ensured primarily by the continuous and compact epithelial cell layer that is held together by tight junctions. Trafficking of molecules through the intestinal epithelial barrier occurs via two routes: paracellular (between cells) controlling ion selectivity, nutrients, and solute permeability and/or transcellular (through cells) allowing for transport of large molecules (antigens, immunoglobulins). Stress or exogenous CRH alter barrier function. Chronic systemic administration of Ucn 3, in rats increases both paracellular and transcellular colonic permeability. By contrast, chronic peripheral administration to rats of the selective CRH_1 ligand stressin-1 only increases paracellular permeability.[16] In human colonic biopsies mounted in Ussing chambers, CRH serosal application induces increased transcellular permeability via activation of both CRH_1 and CRH_2, but does not affect paracellular permeability.[16] This result contrasts with the increase in both paracellular and transcellular permeability observed in rat colonic tissue after exposure to CRH and sauvagine *in vitro*, suggesting a role for both CRH_1 and CRH_2.[16] Together with mast cells, alterations in paracellular permeability are mediated by adrenergic and nicotinic nerves, whereas cholinergic, adrenergic, and nicotinic nerves participate in the alterations of transcellular permeability.[16]

Visceral Pain

The modulatory effect of the CRH peptides on visceral pain is primarily evidenced through the use of CRH and CRH receptor antagonists. Much evidence shows that CRH exerts pronociceptive action via CRH_1 receptors at both central and peripheral levels.[16,31] Very few studies have addressed the role of urocortins in the modulation of visceral pain response. The presence of Ucn 1 and

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Ucn 3 immunoreactive nerve terminals in association with CRH_2 receptors in the dorsal root ganglia and periaqueductal gray suggests, however, a modulatory influence of these receptors in visceral pain. In rats, iv injection of hUcn 2 reduces colorectal distension (CRD)-induced visceral hyperalgesia, and ip administration also has a similar antinociceptive effect in a model of duodenal distension-induced visceral pain. The Ucn 2 antinociceptive effects are blocked by peripheral administration of selective CRH_2 peptide antagonists, astressin$_2$-B and antisauvagine-30, suggesting CRH_2 mediation. Peripheral antinociceptive effects of hUcn 2 has been associated with the inhibition of splanchnic afferent spike activity, as observed in an *in vitro* colonic preparation and the phosphorylation process of ERK in the dorsal horn of the spinal cord.[24] Preliminary evidence, so far, is consistent with the activation of peripheral CRH_2 receptors exerting an antinociceptive modulatory effect while activation of CRH_1 receptors enhancing nociception. The detailed mechanisms as to how urocortins are released during stress and how CRH receptors are recruited to modulate visceral sensitivity require further studies.

Intestinal Inflammation

The modulatory role of CRH and Ucns in intestinal inflammation is shown by the fact that glucocorticoids are the mainstay of therapy against colitis. In the periphery, increased CRH immunoreactivity and mRNA expression in the mucosal biopsy of ulcerative colitis (UC) patients suggest that colonic CRH plays a role in colitis.[13] Ucn 1 IR increases in the colonic mucosa of UC patients that is reduced by glucocorticoid therapy.[30] There is also increased expression of Ucn 2 in colonic biopsies of both UC and CD patients.[25] In the upper gut, the upregulation of Ucn 1 but not CRH is reported in the biopsies of patients with *Helicobacter pylori*-induced gastritis.[4] That Ucns have a modulatory role in the gastrointestinal inflammation is now documented. However, the specific role they play in the prevention, maintenance, and/or recovery of inflammation is yet to be determined. For instance, local deletion of CRH, but not Ucn 2, ameliorates *Clostridium deficile* toxin-induced ileal inflammation. On the other hand, Ucn 2 enhances proinflammatory markers in human colonic epithelial cells, whereas CRH, Ucn 1, and Ucn 2 exert an anti-inflammatory effect in freshly isolated LPS-activated primary murine macrophages and macrophage cell line through Cox2 and PGE_2 pathways[2]

Food Intake

Ucns induce anorexic effect, in experimental animals, when injected peripherally (ip or iv). Intraperitoneally injected Ucns in mice and rats induce inhibitory effect on food intake and activate brain regions implicated in appetite regulation.[34] Differences in potency, however, exist between the CRH peptides. For instance, in rodents, peripherally injected Ucn 1 is more potent than CRH, Ucn 2, or Ucn 3 in appetite suppression. Likewise, although ip administration of Ucn 1 or Ucn 2 inhibits food intake, Ucn 2 does so without inducing malaise while Ucn 1 shows conditioned taste aversion and interoceptive stress with increased corticosterone levels in rats.[6] Mechanisms through which Ucns exert differential effects on food intake are not yet fully known. However, evidence exists supporting peripherally initiated effects through direct modulation of afferent vagal pathways and/or modulation of appetite- and energy balance-regulatory hormones such as ghrelin, insulin, and PYY exists. For instance, in humans, iv infusion of Ucn 1 blocks the increase of ghrelin in the circulation during a fast. Given that ghrelin is mainly secreted by gastric mucosa and has been implicated in the regulation of eating behavior, the results suggest that peripheral Ucn inhibitory effect on food intake may involve a local modulation of gastric ghrelin production. Similarly, Ucn 3 and CRH_2 are abundantly expressed in the pancreatic islets and regulate insulin release, which affects energy metabolism.[15,19]

PATHOPHYSIOLOGICAL IMPLICATIONS

As outlined above, preclinical and clinical evidence suggests that the activation of peripheral Ucn signaling exerts a profound effect on the gastrointestinal functions in health and diseases. The inhibitory effects of Ucns on upper gut secretomotor responses are mediated primarily by peripheral CRH_2, whereas stimulation of the lower gut secretomotor function involves CRH_1. Results mainly from CRH and also from Ucn studies show that Ucns modulate mucosal barrier function through both CRH_1 and CRH_2. The effect of Ucns on gut inflammation is now well documented although a full understanding of the specific effects awaits future studies. Finally, CRH and Ucn 1 enhance stress-related colonic distention-induced pain primarily through CRH_1, whereas CRH_2 exerts antihyperalgesic effects. These results clearly show that Ucns and their receptors are positioned to play a role in the gut secretomotor, mucosal barrier, inflammation, and visceral pain pathophysiology. Overall, available evidence suggests that CRH and Ucn 1, by acting on CRH_1, mediate most of the stress responses. Although not yet conclusive, Ucn 2 and perhaps Ucn 3 by acting on CRH_2 dampen the lower gut responses to stress. The relevance of Ucns in gut pathophysiology is exemplified by the ongoing clinical trials involving selective CRH_1 antagonists against irritable bowel syndrome, a condition known to be highly associated with stressful life events.

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ACKNOWLEDGMENTS

This work is supported by NIH grants RO1 DK078676 (M. Milion) and K01 DK088937 (M. Larauche).

REFERENCES

1. Bittencourt JC, Vaughan J, Arias C, Rissman RA, Vale WW, Sawchenko PE. Urocortin expression in rat brain: evidence against a pervasive relationship of urocortin-containing projections with targets bearing type 2 CRF receptors. *J Comp Neurol* 1999;**415**:285–312.
2. Buckinx R, Adriaensen D, Nassauw LV, Timmermans JP. Corticotrophin-releasing factor, related peptides, and receptors in the normal and inflamed gastrointestinal tract. *Front Neurosci* 2011;**5**:54.
3. Chang J, Hoy JJ, Idumalla PS, Clifton MS, Pecoraro NC, Bhargava A. Urocortin 2 expression in the rat gastrointestinal tract under basal conditions and in chemical colitis. *Peptides* 2007;**28**:1453–60.
4. Chatzaki E, Charalampopoulos I, Leontidis C, Mouzas IA, Tzardi M, Tsatsanis C, et al. Urocortin in human gastric mucosa: relationship to inflammatory activity. *J Clin Endocrinol Metab* 2003;**88**:478–83.
5. Donaldson CJ, Sutton SW, Perrin MH, Corrigan AZ, Lewis KA, Rivier JE, et al. Cloning and characterization of human urocortin. *Endocrinology* 1996;**137**:2167–70.
6. Fekete EM, Zhao Y, Szucs A, Sabino V, Cottone P, Rivier J, et al. Systemic urocortin 2, but not urocortin 1 or stressin(1)-A, suppresses feeding via CRF(2) receptors without malaise and stress. *Br J Pharmacol* 2011. doi:10.1111/j.1476-5381.2011.01512.x. [Epub ahead of print].
7. Fekete EM, Zorrilla EP. Physiology, pharmacology, and therapeutic relevance of urocortins in mammals: ancient CRF paralogs. *Front Neuroendocrinol* 2007;**28**:1–27.
8. Gourcerol G, Wu SV, Yuan PQ, Pham H, Miampamba M, Larauche M, et al. Activation of corticotropin-releasing factor receptor 2 mediates the colonic motor coping response to acute stress in rodents. *Gastroenterology* 2011;**140**:1586–96.
9. Grammatopoulos DK. Insights into mechanisms of corticotropin-releasing hormone receptor signal transduction. *Br J Pharmacol* 2011. doi:10.1111/j.1476-5381.2011.01631.x. [Epub ahead of print].
10. Guzman EA, Zhang W, Lin TR, Mulholland MW. Stimulation of rat pancreatic exocrine secretion by urocortin and corticotropin releasing factor. *Peptides* 2003;**24**:727–34.
11. Hauger RL, Grigoriadis DE, Dallman MF, Plotsky PM, Vale WW, Dautzenberg FM. International union of pharmacology. XXXVI. Current status of the nomenclature for receptors for corticotropin-releasing factor and their ligands. *Pharmacol Rev* 2003;**55**:21–6.
12. Hsu SY, Hsueh AJ. Human stresscopin and stresscopin-related peptide are selective ligands for the type 2 corticotropin-releasing hormone receptor. *Nat Med* 2001;**7**:605–11.
13. Kawahito Y, Sano H, Mukai S, Asai K, Kimura S, Yamamura Y, et al. Corticotropin releasing hormone in colonic mucosa in patients with ulcerative colitis. *Gut* 1995;**37**:544–51.
14. Kozicz T, Arimura A. Distribution of urocortin in the rat's gastrointestinal tract and its colocalization with tyrosine hydroxylase. *Peptides* 2002;**23**:515–21.
15. Kuperman Y, Chen A. Urocortins: emerging metabolic and energy homeostasis perspectives. *Trends Endocrinol Metab* 2008;**19**:122–9.
16. Larauche M, Kiank C, Taché Y. Corticotropin releasing factor signaling in colon and ileum: regulation by stress and pathophysiological implications. *J Physiol Pharmacol* 2009;**60**(Suppl. 7):33–46.
17. Lenz HJ. Regulation of duodenal bicarbonate secretion during stress by corticotropin-releasing factor and beta-endorphin. *Proc Natl Acad Sci U S A* 1989;**86**:1417–20.
18. Lewis K, Li C, Perrin MH, Blount A, Kunitake K, Donaldson C, et al. Identification of urocortin III, an additional member of the corticotropin-releasing factor (CRF) family with high affinity for the CRF2 receptor. *Proc Natl Acad Sci U S A* 2001;**98**:7570–5.
19. Li C, Chen P, Vaughan J, Blount A, Chen A, Jamieson PM, et al. Urocortin III is expressed in pancreatic beta-cells and stimulates insulin and glucagon secretion. *Endocrinology* 2003;**144**: 3216–24.
20. Liu S, Ren W, Qu MH, Bishop GA, Wang GD, Wang XY, et al. Differential actions of urocortins on neurons of the myenteric division of the enteric nervous system in guinea pig distal colon. *Br J Pharmacol* 2010;**159**:222–36.
21. Lovejoy DA, Balment RJ. Evolution and physiology of the corticotropin-releasing factor (CRF) family of neuropeptides in vertebrates. *Gen Comp Endocrinol* 1999;**115**:1–22.
22. Maillot C, Million M, Wei JY, Gauthier A, Taché Y. Peripheral corticotropin-releasing factor and stress-stimulated colonic motor activity involve type 1 receptor in rats. *Gastroenterology* 2000;**119**:1569–79.
23. Martinez V, Wang L, Million M, Rivier J, Taché Y. Urocortins and the regulation of gastrointestinal motor function and visceral pain. *Peptides* 2004;**25**:1733–44.
24. Million M, Wang L, Wang Y, Adelson DW, Yuan PQ, Maillot C, et al. CRF2 receptor activation prevents colorectal distension-induced visceral pain and spinal ERK1/2 phosphorylation in rats. *Gut* 2006;**55**:172–81.
25. Moss AC, Anton P, Savidge T, Newman P, Cheifetz AS, Gay J, et al. Urocortin II mediates pro-inflammatory effects in human colonocytes via corticotropin-releasing hormone receptor 2alpha. *Gut* 2007;**56**:1210–7.
26. Muramatsu Y, Fukushima K, Iino K, Totsune K, Takahashi K, Suzuki T, et al. Urocortin and corticotropin-releasing factor receptor expression in the human colonic mucosa. *Peptides* 2000;**21**:1799–809.
27. Pal K, Swaminathan K, Xu HE, Pioszak AA. Structural basis for hormone recognition by the human CRFR2{alpha} G protein-coupled receptor. *J Biol Chem* 2010;**285**:40351–61.
28. Reyes TM, Lewis K, Perrin MH, Kunitake KS, Vaughan J, Arias CA, et al. Urocortin II: a member of the corticotropin-releasing factor (CRF) neuropeptide family that is selectively bound by type 2 CRF receptors. *Proc Natl Acad Sci U S A* 2001;**98**:2843–8.
29. Sand E, Themner-Persson A, Ekblad E. Corticotropin releasing factor-distribution in rat intestine and role in neuroprotection. *Regul Pept* 2011;**166**:68–75.
30. Saruta M, Takahashi K, Suzuki T, Torii A, Kawakami M, Sasano H. Urocortin 1 in colonic mucosa in patients with ulcerative colitis. *J Clin Endocrinol Metab* 2004;**89**:5352–61.
31. Taché Y, Bonaz B. Corticotropin-releasing factor receptors and stress-related alterations of gut motor function. *J Clin Invest* 2007;**117**:33–40.
32. Todisco A, Park J, Lezoche E, Debas H, Taché Y, Yamada T. Peripheral acid inhibitory action of corticotropin releasing factor in rats: mediation by non-gastric mechanisms. *Gastroenterology* 1987;**92**:919–24.

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33. Vaughan J, Donaldson C, Bittencourt J, Perrin MH, Lewis K, Sutton S, et al. Urocortin, a mammalian neuropeptide related to fish urotensin I and to corticotropin-releasing factor. *Nature* 1995;**378**:287–92.
34. Wang L, Martinez V, Rivier JE, Taché Y. Peripheral urocortin inhibits gastric emptying and food intake in mice: differential role of CRF receptor 2. *Am J Physiol Regul Integr Comp Physiol* 2001;**281**:R1401–10.
35. Wu SV, Yuan PQ, Lai J, Wong K, Chen MC, Ohning GV, et al. Activation of Type 1 CRF receptor isoforms induces serotonin release from human carcinoid BON-1N cells: an enterochromaffin cell model. *Endocrinology* 2011;**152**:126–37.

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Chapter 184

VIP

Karnam S. Murthy and John R. Grider

ABSTRACT

Relaxation of smooth muscle of the gastrointestinal tract reflects the interplay of nitric oxide (NO), and peptide transmitters, mainly vasoactive intestinal peptide (VIP) and its homolog, pituitary adenylyl cyclase-activating polypeptide (PACAP). The role of VIP is most evident as mediator of the relaxation component of the peristaltic reflex. In smooth muscle cells, VIP and PACAP interact with cognate seven-transmembrane $VPAC_2$ receptors to generate cAMP and with the single-transmembrane NPR-C to generate NO and cGMP. Concurrent generation of cAMP and cGMP, and activation of cAMP-dependent protein kinase (PKA) and cGMP-dependent protein kinase (PKG), respectively, is the physiological norm. Both PKA and PKG act on various downstream targets to inhibit Ca^{2+} mobilization and MLC_{20} phosphorylation and thus induce muscle relaxation.

DISCOVERY

Sami Said, who was investigating the vasodilating substance responsible for systemic hypotension in acute respiratory syndrome and massive pulmonary embolism, joined Viktor Mutt, a well-known peptide biochemist, at the Karolinska Institute to isolate the substance from lung extracts.[29] Although a peptide extract with vasodilator activity was identified from the extracts from porcine lung, purification was hindered because of the paucity of a sufficient quantity of fresh tissue from the slaughterhouses in Stockholm. Based on the vasodilatory actions, a vasoactive peptide was isolated from pig small intestine, an organ that develops from the same embryonic bud as the lungs. As they were not sure about the existence of the peptide outside the intestine, it was named "vasoactive intestinal peptide," and initially classified as a gut hormone. Further studies using immunohistochemical and radioimmunoassy have shown that VIP has a widespread distribution, and the predominant localization of VIP in central and peripheral neurons led to the identification of neurotransmitter or neuromodulator functions. Said and Mutt characterized the 28 amino acids sequence of VIP, and this led to the synthesis of this peptide by Miklos Bodansky. The synthetic peptide was equipotent with VIP and had similar biological activities. The amino acid composition of VIP reveals that it is related to the secretin family of peptides, which includes pituitary adenylyl cyclase-activating peptide (PACAP), secretin, glucagon, helodermin, peptide with histidine and isoleucine in the N-terminal, and C-terminal respectively [peptide histidine isoleucine (PHI) and its human homolog peptide histidine methionine (PHM)], gastric inhibitory peptide (GIP), growth-hormone releasing hormone (GHRH), and glucagon-like peptides 1 and 2 (GLP-1 and GLP-2).[29] VIP is a more basic peptide than secretin and glucagon. The sequence of VIP is highly conserved between species including human and in terms of structure, cellular signaling, and function, VIP is most closely related to PACAP.[14]

STRUCTURE OF THE GENE AND PROCESSING OF THE PRECURSOR

The human VIP gene was cloned in 1985 by Gozes using synthetic oligodexoynucleotides based on human VIP-cDNA sequence. Further studies showed that the intron–exon organization of the VIP gene is similar to the genes of VIP-related peptides.[29] The human VIP gene contains seven exons and six introns. Each exon encodes a distinct functional domain of the VIP precursor or its mRNA: 5′ untranslated regions of the mRNA (exon I), signal peptide of preproVIP (exon II), N-terminal peptide (exon III), PHM (exon IV), VIP (exon V), and C-terminal of preproVIP (exon VI), and 3′ untranslated region of the mRNA (exon VII). The peptides VIP and PHI/PHM, which share 48% amino acid sequence identity are located on two adjacent exons. The sequence around VIP-coding and PHM-coding exons and their adjacent introns are highly conserved. The rodent VIP gene is similar in organization to human VIP gene with seven exons and six introns. Using chromosomal mapping and *in situ* hybridization, the human VIP gene was localized to chromosome 6 region 25. Using RNA blot hybridization, S1-nuclease analysis and cDNA cloning, VIP-gene transcripts were characterized. The rate of transcription of the VIP gene is regulated by many transcription factors such

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00184-6
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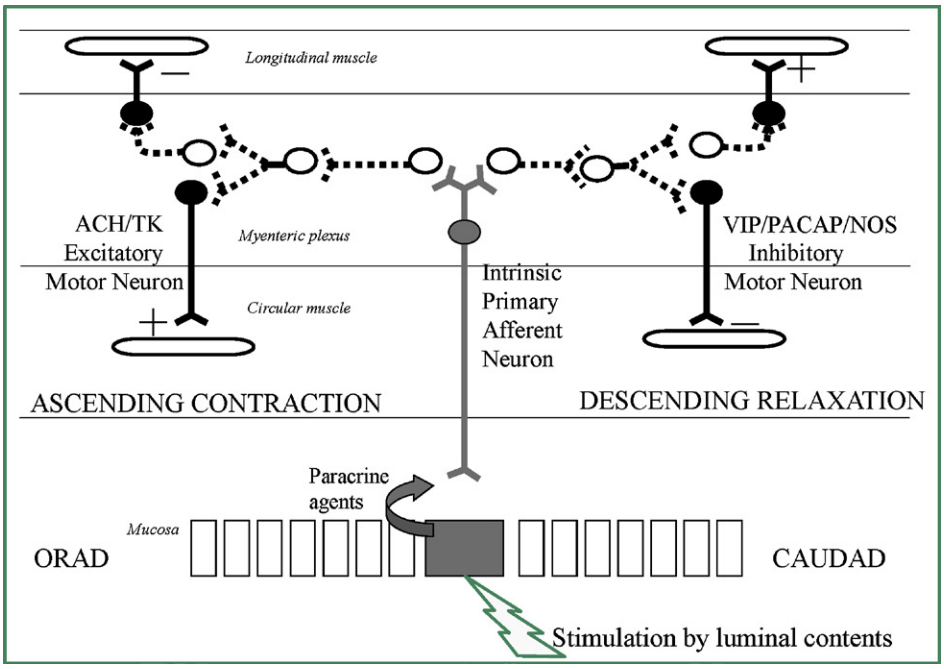

FIGURE 1 Model of peristaltic reflex underlying propulsive peristalsis. Components of chyme or mucosal mechanical stimulation activate specialized enteroendocrine cells (gray square) of the mucosa, which release paracrine agents such as serotonin (5-HT). The paracrine agent then activates intrinsic calcitonin-gene-related peptide containing intrinsic primary afferent neurons (IPAN) (gray neuron) to initiate the reflex. The IPAN activates chains of ascending and descending interneurons (open-dashed neurons), which release a variety of neuropeptides including somatostatin, enkephalin, and vasoactive intestinal peptide (VIP). The ascending interneurons terminate on excitatory motor neurons (solid neurons) that release acetylcholine (ACH) and tachykinins (TK) causing contraction of circular muscle (ascending contraction) orad to the site of stimulation. The descending interneurons terminate on inhibitory motor neurons (solid neurons) that release VIP, pituitary adenylate cyclase-activating peptide (PACAP), and nitric oxide (NO) causing relaxation of circular muscle (descending relaxation) caudad to the site of stimulation. Longitudinal muscle relaxes during ascending contraction and contacts during descending relaxation in a reciprocal manner to circular muscle (see Ref. 10 for details). Plus sign (+) indicates contraction and minus sign (–) indicates relaxation.

as CREB (cAMP response element binding protein). Translation of mRNA of human VIP gene transcription yields a 170-amino-acid precursor, preproVIP, and the posttranslational processing of the precursor yields the 28-amino-acid VIP and 27-amino-acid PHI in rodents and PHM in humans. VIP and PHI are present in a 1:1 molar ratio and released in roughly equimolar amounts. PreproVIP is also processed to preproVIP22–179, preproVIP111–122 and preproVIP156–170. All these preproVIP-derived peptides have been identified albeit with unequal amounts in human gastrointestinal tract and in VIP-producing cell lines.[29] Differences in the posttranslational processing of preproVIP exist in subpopulations of neurons in the gastrointestinal tract.

DISTRIBUTION IN THE GASTROINTESTINAL TRACT

In the gastrointestinal tract, VIP is localized in different populations of enteric neurons with different projections and is colocalized with different combinations of transmitters. The enteric nervous system is composed of two ganglionated plexuses: the myenteric plexus, which is present between the muscle layers and the submucosal plexus, which lies in the submucosa.[6] In the submucosal plexus, VIP is present in secretomotor neurons where it acts to stimulate secretion. In the myenteric plexus, VIP is present in about 25% of enteric neurons, which typically project in a caudad direction to innervate other neurons or smooth muscle cells of the circular muscle layer.[3,6] These neurons typically also contain the homologous peptides PACAP, PHI in animals or PHM in humans, and neuronal nitric oxide synthase (nNOS), an enzyme responsible for generation of nitric oxide (NO) (see the chapters by the discoverers of these peptides elsewhere in this book). Although single enteric neurons contain a variety of transmitters, VIP neurons never contain the tachykinin contractile transmitters, and thus these two peptides mark distinct, nonoverlapping populations of neurons.[3,6,7]

RECEPTORS AND THEIR SIGNALING PATHWAYS

The biological actions of VIP and PACAP are mediated by a family of G-protein-coupled receptors, which are designated $VPAC_1$, $VPAC_2$, and PAC_1 receptors.[14,17] These receptors belong to family B of the G-protein-coupled receptors also known as the secretin receptor family, which includes

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receptors for secretin, GIP, glucagon, glucagon-like peptide 1, calcitonin, parathyroid hormone, GHRH, and corticotrophin-releasing hormone. The PAC_1 receptors exhibit high affinity for PACAP and low affinity for VIP, whereas $VPAC_1$ and $VPAC_2$ receptors exhibit equal affinity for VIP and PACAP. Although $VPAC_1$, $VPAC_2$, and PAC_1 are expressed throughout the gastrointestinal tract, the various VIP/PACAP receptor subtypes exhibit differential expression patterns. $VPAC_1$ receptors are expressed in the mucosa and myenteric neurons; $VPAC_2$ receptors are expressed in neuroendocrine cells, blood vessels, and smooth muscle; and PAC_1 receptors are expressed in myenteric neurons.[14,17] Neural VIP/PACAP receptors are present in two locations: on nerve terminals where they act as autoinhibitory receptors to suppress further neurotransmitter release and on cholinergic/tachykinin neurons that innervate the longitudinal muscle layer. Activation of these receptors by VIP/PACAP released from myenteric neurons stimulates the release of ACh and tachykinins and induces contraction of longitudinal muscle.[10]

In gastrointestinal smooth muscle, VIP and PACAP interact with two distinct classes of receptors: a cognate $VPAC_2$ receptor coupled via G_s and stimulation of adenylyl cyclase V/VI resulting in cAMP formation and activation of cAMP-dependent protein kinase (PKA), and a single-transmembrane natriuretic peptide clearance receptor (NPR-C) coupled via G_{i1}/G_{i2} and stimulation of Ca^{2+} influx and sequential activation of Ca^{2+}/calmodulin-dependent eNOS and NO-dependent soluble guanylyl cyclase[23,24] (Fig. 2). In the smooth muscle of the gut, NPR-C is coexpressed with NPR-B but not NPR-A. Unlike NPR-A and NPR-B, NPR-C is devoid of intracellular cyclase-activating domain and consists of a short 37-amino acid sequence capable of activating pertussis toxin-sensitive G_{i1} and G_{i2}.[24] Using receptor-derived synthetic peptides, a 17-amino-acid G-protein-activating sequence has been identified in the intracellular domain of NPR-C; the sequence (^{469}RRNHQEESNIGKHREL^{485}R) is characterized by two NH_2-termial basic residues and a COOH-terminal motif BBXXB, where B and X represent basic and non-basic residues, respectively[24] (Fig. 2). Similar sequences have been identified in other G-protein-coupled seven-transmembrane receptors such as muscarinic and adrenergic receptors.[26]

In gastrointestinal smooth muscle, $VPAC_2$ receptors are desensitized and internalized by a mechanism distinct from those used by $VPAC_1$ or secretin receptors. $VPAC_2$ receptors are phosphorylated by G-protein-coupled receptor kinase 2 (GRK2) resulting in desensitization and internalization of receptors. GRK2 is phosphorylated by PKA at Ser^{685} and this phosphorylation greatly augments the ability of GRK2 to phosphorylate the receptor and induce receptor internalization.[25] $VPAC_1$ receptor phosphorylation, internalization, and desensitization are inhibited by concomitant activation

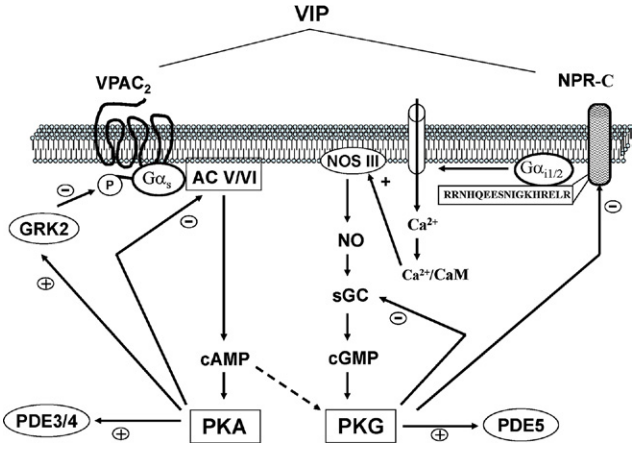

FIGURE 2 Signaling pathways initiated by VIP in gastrointestinal smooth muscle. In gastrointestinal smooth muscle, VIP activates cognate seven-transmembrane $VPAC_2$ receptors coupled via G_s to adenylyl cyclase V/VI, cAMP generation and activation of cAMP-dependent protein kinase (PKA). They also activate a single-transmembrane natriuretic peptide receptor C (NPR-C) coupled via $G\alpha_{i1}$ and $G\alpha_{i2}$ to Ca^{2+} influx and Ca^{2+}/calmodulin activation of NOS III expressed in smooth muscle cells, leading to NO-dependent activation of soluble guanylyl cyclase (sGC), cGMP generation and activation of cGMP-dependent protein kinase (PKG). Activation of $G\alpha_{i1/2}$ is mediated by a 17-amino-acid sequence in the middle region of the intracellular domain of NPR-C (inset). cAMP activates PKA and cGMP activates PKG, but in the presence of cGMP, cAMP crossactivates PKG also. Both PKA and PKG act separately upstream to induce desensitization of $VPAC_2$ and NPR-C receptors, inhibition adenylyl cyclase and soluble guanylyl cyclase, and activation of cAMP-specific PDE3A and PDE4 and cGMP-specific PDE5. Stimulation (+); inhibition (−).

of Gq-coupled muscarinic m_3 receptors. The mechanism involves phosphorylation of RKIP (Raf kinase inhibitory protein) by PKC and subsequent dissociation of RKIP from Raf-1 and association with GRK2 and inhibition of GRK2 activity.[16] The functional significant of this crossregulation may reside when there is overlap in the release of both Gq-coupled excitatory transmitters with the VIP.

In gastric and intestinal smooth muscle cells, $VPAC_2$ receptors exhibit equal affinity for both VIP and PACAP. In tenia coli smooth muscle cells, however, VIP and PACAP interact with distinct receptors.[15] These cells express a VIP-specific receptor that does not recognize PACAP and a PACAP-specific receptor that does not recognize VIP. The VIP-specific receptor, but not the PACAP-specific receptor, is coupled to activation of adenylyl cyclase. The PACAP-specific receptor, but not the VIP-specific receptor, is coupled to the activation of apamin-sensitive K^+ channels.

The cDNA of the VIP-specific receptor cloned from guinea pig tenia coli encodes a 437-amino-acid protein with a calculated molecular weight of 49,560 Da.[35] All amino acid residues required for VIP binding including aspartate, tryptophan, and glycine residues (corresponding to D68, W73, and G109 of human $VPAC_1$ receptor) and all N-terminal cysteine residues are conserved. The amino acid

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sequence differs from that of the cloned guinea pig gastric receptor by only two amino acid residues (Phe^{40}/Phe^{41} in lieu of Leu^{40}/Leu^{41}) in the ligand-binding N-terminal, and showed 87% homology to the sequence of rat and mouse $VPAC_2$ receptors. Mutation of these two residues to Leu^{40}/Leu^{41} so as to mimic gastric VIP receptor results in an equal high affinity for VIP and PACAP suggesting that the ligand specificity in this species is determined by a pair of adjacent phenylalanine residues in the NH_2-terminal ligand-binding domain.[35]

BIOLOGICAL ACTION WITHIN THE GASTROINTESTINAL TRACT

The main function of gastrointestinal motility is to propel ingested food and to mix the food with digestive enzymes for optimal digestion and absorption and excretion of undigested waste. The process of propulsion is initiated by luminal signals generated by components released from chyme, by mechanical stimulation of mucosa, or by distention of the gut wall.[3,6,7] These stimuli trigger a propagated peristaltic reflex consisting of two components: a contraction orad (ascending contraction) and a relaxation caudad (descending relaxation) to the site of stimulation. The reflex is mediated wholly by neurons of the enteric nervous system that are contained within the wall of the gut. The reflex is initiated on activation of intrinsic sensory neurons and is relayed by interneurons to motor neurons, which mediate the final contraction of circular muscle orad and relaxation of circular muscle caudad (Fig. 1). The excitatory motor neurons release acetylcholine and tachykinins, and the relaxant or inhibitory motor neurons release VIP, PACAP, and NO. This role as main relaxant transmitter of inhibitory motor neurons represents the major physiological action of VIP with regard to gut smooth muscle function.

VIP as Mediator of Descending Relaxation of Smooth Muscle

Gastrointestinal motility is mediated by the contractile activity of the smooth muscle cells that line the gastrointestinal wall. This contractile activity is regulated by enteric neurons. Increased contractility is meditated by acetylcholine and tachykinins, and decreased contractility is mediated by VIP/PACAP/NO. Decreased contractility can be reflected in a variety of ways: relaxation of tone or inhibition of ongoing rhythmic activity depending on the distinct regional and functional properties of gut smooth muscle.[19] Smooth muscle of the proximal stomach and gall bladder exhibits sustained tone with some rhythmic contractions, whereas smooth muscle of the distal stomach, small intestine, and colon exhibits variable tone on which are superimposed rhythmic contractions. The smooth muscle of the sphincters, however, is purely tonic in nature.[27] For peristalsis to propel luminal content in an anal direction, not only must there be an increase in contraction orad to a luminal stimulus but there must also be a concomitant relaxation or inhibition of contractility caudad to the stimulus. This same mechanism comes into play as a peristaltic reflex reaches a tonically contracted sphincter where release of VIP in conjunction with NO acts to allow propulsion of luminal material from one section of the gut to another or during the expulsions of material from the gut.[27]

VIP, its homologous peptide transmitters, and NO fulfill the criteria for inhibitory transmitters: they are present in the neurons innervating the muscle of stomach, intestine, and various sphincters and are released by pharmacological and electrical field stimulation of myenteric neurons.[1,2,8,9,11] Additionally, their release is activated by physiological stimuli and is coupled to the peristaltic reflex and muscle relaxation in a variety of species including human.[11,12] Also, in support of a role as relaxant transmitter, exogenous addition of VIP, PACAP, or NO induces muscle relaxation mimicking the response induced by nerve stimulation.

VIP-containing neurons also innervate epithelial cells, exocrine glands, and other neurons of the gastrointestinal tract.[6] In addition to smooth muscle relaxation, the biological effects of VIP on gastrointestinal system include stimulation of water and electrolyte secretion, enzyme secretion, and mucus secretion, as well as inhibition of absorption and of acid secretion. Although not the focus of this chapter, the stimulation of these secretions facilitates the digestion and propulsion of chyme through the gut and results in a coordinated response of the gut to a meal.

Interplay of Inhibitory Neurotransmitters

The neuropeptides VIP and PACAP, and nNOS, the enzyme responsible for formation of NO by enteric nerves, are colocalized in a subset of myenteric neurons that innervate the muscle and their release is functionally linked.[3,6,7,27,30] VIP and PACAP are stored in vesicles in the nerve endings, whereas NO is synthesized on demand by the nNOS. NO formed in the nerve terminals by nNOS regulates VIP and PACAP release, and in turn, VIP and PACAP stimulate smooth muscle eNOS to generate NO within the smooth muscle.[12,13,24]

In isolated myenteric ganglia devoid of smooth muscle, the release of VIP and PACAP, and NO production are inhibited by the blockade of nNOS activity implying NO-dependent VIP and PACAP release.[12] Consistent with this notion, exogenous NO increases VIP and PACAP release, whereas exogenous VIP or PACAP had no effect on NO production. In contrast, in isolated smooth muscle cells devoid of neural elements, VIP and PACAP induce NO production.[13,23,31] The ability of NO to stimulate VIP and

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PACAP from nerve terminals, and the ability of VIP and PACAP to stimulate NO production in smooth muscle was evident in studies with innervated muscle preparations. In these preparations, nerve stimulation elicited stimulation of VIP (and PACAP) release, NO formation, and relaxation.[9] NOS inhibitors abolished NO formation and partly inhibited VIP release and relaxation, whereas VIP antagonist partly inhibited NO formation and relaxation. The ability of NOS inhibitors to block relaxation reflect inhibition of NOS in both myenteric neurons and smooth muscle cells, and inhibition of NO-dependent VIP and PACAP release from the myenteric neurons. The residual relaxation in the absence of NO production reflects the direct effect of VIP and PACAP that is independent of NOS activity in the smooth muscle. The ability of a VIP antagonist to block relaxation reflects suppression of VIP-induced stimulation of NOS and adenylyl cyclase activities in smooth muscle. The residual relaxation, in the absence of NO production and cAMP generation in smooth muscle, reflects the direct effect of NO derived from myenteric neurons on smooth muscle cells. The interplay between VIP and NO during relaxation can be summarized by the following results: (i) NO stimulates VIP from nerve terminals and VIP, in turn, stimulates NO formation in muscle, (ii) relaxation is mediated by NO and VIP release from nerve terminals and by NO formation in smooth muscle cells, and (iii) the effect of NOS inhibitors and VIP antagonists reflect their effects on both VIP release and NO formation.

Rattan et al.[4,5] have demonstrated that in the internal anal sphincter of the opossum VIP acts on both myenteric neurons and smooth muscle cells to generate NO. The major source of NO in response to VIP during relaxation, however, is from myenteric neurons with some contribution from the smooth muscle cells. Studies by Goyal et al.[20] using knockout animals have shown that VIP-induced hyperpolarization in the lower esophageal sphincter is abolished in nNOS deficient mice but is not affected in endothelial NOS deficient mice suggesting that nNOS is the major source of NO in the hyperpolarizing actions of VIP.

Mechanism of Smooth Muscle Relaxation Induced by VIP

VIP- and PACAP-induced activation of adenylyl cyclase by G_s-coupled $VPAC_2$ receptors and soluble guanylyl cyclase by $Gi_{1/2}$-coupled to NPR-C via NO generation leads to concurrent formation of cAMP and cGMP and activation of PKA and cGMP-dependent protein kinase (PKG). The intracellular levels of cAMP and cGMP, and the strength and duration of their physiologic effects depend on the rates of their synthesis by cyclases and degradation of phosphodiesterases (PDEs). The activity of these enzymes, in turn, is regulated by PKA and PKG, the downstream kinase of cAMP and cGMP. Cyclic AMP levels are regulated by feedback activation of cAMP-specific PDE3A and PDE4 and inhibition of adenylyl cyclase V/VI via PKA.[22] Cyclic GMP levels are regulated by feedback activation cGMP-specific PDE5 and inhibition of soluble guanylyl cyclase via PKG.[22] When there is concurrent generation of cAMP and cGMP, cGMP increases cAMP levels by inhibiting its degradation via PDE3A, whereas cAMP decreases cGMP levels by augmenting its degradation via PKA-mediated phosphorylation of PDE5.[21,22] Thus cAMP and cGMP levels are regulated by coordinated interplay between cyclases, phosphodiesterases, and protein kinases.

cGMP selectively activates PKG, whereas cAMP at lower concentrations activates PKA and at higher concentrations crossactivates PKG.[22] Autophosphorylation of PKG in the presence of cGMP greatly augments the affinity of cAMP for PKG. The ability of cAMP to crossactivate PKG is potentiated by concurrent generation of cGMP. Thus, concurrent generation of cAMP and cGMP and activation of PKA and PKG are the physiological norm in smooth muscle.

PKA and PKG act on several downstream targets in the cascades that mediate initial and sustained contraction, resulting in MLC_{20} (20-kDa regulatory light chain of myosin) dephosphorylation and muscle relaxation[22] (Fig. 3).

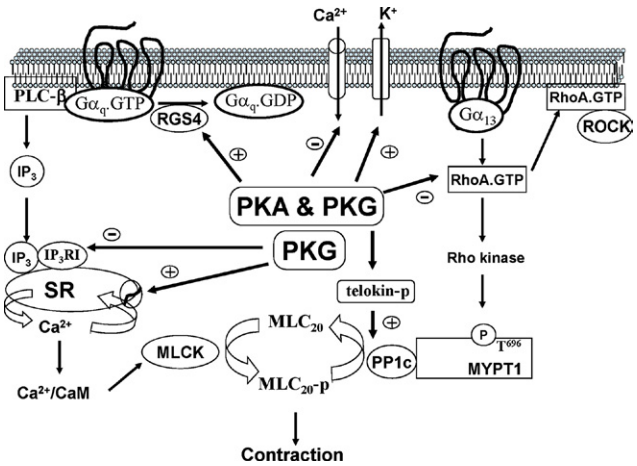

FIGURE 3 Downstream targets of PKA and PKG during VIP-induced relaxation of smooth muscle. PKG and PKA induce relaxation by targeting various components of the contractile signaling pathways. Both PKA and PKG phosphorylate RGS4 and stimulate inactivation of $G\alpha_q$, leading to inhibition of PLC-β1 activity and IP_3 formation. In addition, only PKG can phosphorylate IP_3R-I and the sarcoplasmic Ca^{2+}/ATPase pump, leading to inhibition of Ca^{2+} release and stimulation Ca^{2+} re-uptake. These mechanisms act to reduce intracellular Ca^{2+} and thus inhibit MLCK activity, MLC_{20} phosphorylation and initial contraction. Both PKA and PKG inactivate RhoA by phosphorylating RhoA at Ser^{188} leading to blockade of inhibition of MLC phosphatase via PKC/CPI-17 and Rho kinase/MYPT1. A distinct mechanism involves phosphorylation of telokin, an endogenous activator of MLC phosphatase, by both PKA and PKG leading to dephosphorylation of MLC_{20}.

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Relaxation of initial Ca^{2+}-dependent phase by PKA and PKG correlates with the decrease in $[Ca^{2+}]_i$, which is achieved by decreasing inositol 1,4,5-trisphosphate (IP_3) generation and/or decreasing IP_3-dependent Ca^{2+} release. Both PKA and PKG inhibit $G\alpha_q$-dependent phospholipase C (PLC)-β1 activity. However, neither $G\alpha_q$ nor PLC-β1 is directly phosphorylated by PKA or PKG. The mechanism of inhibition involves acceleration of $G\alpha_q$ inactivation by phosphorylation of two regulatory proteins, RGS4 (regulator of G-protein signaling 4) and GRK2 (G-protein-coupled receptor kinase 2). Phosphorylation of RGS4 and GRK2 increases their binding to $G\alpha_q$ and enhances the inactivation of $G\alpha_q$ leading to inhibition of PLC-β1 activity and IP_3 generation.[25] PKG, but not PKA, in addition, induces phosphorylation of type IP_3 receptors expressed in smooth muscle resulting in inhibition of Ca^{2+} release and relaxation of initial Ca^{2+}-dependent contraction[22] (Fig. 3).

Relaxation of sustained Ca^{2+}-independent phase of muscle contraction is mediated by disinhibition of myosin light chain (MLC) phosphatase via PKA- and PKG-mediated phosphorylation of membrane-bound RhoA at Ser^{188} [22] (Fig. 3). Phosphorylation of RhoA stimulates its translocation back to the cytosol and inhibits the activity of downstream membrane-bound targets, Rho kinase, and phospholipase D (PLD). Inhibition of Rho kinase blocks phosphorylation of the regulatory subunit of MLC phosphatase (MYPT1) at Thr^{696}, whereas inhibition of PLD results in the decrease in diacylglycerol formation, protein kinase C (PKC) activity and PKC-mediated CPI-17 (17-kDa endogenous inhibitor of MLC phosphatase) phosphorylation at Thr^{38}. Thus, the blockade of two pathways, Rho kinase/MYPT1 and PLD/PKC/CPI-17 that converge to inhibit MLC phosphatase leads to the activation of MLC phosphatase and dephosphorylation of MLC_{20} leading to relaxation of sustained Ca^{2+}-independent, RhoA-dependent contraction. Recent studies have shown that PKA and PKG can directly phosphorylate telokin, an endogenous 17-kDa activator of MLC phosphatase, which results in dephosphorylation of MLC_{20} and muscle relaxation[22] (Fig. 3).

PATHOPHYSIOLOGICAL IMPLICATIONS

The significance of the physiological actions of VIP was initially revealed with classical physiological and pharmacological approaches.[29] The importance of VIP as a mediator of gut relaxation is also confirmed by pathological studies and more recent transgenic studies. Thus, in a recent study of Lelievre et al.,[18] $VIP^{-/-}$ mice were shown to have a distinctive gut phenotype that included reduced goblet cell mucus secretion, increased thickness of the circular muscle layer, reduced intestinal length, and a significant 36% reduction in the ability of the intestine to propel luminal contents. The authors conclude that the decreased length is the result of a state of enhanced tonic contraction resulting from the loss of relaxant VIP-ergic tone and that the failure to propel intestinal contents is the result of the loss of the relaxation component of peristalsis. Consistent with this notion, 10–15% of the mice die within the first year and have "an abrupt blockade in movement of intestinal contents" and a site of gut stenosis. Similarly, a role for VIP as a major relaxant in normal physiology is suggested from finding in pathologies such as intestinal ileus, Hirschsprung's disease, Chagas' disease, and achalasia. These diseases have in common loss of inhibitory relaxant neurons, which contain VIP and NO leading to impaired motility and propulsion.[28,34] Similarly, in studies of small bowel transplants in Lewis rats,[32–34] there is a significant impairment of the intestinal motility, which is the result of two compounding effects: an enhancement of the excitatory component of the peristaltic reflex mediated by tachykinin and a decrease in the relaxation component mediated by VIP. Thus alterations in neuropeptide content in general and VIP in particular may play a role in the changes in gut function after small bowel transplant. Based on the primary role of VIP as a key relaxant transmitter and stimulant of intestinal secretion, a role of VIP in the etiology constipation and diarrhea in several conditions has been postulated. These include inflammatory bowel disease, irritable bowel syndrome, diabetes, and aging-related changes in gut motility. The results have been widely variable depending on species and region of study. For example, VIP levels have been reported to be increased in patients with IBS and decreased in pediatric patients with slow transit constipation. These changes are consistent with the established physiology of VIP as outlined in this chapter and likely point to a key role of VIP in the genesis of motility changes which are symptomatic of many gut pathologies.

ACKNOWLEDGMENTS

Supported by grants DK15564 and DK28300 (to K.S. Murthy), and DK34153 (to J.R. Grider) from the National Institute of Diabetes, and Digestive and Kidney Diseases.

REFERENCES

1. Biancani P, Walsh JH, Behar J. Vasoactive intestinal peptide. A neurotransmitter for lower esophageal sphincter. *J Clin Invest* 1985;**73**:963–7.
2. Bitar KN, Makhlouf GM. Relaxation of isolated gastric smooth muscle cells by vasoactive intestinal peptide. *Science* 1982;**216**:531–3.
3. Bornstein JC, Costa M, Grider JR. Enteric motor and interneuronal circuits controlling motility. *Neurogastroenterol Motil* 2004;**16**:34–8.
4. Chakder S, Rattan S. Distribution of VIP binding sited in opossum in anal sphincter circular smooth muscle sites of actions. *J Pharmacol Exp Ther* 1995;**272**:385–91.
5. Chakder S, Rattan S. Evidence for VIP-induced increase in NO production in myenteric neurons of opossum internal anal sphincter. *Am J Physiol* 1996;**270**:G492–7.

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6. Furness JB, Young HM, Pompolo S, Bornstein JC, Kunze WAA, McConalogue K. Plurichemical transmission and chemical coding of neurons in the digestive tract. *Gastroenterology* 1995;**108**:554–63.
7. Goyal RK, Hirano L. The enteric nervous system. *New Engl J Med* 1996;**334**:1106–15.
8. Goyal RK, Rattan S, Said SI. VIP as a possible neurotransmitter of non-cholinergic and non-adrenergic inhibitory neurons. *Nature* 1980;**288**:378–80.
9. Grider JR. Interplay of the inhibitory neurons in the regulation of VIP release and NO production during peristalsis. *Am J Physiol* 1994;**267**:G696–701.
10. Grider JR. Reciprocal activity of longitudinal and circular muscle during intestinal peristaltic reflex. *Am J Physiol* 1994;**284**:G768–75.
11. Grider JR, Cable MB, Bitar KN, Said SI, Makhlouf GM. Vasoactive intestinal peptide: relaxant neurotransmitter in tenia coli of the guinea pig. *Gastroenterology* 1985;**89**:36–42.
12. Grider JR, Jin JG. VIP release and L-citrulline production from isolated ganglia of the myenteric plexus: evidence for regulation of VIP release by NO. *Neuroscience* 1993;**54**:521–6.
13. Grider JR, Murthy KS, Jin J-G, Makhlouf GM. Postjunctional stimulation of nitric oxide in muscle cells by the relaxant neurotransmitter VIP. *Am J Physiol* 1992;**262**:G774–8.
14. Harmar AJ, Arimura A, Gozes I, Journot L, Laburthe M, Pisegna JR, et al. International union of pharmacology. XVIII. Nomenclature of receptors for vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide. *Pharmacol Rev* 1998;**50**:265–70.
15. Jin J-G, Katsoulis S, Schmidt WE, Grider JR. Inhibitory transmission in tenia coli mediated by distinct vasoactive intestinal peptide and apamin-sensitive pituitary adenylyl cylcase activating peptide. *J Pharmacol Exp Ther* 1994;**270**:433–9.
16. Huang J, Mahavadi S, Grider JR, Murthy KS. Cross-regulation of $VPAC_2$ receptor desensitization by m3 receptors via PKC-mediated phosphorylation of RKIP and inhibition of GRK2. *Am J Physiol Gastrointest Liver Physiol* 2007:G867–74.
17. Laburthe M, Couvineau A, Voisin T. Receptors for peptides of the VIP/PACAP and PYY/NPY/PP. *Gastrointest Endocrinol* 1999;**9**:125–57.
18. Lelievre V, Favrais G, Abad C, Adle-Biassette H, Lu Y, Germano PM, et al. Gastrointestinal dysfunction in mice with a targeted mutation in the gene encoding vasoactive intestinal peptide: A model for the study of intestinal ileus and Hirschsprung's disease. *Peptides* 2007;**28**:1688–99.
19. Makhlouf GM. Smooth muscle of the gut. In: Yamada T, editor. *Textbook of Gastroenterology*. 4th ed. Philadelphia: Lippincott Williams and Wilkins; 2009, [Chapter 6], p. 103–32.
20. Mashimo H, He XD, Huang PL, Fishman MC, Goyal RK. Neuronal constitutive nitric oxide synthase is involved in murine enteric inhibitory neurotransmission. *J Clin Invest* 1996;**98**:8–13.
21. Murthy KS. Activation of PDE5 and inhibition of guanylyl cyclase by cGMP-dependent protein kinase in smooth muscle. *Biochem J* 2001;**360**:199–208.
22. Murthy KS. Signaling for contraction and relaxation in smooth muscle of the gut. *Annu Rev Physiol* 2006;**68**:345–74.
23. Murthy KS, Makhlouf GM. VIP/PACAP-mediated activation of membrane-bound NO synthase in smooth muscle is mediated by pertussis toxin-sensitive G_{i1-2}. *J Biol Chem* 1994;**269**:15977–80.
24. Murthy KS, Makhlouf GM. Identification of the G protein-activating domain of the natriuretic peptide clearance receptor (NPR-C). *J Biol Chem* 1999;**274**:17,587–92.
25. Murthy KS, Mahavadi S, Huang J, Zou H, Sriwai W. Phosphorylation of GRK2 by PKA augments GRK2-mediated phosphorylation, internalization, and desensitization of $VPAC_2$ receptors in smooth muscle. *Am J Physiol Cell Physiol* 2008;**294**:C477–87.
26. Okamoto T, Nishimoto I. Detection of G protein-activator regions in m4 subtype muscarinic, cholinergic, and α2 adrenergic receptors based on upon characteristics in primary structure. *J Biol Chem* 1992;**267**:8342–6.
27. Rattan S. The internal anal sphincter: regulation of smooth muscle tone and relaxation. *Neurogastroenterol Motil* 2005;**17**(Suppl. 1):50–9.
28. Rivera LR, Poole DP, Thacker M, Furness JB. The involvement of nitric oxide synthase neurons in enteric neuropathies. *Neurogastroenterol Motil* 2011;**23**:980–88.
29. Said SI. Vasoactive intestinal peptide. Biologic role in health and disease. *Trends Endocrinol Metab* 1991;**2**:107–12.
30. Shah V, Lyford G, Gores G, Farrugia G. Nitric oxide in gastrointestinal health and disease. *Gastroenterology* 2004;**126**:903–13.
31. Teng B-Q, Murthy KS, Kuemmerle JF, Grider JR, Michel T, Makhlouf GM. Constitutive endothelial nitric oxide synthase: expression in human and rabbit gastrointestinal smooth muscle cells. *Am J Physiol* 1998;**275**:G342–51.
32. Tomita R, Fujisaki S, Park E, Kimizuka K, Ikede T, Koshinaga T, et al. Physiological studies on vasoactive intestinal peptide in rat small bowel isografts. *Transplant Proc* 2004;**36**:367–9.
33. Tomita R, Fujisaki S, Park E, Ikede T, Koshinaga T. Substance P and vasoactive intestinal peptide in rat small bowel isografts. *Am J Surg* 2003;**189**:63–70.
34. Tomita R, Morita K, Tanjoh K, Munakata K. A role of peptidergic nerves in the internal anal sphincter of Hirschsprung's disease. *Surgery* 1999;**125**:60–6.
35. Zhou H, Huang J, Murthy KS. Molecular cloning and functional expression of a VIP-specific receptor. *Am J Physiol Gastrointest Liver Physiol* 2006;**291**:G728–34.

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Chapter 185

Adrenomedullin Peptides

Johji Kato and Kazuo Kitamura

ABSTRACT

Isolated first from human pheochromocytoma, adrenomedullin (AM) is a 52 amino acid peptide with pleiotropic cardiovascular actions. Sequence analysis of the AM precursor preproAM revealed that proadrenomedullin N-terminal 20 peptide (PAMP) is processed from the N-terminal of proAM. AM belongs to the calcitonin-gene-related peptide (CGRP) superfamily, and the discovery of AM led to the identification of another member of this peptide family, AM2/intermedin. Numerous basic and clinical studies have been carried out to explore the actions or roles of AM and AM-related peptides in the heart and vasculature. As a result, AM has been shown to play an important role in cardiac or vascular tissues as a local modulator through pleiotropic actions mostly protective of the cardiovascular system. Based also on clinical studies, AM is assumed to be beneficial as a diagnostic or prognostic marker or as a therapeutic tool for cardiovascular diseases.

DISCOVERY AND THE PEPTIDE SEQUENCE OF AM AND RELATED PEPTIDES

In 1993, a biologically active peptide was isolated from the acid extract of human pheochromocytoma tissue by monitoring of the elevation of cAMP levels in rat platelets.[13] This bioactive peptide was named "adrenomedullin" because it was found to be abundantly present in the human adrenal medulla and in pheochromocytoma.[13] Human adrenomedullin (AM) consists of 52 amino acids with a ring structure formed by a disulfide bond and an amidated tyrosine at the C-terminal. The amino acid sequence of AM resembles those of calcitonin-gene-related peptide (CGRP) and amylin, belonging to the CGRP superfamily (Fig. 1). Sequence homology among these bioactive peptides is relatively low, but they share two molecular structures: a disulfide bond and an amidated C-terminal, both of which are essential for exerting biological actions.[3,10,12] In 2004, two independent research groups discovered another member peptide of the CGRP superfamily by searching the genomes of humans and other vertebrates.[31,34] The two groups named this 47-amino-acid peptide adrenomedullin-2 (AM2) and intermedin, respectively. As shown in Table 1, AM has a wide range of actions on the cardiovascular system, as described later in this chapter. The biological property of AM2/intermedin has also been examined since this discovery. According to the findings so far reported, the biological effects of this family peptide are mostly similar to those of AM, while some differences in potency or receptor selectivity have been detected, raising the possibility for a receptor or intracellular signaling system differing from those of AM.[5] AM2/intermedin is further discussed in the Renal Peptides section of this book.

STRUCTURE OF THE mRNA/GENE AND PROCESSING OF THE PRECURSOR

Cloning of the cDNA encoding AM reveals that the AM precursor peptide preproAM consists of 185 amino acids, with the C-terminal followed by a pair of basic amino acids, Arg–Arg, a typical processing signal (Fig. 2).[12] In addition to AM, preproAM contains another bioactive peptide, proadrenomedullin N-terminal 20 peptide (PAMP), in the N-terminal portion.[12] PAMP lowers blood pressure when injected intravenously, probably by the inhibition of catecholamine release from the sympathetic nerve endings, but its hypotensive action is weaker than that of AM, and there is little information available as to the role of PAMP in cardiovascular diseases.[12] In sequence analysis of genomic DNA for human preproAM, AM is encoded in the fourth exon and PAMP in the second and third exons (Fig. 2).[12] When processed from preproAM, AM-Gly, an intermediate form (iAM), is produced, and then iAM is converted by the amidation enzyme into the mature form of AM (mAM) having an amide structure at the C-terminal.[14] The mature form of PAMP is believed to be produced by a similar process (Fig. 2).

Various humoral or mechanical factors are involved in regulating the expression of the AM gene. In the 5′-flanking region of the genomic AM gene of humans, there are many consensus sequences of binding sites for transcription factors including NF-κB, ADRE-AR, AP-1, AP-2, C/EBP, Sp1, HIF-1, and NF-IL6.[17] Both the NF-IL6 and AP-2 sites are

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00185-8
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Adrenomedullin   H2N-YRQSMNNFQGLRSFGCRFGTCTVQKLAHQIYQFTDKDKDNV-APRSKISPQGY-CONH2

CGRP                      H2N-ACDTATCVTHRLAGLLSRSGGVVKNNF-VPTNVGSKA-F-CONH2
                               *   **    **          * *   *    *

Amylin                    H2N-KCNTATCATQRLANFLVHSSNNFGAIL-SSTNVGSNT-Y-CONH2
                               *   **  * **                     *   *

Adrenomedullin-2/
Intermedin          H2N-TQAQLLRVGCVLGTCQVQNLSHRLWQLMGPAGRQDSAPVDPSSPHSY-CONH2
                          *   **  *** ** * *   *          **    **  *
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FIGURE 1 Amino-acid sequences of human adrenomedullin (AM), calcitonin-gene-related peptide (CGRP), amylin, and AM2/intermedin. Asterisks indicate amino acids identical to those of AM.

TABLE 1 Biological Actions of AM-related to Cardiovascular Functions

Systemic actions	Reduction of blood pressure
	Suppression of oxidative stress
	Anti-inflammation
Blood vessels	Vasodilatation
	Stimulation of nitric oxide (NO) production
	Inhibition of apoptosis of vascular endothelial cells
	Inhibition of proliferation and migration of smooth muscle cells
	Inhibition of adventitial fibrosis
	Angiogenesis and lymphoangiogenesis
Heart	Inhibition of myocardial hypertrophy and fibrosis
	Positive or negative inotropic action
Kidney	Diuresis and natriuresis
	Inhibition of proliferation of mesangial cells
Lung	Pulmonary vasodilatation
	Bronchodilation
Endocrine system	Inhibition of aldosterone production
	Inhibition of ACTH and vasopressin secretion
Brain	Reduction of water and salt intake

essential for promoter activity in human vascular endothelial cells, which actively produce and secrete the AM peptide. The transcriptional factor NF-IL6 plays an important role in mediating inflammation by inducing acute phase proteins. Consistent with this, AM production is augmented by such inflammatory cytokines as interleukin-1 and tumor necrosis factor-α.[10] In cardiac myocytes, heterodimetric binding of α and β subunits of hypoxia-inducible factor-1 (HIF-1) protein to the HIF-1 site is assumed to be a mechanism for augmented AM production under hypoxic conditions.[11] Thus, various transcriptional mechanisms, which may differ depending on species, tissue, or type of cell, seem to be involved in controlling the expression of the AM gene.

DISTRIBUTION OF THE mRNA/PEPTIDE AND AM CIRCULATING IN THE BLOOD STREAM

Although isolated from pheochromocytoma tissue, substantial levels of preproAM gene expression are present in the cardiovascular organs or tissues, including cardiac atria and ventricles, blood vessels, kidneys, and lungs, where the AM peptide has been detected by radioimmunoassay and immunohistochemial methods.[3,10,12] In addition, various types of cultured cells isolated from these cardiovascular tissues express preproAM mRNA and secrete the AM peptide from the cells.[3,10–12] Despite the substantial expression of preproAM mRNA, the AM peptide concentrations in these tissues or cultured cells are relatively low, suggesting constitutive secretion of AM with little intracellular storage.[3,10–12]

Because AM circulates in human plasma at concentrations in the order of femtomoles per milliliter, efforts were made to identify the organ or tissue contributing to the AM peptide in human blood. We measured plasma AM concentrations in the adrenal vein as AM is abundantly detected in the adrenal medulla, but found no step-up compared with that of the inferior vena cava.[9] Moreover, no site in the blood vessels showing higher plasma AM concentrations than others could be detected in patients with ischemic heart disease.[9] Meanwhile, active production and secretion are observed in cultured cells derived from the cardiovascular tissues, such as cardiomyocytes and vascular endothelial cells.[10,11] According to our previous study of patients with ischemic heart disease, significant step-ups are observed in plasma mature AM (mAM) concentrations between the femoral artery and vein and between the aortic root and coronary sinus, suggesting AM secretion from the vasculature

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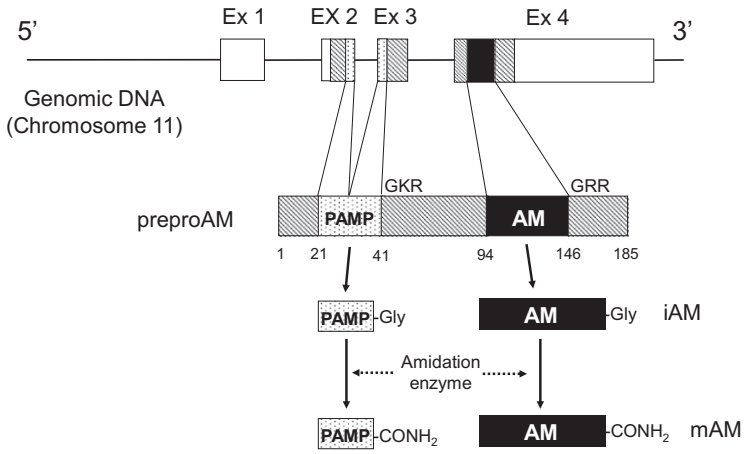

FIGURE 2 The AM gene and processing of AM and PAMP from preproAM. Ex, exon; PAMP, proadrenomedullin N-terminal 20 peptide; iAM and iPAMP, intermediate forms of AM and PAMP; mAM and mPAMP, mature forms of AM and PAMP.

and heart in humans.[10,11] In addition, either adipocytes or adipose tissue are recognized to be significant sources of circulating AM in humans.[9] Thus, various organs or tissues, including cardiovascular tissues, may contribute to plasma AM in humans.

We were able to measure mAM and total AM (mAM plus iAM) concentrations separately, showing the presence of both molecular forms in the plasma and tissues of humans and rats.[14] As mentioned earlier, the amidated C-terminal is essential for the biological action of AM; therefore, iAM itself is inactive. In human plasma, the concentration of mAM is lower than that of iAM; however, according to the *ex vivo* experiment, iAM exerts vasodilator action after conversion to mAM in blood vessels, raising the possibility of iAM serving as a reservoir hormone in plasma or tissues.[10,14] The ratios of mAM to iAM or to total AM concentrations have been examined in healthy volunteers and in patients with cardiovascular diseases, but there is no condition clearly showing a preferential increase of mAM concentrations as compared with that of iAM.

RECEPTOR AND SIGNALING CASCADES

AM was first isolated by the monitoring of an elevation of cAMP in rat platelets,[13] and indeed, an AM-induced increase in intracellular cAMP levels is widely seen in many tissues and cells derived from the cardiovascular system, while there had been controversy over the receptor subtypes of AM. In 1998, McLatchie et al. identified three subtypes of receptor-activity-modifying protein (RAMP1–3), an accessory protein required for the transport of calcitonin-receptor-like receptor (CLR) to the cell membrane.[22] This discovery reveals the receptor system for CGRP and AM: CLR can function as either an AM receptor or a CGRP receptor, depending on the subtype of RAMP expressed. CLR serves as a CGRP receptor when coexpressed with RAMP1, while it functions as an AM type 1 or 2 (AM1 or AM2) receptor when coexpressed with either RAMP2 or 3, respectively.[22] These receptor components are widely expressed in various tissues and organs, including those of the cardiovascular system.[15,22] The CGRP receptor of CLR/RAMP1 heterodimer has a high affinity for CGRP, whereas AM can interact with this receptor at a high concentration of peptide.[15] The AM1 receptor (CLR/RAMP2) is highly specific to AM in humans and rats, but in contrast, AM and AM2/intermedin show similar affinities to the AM2 receptor (CLR/RAMP3).[15]

Both the AM1 and AM2 receptors are linked to the intracellular system of cAMP generation and phosphatidylinositol (PI) hydrolysis, which may activate a PKA- or calcium-dependent pathway, respectively.[15,22] As shown in Table 1, AM has a wide range of cardiovascular effects, which seem to be mediated by different intracellular signaling systems depending on the type of cell or tissue. A study using RAMP2-transgenic mice revealed that the inhibitory effects of AM on vascular hypertrophy and inflammation are mediated by the AM1 receptor, CLR/RAMP2 heterodimer,[18] while there is little information on the role of the AM2 receptor in mediating the cardiovascular protective actions of AM. Interestingly, as discussed later, the generation of gene-manipulated animals reveals that RAMP2 is essential for the angiogenesis action of AM, and RAMP3 is unable to compensate for the role of RAMP2. Meanwhile, there is no report showing distinct intracellular signaling for RAMP2 and RAMP3,[15,22] suggesting an unknown intracellular mechanism mediated by the AM1 receptor.

There are many reports about the intracellular signaling systems for AM in the cardiovascular system, particularly those in the vascular endothelium and ventricular myocardium.[3,10–12,15] Acting on vascular endothelial cells, AM seems to activate phospholipase C, forming inositol

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1,4,5-triphosphate, which stimulates nitric oxide (NO) production by endothelial NO synthase (eNOS). It is also probable that eNOS is activated by AM via the phosphatidylinositol 3′-kinase (PI3K)/Akt-dependent pathway. Then, generated NO activates soluble guanylate cyclase in vascular smooth muscle cells, leading to increased intracellular cGMP and vasodilatation.[10] In contrast, AM has been shown to induce endothelium-independent vasodilatation via the elevation of intracellular cAMP levels in vascular smooth muscle cells by directly acting on those cells.[10]

AM has direct actions on the heart myocardium.[11] *In vitro* experiments reveal that AM inhibits the hypertrophy of cardiomyocytes and the proliferation and collagen synthesis of cardiac fibroblasts, a finding supported by an *in vivo* study using heterozygotes of AM gene knockout mice.[29] The inhibitory action of AM on proliferation and collagen synthesis in cardiac fibroblasts is mediated by a cAMP-dependent pathway, while the mechanism of action in cardiomyocytes appears to be independent of cAMP and remains to be elucidated.[11] AM also affects contractility of the myocardium. AM induces reduction of the contractility and calcium concentration in rabbit cardiomyocytes, an effect mediated by the NO-cGMP pathway; AM also exerts a cAMP-independent positive inotropic action of AM in rat hearts.[11] Thus, it is probable that the intracellular mechanism by which AM exerts cardiovascular actions differs depending on the species, organ, tissue, and type of cell.

BIOLOGICAL ACTIONS WITHIN THE CARDIOVASCULAR SYSTEM

Cardiovascular Protective Effects

The biological actions of AM involved in cardiovascular functions are listed in Table 1. Not all the effects in this table have been proved to occur at a physiological concentration of AM, although AM exerts a wide range of actions in various organs and tissues, which are mostly protective of the cardiovascular system.[3,10,12] To investigate whether AM exerts cardiovascular actions *in vivo*, three experimental approaches have so far been adopted: long-term administration of AM, virally mediated overexpression of AM, and genetic manipulation of the AM gene.

Using the first method, Nishikimi and colleagues showed that in Dahl-salt-sensitive hypertensive rats fed a high-salt diet, AM has a minimal effect on blood pressure, but significantly inhibits left ventricular hypertrophy (LVH) and the transition from LVH to heart failure.[9,28] Long-term infusion of AM also lessens pulmonary congestion and the deterioration of left ventricular (LV) function, improving the survival of this animal model of hypertension and heart failure. The inhibitory effect of AM on LVH without a significant reduction of blood pressure suggests a possibly direct action on cardiac myocytes. Either preload or afterload on the heart results not only in LVH but also in cardiac fibrosis, where excessive fibroblast proliferation and collagen deposition occur, leading to impairment of diastolic dysfunction. To test the antifibrotic action of AM, we infused recombinant human AM into a rat model of acute myocardial infarction (MI), showing the reduction of myocardial collagen deposition after MI.[9] In addition, long-term administration of AM reduces perivascular fibrosis of the coronary arteries in rats with hypertension induced by chronic infusion of angiotensin II.[10] This effect is accompanied by the suppression of fibroblast activation and transforming growth factor-β1 expression, but not by a significant reduction of blood pressure, suggesting an AM action independent of hemodynamic factors. Renoprotective effects of AM have also been implied by *in vitro* experiments. Long-term infusion of AM reduces glomerular and arterio-arteriolar scleroses in Dahl-salt-sensitive rats given a high-salt diet and in spontaneously hypertensive rats (SHR) administered deoxycorticosterone acetate (DOCA) and salt.[9,27] As a result, urinary protein excretion is reduced and creatinine clearance preserved by AM infusion. These renoprotective effects occur without a significant reduction of blood pressure, suggesting direct actions of AM on the kidneys. Increased urinary excretion of cAMP and cGMP suggests roles for these cyclic nucleotides in mediating the actions of AM, but the detailed mechanisms involved remain to be specified. Regardless of the mechanisms, the plasma concentrations of exogenous AM lie within the physiological range in the above-mentioned long-term infusion studies, where 0.2–1.0 μg/h AM was used.[9] It is therefore possible that endogenous AM functions to inhibit cardiac and renal damage in rat models of cardiovascular and renal diseases.

Chao et al. showed that adenovirus-mediated systemic delivery of the human AM gene ameliorated cardiac and renal damage in rats with DOCA salt-induced and 2 kidney–1clip renovascular hypertension and in Dahl-salt-sensitive hypertensive rats fed a high-salt diet.[2] Similar to the results of long-term infusion experiments, the overexpression of AM lessened cardiac hypertrophy and fibrosis and glomerular sclerosis, improving urinary protein excretion and creatinine clearance. Urinary excretion of cAMP is increased by overexpression, whereas systolic blood pressure is reduced by about 20–30 mmHg; therefore, blood pressure-dependent mechanisms may have contributed to the beneficial effects of AM. In addition to systemic overexpression of the AM gene, the same group reported that the local delivery of AM to balloon-injured carotid artery inhibits neointimal formation in rats.[2] Leskinen et al.[16] showed that adenovirus-mediated AM gene delivery elevates AM

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mRNA expression by 20.9-fold, attenuating angiotensin II-induced LVH. Meanwhile, they observed deteriorated LV function by the overexpression of AM, dilating the left ventricle and thinning the anterior wall in rats with myocardial infarction, raising the possibility that protective effects depend on the level of overexpressed AM or the time of delivering the gene in myocardial infarction.

Third, cardiovascular and renal protective effects have been suggested by genetic manipulation of the AM gene in mice. Shimosawa et al. showed that, when given angiotensin II and excessive salt, heterozygotes of AM knockout mice (AM+/−) show more severe perivascular fibrosis and intimal thickening of coronary arteries than do their wild-type littermates, despite a similar elevation of blood pressure between knockout and wild-type mice.[32] Based on increases in the production of reactive oxygen species (ROS) and in NAD(P)H oxidase expression in AM knockout mice, the possibility of augmented oxidative stress was raised as the mechanism responsible for the severe vascular lesions. Periarterial cuff-induced intimal thickening of the femoral artery is also more severe in knockout mice compared with that in control mice.[10,32] Enhanced neointimal formation is reversed by delivery of the AM gene and by an NAD(P)H oxidase inhibitor or tempol, a superoxide dismutase mimetic, further suggesting an inhibitory action of endogenous AM on oxidative stress. Niu et al. subjected heterozygotes of another strain of AM gene knockout mice to the cardiovascular stress of aortic constriction or angiotensin II infusion, resulting in LVH and perivascular fibrosis, changes that were more pronounced in knockout mice than in controls.[29] In addition, renal damage characterized by reduced creatinine clearance and glomerular sclerosis is worse in knockout mice than in controls. Caron et al. showed exacerbates LVH and renal damage in male mice with one copy of the AM gene, where AM mRNA is reduced by 50% in kidneys, but not in females with the same transgene, raising the possibility of a gender difference in AM effects.[1]

Effects on Pulmonary Vascular Bed

AM exerts a protective action on the pulmonary vascular bed.[24] In addition to the pulmonary vasodilator effect, prolonged subcutaneous infusion of AM inhibits medial thickening of the pulmonary artery of rats with pulmonary hypertension induced by monocrotaline; however, when infused intravenously, AM lowers not only pulmonary artery pressure but also systemic blood pressure. In an attempt to avoid an effect on the systemic circulation, Nagaya et al. administered AM as an aerosol with an ultrasonic nebulizer in rats with pulmonary hypertension.[24] Repeated inhalation effectively inhibits medial thickening of pulmonary arteries, reducing pulmonary artery pressure and total pulmonary resistance, without affecting systemic arterial pressure or the heart rate. Further, the same group reported that in patients with idiopathic pulmonary arterial hypertension, inhalation of AM lowers pulmonary artery pressure and resistance, improving exercise tolerance. Although the long-term outcomes of these beneficial actions need to be examined, this approach seems promising for the use of AM in the treatment of primary pulmonary hypertension, for which few effective medical treatments are currently available.

Angiogenesis Effects

A cardiovascular action recently described not only for AM but also PAMP is angiogenesis or lymphoangiogenesis.[21,25] In a mouse model of hind-limb ischemia, heterozygotes of AM knockout mice (AM+/−) showed impaired blood flow recovery with less collateral capillary development as compared with that of their wild-type littermates.[6] According to recent reports of gene manipulation, the AM-CRL/RAMP2 signaling system is essential for lymphatic vascular development: homozygotes of AM-, CRL-, or RAMP2-knockout mice (AM−/−, CRL−/−, RAMP2−/−) are all embryo lethal because of impaired lymphoangiogenesis.[4] Similar to AM, AM2/intermedin has angiogenesis action, an effect assumed to be mediated by the Akt- and NO-dependent pathway.[33] The next question is why the AM2/intermedin system is unable to compensate for deletion of the AM gene, and this important issue needs to be addressed in future research. In contrast to the loss of function experiments for angiogenesis, overexpression of the AM gene is beneficial in animal models of ischemic cardiovascular diseases, enhancing the angiogenesis action. For example, in a mouse model of chronic cerebral hypoperfusion, overexpression of AM results in faster cerebral perfusion recovery, lessening the decline of cognitive function.[20] These findings suggest that augmentation of the actions of AM is a therapeutic strategy for ischemic cardiovascular or cerebrovascular diseases.

Angiogenesis is also an important process for the growth of solid tumors. Several reports show the expression of AM receptor components of CLR and RAMPs and production of the AM peptide in various tissues and cells including vascular endothelium and tumor cells. Martinez et al. carried out a series of experiments in which PAMP promotes angiogenesis even more potently than AM, increasing migration and cord formation of human microvascular endothelial cells.[21] In addition, they showed that PAMP(12–20), a PAMP antagonist, inhibits tumor growth by a xenograft model. The intracellular signaling system for angiogenesis actions of PAMP remains to be clarified, although those of AM seem to be mediated by the PI3K/Akt cascade.[6] It is possible that, in contrast to ischemic cardiovascular diseases, blocking the actions of AM or PAMP

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can be a therapeutic strategy to suppress the progression of malignant tumor.[6,21]

PATHOPHYSIOLOGICAL IMPLICATIONS

Tissue Expressions of AM and Receptors

The AM peptide is present at substantial levels in the cardiovascular or renal tissues, where the AM receptor components CLR and RAMPs are expressed.[3,10,12,15,22] Cultured cells isolated from those tissues actively secrete AM. Based on these findings, AM is assumed to function as a local regulator or modulator acting in an autocrine or paracrine manner in cardiovascular or renal tissues. To investigate the pathophysiological roles of AM, the tissue concentrations and gene expressions of AM have been examined in human patients or animal models of cardiovascular diseases.[9,28] In heart tissues, the results of these studies share a common finding of changes in the AM and its receptor system: the expression levels of mRNA for preproAM, CLR, RAMP2, or RAM3 are upregulated in the cardiac ventricles of a hypertrophied or failing heart of hypertension, heart failure, or myocardial infarction.[9,15,28] In addition to the heart, AM levels were shown to increase in atherosclerotic coronary plaques of unstable angina pectoris or renal medulla of rats with severe hypertension.[10,27] Taking the cardiovascular or renal protective effects of AM into consideration, these results suggest the roles of endogenous AM and its effecter system in acting against deterioration of the heart or renal diseases.

The mechanisms for augmented AM production in the cardiovascular tissues are not totally understood, but we can discuss this important issue based on the findings from cell culture studies.[10,11] According to our previous studies, AM production in cultured cardiomyocytes and cardiac fibroblasts is increased by the pressor peptides angiotensin II and endothelin-1 or simply by mechanical stretching of the cells.[11] The production of AM in cultured cardiomyocytes is also increased by hydrogen peroxide (H_2O_2), suggesting the involvement of oxidative stress. The production of AM by cultured mesangial cells and vascular smooth muscle cells is increased in response to such humoral factors or cytokines as angiotensin II, aldosterone, interleukin-1β, and tumor necrosis factor-α.[9,10] In vascular endothelial cells, AM production is modulated by glucocorticoid or the alteration of shear stress.[10] Thus, a number of factors seem to be involved in augmented AM production in cardiovascular and renal diseases.

Plasma AM Level as a Diagnostic Marker

To clarify the roles of AM in humans, plasma levels of the peptide have been measured in various cardiovascular diseases, such as hypertension, heart failure, acute MI, and peripheral vascular diseases.[3,9,10,12] There may be some differences in the mechanisms by which plasma AM levels were elevated, depending on the diseases, although a consistent finding is that the more severe the cardiovascular disease is, the higher the level of plasma AM.[3,9,10,12] For example, in patients with chronic heart failure, the plasma level of AM is elevated progressively in proportion to the severity of the New York Heart Association (NYHA) functional class.[3,9] In addition, plasma AM is closely correlated with other parameters indicative of disease severity, including LV ejection fraction and LV end-diastolic pressure. It is intriguing to see whether elevated AM is reduced by successful treatment for the above-mentioned cardiovascular diseases. Plasma AM levels in patients with essential hypertension remained unchanged after short- or long-term antihypertensive treatment, and instead, these levels are closely correlated with target organ damage.[9] Meanwhile, in patients with malignant hypertension or acute heart failure, the elevated concentrations of plasma AM are reduced after successful treatment. To see whether increased plasma AM precedes elevation of blood pressure, we conducted a prospective, cohort study with normotensive local residents.[8] As shown in Fig. 3, the rate of the future development of hypertension in normotensive residents with plasma AM concentrations above the median value is higher than in those with lower levels. Both atrial and brain natriuretic peptides (ANP and BNP) are endogenous, blood pressure-lowering hormones, but unlike AM, no such difference was noted between high and low ANP or BNP groups.

Many prospective studies have shown diagnostic or prognostic values of measuring plasma levels of AM or AM-related peptides in patients with cardiovascular diseases. According to the report by Richards and coworkers, the plasma AM level has a greater power in predicting the mortality and hospitalization of patients with chronic ischemic LV dysfunction than does LV ejection fraction.[30] In this study, carvedilol, a beta-adrenergic blocker, reduced mortality in patients with higher AM than the median value but not in those with lower AM. Plasma AM levels may predict LV reverse remodeling after cardiac resynchronization therapy (CRT): patients with higher plasma AM show an improvement of the NYHA functional class after CRT, but those with lower AM do not.[23] In a cohort of high-risk patients with multiple risk factors without heart failure, AM has superior power in predicting future cardiovascular events compared with C-reactive protein or adiponectin.[26] Midregional proAM (MR-proAM), a stable fragment peptide derived from the N-terminal portion of the proAM peptide, has been examined for diagnostic or predictive values in patients with heart failure or acute MI. Similar to AM, plasma MR-proAM levels are increased in relation to the severity of heart failure, providing prognostic information about the patients.[35] According to the Biomarkers in Acute Heart Failure (BACH) study, MR-proAM, like BNP, is useful in diagnosing acute

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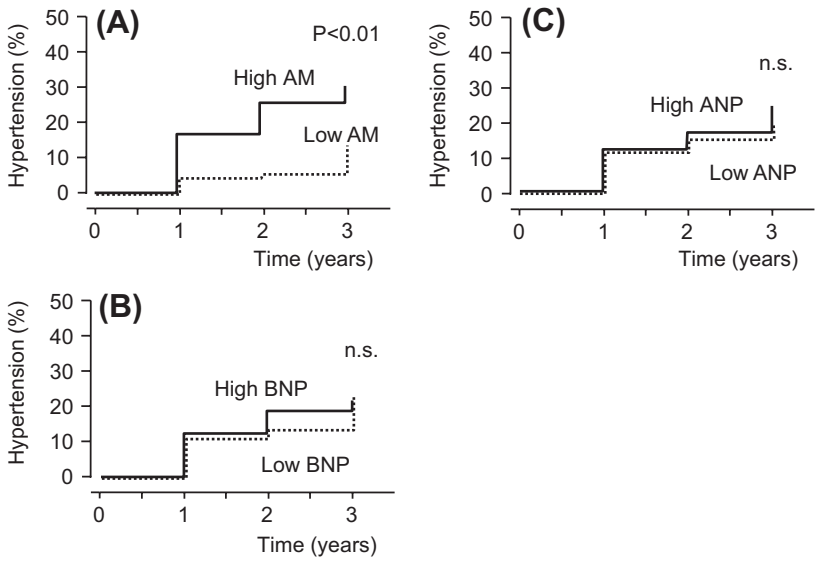

FIGURE 3 Development of hypertension in groups with high and low plasma levels of AM (A), ANP (B) and BNP (C) during a 3-year follow-up period. Data were analyzed by the Kaplan–Meier method and log-rank test. n.s., Not significant. Reprinted from Ref. 8.

heart failure in patients with acute dyspnea, providing a prognostic value to predict the 90-day survival of those patients.[19] Thus, MR-proAM, as well as AM itself, seems to be a useful biomarker of diagnostic or prognostic values for patients with cardiovascular diseases, particularly for those with heart failure.

AM as a Possible Therapeutic Tool

Lastly, we discuss the AM effects in healthy volunteers and patients with cardiovascular diseases based on the results of clinical studies performed to verify the cardiovascular protective effects of AM. Intravenous infusion of AM decreases blood pressure, total peripheral resistance, and pulmonary arterial and capillary wedge pressure, whereas it increases heart rate, cardiac index, urinary volume, and sodium excretion in patients with heart failure as well as in healthy volunteers.[3,10,12] Although plasma renin activity is elevated, possibly by sympathetic nerve activation resulting from the reduction of blood pressure, plasma aldosterone levels are reduced or remain unchanged. These hemodynamic or hormonal responses, which should be preferable for heart failure patients, are consistent with those observed in animal studies and cell culture experiments. Kataoka et al. performed a clinical study of patients with acute MI, in which reduction of infarct size and improvement of cardiac contractility occurred after intravenous infusion of AM after reperfusion therapy.[7] Additionally, as described earlier, in patients with idiopathic pulmonary hypertension, inhalation of aerosolized AM lowers pulmonary arterial pressure and pulmonary vascular resistance, improving exercise capacity.[24] Taken together, it is possible that AM can exert beneficial effects as a therapeutic agent for patients with cardiovascular diseases including heat failure, acute MI, and idiopathic pulmonary hypertension.

REFERENCES

1. Caron K, Hagaman J, Nishikimi T, Kim HS, Smithies O. Adrenomedullin gene expression differences in mice do not affect blood pressure but modulate hypertension-induced pathology in males. *Proc Natl Acad Sci U S A* 2007;**104**:3420–5.
2. Chao J, Chao L. The role of adrenomedullin in cardiovascular and renal function. *Drug News Perspect* 2002;**15**:511–8.
3. Eto T, Kitamura K, Kato J. Biological and clinical roles of adrenomedullin in circulation control and cardiovascular diseases. *Clin Exp Pharmacol Physiol* 1999;**26**:371–80.
4. Fritz-Six KL, Dunworth WP, Li M, Caron KM. Adrenomedullin signaling is necessary for murine lymphatic vascular development. *J Clin Invest* 2008;**118**:40–50.
5. Hong Y, Hay DL, Quirion R, Poyner DR. The pharmacology of adrenomedullin 2/intermedin. *Br J Pharmacol* 2012;**166**:110–20.
6. Iimuro S, Shindo T, Moriyama N, Amaki T, Niu P, Takeda N, et al. Angiogenic effects of adrenomedullin in ischemia and tumor growth. *Circ Res* 2004;**95**:415–23.
7. Kataoka Y, Miyazaki S, Yasuda S, Nagaya N, Noguchi T, Yamada N, et al. The first clinical pilot study of intravenous adrenomedullin administration in patients with acute myocardial infarction. *J Cardiovasc Pharmacol* 2010;**56**:413–9.
8. Kato J, Kitamura K, Eto T. Plasma adrenomedullin level and development of hypertension. *J Hum Hypertens* 2006;**20**:566–70.
9. Kato J, Kuwasako K, Kitamura K. Adrenomedullin in hypertension and obesity. *Curr Hypertens Rev* 2011;**4**:257–67.
10. Kato J, Tsuruda T, Kita T, Kitamura K, Eto T. Adrenomedullin: a protective factor for blood vessels. *Arterioscler Thromb Vasc Biol* 2005;**25**:2480–7.
11. Kato J, Tsuruda T, Kitamura K, Eto T. Adrenomedullin: a possible autocrine or paracrine hormone in the cardiac ventricles. *Hypertens Res* 2003;(Suppl. 26):S113–9.

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12. Kitamura K, Kangawa K, Eto T. Adrenomedullin and PAMP: discovery, structures, and cardiovascular functions. *Microsc Res Technol* 2002;**57**:3–13.
13. Kitamura K, Kangawa K, Kawamoto M, Ichiki Y, Nakamura S, Matsuo H, et al. Adrenomedullin: a novel hypotensive peptide isolated from human pheochromocytoma. *Biochem Biophys Res Commun* 1993;**192**:553–60.
14. Kitamura K, Kato J, Kawamoto M, Tanaka M, Chino N, Kangawa K, et al. The intermediate form of glycine-extended adrenomedullin is the major circulating molecular form in human plasma. *Biochem Biophys Res Commun* 1998;**244**:551–5.
15. Kuwasako K, Kitamura K, Nagata S, Hikosaka T, Takei Y, Kato J. Shared and separate functions of the RAMP-based adrenomedullin receptors. *Peptides* 2011;**32**:1540–50.
16. Leskinen H, Rauma-Pinola T, Szokodi I, Kerkelä R, Pikkarainen S, Uusimaa P, et al. Adaptive or maladaptive response to adenoviral adrenomedullin gene transfer is context-dependent in the heart. *J Gene Med* 2008;**10**:867–77.
17. Li Y, Jiang C, Wang X, Zhang Y, Shibahara S, Takahashi K. Adrenomedullin is a novel adipokine: adrenomedullin in adipocytes and adipose tissues. *Peptides* 2007;**28**:1129–43.
18. Liang L, Tam CW, Pozsgai G, Siow R, Clark N, Keeble J, et al. Protection of angiotensin II-induced vascular hypertrophy in vascular smooth muscle-targeted receptor activity-modifying protein 2 transgenic mice. *Hypertension* 2009;**54**:1254–61.
19. Maisel A, Mueller C, Nowak R, Peacock WF, Landsberg JW, Ponikowski P, et al. Mid-region pro-hormone markers for diagnosis and prognosis in acute dyspnea: results from the BACH (Biomarkers in Acute Heart Failure) trial. *J Am Coll Cardiol* 2010;**55**:2062–76.
20. Maki T, Ihara M, Fujita Y, Nambu T, Miyashita K, Yamada M, et al. Angiogenic and vasoprotective effects of adrenomedullin on prevention of cognitive decline after chronic cerebral hypoperfusion in mice. *Stroke* 2011;**42**:1122–8.
21. Martínez A. A new family of angiogenic factors. *Cancer Lett* 2006;**236**:157–63.
22. McLatchie LM, Fraser NJ, Main MJ, Wise A, Brown J, Thompson N, et al. RAMPs regulate the transport and ligand specificity of the calcitonin-receptor-like receptor. *Nature* 1998;**393**(6683):333–9.
23. Morales MA, Maltinti M, Piacenti M, Turchi S, Giannessi D, Del Ry S. Adrenomedullin plasma levels predict left ventricular reverse remodeling after cardiac resynchronization therapy. *Pacing Clin Electrophysiol* 2010;**33**:865–72.
24. Murakami S, Kimura H, Kangawa K, Nagaya N. Physiological significance and therapeutic potential of adrenomedullin in pulmonary hypertension. *Cardiovasc Hematol Disord Drug Targets* 2006;**6**:125–32.
25. Nagaya N, Mori H, Murakami S, Kangawa K, Kitamura S. Adrenomedullin: angiogenesis and gene therapy. *Am J Physiol Regul Integr Comp Physiol* 2005;**288**:R1432–7.
26. Nishida H, Horio T, Suzuki Y, Iwashima Y, Kamide K, Kangawa K, et al. Plasma adrenomedullin as an independent predictor of future cardiovascular events in high-risk patients: comparison with C-reactive protein and adiponectin. *Peptides* 2008;**29**:599–605.
27. Nishikimi T. Adrenomedullin in the kidney-renal physiological and pathophysiological roles. *Curr Med Chem* 2007;**14**:1689–99.
28. Nishikimi T, Matsuoka H. Cardiac adrenomedullin: its role in cardiac hypertrophy and heart failure. *Curr Med Chem Cardiovasc Hematol Agents* 2005;**3**:231–42.
29. Niu P, Shindo T, Iwata H, Iimuro S, Takeda N, Zhang Y, et al. Protective effects of endogenous adrenomedullin on cardiac hypertrophy, fibrosis, and renal damage. *Circulation* 2004;**109**:1789–94.
30. Richards AM, Doughty R, Nicholls MG, MacMahon S, Sharpe N, Murphy J, et al. Plasma N-terminal pro-brain natriuretic peptide and adrenomedullin: prognostic utility and prediction of benefit from carvedilol in chronic ischemic left ventricular dysfunction. *J Am Coll Cardiol* 2001;**37**:1781–7.
31. Roh J, Chang CL, Bhalla A, Klein C, Hsu SY. Intermedin is a calcitonin/calcitonin gene-related peptide family peptide acting through the calcitonin receptor-like receptor/receptor activity-modifying protein receptor complexes. *J Biol Chem* 2004;**279**:7264–74.
32. Shimosawa T, Fujita T. Adrenomedullin as a potent antioxidative and antiatherosclerotic substance. *Drug News Perspect* 2005;**18**:185–9.
33. Smith Jr RS, Gao L, Bledsoe G, Chao L, Chao J. Intermedin is a new angiogenic growth factor. *Am J Physiol Heart Circ Physiol* 2009;**297**:H1040–7.
34. Takei Y, Inoue K, Ogoshi M, Kawahara T, Bannai H, Miyano S. Identification of novel adrenomedullin in mammals: a potent cardiovascular and renal regulator. *FEBS Lett* 2004;**556**:53–8.
35. von Haehling S, Filippatos GS, Papassotiriou J, Cicoira M, Jankowska EA, Doehner W, et al. Mid-regional pro-adrenomedullin as a novel predictor of mortality in patients with chronic heart failure. *Eur J Heart Fail* 2010;**12**:484–91.

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Chapter 186

Angiotensin II Peptides

Yasukatsu Izumi and Hiroshi Iwao

ABSTRACT

Much evidence supports the notion that angiotensin II (Ang II), the central product of the renin–angiotensin system (RAS), may play a central role not only in the etiology of hypertension but also in the pathophysiology of cardiovascular diseases in humans. Ang II, via the Ang II type 1 receptor, directly causes cellular phenotypic changes and cell growth, regulates the gene expression of various bioactive substances, and activates multiple intracellular signaling cascades in cardiac myocytes and fibroblasts, as well as vascular endothelial and smooth muscle cells. Recently, new factors have been discovered, such as angiotensin-converting enzyme 2, angiotensin-(1-7), and its receptor Mas. This section summarizes the current knowledge about the broad RAS in the pathophysiology of cardiac hypertrophy and remodeling, heart failure, vascular thickening, and atherosclerosis.

DISCOVERY

Research on the renin–angiotensin system (RAS) began with the discovery of renin from the kidney by Tiegerstedt and Bergman in 1898. In 1940, a peptide that had vasoconstrictive effects in the RAS was discovered, and it was named *hypertensin* by Braun-Menendez in Argentina, and *angionin* by Page and Helmer in the United States. These two terms persisted for about 20 years, until it was agreed to rename the pressor substance *angiotensin*. In the 1950s, two forms of angiotensin were recognized, the first a decapeptide (angiotensin I: Ang I) and the second an octapeptide (angiotensin II: Ang II). Skeggs et al. in the United States reported that Ang II was formed by the enzymatic cleavage of Ang I by another enzyme, termed *angiotensin-converting enzyme* (ACE). Schwyzer and Bumpus succeeded in the synthesis of Ang II in 1957. Gross suggested, in 1958, that the RAS was involved in the regulation of aldosterone secretion, and then Davis, Genet, Laragh et al. proved his hypothesis.

In the early 1970s, polypeptides either inhibiting the formation of Ang II or blocking Ang II receptors were discovered, but these were not orally active. Cushman and Ondetti succeeded in the development of captopril, the orally active ACE inhibitor in 1977. After that, many experimental and clinical studies with ACE inhibitors uncovered additional roles for the RAS in the pathophysiology of hypertension, heart failure, and vascular diseases. Furthermore, losartan (Dup 753), an orally active, highly selective and potent nonpeptide Ang II receptor blocker (ARB), was developed in 1988, and the cloning of Ang II receptors, type 1 (AT_1R) and type 2 (AT_2R) was accomplished in the early 1990s.

Angiotensin-(1-7) [Ang-(1-7)] was discovered in 1988 by Santos et al., and angiotensin-converting enzyme 2 (ACE2) was identified in 2000, which catalyzes the conversion of Ang I [Ang-(1-10)] to Ang-(1-9) by the removal of a single carboxy-terminal amino acid. ACE2 is an essential regulator of heart function and a functional receptor for the SARS coronavirus.

STRUCTURE OF THE PEPTIDES AND COMPONENT OF RAS

The RAS plays an important role in the regulation of arterial blood pressure. Renin is an enzyme that acts on angiotensinogen to catalyze the formation of Ang I. Ang I is then cleaved by ACE to yield Ang II. A representation of the biochemical pathways of RAS is shown in Fig. 1.

The major element of the rate of Ang II production is the amount of renin released by the kidney. Renin is synthesized, stored, and secreted into the renal arterial circulation by the granular juxtaglomerular cells. The secretion of renin is controlled predominantly by three pathways: The first mechanism controlling renin release is the intrarenal macula densa pathway and the second is the intrarenal baroreceptor pathway. The third mechanism is the β-adrenergic receptor pathway, which is mediated by the release of norepinephrine from postganglionic sympathetic nerve terminals. An increase in renin secretion enhances the formation of Ang II, and Ang II stimulates the AT_1R on juxtaglomerular cells to inhibit renin release.

The substrate for renin is angiotensinogen, an abundant α_2-globulin that circulates in the plasma. The primary

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00186-X
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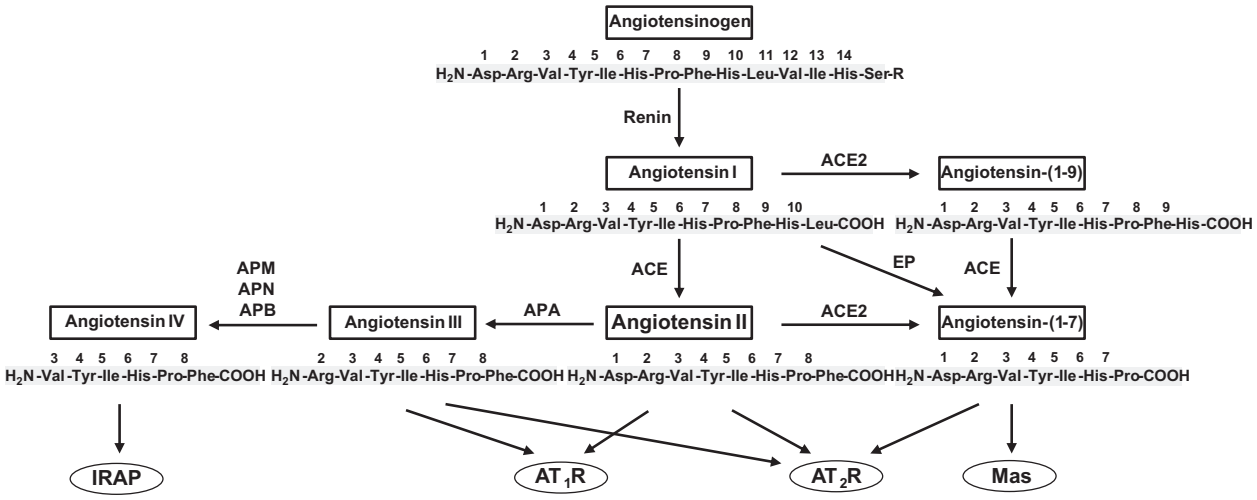

FIGURE 1 Formation of RAS peptides. ACE, angiotensin-converting enzyme; EP, endopeptidase; APA, B, M, N; aminopeptidase A, B, M, N; IRAP, insulin-regulated aminopeptidases.

structure of angiotensinogen has been deduced by molecular cloning. Angiotensinogen is synthesized primarily in the liver, although mRNA that encodes the protein is abundant in fat, certain regions of the central nervous system, and the kidney. The rate of Ang II synthesis can be influenced by changes in angiotensinogen levels.

ACE was discovered in plasma as the factor responsible for conversion of Ang I to Ang II. The ACE gene contains, in intron 16, an insertion/deletion polymorphism that explains 47% of the phenotypic variance in serum ACE levels. Individuals homozygous for the deletion allele have higher levels of serum ACE.

Ang I is rapidly converted to Ang II, when given intravenously. Ang III [Ang-(2-8)] can be formed by the action of aminopeptidase on Ang II. Ang III Ang II cause qualitatively similar effects. Ang III is approximately as potent as Ang II in stimulating the secretion of aldosterone. However, Ang III is only 25% as potent as Ang II in elevating blood pressure. Ang IV [Ang-(3-8)] is generated by the sequential cleavage of two amino acid residues from the amino terminus of Ang II by aminopeptidases localized to the endothelial surface.

There are bypass pathways in the RAS to produce Ang II besides the main enzymes such as renin and ACE. Arakawa et al. showed that trypsin and kallikrein can produce Ang II. One of the most famous enzymes in such bypass pathways is a heart chymase that is secreted by mast cells.

DISTRIBUTION OF RAS, PROCESSING, AND ENDOGENOUS FORM IN THE CARDIOVASCULAR SYSTEM

Circulating renin of renal origin acts on circulating angiotensinogen of hepatic origin to produce Ang I in the plasma, and circulating Ang I is converted by plasma ACE and pulmonary endothelial ACE to Ang II. Then, Ang II is delivered to its target organs via blood flow. This traditional pathway is called the circulating RAS. However, heart, blood vessels, and several other tissues contain and/or synthesize components of the RAS, called the tissue (local) RAS.

Many tissues, including heart, blood vessels, brain, kidney, and adrenal gland, express mRNAs for renin, angiotensinogen, and/or ACE. Furthermore, several other Ang I cleavage products have been found in the cultured cell types from these tissues, for example, Ang-(1-9), Ang-(1-7), Ang III, and Ang IV (Fig. 1). Ang I and Ang II have been localized in atria and ventricles. Therefore, it seems that the tissue RAS exists independently of the renal/hepatic-based system.

Several serine proteases, such as tonin and cathepsin G, have been shown to hydrolyze Ang II precursors. An aspartyl protease with cathepsin D-like properties was shown to convert angiotensinogen to Ang I. Conversion of Ang I to Ang II in human and dog cardiac ventricles may occur by heart chymase. Unlike ACE, human heart chymase shows high specificity for Ang I and does not degrade bradykinin or vasoactive intestinal peptide. It is likely that the relative contribution of ACE and chymase to cardiac Ang II formation varies with the cardiac chamber in the human heart, because ACE levels are the highest in the atria and chymase levels are the highest in ventricles. It is recently believed that chymase plays a crucial role in various tissues because chymase can not only produce Ang II but also convert precursors of transforming growth factor (TGF)-β and matrix metalloproteinase-9 to their active forms, whose increases damage various organs.[30]

RECEPTORS AND THEIR DISTRIBUTION IN THE CARDIOVASCULAR SYSTEM

The effects of angiotensins are exerted through specific cell surface receptors. There are two subtypes of Ang II receptors, types 1 and 2 (AT_1R and AT_2R). The successful

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cloning of the AT_1R in 1991 and the AT_2R in 1993 allowed the development of further research on the structure and function of this receptor.

The AT_1R consists of two subtypes, $AT_{1a}R$ and $AT_{1b}R$, which have 94% homology with regard to amino acid sequence and have similar pharmacological properties and tissue distribution patterns. The AT_1R is a member of the seven-transmembrane-spanning, G protein-coupled receptor (GPCR) family; it binds to heterotrimeric G proteins and lacks intrinsic tyrosine kinase activity. The human AT_1R gene is mapped to chromosome 3, and the $AT_{1a}R$ and $AT_{1b}R$ genes in rats are mapped to chromosomes 17 and 2, respectively. The AT_1R is ubiquitously and abundantly distributed in adult tissues, including blood vessel, heart, kidney, adrenal gland, liver, brain, and the lung. The AT_1R mediates all the classic well-known effects of Ang II, such as elevation of blood pressure, vasoconstriction, increase in cardiac contractility, aldosterone release from the adrenal gland, facilitation of catecholamine release from nerve endings, and renal sodium and water absorption. Therefore, the AT_1R has a role in atherosclerosis, congestive cardiac failure, and several acute and chronic inflammatory diseases, conditions in which inflammation is known to play a significant role (Fig. 2). Numerous selective and potent nonpeptide AT_1R antagonists, such as losartan, candesartan, valsartan, irbesartan, eprosartan, telmisartan, tasosartan, have been developed.

In contrast, the AT_2R has high affinity for PD123177, PD123319, CGP42112, L-162,686, L-162,638, and CGP42112A. The cDNA and genomic DNAs of human, rat, and mouse AT_2Rs have been cloned. The AT_2R is mainly expressed in developing fetal tissues, and it is believed to have an essential role in physiological vascular development. The AT_2R expression rapidly decreases after birth,[19] and, in the adult, expression of this receptor is limited mainly to the uterus, ovary, certain brain nuclei, heart, and adrenal medulla. Unlike the AT_1R, which has been shown to have subtypes in rats and mice, there is no evidence for subtypes of the AT_2R. Although a comparison of amino acid sequences of $AT_{1a}R$ and AT_2R in rats, deduced from nucleotide sequences, shows a low homology between these receptors (32%), The AT_2R is also a seven-transmembrane domain receptor. In various cell lines, the AT_2R-activated protein tyrosine phosphatase was shown to inhibit cell growth or induce programmed cell death (apoptosis). The AT_2R inhibited AT_1R-mediated cell growth, demonstrating an antagonistic action. It is believed that the expression balance of AT_1 and the AT_2 is important for cardiovascular diseases.[8] There have also been conflicting findings regarding these receptors. For example, it is controversial whether cardiac hypertrophy is promoted by the AT_2R. However, the AT_2R plays a cardioprotective role on left ventricular function after myocardial infarction. In contrast to extensive data on the molecular and cellular functions and pathophysiological significance of the AT_1R, the role of the AT_2R in cardiovascular diseases remains to be defined. At present, the implications of overstimulation of AT_2R by long-term use of ARB are being investigated in clinical trials.[15]

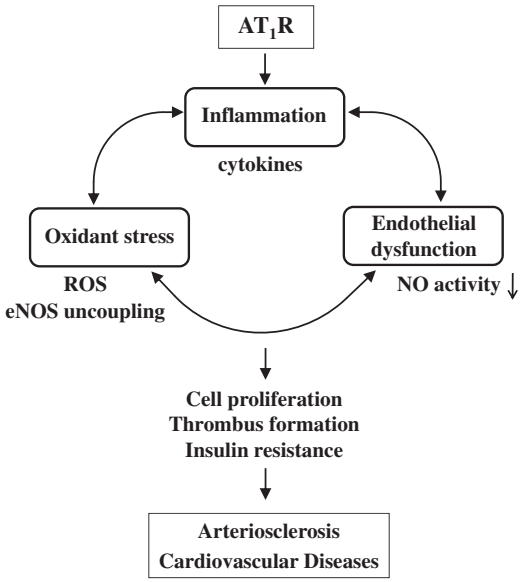

FIGURE 2 Proposed mechanism of the Ang II-mediated pathological cardiac hypertrophy. ROS, reactive oxygen species; eNOS, endothelial nitric oxide synthase.

It has been suggested that Ang-(1-7) mediates its effects by interacting with the GPCR Mas, a prototypic seven-transmembrane domain receptor, which is predominantly expressed not only in the brain and testis but is also found in the kidney, heart, and blood vessels.

The Ang AT_4 receptor was originally defined as the specific, high-affinity binding site for Ang IV. Therefore, it was suspected to be classically a GPCR. And then, Albiston et al. have identified it as the transmembrane enzyme, insulin-regulated aminopeptidase (IRAP),[3] an abundant protein that is found in specialized vesicles containing the insulin-sensitive glucose transporter GLUT4.

BIOLOGICAL ACTIONS AND PATHOPHYSIOLOGICAL IMPLICATION IN THE CARDIOVASCULAR SYSTEM

Molecular Characteristics of Pathological Cardiac Hypertrophy

Generally, pathological left ventricular hypertrophy is characterized not only by an increase in myocyte size (quantitative change) but also by myocyte gene reprogramming (qualitative change), as shown by enhanced expression of fetal phenotypes of genes such as β-myosin heavy chain (β-MHC), skeletal α-actin, and atrial natriuretic peptide (ANP). In the cardiac ventricle of most mammalian species, MHC consists of two isoforms, α- and β-MHCs.

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In the rat, α-MHC is the predominant isoform in adult hearts, whereas β-MHC is the predominant isoform in fetal hearts. Therefore, changes in the ratio of β-MHC to α-MHC in the cardiac ventricle significantly alter the contractile properties of the heart. Cardiac sarcomeric actin is also composed of two isoforms: cardiac α-actin and skeletal α-actin. Cardiac α-actin is predominantly expressed in adult rat hearts, whereas skeletal α-actin is normally expressed in fetal and neonatal rat hearts. The ratio of skeletal α-actin to cardiac α-actin in the ventricle plays a significant role in cardiac function, because skeletal α-actin has greater contractility than cardiac α-actin. Furthermore, in addition to showing enhanced expression of β-MHC and skeletal α-actin, hypertrophic ventricular myocytes are also characterized by significant upregulation of ANP, which is scarcely expressed in normal adult ventricular myocytes.

Another important property of pathological cardiac hypertrophy is increased accumulation of extracellular matrix (ECM) proteins such as collagen (particularly collagen types I and III) and fibronectin in the interstitium and around blood vessels within the heart. These changes play a central role in ventricular fibrosis or remodeling.[21,34] Increased interstitial collagen deposition in the heart enhances cardiac stiffness and results in diastolic dysfunction. Fibronectin is localized on the surface of cardiac myocytes, connects cardiac myocytes to perimyocytic collagen and is believed to affect cardiac systolic and diastolic functions. Thus, increased ECM accumulation, and the abovementioned ventricular myocyte gene reprogramming play critical roles in the impairment of cardiac performance and pathophysiology of cardiac failure. ECM proteins within the heart are predominantly produced by fibroblasts. Unlike cardiac myocytes, cardiac fibroblasts proliferate and increase the production of ECM proteins when the heart is exposed to hypertrophic stimuli such as hemodynamic overload. Thus, cardiac fibroblasts and cardiac myocytes play key roles in the development of pathological cardiac hypertrophy and dysfunction. Accumulating *in vitro* and *in vivo* evidence supports the concept that Ang II is involved in all these important processes of pathological cardiac hypertrophy, including myocyte hypertrophy, myocyte gene reprogramming, fibroblast proliferation, and ECM protein accumulation.

Effect of AT_1R on Cardiac Remodeling (Fig. 2)

Activated AT_1R can activate multiple signaling pathways. The acute vasoconstrictor function of Ang II is primarily mediated through AT_1R by classical G protein-dependent signaling mechanisms. Investigations of the effects of Ang II on cardiac intracellular signaling cascades are essential to elucidate the molecular mechanism underlying Ang II-induced pathological cardiac hypertrophy. Accumulating *in vitro* evidence on cultured cardiac myocytes or fibroblasts suggests that mitogen-activated protein (MAP) kinases, including extracellular signal-regulated kinase (ERK), c-jun amino terminal kinase (JNK) and p38MAP kinase (p38MAPK), may be responsible for myocyte hypertrophy and gene reprogramming or fibroblast proliferation. Ang II-induced cardiac activation of JNK occurs in a more sensitive manner than that of ERK.

The ERK cascade activates platelet-derived growth factor (PDGF), epidermal growth factor receptor (EGFR), insulin receptor pathways, and nonreceptor tyrosine kinases belonging to the c-Src family, prolinerich tyrosine kinase 2, focal adhesion kinase, and janus kinases (JAKs).[18] Besides the G protein-dependent effects of Ang II activated AT_1R, the activated AT_1R can also stimulate G protein-independent signal transduction mechanisms by directly associating with signaling molecules, such as β-arrestins, JAK, Cdc42, and Src.[10]

JNK activation by Ang II without ERK activation is followed by activation of activator protein-1 (AP-1). Importantly, AP-1 regulates the expression of various genes by binding the AP-1 consensus sequence present in their promoter regions. Interestingly, fetal phenotypes of cardiac genes such as skeletal α-actin and ANP, and cardiac fibrosis-associated genes such as TGF-β1 and collagen type I, have AP-1 responsive sequences in their promoter regions. Indeed, AP-1 activation has been demonstrated to lead to increased promoter activity of skeletal α-actin and TGF-β1. Therefore, it is intriguing to postulate that JNK activation, in part through the activation of AP-1, may be implicated in Ang II-induced cardiac hypertrophic response *in vivo*. An *in vivo* study showed that apoptosis signal-regulating kinase 1 (ASK1), one of the MAP kinase kinase kinases, plays an important role in Ang II-induced cardiac hypertrophy and remodeling, including cardiomyocyte hypertrophy, cardiac hypertrophy-related mRNA upregulation, cardiomyocyte apoptosis, interstitial fibrosis, coronary arterial remodeling, and collagen gene upregulation.[11]

The RAS, TGF-β, and β-adrenergic system network mediates this myocardial remodeling. TGF-β1 mRNA and protein expression is augmented in cardiomyocytes and cardiac fibroblasts, which in these situations transdifferentiate to myofibroblast phenotype, following progressive diastolic dysfunction. Ang II caused a significant increase in TGF-β1 mRNA. Chronic administration of Ang II-induced TGF-β1 protein expression in myocardium.[14] Ang II-induced hypertrophic growth of cardiomyocytes is mediated by TGF-β1, which is downstream to Ang II in network.[26] Mice expressing constitutively hyperactive TGF-β1 had a chronic hypertrophy, fibrosis, and cardiac dysfunction that was resistant to the ARB telmisartan but blocked by TGF-β antagonist.[24] The Ang II-mediated cardiac remodeling mechanism is thus dependent on TGF-β1. From all the studies performed so far, it is clear that Ang II/TGF-β1 induced autocrine–paracrine cellular responses in cardiac fibroblasts, in the myocardial

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interstitium, and cardiomyocytes cause cardiac hypertrophy. Ang II-induced TGF-β1 signaling also induces translocation of Smad proteins into the nucleus to drive transcription of fibrotic marker proteins such as collagen, fibronectin, and connective tissue growth factor (CTGF).[17] CTGF is a profibrotic factor that stimulates TGF-β1 responses mediating fibrosis and apoptosis.[22] The inflammatory cytokines such as interleukin-8, interleukin-6, vascular cell adhesion molecule-1, and monocyte chemoattractant protein-1, are believed to have important roles in the coupling acute ischemic episodes and chronic myocardial remodeling with chronic angiotensin receptor stimulation.[9]

Ang II facilitates the release of norepinephrine from cardiac sympathetic nerve terminals. In Ang II-infused rats, surgical cardiac sympathectomy or treatment with β_1-adrenergic receptor blocker significantly prevented cardiac myocyte necrosis, showing that Ang II-induced cardiac damage is, at least in part, mediated by catecholamine release from cardiac sympathetic neurons. Thus, the activation of cardiac sympathetic neurons by Ang II also contributes to pathological cardiac hypertrophy.

Ang II, via the AT_1R, is also known to be a potent mediator of oxidative stress and reactive oxygen species (ROS)-mediated signaling. A large body of evidence indicates that ROS plays a major part in the initiation and progression of cardiovascular dysfunctions associated with diseases such as hyperlipidemia, diabetes mellitus, hypertension, ischemic heart disease, and chronic heart failure.[32] In addition, Ang II signaling activates membrane NAD(P)H oxidases in VSMCs to produce ROS, which mediate the pleiotrophic effects of Ang II.[28] Ang II signaling mediated activation of NAD(P)H oxidases involves the upstream mediators Src/EGFR/PI3K/Rac-1 and PLD/PKC/p47phox phosphorylation.[28,33] Transcription factors such as nuclear factor-κB, AP-1, and Nrf2 that are implicated in the pathogenesis of atherosclerosis are activated by ROS.[20] ROS may cause vessel inflammation by inducing the release of cytokines and causing an increase in the expression of the leukocyte adhesion molecules in the cell membranes. This may increase recruitment of monocytes to the area of endothelial damage. Thus, activated AT_1R-induced ROS production can lead to changes in structural and functional properties of the vasculature and is the central aspect of vascular pathobiology in hypertension, diabetes, and cardiovascular diseases.[7]

Effect of AT_1R on Vascular Remodeling

Numerous *in vivo* experiments have shown that Ang II can induce VSMC proliferation *in vivo*.[13] Ang II infusion increased mesenteric vascular media width, media cross-sectional area, and media/lumen ratio. Despite detailed investigations into the molecular mechanism of Ang II-mediated VSMC growth *in vitro*, this process is poorly understood. Ang II infusion, at least in part independent of its blood pressure-elevating effect, increased aortic mRNA and protein expression of fibronectin, which is an ECM protein that induces phenotypic change of VSMCs from a contractile to a synthetic phenotype. Basic fibroblast growth factor (bFGF) may play a key role in Ang II-mediated VSMC replication *in vivo*, as shown by the observation that the injection of anti-bFGF antibody significantly inhibited the mitogenic effect of Ang II infusion on rat carotid arteries. Ang II infusion in rats doubled superoxide production in rat aorta by activation of NAD(P)H oxidase. On the other hand, norepinephrine infusion did not increase vascular superoxide production, despite a hypertensive effect comparable to that of Ang II, suggesting that VSMC growth due to Ang II may be specifically mediated by increased superoxide generation. Ang II infusion in rats increased heme oxygenase-1 (HO-1) mRNA and protein in the endothelium.[16] Because HO-1 is an oxidant-sensitive gene, it is possible that increased oxidative stress is a trigger for HO-1 mRNA upregulation in the Ang II-infused rat aorta and that HO-1 may serve to abrogate this increased stress caused by Ang II. Ang II infusion stimulated aortic thrombin receptor mRNA expression in rats, which was blocked by either ARB or the heparin-binding chimera of human Cu/Zn superoxide dismutase but not by normalization of blood pressure with hydralazine treatment, suggesting that Ang II increases vascular thrombin receptor by AT_1R-mediated superoxide production and may be implicated in the pathophysiology of atherosclerosis by thrombin cascade activation. The injection of Ang II resulted in increased oxidized low-density lipoprotein uptake by peritoneal macrophages and increased macrophage proteoglycan content, suggesting that Ang II may accelerate atherosclerosis by promoting foam cell formation and cholesterol accumulation in the vascular wall.

Endothelial dysfunction in the context of hypertension and cardiovascular diseases is partly dependent on the production of ROS. AT_1R-induced ROS not only burns nitric oxide (NO) but also reduces NO formation in the endothelium by oxidizing tetrahydrobiopterin, a crucial cofactor for endothelial NO synthase (eNOS).[27] Superoxide anion, a highly reactive ROS, is known to cause breakdown of endothelial-derived relaxing factor and dysfunction of endothelium. Regenerated endothelium has an impaired ability to release endothelial-derived relaxing factors, in particular NO. Activation of angiotensin receptors triggers an efflux of cytosolic calcium in endothelial cells (ECs). Calcium efflux activates PLA-2 to release arachidonic acid, which is metabolized by cyclooxygenase (COX) to generate various eicosanoids including endoperoxides and various prostaglandins that activate thromboxane A2/prostanoid receptors located on the VSMCs causing contractions. Endothelial COX activity also produces ROS. In addition, uric acid, despite its known antioxidant effects, may cause

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human vascular EC dysfunction by local activation of RAS, inducing oxidative stress. ROS may, in turn, activate local RAS of whole vasculature, including EC, smooth muscle cells, fibroblast-enhancing Ang II production and concomitant stimulation of AT_1R to create a positive feedback loop between local RAS and ROS. Thus, in hypertensive cardiovascular diseases, endothelial dysfunction is characterized by blunted endothelium-dependent vasodilations or enhanced endothelium-dependent contractions.[7,31]

Effects of in AT_2R on Cardiovascular System

Generally, AT_2R is believed to promote apoptosis and inhibit proliferation and hypertrophy. Unlike AT_1R, AT_2R contributes to maintenance of blood pressure by controlling the vascular tone through vasodilatation. Available evidence indicates links between AT_2R and bradykinin B_2 receptor (B_2R) in NO production in vasodilation.[5,12] AT_2R stimulation by Ang II leads to an increase in cGMP levels through a mechanism involving B_2R, causing NO release. AT_2R activation stimulates bradykinin formation by activating kininogenases. Chronic infusion of Ang II into AT_2R overexpressing mice completely abolished the AT_1R-mediated pressor effect, suggesting vasodilatation by AT_2R. This was blocked by one of the inhibitors of B_2R and NOS. In addition, aortic explants from these transgenic mice overexpressing AT_2R showed greatly increased cGMP/NO production and diminished AT_1R-induced vascular constriction. Removal of endothelium or treatment with the inhibitors of B_2R and NOS abolished these AT_2R-mediated effects, indicating that endothelium is the primary site of effect manifestation by AT_2R. The AT_2R in aortic SMCs also stimulates the production of bradykinin, which stimulates the NO/cGMP system to promote vasodilation. In the AT_2R-deficient mice, bradykinin-induced dilation is seriously impaired. Bradykinin-induced dilation is inhibited by AT_2R antagonist but not by Losartan, an ARB. Thus, AT_2R and B_2R may form functional heterodimers, as recently shown, and this might lead to increased NO and cGMP production.[1] The physical association between the dimerized AT_2R and B_2R initiates changes in intracellular phosphoprotein signaling activities and enhanced production of NO and cGMP.[7,15]

Growth and apoptosis are also two opposite components of tissue remodeling. Cardiac remodeling happens in certain physiological or pathological circumstances, such as exercise, aging, hypertension, and myocardial infarction. It is known that although AT_2R expression decreases sharply after birth, it can increase again in some pathophysiological conditions. Stimulation of AT_2R expression may inhibit neointima formation, cell proliferation, inflammation in vascular injury, myocardial infarction, and ischemic diseases, suggesting its protective role. Conversely, in AT_2R-deficient mice, recovery from acute myocardial infarction was reduced compared to the wild-type mice.[2] Although neointima formation and VSMC proliferation after vascular injury were exaggerated in AT_2R-deficient mice, they were suppressed in $AT_{1a}R$-deficient mice. AT_2R possibly exerts antiproliferative effects and proapoptotic effects in VSMCs by controlling $AT_{1a}R$.[29] The number of apoptotic cells in the injured artery was increased significantly in $AT_{1a}R$-deficient mice but decreased in AT_2R-deficient mice, indicating that AT_2R mediates apoptosis. Selective AT_2R stimulation also inhibited restenosis after balloon angioplasty. Thus, experimental studies have shown a beneficial role for the reexpression of the AT_2R after vascular injury leading to inhibition of cell growth and promotion of apoptosis. Gene transfer studies *in vivo* have also supported that AT_2R expression attenuated neointima accumulation in injured carotid arteries by antagonizing growth-promoting effects of AT_1R. Vascular injury studies using mice with disrupted AT_2R expression showed enhanced neointimal formation compared with wild-type mice, suggesting a protective role for AT_2R.

AT_2R expression is upregulated in several disease conditions. In the left ventricle-specific AT_2R overexpression mice, elevated left ventricular endodiastolic pressure, increased systolic and diastolic dimensions, severely depressed left ventricular fractional shortening, and wall thinning were observed, suggesting that ventricular myocyte-specific expression of AT_2R promotes the development of dilated cardiomyopathy and heart failure *in vivo*. *In vivo* ectopic AT_2R overexpression in postnatal left ventricular myocardium is thus detrimental, explaining the sharp decline of AT_2R after birth. If prolonged ARB treatment turns out to be completely beneficial through clinical trials, then it is either a dosage effect or it depends on the time and site of AT_2R expression. The implications of overstimulation of AT_2R by long-term use of ARB are being investigated in clinical trials.

Effects of in Mas on Cardiovascular System

Ang-(1-7) mediates its effects by interacting with the GRCR, Mas,[25] a prototypic seven-transmembrane domain receptor, which is not only predominantly expressed in the brain and testis but is also found in the kidney, heart, and blood vessels.[4] Moreover, several studies have shown that the interaction of Ang-(1-7) with Mas induces the protective cardiovascular actions such as NO release, Akt phosphorylation and vasodilation.

Ang-(1-7) also functions as an antithrombotic, antiproliferative, and antioxidative agent. Although the mechanisms of action in the vascular system has not yet been well established, several studies have focused on control of the NO and O_2^- balance, which regulates cardiovascular function. A reduction in superoxide dismutase and catalase activity in Mas-deficient mice demonstrates impaired antioxidant properties in these animals.[35] Moreover, ROS

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levels are increased in Mas-deficient animals. The Mas-deficient mice exhibit impaired *in vivo* endothelial function and increased blood pressure. The transgenic rats overexpressing human ACE2 in VSMCs, showed improved endothelial function and decreased vasoconstriction response to Ang II. Furthermore, the infusion of AVE 0991, a Mas agonist, increases the hypotensive effect of bradykinin in rats; one plausible mechanism for this effect involves an increase in NO levels.[6]

ACE2 protects endothelial cells from Ang II-mediated macrophage infiltration and oxidative stress in an Ang-(1-7)-dependent manner in atherosclerosis. The long-term Ang-(1-7) treatment induces atheroprotective effects in ApoE-deficient mice, an animal model for atherosclerosis. The improvement in endothelial function resulted from an increase in NO bioavailability, as the heptapeptide increased NOS expression and decreased O_2^- production. The genetic deletion of ACE2 significantly increases plaque formation in ApoE-deficient mice, which is decreased by targeted vascular ACE2 overexpression. Consequently, these actions of the ACE2-Ang-(1-7)-Mas system may be exploited in the treatment of atherosclerosis and other vascular diseases.

Thus, the ACE2-Ang-(1-7)-Mas system has opposite functions of the classical RAS in tissues responsible for blood pressure regulation and cardiovascular homeostasis. It decreases the production of ROS and increases eNOS, improving the antioxidant capacity of cardiovascular tissues. The ACE2-Ang-(1-7)-Mas system may become an important therapeutic target for cardiovascular protection.[23]

REFERENCES

1. Abadir PM, Periasamy A, Carey RM, Siragy HM. Angiotensin II type 2 receptor-bradykinin B2 receptor functional heterodimerization. *Hypertension* 2006;**48**:316–22.
2. Adachi Y, Saito Y, Kishimoto I, Harada M, Kuwahara K, Takahashi N, et al. Angiotensin II type 2 receptor deficiency exacerbates heart failure and reduces survival after acute myocardial infarction in mice. *Circulation* 2003;**107**:2406–8.
3. Albiston AL, McDowall SG, Matsacos D, Sim P, Clune E, Mustafa T, et al. Evidence that the angiotensin IV (AT(4)) receptor is the enzyme insulin-regulated aminopeptidase. *J Biol Chem* 2001;**276**:48623–6.
4. Alenina N, Xu P, Rentzsch B, Patkin EL, Bader M. Genetically altered animal models for Mas and angiotensin-(1-7). *Exp Physiol* 2008;**93**:528–37.
5. Bergaya S, Hilgers RH, Meneton P, Dong Y, Bloch-Faure M, Inagami T, et al. Flow-dependent dilation mediated by endogenous kinins requires angiotensin AT2 receptors. *Circ Res* 2004;**94**:1623–9.
6. Carvalho MB, Duarte FV, Faria-Silva R, Fauler B, da Mata Machado LT, de Paula RD, et al. Evidence for Mas-mediated bradykinin potentiation by the angiotensin-(1-7) nonpeptide mimic AVE 0991 in normotensive rats. *Hypertension* 2007;**50**:762–7.
7. Dasgupta C, Zhang L. Angiotensin II receptors and drug discovery in cardiovascular disease. *Drug Discov Today* 2011;**16**:22–34.
8. de Gasparo M, Catt KJ, Inagami T, Wright JW, Unger T. International union of pharmacology. XXIII. The angiotensin II receptors. *Pharmacol Rev* 2000;**52**:415–72.
9. Gabrielsen A, Lawler PR, Yongzhong W, Steinbruchel D, Blagoja D, Paulsson-Berne G, et al. Gene expression signals involved in ischemic injury, extracellular matrix composition and fibrosis defined by global mRNA profiling of the human left ventricular myocardium. *J Mol Cell Cardiol* 2007;**42**:870–83.
10. Hunyady L, Catt KJ. Pleiotropic AT1 receptor signaling pathways mediating physiological and pathogenic actions of angiotensin II. *Mol Endocrinol* 2006;**20**:953–70.
11. Izumiya Y, Kim S, Izumi Y, Yoshida K, Yoshiyama M, Matsuzawa A, et al. Apoptosis signal-regulating kinase 1 plays a pivotal role in angiotensin II-induced cardiac hypertrophy and remodeling. *Circ Res* 2003;**93**:874–83.
12. Katada J, Majima M. AT(2) receptor-dependent vasodilation is mediated by activation of vascular kinin generation under flow conditions. *Br J Pharmacol* 2002;**136**:484–91.
13. Kim S, Iwao H. Molecular and cellular mechanisms of angiotensin II-mediated cardiovascular and renal diseases. *Pharmacol Rev* 2000;**52**:11–34.
14. Kupfahl C, Pink D, Friedrich K, Zurbrugg HR, Neuss M, Warnecke C, et al. Angiotensin II directly increases transforming growth factor beta1 and osteopontin and indirectly affects collagen mRNA expression in the human heart. *Cardiovasc Res* 2000;**46**:463–75.
15. Lemarie CA, Schiffrin EL. The angiotensin II type 2 receptor in cardiovascular disease. *J Renin Angiotensin Aldosterone Syst* 2010;**11**:19–31.
16. Li Volti G, Seta F, Schwartzman ML, Nasjletti A, Abraham NG. Heme oxygenase attenuates angiotensin II-mediated increase in cyclooxygenase-2 activity in human femoral endothelial cells. *Hypertension* 2003;**41**:715–9.
17. Lim H, Zhu YZ. Role of transforming growth factor-beta in the progression of heart failure. *Cell Mol Life Sci* 2006;**63**:2584–96.
18. Mehta PK, Griendling KK. Angiotensin II cell signaling: physiological and pathological effects in the cardiovascular system. *Am J Physiol Cell Physiol* 2007;**292**:C82–97.
19. Miura S, Karnik SS. Ligand-independent signals from angiotensin II type 2 receptor induce apoptosis. *Embo J* 2000;**19**:4026–35.
20. Papaiahgari S, Zhang Q, Kleeberger SR, Cho HY, Reddy SP. Hyperoxia stimulates an Nrf2-ARE transcriptional response via ROS-EGFR-PI3K-Akt/ERK MAP kinase signaling in pulmonary epithelial cells. *Antioxid Redox Signal* 2006;**8**:43–52.
21. Pelouch V, Dixon IM, Golfman L, Beamish RE, Dhalla NS. Role of extracellular matrix proteins in heart function. *Mol Cell Biochem* 1993;**129**:101–20.
22. Perbal B. CCN proteins: multifunctional signalling regulators. *Lancet* 2004;**363**:62–4.
23. Rabelo LA, Alenina N, Bader M. ACE2-angiotensin-(1-7)-Mas axis and oxidative stress in cardiovascular disease. *Hypertens Res* 2011;**34**:154–60.
24. Rosenkranz S. TGF-beta1 and angiotensin networking in cardiac remodeling. *Cardiovasc Res* 2004;**63**:423–32.
25. Santos RA, Brosnihan KB, Chappell MC, Pesquero J, Chernicky CL, Greene LJ, et al. Converting enzyme activity and angiotensin metabolism in the dog brainstem. *Hypertension* 1988;**11**:I153–7.
26. Schultz Jel J, Witt SA, Glascock BJ, Nieman ML, Reiser PJ, Nix SL, et al. TGF-beta1 mediates the hypertrophic cardiomyocyte growth induced by angiotensin II. *J Clin Invest* 2002;**109**:787–96.

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27. Schulz E, Jansen T, Wenzel P, Daiber A, Munzel T. Nitric oxide, tetrahydrobiopterin, oxidative stress, and endothelial dysfunction in hypertension. *Antioxid Redox Signal* 2008;**10**:1115–26.
28. Seshiah PN, Weber DS, Rocic P, Valppu L, Taniyama Y, Griendling KK. Angiotensin II stimulation of NAD(P)H oxidase activity: upstream mediators. *Circ Res* 2002;**91**:406–13.
29. Suzuki J, Iwai M, Nakagami H, Wu L, Chen R, Sugaya T, et al. Role of angiotensin II-regulated apoptosis through distinct AT1 and AT2 receptors in neointimal formation. *Circulation* 2002;**106**:847–53.
30. Takai S, Jin D, Miyazaki M. New approaches to blockade of the renin-angiotensin-aldosterone system: chymase as an important target to prevent organ damage. *J Pharmacol Sci* 2010;**113**:301–9.
31. Tang EH, Vanhoutte PM. Endothelial dysfunction: a strategic target in the treatment of hypertension? *Pflugers Arch* 2010;**459**:995–1004.
32. Taniyama Y, Griendling KK. Reactive oxygen species in the vasculature: molecular and cellular mechanisms. *Hypertension* 2003;**42**:1075–81.
33. Touyz RM, Yao G, Quinn MT, Pagano PJ, Schiffrin EL. p47phox associates with the cytoskeleton through cortactin in human vascular smooth muscle cells: role in NAD(P)H oxidase regulation by angiotensin II. *Arterioscler Thromb Vasc Biol* 2005;**25**:512–8.
34. Weber KT. Extracellular matrix remodeling in heart failure: a role for de novo angiotensin II generation. *Circulation* 1997;**96**:4065–82.
35. Xu P, Costa-Goncalves AC, Todiras M, Rabelo LA, Sampaio WO, Moura MM, et al. Endothelial dysfunction and elevated blood pressure in MAS gene-deleted mice. *Hypertension* 2008;**51**:574–80.

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Chapter 187

Apelin

Ábel Perjés, Klára Farkasfalvi, Heikki Ruskoaho and István Szokodi

ABSTRACT

Apelin and its cognate G protein-coupled APJ receptor are emerging as an important regulator of cardiovascular homoeostasis. The expression of apelin and APJ receptor is widely abundant throughout the cardiovascular system. Soon after its discovery, potent vasodilator and positive inotropic effects of the peptide were revealed, a unique combination among endogenous agents. Despite sequence homologies with angiotensin II and its receptor, the apelin–APJ system seemingly antagonizes many potentially deleterious effects of angiotensin II. Further investigations show that the peptide may play a role in the regulation of cardiovascular development and angiogenesis. Apelin signaling is supposed to involve G_i and G_q proteins, activating protein kinase C, mitogen-activated protein kinases, and the phosphoinositide 3-kinase-Akt-nitric oxide pathway. Preclinical and preliminary clinical studies show beneficial effects of apelin in various cardiovascular diseases, such as heart failure, ischemia–reperfusion injury, and atherosclerosis. This chapter summarizes the rapidly growing literature of the apelin–APJ system in the cardiovascular field.

THE APELIN–APJ SYSTEM

Apelin was discovered as the endogenous ligand of the previously orphan G protein-coupled receptor (GPCR) APJ.[31] The APJ receptor shares sequence identity (30% in total, 54% in the transmembrane regions) with the angiotensin II type-1 receptor (AT_1-R).[25] The human apelin gene, encoding the 77-amino acid preproapelin is located on chromosome Xq25–26.1. The gene shows great sequence homology across all species examined, with the last 23 residues of the C terminus being identical in mammals. Sequence analysis of the mature peptide reveals identity, albeit limited, to angiotensin II.[22] The 77-amino acid preproprotein is cleaved to shorter active peptides, such as apelin-36, apelin-17, apelin-13, apelin-12, and the post-translationally modified (Pyr1)apelin-13. All these are C-terminal remnants of the original preproapelin. They are all agonists of the APJ receptor, but binding affinity and biological efficacy differ from isoform to isoform, with the shorter fragments being more potent.[32] (Pyr1)apelin-13 is the most potent and abundant form in cardiac tissue.[23] Fragments shorter than 12 amino acids are biologically inert.[32] For a more detailed review of the discovery, structure, and metabolism of apelin and APJ receptor, please refer to the Apelin chapters in the Brain Peptides and Gastrointestinal Peptides sections of this book.

DISTRIBUTION WITHIN THE CARDIOVASCULAR SYSTEM

Apelin and APJ receptor are expressed widely through the organism. In humans, preproapelin and APJ mRNA are abundant in the central nervous system, heart, lung, kidney, pancreas, and placenta. In human vasculature, both apelin and its receptor, APJ, are detectable immunohistologically in endothelial cells and vascular smooth muscle cells of large conduit vessels, small arteries, and veins. In the heart, APJ-like immunoreactivity is present in the endocardial endothelium and, to a lesser extent, also in the myocardium (reviewed in Ref. [19]). The apelin peptide is present in normal human plasma with a brief half-life of <5 min.[17] The plasma concentration of immunoreactive apelin is approximately 10^{-10} g/ml. Apelin-like immunoreactivity is present in the heart, being >200-fold higher in the right atria than in the left ventricles.[12] This distribution pattern, low plasma concentration, and short lifespan of circulating peptide suggest an autocrine or paracrine way of action in the cardiovascular system.

SIGNALING CASCADES

Apelin inhibits forskolin-induced cAMP production in cells expressing APJ. This finding suggests that APJ couples to inhibitory G proteins (G_i). Apelin peptides activate p70S6 kinase, a regulator of cell cycle progression, in umbilical endothelial cells through two separate phosphorylation signals. One regulator is the phosphoinositide 3-kinase (PI3 K)-Akt cascade and the other pathway is mediated via extracellular signal-regulated kinases (ERKs). These apelin-induced signaling cascades are pertussis toxin sensitive, strengthening the hypothesis that the APJ receptor is linked to G_i proteins.[24] On the contrary, pertussis toxin can

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00187-1
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only partially inhibit the biological effect of apelin in rat hearts. The apelin signaling appears to involve activation of phospholipase C and protein kinase C, a pathway known to be mediated through G_q proteins. Therefore, APJ receptor may be linked to G_q proteins as well.[29]

BIOLOGICAL ACTIONS IN THE CARDIOVASCULAR SYSTEM

Effects on Vasculature

Vascular Tone

Given the peptide's and its receptor's similarity in sequence and in tissue distribution profile with the Ang II/AT_1-R system, it is not surprising that apelin is a potent regulator of the cardiovascular system. Despite the shared sequences, Ang II does not bind to APJ receptors,[25] and apelin modulates blood pressure oppositely to Ang II. When administered to rats *in vivo*, apelin reduces systolic and diastolic arterial blood pressure significantly, with a slight or no increase in heart rate. The drop is transient, lasting for 3–4 min.[22,32] Besides arterial vasodilatation, intravenous bolus apelin injection to rats causes a drop in the mean circulatory filling pressure, an indicator of systemic venous pressure, suggesting that apelin is a potent venodilator as well.[7] The arterial vasodilator effect of apelin exists also in humans both *in vivo*[17] and ex vivo.[23] Furthermore, direct vasodilatation of the coronary arteries is also detected. On the contrary, apelin does not seem to affect peripheral venous tone in humans.[17] Conversely, apelin exerts a biphasic hemodynamic response in sheep, characterized by an acute fall in arterial pressure and an increase in the heart rate, followed immediately by an increase in arterial pressure and a fall in heart rate. The secondary hypertensive phase is associated with a fall in cardiac output.[4]

The APJ is the single receptor involved in the depressor effect of apelin, because genetic knockout of APJ abolishes the blood pressure lowering effect of exogenous apelin administration in mice.[15] The apelin-induced reduction in blood pressure is most probably mediated by nitric oxide (NO), because it is almost completely abolished by pretreatment with the NO synthase (NOS) inhibitor L-NAME. Moreover, plasma nitrite and nitrate concentrations are significantly increased after apelin administration.[32] Endothelial NOS (eNOS) is supposed to be the main source of NO during the apelin-induced vascular response, as eNOS regulatory phosphorylation (on Ser^{1176}) is markedly increased after apelin treatment in wildtype mouse endothelial cells, an effect not seen in endothelial cells from APJ knockout mice.[15] Parallel to this, the net NO bioavailability significantly increases in apelin-treated mice compared with controls. L-NAME administration abolishes this effect, further strengthening the hypothesis that apelin stimulates NOS activity.[8] Therefore, the depressor effect of apelin seems to be mediated by the well-described Akt-eNOS pathway, leading to NO formation, which activates the soluble guanylate cyclase in vascular smooth muscle cells and consequently relaxes vessels (Fig. 1).

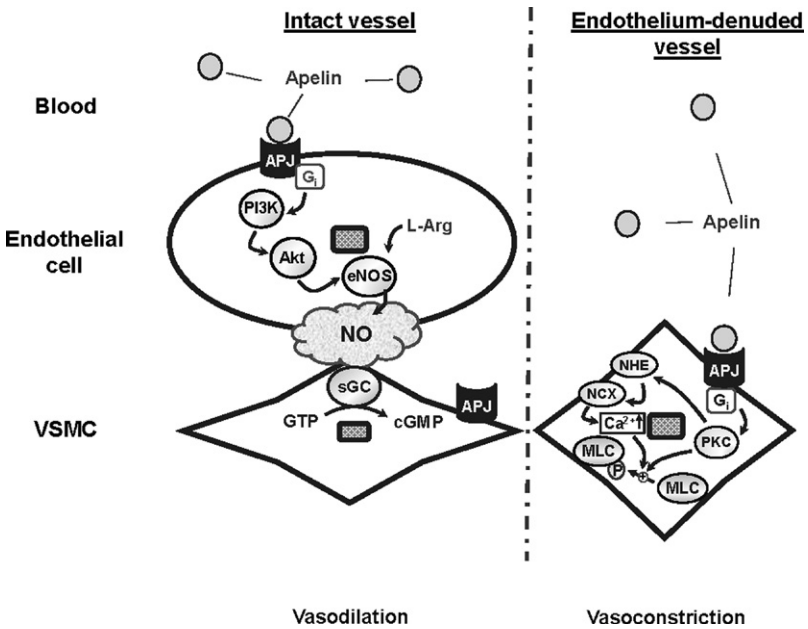

FIGURE 1 The effect of apelin on vascular tone. In intact vessels, apelin activates the PI3 K/Akt pathway in endothelial cells, leading to NO formation by eNOS. NO diffuses to VSMCs and activates soluble guanylate cyclase (sGC), inducing cGMP production, which results in vasodilatation. On the contrary, apelin generates vasoconstriction in the absence of endothelium. MLC is the key regulator of contraction in smooth muscles. Activated APJ on VSMC induces MLC phosphorylation via PKC, NHE, and NCX.

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Interestingly, apelin acts as a potent vasoconstrictor on isolated, endothelium-denuded human saphenous veins, and mammary arteries.[23] These results suggest that the endothelium is essential for the vasodilator effect of apelin in the vasculature. The peptide supposedly triggers different signaling pathways when binding to receptors directly on vascular smooth muscle cells (VSMCs), resulting in vasoconstriction. The possible mechanisms involved in vascular contraction have been examined by Hashimoto et al.[14] They show that apelin stimulates myosin light chain (MLC) phosphorylation in VSMCs, an event responsible for smooth muscle contraction. Apelin-induced phosphorylation of MLC is accompanied by myosin phosphatase target subunit phosphorylation, which inhibits MLC phosphatase activity and induces phosphorylation of MLC. Their results suggest that apelin may cause vasoconstriction in VSMC by inducing MLC phosphorylation, a process that involves pertussis toxin-sensitive G proteins and activation of protein kinase C (PKC), sodium–proton exchanger (NHE), sodium–calcium exchanger (NCX), and myosin phosphatase target subunit but not phospholipase C (PLC)[14] (Fig. 1). Nevertheless, according to the *in vivo* results with the overall vasodilatation observed, vasoconstriction is overpowered by a more dominant NO-signaling, either because NO is more potent than apelin on the VSMCs or local tissue distribution of apelin favors the endothelium-derived vascular response.

Angiogenesis

Apelin signaling is necessary and sufficient to trigger embryonic angiogenesis in Xenopus,[21] and apelin-deficient mice have impaired retinal vascularization.[18] Apelin acts as a potent mitogenic[24] and chemotactic factor[21] *in vitro* for human primary endothelial cells. The mitogenic effect is associated with the activation of p70S6 K, suggesting the involvement of ERK and Akt-dependent cascades.[24] Apelin seemingly acts as a downstream mediator of vascular endothelial growth factor in Xenopus and mice as well, most probably in an autocrine or paracrine fashion.[21,18] These results implicate a possibly important role for the apelin–APJ system in embryonic and tumor angiogenesis.

Apelin and the Renin–Angiotensin System

The relationship between Ang II and apelin is not limited to structural similarity and shared localization within the organism, but the two-peptide systems interfere with each other on multiple sites of function. Ishida et al. provided evidence for this interaction: APJ knockout mice, in contrast to the wild-type animals, are highly sensitive to low dose Ang II treatment, suggesting a possible regulatory effect of the endogenous apelin system to counterbalance the pressor effects of Ang II. These findings are further supported by other experiments, in which the blood pressure values of APJ/AT_1-R double knockout mice show partial normalization compared with those of AT_1-R single knockout mice.[15] Moreover, studies on rat primary aortic smooth muscle cells show that apelin has direct inhibitory effects on Ang II-mediated increases in gene expression. Interestingly, APJ and AT_1-R are physically associated in HEK293 cells expressing both receptors, and this association of APJ seems to inhibit AT_1-R signaling in a stoichiometric fashion, independent of the presence of apelin, implicating a direct receptor–receptor crosstalk.[8]

Effects on Heart

Contractility

Apelin is a potent inotropic agent in isolated rat heart preparations in a dose-dependent manner.[29] Similar results are observed in isolated adult rat ventricular myocytes.[10] Although the ex vivo positive inotropic effect of apelin has been repeatedly shown, its effect *in vivo* is less clear. For instance, although *in vivo* acute administration of apelin increases certain load-dependent and load-independent (e.g. preload recruitable stroke work) parameters of cardiac contractility, the increase in cardiac output is not significant in rats.[2] Apelin treatment causes a reduction in left ventricular end-diastolic area and increased left ventricular elastance; however, it fails to increase cardiac output in mice. Therefore, the change in loading conditions after acute apelin administration, together with changes in the viscoelastic properties of the ventricle, may influence the net effect of apelin on cardiac output. Chronic administration of apelin (two-week continuous infusion) to mice results in significant increase in the velocity of circumferential shortening and cardiac output. Importantly, these hearts show no evidence of left ventricular hypertrophy, which is frequently seen after chronic administration of other positive inotropic substances.[1] Apelin and APJ knockout mice develop a modest impairment of baseline cardiac contractility *in vivo* in both load-dependent and load-independent measures. Moreover, in isolated left ventricular myocytes from apelin$^{-/-}$ and APJ$^{-/-}$ mice, impaired sarcomere function is observed with no change in baseline sarcomere length.[5] Finally, acute administration of apelin could increase cardiac output in healthy humans. Because apelin causes a simultaneous decrease in peripheral resistance, its effects on cardiac function may be partly attributable to a reduction in afterload. However, an additional direct inotropic effect is probable, as the increase in cardiac output occurs without significant changes in the heart rate or the blood pressure.[17]

Being active in the subnanomolar range (EC_{50} values: 33 pmol/L), apelin seems to be one of the most potent endogenous positive inotropic agent yet identified. The maximum increase in developed tension induced by apelin is approximately 70% of that of isoproterenol response, and it is comparable in magnitude to the results seen previously with

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the inotropic peptides endothelin and adrenomedullin. The inotropic effect of apelin develops slower than that of the beta-adrenergic response, but lasts longer ex vivo, having its peak between 20 and 30 min after the initiation of treatment. The apelin-induced increase in cardiac contractility develops independently of the release of catecholamines, other vasoactive peptides (endothelin, Ang II) or NO.[29]

Szokodi et al. also investigated the intracellular mechanisms underlying the inotropic effect of apelin. Their results show that pertussis toxin treatment only partly reduces the apelin-induced positive inotropic response, suggesting that apelin may act via both pertussis toxin-insensitive G_q and —sensitive G_i proteins. Experiments with a variety of pharmacological inhibitors imply the involvement of PLC, PKC, NHE, and NCX in the positive inotropy-related signaling.[29] The role of NHE in this process is confirmed by the observation that apelin increases intracellular pH and NHE activity in adult cardiomyocytes.[10] Stimulation of the NHE can lead to intracellular alkalization and sensitization of cardiac myofilaments to intracellular Ca^{2+}. However, NHE-mediated accumulation of intracellular Na^+ can indirectly promote a rise in intracellular levels of Ca^{2+} via reverse mode NCX. Concomitant inhibition of NCX and NHE supports the hypothesis that NHE and NCX are proximal and distal components in this signaling pathway[29] (Fig. 2). However, the impact of apelin on intracellular Ca^{2+} homeostasis is still controversial. Wang et al. reported a double effect of apelin on intracellular Ca^{2+} concentrations in isolated rat cardiac myocytes, such as systolic increase and diastolic decrease via PKC-dependent mechanism. Additionally, they found that apelin enhances the activity of NCX and SERCA.[33] On the contrary, apelin stimulation in perforated patch-clamp experiments does not modulate Ca^{2+} flux through L-type Ca^{2+} channels,[29] moreover, no apelin-induced changes of Ca^{2+} transients could be detected with fluorescent indicators.[10] These results suggest that apelin may exert its positive inotropic effect by enhancing the sensitivity of myofilaments to Ca^{2+} rather than increasing intracellular Ca^{2+} concentrations. Overall, these results indicate that apelin may contribute to the regulation of physiological cardiac contractile function.

Electrophysiology

The localization of APJ receptor in the intercalated disc region, the cellular structure involved in the electric coupling between cardiomyocytes, makes apelin likely to influence electrophysiological properties of cardiac tissue. Farkasfalvi et al. could show in monolayers of cultured neonatal cardiomyocytes that apelin increases the frequency of spontaneous activation and conduction velocity, and

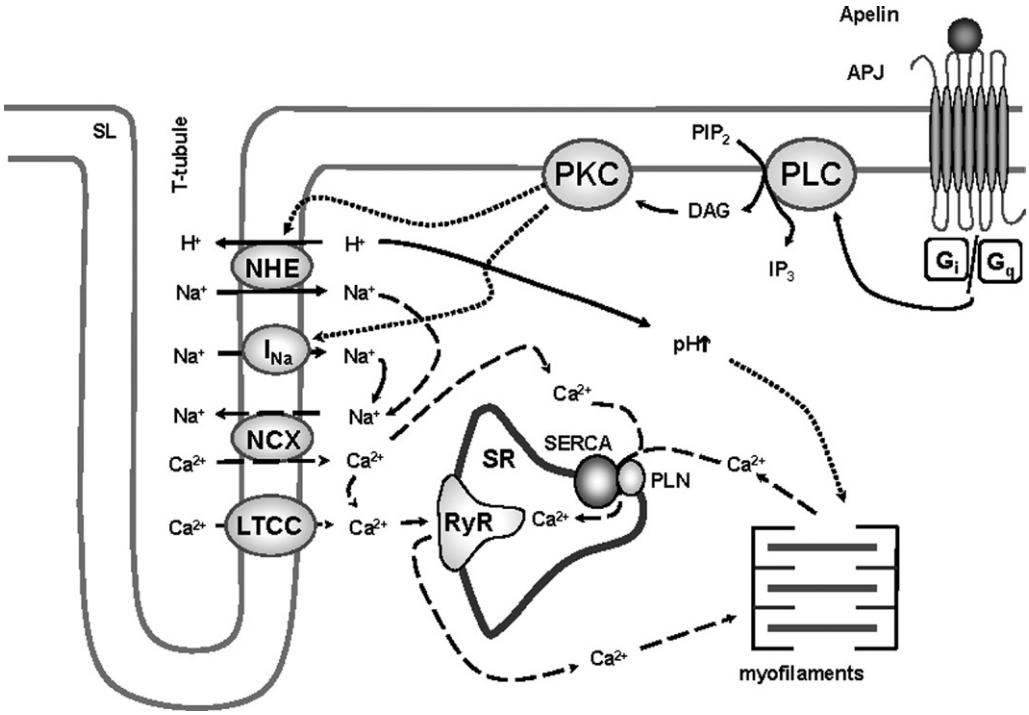

FIGURE 2 Apelin increases cardiac contractility. Apelin may exert positive inotropic effect via both Gq and Gi proteins. PKC can induce NHE activity. Stimulation of the NHE can lead to intracellular alkalization and consequent sensitization of cardiac myofilaments to intracellular Ca^{2+}. Furthermore, NHE-mediated accumulation of intracellular Na^+ can indirectly promote a rise in intracellular levels of Ca^{2+} via reverse mode NCX. Continuous and dotted arrows represent apelin-mediated changes. Dotted lines mark indirect evidences. Dashed arrows stand for mechanisms of regular excitation of cardiomyocytes.

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decreases the duration of field potential.[10] In canine cardiomyocytes, apelin increased cardiac peak sodium current (I_{Na}) and shifted channel opening probability to more negative voltage. In line with these findings, apelin increases cardiac excitability. These results suggest that apelin-related changes in I_{Na} may contribute to the positive inotropic effect of the peptide. Moreover, apelin slows I_{Na} recovery from inactivation, thereby prolonging the refractory period. This latter effect may confer antiarrhythmic properties to apelin.[3]

There are controversial results about apelin's effect on the heart rate *in vivo*. Usually, no significant change in the heart rate can be detected in anesthetized animals,[22,32] while conscious animals or animals in superficial narcosis produce a slight increase in response to acute apelin administration.[1,7] However, apelin causes reductions in mean arterial pressure without changing heart rate in the sympathetic ganglionic-blocked rats, implying that the increase of the heart rate in intact animals probably results from a vegetative reflex to hypotension, and apelin has no significant independent chronotropic effect.[7] Suppression of the same reflex in deep anesthesia could also explain the different results obtained.

Cardiomyogenesis

Results in zebrafish provide evidence that the apelin–APJ pathway is required to mediate migration of myocardial progenitors to the correct position at the onset of gastrulation.[35] D'Aniello et al. identified APJ and apelin as effectors of the Cripto/Smad2 pathway, a mechanism necessary in the early phase of mammalian cardiac differentiation. Their results suggest that apelin activates ERK-, but not Akt-dependent p70S6 K cascade in cardiac development.[9] The importance of the apelin–APJ system in cardiomyogenesis is also shown by the deletion of APJ gene. Homozygous APJ null-mutant mice have significantly lower birth rates, whereas early stage embryos produce multiple cardiac developmental defects.[5]

PATHOPHYSIOLOGICAL IMPLICATIONS

Apelin and Atherosclerosis

Atherosclerosis is a major cause of morbidity and mortality in the western world. Evidence suggests an important regulatory role of the apelin–APJ system in the pathophysiology of atherosclerosis; however, the exact function of apelin is not entirely clear yet. Chun et al. reported that apelin–apolipoprotein E (ApoE) double knockouts develop significantly greater atherosclerotic lesion areas compared with that in ApoE-knockout mice, irrespective of the type of diet that was administered. It indicates that endogenous apelin has antiatherogenic properties. As seen above, apelin can antagonize some Ang II-induced cardiovascular effects. Consequently, concomitant apelin administration completely blocks exogenous Ang II-induced atherosclerosis and abdominal aortic aneurysm formation in ApoE-KO mice. This antiatherogenic feature of apelin is NO-dependent, and it cannot be explained solely by its blood pressure lowering effect, because another antihypertensive drug (hydralazine) does not reduce plaque size formation during Ang II treatment. Proatherogenic effect of Ang II is, at least partly, linked to excessive reactive oxygen species (ROS) production. It is, therefore, interesting, that apelin can also limit Ang II-induced elevation in vascular ROS production.[8]

On the contrary, another study by Hashimoto et al. shows that APJ and ApoE double knockout mice on high cholesterol diet have atherosclerotic lesion significantly smaller in size compared with that in the $APJ^{+/+}/ApoE^{-/-}$ animals. This strongly contrasts with the findings of Chun et al., because it proposes that the presence of apelin signaling is proatherogenic. Furthermore, Hashimoto et al. found that vascular production of superoxide radicals is decreased in $APJ^{-/-}/ApoE^{-/-}$ mice compared with that in $APJ^{+/+}/ApoE^{-/-}$ mice, suggesting that activation of APJ receptors by endogenous apelin potentially increases ROS generation.[13] Further investigations are needed to clarify these controversial findings.

Even though the net atherogenic effect of the endogenous apelin–APJ system remains to be determined, Peltonen et al. reported that aortic valve stenosis in humans is characterized by overexpression of both apelin and APJ in the sclerotic plaques of the valves. They suggest that the peptide and its receptor may play a role in neovessel formation, macrophage chemotaxis and activation, and fibroblast proliferation, all of which are well-documented phenomena in the atherosclerotic disease process.[26]

Apelin in Ischemia and Reperfusion

Simpkin et al. provided the first evidence for the direct cardioprotective effect of apelin against ischemia–reperfusion injury. The peptide, given in pharmacological doses during reperfusion, can reduce infarct size in ex vivo and *in vivo* murine ischemia–reperfusion models. In addition, in isolated cardiomyocytes apelin delays the opening of the mitochondrial permeability transition pore, the major determinant of cellular death or survival. These apelin-induced cardioprotective effects are all mediated via PI3 K- and ERK-dependent pathways,[28] the components of the reperfusion injury salvage kinase (RISK) cascade. Apelin-related reduction of infarct size was also seen in rats *in vivo* by Tao et al. They found that apelin blocks ischemia–reperfusion injury via modulating endoplasmic reticulum (ER) stress-dependent apoptosis with a strong correlation within activation of ER-stress induced apoptotic pathways, proportion of apoptotic cells, and cardiac infarct size. Intact PI3 K/Akt,

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ERK, and AMP-activated kinase (AMPK) function was necessary for the apelin-derived inhibition of ER stress and in line with this, also for the reduction of infarct size. It is remarkable, that unlike numerous other apelin-induced cardiovascular effects, blockade of ER stress was independent from eNOS. However, suppression of eNOS activity abolishes the apelin-induced reduction of infarct size after 2 h of reperfusion, but in a later phase (between 2 and 24 h), apelin develops further cardioprotection without functional eNOS, indicating that NO contributes only to the early phase of the cardioprotective effect of apelin unrelated to ER stress. Further interesting findings of Tao et al. suggest interactions among the above-mentioned signaling cascades. PI3 K/Akt and AMPK phosphorylation levels are partially blocked by inhibition of the other. In addition, the inhibition of either PI3 K/Akt or AMPK also partially attenuates eNOS activation, indicating that both signaling pathways function upstream to eNOS. ERK activation seems to be independent of PI3 K/Akt, AMPK, or eNOS signaling.[30] Thus, apelin can be considered as a postconditioning agent in ischemia reperfusion. Of note, the effect of apelin on preconditioning is not known.

These experimental findings suggest that the apelin–APJ system could be a potential target in the treatment of acute coronary syndrome. Furthermore, Weir et al. report that plasma apelin is reduced in man after myocardial infarction,[34] indicating that apelin system may be involved in the pathophysiology of acute cardiac ischemia.

The Apelin–APJ System in Heart Failure

Cardiac Expression and Plasma Levels in Heart Failure

As described above, apelin is one of the most potent endogenous inotropic substances known so far. Moreover, it causes vasodilatation in both arterial and venous systems, thereby reducing cardiac afterload and preload, making apelin unique among inotropic agents. These characteristics brought the apelin–APJ system into the focus of heart failure research.

Chen et al. reported that apelin plasma concentrations are elevated significantly in humans in early stages of heart failure, but fall below baseline level in patients suffering from severe heart failure.[6] This is in line with the findings of Földes et al., who observed that plasma concentrations of apelin are significantly decreased in patients with advanced heart failure.[12]

Expression of apelin and APJ receptor changes also within the failing heart. Left ventricular apelin mRNA levels are significantly increased in humans with heart failure resulting from coronary heart disease or idiopathic dilated cardiomyopathy; however, atrial apelin mRNA levels are similar in failing and nonfailing hearts. Simultaneously, cardiac APJ mRNA expression is significantly decreased in heart failure. Thus, one could propose that induction of apelin gene expression in the failing ventricles constitutes an adaptive mechanism triggered by increased cardiac overload. Downregulation of the APJ receptor, however, could be maladaptive, because lower expression level could lead to lower receptor density and to consequent impaired apelin efficacy.[12] This hypothesis is supported by the findings of Chen et al. showing that the APJ receptor is one of the most upregulated genes in the myocardium after placement of left ventricular assist device. Moreover, mechanical offloading of the failing left ventricle also increases cardiac tissue apelin levels.[6] Thus, although the apelin–APJ signaling cascade is capable of increasing inotropy and reducing peripheral resistance, both beneficial in heart failure, this pathway is probably endogenously downregulated in severe heart disease.

Effect on Cardiac Function in the Failing Heart

Because the APJ receptor is downregulated in heart failure, one may speculate that apelin would not increase cardiac contractility in failing hearts as much as seen previously in normal, healthy myocardium. There are, however, several studies showing that the positive inotropic effect of apelin is conserved in heart failure. Acute apelin administration increases cardiac contractility *in vivo* in rats with myocardial infarction-related heart failure similar to that in control animals.[2] These results could be reproduced in adult ventricular myocytes, when the rate of increase in sarcomere shortening is the same in cells isolated either from normal or failing hearts.[10] Additionally, short-term apelin infusion increases cardiac output and reduces cardiac preload *in vivo* in humans, with the hemodynamic effects being preserved in patients with heart failure.[17]

Loss-of-function mutant animal models can also provide interesting features from the role of apelin–APJ system in preventing pathophysiological processes. For instance, mice lacking apelin gene, and, therefore, the functional peptide, develop impaired cardiac function with age. Older apelin$^{-/x}$ knockout animals have progressive systolic dysfunction in the absence of histological abnormalities, while systolic function is maintained in wildtype littermates. It is remarkable that two weeks of apelin infusion restores this loss of systolic function. Moreover, the same apelin$^{-/x}$ knockout mice develop more serious systolic and diastolic heart failures than the wildtype animals in the chronic pressure overload model of aortic constriction. The loss of apelin has no marked effect on pressure overload-induced adaptive cardiac hypertrophy, whereas it results in increased interstitial fibrosis.[20] Like the lack of apelin peptide, deletion of the APJ receptor does not cause marked changes of basal contractile function in adult mice. However, with exercise stress both mutant lines show consistent decreases in exercise capacity.

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In addition, isolated left ventricular cardiomyocytes lacking either apelin or APJ manifest less sarcomere shortening and impaired velocity of contraction and relaxation with no difference in calcium transient.[5] These results propose that endogenous apelin–APJ system has a modest role in maintaining basal cardiac contractility, but this cascade possesses more substantial role during stressed conditions.

Apelin Influences Fibrosis

Chronic heart failure is characterized by the progressive activation of the renin–angiotensin–aldosterone system. Ang II is responsible for many deleterious processes during the progression of the disease, including stimulation of growth in cardiomyocytes and proliferation in smooth muscle cells, and increase in extracellular matrix formation. The apelin–APJ cascade can affect Ang II signaling and vice versa[15,8] (see Section 4.2). Double transgenic rats harboring both the human angiotensinogen and human renin genes develop severe hypertension and cardiac hypertrophy. Apelin expression is significantly reduced in those animals.[29] Parallel with this, Iwanaga et al. report that apelin and APJ mRNA levels are markedly downregulated in Dahl salt-sensitive hypertensive rats with decompensated heart failure. This downregulation is restored after treatment with AT_1-R blocker (ARB).[16] According to Siddiquee et al., apelin attenuates, but does not entirely block Ang II-induced cardiac and perivascular fibrosis *in vivo* in wildtype mice.[27] This is in line with the observations of Kuba et al., who found excessive fibrosis in chronic pressure overloaded apelin$^{-/x}$ mice. The lack of apelin changes the gene expression profile of the heart during chronic pressure overload, upregulating sets of genes involved in extracellular matrix remodeling and fibrosis.[20] The antifibrotic effects of apelin are similar to those reported for ARBs. Activation of plasminogen activator inhibitor-1 (PAI-1) is a key step in promoting Ang II-induced fibrosis. Consequent with the observations of decreased fibrosis *in vivo*, apelin inhibits Ang II-linked upregulation of PAI-1, as well as several other Ang II-target genes that contribute to the development of fibrosis *in vivo*. Moreover apelin alone significantly decreases the cardiac expression of PAI-1. Of note, apelin alone increases the expression of the collagen-degrading enzyme matrix metalloproteinase-2, while simultaneously reducing the expression of collagen *in vitro*. Ang II stimulates PAI-1 through Rho kinase. Apelin decreases phosphorylation of MLC and myosin phosphatase subunit, two targets downstream of Rho kinase.[27] This, however, conflicts with another report showing that apelin administration could increase phosphorylation of MLC.[14] Siddique et al. have proposed that inhibition of PAI-1 expression by apelin results from the antagonism of Ang II-induced activation of Rho-kinase pathway. NO synthase-inhibitor L-NAME exerts effects similar to that of Ang II under the same conditions regarding fibrosis and PAI-1 upregulation; however, apelin could not reduce these changes at all. Moreover, administration of L-NAME abolishes the apelin-related inhibition of Ang II-induced upregulation of PAI-1 or fibrosis, suggesting that the beneficial effects of apelin are mediated through NOS. In addition, relative levels of NOS mRNA are significantly increased in the hearts of mice receiving apelin. Interestingly, addition of Ang II together with apelin further increase NOS mRNA levels. Conversely, NOS mRNA is significantly decreased in the hearts of mice receiving apelin plus L-NAME or apelin plus Ang II plus L-NAME. These findings propose a positive feedback loop, where apelin enhances NOS expression in a nitric oxide-dependent manner.[27]

Role in Cardiac Hypertrophy

Besides fibrosis, hypertrophy also occurs during cardiac remodeling. It is believed that ventricular hypertrophy represents a compensatory mechanism in response to an increased workload on cardiac muscle from different etiologies, such as exercise stress, myocardial infarction or pressure overload. However, the compensative mechanisms of remodeling could become unbalanced, leading to excessive hypertrophy or dilated cardiomyopathy, both deleterious for cardiac function and potentially resulting in heart failure. It is therefore very important to note that *in vivo* chronic apelin treatment can improve cardiac contractility in wildtype mice, without any signs of cardiac hypertrophy.[1] ROS have emerged as key mediators of hypertrophic remodeling. In cardiomyocytes, excessive ROS generation mediates the hypertrophic response to various forms of mechanical or neurohumoral stress. Apelin is able to abolish serotonin-induced hypertrophic response in isolated rat cardiomyocytes, in which the signaling pathway is known to involve monoamine oxidase-dependent hydrogen peroxide (H_2O_2) generation. Apelin completely blocks serotonin-related H_2O_2 formation as well. It was suggested that upregulation of H_2O_2-degrading enzymes are responsible for this effect, because apelin increases catalase mRNA expression and enzyme activity in rat cardiomyocytes. Parallel results are obtained in the murine model of pressure overload-induced cardiac hypertrophy, where apelin blunts cardiac hypertrophic growth significantly. Heart weight to body weight comparisons, histological analysis of cardiac tissues, and echocardiographic measurements all show reduced cardiac hypertrophy in apelin-treated animals. The antihypertrophic effect of apelin is associated with significant suppression of oxidative stress *in vivo*.[11]

PERSPECTIVE

Apelin and its cognate G protein-coupled APJ receptor are emerging as an important regulator of the cardiovascular homoeostasis. The expression of apelin and APJ receptor

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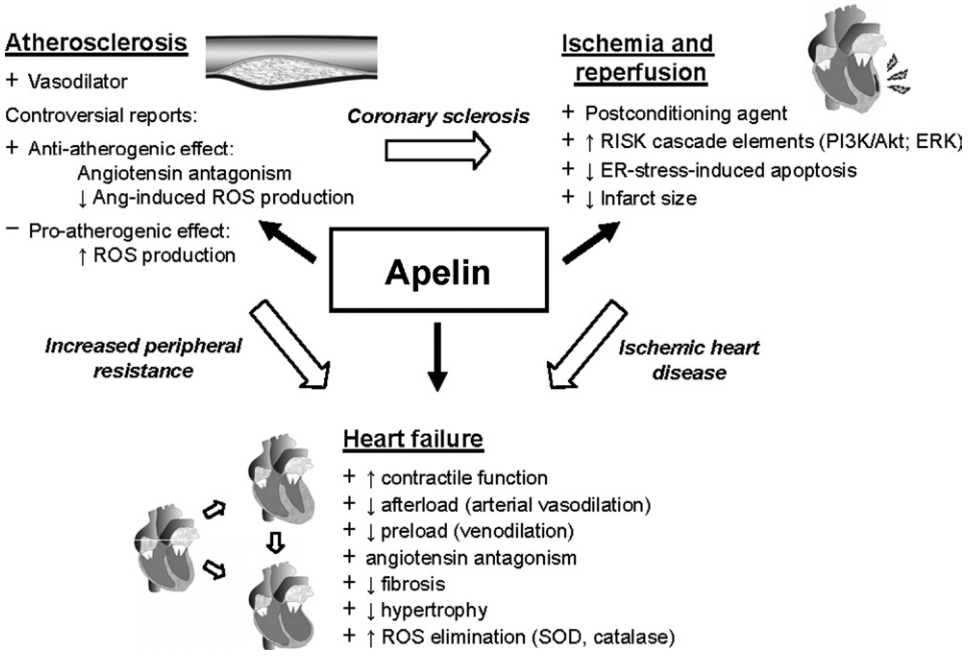

FIGURE 3 Potential role of apelin at pathological cardiovascular conditions. "+" and "−" mark beneficial and deleterious effects, respectively. Empty arrows represent connections between pathological entities.

is widely abundant throughout the cardiovascular system. In view of the putative antiatherogenic effect of apelin, the vasodilator effect on both arterial and venous side of the circulation, the involvement in regulation of cardiac contractile function, the antagonism of the renin–angiotensin system, the inhibition of cardiac hypertrophy and fibrosis, and the cardioprotective effect in ischemia and reperfusion (Fig. 3), the apelin–APJ system represents a promising therapeutic target in cardiovascular disease states, such as ischemic heart disease, myocardial infarction, and heart failure.

ACKNOWLEDGMENTS

This work was supported by grants from the Hungarian Scientific Research Fund (K69118), the Foundation for the Development of Interventional Cardiology, the Academy of Finland (Center of Excellence), Sigrid Juselius Foundation, Finnish Foundation for Cardiovascular Research, and Biocenter Oulu.

REFERENCES

1. Ashley EA, Powers J, Chen M, Kundu RK, Finsterbach TP, Caffarelli A, et al. The endogenous peptide apelin potently improves cardiac contractility and reduces cardiac loading in vivo. *Cardiovasc Res* 2005;**65**:73–82.
2. Berry MF, Pirolli TJ, Jayasankar V, Burdick J, Morine KJ, Gardner TJ, et al. Apelin has in vivo inotropic effects on normal and failing hearts. *Circulation* 2004;**110**:II-187–93.
3. Chamberland C, Barajas-Martinez H, Haufe V, Fecteau M-H, Delabre J-F, Burashnikov A, et al. Modulation of canine cardiac sodium current by apelin. *J Mol Cell Cardiol* 2010;**48**:694–701.
4. Charles CJ, Rademaker MT, Richards AM. Apelin-13 induces a biphasic haemodynamic response and hormonal activation in normal conscious sheep. *J Endocrinol* 2006;**189**:701–10.
5. Charo D, Ho M, Fajardo G, Kawana M, Kundu RK, Sheikh AY, et al. Endogenous regulation of cardiovascular function by apelin. *Am J Physiol Heart Circ Physiol* 2009;**297**:1904–13.
6. Chen MM, Ashley EA, Deng DXF, Tsalenko A, Deng A, Tabibiazar R, et al. Novel role for the potent endogenous inotrope apelin in human cardiac dysfunction. *Circulation* 2003;**23**(108):1432–9.
7. Cheng X, Cheng X, Pang C. Venous dilator effect of apelin, an endogenous peptide ligand for the orphan APJ receptor, in conscious rats. *Eur J Pharmacol* 2003;**470**:171–5.
8. Chun HJ, Ali ZA, Kojima Y, Kundu RK, Sheikh AY, Agrawal R, et al. Apelin signaling antagonizes Ang II effects in mouse models of atherosclerosis. *J Clin Invest* 2008;**118**:3343–54.
9. D'Aniello C, Lonardo E, Iaconis S, Guardiola O, Liguoro AM, Liguori GL, et al. G protein-coupled receptor APJ and its ligand apelin act downstream of Cripto to specify embryonic stem cells toward the cardiac lineage through extracellular signal-regulated kinase/p70S6 kinase signaling pathway. *Circ Res* 2009;**105**:231–8.
10. Farkasfalvi K, Stagg MA, Coppen SR, Siedlecka U, Lee J, Soppa GK, et al. Direct effects of apelin on cardiomyocyte contractility and electrophysiology. *Biochem Biophys Res Commun* 2007;**357**:889–95.
11. Foussal C, Lairez O, Calise D, Pathak A, Guilbeau-Frugier C, Valet P, et al. Activation of catalase by apelin prevents oxidative stress-linked cardiac hypertrophy. *FEBS Lett* 2010;**584**:2363–70.
12. Földes G, Horkay F, Szokodi I, Vuolteenaho O, Ilves M, Lindstedt KA, et al. Circulating and cardiac levels of apelin, the novel ligand of the orphan receptor APJ, in patients with heart failure. *Biochem Biophys Res Commun* 2003;**308**:480–5.
13. Hashimoto T, Kihara M, Imai N, Yoshida S-I, Shimoyamada H, Yasuzaki H, et al. Requirement of apelin–apelin receptor system for oxidative stress-linked atherosclerosis. *Am J Pathol* 2007;**171**:1705–12.

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14. Hashimoto T, Kihara M, Ishida J, Imai N, Yoshida S, Toya Y, et al. Apelin stimulates myosin light chain phosphorylation in vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol* 2006;**26**: 1267–72.
15. Ishida J, Hashimoto T, Hashimoto Y, Nishiwaki S, Iguchi T, Harada S, et al. Regulatory roles for APJ, a seven-transmembrane receptor related to angiotensin-type 1 receptor in blood pressure in vivo. *J Biol Chem* 2004;**279**:26274–9.
16. Iwanaga Y, Kihara Y, Takenaka H, Kita T. Down-regulation of cardiac apelin system in hypertrophied and failing hearts: possible role of angiotensin II-angiotensin type 1 receptor system. *J Mol Cell Cardiol* 2006;**41**:798–806.
17. Japp AG, Cruden NL, Barnes G, van Gemeren N, Mathews J, Adamson J, et al. Acute cardiovascular effects of apelin in humans: potential role in patients with chronic heart failure. *Circulation* 2010;**121**:1818–27.
18. Kasai A, Shintani N, Kato H, Matsuda S, Gomi F, Haba R, et al. Retardation of retinal vascular development in apelin-deficient mice. *Arterioscler Thromb Vasc Biol* 2008;**28**:1717–22.
19. Kleinz MJ, Davenport AP. Emerging roles of apelin in biology and medicine. *Pharmacol Therapeut* 2005;**107**:198–211.
20. Kuba K, Zhang L, Imai Y, Arab S, Chen M, Maekawa Y, et al. Impaired heart contractility in apelin gene deficient mice associated with aging and pressure overload. *Circ Res* 2007;**101**:e32–42.
21. Kälin RE, Kretz MP, Meyer AM, Kispert A, Heppner FL, Brändli AW. Paracrine and autocrine mechanisms of apelin signaling govern embryonic and tumor angiogenesis. *Dev Biol* 2007;**305**:599–614.
22. Lee DK, Cheng R, Nguyen T, Fan T, Kariyawasam AP, Liu Y, et al. Characterization of apelin, the ligand for the APJ receptor. *J Neurochem* 2000;**74**:34–41.
23. Maguire JJ, Kleinz MJ, Pitkin SL, Davenport AP. [Pyr1] Apelin-13 identified as the predominant apelin isoform in the human heart. Vasoactive mechanisms and inotropic action in disease. *Hypertension* 2009;**54**:598–604.
24. Masri B, Morin N, Cornu M, Knibiehler B, Audigier Y. Apelin (65-77) activates p70 S6 kinase and is mitogenic for umbilical endothelial cells. *FASEB J* 2004;**26**:1–26.
25. O'Dowd BF, Heiber M, Chan A, Heng HH, Tsui LC, Kennedy JL, et al. A human gene that shows identity with the gene encoding the angiotensin receptor is located on chromosome 11. *Gene* 1993;**136**:355–60.
26. Peltonen T, Näpäkangas J, Vuolteenaho O, Ohtonen P, Soini Y, Juvonen T, et al. Apelin and its receptor APJ in human aortic valve stenosis. *J Heart Valve Dis* 2009;**18**:644–52.
27. Siddiquee K, Hampton J, Khan S, Zadory D, Gleaves L, Vaughan DE, et al. Apelin protects against angiotensin II-induced cardiovascular fibrosis and decreases plasminogen activator inhibitor type-1 production. *J Hypertens* 2011;**29**:724–31.
28. Simpkin JC, Yellon DM, Davidson SM, Lim SY, Wynne AM, Smith CCT. Apelin-13 and apelin-36 exhibit direct cardioprotective activity against ischemia-reperfusion injury. *Basic Res Cardiol* 2007;**102**:518–28.
29. Szokodi I, Tavi P, Földes G, Voutilainen-myllylä S, Ilves M, Tokola H, et al. Apelin, the novel endogenous ligand of the orphan receptor APJ, regulates cardiac contractility. *Circ Res* 2002;**91**:434–40.
30. Tao J, Zhu W, Li Y, Xin P, Li J, Liu M, et al. Apelin-13 protects the heart against ischemia-reperfusion injury through inhibition of ER-dependent apoptotic pathways in a time-dependent fashion. *Am J Physiol Heart Circ Physiol* 2011:Epub ahead of print.
31. Tatemoto K, Hosoya M, Habata Y, Fujii R, Kakegawa T, Zou MX, et al. Isolation and characterization of a novel endogenous peptide ligand for the human APJ receptor. *Biochem Biophys Res Commun* 1998;**251**:471–6.
32. Tatemoto K, Takayama K, Zou MX, Kumaki I, Zhang W, Kumano K, et al. The novel peptide apelin lowers blood pressure via a nitric oxide-dependent mechanism. *Regul Peptides* 2001;**99**:87–92.
33. Wang C, Du J-F, Wu F, Wang H- C. Apelin decreases the SR Ca^{2+} content but enhances the amplitude of $[Ca^{2+}]i$ transient and contractions during twitches in isolated rat cardiac myocytes. *Am J Physiol Heart Circ Physiol* 2008;**294**:H2540–6.
34. Weir RAP, Chong KS, Dalzell JR, Petrie CJ, Murphy CA, Steedman T, et al. Plasma apelin concentration is depressed following acute myocardial infarction in man. *Eur J Heart Fail* 2009;**11**:551–8.
35. Zeng X-XI, Wilm TP, Sepich DS, Solnica-Krezel L. Apelin and its receptor control heart field formation during zebrafish gastrulation. *Dev Cell* 2007;**12**:391–402.

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Chapter 188

Bradykinin Peptides

Duncan John Campbell

ABSTRACT

The kallikrein–kinin system (KKS) generates a family of bioactive peptides with varying biological activities. These include hydroxylated and nonhydroxylated bradykinin and kallidin (Lys^0-bradykinin) peptides and their respective carboxypeptidase metabolites, des-Arg^9-bradykinin and des-Arg^{10}-kallidin. Although bradykinin and kallidin act mainly via the bradykinin type 2 (B_2) receptor, des-Arg^9-bradykinin and des-Arg^{10}-kallidin act mainly via the bradykinin type 1 (B_1) receptor. Kinins are potent vasodilators, promote natriuresis and diuresis, and have beneficial cardiovascular effects; however, kinins may also promote inflammation. Peptidases are important determinants of kinin levels, and increased kinin levels may contribute to both the beneficial and adverse effects of peptidase inhibitor therapy.

DISCOVERY

Bradykinin and its related peptides are generated by the action of kallikrein enzymes on kininogen substrates. Initial discoveries of the kallikrein–kinin system (KKS) took place in the laboratories of Frey and Kraut in Germany (reviewed by Werle[35]) and Roche e Silva in Brazil.[30] In the laboratory of Frey and Kraut, kallikrein-like activity was initially revealed by the hypotensive effects of intravenous injection of urine.[35] It was also found that plasma and serum deactivate this activity. Subsequent isolation of this hypotensive material in large amounts from the pancreas led to it being named kallikrein, although it was evident that the pancreas was not the only source of kallikrein. Kallikrein could be extracted from the pancreas in both the active and inactive forms. A similar hypotensive material was also isolated from plasma in both the active and inactive forms and was named plasma kallikrein. Moreover, the inactive forms of kallikrein were activated by trypsin. The biological activity of kallikrein extracted from the submandibular salivary gland was found to be due to its enzymatic release of a low-molecular-weight, thermostable compound from a serum protein. This low-molecular-weight compound, believed to be a polypeptide, was initially called DK, and later called kallidin, and its inactive precursor was called kallidinogen.[35]

In the laboratory of Roche e Silva, bradykinin was initially identified as a hypotensive and smooth muscle-stimulating factor released from plasma globulin by snake venoms and by trypsin.[30] It was named bradykinin because it produced a slow contraction of the guinea pig ileum.[30] Trypsin digestion of an ammonium sulfate fraction from ox serum produced the nonapeptide bradykinin, the sequence of which was reported by Elliott et al. in 1960.[13] However, digestion of human plasma with human urinary kallikrein produced two peaks of bioactivity, one (named kallidin I) corresponding to bradykinin and a second (named kallidin II) that corresponded to lysine bradykinin.[27] Because human urinary kallikrein can hydrolyze synthetic arginine esters but not lysine esters, Pierce and Webster concluded that human urinary kallikrein produced lysine bradykinin (now known as kallidin).[27]

STRUCTURE OF KININOGEN mRNA AND GENE

Kininogens are glycoproteins that contain the bradykinin sequence in their midportion. There are two forms of kininogen that differ in structure, size, and susceptibility to cleavage by plasma and tissue kallikreins.[3] Human high-molecular-weight kininogen (HMWK) has 626 amino acids, with a mass of 88–120 kDa, whereas human low-molecular-weight kininogen (LMWK) has 409 amino acids, with a mass of 50–68 kDa.[32] The human kininogen gene encompasses 11 exons spanning approximately 27 kb and codes for both HMWK and LMWK by differential splicing of the initial messenger RNA (mRNA) transcript (Fig. 1).[18,32] In the rat, T-kininogen (major acute phase protein) is a potential precursor for Ile,Ser-bradykinin and Met,Ile,Ser-bradykinin.[14] However, there is no evidence that T-kininogen is a precursor of kinin peptides *in vivo*.[9]

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00188-3
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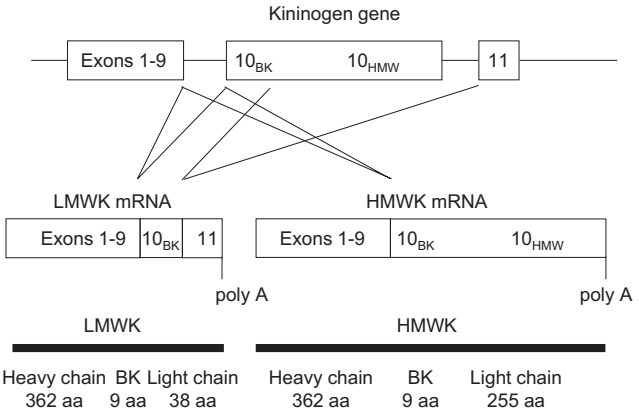

FIGURE 1 Diagrammatic representation of differential splicing of the kininogen gene transcript that produces high-molecular-weight kininogen (HMWK) and low-molecular-weight kininogen (LMWK) mRNA. HMWK represents exons 1–9 spliced to the whole of exon 10, whereas LMWK represents exons 1–9 and the first 78 base pairs of exon 10 spliced to exon 11.[18,32] Both HMWK and LMWK contain the bradykinin (BK) sequence in their midportion, between their respective heavy and light chains. aa, amino acid. Not drawn to scale.

PROCESSING OF THE PRECURSOR

Bradykinin and kallidin are released from kininogens predominantly by the action of the serine proteases plasma kallikrein and tissue kallikrein. Plasma kallikrein is initially secreted as the inactive prekallikrein, and tissue kallikrein is initially secreted as inactive prokallikrein. Both plasma prekallikrein and tissue prokallikrein are activated by serine protease activity. Although a single gene codes for plasma prekallikrein, there is a large family of tissue prokallikrein genes that code for tissue-kallikrein-like serine proteases. In humans, *Klk1* codes for true tissue kallikrein, the only member of the tissue-kallikrein-like family of serine proteases known to generate kinin peptides.[20] However, in rats, true tissue kallikrein is referred to as *Klk1b3*, although Lundwall et al. recommended that this sequence be given the gene symbol *Klk1*.[20]

In humans, plasma kallikrein forms bradykinin from HMWK, whereas tissue kallikrein forms kallidin from HMWK and LMWK (Figs 2 and 3). By contrast, both plasma and tissue kallikrein generate bradykinin in rodents.[3,5] In humans, a proportion of kininogens are hydroxylated on Pro^3 of the bradykinin sequence, leading to the formation of hydroxylated kinin peptides (Hyp^3-bradykinin and Hyp^4-kallidin). Hydroxylated kinins have biological activities that are similar to those of nonhydroxylated kinins.[29]

Alternative pathways of kinin formation involving enzymes other than kallikreins may operate in disease states.[5] Although LMWK is a poor substrate for plasma kallikrein, cleavage by neutrophil elastase renders it much more susceptible to cleavage by plasma kallikrein to release bradykinin. Moreover, the combination of mast cell tryptase and neutrophil elastase releases bradykinin from oxidized kininogens that are resistant to cleavage by kallikreins.

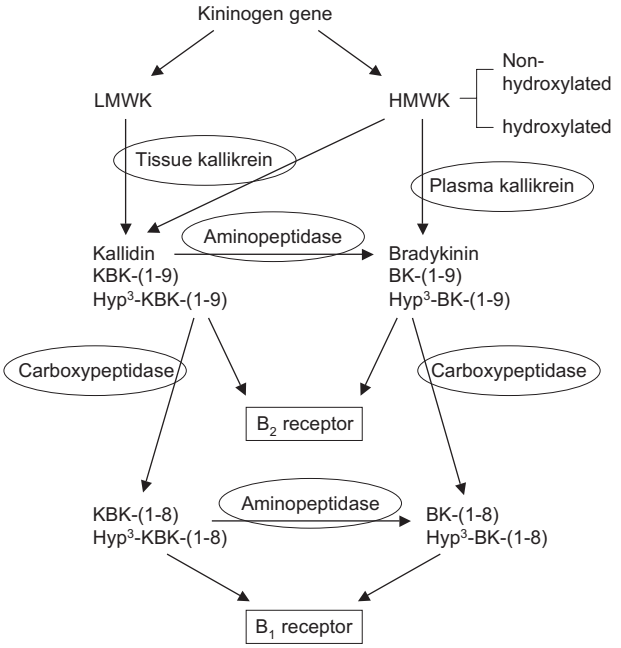

FIGURE 2 Overview of the kallikrein–kinin system in humans. LMWK, low-molecular-weight kininogen; HMWK, high-molecular-weight kininogen; Hyp, hydroxyproline. A proportion of kininogen is hydroxylated on the third amino acid (Hyp^3) of the bradykinin sequence of the precursor. Thus, bradykinin peptides may be nonhydroxylated [BK-(1-9)] or hydroxylated [Hyp^3-BK-(1-9)]. Kallidin is Lys^0-bradykinin and may also be either non-hydroxylated [KBK-(1-9)] or hydroxylated [Hyp^3-KBK-(1-9)]. Similarly, the carboxypeptidase metabolites des-Arg^9-BK-(1-9) and des-Arg^9-KBK-(1-9) may also be either non-hydroxylated [BK-(1-8), KBK-(1-8)] or hydroxylated [Hyp^3-BK-(1-8), Hyp^3-KBK-(1-8)].

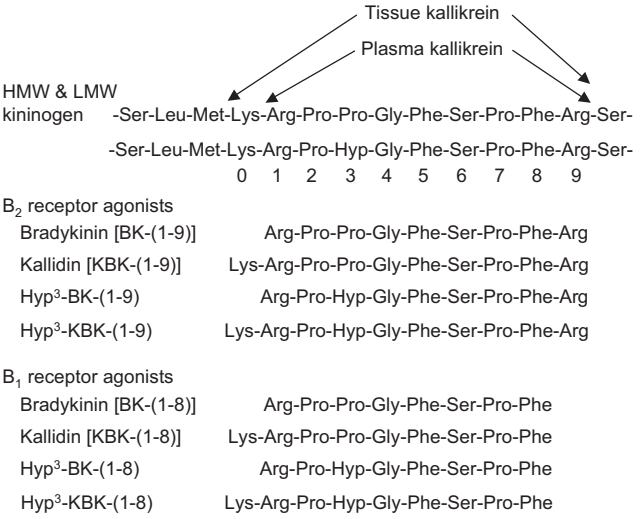

FIGURE 3 Amino acid sequences of the agonists of the type 1 (B_1) and type 2 (B_2) bradykinin receptors. Hyp, hydroxyproline. Bradykinin [BK-(1-9)] and kallidin [Lys^0-bradykinin, KBK-(1-9)] are excised from the interior of the kininogen molecules. Tissue kallikrein cleaves both HMWK and LMWK between Met^{379} and Lys^{380} (the same numbering for human HMWK and LMWK precursors) to produce the amino-terminus and between Arg^{389} and Ser^{390} to produce the carboxy-terminus of kallidin. Plasma kallikrein cleaves HMWK between Lys^{380} and Arg^{381} to produce the amino-terminus and between Arg^{389} and Ser^{390} to produce the carboxy-terminus of bradykinin.

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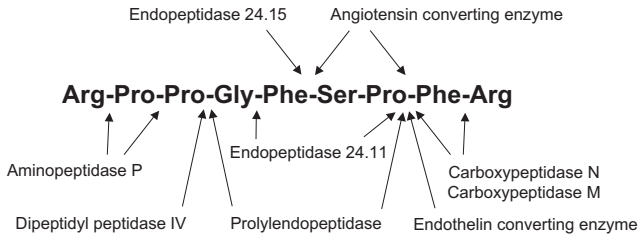

FIGURE 4 Metabolism of bradykinin. Carboxypeptidase N is known as kininase I and angiotensin-converting enzyme is also known as kininase II. Carboxypeptidases M and N produce sequential cleavage of amino acids from the carboxy-terminus of the peptide. Aminopeptidase P produces sequential cleavage of amino acids from the amino-terminus of the peptide. Angiotensin-converting enzyme is a carboxy dipeptidase that produces sequential cleavage of dipeptides from the carboxy-terminus of the peptide. Dipeptidyl peptidase IV cleaves des-Arg[1]-bradykinin.

Kinin production *in vivo* is controlled partly by endogenous inhibitors of the kallikrein enzymes. The main inhibitors of plasma kallikrein are C1 esterase inhibitor, α_2-macroglobulin, and antithrombin III.[3] An important inhibitor of tissue kallikrein is kallistatin, although there is continuing uncertainty about the function of kallistatin *in vivo*.[5]

Kinin peptide metabolism is an important determinant of kinin levels in blood and tissue. Many different peptidases participate in the conversion of bradykinin and kallidin to alternative bioactive peptides and their degradation (Fig. 4). Aminopeptidase-mediated cleavage of kallidin generates bradykinin. Carboxypeptidase (kininase I)-mediated cleavage converts bradykinin and kallidin, which are more potent on the type 2 (B_2) bradykinin receptor, to des-Arg[9]-bradykinin and des-Arg[10]-kallidin, respectively, which are also bioactive and more potent on the type 1 (B_1) receptor (Fig. 2).[29] Thus, there are 8 bioactive kinin peptides in humans, hydroxylated and nonhydroxylated bradykinin and kallidin peptides, and their carboxypeptidase metabolites (Fig. 3).

Angiotensin-converting enzyme (ACE) plays an important role in bradykinin metabolism, producing bradykinin-(1-5) [BK-(1-5)], the most abundant bradykinin metabolite,[23] by sequential cleavage of dipeptides from the carboxy-terminus of bradykinin. Other peptidases play a greater role in kinin peptide metabolism during ACE inhibition.

DISTRIBUTION OF KININOGEN AND KALLIKREIN mRNA, AND KININ PEPTIDES

The liver is the main site of kininogen production and, in humans, HMWK mRNA is also found in the kidney, pancreas, placenta, heart, and colon, whereas LMWK mRNA is found in the kidney, brain, placenta, testis, pancreas, thymus, heart, spleen, lung, colon, and small intestine.[24]

Plasma prekallikrein mRNA is present in all tissues examined and is most abundantly present in the liver and pancreas.[24] In humans, the *Klk1* gene is highly expressed in the kidney, pancreas, salivary gland, endometrium, ovary, skin, and pituitary.[12] Other sites of *Klk1* gene expression include the colon, heart, blood vessels, and adrenal.[25,26,28]

TABLE 1 Blood Kinin Peptide Levels in Humans

Peptide (fmol/mL)	Placebo
BK-(1-7)	1.4 (0.5–4.3)
BK-(1-8)	0.08 (0.02–0.41)
BK-(1-9)	0.18 (0.02–1.90)
Hyp[3]-BK-(1-7)	<0.14
Hyp[3]-BK-(1-8)	0.53 (0.12–2.37)
Hyp[3]-BK-(1-9)	0.18 (0.04–0.91)
KBK-(1-7)	0.46 (0.07–3.33)
KBK-(1-8)	0.10 (0.01–1.35)
KBK-(1-9)	0.22 (0.02–2.70)
Hyp[3]-KBK-(1-7)	0.04 (0.01–0.49)
Hyp[3]-KBK-(1-8)	<0.03
Hyp[3]-KBK-(1-9)	0.04 (0.01–0.26)

Data shown as geometric mean (95% confidence interval), n = 19 for bradykinin (BK) peptides; n = 18 for kallidin (KBK) peptides. Hyp, hydroxyproline.
Data from Campbell et al.[10]

Bradykinin peptides are present in all tissues studied (blood, heart, aorta, brown adipose tissue, adrenal, lung, and brain).[9] In humans, circulating levels of kinin peptides are low, usually <3 fmol/mL (Table 1). Studies in rats show that the levels of bradykinin in tissues are higher than in blood,[9] and in humans, bradykinin and kallidin peptide levels are higher in venous than in arterial blood,[5] consistent with the tissue being the main site of formation of these peptides. Bradykinin peptides are more abundant than kallidin peptides in the blood and atrial tissue of humans.[5,10] In human urine, kallidin peptides are more abundant than bradykinin peptides, and the levels are several orders of magnitude higher than that in blood.[5]

RECEPTORS AND SIGNALING CASCADES

In the basal state, the B_2 receptor is constitutively expressed throughout and mediates the majority of the vascular and metabolic actions of kinins, whereas the B_1 receptor is not detectable but is induced by a variety of pathological conditions, including inflammation, toxins, cytokines, and tissue trauma.[19,22] Both B_1 and B_2 receptors can be expressed by the same cell types: vascular endothelial and smooth muscle cells, nonvascular smooth muscle such as the uterus and

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intestine, fibroblasts, epithelial cells, various tumor cells, and also on leukocytes and in the central nervous system.[19] Immunohistochemical studies show a different distribution of B_2 receptors in arteries and arterioles. B_2 receptors are predominant in the endothelium of arteries with minor immunohistochemical staining in smooth muscle cells, whereas there is intense staining of the tunic media and minor staining of the endothelium of arterioles.[5]

The pharmacology of kinin receptors has been reviewed by Leeb-Lundberg et al.[19] The B_1 and B_2 receptors are G protein-coupled, 7-transmembrane-spanning receptors. The B_2 receptor signals through $G\alpha_q$, and also interacts with $G\alpha_s$ and $G\alpha q_{12/13}$. Stimulation of the $G\alpha_q$-sensitive phospholipase Cß produces phosphoinositol hydrolysis and increased intracellular free Ca^{2+}. Moreover, translocation of protein kinase C isoenzymes α, ε, and ζ has been observed in fibroblasts and endothelial cells. B_2 receptor stimulation can also result in phospholipase A_2 and phospholipase D activation.[19] These second messenger systems result in stimulation of endothelial nitric oxide synthase and nitric oxide production through Ca^{2+}-mediated mechanisms in endothelial cells, in addition to cellular release of prostacyclin, reactive oxygen species, and tissue-type plasminogen activator.

The B_1 receptor interacts with $G\alpha_q$, and $G\alpha_i$, through which it stimulates many of the same signaling pathways as the B_2 receptor, including phosphoinositol hydrolysis, elevation of intracellular free Ca^{2+}, arachidonic acid release, eicosanoid production, endothelial nitric oxide synthase activation, and nitric oxide production. Although they seem to couple to similar cellular signaling pathways, the pattern of signaling of B_1 and B_2 receptors may differ. In vascular smooth muscle cells, B_2 receptor stimulation leads to transient phosphoinositol hydrolysis that exhibits little dependence on extracellular Ca^{2+}, whereas B_1 receptor stimulation is more sustained and significantly dependent on extracellular Ca^{2+}. Moreover, the B_2 receptor elicits a transient increase in intracellular Ca^{2+}, whereas the B_1 receptor elicits a sustained signal characterized by a plateau of elevated Ca^{2+}, or baseline oscillation that is dependent on extracellular Ca^{2+} influx.[19]

The interaction between angiotensin type 2 (AT_2) and B_2 receptors is discussed by Izumi and Iwao elsewhere in this volume. Several studies have also suggested interaction between the angiotensin type 1 (AT_1) and B_2 receptors, although others have found no evidence for this interaction.[15]

There is evidence that the B_2 receptor also interacts with ACE.[19] Initial evidence for this interaction came from studies showing that ACE inhibitors potentiate the effects of B_2 receptor agonists that are resistant to cleavage by ACE. Furthermore, ACE inhibitors reactivate the B_2 receptor after desensitization and prevent sequestration of the B_2 receptor to caveolae, and there is evidence for the direct association of the B_2 receptor and ACE in the cell membrane.[19]

In addition to activation by kinin peptides, the B_2 receptor may be directly activated by serine proteases such as kallikreins by a mechanism that involves receptor proteolysis.[6]

BIOLOGICAL ACTIONS WITHIN THE CARDIOVASCULAR SYSTEM

The role of kinins in amphibia is described by Conlon, and in the lung by Couture, in this volume. Kinins are potent vasodilators and also promote diuresis and natriuresis. Most of the circulatory effects of kinins are determined by the stimulation of endothelial cells with subsequent endothelial release of nitric oxide and prostacyclin, which diffuse to the vascular smooth muscle causing vasodilatation.[19]

Evidence for a role for kinins in the regulation of vascular tone and blood pressure in man has come from the studies of the effects of the B_2 receptor antagonist icatibant. Icatibant caused coronary artery vasoconstriction, suggesting a role for basal kinin levels in the control of coronary vascular tone.[5] Moreover, icatibant attenuated the hypotensive effects of ACE inhibition in normotensive and hypertensive patients.[5]

BK-(1-5) has antithrombin activity.[16] However, it is unlikely that endogenous BK-(1-5) levels inhibit thrombin *in vivo* because plasma BK-(1-5) levels (30–40 pmol/L) are at least 6 orders of magnitude below the micromolar concentrations required to inhibit thrombin.[16]

Contact System

The "contact system" comprises HMWK complexed with plasma prekallikrein, Hageman factor (XII, FXII), and FXI. It is so called because its components require contact with artificial, negatively charged surfaces for zymogen activation *in vitro* (Fig. 5).[17] *In vivo*, the contact system is assembled on endothelial and neutrophil cell membranes where it may participate in basal kinin peptide production. This complex associates with endothelial membranes through a binding site formed by the 33 kDa cell-surface receptor for the first component of complement (gC1qR/p33), urokinase plasminogen activator receptor, and cytokeratin.[4] Endothelial prolylcarboxypeptidase and/or heat-shock protein 90 initiates the reciprocal activation of plasma prekallikrein to kallikrein, FXII to FXIIa, and FXI to FXIa. Plasma kallikrein, FXIIa, and FXIa all cleave HMWK to release bradykinin. FXIIa also activates plasminogen to plasmin. Plasmin cleaves HMWK to release bradykinin that may contribute to the angioedema that occurs with administration of recombinant tissue plasminogen activator. In addition, plasma prekallikrein may cleave HMWK directly.[17] C1 esterase inhibitor plays a critical role in the control of bradykinin production by inhibiting plasma kallikrein, FXIIa, FXIa, and plasmin (Fig. 5). Thus, C1 esterase inhibitor deficiency leads

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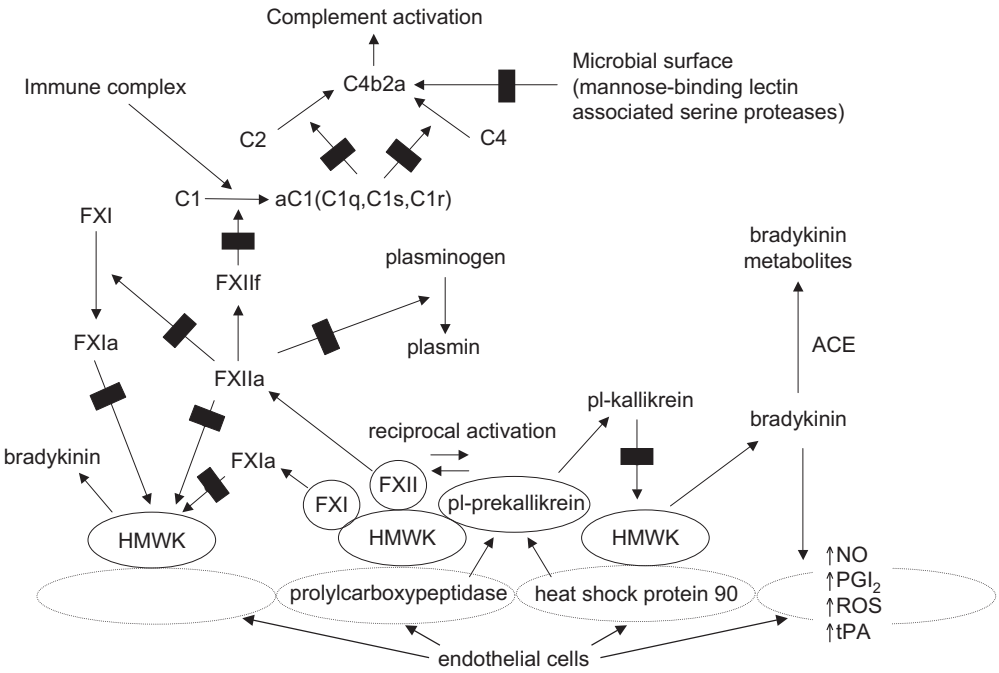

FIGURE 5 Outline of the contact system and its relationship to the complement pathway, which contributes to angioneurotic edema. The black rectangles indicate enzymes inhibited by C1 esterase inhibitor. The contact system comprises high-molecular-weight kininogen (HMWK) complexed with plasma prekallikrein (pl-prekallikrein), Hageman factor (factor XII, FXII), and FXI. This complex associates with endothelial membranes, where endothelial prolylcarboxypeptidase and/or heat-shock protein 90 initiates the reciprocal activation of prekallikrein to kallikrein, FXII to FXIIa, and FXI to FXIa. Kallikrein, FXIIa, and FXIa can all cleave HMWK to release bradykinin. FXIIa also activates plasminogen to plasmin and plasmin cleaves HMWK to release bradykinin. Not shown in this figure is the role of plasma kallikrein and plasmin in the conversion of FXIIa to FXII fragment (FXIIf). FXIIf activates the first component of the complement cascade (C1) that subsequently cleaves C2 and C4. Bradykinin stimulates endothelial cell B_2 receptors to cause the release of nitric oxide (NO), prostacyclin (PGI_2), reactive oxygen species (ROS), and tissue-type plasminogen activator (tPA), which together lead to increased vascular permeability and angioedema. The complement pathway is also activated by the classical pathway (immune complex), and the lectin pathway (microbial surface, mannose-binding lectin pathway). Activation of any of these pathways leads to the reduction in C2 and C4 levels. ACE, angiotensin-converting enzyme.

to increased bradykinin formation, particularly during conditions that increase the activity of the contact system, such as tissue trauma.

In addition to kinin peptide formation, the contact system activates the intrinsic coagulation, complement, and fibrinolytic systems. Moreover, kinin-mediated stimulation of endothelial release of tissue-type plasminogen activator further promotes fibrinolysis.

Tissue prokallikrein may also participate in the contact system by binding to HMWK.[6] Activation of the contact system and kinin peptide formation occurs in cardiopulmonary bypass, whereby both bradykinin and kallidin peptides are increased, indicating activation of both plasma prekallikrein and tissue prokallikrein.[8]

Genetic Models of Deficiency of Components of the Kallikrein–Kinin System

Detection of kininogen-deficient human subjects is based on their severe abnormality in surface-activated intrinsic coagulation, fibrinolytic and kinin-generating pathways, although they have little or no bleeding abnormality.[5] Kininogen deficiency in humans is reported to be relatively asymptomatic, suggesting that kinin peptides may have little role in normal physiology. However, studies in experimental animals provide evidence for an important role for kinin peptides in the regulation of blood pressure and sodium homeostasis, and in contributing to inflammatory processes.[5] The kininogen-deficient Brown–Norway Katholiek strain of rat shows increased sensitivity to the pressor effects of increased dietary salt, mineralocorticoid administration, and angiotensin II infusion, and an impairment of the cardioprotective effects of preconditioning.[5] In addition, the Brown–Norway Katholiek rat shows a reduced response to inflammatory stimuli.[5]

Gene knockout mouse models have also helped demonstrate the role of the KKS in normal physiology and in disease states. There is some controversy about the effects of B_2 receptor gene knockout on blood pressure and cardiac function.[22] However, the B_2 receptor gene knockout mouse has an exaggerated pressor response to angiotensin II infusion and chronic dietary salt loading, and a diminished cardioprotective

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response to ACE inhibitor and AT_1 receptor blocker (ARB) therapies, indicating an important role for endogenous kinins in mediating the effects of these therapeutic agents. B_2 receptor gene knockout mice have reduced insulin sensitivity despite the increased B_1 receptor expression in this model.[19] B_2 receptor gene knockout mice also have less efficient myocardial metabolism with increased propensity to ischemic myocardial injury and increased cardiac oxygen consumption.[22] In addition, B_2 receptor gene knockout mice demonstrate increased urinary concentration in response to vasopressin, indicating that endogenous kinins acting through B_2 receptors oppose the antidiuretic effect of vasopressin.

Studies of the tissue kallikrein gene knockout mouse suggest that tissue kallikrein is the major kinin-forming enzyme in most organs, including the heart and blood vessels, and have identified a role for tissue kallikrein and kinins in protection from acute cardiac ischemia and post-ischemic cardiac failure, and a nephroprotective role for tissue kallikrein in diabetes.[28] However, in contrast to the nephroprotective role of tissue kallikrein, targeted deletion of the B_2 receptor protects against the development of diabetic nephropathy, thereby suggesting that the B_2 receptor contributes to the pathogenesis of diabetic nephropathy.[33]

In a direct comparison of B_2 receptor and tissue kallikrein gene knockout mice, both models had similar blood pressures to wild-type mice and displayed normal hemodynamic and echocardiographic parameters.[34] However, the heart rate was lower in B_2 receptor gene knockout mice than in tissue kallikrein gene knockout or wild-type mice. In addition, B_2 receptor gene knockout mice, but not tissue kallikrein gene knockout mice, exhibited lower coronary and renal blood flows and greater corresponding vascular resistances than did wild-type mice, indicating a tonic physiological vasodilating effect of bradykinin in these vascular beds. However, maximal coronary vasodilator capacity, estimated after dipyridamole infusion, was similar in the 3 groups of mice. B_2 receptor gene knockout mice were significantly more sensitive than were tissue kallikrein gene knockout mice to the vasoconstrictor effects of angiotensin II and norepinephrine. Moreover, renin mRNA levels were significantly higher in B_2 receptor gene knockout mice and lower in tissue kallikrein gene knockout mice, compared with that of wild-type mice. Taken together, these results indicate that under basal conditions, the B_2 receptor is not an important determinant of blood pressure in mice but is involved in the control of regional vascular tone in the heart and kidney.[34] The phenotypic differences observed between B_2 receptor and tissue kallikrein gene knockout mice may represent the contribution of plasma kallikrein to bradykinin production and/or B_1 receptor activation.

Given the vascular and renal actions of kinin peptides, reduced KKS activity has long been a candidate mechanism for essential hypertension.[21] However, heterozygous individuals with a loss of function polymorphism (R53H) of the *Klk1* gene have normal blood pressure, although they have an alteration of the geometry of the brachial artery. Moreover, although the R53H polymorphism was associated with reduced plasma and urinary levels of tissue kallikrein, it was not associated with alteration in urinary kallidin levels.[2]

Gene knockout models also indicate an important role for bradykinin in inflammation and nociception. The B_1 receptor gene knockout mouse has a reduced hypotensive response to bacterial lipopolysaccharide and reduced accumulation of polymorphonuclear leukocytes in carrageenan-induced inflammation, although accumulation of mononuclear cells and endothelial permeabilization are preserved.[5] By contrast, the B_2 receptor gene knockout mouse has reduced paw edema in response to intraplantar injection of carrageenan.[5] Moreover, both the B_1 and B_2 receptor gene knockout mice show evidence of reduced nociception, indicating an important role for endogenous kinins in perception of pain.[5]

PATHOPHYSIOLOGICAL IMPLICATIONS

As noted above, the B_2 receptor is constitutively expressed, whereas the B_1 receptor is not detectable but is induced by a variety of pathological conditions, including inflammation, toxins, cytokines, and tissue trauma.[19,22] Kinin peptides are well recognized for producing cardioprotection mediated by the B_2 receptor, as indicated by the prevention of cardioprotection by icatibant.[22] Cardioprotection mediated by the B_2 receptor includes ischemic preconditioning, the reduction in myocardial infarct size, and the reduction of cardiac hypertrophy and cardiac remodeling post myocardial infarction.[19] Kinins protect against ischemia–reperfusion injury by decreasing endothelial adherence of leukocytes, leading to attenuation of post-ischemic leukocyte adherence and disruption of the microvascular barrier, and reduced tissue injury.[3] Both the B_1 and B_2 receptors are implicated in ischemia-induced angiogenesis.[19,31]

In addition to their induction by inflammation, toxins, cytokines, and tissue trauma, B_1 receptors are induced and upregulated by disturbances in circulatory homeostasis, such as induction of experimental hypertension by salt loading, renal artery clipping, or exogenous angiotensin II infusion, as well as by the normal aging process. But the most important stimulus causing overexpression of the B_1 receptor was found to be the disruption of the B_2 receptor, whether it was due to functional inactivation via a selective B_2 receptor antagonist or to the deletion of the B_2 receptor gene. In such cases, the B_1 receptor can take over some of the vasodilatory functions of the B_2 receptor, but not its metabolic (insulin-sensitizing) function. Patients with end stage cardiac failure have increased cardiac B_1 receptor

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mRNA and protein levels, in contrast to B_2 receptor mRNA and protein levels, which are significantly downregulated.[22] It is unclear whether the upregulation of the B_1 receptor in patients with heart failure contributes to tissue protection by enhancing regional blood flow, or has a detrimental effect by interacting with inflammatory mediators and further exacerbating the inflammatory process.[22]

Both ACE inhibitor and ARB therapies increase kinin peptide levels[5,10] that may contribute to the therapeutic effects of these agents. Recently, the renin inhibitor aliskiren was shown to increase bradykinin levels in the heart and lung of the rat, associated with increases in tissue kallikrein mRNA and protein levels.[11] Studies with tissue kallikrein gene knockout mice confirm the role of the KKS in mediating cardioprotection by ACE inhibitor and ARB therapies.[28] The importance of the B_2 receptor in mediating in part the benefits of ACE inhibitor and ARB therapies is illustrated by the effects of icatibant, which attenuates the hypotensive effects of ACE inhibition in humans,[5] and also attenuates angiogenesis and reduction of cardiac hypertrophy, infarct size, and fibrosis produced by these therapies in animal models. The B_2 receptor is also implicated in the improvement in ischemic preconditioning, and in insulin sensitivity, reported with ACE inhibition.[19,22] By contrast, treatment with a selective B_1 receptor antagonist in the setting of myocardial infarction did not modify myocardial infarct size, but reversed the cardioprotective effects of ARB therapy, an effect attributed to cross-talk between the AT_1 and B_1 receptors.[22] Both the B_1 and B_2 receptors are implicated in the promotion of angiogenesis by ACE inhibition.[19,31]

The potential for the KKS to contribute to inflammation is well illustrated by the clinical condition of angioneurotic edema, whereby C1 esterase inhibitor deficiency results in unpredictable activation of the contact system, leading to angioedema and increased circulating bradykinin levels (Fig. 5).[5] The role of kinins in angioneurotic edema is demonstrated by the therapeutic benefits of icatibant and plasma kallikrein inhibition.[17] Local tissue trauma and sepsis produce local kallikrein activation, possibly involving the contact system, and kinins contribute to the cardinal features of inflammation, producing vasodilatation, increased blood flow, vascular permeability, edema, neutrophil chemotaxis, and pain.[3]

Increased kinin peptide levels may contribute to the increased incidence of angioedema associated with ACE inhibitor and ARB therapies.[5,10] Kinin levels are increased further when neutral endopeptidase 24.11 (NEP) inhibition is combined with ACE inhibition. Consequently, the incidence of angioedema is higher with combined ACE and NEP inhibition than with ACE inhibition alone.[7] Moreover, reduced plasma levels of aminopeptidase P are associated with ACE inhibitor-induced angioedema.[1] Patients with heart failure have reduced circulating levels of bradykinin and kallidin peptides[5] that may account for the lower incidence of angioedema in subjects with heart failure treated with ACE inhibitors or vasopeptidase inhibitors.[7]

In summary, kinin peptides exert a broad range of cardioprotective and inflammatory actions. In general, low physiological levels of kinin peptides play an important role in cardiovascular homeostasis, particularly in relation to sodium homeostasis and in cardioprotection, whereas the high pathological levels of kinin peptides that occur when the contact system is activated and may also occur when key kininase enzymes such as ACE, NEP and aminopeptidase P are inhibited, contribute to inflammation.

ACKNOWLEDGMENTS

Duncan Campbell (Grant ID 395508) is a recipient of a Senior Research Fellowship from the National Health and Medical Research Council of Australia. This work was supported partly by the Victorian Government's Operational Infrastructure Support Program.

REFERENCES

1. Adam A, Cugno M, Molinaro G, Perez M, Lepage Y, Agostoni A. Aminopeptidase P in individuals with a history of angio-oedema on ACE inhibitors. *Lancet* 2002;**359**:2088–9.
2. Azizi M, Emanueli C, Peyrard S, Maddedu P, Alhenc-Gelas F, Campbell DJ. Genetic and dietary control of plasma tissue kallikrein secretion and urinary kinins excretion in man. *J Hypertens* 2008;**26**:714–20.
3. Bhoola KD, Figueroa CD, Worthy K. Bioregulation of kinins: kallikreins, kininogens, and kininases. *Pharmacol Rev* 1992;**44**:1–80.
4. Bossi F, Peerschke EI, Ghebrehiwet B, Tedesco F. Cross-talk between the complement and the kinin system in vascular permeability. *Immunol Lett* 2011;**140**:7–13.
5. Campbell DJ. The kallikrein–kinin system in humans. *Clin Exp Pharmacol Physiol* 2001;**28**:1060–5.
6. Campbell DJ. The renin –angiotensin and the kallikrein–kinin systems. *Int J Biochem Cell Biol* 2003;**35**:784–91.
7. Campbell DJ. Vasopeptidase inhibition: a double-edged sword? *Hypertension* 2003;**41**:383–9.
8. Campbell DJ, Dixon B, Kladis A, Kemme M, Santamaria JD. Activation of the kallikrein–kinin system by cardiopulmonary bypass in humans. *Am J Physiol Regul Integr Comp Physiol* 2001;**281**:R1059–70.
9. Campbell DJ, Kladis A, Duncan A-M. Bradykinin peptides in kidney, blood, and other tissues of the rat. *Hypertension* 1993;**21**:155–65.
10. Campbell DJ, Krum H, Esler MD. Losartan increases bradykinin levels in hypertensive humans. *Circulation* 2005;**111**:315–20.
11. Campbell DJ, Zhang Y, Kelly DJ, Gilbert RE, McCarthy DJ, Shi W, et al. Aliskiren increases bradykinin and tissue kallikrein mRNA levels in the heart. *Clin Exp Pharmacol Physiol* 2011;**38**:623–31.
12. Clements JA, Willemsen NM, Myers SA, Dong Y. The tissue kallikrein family of serine proteases: functional roles in human disease and potential as clinical biomarkers. *Crit Rev Clin Lab Sci* 2004;**41**:265–312.
13. Elliot DF, Lewis GP, Horton EW. The structure of bradykinin – a plasma kinin from ox blood. *Biochem Biophys Res Commun* 1960;**3**:87–91.

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14. Furuto-Kato S, Matsumoto A, Kitamura N, Nakanishi S. Primary structures of the mRNAs encoding the rat precursors for bradykinin and T-kinin. Structural relationship of kininogens with major acute phase protein and a_1-cysteine proteinase inhibitor. *J Biol Chem* 1985;**260**:12054–9.
15. Hansen JL, Hansen JT, Speerschneider T, Lyngso C, Erikstrup N, Burstein ES, et al. Lack of evidence for AT_1R/B2R heterodimerization in COS-7, HEK293, and NIH3T3 cells: how common is the AT_1R/B2R heterodimer? *J Biol Chem* 2009;**284**:1831–9.
16. Hasan AAK, Amenta S, Schmaier AH. Bradykinin and its metabolite, Arg-Pro-Pro-Gly. Phe, are selective inhibitors of a-thrombin-induced platelet activation. *Circulation* 1996;**94**:517–28.
17. Kaplan AP, Ghebrehiwet B. The plasma bradykinin-forming pathways and its interrelationships with complement. *Mol Immunol* 2010;**47**:2161–9.
18. Kitamura N, Kitagawa H, Fukushima D, Takagaki Y, Miyata T, Nakanishi S. Structural organization of the human kininogen gene and a model for its evolution. *J Biol Chem* 1985;**260**:8610–7.
19. Leeb-Lundberg LM, Marceau F, Muller-Esterl W, Pettibone DJ, Zuraw BL. International union of pharmacology. XLV. Classification of the kinin receptor family: from molecular mechanisms to pathophysiological consequences. *Pharmacol Rev* 2005;**57**:27–77.
20. Lundwall A, Band V, Blaber M, Clements JA, Courty Y, Diamandis EP, et al. A comprehensive nomenclature for serine proteases with homology to tissue kallikreins. *Biol Chem* 2006;**387**:637–41.
21. Madeddu P, Emanueli C, El-Dahr S. Mechanisms of disease: the tissue kallikrein–kinin system in hypertension and vascular remodeling. *Nat Clin Pract Nephrol* 2007;**3**:208–21.
22. Manolis AJ, Marketou ME, Gavras I, Gavras H. Cardioprotective properties of bradykinin: role of the B_2 receptor. *Hypertens Res* 2010;**33**:772–7.
23. Murphey LJ, Eccles WK, Williams GH, Brown NJ. Loss of sodium modulation of plasma kinins in human hypertension. *J Pharmacol Exp Ther* 2004;**308**:1046–52.
24. Neth P, Arnhold M, Nitschko H, Fink E. The mRNAs of prekallikrein, factors XI and XII, and kininogen, components of the contact phase cascade are differentially expressed in multiple non-hepatic human tissues. *Thromb Haemost* 2001;**85**:1043–7.
25. Nolly H, Carbini LA, Scicli G, Carretero OA, Scicli AG. A local kallikrein-kinin system is present in rat hearts. *Hypertension* 1994;**23**:919–23.
26. Nolly H, Saed G, Carretero OA, Scicli G, Scicli AG. Adrenal kallikrein. *Hypertension* 1993;**21**:911–5.
27. Pierce JV, Webster ME. Human plasma kallidin: isolation and chemical studies. *Biochem Biophys Res Commun* 1961;**5**:353–7.
28. Pizard A, Richer C, Bouby N, Picard N, Meneton P, Azizi M, et al. Genetic deficiency in tissue kallikrein activity in mouse and man: effect on arteries, heart and kidney. *Biol Chem* 2008;**389**:701–6.
29. Regoli D, Rhaleb N-E, Drapeau G, Dion S, Tousignant C, D'Orléans-Juste P, et al. Basic pharmacology of kinins: pharmacologic receptors and other mechanisms. *Adv Exp Med Biol* 1989;**247A**:399–407.
30. Roche e Silva M, Beraldo WT, Rosenfeld G. Bradykinin, a hypotensive and smooth muscle stimulating factor released from plasma globulin by snake venoms and by trypsin. *Am J Physiol* 1949;**156**:261–73.
31. Sanchez de Miguel L, Neysari S, Jakob S, Petrimpol M, Butz N, Banfi A, et al. B2-kinin receptor plays a key role in B1-, angiotensin converting enzyme inhibitor-, and vascular endothelial growth factor-stimulated *in vitro* angiogenesis in the hypoxic mouse heart. *Cardiovasc Res* 2008;**80**:106–13.
32. Takagaki Y, Kitamura N, Nakanishi S. Cloning and sequence analysis of cDNAs for human high molecular weight and low molecular weight prekininogens. Primary structures of two human prekininogens. *J Biol Chem* 1985;**260**:8601–9.
33. Tan Y, Keum JS, Wang B, McHenry MB, Lipsitz SR, Jaffa AA. Targeted deletion of B_2-kinin receptors protects against the development of diabetic nephropathy. *Am J Physiol Renal Physiol* 2007;**293**: F1026–35.
34. Trabold F, Pons S, Hagege AA, Bloch-Faure M, Alhenc-Gelas F, Giudicelli JF, et al. Cardiovascular phenotypes of kinin B_2 receptor- and tissue kallikrein-deficient mice. *Hypertension* 2002;**40**:90–5.
35. Werle E. Discovery of the most important kallikreins and kallikrein inhibitors. In: Erdos EG, editor. *Handbook of experimental pharmacology*, vol. 25. Bradykinin, kallidin and kallikrein. Berlin: Springer-Verlag; 1970. p. 1–6.

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Chapter 189

CGRP

Ross King and Susan D. Brain

ABSTRACT

Calcitonin gene-related peptide (CGRP) is a 37-amino acid neuropeptide that belongs to a family of structurally related peptides, including adrenomedullin (AM), adrenomedullin II (AM2, or intermedin), and amylin (AMY). CGRP expression is largely concentrated within small-diameter primary sensory afferents that innervate the vasculature. When released into and around the vasculature, CGRP acts as a potent vasodilator. To achieve its effect, CGRP binds to a Class B G protein-coupled receptor, known as the calcitonin receptor-like receptor (CLR), which in turn is coupled to a receptor activity modifying protein 1 (RAMP1) to confer specificity for the CGRP ligand. Abnormal CGRP activity has been identified as a potential causative factor within the pathophysiological setting of migraine and, as such, antagonists of the CGRP receptor are in development for the treatment of this disease. Given its potent activity and ability to be released throughout the body, CGRP has the potential to maintain cardiovascular homeostasis, warranting future work to fully characterize its effects.

DISCOVERY

Calcitonin gene-related peptide (CGRP) is a 37-amino acid neuropeptide that was first identified in 1982 by Amara et al. It was discovered in the thyroid tissue of aging rats, where alternative processing of RNA transcripts from the calcitonin gene was shown to result in the production of distinct mRNAs encoding CGRP. Calcitonin mRNA comprises exons 1, 2, 3, and 4 of the calcitonin gene, whereas CGRP mRNA comprises exons 1, 2, 3, 5, and 6, as shown in Fig. 1. Expression of calcitonin mRNA was shown to be largely confined to the thyroid tissue, while CGRP-specific mRNA is considered to predominate throughout the sensory nervous system.[2] After this discovery, a human form of CGRP was isolated from medullary thyroid carcinomas.[22] Two distinct isoforms of CGRP are now known to exist, termed αCGRP and βCGRP. αCGRP is formed from the alternative splicing of mRNA transcribed from the calcitonin gene located on chromosome 11, whereas the beta isoform is transcribed from its own gene located on the same chromosome.[1] Because of the similar organization of both genes, it is believed that the two separate genes arose by duplication. Both isoforms of the mature peptide are structurally similar, with the β isoform only differing by 3 amino acid residues in humans and 1 amino acid in rodents. Indeed, both isoforms display comparable biological activity and primarily differ in their tissue distribution. αCGRP is most commonly found within peripheral sensory neurons, whereas βCGRP seems to be more restricted to nerves of the enteric plexuses. Little is known about the specific function of the β isoform, and thus from this point, only αCGRP will be discussed.

DISTRIBUTION OF THE mRNA/PEPTIDE IN THE CARDIOVASCULAR SYSTEM

Neuronal Localization of CGRP

CGRP is widely distributed throughout the central and peripheral nervous systems. It is primarily located within unmyelinated, small-diameter sensory C, and thinly myelinated Aδ fibers that are commonly found in close contact with the vasculature, although with a greater degree of innervation on the arterial side of the circulation. Sensory nerve terminals have been shown to protrude as far into the vascular wall as the adventitial–medial border, enabling a direct influence over vascular function. A well-established mechanism by which CGRP and other neuropeptides are released from sensory nerves is after neuronal stimulation with the potent chilli-pepper extract, capsaicin. Capsaicin is a highly selective agonist for the vanilloid subtype of transient receptor potential class of ion channel (TRPV1), and its binding to TRPV1 induces the opening of a central pore that is permeable to the influx of extracellular cations, with a degree of selectivity for divalent ions, such as calcium. An increase in intracellular calcium leads to depolarization of the neuronal membrane and triggering of calcium-dependent exocytotic mechanisms and release of CGRP. Indeed, long-term treatment of rodent neonates with capsaicin leads to neuronal excitotoxicity and subsequent sensory nerve loss. Other activators of TRPV1 include noxious heat and the high concentrations of protons associated with lowered

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00189-5
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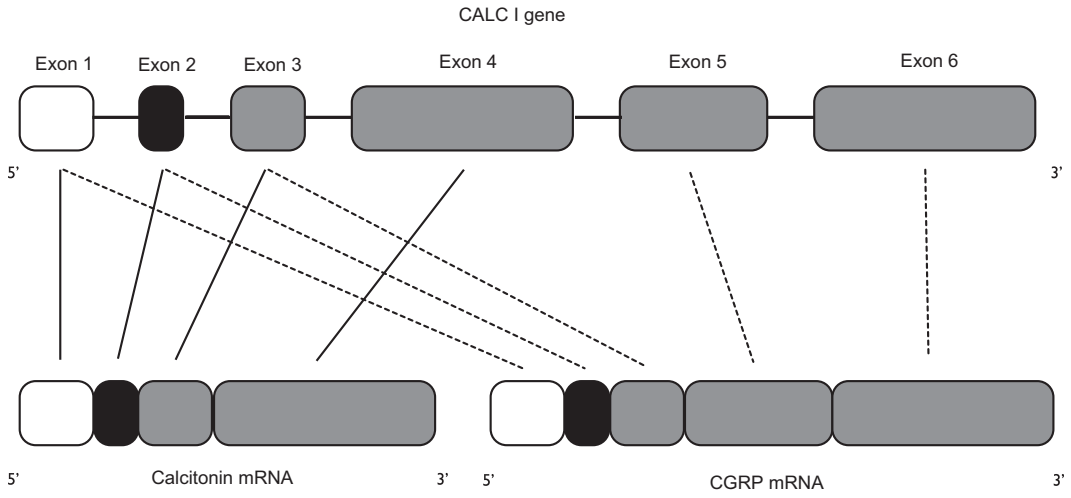

FIGURE 1 Arrangement of CALC I exons encoding either calcitonin or CGRP mRNA.

environmental pH.[28] There has been recent research involving other TRP receptors, one of which is the ankyrin receptor TRPA1. This is found on 50% of sensory nerves and when stimulated is capable of releasing vasodilator CGRP, depending on the circumstance.[24] Physiological plasma levels of CGRP in humans are usually within the low picomolar range, and this low level is generally attributed to overspill from the site of release, as opposed to having a systemic circulatory effect. Once released, it is unclear as to how CGRP may be metabolized to an inactive form, and several removal mechanisms are likely to take place.[4] Mast cell tryptase and other peptidases have been shown to be capable of breaking down CGRP at extravascular sites, and there also exists a role for a matrix metalloprotease II enzyme in CGRP metabolism. Furthermore, there is some evidence to suggest that CGRP is taken up by the nerve terminal following its repolarization, where it may be recycled for reuse or marked for degradation.

Novel, Nonneuronal Sites of CGRP Expression

Aside from the canonical synthesis and release of CGRP from perivascular sensory neurons, other nonneuronal sources of the peptide are slowly beginning to emerge. A range of studies have been performed to investigate endothelial progenitor cell (EPC) function, and this cell type has been shown to actively express and release CGRP.[11] Moreover, it has been suggested that mature endothelial cells (ECs) possess the ability to express both isoforms of CGRP and release CGRP in a TRPV1-dependent manner. This endothelial-derived CGRP has been suggested to play an important role in the protection of endothelial function induced by acute heat stress.[31] Furthermore, a study published by Wang et al. in 2002 suggests that human lymphocytes constitutively express both isoforms of CGRP, although this finding is yet to be confirmed in subsequent experiments.[30]

RECEPTORS AND SIGNALING CASCADES

Our knowledge of the CGRP receptor and its associated signaling cascades has increased in a short span of time. Historically, two distinct receptors for CGRP were initially believed to exist: the $CGRP_1$ and $CGRP_2$ receptors. The idea of heterogeneous CGRP receptor subpopulations was put forward when it was discovered that responses to CGRP in the guinea pig atria could be blocked by the peptide antagonist, $CGRP_{8–37}$, whereas in the rat vas deferens, this fragment was less capable of antagonizing CGRP-induced effects.[9] However, by using molecular and pharmacological techniques, we can now appreciate that apparent differences in CGRP activity may be ascribed to the activation of receptors for other members of the calcitonin family, such as adrenomedullin and amylin.[13] The identity of the CGRP receptor takes the form of a multiprotein oligomer comprising a secretin-like ("Class B") G protein-coupled receptor (GPCR), known as the calcitonin receptor-like receptor (CLR), bound to a receptor activity modifying protein (RAMP)1 molecule. Dimerization of the CLR to other RAMPs confers pharmacological activity on other members of the calcitonin family of peptides: RAMP2 and RAMP3 association leads to the formation of AM_1 and AM_2 receptors, respectively.[29] Fig. 2 illustrates how all CGRP receptor protein subunits combine to form a fully functional receptor.

Assembled CGRP receptors are known to be expressed in a variety of cell types throughout the cardiovascular system, including vascular smooth muscle cells (VSMCs), endothelial cells, cardiac myocytes, some populations of leukocytes, and on the cell bodies of perivascular sensory neurons.

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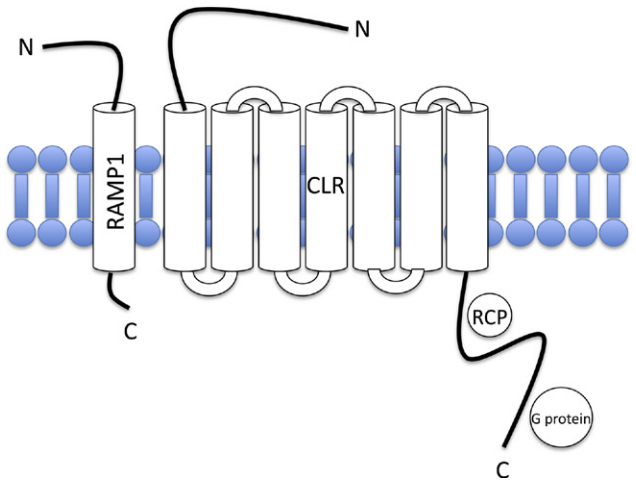

FIGURE 2 Schematic representation of the oligomerization of CGRP receptor components. The CLR binds to RAMP1 to confer ligand specificity to CGRP. CGRP receptor activation typically results in the activation of a G protein, usually Gαs. The RCP positively regulates Gαs-mediated signal transduction.

Critical to the advancing of our insight into receptor function and into the formation of novel therapeutic strategies is the development of a "pharmacological toolkit," including antagonists selective for distinct receptor subpopulations. The first antagonist for the CGRP receptor was developed with the finding that truncation of the first seven amino acid residues of the peptide (resulting in the $CGRP_{8–37}$ peptide fragment) binds to the receptor complex without activating signaling pathways, thereby competing with the full-length peptide for receptor occupancy.[6] However, in keeping with its peptide nature, $CGRP_{8–37}$ is not an ideal tool for interrogating specific signaling pathways. More recently, however, novel nonpeptide antagonists have been designed to selectively target the interface between CLR and RAMP1, binding to the CLR at methionine-42 and RAMP1 at tryptophan-74, and these have been shown to have considerable efficacy in the treatment of migraine-associated headache: olcegepant (BIBN4096; 1-[N2-[3,5-dibromo-*N*-[[4-(3,4-dihydro-2(1H)-oxoquinazolin-3-yl)-1-piperidinyl]carbonyl]-D-tyrosyl]-1-lysyl]-4-(4-pyridinyl)piperazine) and telcagepant (MK-0974; [N-[(3R,6S)-6-(2.3-difluorophenyl)-2-oxo-1-(2,2,2-trifluoroethyl)azepan-3-yl]-4-(2-oxo-2,3-dihydro-1H-imidazo[4,5-b]pyridin-1-yl)piperidine-1-carboxamide]).[21]

CGRP Receptor Components

The main functional component of the CGRP receptor critical for signal transduction following agonist binding is the CLR. Consistent with the highly conserved structure of GPCRs, the CLR contains regions that are critical for GPCR signaling. The CLR possesses a large N-terminal extracellular domain (ECD), three extracellular loops (ECLs), seven transmembrane (TM) domains, three intracellular loops (ICLs) and an intracellular C-terminus. The manner in which peptide ligands bind to Class B GPCRs, including the CLR/RAMP1 complex, is generally described as the "two-domain" model. In this model, the C-terminus of the peptide binds with high affinity to the N-terminal ECD and is subsequently delivered to the ECL and extracellular TM domains of the receptor while also binding to the RAMP molecule, resulting in the activation of the receptor by inducing a change in receptor conformation.[15]

The CLR alone is incapable of responding to CGRP stimulation and requires the heterodimerization of the single transmembrane-spanning RAMP1 to offer full functionality. Indeed, association of RAMP1 with the CLR is critical for the maturation of the CLR via N-terminal glycosylation and subsequent export of the receptor from the endoplasmic reticulum to the plasma membrane, where it is able to interact with its peptide agonist.[19] Coupling of the CLR to RAMP1 produces a receptor that is responsive to CGRP and is blocked by BIBN4096BS/$CGRP_{8–37}$. Association of the CLR with RAMP2 produces a receptor for AM that is blocked by its associated peptide fragment $AM_{22–52}$, and binding of RAMP3 produces a receptor responsive to both CGRP and AM, about which very less is known.

In addition to RAMP1, It is now known that a small intracellular peripheral membrane protein also associates with the CLR, as shown by coimmunoprecipitation studies. This receptor coupling protein (RCP) is important for the amplification of signal generated by CGRP receptor activation. Endogenously expressed by most cell types, molecular knockdown techniques have shown that ablation of RCP activity leads to a decreased production of cAMP secondary to CGRP receptor activation but not from other receptor systems that are coupled to Gαs. Therefore, it seems that the association of RCP with the CLR is important for the regulation of efficient intracellular signal transduction.[10]

CGRP Receptor Signaling

Activation of the CGRP receptor is typically associated with an increase in the formation of the intracellular second messenger molecule cyclic 3′-5′-adenosine monophosphate (cAMP), as a result of the small G protein Gαs associating with the CLR and promoting adenylyl cyclase activity. Accumulation of cAMP within subcellular signaling compartments results in the temporospatial activation of the cAMP-dependent protein kinase (PKA), which itself is capable of targeting multiple protein substrates for phosphorylation, thereby altering their functionality. PKA-dependent events occurring downstream from CGRP receptor stimulation have been shown to include alterations of the activity of critical signaling proteins pertaining to cell excitability, such as ATP-sensitive potassium channels and

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L-type calcium channels, and DNA transcription as a result of the activation of the cAMP response element binding protein (CREB). Furthermore, consistent with other members of the GPCR superfamily, it is likely that the CGRP receptor may couple to multiple additional signaling networks, such as other G proteins including $G_{i/o}$ and $G_{q/11}$. In addition, there is a growing body of evidence to suggest that CGRP receptor stimulation may result in the activation of mitogen-activated protein kinases (MAPKs), which are essential to cell proliferation and survival. It is yet unclear how the receptor might signal to these kinases, though a possible mechanism may be through β-arrestin-mediated signaling; β-arrestin has been shown to associate with the CGRP receptor and facilitates receptor desensitization and internalization. Therefore, it is plausible that the CGRP receptor may transmit a signal in this manner, as it has been shown with other receptor systems.[29]

To be able to maintain efficient control of CGRP activity, the signal generated by the receptor must be terminated in a suitable manner. Although Class B GPCRs are less well characterized than Class A GPCRs, it is known that they both share the ability to undergo agonist-induced homologous desensitization, and this has indeed shown to be the case for the CGRP receptor. In cultured human neuroblastoma cells and VSMCs, pretreatment of cells with CGRP for 20min resulted in an attenuated elevation of cAMP on a secondary exposure. It has since been shown that after the binding of CGRP to its cognate receptor, the CLR undergoes rapid phosphorylation and binds β-arrestin where it is thereby internalized as part of a ternary complex comprising the CLR, RAMP1, and β-arrestin molecules. The association of β-arrestin with the receptor complex is stable and long lasting, showing an internalization profile similar to that of the V_2 vasopressin receptor.[12]

BIOLOGICAL ACTIONS WITHIN THE CARDIOVASCULAR SYSTEM

CGRP may have a variety of roles within the cardiovascular system, but the feature that is best characterized is its ability to act as a potent vasodilator. Often considered the most potent microvascular vasodilator to date, its property was first discovered by Brain et al. in 1985,[3] where intradermal injections of femtomolar concentrations of CGRP into the human forearm was sufficient to induce surface erythema of the skin, attributable to local vasodilatation and, as a result, increased blood flow. Higher doses of CGRP were found to produce a reddening of the skin that lasted for several hours.[3] Intravenous delivery of CGRP into rodents causes systemic hypotension coupled with a positive inotropic and chronotropic response from the heart, though these effects are considered to be secondary to stimulation of the sympathetic nervous system as part of the baroreceptor reflex, rather than a direct action of CGRP on the heart. The vasodilator activity of CGRP is predominantly mediated by the binding of CGRP to the CGRP receptor complex embedded within the plasma membrane of VSMCs and, to an extent, the ECs lining the blood vessels. Within the VSMC, stimulation of the CGRP receptor leads to an increase in the formation of cAMP and subsequent activation of PKA, which has been shown to target the ATP-sensitive potassium channel for phosphorylation, leading to cellular relaxation as a result of membrane hyperpolarization. Alternatively, CGRP may interact with receptors located on ECs, leading to the downstream phosphorylation and activation of endothelial nitric oxide synthase (eNOS) and liberation of nitric oxide (NO) from the EC, which is then capable of diffusing to the VSMC layer and causing relaxation via the stimulation of soluble guanylate cyclase and triggering an accumulation of intracellular cGMP as a result.[4]

CGRP and Control of Blood Pressure

Although it is evident that CGRP possesses clear vasomotor activity, it is less clear as to what sort of effect it has on hemodynamics under normal physiological conditions. A range of studies has been performed using murine knockout models to address this question, with some conflicting results. Some groups report no change in baseline blood pressure in mice with the removal of αCGRP, whereas others have witnessed a significant increase in blood pressure in its absence. Difficulty arises when interpreting these findings, however, as the generation of CGRP knockout mice can differ between groups, where some animals have the complete CALC I gene removed (thereby preventing both calcitonin and αCGRP synthesis) and others lack only αCGRP. It is worth noting, however, that mice holding the sole deletion of CGRP seem to be more likely to have normal resting blood pressures.[27] Adding further credence to the hypothesis that CGRP plays a minor role in baseline blood pressure regulation, studies performed in a variety of species have shown that injection of the CGRP receptor antagonist $CGRP_{8–37}$ or CGRP receptor-directed antibodies has no effect on systemic blood pressure. Furthermore, injection of the selective CGRP receptor antagonist BIBN4096BS has also been shown not to exert an effect on resting heart rate or blood pressure across a range of species, including humans.[23] Therefore, it may be the case that the functional consequences of CGRP are more pronounced within the microcirculation of specific tissues, as opposed to the entire circulation. This is supported by findings that indicate that administration of CGRP at doses below those required to produce systemic hypotension produces facial flushing, indicative of a specific sensitivity to CGRP within the cutaneous microcirculation. Indeed, capsaicin-induced denervation of peripheral sensory nerves in neonatal rats

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typically results in the formation of skin lesions resulting from the removal of neuropeptidergic control of the dermal microcirculation. Furthermore, skin flap survival in sensory nerve-depleted rats is reduced when compared with those with sensory nerves intact and skin flap survival can be rescued with the administration of CGRP. In mice subjected to hind limb ischemia, neovascularization is significantly impaired after blockade of CGRP. These findings are in keeping with the principal role of CGRP as a regulatory microvascular neuropeptide, being released from perivascular sensory nerves at specific sites to produce local vascular control as opposed to behaving in an endocrine manner.[4]

Cardiac Effects of CGRP

The role of CGRP within cardiac physiology is less clear. CGRP-immunoreactive fibers have been shown to innervate regions of the myocardium and the coronary vessels that supply the heart with blood. A range of studies has been performed that propose a cardioprotective role for CGRP. Isolated heart preparations using mice with mixed CGRP/calcitonin gene deletion were found to have an increased susceptibility to ischemia/reperfusion injury, associated with a marked increase in markers of myocardial cell damage, coupled with an increase in reactive oxygen species generation. Recovery of coronary flow after acute myocardial ischemia was also significantly impaired in mice lacking CGRP. Similar results were obtained with pharmacological blockade of the CGRP receptor with $CGRP_{8–37}$.[17] More recently, it has been shown that mice subjected to streptozotocin-induced diabetes mellitus have an impaired ability to produce cardioprotection via ischemic preconditioning, and this is attributed to a loss of CGRP activity. This effect was rescued by CGRP adenoviral gene delivery and, interestingly, post-IR myocardial function was also improved in nondiabetic hearts receiving CGRP gene transfer.[34] Furthermore, in cultured rat cardiomyocytes, CGRP was shown to inhibit the accelerated apoptosis of myocardial cells induced by norepinephrine.[32] This finding may have particular relevance to research into heart failure, as patients often present with an increased activity of the sympathetic nervous system and increased catecholamine release. Although the cardiac effects of CGRP are not as well documented as those in the vasculature, it is conceivable that increasing cardiac CGRP activity by pharmacological or genetic means may represent a new way to prevent the deleterious effects of ischemia/reperfusion injury and associated cell death.

PATHOPHYSIOLOGICAL IMPLICATIONS

Migraine

Despite affording apparent protective effects within the cardiovascular system, CGRP has also been shown to play a key role in the pathophysiology of migraine. Migraine is a debilitating cerebrovascular condition affecting around 15% of people, characterized by a headache comprising a unilateral throbbing sensation with a range of associated symptoms. Migraine may manifest in an episodic or chronic manner, with both instances impacting in a detrimental manner on society and the patient. Current standard treatment for the symptoms of migraine is generally limited to the triptan family of drugs, which possess dual pharmacological activity at $5\text{-}HT_{1B/1D}$ receptors. However, because of their ability to constrict blood vessels, the use of triptans is contraindicated in patients with cardiovascular complications, such as hypertension and coronary artery disease.[14] Interestingly, pharmacological treatment of migraine now seems to be moving in the direction of CGRP receptor antagonism. Soon after the discovery of CGRP, early localization studies showed its presence in cranial blood vessels and in the trigeminal peripheral neurons that innervated this region of the vasculature, where it seemed to function as a protective neurotransmitter that exerted reflex control over hypercontractile vessels. Experiments were also performed in human patients suffering from migraine, in whom collection of blood from the external jugular vein draining the extracerebral tissues was analyzed and found to contain elevated concentrations of CGRP (92 pM versus 40 pM in healthy control subjects), but not of other neuropeptides. Moreover, intravenous delivery of CGRP was shown to dilate cerebral arteries and arterioles concomitant with an increased incidence of migraine-like headache. One theory suggests that the pain associated with migraine is a result of the extracerebral blood vessels (e.g., the middle meningeal artery and its dural arterioles) that supply the dura mater relaxing to an exaggerated extent and thereby stimulating the perivascular nociceptive neurons. These observations together led to the rational development of the nonpeptide CGRP receptor antagonists: olcegepant (BIBN4096BS, administered intravenously) and telcagepant (MK-0974, given orally). Owing to its oral availability, telcagepant has superseded olcegepant in the treatment of migraine.[14]

Raynaud's Phenomenon

Another clinical problem that may benefit from manipulation of the CGRP system is Raynaud's phenomenon. Characterized by inappropriate vasoconstriction and pain within the cutaneous microcirculation (commonly affecting the digits of the hand), the syndrome can restrict adequate tissue perfusion, leading to ischemia and potentially the development of gangrene. Patients suffering from Raynaud's have been shown to have increased levels of the potent pressor agent, endothelin, and decreased levels of CGRP within the digital circulation. Moreover, patients receiving CGRP infusion at room temperature were found to respond in a

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manner similar to that of healthy control subjects. However, at cold temperatures (5 °C), patients were shown to exhibit a significantly attenuated response to CGRP infusion.[5] Therefore, the disease may present as a result of increased responsiveness to endothelin and a lack of responsiveness to CGRP, opening a potential avenue for CGRP receptor agonists in the treatment of the disease. Indeed, recent *in vivo* and ex vivo studies have shown that CGRP is capable of terminating long-lasting vasopressor responses to endothelin, further strengthening a potential therapeutic role for CGRP within this setting.[20]

Hypertension and Vascular Inflammation

Finally, there is developing evidence to show that CGRP activity seems to become more important in resisting the onset of hypertension and its associated vascular dysfunction within several experimental models. Indeed, it has recently been shown that global overexpression of RAMP1 in mice results in a significant attenuation of ATII-induced hypertension and improved baroreceptor reflex activity.[26] Similarly, studies performed with the peptide CGRP receptor antagonist $CGRP_{8–37}$ have shown that blockade of the CGRP receptor exaggerates cardiovascular pathologies induced by hypertensive stimuli, such as salt-induced hypertension and L-NAME–induced hypertension.[27] Moreover, although plasma CGRP concentrations in humans are generally considered to be low, there is evidence to suggest that circulating levels of CGRP can become reduced in patients with essential hypertension. These clinical findings are supported by rodent studies, which have shown that CGRP release and activity may be reduced when levels of angiotensin (AT) are raised. Furthermore, in spontaneously hypertensive rats (SHR—considered a relevant model of essential human hypertension), the level of CGRP mRNA expression by the dorsal root ganglion cells (the principal site of CGRP synthesis) has been shown to decline with age. Therefore, the development of hypertension and associated vasculopathy may be linked to a decrease in CGRP activity within the cardiovascular system. Indeed, there is some evidence to suggest that chronic supplementation of the traditional Chinese medicine and TRPV1 agonist rutaecarpine may have an antihypertensive effect that is attributable to increased CGRP release into the systemic circulation.[8] This protective effect has been documented in hypertension induced by renal phenol injury, two-kidney, one-clip surgery, and in spontaneously hypertensive rats. Additionally, CGRP released from TRPV1 after rutaecarpine stimulation was demonstrated to have a beneficial effect in reducing platelet aggregation by inhibiting the release of platelet-derived tissue factor.[18] Therefore, targeting of TRPV1 with agonists such as rutaecarpine may provide a valid strategy for the treatment of hypertension and its associated conditions.

Hypertension often copresents as an increase in systemic blood pressure and also extensive vascular remodeling and dysfunction. This commonly includes an accelerated rate of hypertrophy and hyperplasia of the VSMCs within the medial wall of the blood vessel. Furthermore, endothelial dysfunction is known to occur, typically characterized by an impaired capability of the endothelial cell to produce the vasoactive mediator, NO. Transgenic overexpression of the RAMP1 component of the CGRP receptor has been shown to protect against ATII-induced endothelial dysfunction, measured by the ability of isolated vessels to relax in response to acetylcholine stimulation of the endothelium to produce NO.[7] Critical to the repair of damaged regions of the endothelium are circulating EPCs: bone marrow-derived circulating cells that are now appreciated to play an important role in vascular repair after injury and also neovascularization. It is believed that a decline in blood EPC content and activity could predispose patients toward future cardiovascular events. CGRP is known to be synthesized and secreted by this cell type, and it has been shown that EPC-derived CGRP can protect against acceleration of EPC senescence induced by ATII and downstream production of oxygen radicals.[35] EPC-derived CGRP also seems to attenuate the degree of VSMC hypertrophy within an ATII model of hypertension.[11] This finding is strengthened by *in vitro* studies examining ATII-induced VSMC proliferation and how this activity can be inhibited by exogenous CGRP application.[25] Furthermore, CGRP-expressing EPCs have been shown to be of some therapeutic value in a model of pulmonary hypertension, where animals receiving CGRP-positive EPCs had a lowered vascular resistance within the pulmonary circulation and decreased smooth muscle thickening.[33] Another characteristic associated with hypertension is an exacerbated incidence of vascular inflammation. Huang et al. showed that exposure of activated microvascular ECs to CGRP significantly attenuated the ratio of inflammatory cells such as neutrophils and monocytes migrating through the endothelial monolayer. This was attributed to a decrease in chemokine expression by the endothelial cell following CGRP stimulation, as a result of a reduced activation of the proinflammatory transcription factor, NFκB.[16] Chronic activation of NFκB is known to predispose arteries to developing atherosclerotic lesions. As interactions between circulating leukocytes and the vasculature are important factors in the etiology of inflammatory diseases such as atherosclerosis, there may be some benefit in manipulating the CGRP signaling pathway to prevent atherogenesis and related inflammatory conditions within the vasculature.

In summary, it is now appreciated that CGRP has a wide range of effects in the cardiovascular system and may be involved in multiple pathophysiological circumstances. It is probable that we will witness the progression of CGRP

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receptor antagonists for the treatment of migraine into the clinic within the coming years. With particular interest to more prevalent and serious cardiovascular diseases, however, it is possible that CGRP plays an important protective role in the pathophysiology of hypertension and its associated vascular disease component. Therefore, future basic and clinical research may lead us in the direction of developing ways by which CGRP activity may be selectively stimulated to develop novel treatments for cardiovascular conditions.

REFERENCES

1. Alevizaki M, Shiraishi A, Rassool FV, Ferrier GJ, MacIntyre I, Legon S. The calcitonin-like sequence of the beta CGRP gene. *FEBS Lett* 1986;**206**:47–52.
2. Amara SG, Jonas V, Rosenfeld MG, Ong ES, Evans RM. Alternative RNA processing in calcitonin gene expression generates mRNAs encoding different polypeptide products. *Nature* 1982;**298**:240–4.
3. Brain SD, Williams TJ, Tippins JR, Morris HR, MacIntyre I. Calcitonin gene-related peptide is a potent vasodilator. *Nature* 1985;**313**:54–6.
4. Brain SD, Grant AD. Vascular actions of calcitonin gene-related peptide and adrenomedullin. *Physiol Rev* 2004;**84**:903–34.
5. Bunker CB, Goldsmith PC, Leslie TA, Hayes N, Foreman JC, Dowd PM. Calcitonin gene-related peptide, endothelin-1, the cutaneous microvasculature and Raynaud's phenomenon. *Br J Dermatol* 1996;**134**:399–406.
6. Chiba T, Yamaguchi A, Yamatani T, Nakamura A, Morishita T, Inui T, et al. Calcitonin gene-related peptide receptor antagonist human CGRP-(8-37). *Am J Physiol* 1989;**256**:E331–5.
7. Chrissobolis S, Zhang Z, Kinzenbaw DA, Lynch CM, Russo AF, Faraci FM. Receptor activity-modifying protein-1 augments cerebrovascular responses to calcitonin gene-related peptide and inhibits angiotensin II-induced vascular dysfunction. *Stroke* 2010;**41**:2329–34.
8. Deng P-Y, Ye F, Cai W-J, Tan G-S, Hu C-P, Deng H-W, et al. Stimulation of calcitonin gene-related peptide synthesis and release: mechanisms for a novel antihypertensive drug, rutaecarpine. *J Hypertens* 2004;**22**:1819–29.
9. Dennis T, Fournier A, Cadieux A, Pomerleau F, Jolicoeur FB, St Pierre S, et al. hCGRP8-37, a calcitonin gene-related peptide antagonist revealing calcitonin gene-related peptide receptor heterogeneity in brain and periphery. *J Pharmacol Exp Ther* 1990;**254**:123–8.
10. Evans BN, Rosenblatt MI, Mnayer LO, Oliver KR, Dickerson IM. CGRP-RCP, a novel protein required for signal transduction at calcitonin gene-related peptide and adrenomedullin receptors. *J Biol Chem* 2000;**275**:31438–43.
11. Fang L, Chen M-F, Xiao Z-L, Liu Y, Yu G-L, Chen X-B, et al. Calcitonin gene-related peptide released from endothelial progenitor cells inhibits the proliferation of rat vascular smooth muscle cells induced by angiotensin II. *Mol Cell Biochem* 2011.
12. Hay DL, Poyner DR, Smith DM. Desensitisation of adrenomedullin and CGRP receptors. *Regul Pept* 2003;**112**:139–45.
13. Hay DL, Poyner DR, Quirion R, International Union of Pharmacology. International Union of Pharmacology. LXIX. Status of the calcitonin gene-related peptide subtype 2 receptor. *Pharmacol Rev* 2008;**60**:143–5.
14. Ho TW, Edvinsson L, Goadsby PJ. CGRP and its receptors provide new insights into migraine pathophysiology. *Nat Rev Neurol* 2010;**6**:573–82.
15. Hoare SRJ. Mechanisms of peptide and nonpeptide ligand binding to Class B G protein-coupled receptors. *Drug Discov Today* 2005;**10**:417–27.
16. Huang J, Stohl LL, Zhou X, Ding W, Granstein RD. Calcitonin gene-related peptide inhibits chemokine production by human dermal microvascular endothelial cells. *Brain Behav Immun* 2011;**25**: 787–99.
17. Huang R, Karve A, Shah I, Bowers MC, Dipette DJ, Supowit SC, et al. Deletion of the mouse alpha-calcitonin gene-related peptide gene increases the vulnerability of the heart to ischemia-reperfusion injury. *Am J Physiol Heart Circ Physiol* 2008;**294**:H1291–7.
18. Li D, Peng J, Xin H-Y, Luo D, Zhang Y-S, Zhou Z, et al. Calcitonin gene-related peptide-mediated antihypertensive and anti-platelet effects by rutaecarpine in spontaneously hypertensive rats. *Peptides* 2008;**29**:1781–8.
19. McLatchie LM, Fraser NJ, Main MJ, Wise A, Brown J, Thompson N, et al. RAMPs regulate the transport and ligand specificity of the calcitonin-receptor-like receptor. *Nature* 1998;**393**:333–9.
20. Meens MJPMT, Mattheij NJA, Nelissen J, Lemkens P, Compeer MG, Janssen BJA, et al. Calcitonin gene-related peptide terminates long-lasting vasopressor responses to endothelin 1 in vivo. *Hypertension* 2011;**58**:99–106.
21. Miller PS, Barwell J, Poyner DR, Wigglesworth MJ, Garland SL, Donnelly D. Non-peptidic antagonists of the CGRP receptor, BIBN4096BS and MK-0974, interact with the calcitonin receptor-like receptor via methionine-42 and RAMP1 via tryptophan-74. *Biochem Biophys Res Commun* 2010;**391**:437–42.
22. Morris HR, Panico M, Etienne T, Tippins J, Girgis SI, MacIntyre I. Isolation and characterization of human calcitonin gene-related peptide. *Nature* 1984;**308**:746–8.
23. Olesen J, Diener H-C, Husstedt IW, Goadsby PJ, Hall D, Meier U, BIBN 4096 BS Clinical Proof of Concept Study Group, et al. Calcitonin gene-related peptide receptor antagonist BIBN 4096 BS for the acute treatment of migraine. *N Engl J Med* 2004;**350**: 1104–10.
24. Pozsgai G, Bodkin JV, Graepel R, Bevan S, Andersson DA, Brain SD. Evidence for the pathophysiological relevance of TRPA1 receptors in the cardiovascular system in vivo. *Cardiovasc Res* 2010;**87**:760–8.
25. Qin X-P, Ye F, Hu C-P, Liao D-F, Deng H-W, Li Y-J. Effect of calcitonin gene-related peptide on angiotensin II-induced proliferation of rat vascular smooth muscle cells. *Eur J Pharmacol* 2004;**488**:45–9.
26. Sabharwal R, Zhang Z, Lu Y, Abboud FM, Russo AF, Chapleau MW. Receptor activity-modifying protein 1 increases baroreflex sensitivity and attenuates angiotensin-induced hypertension. *Hypertension* 2010;**55**:627–35.
27. Smillie S-J, Brain SD. Calcitonin gene-related peptide (CGRP) and its role in hypertension. *Neuropeptides* 2011;**45**:93–104.
28. Tominaga M, Tominaga T. Structure and function of TRPV1. *Pflugers Arch* 2005;**451**:143–50.
29. Walker CS, Conner AC, Poyner DR, Hay DL. Regulation of signal transduction by calcitonin gene-related peptide receptors. *Trends Pharmacol Sci* 2010;**31**:476–83.
30. Wang H, Xing L, Li W, Hou L, Guo J, Wang X. Production and secretion of calcitonin gene-related peptide from human lymphocytes. *J Neuroimmunol* 2002;**130**:155–62.
31. Ye F, Deng P-Y, Li D, Luo D, Li N-S, Deng S, et al. Involvement of endothelial cell-derived CGRP in heat stress-induced protection of endothelial function. *Vascul Pharmacol* 2007;**46**:238–46.

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32. Zhao F-P, Guo Z, Wang P-F. Calcitonin gene related peptide (CGRP) inhibits norepinephrine induced apoptosis in cultured rat cardiomyocytes not via PKA or PKC pathways. *Neurosci Lett* 2010;**482**:163–6.
33. Zhao Q, Liu Z, Wang Z, Yang C, Liu J, Lu J. Effect of preprocalcitonin gene-related peptide-expressing endothelial progenitor cells on pulmonary hypertension. *Ann Thorac Surg* 2007;**84**: 544–52.
34. Zheng L-R, Han J, Yao L, Sun Y-L, Jiang D-M, Hu S-J, et al. Up-regulation of calcitonin gene-related peptide protects streptozotocin-induced diabetic hearts from ischemia/reperfusion injury. *Int J Cardiol* 2011. http://dx.doi.org/10.1016/j.ijcard.2011.04.009.
35. Zhou Z, Hu C-P, Wang C-J, Li T-T, Peng J, Li Y-J. Calcitonin gene-related peptide inhibits angiotensin II-induced endothelial progenitor cells senescence through up-regulation of klotho expression. *Atherosclerosis* 2010;**213**:92–101.

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Chapter 190

Endothelins

Takashi Miyauchi and Katsutoshi Goto

ABSTRACT

A potent vasoconstrictor peptide, endothelin (endothelin-1), was discovered from culture supernatant of porcine endothelial cells in 1988. From human genomic DNA analysis, two other family peptides, endothelin-2 and endothelin-3, were found. They showed different effects and distribution, suggesting that each peptide may play separate roles in different organs. Endothelins act via the activation of two receptor subtypes, ET_A and ET_B receptors, both of which are coupled to various GTP-binding proteins depending on cell types. Endogenous ET-1 may be involved in the progression of various cardiovascular diseases, and ET antagonists are currently used clinically in the treatment of patients with pulmonary hypertension and are considered to have further target diseases such as systemic hypertension, heart failure, cardiac diseases, renal diseases.

DISCOVERY

Vasoconstriction dependent on or enhanced by intact endothelium has been observed in response to various chemical and physical stimuli such as norepinephrine, thrombin, hypoxia, increased transmural pressure, mechanical stretch. These observations lead to the speculation that endothelial cells may release certain vascular constricting substance(s), endothelium-derived contracting factors (EDCF). In 1985, Hickey et al. attempted to test the biological activity of the culture medium of bovine aortic endothelial cells on isolated porcine coronary arteries, and they found that the culture supernatant contained peptide-like factor(s), which triggered a slowly developing and long-lasting contraction of the coronary arteries. Based on gel chromatography analysis, they suggested that this particular EDCF is a peptide with a molecular mass of approximately 8500 Da.[8] At about the same time, Gillespie et al. also detected vascular constricting activity in the culture supernatant of porcine aortic endothelial cells. They subsequently reported that the activity was increased when the cultured endothelial cells were stimulated by thrombin, and they suggested that this EDCF is a peptide with a molecular mass of approximately 3000 Da.[19]

In 1987, we performed experiments similar to those of Hickey et al. and confirmed that the supernatant from confluent monolayer cultures of porcine aortic endothelial cells contained a slowly developing and long-lasting vascular constricting factor(s), peptidic in nature, because the vascular constricting activity was abolished by pretreatment of the conditioned medium with trypsin. The activity was also detected in serum-free conditioned medium, and no appreciable change in activity was observed even after long-term (2–3 weeks) maintenance of the endothelial cell culture in serum-free condition. The successful attempt at serum-free maintenance and detection of vascular constricting activity prompted us to isolate and purify the active peptide in the supernatant because of the absence of interference with proteins and/or peptides in the serum itself.

Cells isolated from porcine thoracic aortas and grown to a confluent monolayer were maintained in serum-free minimum essential medium. The medium was changed every 5 days, and the conditioned medium was pooled at −20 °C. The pooled conditioned medium was first centrifuged at 1000 g for 20 min, and subsequently, the supernatant was desalted and concentrated. The concentrated medium was loaded onto an anion-exchange column and eluted by applying a linear gradient of NaCl. The vascular constricting activity of the eluent was assayed by adding a small amount of each fraction directly into a muscle chamber where a helical strip of porcine coronary artery with the intima denuded was suspended, and the active fraction was collected. The active fraction was subjected to reversed-phase high-performance liquid chromatography (HPLC) and elution with a linear gradient of acetonitrile, and the vascular constricting activity of each fraction was similarly assayed. A second trial of reversed-phase HPLC enabled us to purify the active component. Approximately 3 nmol of the final product was obtained, which was just enough to perform subsequent amino acid analysis.

The purified peptide was subjected to amino acid sequence analysis by means of an automated gas-phase peptide sequencer and carboxy-terminal analysis by hydrazinolysis (Edman's reaction). As a result, the peptide was revealed to be composed of 21 amino acid residues with

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00190-1
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free amino- and carboxy-termini. At first, the carboxy-terminal amino acid (tryptophan) was not detected, and a 20 amino acid peptide synthesized artificially did not exhibit any biological activity. It was soon recognized that the structure of tryptophan (indole ring) is easily degraded during Edman's reaction. The peptide comprising 21 amino acid residues subsequently synthesized did exhibit vascular constricting activity identical to that of natural peptide. The four cysteine residues at the amino-terminal portion were found to form two intramolecular disulfide bonds, the topological analysis of which revealed that the arrangement of disulfide bonds is a coaxial form (1–15 and 3–11). Because it was originally discovered from the culture supernatant of endothelial cells, the peptide was termed "endothelin."[27]

STRUCTURE OF THE mRNA/GENE

Based on the amino acid residues 7–20 of endothelin, several kinds of oligonucleotides were synthesized according to the mammalian codon usage statistics. Using one of the synthesized oligonucleotides as a single "optimal" DNA probe, approximately 2×10^6 clones were screened from aλgt10 cDNA library constructed for porcine endothelial cell mRNA, and 38 hybridization-positive clones were soon identified. Four of these clones were subjected to further characterization, and finally, a complete nucleotide sequence of porcine preproendothelin cDNA and the deduced amino acid sequence were determined.

PROCESSING OF THE PRECURSOR

The 203 residue porcine preproendothelin contains 19 residues of deduced N-terminal amino acid sequence, being characteristic of a secretory signal sequence, that is, a hydrophobic core followed by residues with small polar side chains. As anticipated, paired basic amino acid residues Lys^{51}-Arg^{52}, which are recognized by the usual processing endopeptidase, furin (see the furin chapter in Peptide Biosynthesis/Processing section of the book), directly precede the endothelin sequence, but no dibasic pair is found thereafter until Ara^{92}-Arg^{93}. This indicates that the intermediate peptide of 39 amino acids, called big endothelin (Cys^{53}-Arg^{92}), may be first generated, and subsequently, mature endothelin may be generated via the unusual proteolytic processing between Try^{73} and Val^{74} in big endothelin by an endopeptidase exhibiting chymotrypsin-like specificity, which was putatively termed "endothelin-converting enzyme (ECE)." This presumptive pathway of endothelin biosynthesis in endothelial cells was confirmed later. The human preproendothelin cDNA was soon cloned, and its sequence of 212 amino acid residues containing big endothelin of 38 amino acid sequences was determined (Fig. 1).

The cDNA of ECE was also cloned, and it was revealed that ECE is a 785 amino acid metalloprotease containing a single membrane-spanning sequence with only a 56-residue N-terminal cytoplasmic tail and an extracellular C-terminal of 681 amino acid residues that contains the catalytic domain.[26] Amino acid residues 593–601 match the highly conserved consensus sequence of a zinc-binding motif, HEXXH, which is shared by many known metalloproteases. The ECE protein has ten predicted sites for N-glycosylation in the extracellular domain, suggesting that ECE behaves as a highly glycosylated protein.

DISTRIBUTION OF THE mRNA/PEPTIDE IN THE CARDIOVASCULAR SYSTEM

Southern blot analysis of human genomic DNA under low hybridization stringency with a 42-mer synthetic oligonucleotide probe corresponding to amino acid residues 7–20 of endothelin, showed that three different restriction fragments were always detected regardless of the restriction endonucleases used. The nucleotide sequences encoding amino acid residues of the three endothelins are highly conserved among the three genes, with 77–82% of the nucleotide residues being identical. By contrast, the nucleotide sequences upstream from the mature peptides are very poorly conserved. These observations suggest that although the three genes are evolutionally relatively distant from each other, the genes evolved from a common ancestral gene under strong pressure to preserve mature endothelin sequences. The three peptides were designated endothelin-1, endothelin-2, and endothelin-3.[10] Endothelin-1 is the original peptide corresponding to that detected in the culture medium of porcine aortic endothelial cells. Although vascular endothelial cells are the major source of endothelin-1, Northern blot analysis revealed that the genes encoding the three endothelin isopeptides are expressed with different patterns in a wide variety of cell types including cardiac myocytes, vascular smooth muscle cells, pituitary cells, macrophages, and mast cells, suggesting that the peptides may participate independently in complex regulatory mechanisms in various organs.

The conservation of numerous endothelin-related genes was also observed in several mammalian species examined, including humans, pigs, and rats. Further, four cardiotoxic peptides highly homologous to endothelins, sarafotoxins (S6a–S6d), were isolated from the snake venom of *Atractaspis engaddensis*. Endothelin-like immunoreactivity was also found in several species of invertebrates and fishes, indicating that endothelins found in humans seem to have a long evolutionary history.

Since endothelin-1 was discovered first and showed a wide variety of actions not only on the cardiovascular system but also on various other tissues, much information has accumulated on endothelin-1 compared with the two other peptides. The method for measurement of endothelin-1 levels in plasma or various tissues by means of

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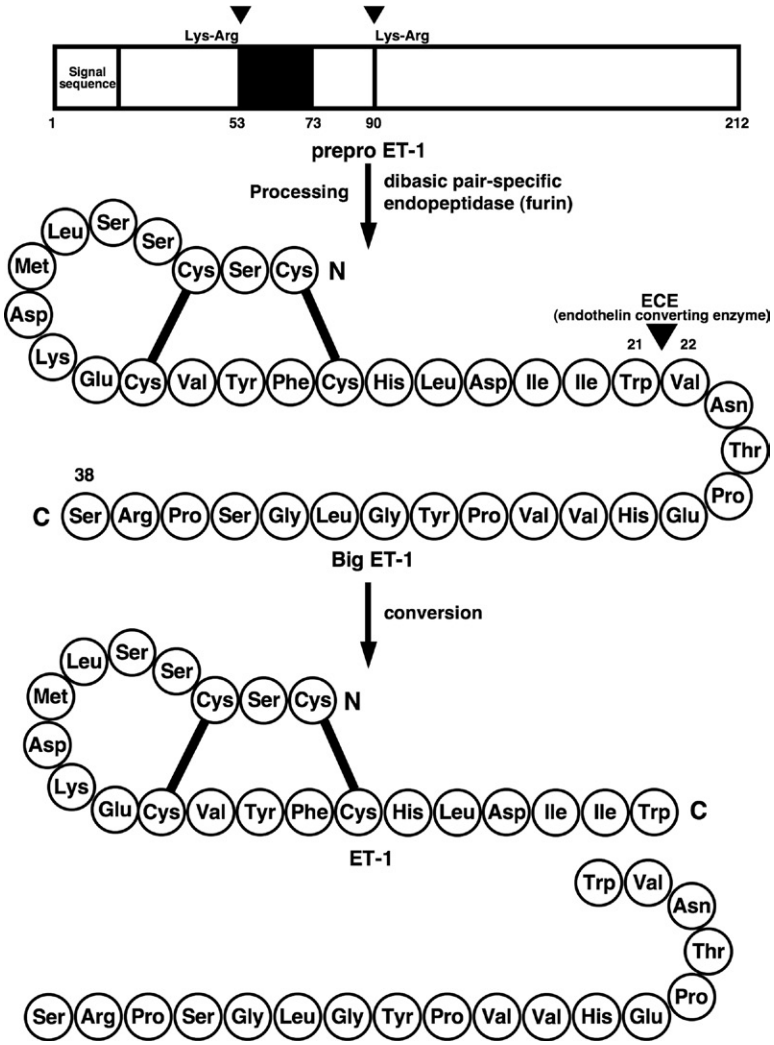

FIGURE 1 The biosynthesis, intracellular processing of endothelin-1 (ET-1) from preproET-1. Endothelin-converting enzyme (ECE) specifically catalyzes the conversion of big ET-1 to ET-1. Cys–Cys represents the intramolecular disulfide bond.

radioimmunoassay (RIA) or enzyme immunoassay (EIA)[25] was established soon after the discovery of endothelin-1. RIA uses only one kind of antibody against endothelin-1, whereas EIA uses two kinds of antibodies, each recognizing the N-terminal and C-terminal portions of endothelin-1, respectively. At an early stage, the reported plasma concentrations of endothelin-1 in humans were fairly divergent, ranging from 0.2 to 18.5pg/ml in healthy subjects. To avoid unnecessary confusion, the reason for the difference in the values of plasma endothelin-1 level was examined thoroughly and, as a result, it was generally agreed that the divergence originated mostly from differences in the specificity of the antibodies used and in the recovery rate during extraction of endothelin-1 from plasma or tissues. It is now accepted that the plasma concentration of endothelin-1 in healthy subjects is 1.0–2.0pg/ml,[13–16] which is at least one order of magnitude lower than that of circulating human natriuretic peptides and several times less than that of angiotensin II. Plasma concentrations of endothelin-1 increase drastically in the case of various cardiovascular diseases.[13–16]

Endothelin-1 may be released in both luminal and abluminal directions from endothelial cells *in vivo*. Luminal released endothelin-1 may be diluted by the blood stream, and its circulating concentration (1.0–2.0pg/ml) is below the threshold concentration producing vasoconstriction. Although the exact concentration in the abluminal space (vascular smooth muscle surface) is not known, endothelin-1 is more likely to be a locally acting rather than a circulating hormone. When endothelin-1 production and secretion are increased drastically in certain sites under pathophysiological conditions, severe local vasoconstriction might be anticipated.

When endothelin-1 is intravenously administered as a bolus, it disappears quite rapidly from the blood stream with a half-life of a few minutes. This rapid removal of endothelin-1 from the circulation results from uptake into

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various tissues, including the lung, kidney, spleen, and liver. The lung seems to be one of the most important tissues for uptake, because approximately 60% of endothelin-1 is removed after a single passage through the pulmonary circulation. In lung tissues, the ET_B receptor is highly expressed and endothelin-1-ET_B receptor complex may be taken up into cells through an internalization process and degraded by lysozomal enzymes, for example, aspartic proteases. Neutral endopeptidase and lysozomal cathepsin G are also concerned with enzymatic degradation of endothelin-1. Despite such a rapid disappearance from the circulation, the blood pressure–elevating effect of endothelin-1 continues for an extremely long period; stabilization of the endothelin-1-ET_A receptor complex may contribute, but the exact reason for this phenomenon is unknown.

RECEPTORS AND THEIR DISTRIBUTION

Endothelin-induced responses can be divided into two major groups, according to the pharmacological potency order of the three isopeptides. In the first group of responses, including vascular constriction, broncho-constriction, uterine smooth muscle constriction, and stimulation of aldosterone secretion, endothelin-1 and endothelin-2 act as far more potent agonists than endothelin-3 do. In the second group, which includes endothelium-dependent vascular relaxation, astrocyte proliferation, and inhibition of ex vivo platelet aggregation, the three isopeptides possess almost equal potency. These findings suggest that there are at least two distinct endothelin receptors mediating these two distinct groups of pharmacological responses. The existence of multiple receptors was also supported by biochemical studies, for example, crosslinking experiments and radioligand-binding affinity study. Subsequently, two cDNAs encoding for endothelin receptors were cloned and their amino acid sequences were deduced.[1,21] The order of affinity of the three endothelin isopeptides for one receptor type, designated ET_A, is endothelin-1≧endothelin-2 ≥ endothelin-3. The other type of receptor, designated ET_B, shows equipotent affinity for all three endothelins. These results are consistent with the results of previous pharmacological and biochemical studies and, thus, definite molecular evidence for the existence of two distinct subtypes of endothelin receptors was provided.

The existence of more subtypes of endothelin receptor has been a matter of controversy. Much pharmacological evidence has accumulated to suggest that there may exist an ET_C receptor specific for endothelin-3, or further sub-subtypes of ET_A and ET_B receptors. However, there has been no report thus far on the isolation of the cDNA clone encoding for such a subtype or sub-subtype of endothelin receptors from mammalian tissues. One possible explanation is posttranslational modification of ET_A or ET_B receptor proteins, but to solve such discrepancies, further pharmacological, biochemical, or molecular biological analyses may be necessary.

In addition to causing constriction of vascular tissue, endothelin isopeptides exert pleiotropic effects on nonvascular tissues, for example, respiratory and gastrointestinal tissues, kidney, endocrine glands, and peripheral and central nervous system. Each endothelin isopeptide has been considered to play specific physiological and/or pathophysiological roles independently in various tissues. The pharmacological effects of endothelin-1 on various tissues and distribution of ET_A and ET_B receptors is collectively shown in Table 1.

TABLE 1 Distribution of ET_A and ET_B Receptors

Tissue (effect)	ET_A	ET_B
Vascular tissues		
Arterial smooth muscle (contraction)	+++	+
Venous smooth muscle (contraction)		++
Endothelial cell (release of EDRF)		+++
Cardiac muscle		
(Positive inotropism)	++	+
(Positive chronotropism)	++	+
Coronary vessel (contraction)	+++	+
Nonvascular smooth muscle (contraction)		
Airway, gastrointestinal tract, bladder, uterus, etc.	++	+
Lung (clearance receptor)		
Other than airways and vascular smooth muscle	+	+++
Endocrine glands		
Adrenal cortex (release of aldosterone)	+	+++
Anterior pituitary (release of ACTH and prolactin)	+	++
Ovary (stimulation of female hormone synthesis)	++	+
Kidney		
Afferent and efferent arterioles (contraction)	+++	++
Renal tubule (Na^+ excretion and diuresis)		+++
Mesangial cell (contraction and proliferation)	+	+++
Central nervous system		
Mesencephalon (control of blood pressure)	+	++
Astrocyte (proliferation)		+++

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BIOLOGICAL ACTIONS WITHIN THE CARDIOVASCULAR SYSTEM

Endothelin-1 causes extremely potent and long-lasting vasoconstriction in most mammalian species including humans, both *in vivo* and *in vitro*. The *in vivo* hemodynamic responses to intravenously injected endothelin-1 are complex, depending on the vascular bed, and include both direct vasoconstriction and indirect endothelium-mediated vasodilatation, and reflex-mediated responses. In most arterial and some venous smooth muscle cells, endothelin-1 causes constrictor responses via the stimulation of ET_A receptors on the cell membrane. By contrast, endothelin-1 stimulates ET_B receptors on endothelial cells and releases EDRF (endothelium-derived relaxing factor: nitric oxide), thereby producing vasodilatation. In cardiac muscle cells, endothelin-1 produces positive inotropic and chronotropic responses via stimulation of mostly ET_A receptors. Both ET_A and ET_B receptors are coupled to various GTP-binding proteins (Gq, Gs, Gi, Go, etc.), suggesting that the downstream signal transduction may differ in various cells depending on the type of GTP-binding proteins coupled.

In addition to vasoconstrictor actions (acute effects), endothelin-1 exerts potent mitogenic actions on vascular smooth muscle cells and cardiac myocytes (chronic effects), hence causing vascular and cardiac hypertrophy.[18,23,24] These effects are mediated via the stimulation of either ET_A or ET_B receptors and may be provoked through the sequential activation of intracellular kinase cascade, including raf-1, mitogen-activated protein kinase (MAPK) kinase, MAPK, and S6 kinase.[6,7,18]

PATHOPHYSIOLOGICAL IMPLICATIONS

The development of ET_A and/or ET_B receptor antagonists and gene-manipulated animals greatly accelerated elucidation of physiological and/or pathophysiological roles of endogenous endothelin-1. In normal states, the release of endothelin-1 seems to be sparse, and it plays no appreciable physiological role in the maintenance of blood pressure.

When the production and release of endothelin-1 are increased, endothelin-1 contributes to the pathogenesis of and stimulates the progression of various cardiovascular diseases.[11,17,18,20] Although the involvement of endothelin-1 in mild to moderate hypertension remains controversial, it is undoubtedly implicated in certain types of hypertension,[12] particularly in malignant stages of the disease. Pulmonary hypertension (PH) is a progressively deteriorating condition characterized by an increase in pulmonary vascular tone and enhanced proliferation of pulmonary vascular smooth muscle cells. There has been no effective therapeutic method for the treatment of this disease. PH is associated with the increase in plasma endothelin-1 levels, which is strongly correlated with the severity of the disease.[16–18] These aspects are discussed in more detail in the Respiratory Peptides section of this book. The endothelin receptor antagonists are remarkably efficacious in all animal models thus far investigated.[17,18] Bosentan (ET_A and ET_B receptor antagonist) is now available for the clinical treatment of PH (Fig. 2).[2,11] In atherosclerosis and in various kinds of vascular remodeling, endothelin-1 plays causing or progressing roles in the diseases. Currently, orally active endothelin receptor antagonists, such as bosentan, ambrisentan, macitentan, are applied to cardiovascular patients.[2,9,12,22] The concept of the cardiovascular continuum and angiotensin II was put forward by Dzau et al.[3–5] As for endothelin, we illustrate the concept of the cardiovascular disease continuum and the target diseases of the endothelin receptor antagonists as in Fig. 3. Endothelin antagonist will be extensively used for cardiac diseases, including cardiac hypertrophy, acute and chronic heart failure.[11,18]

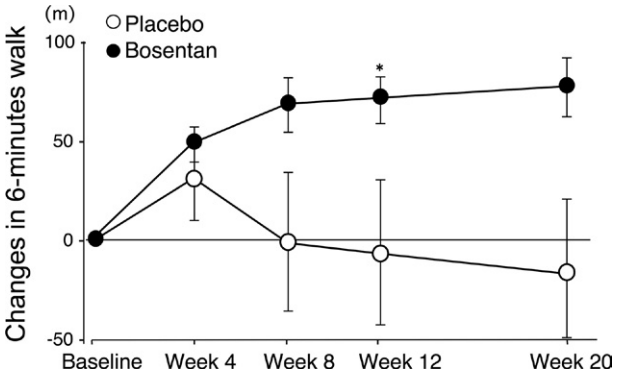

FIGURE 2 Effects of the $ET_{A/B}$ dual receptor antagonist bosentan treatment on 6-min walking distance. In double-blind, placebo-controlled study, 32 patients with pulmonary hypertension were randomly assigned to receiving bosentan (62.5 mg twice daily) for 4 weeks, then 125 mg twice daily or placebo. From baseline to week 20, bosentan increased the mean 6-min walking distance from 360 m to 437 m, whereas the distance was decreased in the placebo group from 355 m to 340 m. *: $P<0.05$ versus baseline, $P=0.021$ versus placebo. (From *The Lancet* 2001;**358**:1119–1123 (Ref. 2). With permission from Elsevier.)

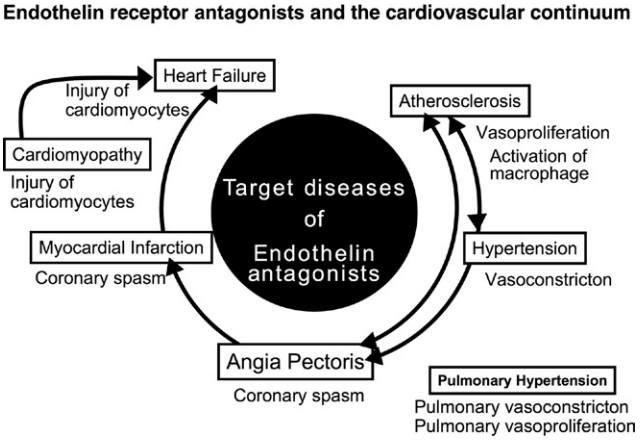

FIGURE 3 The cardiovascular disease continuum and the target diseases of the endothelin receptor antagonists.

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Endogenous endothelin-1 is considered to be involved in the progression of atherosclerosis, hypertension, angina pectoris, myocardial infarction, heart failure, and pulmonary hypertension, and these diseases may become target diseases of the endothelin receptor antagonists (Fig. 3). Endothelin receptor antagonists have been shown to exhibit desirable preventive and/or curative effects in various animal models of these cardiovascular diseases,[11,18] suggesting that they are of potential therapeutic value, and some of them are already under clinical use.

REFERENCES

1. Arai H, Hori S, Aramori I, Ohkubo H, Nakanishi S. Cloning and expression of a cDNA encoding and endothelin receptor. *Nature* 1990;**348**:730–2.
2. Channick RN, Simonneau G, Sitbon O, Robbins IM, Frost A, Tapson VF, et al. Effects of the dual endothelin-receptor antagonist bosentan in patients with pulmonary hypertension: a randomised placebo-controlled study. *Lancet* 2001;**358**(9288):1119–23.
3. Dzau V, Braunwald E. Resolved and unresolved issues in the prevention and treatment of coronary artery disease: a workshop consensus statement. *Am Heart J* 1991;**121**:1244–62.
4. Dzau VJ. The cardiovascular continuum and rennin–angiotensin–aldosterone system blockade. *J Hypertens Suppl* 2005;**23**(1):S9–17.
5. Dzau VJ, Antman EM, Black HR, Hayes DL, Manson JE, Plutzky J, et al. The cardiovascular disease continuum validated: clinical evidence of improved patient outcomes: part II: clinical trial evidence (acute coronary syndromes through renal disease) and future directions. *Circulation* 2006;**114**(25):2871–91.
6. Goto K, Hama H, Kasuya Y. Molecular pharmacology and pathophysiological significance of endothelin. *Jpn J Pharmacol* 1996;**72**:261–90.
7. Goto K. Basic and therapeutic relevance of endothelin-mediated regulations. *Biol Pharm Bull* 2001;**24**:1219–30.
8. Hickey KA, Rubanyi GM, Paul RJ, Highsmith RF. Characterization of a coronary vasoconstrictor produced by cultured endothelial cells. *Am J Physiol* 1985;**252**:C550–6.
9. Iglarz M, Binkert C, Morrison K, Fischli W, Gatfield J, Treiber A, et al. Pharmacology of macitentan, an orally active tissue-targeting dual endothelin receptor antagonist. *J Pharmacol Exp Ther* 2008;**327**(3):736–45.
10. Inoue A, Yanagisawa M, Kimura S, Kasuya Y, Miyauchi T, Goto K, et al. The human endothelin family. Three structurally and pharmacologically distinct isopeptides predicted by three separate genes. *Proc Natl Acad Sci U S A* 1989;**86**:2863–7.
11. Khimiji Al-K, Rockey DC. Endothelin-biology and disease. *Cell Signal* 2010;**22**:1615–25.
12. Krum H, Viskoper RJ, Lacourciere Y, Budde M, Charlon V. The effect of an endothelin-receptor antagonist, bosentan, on blood pressure in patients with essential hypertension. *N Engl J Med* 1998;**338**:784–90.
13. Maeda S, Miyauchi T, Goto K, Matsuda M. Differences in the change in the time course of plasma endothelin-1 and endothelin-3 levels after exercise in humans: the response to exercise of endothelin-3 is more rapid than that of endothelin-1. *Life Sci* 1997;**61**:419–25.
14. Maeda S, Miyauchi T, Kakiyama T, Sugawara J, Iemitsu M, Irukayama-Tomobe Y, et al. Effects of exercise training of 8 weeks and detraining on plasma levels of endothelium-derived factors, endothelin-1 and nitric oxide, in healthy young humans. *Life Sci* 2001;**69**:1005–16.
15. Maeda S, Tanabe T, Miyauchi T, Otsuki T, Sugawara J, Iemitsu M, et al. Aerobic exercise training reduces plasma endothelin-1 concentration in older women. *J Appl Physiol* 2003;**95**:336–41.
16. Miyauchi T, Yanagisawa M, Tomizawa T, Sugishita Y, Suzuki N, Fujino M, et al. Increased plasma concentrations of endothelin-1 and big endothelin-1 in acute myocardial infarction. *Lancet* 1989;**ii**(8653):53–4.
17. Miyauchi T, Yorikane R, Sakai S, Sakurai T, Okada M, Nishikibe M, et al. Contribution of endogenous endothelin-1 to the progression of cardiopulmonary alterations in rat with monocrotaline-induced pulmonary hypertension. *Circ Res* 1993;**73**:887–97.
18. Miyauchi T, Masaki T. Pathophysiology of endothelin in the cardiovascular system. *Annu Rev Physiol* 1999;**61**:391–415.
19. O'Brien RF, Robbins RJ, McMurthy IF. Endothelial cells in culture produce a vasoconstrictor substance. *J Cell Physiol* 1987;**132**:263–70.
20. Sakai S, Miyauchi T, Kobayashi M, Yamaguchi I, Goto K, Sugishita Y. Inhibition of myocardial endothelin pathway improves long-term survival in heart failure. *Nature* 1996;**384**:353–5.
21. Sakurai T, Yanagisawa M, Takuwa Y, Miyazaki H, Kimura S, Goto K, et al. Cloning of a cDNA encoding a non-isopeptide-selective subtype of the endothelin receptor. *Nature* 1990;**348**:732–5.
22. Sidharta PN, van, Giersbergen PL, Halabi A, Dingemanse J. Macitentan: entry-into-humans study with a new endothelin receptor antagonist. *Eur J Clin Pharmacol* 2011;**67**(10):977–84.
23. Shimojo N, Jesmin S, Zaedi S, Maeda S, Soms M, Aonuma K, et al. Eicosapentaenoic acid prevents endothelin-1-induced cardiomyocyte hypertrophy in vitro through the suppression of TGF-beta 1 and phosphorylated JNK. *Am J Physiol Heart Circ Physiol* 2006;**291**:H835–45.
24. Shimojo N, Jesmin S, Zaedi S, Otsuki T, Maeda S, Yamaguchi N, et al. Contributory role of VEGF overexpression in endothelin-1-induced cardiomyocyte hypertrophy. *Am J Physiol Heart Circ Physiol* 2007;**293**:H474–81.
25. Suzuki N, Matsumoto H, Kitada C, Masaki T, Fujino M. A sensitive sandwich-enzyme immunoassay to detect immunoreactive big endothelin in plasma. *J Immunol Methods* 1989;**118**:245–50.
26. Xu D, Emoto N, Giaid A, Slaughter C, Kaw S, deWit D, Yanagisawa M. ECE-1: A membrane-bound metalloprotease that catalyzes the proteolytic activation of big endothein-1. *Cell* 1994;**78**:473–85.
27. Yanagisawa M, Kurihara H, Kimura S, Tomobe Y, Kobayashi M, Mitsui Y, et al. A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature* 1988;**332**:411–5.

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Chapter 191

Ghrelin

Ichiro Kishimoto, Takeshi Tokudome, Hiroshi Hosoda, Mikiya Miyazato and Kenji Kangawa

ABSTRACT

Ghrelin, a peptide isolated from the stomach, exerts potent growth hormone-releasing and orexigenic actions. Recently, several lines of evidence suggest the beneficial effects of ghrelin in cardiovascular diseases. Administration of ghrelin for heart failure improves cardiac dysfunction and remodeling in humans and in animals. In addition, ghrelin treatment early after myocardial infarction effectively reduces fatal arrhythmia and, consequently, mortality. Moreover, ghrelin effectively reduces pulmonary hypertension induced by chronic hypoxia. Ghrelin provides various beneficial effects such as an increase in plasma growth hormone level, an improvement of energy balance, direct actions on cardiovascular cells, and regulation of autonomic nervous activity. It is, therefore, suggested that ghrelin would be a novel promising therapeutic agent for cardiovascular diseases.

INTRODUCTION

Ghrelin was purified from rat stomach by a reverse pharmacology paradigm in a stable cell line overexpressing its receptor.[14] Purification of ghrelin from the stomach indicates that the release of growth hormone (GH) from the pituitary is regulated not only by hypothalamic GH-releasing hormone but also by a peripherally derived hormone. Given the hemodynamic and metabolic effects of GH and its mediator, insulin-like growth factor-1 (IGF-1), ghrelin may have beneficial effects on the cardiovascular system. In addition, ghrelin has several actions on the heart and the vessel independent of GH/IGF-1. This chapter mainly focuses on the biological actions and therapeutic potential of ghrelin in cardiovascular diseases.

DISCOVERY

It has been known that small synthetic compounds called growth hormone secretagogues (GHS) stimulate the release of GH from the pituitary.[31] They act through their receptor (GHS-R), a G protein-coupled receptor (GPCR) with seven-membrane spanning domains.[8] In 1999, by a screening technology that matches orphan GPCRs with unknown cognate ligands by detection of the agonist-induced increase in intracellular calcium levels, a 28-amino acid peptide was purified from the stomach as the endogenous ligand for GHS-R.[14] It was named "ghrelin," from the word root "ghre" in Proto-Indo-European languages meaning "grow." Ghrelin also represents an abbreviation for "GH-release," a characteristic feature of the peptide.[14]

In the brain, ghrelin acts on GHS-R in the pituitary with its potent GH-releasing activity. The discovery of ghrelin shows that the release of GH from the pituitary is regulated not only by hypothalamic GH-releasing hormone (GHRH) but also by a peripherally derived hormone. In addition to its GH-releasing activity, ghrelin stimulates appetite by acting directly or indirectly on the hypothalamic arcuate nucleus, the region known to control food intake in humans and in rodents.[7,26] Furthermore, peripheral roles of ghrelin in the cardiovascular, bone, gastrointestinal, and immune systems have been implicated.[15]

STRUCTURE OF THE mRNA/GENE

The human ghrelin gene consists of five exons, which span 5 kb of genomic DNA on chromosome 3p25-26. Although the first exon encodes the 5′-untranslated region, the second and third exons encode the 28 amino acids of ghrelin peptide.[11] As described previously, the stomach is the major tissue, which produces ghrelin, and in situ analysis revealed that ghrelin and its mRNA are mainly localized in the submucosal layer of the stomach. Among four types of endocrine cells, that is, ECL, D, enterochromaffin, and X/A-like cells in the oxyntic mucosa of the stomach, ghrelin is found in X/A-like cells,[5] which were named so because of its previously undefined function and of the morphological similarity to pancreatic alpha cells. Cells producing ghrelin mRNA are also found in the intestines[5] and in the brain,[26] but at a much lower level than in the stomach.

PROCESSING OF THE PRECURSOR

The 28 amino acids of ghrelin are cleaved from its precursor, preproghrelin, which consists of 117 amino acids in

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00191-3
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rats and in humans.[16] The amino acid sequences of ghrelin precursors are well conserved among mammalian species. The distinguishing structural feature of ghrelin is its fatty acid modification at the third residue (serine in most species including humans).[14,16,31] Interestingly, the acylation, particularly the *n*-octanoyl modification, is conserved among many species including mammals, fish, birds, and amphibians and is necessary for full binding of ghrelin to its receptor, GHS-R, and thus for expressing the biological function.[14] Although an enzyme that catalyzes the acyl modification of ghrelin had been proposed, the attachment of octanoate to the third serine residue of ghrelin is catalyzed by a membrane-bound enzyme, named ghrelin O-acyltransferase (GOAT)[35] (see the chapter on GOAT in the Peptide Biosynthesis/Processing section of this book for details).

REGULATION OF PLASMA GHRELIN CONCENTRATION

From the submucosal layer of the stomach, ghrelin is secreted not into the gastrointestinal tract but into the blood stream. Results from patients with gastrectomy or gastric bypass surgery suggest that a major portion of the circulating ghrelin in plasma is from the stomach.[4,16] The plasma level of ghrelin and mRNA level in the stomach are increased by fasting and decreased by feeding,[3] and it is low in obese people and high in lean people.[4,34] In addition, oral or intravenous administration of glucose or meals with high lipid content decreases plasma ghrelin level. Furthermore, fasting plasma ghrelin concentrations are high in patients with anorexia nervosa. Thus, the nutritional state seems to be a major determinant of release of ghrelin from the stomach. Together with the potent orexigenic action, it is speculated that ghrelin serves as a "hunger hormone."[3]

Although it does not correlate with left ventricular systolic function, plasma ghrelin concentrations are elevated in cachectic patients with congestive heart failure (CHF)[25] or with chronic obstructive pulmonary diseases (COPD).[10] Given the anabolic effects of ghrelin, the increased plasma concentration may represent a compensatory mechanism under conditions of metabolic imbalance in such patients.

DISTRIBUTION OF THE mRNA/PEPTIDE IN THE CARDIOVASCULAR SYSTEM

Studies using polymerase chain reaction reveal that ghrelin mRNA can be found in parts of the body other than the stomach or brain, including heart, lung, kidney, and pancreas, although the significance of the localization and the production of mature ghrelin are not fully known. In brain, GHS-R mRNA is predominantly expressed in the arcuate and ventromedial nuclei and in the hippocampus,[8] although it is also detected in other parts of the neuronal system such as the pituitary and dentate gyrus. In the periphery, GHS-R mRNA can be found in various organs including the heart, lung, liver, kidney, pancreas, and the gastrointestinal tract. In addition, there is expression of ghrelin peptide and mRNA in a cultured cardiomyocyte cell line and in human vascular endothelial cells. Furthermore, although to a much lower extent than in the pituitary, GHS-R is also expressed in human myocardium[9] and in the aorta. Interestingly, in some cultured cells such as H9C2 cardiomyocytes and endothelial cells, a subtype of ghrelin receptor, which is distinct from GHS-R, is speculated to occur, since the binding affinity of either acylated or nonacylated ghrelin to the receptor was similar.[1]

RECEPTORS AND SIGNALING CASCADES

As stated, GHS-R, which was identified by expression cloning in 1996, acts as a receptor for ghrelin and ghrelin-induced GH release and appetite promotion are mediated through it.[32] So far, two types of transcript variants have been identified in several tissues and in several species. One transcript, GHS-R1a, encodes the functionally active protein and defines the effect of ghrelin for GH release. The second transcript, GHS-R1b, is derived only from the first exon by alternative splicing, retains the intron and does not function as a receptor for ghrelin. It might, however, attenuate the activity of GHS-R1a by acting in a dominant negative fashion. The amino acid sequences of the ghrelin receptor are well conserved across all vertebrate species examined from mammals to fish, implicating the important role of ghrelin and its receptor system in basic physiological functions.

In the pituitary, although GHRH stimulates GH release through a cyclic AMP-dependent pathway, ghrelin stimulates GH release through an elevation of intracellular calcium concentration. In different cell types, it is reported that ghrelin activates mitogen-activated protein kinase, Akt, nitric oxide synthase, or AMPK cascades.

BIOLOGICAL ACTIONS WITHIN THE CARDIOVASCULAR SYSTEM

Since the administration of ghrelin decreases blood pressure, reduces cardiac afterload, and increases cardiac output without affecting the heart rate in humans and in animals, the significance of ghrelin in cardiac diseases deserves to be studied.[21] In addition, ghrelin potently stimulates GH release from the pituitary gland, improves energy balance, and modulates the autonomic nervous system, all of which could have potentially beneficial effects on the cardiovascular system. Moreover, the receptor for ghrelin, GHS-R, can be found in cardiac ventricles and in blood vessels, suggesting that ghrelin may have direct cardiovascular actions.

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Growth Hormone Release

Ghrelin was originally discovered as an endogenous ligand for GHS-R and has potent GH-releasing activity (see the ghrelin chapter in the Brain Peptides section of this book). Ghrelin increases GH release from cultured pituitary cells. In addition, intravenous ghrelin administration markedly increases circulating GH in rats and in humans with a potency comparable to that of GHRH. After bolus ghrelin injection, the concentration of GH peaks between 15 and 20 min and the elevation lasts >60 min afterward.[22] Interestingly, ghrelin-induced GH release is abolished by resection of the gastric branch of the vagus nerve, suggesting that the effect of ghrelin is mediated via vagal afferent nerve activity.[6] In fact, the receptor for ghrelin is synthesized in vagal afferent neurons and transported to the afferent nerve terminals.[6]

GH and its mediator, IGF-1, are anabolic hormones that are essential for skeletal and myocardial growth and for metabolic homeostasis. Therefore, through the GH/IGF-1 pathway, ghrelin may have beneficial effects on cardiac function and on energy metabolism in diseases such as CHF. Because GH exerts both direct and indirect effects on cardiac structure and function, the cardiovascular effect of ghrelin might be mediated by the elevation of GH levels. Ghrelin when given in a physiological dose has no acute effects on cardiac function in patients with severe GH deficiency, suggesting that most of its acute actions in individuals with an intact GH system are mediated by GH. In addition, in hypophysectomized rats with GH deficiency, ghrelin plays a minor role in the control of cardiac function after ischemia/reperfusion injury.

However, the therapeutic effect of ghrelin is not accompanied by increases in GH-induced serum IGF-I levels or in heart weight, suggesting that ghrelin has actions independent of GH, especially in disease states. In addition, several lines of evidence showing the GH-independent mechanisms of ghrelin action are accumulating as described below.

Improved Energy Balance

The hypothalamic arcuate nucleus, the center of controlling the appetite, is one of the main sites of the ghrelin action (see the ghrelin chapters in the Brain, Ingestive, and Gastrointestinal Peptides sections of this book). Nakazato et al. first described that exogenously administered ghrelin has a potent appetite-stimulating effect.[26] Because the orexigenic effect of ghrelin can be observed in GH-deficient dwarf rats, the appetite-promoting effect is independent of GH release. In addition, ghrelin induces weight gain by reducing the use of fat.[33] In patients with end-stage CHF, both left ventricular dysfunction and cardiac cachexia are observed. Cardiac cachexia, which is a catabolic state characterized by weight loss and muscle wasting, is associated with hormonal changes and cytokine activation in patients with CHF. The presence of cardiac cachexia is a strong independent factor for mortality in patients with CHF. Therefore, cardiac cachexia and left ventricular dysfunction are therapeutic targets in the treatment of CHF. Because ghrelin causes a positive energy balance through GH-dependent and independent mechanisms, the administration of ghrelin may be of clinical value in cachectic patients with CHF. In fact, in ghrelin-treated patients with CHF, the improvement of cardiac function is associated with increases in body weight, in lean body mass, and in muscle strength.[23] Therefore, the orexigenic action of ghrelin might contribute to improving energy balance in patients with cachexia resulting from severe pathological conditions such as end-stage cardiovascular disease.

Direct Cardiovascular Actions

The presence of GHS-R mRNA in cardiovascular cells suggests direct actions of ghrelin in these cells. Because intra-arterial ghrelin effectively increases forearm blood flow measured by plethysmograph in humans, it is proposed that ghrelin acts directly on blood vessels.[27] Supporting this, in isolated human arteries, it is seen that ghrelin has an endothelium-independent vasodilating property.[13]

In addition, treatment with ghrelin is reported to inhibit apoptosis of cultured cardiomyocytes and endothelial cells possibly through the activation of extracellular signal–regulated kinase-1/2 and Akt serine kinases.[1] Interestingly, des-acyl ghrelin, which lacks the acylation at the third Ser residue, does not bind GHS-R, and is devoid of any endocrine activity, is also effective in inhibiting apoptosis.[1] The findings raise the possibility that ghrelin may act as a survival factor directly on the cardiovascular system possibly through binding to a yet to be identified receptor distinct from GHS-R.

Cardiac Sympathetic Efferent Pathway

Administration of ghrelin significantly suppresses the increased heart rate in cardiac diseases.[30] In addition, in both humans and animals, ghrelin significantly suppresses plasma norepinephrine concentrations. Furthermore, intracerebroventricular administration of ghrelin inhibits sympathetic nerve activity.[19] We investigated the subcutaneous administration of ghrelin in a rat model of myocardial infarction and, by the direct recoding of cardiac sympathetic nerve activity, showed that ghrelin prevents an increase in cardiac sympathetic nerve activity, which was accompanied by the reduction in mortality.[28] In acute myocardial infarction, because it is the initial increase in cardiac sympathetic nerve activity that considerably contributes to ventricular arrhythmia and, consequently, a high mortality, it is suggested that ghrelin injection after myocardial infarction

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would attenuate the early increase in sympathetic nerve activity, which could potentially improve cardiac prognosis.

Cardiac Vagal Afferent Pathway

Peripherally administered ghrelin stimulates hypothalamic neurons, which results in the increase in the food intake. Date et al. showed that ligation of the gastric branch of the vagal nerve suppressed the orexigenic effect of peripherally administered ghrelin.[6] In addition, chemical denervation by capsaicin, a neurotoxin specific for sensory afferents, also blocked the orexigenic effect of ghrelin, indicating that the appetite-promoting effect of peripherally administered ghrelin was mediated via vagal sensory afferent nerves.[6] Furthermore, microinjection of ghrelin into the nucleus of the solitary tract (NTS), the brain region important for controlling the autonomic nervous system, decreases the heart rate and mean arterial pressure.[18] Because cardiac vagal afferent nerves send projections to the NTS, it is speculated that ghrelin might decrease cardiac sympathetic nerve activity through vagal afferent nerves and the NTS. In addition, immunostaining with a specific antibody against GHS-R clearly shows the presence of the ghrelin receptor on the nerve terminals of the infarcted heart.[12] Taken together, these results suggest that the administration of ghrelin might exert its potent sympathetic inhibitory action by acting on the peripheral nerve terminals within the heart, which, through vagal afferent nerves, sends signals to the vasomotor center of the medulla via the NTS, resulting in decreases in sympathetic activity and in the heart rate upregulated in myocardial infarction. The proposed neuronal pathway of peripherally administered ghrelin is summarized schematically in Fig. 1.

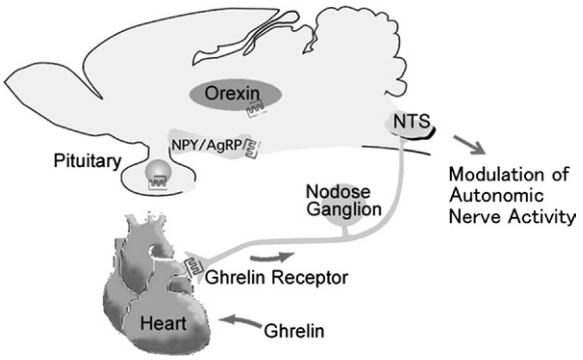

FIGURE 1 Schematic representation of the neuronal pathway in autonomic modulatory actions of ghrelin. Peripheral administration of ghrelin potently inhibits the cardiac sympathetic nerve activity in diseases such as myocardial infarction and heart failure. Ghrelin acts on cardiac vagal afferent nerve terminals, which send signals to the vasomotor center of the medulla through the nucleus of the solitary tract (NTS). The resultant inhibition of sympathetic nerve activity could protect the heart from excessive damage. *(Modified from Ref 12.)*

Anti-inflammatory Action

Ghrelin suppresses the production of proinflammatory cytokines, including interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α). In addition, ghrelin inhibits the activation of nuclear factor-κB (NF-κB), a transcriptional factor regulating the gene expression of proinflammatory cytokines.[17] In patients with pulmonary infections, chronic administration of ghrelin decreases inflammatory cytokine levels. Inhibition of the release of proinflammatory cytokines could be mediated at least partly by the ghrelin-induced activation of the vagus nerve. Because cardiovascular diseases are often accompanied by an augmented inflammatory response, ghrelin may exert its protective actions through these anti-inflammatory responses.

PATHOPHYSIOLOGICAL IMPLICATIONS

Plasma Concentrations of Ghrelin in Cardiopulmonary Diseases

Patients with CHF often exhibit some cachexia, which is an independent risk factor for mortality in such patients. Although plasma ghrelin concentrations do not differ significantly between patients with CHF and control subjects, they are significantly higher in CHF patients with cachexia than in those without cachexia. Similarly, plasma ghrelin is elevated in lean patients with COPD. Considering the known potent anabolic effect of ghrelin, the increase in plasma ghrelin in cachectic patients could be a compensatory mechanism.

Therapeutic Potential of Ghrelin in Heart Failure

A previous animal study revealed that chronic ghrelin treatment improved cardiac performance in rats with chronic heart failure, as shown by increases in cardiac output and left ventricular fractional shortening.[24] In addition, when intravenous bolus ghrelin (2 μg/kg, twice a day) was administered to the patients with CHF for three weeks, left ventricular ejection fraction was significantly improved ($27 \pm 2\%$ to $31 \pm 2\%$; $P < 0.05$), which was accompanied by an increase in peak workload and peak oxygen consumption during exercise.[23] Furthermore, three-week administration of ghrelin resulted in a dramatic decrease in plasma norepinephrine (1132 ± 188 to 655 ± 134 pg/mL; $P < 0.001$). These results, therefore, suggest the therapeutic potential of ghrelin in patients with CHF.

Therapeutic Potential of Ghrelin in Myocardial Infarction

Left ventricular remodeling after myocardial infarction is often associated with subsequent heart failure, which could

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lead to a fatal outcome. In a rat model of experimental myocardial infarction, we examined whether peripheral ghrelin administration attenuates left ventricular dysfunction and remodeling.[30] A two-week subcutaneous administration of ghrelin (100 μg/kg, twice a day) significantly improved left ventricular enlargement induced by myocardial infarction. In addition, there was a substantial decrease in left ventricular end-diastolic pressure and increases in the peak rate of the rise and fall (d*P*/d*t* max and min) of left ventricular pressure in ghrelin-treated rats compared with saline-treated controls. Furthermore, ghrelin attenuated an increase in morphometrical collagen volume fraction of the noninfarct region. Interestingly, the beneficial effects of ghrelin were accompanied by the complete suppression of the infarction-induced increase of heart rate.[30] Because the effect of ghrelin on the heart rate is more marked than on other parameters examined, it is speculated that ghrelin might have an inhibitory action on the infarction-induced activation of the sympathetic nerve independent of the improvement of cardiac function.

We next examined whether a subcutaneous injection of ghrelin (150 μg/kg) 1 min after the infarction leads to a beneficial effect.[28] Surprisingly, the early one shot bolus injection of ghrelin significantly reduced the high mortality rate associated with myocardial infarction. Although the mortality rate after infarction was 61% in vehicle-treated rats, it was significantly reduced to 23% in ghrelin-treated rats.[28] In the ghrelin-treated group, there were significantly fewer arrhythmic insults by the second to third hour after myocardial infarction.[28] Because one of the key factors that contributes to a high mortality after acute myocardial infarction is malignant ventricular arrhythmia, these results indicate that ghrelin treatment early after myocardial infarction improves survival by preventing fatal ventricular arrhythmias.

Effects of Ghrelin on Myocardial Ischemia/Reperfusion Injury

Administration of ghrelin protects the heart against ischemia/reperfusion injury.[2] The cardioprotective effects of ghrelin are independent of GH release and probably involve binding of the peptide to receptors in the heart. The beneficial effect of ghrelin infusion on myocardial ischemia/reperfusion injury might be attributed to the antiapoptotic effect of ghrelin via the ERK 1/2 and PI3 K/Akt-dependent pathway.

Therapeutic Potential of Ghrelin in Pulmonary Hypertension

In COPD, a sustained alveolar hypoxia leads to irreversible remodeling of the pulmonary artery. The consequent elevation of arterial pressure increases the workload of the heart, which can lead to heart failure and to increased morbidity and mortality. We tested whether ghrelin would impede pulmonary arterial hypertension during chronic hypoxia.[29] Under a hypoxic condition (10% of oxygen), conscious male rats received a daily subcutaneous injection of ghrelin (150 μg/kg) and pulmonary arterial pressure was continuously measured by radiotelemetry. In control rats, chronic hypoxia significantly increased pulmonary arterial pressure (110% increase in mean pressure), wall thickness of peripheral pulmonary arteries, and, consequently, right ventricular hypertrophy. In ghrelin-treated animals, the hypoxia-induced development of pulmonary hypertension was significantly attenuated to 48% increase in mean pressure, which was accompanied by improvement of pulmonary vascular remodeling, and right ventricular hypertrophy.[29] In addition, hypoxia-induced overexpression of endothelial nitric oxide synthase and endothelin-1 mRNA were attenuated in ghrelin-treated group. In patients with COPD, it is, therefore, suggested that ghrelin prevents pulmonary hypertension and right ventricular hypertrophy. Furthermore, patients with COPD often show a certain degree of cachexia, which is an independent risk factor for mortality in COPD. The repeated administration of ghrelin improves body composition, muscle wasting, functional capacity, and sympathetic augmentation in cachectic patients with COPD.[20] Taken together, these results indicate that the administration of ghrelin could be an effective therapy to these patients.

Atherosclerosis

Plasma ghrelin level is negatively correlated with atherosclerosis in elderly patients with metabolic syndrome or with hypertension, and in patients with endothelial dysfunction. Administration of exogenous ghrelin can improve endothelial dysfunction, an early marker for atherosclerosis, in patients with metabolic syndrome. It is speculated that the antiatherosclerotic effect of ghrelin could be mediated by increasing the bioavailability of nitric oxide, by decreasing oxidative stress, or by inhibiting inflammatory process.

CONCLUSIONS

Ghrelin is a GH-releasing peptide, isolated from the stomach, which has been identified as an endogenous ligand for GHS-R. Administration of ghrelin improves body composition, muscle wasting, functional capacity, and sympathetic hyperactivation seen in cachectic patients. In addition, ghrelin exerts cardioprotective actions in diseases such as heart failure, myocardial infarction, pulmonary hypertension, and fatal arrhythmias through various mechanisms including GH release, direct actions on cardiovascular cells, and inhibition of the sympathetic nervous activity (Fig. 2).

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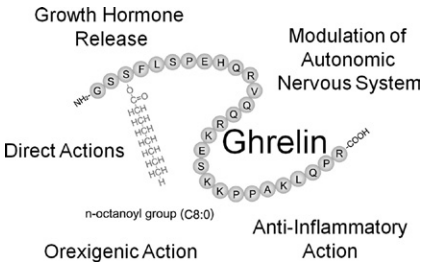

FIGURE 2 Structure of ghrelin and underlying mechanisms of its cardioprotective actions Ghrelin is a 28-amino acid peptide isolated from the stomach. A distinguishing structural feature of ghrelin is its *n*-octanoylation at the third serine residue, which is necessary for its receptor binding and function. Ghrelin potentially protects the diseased heart possibly through growth hormone (GH)-releasing activity, orexigenic action, direct effects on the cardiovascular cells, and inhibition of activated sympathetic nerves in cardiac diseases.

Treatment with ghrelin offers several advantages: ghrelin treatment for a short period of time in the acute setting is sufficient to exert protective actions, it can be administered by the convenient subcutaneous route, and it probably can be used more safely than chemically synthesized drugs because it is an endogenous hormone. Because of these advantages over other medications, ghrelin can be a promising new treatment for cardiac diseases.

REFERENCES

1. Baldanzi G, Filigheddu N, Cutrupi S, Catapano F, Bonissoni S, Fubini A, et al. Ghrelin and des-acyl ghrelin inhibit cell death in cardiomyocytes and endothelial cells through ERK1/2 and PI 3-kinase/AKT. *J Cell Biol* 2002;**159**:1029–37.
2. Chang L, Ren Y, Liu X, Li WG, Yang J, Geng B, et al. Protective effects of ghrelin on ischemia/reperfusion injury in the isolated rat heart. *J Cardiovasc Pharmacol* 2004;**43**:165–70.
3. Cummings DE, Purnell JQ, Frayo RS, Schmidova K, Wisse BE, Weigle DS. A preprandial rise in plasma ghrelin levels suggests a role in meal initiation in humans. *Diabetes* 2001;**50**:1714–9.
4. Cummings DE, Weigle DS, Frayo RS, Breen PA, Ma MK, Dellinger EP, et al. Plasma ghrelin levels after diet-induced weight loss or gastric bypass surgery. *N Engl J Med* 2002;**346**:1623–30.
5. Date Y, Kojima M, Hosoda H, Sawaguchi A, Mondal MS, Suganuma T, et al. Ghrelin, a novel growth hormone-releasing acylated peptide, is synthesized in a distinct endocrine cell type in the gastrointestinal tracts of rats and humans. *Endocrinology* 2000;**141**:4255–61.
6. Date Y, Murakami N, Toshinai K, Matsukura S, Niijima A, Matsuo H, et al. The role of the gastric afferent vagal nerve in ghrelin-induced feeding and growth hormone secretion in rats. *Gastroenterology* 2002;**123**:1120–8.
7. Horvath TL, Diano S, Sotonyi P, Heiman M, Tschop M. Minireview: ghrelin and the regulation of energy balance–a hypothalamic perspective. *Endocrinology* 2001;**142**:4163–9.
8. Howard AD, Fieghner SD, Cully DF, Arena JP, Liberator PA, Rosenblum CI. A receptor in pituitary and hypothalamus that functions in growth hormone release. *Science* 1996;**273**:974–7.
9. Iglesias MJ, Pineiro R, Blanco M, Gallego R, Dieguez C, Gualillo O, et al. Growth hormone releasing peptide (ghrelin) is synthesized and secreted by cardiomyocytes. *Cardiovasc Res* 2003;**62**:481–8.
10. Itoh T, Nagaya N, Yoshikawa M, Fukuoka A, Takenaka H, Shimizu Y, et al. Elevated plasma ghrelin level in underweight patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2004;**170**:879–82.
11. Kanamoto N, Akamizu T, Tagami T, Hataya Y, Moriyama K, Takaya K, et al. Genomic structure and characterization of the 5′-flanking region of the human ghrelin gene. *Endocrinology* 2004;**145**:4144–53.
12. Kishimoto I, Tokudome T, Schwenke DO, Soeki T, Hosoda H, Nagaya N, et al. Therapeutic potential of ghrelin in cardiac diseases. *Expert Rev Endocrinol Metab* 2009;**4**:283–9.
13. Kleinz MJ, Maguire JJ, Skepper JN, Davenport AP. Functional and immunocytochemical evidence for a role of ghrelin and des-octanoyl ghrelin in the regulation of vascular tone in man. *Cardiovasc Res* 2006;**69**:227–35.
14. Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H, Kangawa K. Ghrelin is a growth-hormone-releasing acylated peptide from stomach. *Nature* 1999;**402**:656–60.
15. Kojima M, Kangawa K. Drug insight: the functions of ghrelin and its potential as a multitherapeutic hormone. *Nat Clin Pract Endocrinol Metab* 2006;**2**:80–8.
16. Kojima M, Kangawa K. Ghrelin: structure and function. *Physiol Rev* 2005;**85**:495–522.
17. Li WG, Gavrila D, Liu X, Wang L, Gunnlaugsson S, Stoll LL, et al. Ghrelin inhibits proinflammatory responses and nuclear factor-kappaB activation in human endothelial cells. *Circulation* 2004;**109**:2221–6.
18. Lin Y, Matsumura K, Fukuhara M, Kagiyama S, Fujii K, Iida M. Ghrelin acts at the nucleus of the solitary tract to decrease arterial pressure in rats. *Hypertension* 2004;**43**:977–82.
19. Matsumura K, Tsuchihashi T, Fujii K, Abe I, Iida M. Central ghrelin modulates sympathetic activity in conscious rabbits. *Hypertension* 2002;**40**:694–9.
20. Nagaya N, Itoh T, Murakami S, Oya H, Uematsu M, Miyatake K, et al. Treatment of cachexia with ghrelin in patients with chronic obstructive pulmonary disease. *Chest* 2005;**128**:1187–93.
21. Nagaya N, Kojima M, Kangawa K. Ghrelin, a novel growth hormone releasing peptide in the treatment of cardiopulmonary associated cachexia. *Intern Med* 2006;**45**:127–34.
22. Nagaya N, Kojima M, Uematsu M, Yamagishi M, Hosoda H, Oya H, et al. Hemodynamic and hormonal effects of human ghrelin in healthy volunteers. *Am J Physiol* 2001;**280**:R1483–7.
23. Nagaya N, Moriya J, Yasumura Y, Uematsu M, Ono F, Shimizu W, et al. Effects of ghrelin administration on left ventricular function, exercise capacity, and muscle wasting in patients with chronic heart failure. *Circulation* 2004;**110**:3674–9.
24. Nagaya N, Uematsu M, Kojima M, Ikeda Y, Yoshihara F, Shimizu W, et al. Chronic administration of ghrelin improves left ventricular dysfunction and attenuates development of cardiac cachexia in rats with heart failure. *Circulation* 2001;**104**:1430–5.
25. Nagaya N, Uematsu M, Kojima M, Ikeda Y, Yoshihara F, Shimizu W, et al. Elevated circulating level of ghrelin in cachexia associated with chronic heart failure. relationships between ghrelin and anabolic/catabolic factors. *Circulation* 2001;**104**:2034–8.
26. Nakazato M, Murakami N, Date Y, Kojima M, Matsuo H, Kangawa K, et al. A role for ghrelin in the central regulation of feeding. *Nature* 2001;**409**:194–8.

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27. Okumura H, Nagaya N, Enomoto M, Nakagawa E, Oya H, Kangawa K, et al. Vasodilatory effect of ghrelin, an endogenous peptide from the stomach. *J Cardiovasc Pharmacol* 2002;**39**:779–83.
28. Schwenke DO, Tokudome T, Kishimoto I, Horio T, Shirai M, Cragg PA, et al. Early ghrelin treatment after myocardial infarction prevents an increase in cardiac sympathetic tone and reduces mortality. *Endocrinology* 2008;**149**:5172–6.
29. Schwenke DO, Tokudome T, Shirai M, Hosoda H, Horio T, Kishimoto I, et al. Exogenous ghrelin attenuates the progression of chronic hypoxia-induced pulmonary hypertension in conscious rats. *Endocrinology* 2008;**149**:237–44.
30. Soeki T, Kishimoto I, Schwenke DO, Tokudome T, Horio T, Yoshida M, et al. Ghrelin suppresses cardiac sympathetic activity and prevents early left ventricular remodeling in rats with myocardial infarction. *Am J Physiol* 2008;**294**:H426–32.
31. Smith RG, Cheng K, Schoen WR, Pong SS, Hickey G, Jacks T. A nonpeptidyl growth hormone secretagogue. *Science* 1993;**260**:1640–3.
32. Sun Y, Wang P, Zheng H, Smith RG. Ghrelin stimulation of growth hormone release and appetite is mediated through the growth hormone secretagogue receptor. *Proc Natl Acad Sci U S A* 2004;**101**:4679–84.
33. Tschop M, Smiley DL, Heiman ML. Ghrelin induces adiposity in rodents. *Nature* 2000;**407**:908–13.
34. Tschop M, Weyer C, Tataranni PA, Devanarayan V, Ravussin E, Heiman ML. Circulating ghrelin levels are decreased in human obesity. *Diabetes* 2001;**50**:707–9.
35. Yang J, Brown MS, Liang G, Grishin NV, Goldstein JL. Identification of the acyltransferase that octanoylates ghrelin, an appetite-stimulating peptide hormone. *Cell* 2008;**132**:387–96.

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Chapter 192

Natriuretic Peptides

Naoto Minamino and Toshio Nishikimi

ABSTRACT

The natriuretic peptide family in mammals consists of three homologous peptides: atrial (A-type) natriuretic peptide (ANP), brain (B-type) natriuretic peptide (BNP), and C-type natriuretic peptide (CNP). ANP and BNP bind to natriuretic receptor-A, whereas CNP acts on natriuretic receptor-B. Natriuretic receptor-A and B are receptor-guanylyl cylases, and these peptides dilate blood vessels and induce diuresis/natriuresis by increasing the intracellular cGMP concentration. ANP and BNP are most abundantly synthesized in cardiomyocytes, whereas CNP is expressed in neurons, endothelial cells, and cardiac fibroblasts. Natriuretic peptide receptors are widely expressed in the peripheral tissue, and these peptides not only regulate blood pressure and body fluid volume but also inhibit remodeling of heart and blood vessels. The plasma concentrations of ANP and BNP are increased in the cardiovascular disease. Thus, natriuretic peptides are crucial regulatory factors and biomarkers in the cardiovascular system.

DISCOVERY OF NATRIURETIC PEPTIDES (NPs)

Mammalian atrial cardiac myocytes exhibit the morphology of endocrine cells and contain "atrial specific granules." By the beginning of 1980s, atrial extracts were shown to induce potent diuresis/natriuresis and hypotension when injected into rats. Soon after confirmation of the correlation between diuretic/natriuretic activity and smooth muscle relaxant activity of the atrial extracts, the diuretic/natriuretic and hypotensive factor was isolated, and its structure was determined in 1983–1984. As many research groups competed for its identification, this peptide was first given a variety of names, such as atriopeptin, cardionatrin, and auriculin but is now generally called atrial natriuretic peptide (ANP).[19,21,23]

The second member of the NP family was isolated from pig brain extracts by monitoring of the relaxant activity of chick rectum in 1988 and named brain natriuretic peptide (BNP).[19,21,23] In a few years, this peptide was shown to be a cardiac peptide mainly expressed in the ventricle;[12] the concentration of BNP in brain was extremely low in rodents and human. BNP is now recognized as B-type NP of the NP family.

The third NP was also identified in 1990 in the pig brain extracts by the same procedures and named C-type natriuretic peptide (CNP).[19,21,23] CNP is present in human brain, not at a high concentration but at the highest concentration of the members of the NP family. CNP is considered to be a true brain NP, but this peptide is produced in the vasculature, heart, bone, macrophages, and elsewhere.[19,21,28] CNP is an original and ancestral peptide of the NP family, as described elsewhere in this book.

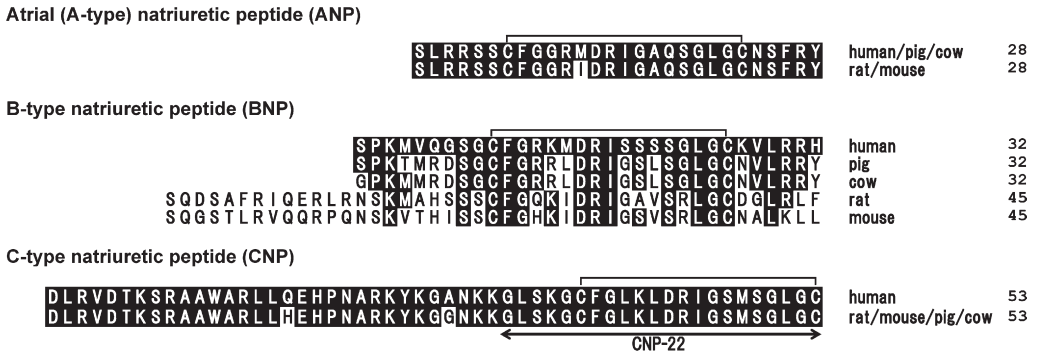

FIGURE 1 Amino acid sequences of major active forms mammalian ANP, BNP, and CNP. Highlighted letters (white in black) indicate amino acids common to human NP in each group, and bracket over top NP sequence of each group indicates a disulfide linkage that forms the ring structure. CNP-22 sequence is shown by arrowed line at the bottom. Number on the right edge is residue number of each peptide.

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00192-5
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Figure 1 shows the amino acid sequences of major active molecular forms of human ANP, BNP, and CNP.[19,21,23,25] All NPs have two cysteine residues in the molecule, and a 17-residue ring structure formed by a disulfide linkage is essential for exerting biological activity. In this ring structure, the amino acid sequence is well conserved among the three NPs. In the case of ANP and BNP, the C-terminal extension from the ring structure is essential for their biological activity, while CNP does not have it. In all NPs, the N-terminal extension does not alter the primary potency of biological activity. In mammalian ANPs, α-ANP of 28-amino-acid residues is an endogenous and active form, and only one amino acid replacement (Met/Ile) is observed. There are many replacements in the case of BNPs, and the length of a major endogenous form is different between rodents (BNP-45) and other mammals (BNP-32). CNP is present as CNP-22 and CNP-53, both of which have a comparable activity. CNP-53 is generally a major endogenous form. The amino acid sequence of CNP-22 is identical in all mammals, and two replacements are observed in the CNP-53 sequence.

STRUCTURE OF THE NP mRNAs/GENES

The exon–intron organization, structure of mRNA, precursor protein, active form, and other endogenous forms of three mammalian NPs are schematically represented in Figure 2. Three mammalian NP genes are each coded in three exons. Human ANP and BNP genes are neighboring genes located at 11,905,766–11,907,840 (reverse strand) and 11,917,521–11,918,988 (reverse strand) in chromosome 1, and no transcript from the 9.7 kb intervening sequence has been recorded. Human CNP gene is located at 232,786,530–232,791,113 (reverse strand) in chromosome 2.

In the 5′-flanking region (FR) of rat ANP gene, GATA, NKE, TBE, AT-rich, and phenylephrine-responsive elements, and CArG boxes/SRE are present as *cis*-acting elements, and the binding of transcriptional factors, GATA4/6, Nkx2.5/Csx, Tbx5, MEF2C, Zfp260, and SRF, to these elements are reported to be important for ANP gene transcription. Neuron-restrictive silencer factor (NRSF), which binds to the 3′-untranslated region (UTR) of the ANP gene, also regulates the ANP gene transcription.[3,10] In the 5′-FR of the BNP gene, GATA, M-CAT, SRE, shear stress- and thyroid hormone-responsive element, and NFAT, NRSF and AP-1/CRE-like binding sites are observed, and binding of transcriptional factors to these elements and sites increases BNP gene transcription. Most of these transcriptional factors in the ANP and BNP gene expression are essential for embryogenesis and morphogenesis of heart tissue.[3,10] Regulation of CNP gene expression has been less well studied. Human CNP gene has an inverted CCAAT box, 2 GC boxes, and a CRE-like sequence in the 5′-FR. Although the GC box is crucial for TGF-β- and shear stress-induced gene expression, the number of GC box is different between human and rodents. The rodent CNP gene in the kidney is positively regulated by the Wnt

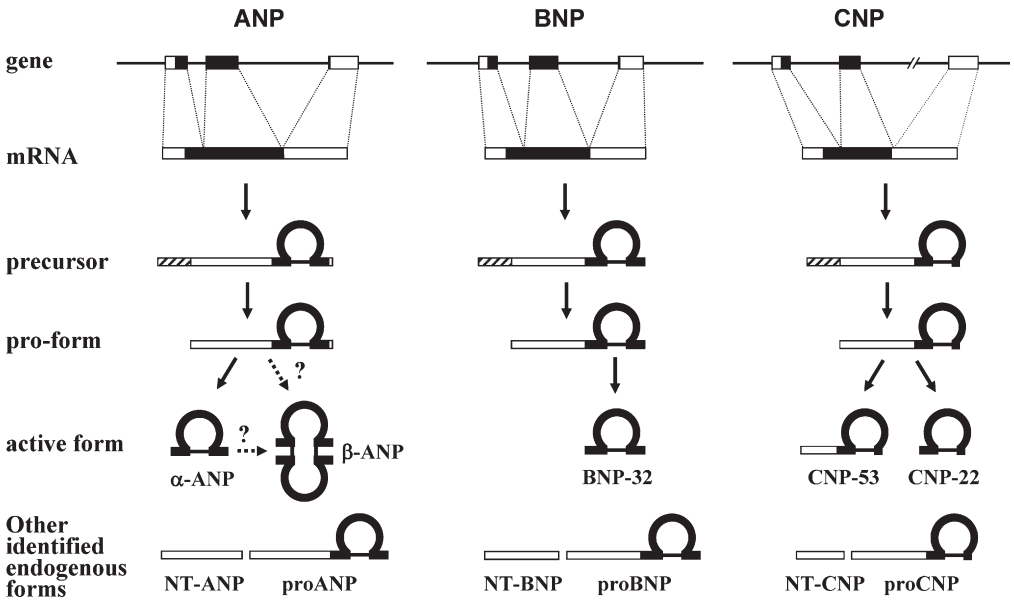

FIGURE 2 Schematic representation of genes, mRNAs, precursors, and active forms of human ANP, BNP and CNP. Boxes in the gene row indicate exons, and black and white regions represent coding and noncoding regions, respectively. In the precursor, proform and active form rows, hatched bar, white bar and ring structure indicate signal peptide, N-terminal intervening peptide and active unit of each NP, respectively. In ANP, 2 arginines are usually inserted after α-ANP as shown by a white bar. In addition to major active forms, identified endogenous forms of NPs are shown at the bottom. The proform of each NP has weak but significant biological activity.

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signaling pathway, operating through β-catenin and T-cell factor/lymphoid enhancer binding factor, of which the binding site is shared with human CNP gene.[3,28]

The apparent length of human NP mRNAs is about 850–900 bp for ANP and BNP, and 1300 bp for CNP, and the difference in the mRNA length mainly results from that in the 3′-UTR. In the 3′-UTR of BNP gene, AUUUA repeats are present, which accelerates mRNA degradation. The BNP gene expression is considered to be posttranscriptionally controlled for adapting to dynamic physiological alterations.[19,21,25]

The precursor proteins of human ANP, BNP, and CNP are 153, 134, and 126 amino acids in length, and the active structure is located at the C-terminus in each precursor. In the cases of the ANP precursor, two arginines are inserted after α-ANP, except for pig and dog.[19,21,23,25] There are two human ANP genes; one has this insertion, whereas another not.

PROCESSING OF THE NP PRECURSORS

ANP is stored in the cardiac myocytes as a 126-residue polypeptide called γ-ANP, which lacks the C-terminal 2 arginines from proANP. In the normal heart tissue, ANP is almost exclusively present as γ-ANP. When secreted, it is cleaved after a Pro-Arg sequence mainly by corin, a specific converting enzyme for ANP, to generate α-ANP and an N-terminal intervening peptide (NT-ANP).[35] In the atrium of patients with heart failure, α-ANP is present in the myocytes along with a unique antiparallel dimer of β-ANP. In the plasma, α-ANP is a major circulating and active form, along with inactive NT-ANP (Fig. 2). In mammals other than humans, γ-ANP and α-ANP are major forms in the heart and plasma, respectively.[21,23]

In the case of human BNP, proBNP is processed probably by furin to BNP-32 and an N-terminal 76-residue peptide (NT-BNP). In heart tissue, BNP is present as both proBNP and BNP-32. BNP-32 is a major molecular form in the atrium, whereas proBNP is a major form in the ventricle.[17] Both proBNP and BNP-32 circulate in the plasma, and proBNP is a major form in severe heart failure. In the pig, BNP is present as proBNP in the heart and as BNP-32 and BNP-26 in the plasma. As expected from its sequence heterogeneity (Fig. 1), an endogenous form of mammalian BNP is different in each animal. In the rat, BNP-45 and proBNP are major forms in the atrium and ventricle, respectively, whereas BNP-45 is a major form in the plasma. NT-BNP is present in the plasma at a much higher concentration than BNP-32.[19,21,25] A high percentage of human plasma proBNP and NT-BNP are *O*-glycosylated, which increases their molecular masses >10 KDa. There are 7 *O*-glycosylated Ser/Thr residues, T36, S37, S44, T48, S53, T58, and T71,[26] and glycosylation of T71 inhibits the conversion by furin from proBNP to BNP-32.[29] (see the Furin chapter in the Peptides Biosynthesis/Processing section of this book).

CNP is present in all mammals in the uniform molecular forms as deduced from its high sequence identity.[19,23] In the cardiovascular system, vascular endothelial cells (ECs), cardiac fibroblasts, and monocyte–macrophage system are known to produce CNP.[19,21,28] For ECs, CNP is mainly present as CNP-53 along with CNP-22 in a manner similar to that in the brain. Furin is also considered to process proCNP to CNP-53, but not convert CNP-53 to CNP-22.

DISTRIBUTION OF THE NP mRNA/PEPTIDE IN THE CARDIOVASCULAR SYSTEM

ANP mRNA is detected in a variety of tissues, including brain, pituitary, and kidney, but it is most abundant in the heart.[19,21,23] Within the heart, the atrium is a major site of ANP production. The primary stimulus for ANP secretion is an increase in atrial-wall tension, which is attributable to elevation of venous blood pressure. Tachycardia also promotes atrial ANP secretion. Several hormones and neurotransmitters, such as endothelin (ET), vasopressin, and catecholamines (especially α-adrenergic agonists), directly stimulate ANP secretion from atrial and ventricular myocytes.[32]

Peptide concentrations of ANP in human, rat, and pig cardiac ventricles are 250- to 1000-fold lower than those in atria. ANP mRNA levels are also much lower in the ventricles than in the atria. Although little ANP is produced by ventricular tissue in normal adults, a high level of ANP is present in the fetus ventricle. Gene expression and synthesis of ANP are enhanced in both atrium and ventricle during early embryogenesis, but its expression in the ventricle is decreased after the prenatal period. ANP expression in the ventricle is reinducible postnatally in hypertrophied and failing hearts.[19,21,23,25]

Although BNP was first identified in pig brain extracts, this peptide is found in the circulation, and its highest concentration in the tissue is observed in heart in all mammals. Tissue concentrations of BNP other than the heart are low, and its distribution is limited to brain, amniotic membrane, and uterine. In human and rat hearts, both atrium and ventricle express the BNP gene at a high level. The total BNP mRNA content is about 3-fold higher in the ventricle than in the atrium, whereas ANP mRNA content in the ventricle is ~10% than that in the atrium. The peptide concentration of BNP in the ventricle is <1% of that in the atrium. In an experiment with isolated perfused rat hearts, however, the majority of BNP secretion (~60%) was maintained even after atrial removal, in contrast to the observation that ANP secretion was reduced to <5% by atrial removal. In the human heart, predominant BNP secretion from the ventricle has been confirmed by a step-up of plasma BNP level in the anterior interventricular vein compared with that in the aorta. Thus, BNP is a cardiac hormone that is predominantly synthesized in and secreted from the ventricle.[12,19,21,25]

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CNP was initially believed to act solely as a neuropeptide, as CNP and its CNP mRNA are found predominantly in the brain and pituitary. However, subsequent studies have shown that CNP is synthesized in peripheral tissues including vasculature, bone, blood cells, heart, kidney, and reproductive system. Significant CNP production is observed in vascular ECs. The expression and secretion of CNP from the ECs is markedly stimulated by transforming growth factor β (TGF-β) and tumor necrosis factor α, and moderately by interleukin-1, basic fibroblast growth factor (bFGF), lipopolysaccharide, ANP and BNP, and shear stress.[28,31] Vascular smooth muscle cells (VSMC) also produce CNP, which is stimulated by TGF-β. Platelet-derived growth factor-BB has a strong effect on the CNP expression, but elicits reverse effects on human and rat VSMCs. CNP is now recognized as an important regulator within the vascular wall under various pathological states.[27,28]

Although much lower than ANP and BNP, CNP is expressed and present in both cardiac atrium and ventricle. Failing heart locally produces CNP at a level higher than in normal heart.[7] An *in vitro* study verified significant gene expression and secretion of CNP from cultured rat cardiac fibroblasts but not from myocytes, indicating a distinct profile of cardiac CNP. The secretion of CNP from cardiac fibroblasts is augmented by TGF-β, bFGF, and ET-1, and the secreted CNP inhibits hypertrophy and remodeling of heart tissue.[6,33]

NP RECEPTORS AND SIGNALING CASCADES

Natriuretic receptor-A (NPR-A) and natriuretic receptor-B (NPR-B) are guanylyl cyclase (GC)-linked receptors, and they use cGMP as the intracellular messenger (Fig. 3). Both ANP and BNP bind preferentially to NPR-A, whereas CNP specifically binds to NPR-B. NPR-B has an extremely low affinity for ANP and BNP, while CNP does not bind to NPR-A even at a high concentration. NPs stimulated cGMP accumulation via NPR-A with a rank order potency of ANP≥BNP>>CNP, whereas that via NPR-B was CNP>ANP≥BNP. All three NPs bind to a third receptor NPR-C, known as a clearance receptor. NPR-C is not linked to guanylyl cyclase, and mainly acts to clear the NPs and control their concentrations.[19,21]

NPR-A and NPR-B transcripts were widely detected in all peripheral tissues. NPR-A is highly expressed in kidney, adrenal gland, terminal ileum, adipose, vasculature, and lung, whereas NPR-B is expressed in vasculature, heart, adrenal gland, kidney, lung, uterus, ovary, and bone, in addition to pituitary and brain including cerebellum. In the vasculature, VSMCs express NPR-B at a high level. NPR-B is desensitized more easily than NPR-A, and sphingosine 1-phosphate is one of desensitizing factors in the serum.[2,19,21]

In the heart, both cardiac myocytes and fibroblasts express NPRs. Myocytes predominantly express NPR-A, whereas fibroblasts express NPR-A and NPR-B. Indeed, ANP and BNP increase an intracellular cGMP level in myocytes, whereas ANP, BNP, and CNP increase it in fibroblasts. These results indicate that the NPs also act as autocrine and/or paracrine factors in the heart tissue.[2,19,21]

All three NPs bind to NPR-C with high affinity. The order of affinity to NPR-C is ANP>CNP>BNP, but their differences in the affinity are much smaller than those to NPR-A and NPR-B. Although NPR-C primarily functions as a clearance receptor, this receptor suppresses adenylyl cyclase and activates phospholipase C through the Gαi-mediated pathway.[22] Osteocrin with limited similarity to the NPs is reported to induce some natriuretic peptide-like effects by blocking the NP binding to NPR-C.[21]

Crystallographic studies reveal that the extracellular domains of NPR-A and NPR-C form 2:1 complexes with ANP, and that the structure of ANP in the complex of NPR-A is similar to that of NPR-C (Fig. 3). As the dimers of the extracellular domains of NPR-A and NPR-C have a 2-fold rotational symmetry and ANP is located along the center of the receptor dimer axis, the interaction between ANP and one of the two NPRs is not symmetrical. It is postulated that ANP binding to the dimers of NPR-A extracellular domains induces rotation of the juxtamembrane domains, which intracellularly induces a similar rotation in each of the catalytic domains to form an active structure of GC domains. ATP is a requisite or at least stabilizing factor for GC activation, and chloride ion binding may modulate an allosteric effect of the NPR-A.[11]

BIOLOGICAL ACTIONS OF NPS IN THE CARDIOVASCULAR SYSTEM

Because NPR-A is widely distributed, ANP and BNP have a wide range of physiological actions. In the kidney, ANP inhibits tubular reabsorption at a low dose, and induces renal vasodilation at a higher dose. Renal functions of the NPs are discussed in detail in that section of this book.

ANP acts on multiple sites to reduce blood pressure. ANP at first dilates the vasculature and shifts fluid to the extravascular compartment. The reduction of blood volume and an increase in venous capacitance are considered to be responsible for the reduced cardiac output. Thus, ANP seems to have a 'depressor' effect rather than a direct vasodilator effect. A lowering of peripheral resistance in response to ANP is not observed in normotensives but is seen in hypertensives associated with an increased vascular tone. This most probably explains the discrepancy in the hemodynamic responses to ANP in normotensives and hypertensives.

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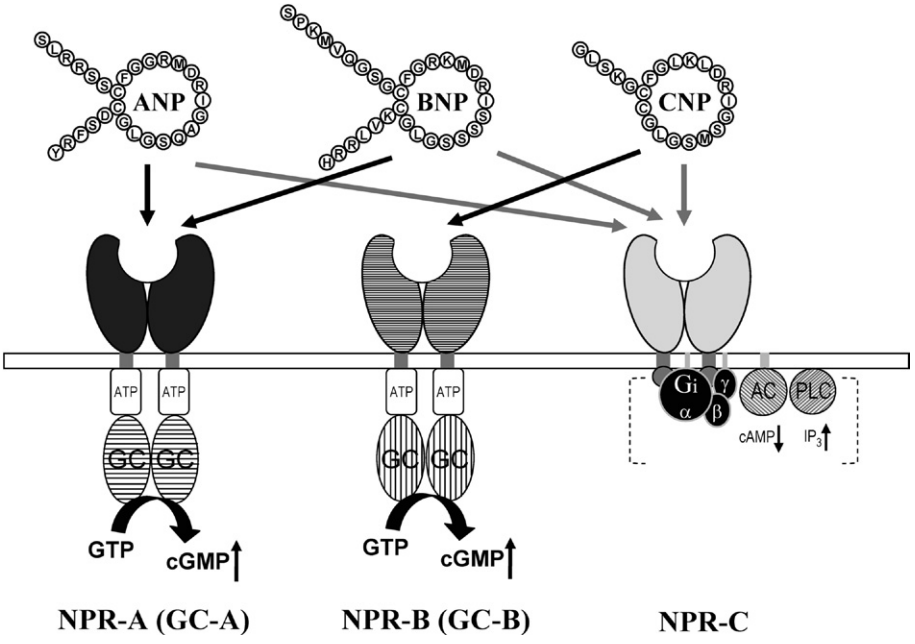

FIGURE 3 Schematic representation of NPRs and ligand–receptor relationship of the NP family. Black arrows indicate interaction with active receptors, NPR-A and NPR-B, and gray arrows interaction with clearance receptor, NPR-C. NPR-A and NPR-B are also called GC-A and GC-B, respectively, because they are membrane-bound guanylyl cyclases (GCs). NPR-C is in some cases coupled with Gi protein, suppresses adenylyl cyclase (AC), or stimulates phospholipase C. One NP molecule binds to each dimer-formed NPR.

In the endocrine/neuroendocrine system, ANP diminishes secretion of renin, aldosterone, adrenocorticotropic hormone, and vasopressin along with reduction of sympathetic tone. All these actions lead to lowering of the blood pressure. Intracerebroventricular administration of ANP attenuates angiotensin II-induced water intake and pressor response. Centrally injected ANP produced a greater reduction of water intake, and 1-week intracerebroventricular infusion of ANP also reduced salt appetite in spontaneously hypertensive rats. Thus, the ANP/NPR-A system antagonizes the renin–angiotensin system in both central and peripheral systems.[21] Effects of the NPs in the endocrine and central nervous systems are discussed in detail elsewhere in this book.

NPs are also potent growth regulators of multiple cell types. ANP inhibits proliferation of mesangial cells induced by angiotensin II or ET-1. The ANP/NPR-A system negatively regulates MAPK/ERK2 activity and proliferation of mesangial cells through a cGMP-dependent-protein kinase pathway. In studies with cultured neonatal myocytes and fibroblasts, NPs elicited marked antihypertrophic and antifibrotic actions. Later endogenous NPs were verified to have antihypertrophic and antifibrotic effects.[5] Mice lacking NPR-A develop ventricular hypertrophy and fibrosis independent of their blood pressure.[8] These results suggest that ANP and BNP are autocrine and/or paracrine antiremodeling factors. In cultured VSMCs, NPs inhibit proliferation and migration induced by growth factors or angiotensin II, suggesting that NPs act as antiatherosclerotic factors. Doubly deficient NPR-A(−/−) and ApoE(−/−) mice showed greater atherosclerotic lesion size and more advanced plaque morphology compared with NPR-A(+/+) and ApoE(−/−) mice. Aortic medial thickness was also increased in NPR-A(−/−) and ApoE(−/−) mice.[1]

Adipose tissues express NPR-A and NPR-C. ANP activates hormone-sensitive lipase in human fat cells *in vitro* via its phosphorylation through a cGMP-mediated mechanism.[30] Hormone-sensitive lipase then breaks down triglycerides into free fatty acids and glycerol. Systemic ANP infusion that increases its plasma concentration to the pathophysiological level is reported to stimulate lipid mobilization and oxidation. These results suggest that NP-induced lipid mobilization might contribute to cardiac cachexia in heart failure.

CNP is a vasorelaxant with particularly venodilator effects, contrasting with the arterial bias of ANP and BNP. CNP causes relaxation of canine saphenous, femoral, and renal venous tissue but does not cause arterial relaxation. Systemic administration of low-dose CNP in man induces no hemodynamic response, but higher-dose CNP lowers blood pressure. CNP does not seem to have any marked influence on renal sodium handling, either. Thus, systemic hemodynamic effects of CNP are much less than those of ANP and BNP.[21,28]

As CNP is produced in ECs, endothelium-derived CNP can elicit direct vasodilation through NPR-B abundantly expressed in VSMCs. CNP suppresses various growth factor-stimulated VSMC migration and proliferation more potently than ANP and BNP. CNP might also inhibit

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ET-1-mediated VSMC proliferation, as CNP directly inhibits secretion of ET-1 from ECs. These findings suggest that CNP regulates vascular tone and growth as an autocrine/paracrine factor in the vascular wall.[27,28]

In vitro studies have shown the direct effects of CNP on cardiac myocytes and fibroblasts.[33] CNP inhibits fibroblast proliferation and extracellular matrix production more potently than ANP and BNP. ET-1 secretion from cardiac fibroblasts and ET-1-induced hypertrophy of cardiac myocytes are also inhibited by CNP. Taken together with the CNP production in the fibroblasts, CNP may also act as a local autocrine/paracrine regulator in the heart. These results indicate that the NP/cGMP system plays a key role in the growth regulation of various cells.[19,21,28]

PATHOPHYSIOLOGICAL IMPLICATIONS OF NPs

It is well known that plasma concentrations of ANP and BNP are increased in various pathological conditions such as heart failure, acute myocardial infarction (AMI), hypertension, left ventricular hypertrophy, coronary artery disease, pulmonary hypertension, and renal failure.

In heart failure, plasma ANP and NT-ANP concentrations are elevated in patients with symptomatic congestive heart failure in proportion to the severity of the disease and also in asymptomatic patients with left ventricular dysfunction. As plasma BNP and NT-BNP concentrations in patients with heart failure change more dynamically and correlate well with the New York Heart Association functional class and hemodynamics, these plasma concentrations are used to evaluate heart failure severity and to identify patients with asymptomatic left ventricular dysfunction.[12,16] Several studies have examined the diagnostic value of ANP, BNP, NT-ANP, and NT-BNP for the detection of left ventricular systolic dysfunction and showed that the levels of these peptides are useful biomarkers for both in the general population and in patients with cardiovascular disease. Further, the measurement of BNP can rule out congestive heart failure in newly symptomatic subjects with a high negative predictive value for heart failure. In the urgent-care setting, BNP is useful to discriminate acute dyspnea resulting from congestive heart failure from other causes. Thus, the measurement of plasma NP concentrations is useful not only for evaluating the severity of heart failure but also to rule out the screening test for left ventricular dysfunction and heart failure. Increased expression and secretion of NPs in heart failure are considered to be a compensatory action of the NPs, which is mediated by their natriuretic actions and not by altering hemodynamics.[14] Further, induction of heart failure in NPR-A(−/−) mice resulted in a more severe outcome than that in NPR-A(+/+) mice, indicating a protecting role of the NPs/NPR-A system against heart failure.[16]

In AMI, plasma ANP concentrations are already increased at the time of admission and decrease thereafter. By contrast, plasma BNP concentrations are increased on admission and reach a peak at 12–24 h after admission. Then, the BNP concentration decreases and has a second peak on day 5–7, after which BNP concentrations gradually decrease.[16] In a rat AMI model, BNP levels are increased in and around the necrotic tissues of the infarcted region, indicating the augmented production of BNP in the remaining myocytes.[16] Thus, the height of the second peak of BNP may be a good index for evaluating left ventricular remodeling in patients with AMI. Because ANP and BNP have potent antihypertrophic and antifibrotic effects, augmented production of NPs in the ventricle may also be aimed to inhibit the remodeling.

Several studies show that plasma ANP and BNP concentrations are increased in patients with hypertension compared with that of normotensive subjects. In hypertension, plasma ANP and BNP concentrations are higher in patients with left ventricular concentric hypertrophy than in those without it or with other types of hypertrophy or remodeling.[15] ANP(−/−) mice exhibit salt-dependent hypertension, suggesting that ANP regulates blood pressure via its natriuretic actions, whereas BNP(−/−) mice show normal blood pressure with slight focal fibrosis in the ventricle.[8] Interestingly, NPR-A(−/−) mice show salt-independent hypertension as well as cardiac hypertrophy and fibrosis.[8] The differences in phenotype between ANP(−/−) mice and BNP(−/−) mice may result from different sites of action and/or different gene expression regulatory systems of ANP and BNP. The increased production of NPs in the hypertrophied ventricle is thought to inhibit cardiac hypertrophy and fibrosis via their direct effects on the myocytes and fibroblasts.[8] Thus, ANP and BNP play pivotal roles in the pathogenesis of hypertension and subsequently occurring left ventricular hypertrophy.

In patients with right ventricular pressure overload resulting from pulmonary hypertension, plasma ANP and BNP concentrations are higher than those in patients with right ventricular volume overload resulting from atrial septal defect. The plasma ANP and BNP concentrations correlate with mean pulmonary artery pressure, right atrial pressure, right ventricular end-diastolic pressure, and total pulmonary resistance in these patients. After long-term vasodilator treatment, their plasma concentrations decrease according to a reduction of total pulmonary resistance.[13] Plasma BNP concentrations are also high in patients with acute pulmonary embolism, in whom BNP levels are associated with right ventricular overload. Thus, both right atrial and ventricular pressure overloads stimulate ANP and BNP secretion independent of etiology, and plasma ANP and BNP concentrations are good indices especially for the evaluation of the severity and treatment efficacy in patients

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with right ventricular overload. Chronic hypoxia produces a greater increase in right ventricular systolic pressure and more muscularization of pulmonary arterioles in NPR-A(−/−) mice compared with NPR-A(+/+) mice.[8] Thus, NPs/NPR-A system regulates tone and remodeling of pulmonary vasculature, and right ventricular hypertrophy in pulmonary hypertension.

Elevated plasma ANP and BNP concentrations are prognostic indicators of heart failure, AMI and pulmonary hypertension. Medical therapy can reduce the ANP and BNP concentrations in parallel with hemodynamic and clinical improvement in these patients. These findings strongly indicate that the measurement of NPs can be used to optimize therapy. After NT-BNP-guided therapy was firstly reported,[34] BNP- or NT-BNP-guided treatment of patients with chronic heart failure has been investigated in a number of randomized controlled trials and showed that this approach leads to fewer hospital readmissions and a lower mortality due to heart failure. However, a meta-analysis shows that the risk of all-cause hospitalization and survival free of any hospitalization is not significantly different between groups.[20] At present, there seems to be a limitation in the usefulness of BNP-guided therapy in chronic heart failure patients.

Infusion of ANP significantly decreases pulmonary capillary wedge pressure and aldosterone concentrations, and increases stroke volume index, urine volume, excretion of sodium, and endogenous creatinine clearance in patients with heart failure.[16] The similar effects of BNP are observed in patients with heart failure.[16] These results indicate the possibility that BNP and ANP could be used as a drug in patients with heart failure. Indeed, ANP and BNP are widely used in clinical setting in many countries. Although Sackner-Bernstein et al. pointed out that BNP may worsen renal function in patients with acutely decompensated heart failure,[24] succeeding studies negated such a harmful effect of BNP.[18]

NPs may also be applicable to AMI. An earlier study showed that ANP infusion group had greater improvement of left ventricular ejection fraction than the nitroglycerin group.[4] A recent prospective, randomized, and multicenter study shows that ANP reduces an infarct size and reperfusion injury compared with controls.[9] As ANP/BNP/NPR-A signaling is deeply involved in the pathophysiology of cardiovascular disease, ANP and BNP can be applied to a series of heart diseases, in addition to their usefulness in the clinical test for the cardiovascular diseases.

CNP potently inhibits VSMC proliferation and migration, suggesting that this peptide may play a protective role against abnormal growth of VSMCs in the disorders such as atherosclerosis and postangioplasty restenosis. CNP can limit neointimal formation of arteries subjected to balloon injury by inhibiting VSMC proliferation and migration as well as rapid reendothelialization of the injured vessel. Elevated circulating concentrations of CNP are found in hypoxemia, sepsis, and chronic renal failure, but not in essential hypertension.[19,21,28] Endothelial damage caused by hypoxia and endotoxin may provoke CNP secretion from ECs. Marked elevation in plasma CNP observed in renal failure is probably attributable to impaired clearance.

No significant increase in the plasma CNP level was reported in patients with chronic heart failure, in contrast to that of ANP and BNP. However, tissue levels of CNP are increased along with ANP and BNP in the ventricular myocardium of patients with heart failure. A significant cardiac production of CNP is observed in chronic heart failure, along with the correlation of coronary sinus CNP concentrations with mean pulmonary capillary wedge pressure.[7] Both cardiac myocytes and fibroblasts express NPR-B, and the NPR-B mRNA level is markedly increased in the hypertrophied left ventricle. Collectively, cardiac CNP may act as a key local regulator in some pathological states of the heart, though further investigations are required.

REFERENCES

1. Alexander MR, Knowles JW, Nishikimi T, Maeda N. Increased atherosclerosis and smooth muscle cell hypertrophy in natriuretic peptide receptor A−/− apolipoprotein E−/− mice. *Arterioscler Thromb Vasc Biol* 2003;**23**:1077–82.
2. Garbers DL, Chrisman TD, Wiegn P, Katafuchi T, Albanesi JP, Bielinski V, et al. Membrane guanylyl cyclase receptors: an update. *Trends Endocrinol Metab* 2006;**17**:251–8.
3. Gardner DG, Chen S, Glenn DJ, Grigsby CL. Molecular biology of the natriuretic peptide system: implications for physiology and hypertension. *Hypertension* 2007;**49**:419–26.
4. Hayashi M, Tsutamoto T, Wada A, Maeda K, Mabuchi N, Tsutsui T, et al. Intravenous atrial natriuretic peptide prevents left ventricular remodeling in patients with first anterior acute myocardial infarction. *J Am Coll Cardiol* 2001;**37**:1820–6.
5. Horio T, Nishikimi T, Yoshihara F, Matsuo H, Takishita S, Kangawa K. Inhibitory regulation of hypertrophy by endogenous atrial natriuretic peptide in cultured cardiac myocytes. *Hypertension* 2000;**35**:19–24.
6. Horio T, Tokudome T, Maki T, Yoshihara F, Suga S, Nishikimi T, et al. Gene expression, secretion, and autocrine action of C-type natriuretic peptide in cultured adult rat cardiac fibroblasts. *Endocrinology* 2003;**144**:2279–84.
7. Kalra PR, Clague JR, Bolger AP, Anker SD, Poole-Wilson PA, Struthers AD, et al. Myocardial production of C-type natriuretic peptide in chronic heart failure. *Circulation* 2003;**107**:571–3.
8. Kishimoto I, Tokudome T, Nakao K, Kangawa K. Natriuretic peptide system: an overview of studies using genetically engineered animal models. *FEBS J* 2011;**278**:1830–41.
9. Kitakaze M, Asakura M, Kim J, Shintani Y, Asanuma H, Hamasaki T, et al. J-WIND investigators. Human atrial natriuretic peptide and nicorandil as adjuncts to reperfusion treatment for acute myocardial infarction (J-WIND): two randomised trials. *Lancet* 2007;**370**:1483–93.

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10. Kuwahara K, Nakao K. Regulation and significance of atrial and brain natriuretic peptides as cardiac hormones. *Endocr J* 2010;**57**:555–65.
11. Misono KS, Philo JS, Arakawa T, Ogata CM, Qiu Y, Ogawa H, et al. Structure, signaling mechanism and regulation of the natriuretic peptide receptor guanylate cyclase. *FEBS J* 2011;**278**:1818–29.
12. Mukoyama M, Nakao K, Hosoda K, Suga S, Saito Y, Ogawa Y, et al. Brain natriuretic peptide as a novel cardiac hormone in humans. *J Clin Invest* 1991;**87**:1402–12.
13. Nagaya N, Nishikimi T, Uematsu M, Satoh T, Kyotani S, Sakamaki F, et al. Plasma brain natriuretic peptide as a prognostic indicator in patients with primary pulmonary hypertension. *Circulation* 2000;**102**:865–70.
14. Nishikimi T, Miura K, Minamino N, Takeuchi K, Takeda T. Role of endogenous atrial natriuretic peptide on systemic and renal hemodynamics in heart failure rats. *Am J Physiol* 1994;**267**:H182–6.
15. Nishikimi T, Yoshihara F, Morimoto A, Ishikawa K, Ishimitsu T, Saito Y, et al. Relationship between left ventricular geometry and natriuretic peptide levels in essential hypertension. *Hypertension* 1996;**28**:22–30.
16. Nishikimi T, Hagaman JR, Takahashi N, Kim HS, Matsuoka H, Smithies O, et al. Increased susceptibility to heart failure in response to volume overload in mice lacking natriuretic peptide receptor-A gene. *Cardiovasc Res* 2005;**66**:94–103.
17. Nishikimi T, Minamino N, Ikeda M, Takeda Y, Tadokoro K, Shibasaki I, et al. Diversity of molecular forms of plasma brain natriuretic peptide in heart failure—different proBNP-108 to BNP-32 ratios in atrial and ventricular overload. *Heart* 2010;**96**:432–9.
18. O'Connor CM, Starling RC, Hernandez AF, Armstrong PW, Dickstein K, Hasselblad V, et al. Effect of nesiritide in patients with acute decompensated heart failure. *New Engl J Med* 2011;**365**:32–43.
19. Pandey KN. Biology of natriuretic peptides and their receptors. *Peptides* 2005;**26**:901–32.
20. Porapakkham P, Porapakkham P, Zimmet H, Billah B, Krum H. B-type natriuretic peptide-guided heart failure therapy: a meta-analysis. *Arch Intern Med* 2010;**170**:507–14.
21. Potter LR, Abbey-Hosch S, Dickey DM. Natriuretic peptides, their receptors, and cyclic guanosine monophosphate-dependent signaling functions. *Endocr Rev* 2006;**27**:47–72.
22. Rose RA, Giles WR. Natriuretic peptide C receptor signalling in the heart and vasculature. *J Physiol* 2008;**586**:353–66.
23. Rosenzweig A, Seidman CE. Atrial natriuretic factor and related peptide hormones. *Annu Rev Biochem* 1991;**60**:229–55.
24. Sackner-Bernstein JD, Kowalski M, Fox M, Aaronson K. Short-term risk of death after treatment with nesiritide for decompensated heart failure: a pooled analysis of randomized controlled trials. *JAMA* 2005;**293**:1900–5.
25. Samson WK, Levin ER, editors. *Natriuretic peptides in health and disease*. Totowa: Humana Press; 1997.
26. Schellenberger U, O'Rear J, Guzzetta A, Jue RA, Protter AA, Pollitt NS. The precursor to B-type natriuretic peptide is an O-linked glycoprotein. *Arch Biochem Biophys* 2006;**451**:160–6.
27. Scotland RS, Ahluwalia A, Hobbs AJ. C-type natriuretic peptide in vascular physiology and disease. *Pharmacol Ther* 2005;**105**:85–93.
28. Sellitti DF, Koles N, Mendonc MC. Regulation of C-type natriuretic peptide expression. *Peptides* 2011;**32**:1964–71.
29. Semenov AG, Postnikov AB, Tamm NN, Seferian KR, Karpova NS, Bloshchitsyna MN, et al. Processing of pro-brain natriuretic peptide is suppressed by O-glycosylation in the region close to the cleavage site. *Clin Chem* 2009;**55**:489–98.
30. Sengenes C, Bouloumie A, Hauner H, Berlan M, Busse R, Laf, et al. Involvement of a cGMP-dependent pathway in the natriuretic peptide-mediated hormone-sensitive lipase phosphorylation in human adipocytes. *J Biol Chem* 2003;**278**:48617–26.
31. Suga S, Nakao K, Itoh H, Komatsu Y, Ogawa Y, Hama N, et al. Endothelial production of C-type natriuretic peptide and its marked augmentation by transforming growth factor-β. *J Clin Invest* 1992;**90**:1145–9.
32. Thibault G, Amiri F, Garcia R. Regulation of natriuretic peptide secretion by the heart. *Annu Rev Physiol* 1999;**61**:193–217.
33. Tokudome T, Horio T, Soeki T, Mori K, Kishimoto I, Suga S, et al. Inhibitory effect of C-type natriuretic peptide (CNP) on cultured cardiac myocyte hypertrophy: interference between CNP and endothelin-1 signaling pathways. *Endocrinology* 2004;**145**:2131–40.
34. Troughton RW, Frampton CM, Yandle TG, Espiner EA, Nicholls MG, Richards AM. Treatment of heart failure guided by plasma amino-terminal brain natriuretic peptide (NT-BNP) concentrations. *Lancet* 2000;**355**:1126–30.
35. Yan W, Wu F, Morser J, Wu Q. Corin, a transmembrane cardiac serine protease, acts as a pro-atrial natriuretic peptide-converting enzyme. *Proc Natl Acad Sci USA* 2000;**97**:8525–9.

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Chapter 193

Salusins

Masayoshi Shichiri, Hajime Izumiyama and Takuya Watanabe

ABSTRACT

Salusin-α and salusin-β, endogenous peptides biosynthesized from the same precursor – prosalusin – exert a variety of cardiovascular activities. Initially, the presence of salusins was predicted from a full-length-enriched cDNA library. Later salusins were discovered in human biological fluids. Thus, they were the first peptides to be successfully discovered using an "in silico" approach. Salusins exhibit similar mitogenic and antiapoptotic properties but exert contrasting activities on atherosclerosis. The salusin-β peptide causes potent hemodynamic effects by parasympathomimetic actions. Attempts to elucidate the bioactivity of salusin-β were enormously difficult because of its physicochemical property of adhering firmly to the experimental apparatus material, such as polypropylene, polystyrene, and glass. Despite its potent bioactivity, because of this adherent feature, salusin-β was not discovered by conventional methods, including reverse pharmacology. We speculate that other unidentified ligands possessing similar or other characteristics preventing their isolation/discovery may be discovered by in silico analyses.

DISCOVERY

Discovery of a novel endogenous bioactive peptide in humans has been a frustrating and lengthy process. Because circulating bioactive peptides in mammals usually exist at very low concentrations, purification and identification of such peptides buried by vast amounts of more abundant proteins still remain an extremely difficult task. In the history of bioactive peptide research, the reverse pharmacology approach has come to the fore relatively recently and has successfully unraveled many important endogenous peptides. However, this method requires sequence information on ligand candidates to test their responsiveness in each orphan G protein–coupled receptor, and the number of bioactive peptides with unidentified receptors is limited. We initiated bioinformatics analyses using full-length cDNA and genomic DNA information anticipating that this novel approach could markedly shorten the lengthy identification process for novel endogenous bioactive peptides, and this resulted in successful identification of salusin peptides with cardiovascular activities.

Our prediction of salusin-α and salusin-β derived from a search of a large collection of full-length, enriched cDNA libraries owned by the Helix Research Institute, a joint research project principally funded by the Japanese Ministry of International Trade and by the major pharmaceutical industries in Japan.[33] The library included human clones that had been constructed with a mammalian expression vector driven by the SRα promoter, pME18S-FL3, by the oligo-capping method[24] and sequenced in the governmental New Energy and Industrial Technology Development Organization Human cDNA Sequencing Project.[33] We first chose putative secretory polypeptides from the human cDNA library using the SignalP server (http://www.cbs.dtu.dk/services/SignalP/), which predicts the presence and location of signal peptide cleavage sites in amino acid sequences.[8] Selected cDNAs that encode preproproteins having cleavable signal peptide sequences were further assessed with ATGpr[13] and PSORT algorithms,[6] which provide information as to whether translated proteins could be released extracellularly. Promising candidates were subjected to further scrutiny on the basis of common structural features characteristic of endogenous bioactive peptides.

Major bioactive peptides are biosynthesized from their precursor preproproteins with amino acid lengths ranging from 90 to 250. After cleavage of their signal sequence, the proproteins can be further digested at the C-terminal site of dibasic amino acid residues by several types of prohormone convertases.[21] The resultant C-terminal dibasic amino acid residues are usually removed by carboxypeptidase E, and the C-terminal glycine residue is converted to NH_2 by peptidylglycine α-amidating monooxygenase to produce an amidated peptide[1] (see the chapters on all these enzymes in the Peptide Biosynthesis/Processing section of this book). Our initial selection of bioactive peptide candidates from the cDNA library information used the above features, which are characteristic of human bioactive peptides and predicted processed peptide sequences of <30 amino acid residues. We did not restrict endogenous peptide candidates to those containing disulfide bonds or to those containing a C-terminal NH_2. Predicted candidate sequences were synthesized and

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00193-7
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tested for the ability to increase intracellular free Ca^{2+} ($[Ca^{2+}]_i$) in cultured mammalian cells derived from the vasculature. Because synthetic salusin-α and salusin-β induced potent and sustained increases in $[Ca^{2+}]_i$ in human and rat vascular smooth muscle cells and rat fibroblasts, they were subjected to subsequent studies to further clarify their bioactivities. The cleavage prediction approaches we used in discovering salusin peptides have recently been shown to predict prohormone cleavage sites with high accuracy even in genome libraries.[20]

The discovery process of novel bioactive peptides using in silico selection of cDNAs and cleavage prediction strategies are not complete until the predicted peptide sequences are proven to exist in the circulation or in other biological fluids. The specific radioimmunoassay for salusin-α was relatively easily established, which allowed confirmation of the existing molecular forms in humans. Immunoreactive salusin-α in serum and urine was analyzed by high-performance liquid chromatography and gel permeation chromatography, and the findings coincided with the predicted authentic salusin-α sequence.[15] However, confirmation of salusin-β in human plasma required several years because of its peculiar physicochemical characteristics.

We originally described that salusin-β exerts very potent and unique hemodynamic activities,[19] which aroused wide interest. We soon received a number of personal communications that noted varying degrees of biological activities of salusin-β. Some scientists could not reproduce our results at all,[2] others found less potent effects,[12] and several other investigators successfully unraveled novel actions and/or mechanisms.[28,31,32] Especially puzzling were the findings that many investigators shared in common: The effects of salusin-β sometimes varied from experiment to experiment, making it difficult to finish a series of planned procedures. Major commercial testing laboratories in Japan failed to establish an assay because of extraordinarily unstable results when determining plasma and urine salusin-β concentrations. This mystery was soon resolved after we found that the salusin-β peptide sticks to commonly used laboratory tubes, tips, plates and pipettes, whether they are made of polypropylene, polystyrene, or glass.[18] Addition of a very low amount of NP-40 or Tween 20 when reconstituting purified salusin-β avoids this property and allowed us to successfully establish an assay system and stably reproduce experiments.[16] These procedures confirm that salusin-β is also present in human serum and urine as was originally predicted. It is probable that published results performed without this knowledge had either required a far larger dose than it should have[12] or failed to achieve positive results.[2,32]

STRUCTURE OF THE mRNA/GENE

Salusins derive from an alternatively spliced product of TOR2a, a torsin dystonia-family gene. TOR2a has 5 exons, is located at 9q34.11, and encodes a protein of 321 amino acids[9,10] Its splice variant, preprosalusin (PSEC0218, HEMBA 1005096, AK075520), contains a frameshift resulting from the deletion of exon 4 that results in a deduced 242-amino acid protein with an alternate C terminus (Fig. 1).

PROCESSING OF THE PRECURSOR

Preprosalusin, the precursor of salusins, has a 26-amino acid signal peptide at the N terminus, and the resultant proprotein of 216 amino acids is termed prosalusin. The C-terminal end of prosalusin tandemly contains salusin-β and salusin-α, which should be cleaved by prohormone convertases. Carboxypeptidase E probably removes the resultant C-terminal dibasic amino acids between salusin-β and salusin-α to create the 20-amino acid salusin-β. Peptidylglycine α-amidating monooxygenase converts the glycine residue at the C-terminal end to NH_2 to yield the 28-amino acid salusin-α with C-terminal amidation. The processing

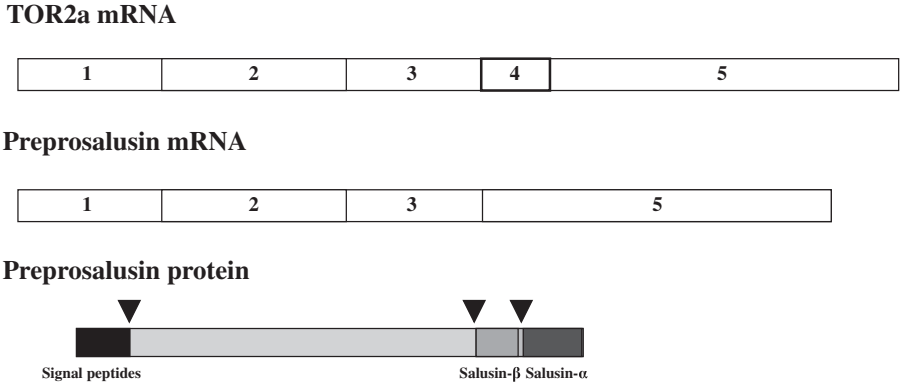

FIGURE 1 Schema showing transcripts of TOR2a and preprosalusin and biosynthesis of the salusin peptides from their precursor, preprosalusin. Open boxes represent mRNAs, and numbers indicate exons. Salusin-α and salusin-β in the C-terminal ends of preprosalusin are cleaved out. Arrowheads show signal peptide cleavage sites and two dibasic amino acid sites.

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of preprosalusin into salusin-α and salusin-β may differ in various cell types.[7,14]

DISTRIBUTION OF THE mRNA/PEPTIDE IN THE CARDIOVASCULAR SYSTEM

TOR2a and preprosalusin are coexpressed almost ubiquitously throughout tissues, with preprosalusin transcripts expressed at quantitatively lower levels than TOR2A in most human and rat tissues.[19,22] Because semiquantification of the alternatively spliced product by RT-PCR is often misleading because of the low amplification efficiency of spliced transcripts, we quantified preprosalusin transcripts using the TaqMan hybridization probe format. In humans, preprosalusin mRNA is abundant in spleen, peripheral leucocytes, lung, kidney, and brain. Additionally, preprosalusin mRNA is abundant in the endocrine glands, including the pancreas, testis, ovary, and thymus, while the expression level is low in cardiac and skeletal muscle.[19] The low mRNA expression level of preprosalusin in the cardiovascular system, however, does not lessen the significance of salusins as cardiovascular peptides.

Similar to the ubiquitous distribution of preprosalusin mRNA, immunoreactive salusin-α and salusin-β are also widely expressed, but show distinct systemic distributions, which give an insight into their potential biological activities.[22,23] Salusin-α is conserved in mice and rats with a shorter amino acid length. In rats, it is detected abundantly in the epithelial tissues throughout the organs, in the gastrointestinal and immune systems, and in submucosal and myenteric plexuses in the stomach.[23] Salusin-β is undetectable in any of these tissues. By contrast, salusin-α and salusin-β are concomitantly present in macrophages in the spleen and lymph nodes, immature myeloid cells and megakaryocytes of the bone marrow, and proximal tubules of the kidney. Salusins are barely detected in the heart, and only a small amount of the immunoreactivity is present in the intact vasculature. Salusin-β-like immunoreactivity is most prominently present in the hypothalamus and posterior pituitary and coexists in vasopressin-expressing neurons,[22,25] suggesting its possible neural secretion into the systemic circulation via axon terminals. Salusin-β positive neurons and fibers in the supraoptic nucleus and the paraventricular nucleus are markedly increased after chronic salt loading and dehydration.[12] Thus, despite the limited expression levels of preprosalusin mRNA and salusin peptides in the heart, salusin peptides released into circulation from the hypothalamo-neurohypophyseal system and/or other systemic organs could contribute to the pathophysiologic condition of the cardiovascular system.

Further, the expression of salusin-α and salusin-β in the vasculature may be markedly modified in a variety of cardiovascular diseases associated with atherosclerosis. The exaggerated expression of salusin-β in atherosclerotic lesions in the vasculature and amelioration of vascular changes by antisalusin-β antibody suggests a mediatory role for endogenous salusin-β in the development and/or maintenance of atherosclerosis.[28] Especially remarkable is the marked immunoreactivity of salusin-β in contrast to trace amounts of salusin-α in human coronary atherosclerotic plaques. This study showed an especially predominant expression of salusin-β in macrophages, vascular smooth muscle, and fibroblasts.[28]

RECEPTORS AND SIGNALING CASCADES

Selective receptors for salusins still remain unidentified, although salusin-β is a surrogate ligand of the mas-like G protein–coupled receptor MrgA1.[27] Salusins can induce intracellular signaling and subsequent cellular responses, which are downstream to many G protein–coupled receptors. Thus, it is possible that a yet-unidentified G protein–coupled receptor directly or indirectly mediates the signaling cascades elicited by salusins. For example, salusin-β, released from a nerve ending in the neurohypophysis, increases intracellular free Ca^{2+} via influx through voltage-dependent Ca^{2+} channels, and stimulates the release of vasopressin and oxytocin from the posterior pituitary in an autocrine/paracrine fashion.[12] In neonatal rat cardiomyocytes, both salusin-α and salusin-β stimulate protein synthesis through the calcium, calcineurin, mitogen-activated protein kinase, and protein kinase C signal pathways.[32] They also protect cardiomyocytes against serum deprivation-induced apoptosis by the upregulation of GRP78.[31] Opposing effects of salusin-α and salusin-β in regulating human macrophage foam cell formation are mediated via G-protein, c-Src tyrosine kinase, protein kinase C, and mitogen-activated protein kinases.[28] Salusins are initially identified by their ability to induce increases in $[Ca^{2+}]_i$ and the expression of the immediate early oncogene, c-myc, and subsequent proliferation of vascular smooth muscle cells and fibroblasts.[19] The above signaling elicited by salusins implies direct and/or indirect mediation by their unidentified receptors.

BIOLOGICAL ACTIONS WITHIN THE CARDIOVASCULAR SYSTEM

The cardiovascular activities of the two salusin peptides can roughly be categorized into systemic and local actions. Remarkable systemic bioactivities of salusin-β include its potent hemodynamic effects. Intravenous salusin-β causes rapid, temporary, and profound decreases in the blood pressure and heart rate in rats.[19] These effects are mainly mediated by parasympathetic stimulation rather than direct suppression of cardiac contractility, because the effects are almost completely abrogated by pretreatment with atropine

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sulfate.[3] In addition to its potent effects to facilitate vagal outflows to the heart, however, salusin-β induces negative inotropism via a direct myotropic effect as well.[3] Because salusin-β is found in human circulation in its authentic form,[16] it probably contributes to the maintenance and/or regulation of cardiovascular homeostasis. Salusin-α, on the other hand, may also reduce blood pressure, but the magnitude is far less: the salusin-β-like systemic hemodynamic effects are not evident.[19]

In contrast to the systemic actions, the two salusins exert a variety of local activities in the cardiovascular systems. In isolated rat cardiomyocytes, salusins promote hypertrophy,[32] whereas salusin-β inhibits L-type calcium currents in accordance with its negative inotropic effect.[17] Salusins also stimulate the proliferation of vascular smooth muscle cells and fibroblasts,[19] and protect against apoptotic death of cardiomyocytes.[31] Despite the similar *in vitro* effects of salusin-α and salusin-β on mitogenesis and antiapoptosis, they exert contrasting effects on atherosclerotic lesion development in apolipoprotein E-knockout mice.[5] These remarkable effects are probably mediated by the modulation of acyl-coenzyme A: cholesterol acyltransferase-1 (ACAT-1) expression and subsequent macrophage foam cell formation, which are also induced by salusins in an opposing manner.[28] Salusin-α not only inhibits ACAT1 expression and macrophage foam cell formation but it also reduces serum total cholesterol levels, thereby exerting antiatherosclerotic activities.[5] These results, together with the clinical observation that decreased serum salusin-α levels are associated with atherosclerosis in humans, leads to the hypothesis that salusin-α protects against the development of atherosclerosis.[30]

PATHOPHYSIOLOGICAL IMPLICATIONS

As delineated above, recent studies have exploited the evidence that salusin-α participates in the pathophysiology of cardiovascular diseases, presumably as a result of its potent antiatherosclerotic effects. Decreases in serum salusin-α levels occur in human cardiovascular diseases, such as hypertension[30] and chronic renal failure.[4] The decrease in essential hypertension is associated with the development of carotid atherosclerosis[30] and left ventricular diastolic dysfunction,[26] whereas in patients with coronary arteriography-proven acute coronary syndrome, the magnitude of serum salusin-α decrease is markedly exaggerated in accordance with the severity of coronary artery lesions.[28] Together with the potent antiatherosclerotic activities of salusin-α,[5] a predictive value of low serum salusin-α for hidden coronary artery diseases has been proposed.[29] However, a recent report shows that the negative correlation between serum salusin-α and common carotid intima-media thickness is lost in the presence of inflammatory diseases, such as rheumatoid arthritis.[11] Chronic renal insufficiency decreases serum salusin-α, but other conditions affecting serum salusin-α remain largely uninvestigated.[4]

Salusin-β, however, is implicated in the regulation of hemodynamics and atherosclerosis in the pathophysiology of cardiovascular diseases. Salusin-β exerts its hypotensive and bradycardic actions by parasympathetic stimulation.[3] Salusin-β that is biosynthesized in the neuroendocrine system may be released into the systemic circulation from the posterior pituitary, but the exact contribution of pituitary-derived salusin-β to circulating salusin-β levels remains unknown. Pituitary-derived salusin-β also acts on adjacent neurohypophyseal cells to stimulate the release of vasopressin and oxytocin. Thus, salusin-β is involved in the hemodynamic regulation not only by acting as an endogenous humoral factor to directly modulate the parasympathetic nervous system but also as an autocrine/paracrine factor involved in the regulation of water diuresis by regulating the secretion of vasopressin. However, a recently established radioimmunoassay to detect human salusin-β was not sensitive enough to directly determine its circulating levels. Because salusin-β in the blood could bind to common circulating proteins, exact roles for free and bound salusin-β need to be determined to elucidate its pathophysiological roles in human cardiovascular diseases.

Finally, human urine contains salusin-α- and salusin-β–like immunoreactivity that are immunologically indistinguishable from their authentic sequences.[15,16] Excreted salusins seem to derive from renal tissues, because renal epithelial cells abundantly express salusin-α- and salusin-β-like immunoreactivity.[19,22] Further, end-stage renal failure patients not on hemodialysis exhibit higher renal salusin-α clearance values exceeding their creatinine clearance.[4,16] These findings show that urinary salusin-α is, at least in part, of renal origin.

REFERENCES

1. Eipper BA, Milgram SL, Husten EJ, Yun HY, Mains RE. Peptidylglycine alpha-amidating monooxygenase: a multifunctional protein with catalytic, processing, and routing domains. *Protein Sci* 1993;**2**:489–97.
2. Gardiner SM, Keyte J, Bennett T. Reply to Salusins: newly identified bioactive peptides with hemodynamic and mitogenic activities. *Nat Med* 2007;**13**:661:author reply -2.
3. Izumiyama H, Tanaka H, Egi K, Sunamori M, Hirata Y, Shichiri M. Synthetic salusins as cardiac depressors in rat. *Hypertension* 2005;**45**:419–25.
4. Kimoto S, Sato K, Watanabe T, Suguro T, Koyama T, Shichiri M. Serum levels and urinary excretion of salusin-α in renal insufficiency. *Regul Pept* 2010;**162**:129–32.
5. Nagashima M, Watanabe T, Shiraishi Y, Morita R, Terasaki M, Arita S, et al. Chronic infusion of salusin-α and -β exerts opposite effects on atherosclerotic lesion development in apolipoprotein E-deficient mice. *Atherosclerosis* 2010;**212**:70–7.

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6. Nakai K, Horton P. PSORT: a program for detecting sorting signals in proteins and predicting their subcellular localization. *Trends Biochem Sci* 1999;**24**:34–6.
7. Nakayama C, Shichiri M, Sato K, Hirata Y. Expression of prosalusin in human neuroblastoma cells. *Peptides* 2009;**30**:1362–7.
8. Ota T, Nishikawa T, Salamov A, Nakai K, Kawai Y, Ishii S, et al. High throughput screening of genes encoding secreted and membrane proteins in silico from full length-enriched cDNA libraries. *Microb Comp Genomics* 1988;**C**:87.
9. Ozelius LJ, Hewett JW, Page CE, Bressman SB, Kramer PL, Shalish C, et al. The early-onset torsion dystonia gene (DYT1) encodes an ATP-binding protein. *Nat Genet* 1997;**17**:40–8.
10. Ozelius LJ, Page CE, Klein C, Hewett JW, Mineta M, Leung J, et al. The TOR1A (DYT1) gene family and its role in early onset torsion dystonia. *Genomics* 1999;**62**:377–84.
11. Ozgen M, Koca SS, Dagli N, Balin M, Ustundag B, Isik A. Serum salusin-alpha level in rheumatoid arthritis. *Regul Pept* 2010;**167**:125–8.
12. Saito T, Dayanithi G, Saito J, Onaka T, Urabe T, Watanabe TX, et al. Chronic osmotic stimuli increase salusin-β-like immunoreactivity in the rat hypothalamo-neurohypophyseal system: possible involvement of salusin-beta on $[Ca^{2+}]_i$ increase and neurohypophyseal hormone release from the axon terminals. *J Neuroendocrinol* 2008;**20**:207–19.
13. Salamov AA, Nishikawa T, Swindells MB. Assessing protein coding region integrity in cDNA sequencing projects. *Bioinformatics* 1998;**14**:384–90.
14. Sato K, Koyama T, Shichiri M. Biosynthesis and secretion of salusin-α from human cells. *Peptides* 2008;**29**:2203–7.
15. Sato K, Koyama T, Tateno T, Hirata Y, Shichiri M. Presence of immunoreactive salusin-α in human serum and urine. *Peptides* 2006;**27**:2561–6.
16. Sato K, Sato T, Susumu T, Koyama T, Shichiri M. Presence of immunoreactive salusin-β in human plasma and urine. *Regul Pept* 2009;**158**:63–7.
17. Shi JS, Li D, Li N, Lin L, Yang YJ, Tang Y, et al. Inhibition of L-type calcium currents by salusin-β in rat cardiac ventricular myocytes. *Peptides* 2010;**31**:1146–9.
18. Shichiri M. Reply to Salusins; newly identified bioactive peptides with hemodynamic and mitogenic activities. *Nat Med* 2007;**13**:661–2.
19. Shichiri M, Ishimaru S, Ota T, Nishikawa T, Isogai T, Hirata Y. Salusins: newly identified bioactive peptides with hemodynamic and mitogenic activities. *Nat Med* 2003;**9**:1166–72.
20. Southey BR, Rodriguez-Zas SL, Sweedler JV. Characterization of the prohormone complement in cattle using genomic libraries and cleavage prediction approaches. *BMC Genomics* 2009;**10**:228.
21. Steiner DF. The proprotein convertases. *Curr Opin Chem Biol* 1998;**2**:31–9.
22. Suzuki N, Shichiri M, Akashi T, Sato K, Sakurada M, Hirono Y, et al. Systemic distribution of salusin expression in the rat. *Hypertens Res* 2007;**30**:1255–62.
23. Suzuki N, Shichiri M, Tateno T, Sato K, Hirata Y. Distinct systemic distribution of salusin-α and salusin-β in the rat. *Peptides* 2011;**32**:805–10.
24. Suzuki Y, Yoshitomo NK, Maruyama K, Suyama A, Sugano S. Construction and characterization of a full length-enriched and a 5′-end-enriched cDNA library. *Gene* 1997;**200**:149–56.
25. Takenoya F, Hori T, Kageyama H, Funahashi H, Takeuchi M, Kitamura Y, et al. Coexistence of salusin and vasopressin in the rat hypothalamo–hypophyseal system. *Neurosci Lett* 2005;**385**:110–3.
26. Ti Y, Wang F, Wang ZH, Wang XL, Zhang W, Zhang Y, et al. Associations of serum salusin-α levels with atherosclerosis and left ventricular diastolic dysfunction in essential hypertension. *J Hum Hypertens* 2011.
27. Wang Z, Takahashi T, Saito Y, Nagasaki H, Ly NK, Nothacker HP, et al. Salusin-β is a surrogate ligand of the mas-like G protein-coupled receptor MrgA1. *Eur J Pharmacol* 2006;**539**:145–50.
28. Watanabe T, Nishio K, Kanome T, Matsuyama TA, Koba S, Sakai T, et al. Impact of salusin-α and -β on human macrophage foam cell formation and coronary atherosclerosis. *Circulation* 2008;**117**:638–48.
29. Watanabe T, Sato K, Itoh F, Iso Y, Nagashima M, Hirano T, et al. The roles of salusins in atherosclerosis and related cardiovascular diseases. *J Am Soc Hypertens* 2011;**5**:359–65.
30. Watanabe T, Suguro T, Sato K, Koyama T, Nagashima M, Kodate S, et al. Serum salusin-α levels are decreased and correlated negatively with carotid atherosclerosis in essential hypertensive patients. *Hypertens Res* 2008;**31**:463–8.
31. Xiao-Hong Y, Li L, Yan-Xia P, Hong L, Wei-Fang R, Yan L, et al. Salusins protect neonatal rat cardiomyocytes from serum deprivation-induced cell death through upregulation of GRP78. *J Cardiovasc Pharmacol* 2006;**48**:41–6.
32. Yu F, Zhao J, Yang J, Gen B, Wang S, Feng X, et al. Salusins promote cardiomyocyte growth but does not affect cardiac function in rats. *Regul Pept* 2004;**122**:191–7.
33. Yudate HT, Suwa M, Irie R, Matsui H, Nishikawa T, Nakamura Y, et al. HUNT: launch of a full-length cDNA database from the helix research institute. *Nucleic Acids Res* 2001;**29**:185–8.

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Chapter 194

Urocortins

Mark Richards and Miriam Rademaker

ABSTRACT

Since the discovery of Urocortin 1 (Ucn 1) in 1995 and identification of Ucns 2 and 3 in 2001, evidence of a cardiovascular role for these paralogs of corticotropin-releasing hormone (CRH) has grown rapidly. Ucns act through CRH-R2 G-protein-coupled receptors distributed widely in organs relevant to pressure and volume homeostasis including the heart, kidneys, adrenals, and vasculature. Effects occur via multiple signaling cascades including adenylyl cyclase/cAMP and MAP kinase pathways. Ucns lower blood pressure in animal models of hypertension and are cardioprotective in experimental ischemia reperfusion. Plasma concentrations of Ucn 1 are elevated in heart failure. Ucns 1, 2, and 3 exert beneficial hemodynamic, neurohormonal, and renal effects in experimental heart failure, and Ucn 2 has hemodynamic efficacy in human heart failure. Further research on the Ucns promises improved understanding of the pathophysiology of ischemia-reperfusion injury, hypertension, and heart failure with the attendant potential for novel therapeutic applications.

DISCOVERY

The Ucns are members of the corticotropin-releasing hormone (CRH) family of peptides, which includes CRH, fish urotensin 1, frog sauvagine, and urocortins 1, 2, and 3. In 1995, Vaughan et al.[32] discovered urotensin 1-like immunoreactivity in rat brain (Edinger–Westphal nucleus and lateral superior olive region). On analysis, the novel peptide concerned was named urocortin (now urocortin 1) in view of its structural and biological similarities to urotensin and CRH. Two further urocortins have been subsequently discovered in 2001 and named urocortin 2 (also known as stresscopin-related peptide) and urocortin 3 (also known as stresscopin). Murine and human cDNAs for Ucns 2 and 3 were identified from sequence homology searches of the respective genomes.[19,29]

STRUCTURE OF UROCORTINS mRNA/GENE

Human CRH and Ucn 1 genes are located on chromosomes 8(8q13) and 2 (2p23–p21) respectively.[13,16] The Ucn 1 gene has two exons with the coding region confined to the second exon. It codes for a 122 residue preprotein with the mature Ucn 1 within the carboxyl terminus. Several possible transcription-factor-binding sites have been identified in the Ucn 1 promoter but not tested for actual promoter function. Regulatory elements include a TATA box, a CCAAT enhancer-binding protein (C/EBP) binding site, GATA binding sites, a POU domain transcription factor Brn-2 and a cyclic adenosine monophosphate (cAMP) responsive element (CRE). Upstream of the CRE, the Ucn 1 promoter contains a glucocorticoid response element (GRE) consistent with the ability of glucocorticoids to upregulate Ucn 1 expression. Genes for Ucn 2 and Ucn 3 reside on chromosomes 3 (3p21.3–4) and 10 (10p15.1) respectively.

PROCESSING OF PRECURSOR PEPTIDES

Mature Ucns are about 40 amino acids in length. The Ucn 1 precursor is a122-amino-acid protein with an N terminal methionine and an amidated carboxy terminal. The amidation is necessary for bioactivity of the mature peptide.

Ucns 2 and 3 are derived from precursor proteins of 112 and 161 residues, respectively. There is some uncertainty about the mature forms of Ucns 2 and 3, and some investigators suggest amino-terminally extended versions of the putative 38 and 40 amino acid candidate forms. In the case of Ucn 2, this reflects possible cleavage at either serine–arginine or threonine–arginine sites with resultant 38- and 45-amino-acid mature forms respectively. For Ucn 3, a dibasic (arginine–arginine) cleavage site present in several species suggests a 40-amino-acid peptide (as initially predicted for Ucn 3 or Stresscopin), but this site is missing in humans, leading to the prediction of cleavage at a threonine–lysine site with production of a 38-amino-acid peptide. As mature Ucn 2 and 3 peptides are yet to be isolated and sequenced, this uncertainty remains for the time being.[13]

The amino acid sequence (primary structure) of Ucn 1 resembles that of CRH as much as it does that of Ucns 2 and 3. It has 63% homology to urotensin 1 and 45% homology to CRH (Table 1). CRH analog peptides have an alpha

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00194-9
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TABLE 1 Amino Acid Sequences for Human CRF and Ucns 1, 2 and 3

C	**R**	**F**		**S**	**E**	**E**	**P**	**P**	**I**	**S**	**L**	**D**	**L**	**T**	**F**	**H**	**L**	**L**	**R**	**E**	**V**	**L**	**E**	**M**	**A**	**R**	**A**	**E**	**Q**	**L**	**A**	**Q**	**Q**	**A**	**H**	**S**	**N**	**R**	**K**	**L**	**M**	**E**	**I**	**I**
U	C	n	1		D	N	P	S	L	S	I	D	L	T	F	H	L	L	R	T	L	L	E	L	A	R	T	O	S	Q	R	E	R	A	E	Q	N	R	I	I	F	D	S	V
U	C	n	2				I	V	L	S	L	D	V	P	I	G	L	L	Q	I	L	L	E	Q	A	R	A	R	A	A	R	E	Q	A	T	T	N	A	R	I	L	A	R	V
U	C	n	3		T	K	F	T	L	S	L	D	V	P	T	N	I	M	N	L	L	F	N	I	A	K	A	K	N	L	R	A	Q	A	A	A	N	A	H	L	M	A	Q	I

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helical conformation with varying amphipathicity, which may be relevant to their selectivity for CRH-R1 but not relevant to CRH-R2 binding. Ucn 2 (34 and 43 and 37–40% homology with CRH, Ucn 1 and Ucn 3, respectively) and Ucn 3 resemble each other more than CRH. It is hypothesized an ancient gene duplication event, predating evolutionary divergence of teleost fish from tetrapods, resulted in separate type 1 (CRH/Ucn 1) and type 2 (Ucn 2/Ucn 3) lineages.[13]

DISTRIBUTION OF UROCORTINS mRNA AND PEPTIDE IN THE CARDIOVASCULAR SYSTEM

The Ucns are widely expressed in the central nervous system, gastrointestinal tract, skeletal muscle, skin, immune system, and the heart.[18,21] Ucn 1 is expressed in vascular smooth muscle cells and cardiomyocytes. Ucn 1 is produced in the heart and stored there in larger molecular weight forms. Ucns 2 and 3 are expressed in the heart. Unlike Ucn 1, Ucns 2 and 3 do not seem to have any role in the regulation of the hypothalamic–pituitary–adrenal axis. Ucn 1 mRNA or immunoreactivity (i.e., peptide) has been found in the central nervous system (CNS) including the cerebellum, hypothalamus, and colocalizing with dopamine in the basal ganglia and hypothalamus. Ucn 2 is present in the tissues of the cardiovascular system (including the heart, peripheral blood vessels, and adrenals) and the central nervous system, stomach, skin, ovary, uterine smooth muscle, and skeletal muscle. Ucn 3 is distributed where CRH-R2 expression is high including the heart, adrenals, and kidneys (distal tubules).

RECEPTORS AND SIGNALING CASCADES

The actions of the Ucns are mediated via the 7 transmembrane, G-protein-coupled CRH receptors, CRH-R1 and CRH-R2 both of which have multiple splice variants.[13,16] Ucn 1 is a ligand for both receptors, but it has higher affinity for CRH-R2. In contrast, Ucns 2 and 3 bind exclusively to CRH-R2. CRH-R1 is found predominantly in the CNS, whereas CRH-R2 receptors appear in the CNS and in peripheral tissues including the heart, adrenals, gut, and lymphocytes. In humans, Ucn 1 and CRH-R2 alpha are present in the aorta and the myocardium and arterioles of all four chambers of the heart. Within the cardiovascular system, CRH-R2 alpha is the relevant receptor mediating Ucn bioactivity.

CRH-Binding Protein

CRH-binding protein (CRH-BP) was isolated from human blood in 1989.[3] This 37-kDa protein binds both CRH and Ucn 1 in humans. Its function seems to be restriction of access of these ligands to the CRH receptors. The half-life of Ucn 1 in sheep is markedly prolonged, and in humans, it exceeds that of Ucns 2 and 3 (~50 min compared with 10 minutes for Ucn 2; the half-life of Ucn 3 remains uncertain) and the binding protein which is selective for Ucn 1 may contribute to this difference in kinetics. In sheep, unpublished data (from Patel et al.) comparing Ucn pharmacokinetics indicate clearances of 0.486, 21.7, and 220 L/h for Ucns 1, 2, and 3 respectively. Steady-state volumes of distribution were similar for Ucn 1 and Ucn 2 (~8 L) but substantially larger for Ucn 3 (23.5 L). Disposition was adequately described by a two-compartment model for Ucn 1 and one-compartment models for Ucn 2 and Ucn 3. Elimination half-life for Ucn 1 was 8.3 h (α phase) and 2.9 h (β phase), and 15.7 and 4.4 min for Ucn 2 and Ucn 3, respectively.

Signaling Cascades

The cardioprotective effects of Ucns are CRH-R2-dependent and seem to involve multiple signal transduction pathways.[13] Generally, in most *in vitro* settings, this involves adenylyl cyclase and the second messenger cyclic AMP. Relaxation of VSMC via CRH-R2 is associated with adenyl cyclase activation and accumulation of cyclic AMP.[17] Ucn 1 may modulate cardiac hypertrophy at least in part through cyclic AMP.

Ucn 1-, 2-, and 3- induced cardioprotection involves MAP kinase signaling, as each peptide induces phosphorylation of Erk1/2-p42, 44 in neonatal cardiomyocytes and reduces postreperfusion infarct size in rodents via CRH-R2-dependent mechanisms.[31] These effects are abolished by inhibitors of MEK1, Ras, or Raf-1. Ucn 1 increases expression and activation of protein kinase C epsilon in primary cultures of rat cardiomyocytes and in the isolated perfused heart. The antiapoptotic effects of Ucn 1 on isolated cardiomyocytes subjected to ischemia reperfusion seems to be dependent on the presence of this PKC isozyme, and Ucn-1-induced protection of isolated whole hearts was absent in PKC-epsilon-deficient mice. Further, chelerythrine, a PKC inhibitor, prevented Ucn-1-related cardioprotection of the isolated rat heart after ischemia reperfusion.

In vitro (isolated cardiomyocytes) Ucn 1 inhibits ischemia-induced increases in the calcium-insensitive phospholipase A2 enzyme (iPLA2) and its toxic metabolite lysophosphatidylcholine.

Ucn 1 increases synthesis of the mitochondrial KATP potassium channel subunit Kir 6.1 in cardiomyocytes. General and mitochondrial-specific KATP channel blockers abolish the cardioprotective effects of Ucn 1 in isolated cardiac cells and in the intact heart. Finally, cardioprotection from Ucns also involves activation of the phosphatidylinositol-3 (PI-3) kinase/Akt-dependent pathway.[13]

Barry et al.[2] performed microarray experiments to provide an expanded view of molecular mechanisms potentially underlying the protection offered by Ucns in myocardial

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ischemia reperfusion. In a rat coronary occlusion model of cardiac ischemia-reperfusion injury, Ucn 1 and Ucn 2 administered before reperfusion, differentially regulated 66 and 141 genes, respectively. Most of these effects on gene expression had not been previously described. Functional analysis demonstrated that Ucn-regulated genes are involved in many biological responses, including cell death, oxidative stress (e.g., nuclear factor erythroid derived 2-related factor 1/nuclear factor erythroid derived 2-like 1) and metabolism (e.g., Prkaa2/AMPK). Both Ucn 1 and Ucn 2 modulate expression of many genes involved in G-protein-coupled receptor (GPCR) signaling including Rac2, Gnb1, Dab2ip (AIP1), Ralgds, Rnd3, Rap1a, and PKA, indicating involvement of many previously unrecognized signaling intermediates downstream of CRH receptors. Moreover, several of these GPCR-related genes are involved in MAP kinase activation, further confirming links between CRH receptors and induction of MAPKs.

BIOLOGICAL ACTIONS WITHIN THE CARDIOVASCULAR SYSTEM

Ucns 1, 2, and 3 cause vasodilation and have positive chronotropic, inotropic, and lusitropic effects on the heart. Ucns lower blood pressure in hypertensive animal models. Ucns have protective effects in cardiac ischemia reperfusion and are anti-inflammatory. Finally, Ucns have powerful multifaceted beneficial effects in heart failure.

Blood Pressure Regulation

Although central administration of CRH in rats induces a pressor effect mediated through CRH-R1 receptors, peripheral CRH lowers blood pressure, an effect blocked by a nonspecific CRH antagonist but not by a selective CRH-R1 receptor antagonist indicating probable mediation of the peripheral depressor effect via CRH-R2 receptors. In genetically modified mice lacking CRH-R2 receptors, the marked hemodynamic effects of infused Ucns are lost indicating dependence on this receptor.[8] In this knockout model, the mice are hypertensive implying a role for the Ucns in regulation of basal vascular tone and arterial pressure; raising the possibility that the same is true in humans. A similar vasodilation and decrease in blood pressure is observed on intravenous administration of CRH to rats. Both Ucn 1 and 2 cause sustained lowering of blood pressure in rats when given intravenously. Ucn 2 produces substantial and sustained falls in blood pressure in hypertensive rat models.[11,15,20] Ucn 2 given twice daily to Dahl-salt-sensitive rats reduces blood pressure and diminishes left ventricular hypertrophy over five weeks of treatment.[11] Isolated perfused hearts from Dahl rats respond to perfusion with Ucn 2 with changes in intracellular calcium cycling, improved left ventricular systolic and diastolic function, and a raised ventricular fibrillation threshold.[15] In the chromogranin A-null mouse, characterized by raised catecholamine production and hyperadrenergic hypertension, Ucn 2 reduced blood pressure in association with reduced catecholamine synthesis and secretion.[20]

Ucn-induced decreases in blood pressure may involve both direct effects on VSMC tone and secondary effects via reduction in vasoconstrictor systems including endothelin 1 (ET-1), arginine vasopressin, and angiotensin II (AngII). Direct vascular actions seem to depend on both endothelium-dependent and independent mechanisms. Studies in isolated human internal mammary arteries and rat coronary arteries indicate endothelium-dependent Ucn 1-induced dilation involves both nitric oxide (via second messenger cGMP) and barium sensitive potassium channels. Vasodilation is blunted but not abolished in endothelium-denuded vessel preparations and both calcium independent phospholipase A1 and store-operated calcium flux have been invoked in this setting.[33]

Urocortins and Endothelial Function

Results on the effect of Ucn on the endothelium generally favor a protective role for this peptide family with respect to this crucial vasoregulatory organ.[14] The endothelium has important roles in regulation of vessel tone, blood pressure, and vascular remodeling. Endothelial dysfunction (ED) seems to be a universal precursor and accompaniment to atherosclerosis being associated with common vasculopathic factors such as hypertension, dyslipidemia, and smoking. Endothelial cells (EC) generate numerous autacoids, including NO, prostacyclin (PGI2), endothelium-derived hyperpolarizing factor (EDHF), bradykinin (BK), adrenomedullin, C-type natriuretic peptide, ET-1, AngII, thromboxane A2 (TXA2), hydrogen peroxide (H2O2), and free radicals. Hence, endothelium hosts both vasoconstrictor and vasodilator substances, which encompass trophic and antiproliferative, prothrombotic and thrombolytic, oxidative, and antioxidant effects. NO synthesized and released from EC is a fundamental vasodilator determinant of vascular tone. It also inhibits proinflammatory cytokines and chemokines, leukocyte adhesion molecules, proliferation of vascular smooth muscle cells (VSMCs), activation and aggregation of platelets, and the production of tissue factor. In addition to NO, ECs also releases the vasodilator prostaglandin, PGI2 in response to shear stress and hypoxia. Endothelium-derived vasoconstrictors include ET-1, the vasoconstrictor prostanoids TXA2 and PGH2, and AngII. AngII facilitates superoxide anion production by activation of vascular NADH/NADPH oxidase. Where do the Ucns fit in this delicate and complex meshwork of factors that

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modulate endothelial function? It has been reported that the rat aortic ECs predominantly express CRH-R2b. The partially endothelium-dependent effects of Ucns on blood pressure and vascular tone have been mentioned already. Hence, Ucns exert part of their vascular effect via endothelial mediators. Ucn seems to enhance endothelial responses in several experimental settings. Ucn increases the coronary vasodilator response to acetylcholine when given either before ischemia or during reperfusion. Ucn 2 induces nitric oxide production via cAMP and calcium-related pathways in endothelial cells.[14] In studies of ischemia-reperfusion injury in isolated rat hearts, Ucn enhances synthesis of NO and decreases the serum superoxide dismutase (SOD) activity. Rademaker et al.[22–24] reported endogenous and exogenous Ucn reduce plasma AngII and ET-1 concentrations in ovine experimental heart failure. Ucns may also have antitrophic effects with an antiproliferative effect on cultured ECs. Concordant with this, mice deficient for CRH-R2 become hypervascularized.[1] Ucn treatment inhibits the formation of capillary-like tubes in three-dimensional collagen gels containing rat aortic ECs. Together, these findings support a protective role for Ucns with respect to endothelium-mediated vasodilator function and suggest Ucns inhibit vascular hypertrophy.

However, CRH and Ucn have exhibited proinflammatory effects in some settings. Ucn induces CRH-R2-dependent mast cell degranulation with abnormal increases in vascular permeability.[30] *In vitro* Ucns may also stimulate interleukin-1β (IL-1β) and interleukin-6 (IL-6) secretion. It can be speculated that such effects may trigger ECs to switch phenotype from their normal net antithrombotic character to a procoagulant state. Further, CRH-related peptides can abolish IL-1α-induced synthesis of PGI2 in bovine aortic ECs.

The endothelium seems to be an important target tissue for Ucns. Although in specific pathological settings Ucn actions may be deleterious, the weight of evidence favors an endothelial-protective role for Ucns in health and in most cardiovascular pathologies.

Urocortins and Cardiac Sympathetic Traffic

Intravenous administration of Ucns 1, 2, and 3 in conscious, intact sheep reduces cardiac sympathetic efferent traffic[6,7] as shown by direct recordings of sympathetic burst activity via electrodes implanted in the cardiac sympathetic efferent nerves in sheep. This unique biological action of the Ucns is apparent at doses of Ucn below the threshold inducing readily recorded changes in blood pressure. The effect remains evident at doses sufficient to lower arterial pressure and increase heart rate. This implies that the effects on heart rate do not reflect baroceptor responses mediated by the sympathetic afferent-efferent loop but are due to direct effects of Ucn on heart rate. This sympatholytic mechanism may be a further contributor to the blood pressure lowering effect of Ucns. Although not yet tested, if suppression of sympathetic traffic extends to renal sympathetic drive this may also contribute to reductions in renal vascular resistance, enhanced renal filtration, and Ucn-associated suppression of renin production, as observed in ovine experimental heart failure. It also raises the possibility of specific therapeutic benefit from Ucns in cardiac injury and/or heart failure, conditions in which sympathetic overdrive is both proarrhythmic and contributes to progressive cardiac dysfunction. The intracellular signaling pathways mediating the antisympathetic effect of the Ucns are as yet unknown.

Cardiac Actions of the Urocortins

The Ucns have direct CRH-R2-mediated positive effects on cardiomyocyte contractility as observed on application of Ucn 2 to isolated rabbit ventricular cardiomyocytes. Intracellular pathways induced by Ucn in this setting include increased PKA activity and subsequent L-type calcium channel and sarcoplasmic and endoplasmic reticulum calcium ATPase (SERCA)-mediated calcium uptake into the sarcoplasmic reticulum.[34] Ucn 2 injected into intact rats induced immediate onset of subsequently long-sustained enhancement of left ventricular shortening. Ucn 1 injection in sheep caused dose-dependent increases in cardiac output and dp/dt (index of contractility) lasting to 24 h after a single bolus intravenous dose.[22]

Urocortin Effects in Models of Ischemia-reperfusion Injury

Ucn 1 precursor is found in rat neonatal cardiomyocytes and is released from cells exposed to hypoxia. *In vitro* secretion is also triggered by inflammatory cytokines including interleukins 1 and 6 and tumor necrosis factor, local and circulating factors increased in cardiac ischemia and in heart failure. These findings raise the possibility of local, direct cardiac actions of Ucns mediated by cardiac CRH-R2 receptors. Ucn 1 reduces cardiomyocyte death in ischemia-reperfusion injury.[5] Ucns are protective both *in vitro* when added to postischemic/hypoxic cardiomyocytes and to isolated intact hearts during postischemic reperfusion. Ucn 1 enhances hemodynamic and bioenergetic recovery of isolated, paced rat hearts with improved ventricular performance postischemia reperfusion. Ucn 2 and Ucn 3 reduce infarct size in isolated rat hearts postischemia reperfusion.[4] Furthermore, sustained treatment with Ucn 2 in a mouse coronary ligation model of myocardial infarction protects against adverse postinfarction left ventricular remodeling and sustains cardiac function in the intact mouse.[12]

Urocortins in Experimental Heart Failure

Since 2002 the Christchurch Cardioendocrine research group has provided a series of pivotal studies documenting

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the effects of Ucns 1, 2, and 3 in experimental ovine heart failure.[22–28] This group has also provided the only results available to date on the bioactivity of exogenous Ucns administered to both normal volunteers and to patients with stable heart failure.[9,10] All 3 Ucns produce a salutary array of powerful hemodynamic, neurohormonal, and renal effects in an ovine model of severe heart failure induced by chronic overpacing (Figure 1). This model in an intact, conscious large animal is one of the few which provides a faithful analog of many aspects of severe human heart failure and permits the observation of developing and resolving heart failure over periods of weeks with serial measurement of key hemodynamic, hormonal, and renal variables. In this model bolus intravenous doses of 25–100 μg of Ucns 1, 2, and 3 all markedly augment cardiac output, lower cardiac filling pressures to near normal levels and reduce systemic vascular resistance with relatively modest falls in arterial pressure.[22,23,25] These salutary hemodynamic effects are accompanied by significant falls in abnormally high levels of neurohormones including suppression of renin, AngII, aldosterone, ET-1, and AVP. The hemodynamic relief provided from Ucns leads to reduced plasma ANP and BNP plasma concentrations in spite of the known direct actions of Ucns stimulating release of natriuretic peptides *in vitro*. Markedly reduced renal filtration and avid antinatriuresis, both consistent features of this model are essentially returned to normal by administration of Ucns. This beneficial profile of effects is sustained over 4 days of dosing with Ucn 1 or Ucn 2.[24] Administration of Ucn from the outset of pacing stress markedly delays onset of heart failure in this model and a role for endogenous Ucns as a powerful beneficial compensatory response in heart failure is further supported by the exacerbation of hemodynamic, neurohormonal, and renal abnormalities in this model on administration of the Ucn blocker CRH[9–41].[26] As any new potential therapy in heart failure will probably function in addition to existing proven treatments, the combination of Ucn with diuretics, angiotensin-converting enzyme inhibitors, and beta adrenoceptor blockers (all mainstay therapy in clinical management of human heart failure) have all been studied in the ovine model.[27,28] Without exception the combinations have resulted in enhancement of beneficial hemodynamic effects compared with either agent alone together with blunting of undesirable effects of current therapies. The combination of Ucn 2 with the loop diuretic frusemide enhanced renal responses with less renin activation and no exacerbation of potassium loss or additional fall in arterial blood pressure.[27] Ucn 2 together with angiotensin-converting enzyme inhibitors, enhanced reductions in aldosterone, reduced increases in renin, enhanced increases in cardiac output, and further lowered systemic resistance without further fall in blood pressure.[28] Ucn 2 combined with beta blockade prevented the initial falls in cardiac output and renal function attendant upon introducing beta blockade in severe heart failure, in association with significantly enhanced suppression of plasma aldosterone.

Concordant with the extensive findings in the ovine model of heart failure, Ucn 2 infusion in a mouse model of dilated cardiomyopathy, because of deficiency of muscle specific LIM protein, markedly improves cardiac output and left ventricular function.[33]

Urocortins in Human Heart Failure

Plasma concentrations of Ucn 1 in human heart failure have been studied and reported by two groups with somewhat discrepant findings.[33] Ng et al developed an immunoassay for Ucn 1 and reported that levels are increased overall in human heart failure but puzzlingly exhibit a direct rather than inverse relationship with left ventricular ejection fraction and that although levels are elevated in milder heart failure (NYHA functional classes I and II) they tend to fall in more severely symptomatic heart failure. By contrast, Wright et al. using a separate locally developed radioimmunoassay reported elevated concentrations in heart failure with continuing increments in plasma concentrations with worsening functional class, an inverse rather than direct relationship with LVEF and significant direct correlations with other circulating markers of severity in heart failure including plasma BNP.[33] Plasma Ucn 1 concentrations are an independent predictor of a final diagnosis of heart failure in patients presenting to their family doctors with new onset of peripheral edema and/or breathlessness on exertion. Plasma Ucn 1 underperformed NTproBNP as a potential individual diagnostic marker for heart failure but, in combination with NTproBNP levels, it enhanced risk stratification for this diagnosis. Using the assay from Wright et al., regional blood sampling was undertaken in the course of cardiac catheterization procedures, in patients without acute disease and normal cardiac function, undergoing clinically indicated coronary angiography. These studies in 25 patients indicate net production of Ucn 1 from heart, kidney, liver, head, and neck, and musculoskeletal tissue (leg) but not from the pulmonary circulation. This suggests widespread synthesis and release of Ucn 1 concordant with the wide distribution of tissue immunoreactivity in preclinical *ex vivo* studies.

Biological Actions of Urocortins in Humans

Controlled short-term studies of the hemodynamic, hormonal, and renal effects of Ucns 1 and 2, administered at total doses between 25 and 100 μg, have been undertaken in both normal volunteers and patients with stable heart failure.[9,10]

Ucn 1 studies reveal little major effect at these doses. Modest increments in ACTH and cortisol are observed in normal but not in heart failure subjects with no significant hemodynamic or renal effects.

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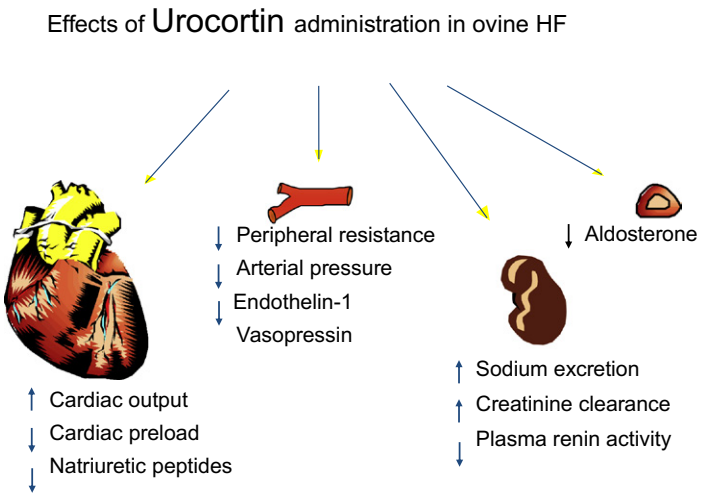

FIGURE 1 Biological actions of urocortins in experimental heart failure.

Both doses of Ucn 2 induce clear increases in cardiac output in both normal and heart failure groups.[9,10] This effect is associated with decreases in systemic vascular resistance that are sustained out to 3 and 6 h postdose for 25 and 100 μg doses, respectively, in heart failure. At the conclusion of the 1 h Ucn 2 infusions, cardiac ultrasound indicates decreases in left ventricular dimensions and a dose-related increase in LVEF in the heart failure group.[10] Calculated cardiac work is reduced by Ucn 2. Renal and neurohormonal effects are only minor, which is notable in that there is no renin or catecholamine rise despite significant falls in blood pressure. However, this latter finding contrasts with the accumulated results from the ovine model of severe untreated heart failure in which the neurohormonal and renal effects of administered Ucns are profound. Such effects in normal sheep are markedly blunted compared with sheep in heart failure, suggesting the full expression of Ucn-induced renal and neurohormone effects requires the markedly activated and abnormal milieu present in severe decompensated heart failure. Heart failure patients recruited to the Ucn studies to date have been stable on long-term therapy and only mildly symptomatic (despite a clear history of previous frank cardiac decompensation and clearly abnormal cardiac function). In this treated stable state, Ucns may not elicit substantial effects on kidney function or hormone levels acutely. However, the lack of neurohormonal response to the hypotensive effects of Ucn 2 is notable. At present, a clinical trial of 4-h infusions of Ucn 2 in recently decompensated heart failure is in progress and may answer some of these questions.

PATHOPHYSIOLOGICAL AND THERAPEUTIC IMPLICATIONS

Ucns have a significant role in the regulation of the heart and circulation in disease states. Their roles in normal health are more subtle although CRH-R2 knockout mice are hypertensive. Ucns are produced in a wide array of tissues and act via receptors that are widely distributed in the cardiovascular system and other organs with key functions in circulatory regulation including the kidneys and adrenal glands. The signaling pathways mediating Ucn's actions are multiple and complex suggesting the potential for tissue- and disease-specific derangements of local and systemic Ucn function with attendant potential multiple points at which Ucn signaling and bioactivity might be manipulated for therapeutic purposes.

Notable cardiovascular effects include vasodilation with lowering of blood pressure. In hypertensive animal models, Ucn 2 is a powerful blood pressure lowering agent. Ucns exert positive inotropic, chronotropic, and lusitropic effects on cardiac ventricular function. The most compelling results on the endogenous compensatory role of Ucns are found in the setting of heart failure. In both experimental and mild human heart failure, Ucns display powerful beneficial effects on the hemodynamic status. Despite the implicit therapeutic potential of the salutary beneficial effects of Ucns 1, 2, and 3 on neurohormones and renal function observed in an ovine model of severe heart failure, these are yet to be reproduced in the clinical setting. Further conclusions and developments await outcomes of exploratory trials in patients with recently decompensated heart failure.

ACKNOWLEDGMENTS

The National Heart Foundation of New Zealand and the Health Research Council of New Zealand supported research by the Christchurch Cardioendocrine Research Group University of Otago Christchurch as reported in this review (references with first authors Charles, Rademaker and Davis).

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REFERENCES

1. Bale TL, Giordano FJ, Hickey RP, Huang Y, Nath AK, Peterson KL, et al. Corticotropin-releasing factor receptor 2 is a tonic suppressor of vascularization. *Proc Natl Acad Sci USA* 2002;**99**:7734–9.
2. Barry SP, Lawrence KM, McCormick J, Soond SM, Hubank M, Eaton S, et al. New targets of urocortin-mediated cardioprotection. *J Mol Endocrinol* 2010;**45**:69–85.
3. Behan DP, Linton EA, Lowry PJ. Isolation of the human plasma corticotrophin-releasing factor-binding protein. *J Endocrinol* 1989;**122**:23–31.
4. Brar B, Jonassen A, Egorina E, Chen A, Negro A, Perrin M, et al. Urocortin-II and urocortin-III are cardioprotective against ischemia reperfusion injury: an essential endogenous cardioprotective role for corticotropin releasing factor receptor type 2 in the murine heart. *Endocrinology* 2004;**145**:24–35.
5. Brar BK, Stephanou A, Okosi A, Lawrence KM, Knight RA, Marber MS, et al. CRH like peptides protect cardiac myocytes from lethal ischaemic injury. *Mol Cell Endocrinol* 1999;**158**:55–63.
6. Charles CJ, Jardine DL, Nicholls MG, Rademaker MT, Richards AM. Urocortin 1 exhibits potent inhibition of cardiac sympathetic nerve activity in conscious sheep. *J Hypertens* 2008;**26**:53–60.
7. Charles CJ, Jardine DL, Rademaker MT, Richards AM. Urocortin 3 inhibits cardiac sympathetic nerve activity in conscious sheep. *J Cardiovasc Pharmacol* 2011;**58**:25–31.
8. Coste SC, Kesterson RA, Heldwein KA, Stevens SL, Heard AD, Hollis JH, et al. Abnormal adaptations to stress and impaired cardiovascular function in mice lacking corticotropin-releasing hormone receptor-2. *Nat Genet* 2000;**24**:403–9.
9. Davis ME, Pemberton CJ, Yandle TG, Fisher SF, Lainchbury JG, Frampton CM, et al. Urocortin 2 infusion in healthy humans: haemodynamics, neurohormonal and renal responses. *J Am Coll Cardiol* 2007;**49**:461–71.
10. Davis ME, Pemberton CP, Yandle TG, Fisher SF, Lainchbury JG, Frampton CM, et al. Urocortin 2 infusion in human heart failure. *Eur Heart J* 2007;**28**:2589–97.
11. Dieterle T, Meili-Butz S, Bühler K, Morandi C, John D, Buser PT, et al. Immediate and sustained blood pressure lowering by urocortin 2 A novel approach to antihypertensive therapy? *Hypertension* 2009;**53**:739–44.
12. Ellmers L, Scott N, Cameron V, Richards M, Rademaker M. Chronic urocortin2 treatment significantly improves cardiovascular function and attenuates cardiac injury and remodeling after experimental myocardial infarction (Abstr 117). Abstracts for the 58th Scientific Meeting of the Cardiac Society of Australia and New Zealand *Heart Lung Circulation* 2010;**19**(Suppl. 2):s49.
13. Fekete EM, Zorrilla EP. Physiology, pharmacology, and therapeutic relevance of urocortins in mammals: ancient CRF paralogs. *Front Neuroendocrinol* 2007;**28**:1–27.
14. Grossini E, Molinari C, Mary DASG, Uberti F, Ribichini F, Caimmi PP, et al. Urocortin II induces nitric oxide production through cAMP and Ca2+ related pathways in endothelial cells. *Anni Vacca Cell Physiol Biochem* 2009;**23**:87–96.
15. Gu Y, Zhang K, Biswas N, Friese RS, Lin DH, Mahata SK, et al. Urocortin2 lowers blood pressure and reduces catecholamine levels in mice with hyperadrenergic activity. *Endocrinology* 2010;**151**:4820–9.
16. Hashimoto K, Nishiyama M, Tanaka Y, Noguchi T, Asaba K, Hossein PN, et al. Urocortins and corticotropin releasing factor type 2 receptors in the hypothalamus and the cardiovascular system. *Peptides* 2004;**25**:1711–21.
17. Kageyama K, Furukawa K-I, Miki I, Terui K, Motomura S, Suda T. Vasodilative effects of urocortin II via protein kinase A and a mitogen-activated protein kinase in rat thoracic aorta. *J Cardiovasc Pharmacol* 2003;**42**:561–5.
18. Kimura Y, Takahashi K, Totsune K, Muramatsu Y, Kaneko C, Darnel AD, et al. Expression of urocortin and corticotropin-releasing factor receptor subtypes in the human heart. *J Clin Endocrinol Metab* 2002;**87**:340–6.
19. Lewis K, Li C, Perrin MH, Blount A, Kunitake K, Donaldson C, et al. Identification of urocortin III, an additional member of the corticotropin-releasing factor (CRF) family with high affinity for the CRF2 receptor. *Proc Natl Acad Sci USA* 2001;**98**:7570–5.
20. Meili-Butz S, Buhler K, John D, Buser P, Vale WW, Peterson KL, et al. Acute effects of urocortin 2 on cardiac function and propensity for arrhythmias in an animal model of hypertension-induced left ventricular hypertrophy and heart failure. *Eur J Heart Failure* 2010;**121**:797–804.
21. Nishikimi T, Miyata A, Horio T, Yoshihara F, Nagaya S, Takishita S, et al. Urocortin, a member of the corticotropin-releasing factor family, in normal and diseased heart. *Am J Physiol Heart Circ Physiol* 2000;**279**:H3031–9.
22. Rademaker MT, Cameron VA, Charles CJ, Richards AM. Integrated hemodynamic, hormonal and renal actions of urocortin 2 in normal and paced sheep: beneficial effects in heart failure. *Circulation* 2005;**112**:3624–32.
23. Rademaker MT, Cameron VA, Charles CJ, Richards AM. Urocortin 3: hemodynamic, hormonal and renal effects in experimental heart failure. *Eur Heart J* 2006;**27**:2088–98.
24. Rademaker MT, Charles CJ, Ellmers LJ, Lewis L, Nicholls MG, Richards AM. Prolonged urocortin 2 administration in experimental heart failure: sustained hemodynamic, endocrine and renal effects. *Hypertension* 2011;**57**:1136–44.
25. Rademaker MT, Charles CJ, Espiner EA, Fisher S, Frampton CM, Kirkpatrick CM, et al. Beneficial hemodynamic, endocrine, and renal effects of urocortin in experimental heart failure: comparison with normal sheep. *J Am Coll Cardiol* 2002;**40**:1495–505.
26. Rademaker MT, Charles CJ, Espiner EA, Frampton CM, Lainchbury JG, Richards AM. Endogenous urocortins reduce vascular tone and renin–aldosterone/endothelin activity in experimental heart failure. *Eur Heart J* 2005;**26**:2046–54.
27. Rademaker MT, Charles CJ, Nicholls GN, Richards AM. Urocortin 2 inhibits furosemide-induced activation of renin and enhances renal function and diuretic responsiveness in experimental heart failure. *Circ Heart Fail* 2009;**2**:532–40.
28. Rademaker MR, Charles CJ, Nicholls MG, Richards AM. Urocortin2 combined with angiotensin-converting enzyme inhibition in experimental heart failure. *Clin Sci* 2008;**114**:635–42.
29. Reyes TM, Lewis K, Perrin MH, Kunitake KS, Vaughan J, Arias CA, et al. Urocortin II: A member of the corticotropin-releasing factor (CRF) neuropeptide family that is selectively bound by type 2 CRF receptors. *Proc Natl Acad Sci USA* 2001;**98**:2843–8.
30. Singh LK, Boucher W, Pang X, Letourneau R, Seretakis D, Green M, et al. Potent mast cell degranulation and vascular permeability triggered by urocortin through activation of corticotropin-releasing hormone receptors. *J Pharmacol Exp Ther* 1999;**288**:1349–56.
31. Schulman D, Latchman DS, Yellon DM. Urocortin protects the heart from reperfusion injury via upregulation of p42/p44 MAPK signaling pathway. *Am J Physiol Heart Circ Physiol* 2002;**283**:H1481–8.

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32. Vaughan J, Donaldson C, Bittencourt J, Perrin MH, Lewis K, Sutton S, et al. Urocortin, a mammalian neuropeptide related to fish urotensin I and to corticotropin-releasing factor. *Nature* 1995;**378**:287–92.
33. Venkatasubramanian S, Newby DE, Lang NN. Urocortins in heart failure. *Biochem Pharmacol* 2010;**80**:289–96.
34. Yang L-Z, Kockskämper J, Heinzel FR, Hauber M, Walther S, Spiess J, et al. Urocortin II enhances contractility in rabbit ventricular myocytes via CRF(2) receptor-mediated stimulation of protein kinase A. *Cardiovasc Res* 2006;**69**:402–11.

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Chapter 195

Urotensin Peptides

Kazuhiro Takahashi and Kazuhito Totsune

ABSTRACT

Urotensins, peptide hormones originally discovered in the fish neuroendocrine organ urophysis, consist of urotensin I (UI), a corticotropin-releasing hormone-like fish peptide, and urotensin II (UII), a somatostatin-like fish peptide. Human UII, a cyclic peptide consisting of 11 amino acids, is widely distributed in various organs including the cardiovascular system, together with its UT receptor. Urotensin-II-related peptide (URP) is an 8-amino acid peptide that has a common ring structure with that of UII. UII and URP bind to the UT receptor with a similar affinity. UII is a potent vasoconstrictor peptide, whereas UII causes endothelium-dependent relaxation that is mediated through NO production. Furthermore, UII has various actions, such as stimulation of cell proliferation and hypertrophy, a positive inotropic action and CNS actions on cardiovascular control. Plasma concentrations of UII are elevated in patients with congestive heart failure, hypertension, end-stage renal disease, and diabetes mellitus. UII and the UT receptor may therefore play important pathophysiological roles and provide novel targets for the treatment of these diseases.

DISCOVERY

Urotensins were originally discovered from urophysis, a fish neuroendocrine organ located in the caudal spinal cord.[26,30] There are at least two types of urotensins: urotensin I (UI), a corticotropin-releasing hormone (CRH)-like fish peptide and urotensin II, a somatostatin-like fish peptide. Urocortins (urocortins 1, 2, and 3) are human homologs of UI and belong to the CRH family of peptides. Urocortins are described in the previous chapter.

Human UII is a cyclic peptide consisting of 11 amino acids (Fig. 1)[1,7] Molecular cloning of human UII and frog UII cDNAs showed that human UII consists of only 11 amino acid residues, whereas fish and frog UII possess 12 and 13 amino acid residues, respectively.[1,7,30] The cyclic region of UII, which is responsible for the biological activity of the peptide, has been fully conserved from fish to human. Reverse pharmacology has revealed that human UII is an agonist for the orphan receptor GPR14 (now called urotensin II receptor or UT receptor).[1] Furthermore, human UII is a potent vasoconstrictor peptide and its vasoconstrictive potency is one order of magnitude greater than that of endothelin-1.[1]

Urotensin-II-related peptide (URP) is an 8-amino acid peptide identified as the UII-immunoreactive molecule in the rat brain [25](Fig. 1). In contrast to human and porcine UII, the amino acid sequences of the tentative rat or mouse precursor proteins for UII, which show high homology to human or porcine ortholog proteins, lack typical cleavage sites to generate mature UII peptides. Therefore, it has not been clarified whether mouse or rat UII peptide is present as an endogenous ligand for UT receptor in mouse and rat. URP was identified as an endogenous ligand for UT receptor in rats. The amino acid sequence of human and mouse URP is the same as that of rat URP. UII and URP genes originate from the same ancestral gene as the somatostatin and cortistatin genes.[28]

DISTRIBUTION OF THE mRNA/PEPTIDE IN THE CARDIOVASCULAR SYSTEM

UII mRNA and peptide are distributed widely in the cardiovascular system, including the heart and vascular vessels.[1,7,26,30] UII is expressed in the myocardium, and vascular endothelial and smooth muscle cells in various organs. In the kidney, UII is expressed in the epithelial cells of tubules and ducts, with greater intensity in the distal convoluted tubules, in addition to vascular vessels.[27] UII is also expressed in the endocrine organs, such as adrenal cortex and medulla, which have important physiological roles in the cardiovascular control.[12,19]

The expression of UII is enhanced in the cardiac tissue obtained from patients with heart failure.[10] Furthermore, strong UII immunostaining is observed in the blood vessels with atherosclerotic lesions, including endothelial, smooth muscle, and inflammatory cells and the atherosclerotic plaques.[4]

Real-time RT-PCR showed that URP mRNA is expressed in peripheral and central tissues of humans and rats, with higher expression found in the ovary, testis, and placenta in humans, and the thymus, spleen, testis, and spinal cord in

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00195-0
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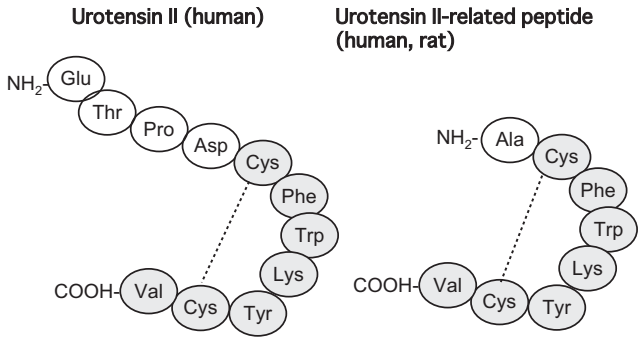

FIGURE 1 Amino acid sequences of urotensin II (human) and urotensin II-related peptide (human, rat). The amino acids common between these two peptides are shown in gray. *(From Takahashi K, Hirose T, Mori N, Morimoto R, Kohzuki M, Imai Y, et al.* Peptides *2009;*30*:1575–1585 with kind permission from the Elsevier Inc. Copyright 2009 Elsevier Inc.)*

rats.[25] Expression levels of UII mRNA are particularly high in the spinal cord of human and rat, whereas the expression of URP mRNA in the spinal cord is not significant. URP mRNA is also expressed in cardiovascular tissues, such as the heart, aorta, and kidney.[14,18,20,25] URP mRNA levels are increased in the heart tissue obtained from rats with congestive heart failure resulting from coronary ligation,[20] and in the kidney and aorta from spontaneously hypertensive rats (SHR).[14]

RECEPTORS AND SIGNALING CASCADES

Both UII and URP bind to UT receptor with a similar affinity.[25] The UT receptor has 7 transmembrane domains, belongs to the class A family of G protein-coupled receptor, and is widely expressed in various organs including the heart and vascular vessels.[1,30] UT is expressed in various human tissues, including heart (atria and ventricles), coronary artery, aorta, kidney, adrenal, brain, and pituitary.[29] Immunocytochemical studies showed that UT receptor is expressed in the myocardium, and smooth muscle cells and endothelial cells of arteries and veins.[20] Expression levels of UT receptor mRNA are elevated in the atrium of rats with congestive heart failure resulting from coronary artery ligation,[20] and the atrium, ventricle, and aorta of SHR. In the kidney, UT receptor is expressed in the renal tubular cells and vascular endothelial cells.[18] Expression levels of UT receptor mRNA are elevated in the kidney of rats with hypertension or chronic renal failure due to 5/6 nephrectomy.

The dissociation of UII from the UT receptor is essentially irreversible.[9] Only about 15% of [^{125}I]UII is dissociated after 90 min by the addition of an excess of unlabeled UII in human SJRH30 rhabdomyosarcoma cells. Moreover, the UII/UT complex is internalized in a dynamin-dependent but arrestin-independent manner.[11] After dissociation from UII, the UT receptor recycles back to the plasma membrane. The low dissociation and the continuous externalization of the UT receptor may explain the repetitive and lasting UII-mediated vasoconstriction.

The main transduction mechanism associated with UT receptor is the coupling and activation of the $G_{\alpha q/11}$ subtype of heterotrimeric G proteins.[13,30] UT receptor is coupled to the phospholipase C/protein kinase C pathway. UII stimulates polyphosphoinositide turnover and provokes mobilization of Ca^{2+} from intracellular stores. UT may also couple to $G_{\alpha i/0}$ proteins. Furthermore, the UT receptor can activate several other signaling pathways, including the phospholipase A2, the MAP kinases ERK1/2, the NO synthase, and the Rho A/Rho kinase pathways.

BIOLOGICAL ACTIONS WITHIN THE CARDIOVASCULAR SYSTEM

Table 1 shows the biological actions of UII in the cardiovascular, endocrine, and renal systems. It was initially reported that human UII is a potent vasoconstrictor peptide, and its vasoconstrictive potency is one order of magnitude greater than that of endothelin-1.[1] UII and URP exert a potent contractile effect on endothelium-denuded rat thoracic aorta, monkey coronary artery, and human pulmonary artery.[1,6,17,30] By contrast, in intact rat small mesenteric arteries, UII causes endothelium-dependent relaxation that is mediated through NO production.[3] The vasoactive profile of UII is species dependent and varies substantially according to the type of blood vessel even within each single species.[8]

Infusion of UII into healthy human volunteers had no effect on forearm blood flow in one study,[34] whereas dose-dependent reductions in forearm blood flow were reported by another group.[2] Measurements of skin microvascular tone showed that UII produced a dose-dependent vasodilator response in normal subjects, whereas vasoconstriction was noted in patients with chronic heart failure, essential hypertension, and diabetes mellitus.[16,27]

UII has a strong inotropic action on the myocardium,[21] whereas bolus injection of UII in the cynomolgus monkey reduces myocardial contractility and provokes circulatory collapse and death.[1] UII stimulates the proliferation of vascular smooth muscle cells, fibroblasts, and some cancer cells.[27,30] It also stimulates the hypertrophy of cardiomyocytes and induces angiogenesis.[13,30]

UII and the UT receptor are expressed in the brain,[15,29,30] as described in the Brain Peptides section of this book. UII may also regulate the cardiovascular system via the brain. Injection of UII into the brain causes significant hemodynamic changes and plasma levels of hormones in rats, depending on the sites of injection,[15,30,33] as shown in Table 1. Intracerebroventricular injection of UII increases the heart rate and arterial blood pressure in rats. These

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TABLE 1 Biological Actions of Urotensin II in Cardiovascular, Endocrine, and Renal Systems

1.	Vasoconstrictor action
2.	Vasodilator action through the release of nitric oxide
3.	Positive inotropic and chronotropic actions on the myocardium
4.	Proliferation of vascular smooth muscle cells, fibroblasts, and others
5.	Hypertrophic action on cardiomyocytes
6.	CNS action on the cardiovascular system and other systems Increase in the heart rate and arterial blood pressure (i.c.v., or microinjection into either the arcuate or paraventricular nuclei.) Depressor and bradycardiac responses (microinjection into the A1 area) Elevation of plasma ACTH, adrenalin, and glucose (i.c.v.)
7.	Modulatory roles on hormone production and secretion Inhibition of insulin secretion Inhibition of corticosterone secretion Increase in immunoreactive aldosterone synthase in adrenal cortex
8.	Regulation of glomerular filtration rate and renal sodium and water handling in the kidney

responses are significantly attenuated by pretreatment with intravenous pentolinium (ganglion blocker), suggesting that UII-mediated responses partly result from increases in sympathetic activity. Intracerebroventricular injection of UII elevates plasma ACTH, epinephrine, and glucose levels. By contrast, microinjection of UII into the A1 area of the medulla produces dose-dependent depressor and bradycardiac responses. However, there is no effect of UII microinjection into the A2 area on mean arterial blood pressure or heart rate. Moreover, significant pressor and tachycardiac responses are observed on microinjection of UII into either the arcuate or paraventricular nuclei.

UII modulates the production and secretion of certain hormones.[27,30] UII may therefore affect the cardiovascular control and the pathophysiology of cardiovascular diseases via the effects on hormone secretion. UII inhibits insulin release from the perfused rat pancreas. UII suppresses basal secretion of corticosterone from *in vitro* cultured adrenocortical cells of Sprague–Dawley rats. By contrast, UT receptor activation *in vivo* in rats enhances the adrenocortical expression of immunoreactive aldosterone synthase.[12]

In the kidney, UII regulates glomerular filtration rate (GFR) and renal sodium and water handling. Injection of rat UII induces reductions in GFR, urine flow, and sodium excretion.[24] Infusion of a UT receptor antagonist, urantide, results in increases in these variables. Endogenous UII therefore seems to contribute to the regulation of GFR and renal sodium and water handling in the rat. UII infusion in anesthetized SHR and control Wistar Kyoto (WKY) rats produces marked reductions in GFR, urine flow, and sodium excretion rates, which are greater in SHR by comparison with WKY rats.[27] WKY rats also show an increase in fractional excretion of sodium in contrast to SHR in which no such change is observed. Blockade of the UII receptor with urantide evokes an increase in GFR, which is greater in SHR compared with that in WKY rats and is accompanied by a diuresis and natriuresis. By contrast, Zhang et al. reported that UII produces vasodilator and natriuretic effects in the kidney and that UII-induced vasodilation is associated with increased endothelial NO in the renal artery.[27]

There are a limited number of reports on the biological actions of URP. URP and UII share the same receptor, UT.[25] The biological actions of URP may therefore be similar to those of UII. URP has about the same binding affinity, but less potency, than UII in contracting rat aortic strips *in vitro*.[6] *In vivo*, intravenous injection of URP produces a long-lasting reduction of blood pressure.[13,27,30] URP dilates rat coronary arteries with 10-fold less potency compared with rat UII.[13,27,30]

PATHOPHYSIOLOGICAL IMPLICATIONS

Plasma concentrations of UII are elevated in patients with heart failure, hypertension, end-stage renal disease, diabetes mellitus, and portal hypertension[13,27,29,30] (Table 2). UII and UT receptor may therefore play important roles of the pathophysiology of these diseases.

Elevated concentrations of plasma UII were first observed in patients with chronic renal failure (Fig. 2). The mean concentration of urotensin II-like immunoreactivity in plasma is 4.4 ± 1.0 fmol/mL in healthy individuals ($n = 24$, mean ± S.D.). The very low concentration of UII in plasma suggests that the main (patho)physiological roles of UII are mediated in an autocrine or paracrine fashion, rather than in an endocrine fashion. Plasma concentrations of UII are 2-fold higher in patients not on dialysis and 3-fold higher in patients on hemodialysis by comparison with controls. These observations suggested that UII might act as an important regulator in the cardiovascular and renal systems. Urotensin II antagonists could, therefore, be useful in the treatment of diseases affecting these organs.

Plasma UII concentrations are elevated in patients with congestive hear failure, and the expression levels of UII and binding sites of UII are increased in the myocardium obtained from patients with end-stage heart failure.[10] UII may contribute to cardiac remodeling after myocardial infarction.[5] A selective antagonist of UT, SB-611812 significantly attenuates both myocardial and endocardial interstitial fibrosis, and reduces collagen type I:III ratio

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TABLE 2 Diseases Associated with Elevated Plasma Concentrations of Urotensin II

Congestive heart failure
Essential hypertension
End-stage renal disease
Diabetes mellitus
Portal hypertension

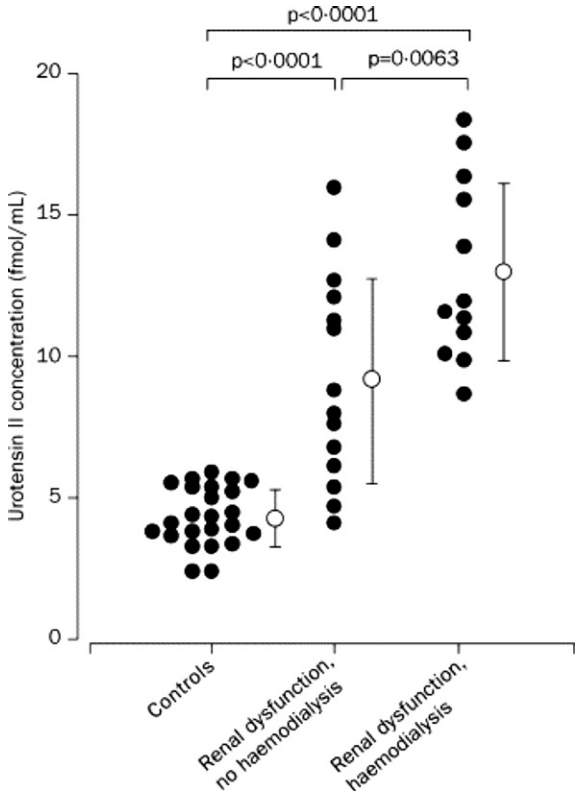

FIGURE 2 Plasma concentrations of urotensin II in patients with chronic renal failure. Open circles and bars show mean and SD, respectively. *(From Totsune K, Takahashi K, Arihara Z, Sone M, Satoh F, Ito S et al.* Lancet *2001;358:810–811 with kind permission from the Elsevier Inc. Copyright 2001 Elsevier Inc.)*

in rats with coronary ligation. UII induces proliferation of cardiac fibroblasts and this mitogenic effect is significantly inhibited with 1 μM of SB-611218. Thus, the blockade of UII action attenuates remodeling in experimental ischemic heart disease.

There are several reports that suggest that UII promotes progression of atherosclerosis.[4,13,27,32] UII is abundantly expressed in endothelial cells, and myointimal and medial smooth muscle cells of atherosclerotic human coronary arteries in comparison with healthy arteries.[4] UII stimulates the proliferation of vascular smooth muscle cells and fibroblasts. Particularly, UII acts synergistically with mildly oxidized low-density lipoprotein to induce vascular smooth muscle cell proliferation.[32] UII activates migration and differentiation of adventitial fibroblasts from rat aorta. Targeted overexpression of the human UT transgene in smooth muscle cells shows a significant increase in atherosclerotic lesion in aorta, suggesting an important role for UT in the pathogenesis of atherosclerosis.[27]

Plasma concentrations of UII are elevated in patients with diabetes mellitus without apparent renal dysfunction.[27] Diabetic mice exhibit higher concentrations of UII and UT mRNAs in skeletal muscle compared with control.[30] Expression levels of UII and UT are enhanced in diabetic kidneys of rats and human compared with nondiabetic control.[27] Furthermore, single nucleotide polymorphism in the UII gene is associated with a higher risk of developing type 2 diabetes mellitus.[27,30] These reports suggest that UII plays roles in carbohydrate metabolism and the pathophysiology of diabetes mellitus and its complications, such as diabetic nephropathy.

In an accelerated rat model of diabetes mellitus prepared by injection of streptozotocin and unilateral nephrectomy, effects of palosuran (a UT receptor antagonist) have been studied.[27] Long-term treatment of these diabetic rats with palosuran improves survival, increases insulin, and slows down the increase in glycemia, glycosylated hemoglobin, and serum lipids. Furthermore, palosuran increases renal blood flow and delays the development of proteinuria and renal damage. These findings suggest that the blockade of UII has protective roles against the progression of diabetic nephropathy. Pharmacodynamics and pharmacokinetics of palosuran were studied in 19 macroalbuminuric, diabetic patients.[22] These 19 patients were treated with oral doses of 125 mg palosuran twice daily for 13.5 days in addition to treatment with either an ACE inhibitor or an angiotensin receptor blocker. An overall clinically significant reduction of 24.3% in the 24-h urinary albumin excretion rate was observed after 13.5 days of treatment. No effect was observed on renal hemodynamic parameters. In contrast to these two reports, palosuran did not affect albuminuria, blood pressure, GFR, or renal plasma flow significantly in 54 hypertensive patients with type 2 diabetic nephropathy treated with a single blocker of the renin–angiotensin–aldosterone system.[31] The antagonism of the UII system by palosuran did not influence insulin secretion or sensitivity or daily blood glucose levels in diet-treated patients with type 2 diabetes mellitus.[23]

Some studies suggest a protective role of UII by showing that plasma UII levels are lower in patients with acute cardiac ischemia, and that plasma UII levels predict better cardiovascular outcomes in patients with end-stage renal disease.[35] It is noteworthy, however, that there are very large variations in plasma UII levels of three orders of magnitude among the reports because of differences in the analytical methods.[27]

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Some assay methods of UII may detect not only UII but also the UII precursor, its fragment peptides, or nonspecific materials in plasma. Thus, difficulty remains in interpreting results of plasma UII levels reported by various research laboratories.

REFERENCES

1. Ames RS, Sarau HM, Chambers JK, Willette RN, Aiyar NV, Romanic AM, et al. Human urotensin-II is a potent vasoconstrictor and agonist for the orphan receptor GPR14. *Nature* 1999;**401**:282–6.
2. Böhm F, Pernow J. Urotensin II evokes potent vasoconstriction in humans in vivo. *Br J Pharmacol* 2002;**135**:25–7.
3. Bottrill FE, Douglas SA, Hiley CR, White R. Human urotensin-II is an endothelium-dependent vasodilator in rat small arteries. *Br J Pharmacol* 2000;**130**:1865–70.
4. Bousette N, Patel L, Douglas SA, Ohlstein EH, Giaid A. Increased expression of urotensin II and its cognate receptor GPR14 in atherosclerotic lesions of the human aorta. *Atherosclerosis* 2004;**176**:117–23.
5. Bousette N, Pottinger J, Ramli W, Ohlstein EH, Dhanak D, Douglas SA, et al. Urotensin-II receptor blockade with SB-611812 attenuates cardiac remodeling in experimental ischemic heart disease. *Peptides* 2006;**27**:2919–26.
6. Chatenet D, Dubessy C, Leprince J, Boularan C, Carlier L, Ségalas-Milazzo I, et al. Structure–activity relationships and structural conformation of a novel urotensin II-related peptide. *Peptides* 2004;**25**:1819–30.
7. Coulouarn Y, Lihrmann I, Jegou S, Anouar Y, Tostivint H, Beauvillain JC, et al. Cloning of the cDNA encoding the urotensin II precursor in frog and human reveals intense expression of the urotensin II gene in motoneurons of the spinal cord. *Proc Natl Acad Sci U S A* 1998;**95**:15803–8.
8. Douglas SA, Sulpizio AC, Piercy V, Sarau HM, Ames RS, Aiyar NV, et al. Differential vasoconstrictor activity of human urotensin-II in vascular tissue isolated from the rat, mouse, dog, pig, marmoset and cynomolgus monkey. *Br J Pharmacol* 2000;**131**:1262–74.
9. Douglas SA, Naselsky D, Ao Z, Disa J, Herold CL, Lynch F, et al. Identification and pharmacological characterization of native, functional human urotensin-II receptors in rhabdomyosarcoma cell lines. *Br J Pharmacol* 2004;**142**:921–32.
10. Douglas SA, Tayara L, Ohlstein EH, Halawa N, Giaid A. Congestive heart failure and expression of myocardial urotensin II. *Lancet* 2002;**359**:1990–7.
11. Giebing G, Tölle M, Jürgensen J, Eichhorst J, Furkert J, Beyermann M, et al. Arrestin-independent internalization and recycling of the urotensin receptor contribute to long-lasting urotensin II-mediated vasoconstriction. *Circ Res* 2005;**97**:707–15.
12. Giuliani L, Lenzini L, Antonello M, Aldighieri E, Belloni AS, Fassina A, et al. Expression and functional role of urotensin-II and its receptor in the adrenal cortex and medulla: novel insights for the pathophysiology of primary aldosteronism. *J Clin Endocrinol Metab* 2009;**94**:684–90.
13. Guidolin D, Albertin G, Ribatti D. Urotensin-II as an angiogenic factor. *Peptides* 2010;**31**:1219–24.
14. Hirose T, Takahashi K, Mori N, Nakayama T, Kikuya M, Ohkubo T, et al. Increased expression of urotensin II, urotensin II-related peptide and urotensin II receptor mRNAs in the cardiovascular organs of hypertensive rats: comparison with endothelin-1. *Peptides* 2009;**30**:1124–9.
15. Hunt BD, Ng LL, Lambert DG. A rat brain atlas of urotensin-II receptor expression and a review of central urotensin-II effects. *Naunyn Schmiedebergs Arch Pharmacol* 2010;**382**:1–31.
16. Lim M, Honisett S, Sparkes CD, Komesaroff P, Kompa A, Krum H. Differential effect of urotensin II on vascular tone in normal subjects and patients with chronic heart failure. *Circulation* 2004;**109**:1212–4.
17. Maguire JJ, Kuc RE, Davenport AP. Orphan–receptor ligand human urotensin II: receptor localization in human tissues and comparison of vasoconstrictor responses with endothelin-1. *Br J Pharmacol* 2000;**131**:441–6.
18. Mori N, Hirose T, Nakayama T, Ito O, Kanazawa M, Imai Y, et al. Increased expression of urotensin II-related peptide and its receptor in kidney with hypertension or renal failure. *Peptides* 2009;**30**:400–8.
19. Morimoto R, Satoh F, Murakami O, Totsune K, Arai Y, Suzuki T, et al. Immunolocalization of urotensin II and its receptor in human adrenal tumors and attached non-neoplastic adrenal tissues. *Peptides* 2008;**29**:873–80.
20. Nakayama T, Hirose T, Totsune K, Mori N, Maruyama Y, Maejima T, et al. Increased gene expression of urotensin II-related peptide in the hearts of rats with congestive heart failure. *Peptides* 2008;**29**:801–8.
21. Russell FD, Molenaar P, O'Brien DM. Cardiostimulant effects of urotensin-II in human heart in vitro. *Br J Pharmacol* 2001;**132**:5–9.
22. Sidharta PN, Wagner FD, Bohnemeier H, Jungnik A, Halabi A, Krähenbühl S, et al. Pharmacodynamics and pharmacokinetics of the urotensin II receptor antagonist palosuran in macroalbuminuric, diabetic patients. *Clin Pharmacol Ther* 2006;**80**:246–56.
23. Sidharta PN, Rave K, Heinemann L, Chiossi E, Krähenbühl S, Dingemanse J. Effect of the urotensin-II receptor antagonist palosuran on secretion of and sensitivity to insulin in patients with type 2 diabetes mellitus. *Br J Clin Pharmacol* 2009;**68**:502–10.
24. Song W, Abdel-Razik AE, Lu W, Ao Z, Johns DG, Douglas SA, et al. Urotensin II and renal function in the rat. *Kidney Int* 2006;**69**:1360–8.
25. Sugo T, Murakami Y, Shimomura Y, Harada M, Abe M, Ishibashi Y, et al. Identification of urotensin II-related peptide as the urotensin II-immunoreactive molecule in the rat brain. *Biochem Biophys Res Commun* 2003;**310**:860–8.
26. Takahashi K. Translational medicine in fish-derived peptides: from fish endocrinology to human physiology and diseases. *Endocr J* 2004;**51**:1–7.
27. Takahashi K, Hirose T, Mori N, Morimoto R, Kohzuki M, Imai Y, et al. The renin-angiotensin system, adrenomedullins and urotensin II in the kidney: possible renoprotection via the kidney peptide systems. *Peptides* 2009;**30**:1575–85.
28. Tostivint H, Joly L, Lihrmann I, Parmentier C, Lebon A, Morisson M, et al. Comparative genomics provides evidence for close evolutionary relationships between the urotensin II and somatostatin gene families. *Proc Natl Acad Sci U S A* 2006;**103**:2237–42.
29. Totsune K, Takahashi K, Arihara Z, Sone M, Satoh F, Ito S, et al. Role of urotensin II in patients on dialysis. *Lancet* 2001;**358**:810–1.
30. Vaudry H, Do Rego JC, Le Mevel JC, Chatenet D, Tostivint H, Fournier A, et al. Urotensin II, from fish to human. *Ann N Y Acad Sci* 2010;**1200**:53–66.
31. Vogt L, Chiurchiu C, Chadha-Boreham H, Danaietash P, Dingemanse J, Hadjadj S, et al. PROLONG (PROteinuria Lowering with urOteNsin receptor antaGonists) Study Group. Effect of the urotensin receptor antagonist palosuran in hypertensive patients with type 2 diabetic nephropathy. *Hypertension* 2010;**55**:1206–9.

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32. Watanabe T, Pakala R, Katagiri T, Benedict CR. Synergistic effect of urotensin II with mildly oxidized LDL on DNA synthesis in vascular smooth muscle cells. *Circulation* 2001;**104**:16–8.
33. Watson AM, McKinley MJ, May CN. Effect of central urotensin II on heart rate, blood pressure and brain Fos immunoreactivity in conscious rats. *Neuroscience* 2008;**155**:241–9.
34. Wilkinson IB, Affolter JT, de Haas SL, Pellegrini MP, Boyd J, Winter MJ, et al. High plasma concentrations of human urotensin II do not alter local or systemic hemodynamics in man. *Cardiovasc Res* 2002;**53**:341–7.
35. Zoccali C, Mallamaci F. Urotensin II: a cardiovascular and renal update. *Curr Opin Nephrol Hypertens* 2008;**17**:199–204.

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Chapter 196

VIP

Robert J. Henning

ABSTRACT

Exogenous vasoactive intestinal peptide (VIP) causes coronary dilation with an EC_{50} of 4×10^{-11} M, mediated by VPAC1 and VPAC2 receptors, adenylyl cyclase, and potassium channels. At greater concentrations, VIP can increase atrial and ventricular contractility and heart rate and decrease atrial ventricular refractory periods and conduction time. Endogenous VIP, released from vagal nerves, increases left coronary artery blood flow, right atrial and right ventricular contractility, and heart rate. Cardiac VIP concentration reflects a balance between local neurogenic synthesis and removal by veins and lymphatics. Circulating VIP is metabolized in the lung and liver by neutral endopeptidase and by mast cell chymase and tryptase. With coronary occlusion, VIP is released from coronary and myocardial neurons and acts as a vasodilator and free-radical scavenger that limits injury. With cardiomyopathies, hypertension, obesity, diabetes, and hypothyroidism the cardiac concentration of VIP, VIP receptor density, and coupling to adenylyl cyclase decreases with resultant increases in coronary vascular resistance and myocardial fibrosis and reductions in myocardial contractility and heart rate.

INTRODUCTION

Vasoactive intestinal peptide (VIP) is a 28-amino-acid neuropeptide discovered by Said and Mutt in 1970 with diverse cardiovascular effects that include vasodilation of the cerebral and coronary artery circulations and an increase in myocardial contractile performance [see the Chapter by S.I. Said in the Respiratory Peptides section of the book and Refs. 14–16, 28, 29]. VIP exerts its effects by activating signaling processes involving VPAC1 and VPAC2 receptors, adenylyl cyclase, guanylyl cyclase, and potassium channels.[16,28,29] VIP immunoreactive nerve fibers originate from the central, peripheral, and intrinsic nervous systems and innervate blood vessels in the myocardium and throughout the body.[11,28] VIP is metabolized in the circulation by neutral endopeptidase and by mast cell chymase and tryptase.[8] The VIP gene has been cloned, sequenced, and localized to chromosome 6q24. VPAC1 and VPAC2 genes are located on chromosomes 3p22 and 7q36.3, respectively.

DISCOVERY

After the seminal discovery in 1902 by Bayliss and Starling of an intestinal extract that had vasodepressor properties, Said and Mutt isolated and sequenced an acid-extractable peptide from the duodenum that produced in anesthetized dogs a decrease in the systemic arterial pressure and an increase in the stroke volume, heart rate, mesenteric, and femoral arterial blood flow. Because the action of the endogenous peptide was initially believed not to extend beyond the intestine because of metabolism by the liver, the peptide was named "Vasoactive Intestinal Peptide." Subsequent studies found this peptide in high concentrations in the plasma and tumor tissues of patients with pheochromocytoma and ganglioneuroblastoma, in neuroblastoma and astrocytoma cell lines, and also widely distributed in the central and peripheral nervous systems. In addition, numerous VIP immunoreactive cell bodies have been discovered in the heart, tracheobronchial tree, gastrointestinal and genitourinary tracts, and pancreas, which indicate that there is also a peripheral intrinsic VIP-ergic system.[28] The observation that VIP immunoreactive fibers innervate blood vessels in the atrial and ventricular myocardium, the conduction system of the heart and small intestine and brain led to additional studies to determine the role of VIP in the regulation of blood flow and cardiac performance.

DISTRIBUTION

VIP immunoreactive nerve fibers innervate blood vessels throughout the central and peripheral nervous systems, as well as the heart. In the heart of guinea pigs, rats, and dogs, VIP-ergic fibers primarily innervate the sinoatrial node, cardiac glomeruli of the proximal coronary arteries, intramural and terminal arteries of the atria, ascending aorta and pulmonary trunk.[28] A moderate number of VIP-ergic fibers innervate the atrioventricular node, perivascular nerve plexuses of the major coronary arteries, and atrial myocytes. VIP-ergic fibers also innervate the intracardiac ganglia and intramural and terminal vasculature

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00196-2
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of the ventricles. In addition, intracardiac ganglia contain VIP immunoreactive cell bodies.[28] Although these fibers may be primarily of intrinsic origin, some extrinsic parasympathetic preganglionic efferent, parasympathetic and sympathetic postganglionic efferent, and parasympathetic and sympathetic afferent VIP-ergic fibers also exist.[11] An extrinsic VIP-ergic innervation exists, particularly in the canine right ventricle.[1,14,28] The concentration of VIP in the left ventricular epicardial coronary arteries varies between 0.7 and 2.2 pmol g^{-1} tissue, with the highest concentrations in the proximal coronary regions.[4] A VIP-ergic innervation of the epicardial coronary arteries may play a role in maintaining coronary vasorelaxation. In the cerebral circulation, interneurons containing VIP innervate arterioles and receive input from acetylcholine and serotonin containing neural projections that contact cell soma and proximal dendrites.[28] Electrical stimulation of these interneurons produces vasodilation of adjacent arterioles, supporting a role for VIP-ergic neurons in the control of cerebral blood flow. VIP, on a molar basis, is 50–100 times more potent than acetylcholine as a vasodilator.[16]

PROCESSING

The VIP peptide is derived from preproVIP, which consists of 170-amino-acid residues. Proteolytic cleavage of preproVIP yields preproVIP (22–79), peptide histidine isoleucine/methionine (PHI/PHM 81–107), preproVIP (111–122), VIP (125–152), and preproVIP (156–170). Peptide histidine isoleucine/methionine is structurally related to VIP and shares many of VIP's biological actions but is generally less potent than VIP. Other peptide sequences have been identified during the proteolytic cleavage of the VIP precursor but have limited biological effects. Nevertheless, the bioactivity of the partial sequences of VIP increases with increasing amino acid chain length.

VIP is metabolized by neutral endopeptidase (NEP), a transmembrane metallopeptidase that is abundant in the central nervous system, kidneys, liver, lung, and intestinal epithelium.[27] This enzyme cleaves a variety of peptides in addition to VIP, including enkephalins, angiotensin I, angiotensin II, substance P, neurotensin, and atrial natriuretic peptides. Topical application of two NEP inhibitors, phosphoramidon and thiorphan, enhances the vasodilatory effect of VIP in resistance vessels, showing a role for vascular NEP in limiting the vasoreactivity of VIP.[8,16,28] The plasma concentration of VIP increases 3.6-fold, from 30 to 108 fmol ml^{-1}, after a single dose of the NEP blocker UK77-568, but then returns to baseline after four consecutive daily doses of the NEP blocker, because of feedback inhibition of VIP release.[8] The major sites of VIP metabolism in circulation are the lungs, liver, and probably kidneys, which contribute to a VIP half-life of 2–5 min.[18] Other tissues, such as the intestine, brain, and heart, play little role in VIP metabolism. In the heart, the concentration of VIP reflects a balance between local neurogenic synthesis and release in the myocardium and removal by the circulatory and lymphatic systems. During chronic NEP inhibition, the myocardial VIP concentration can increase 2-fold.[8] Angiotensin converting enzyme (ACE) inhibition can increase myocardial VIP concentration by as much as 4.5-fold because of local VIP synthesis and release in the myocardium.[28] This increased concentration of VIP in myocardium can contribute to improvement of cardiac function in patients with cardiomyopathy who are treated with ACE inhibitors. However, chronic ACE inhibition does not change the concentration of VIP in the plasma, kidneys, and lungs because it increases the metabolism of VIP.[28]

RECEPTORS

Two subtypes of VIP receptor, VPAC1 and VPAC2, have been identified in the cardiovascular system.[13,16,28,29] These G-protein-coupled receptors are members of the secretin receptor family and bind VIP and pituitary adenylate cyclase activating peptide (PACAP) with equal affinity.[16] VIP receptors are present in heart and blood vessels and have a molecular weight of 43,000–80,000 Da.[16] In general, the density of VIP receptors in arteries is greater than that in veins. This accounts for VIP's greater vasodilatory effect on arteries than veins.[16] The N-terminal region of VIP plays an important role in selective recognition of VPAC or PAC1 receptors, while the formation of the α-helical structure in the C terminus of VIP seems to be important in VIP binding to VPAC receptors. VPAC1 and VPAC2 receptors have been identified in endothelium and smooth muscle of major arteries and arterioles of the right and left ventricle[29] (see Figure 1). VPAC1 and VPAC2 receptors are also present in endothelium and smooth muscle of cerebral arteries and arterioles.[28] Consequently, both receptors seem to be involved in the control of coronary and cerebral arterial blood flow. Involvement of VPAC1 and VPAC2 receptors in vascular regulation has been recently shown by blockade of pressure-induced cutaneous vasodilation with the VPAC1 or VPAC2 receptor antagonists PG97-269 or PACAP6-38, respectively.[28,29] This implies a series relationship in which both receptors are necessary for the full expression of this neurally mediated cutaneous vasodilatory reflex. VPAC2 and to a lesser extent VPAC1 receptors are also present in atrial myocardium.[34] The expression of both VPAC2 and VPAC1 receptors in coronary arteries, arterioles and myocardium contrasts with

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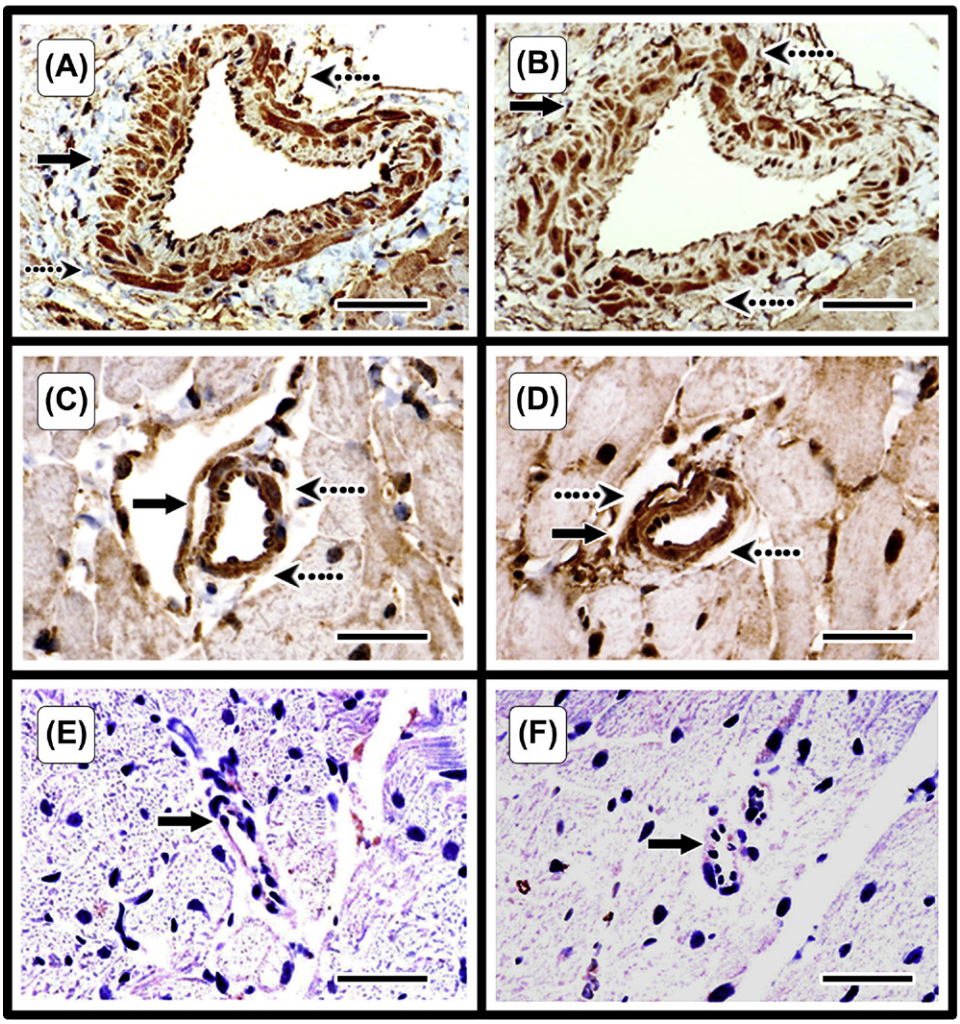

FIGURE 1 Presence of VPAC1 (A, C) and VPAC2 receptors (B, D) in the left ventricular coronary artery (A, B) and coronary arterioles of the left ventricle (C, D) of the rat as determined by immunohistochemistry. Negative controls are also shown (E, F). The primary stain is Dako DAB (*brown*) and the counterstain is hematoxylin (*blue*). Scale bar represents 50 μm for panels A and B and 25 μm for panels C, D, E, and F. Large arrow denotes location of artery or arteriole. Dashed arrow denotes regions expressing high levels of VPAC1 or VPAC2. See color plate 52.

that in intrinsic cardiac neurons, where primarily VPAC2 receptors are expressed.[16]

BIOLOGICAL ACTIONS

VIP produces vasodilation in the coronary, cerebral, mesenteric, pulmonary, and cutaneous circulations. In isolated coronary arterial strips and whole arteries, VIP produces vasorelaxation independent of endothelium.[3,11] VIP also produces coronary vasodilation and increases coronary blood flow in intact animals and in isolated heart preparations, which is mediated by a direct action on coronary vasculature. In isolated perfused rat hearts, for example, VIP produces coronary vasodilation with an EC_{50} of $4–5 \times 10^{-11}$ M without increasing heart rate, myocardial contractility, or myocardial oxygen consumption.[28,29] VIP is present in the proximal coronary arterial walls in concentrations of 1.2–2.2×10^{-12} mol/g tissue ($1.2–2.1 \times 10^{-9}$ M).[4,16] As a coronary vasodilator, VIP is 200-fold more potent than adenosine and 8000-fold more potent than sodium nitroprusside.[29] However, decreases in VIP concentrations in the coronary arteries may contribute to coronary spasm. In anesthetized dogs maintained with a fixed cardiac output, systemic arterial pressure, and heart rate, injection of VIP into the left main coronary artery increases coronary blood flow by as much as 84%, while increasing myocardial oxygen consumption by only 32%.[1,16] In patients, infusion of VIP at rates of 9 to 30 pmole min^{-1} into the left coronary artery decreases arterial-coronary sinus O_2 content difference and myocardial O_2 extraction by as much as 41% without changing left

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ventricular preload, afterload, contractility, or heart rate.[24] In addition to a direct coronary arterial vasodilatory effect, intravenous VIP may also increase coronary blood flow in patients due to increases in heart rate and stroke volume, probably resulting from reduction in afterload.[31] Additional studies indicate that endogenous VIP is released in the coronary vasculature after parasympathetic nerve stimulation and produces significant coronary artery vasodilation. The magnitude of the coronary dilation is directly dependent on the frequency of vagal nerve stimulation. In this regard, VIP is most readily released during high frequency vagal stimulation in contrast to the classical neurotransmitter acetylcholine, which is released during low frequency nerve stimulation.[10,15,16] Vagal nerve stimulation in anesthetized dogs, after blockade of muscarinic and β-adrenergic receptors, can increase left coronary artery blood flow by as much as 62% without changing left ventricular contractility.[9,10] The increase in coronary artery blood flow is equivalent to the increase in blood flow that occurs during intracoronary administration of 9.0×10^{-11} mol of VIP or 1.0×10^{-8} mol of nitroglycerin.[16] This increase in blood flow is blocked by [4Cl-D-Phe6,Leu17]VIP, a sensitive and selective antagonist of VIP receptors.

The coronary vasodilatory response to VIP involves activation of calcium activated potassium channels and voltage sensitive potassium channels, sequestration of calcium in thapsigargin-sensitive stores and a decrease in the calcium sensitivity of the contractile apparatus.[20,28] Cyclic AMP is involved during maximal VIP-elicited coronary vasodilation however the vasodilatory response to VIP does not seem to involve cyclic GMP[28,29] (see Figure 2). VIP-elicited vasodilation in the cerebral, pulmonary, mesenteric, ovarian circulations may also involve activation of adenylyl cyclase.[16] However, the degree of VIP-elicited activation of adenylyl cyclase varies with the type of vessel studied, as well as species (rat, guinea pig, rabbit, cat, cow, or pig). Moreover, the vasodilatory effect of VIP may not be precisely correlated with an increase in cyclic AMP. VIP-elicited vasodilation in the cerebral circulation also involves nitric oxide and cyclic GMP through a direct action on vascular smooth muscle and in the mesenteric circulation involves inhibition of intracellular calcium mobilization independent of cyclic AMP.[16] In the pulmonary circulation, VIP-elicited vasodilation is dependent on endothelium and involves both cyclic AMP and cyclic GMP, and is probably increased by prostacyclin and nitric oxide, respectively.[19] VIP is currently being investigated for treatment of pulmonary hypertension in patients because of its vasodilator properties and the ability of VIP to inhibit pulmonary vascular endothelial cell proliferation.

At concentrations greater than that required for coronary vasodilation, VIP can increase myocardial contractility

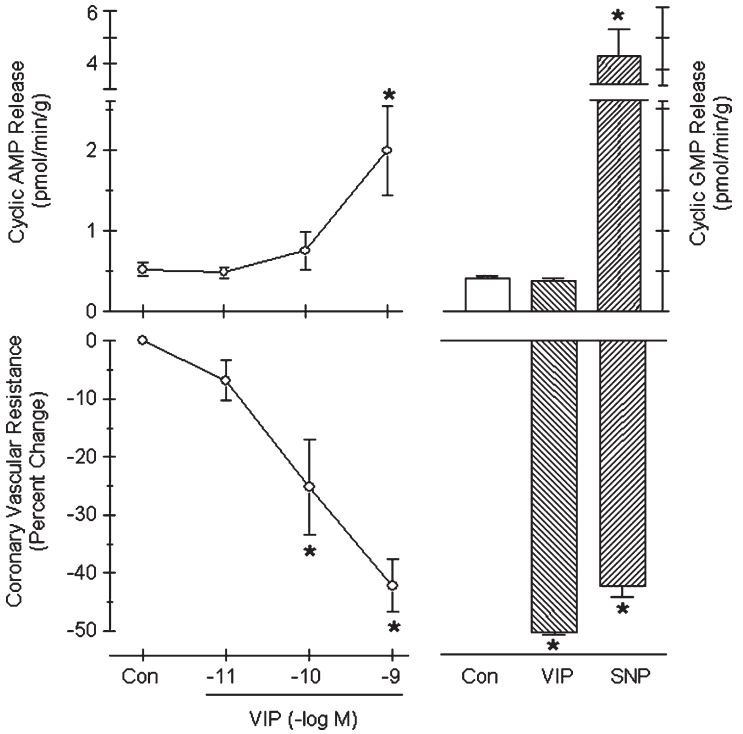

FIGURE 2 Effect of VIP at 1×10^{-11}, 1×10^{-10}, and 1×10^{-9} M on cyclic AMP release and the effect of VIP at 1×10^{-8} M and sodium nitroprusside (SNP) at 1×10^{-5} M on cyclic GMP release from the heart. Also shown are the effects of these agents on coronary vascular resistance. Values are mean ± SE of three hearts for cyclic AMP and three hearts for cyclic GMP. Asterisk indicates $P<0.05$.

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and heart rate. In canine trabeculae, isolated from the right and left atria and ventricles, VIP at concentrations greater than 1×10^{-9} M can increase contractile force by as much as 100%.[16,25,28] Similar concentrations of VIP increase contractility of human atrial and ventricular trabeculae by 15–25%.[14–16] This increase in contractility appears to be mediated by the activation of adenylyl cyclase.[5,16] In anesthetized dogs, VIP release elicited by vagal nerve stimulation, after blockade of muscarinic and β-adrenergic receptors, increases right atrial and right ventricular contractility by as much as 28%, but does not significantly increase left ventricular contractility.[14,15] The significant increase in right ventricular inotropic responses to VIP is best explained by the fact that abundant VIP immunoreactive fibers innervate the right but not the left ventricle in the dog.[1,14,15,28] The increase in myocardial contractility elicited by exogenous VIP, but not the increase in coronary blood flow, can decrease after repetitive intracoronary infusions of VIP due to VIP receptor desensitization.[1,28] This observation suggests that the myocardial and coronary vascular effects of VIP may be mediated by different receptors and/or signaling mechanisms. Additional studies indicate that VIP increases adenylyl cyclase activity, L-type calcium channel current, and contractility in rat ventricular myocytes.[2,32] However, the increase in cyclic AMP is small compared with that elicited by isoproterenol and not all cells respond to VIP. These effects of VIP on adenylyl cyclase activity and contractility in rat ventricular myocytes appear to be mediated by activation of receptors that recognize both VIP and secretin.[2,5]

The discovery that VIP immunoreactive nerve fibers occur in high density in and around the sinus node and atrioventricular node of the heart strongly suggests that VIP affects heart rate. In the anesthetized dog, injection of VIP into the sinoatrial artery, after bilateral vagotomy, stellectomy, and blockade of muscarinic receptors, increases heart rate in a dose dependent manner by as much as 96%.[16,25,28] This increase in the heart rate is similar to that produced by norepinephrine; however, VIP is longer acting and twice as potent as norepinephrine. VIP also decreases the atrioventricular conduction time by as much as 30% and decreases atrial and ventricular refractory periods by 4–15%.[25,28] In addition, VIP can induce a shift of the pacemaker site to Bachman's bundle and can potentiate sinus arrhythmia.[22,23] After blockade of muscarinic and β-adrenergic receptors, vagal nerve stimulation increases the heart rate in a frequency dependent manner by as much as 50%.[14,15] This increase in heart rate is accompanied by an increase in VIP release from the right atrium and is attenuated or blocked by several VIP antagonists, including [4Cl-D-Phe6,Leu17] VIP. This heart rate response to VIP plays a role in "excess tachycardia," which is the increase in heart rate observed after muscarinic receptor blockade minus that observed after vagotomy. VIP also plays a role in "postvagal tachycardia," which is characterized by an increase in the heart rate after the initial bradycardia due to intense vagal stimulation has subsided.[14]

PATHOPHYSIOLOGY

Early investigations showed that hypertension, obesity, diabetes, and hypothyroidism reduce the ability of VIP to increase myocardial adenylyl cyclase activity.[5,26,28] This attenuation seems to result from a specific decrease in density of VIP receptors or uncoupling of receptors from adenylyl cyclase. In hearts isolated from 10-week-old spontaneously hypertensive rats, VIP-elicited adenylyl cyclase activity is reduced by as much as 69% compared with that of normotensive controls.[28] By contrast, adenylyl cyclase activity elicited by the β-adrenergic agonist, isoproterenol, is reduced by only 17%. These reductions are somewhat more pronounced in 14- and 30-week-old spontaneously hypertensive rats. Further, the reduction of VIP-elicited adenylyl cyclase activity precedes the reduction of Isoproterenol-elicited adenylyl cyclase activity. This selective impairment of VIP-elicited adenylyl cyclase activity is specific for the heart and occurs concomitantly with the development of hypertension and myocardial hypertrophy.[26,28] An impairment of VIP-elicited adenylyl cyclase activity can reduce VIP-elicited vasodilation and VIP affects on myocardial contractility and heart rate and can contribute to the development of heart failure.

During coronary artery occlusion and subsequent reperfusion, the release of VIP from the heart and the VIP concentration in the coronary sinus blood is increased. In the isolated perfused hearts of rats, interruption of coronary perfusion for 30 min increases the VIP concentration in the coronary effluent by as much as 250% during the ensuing 60-min reperfusion period.[16] Moreover, coronary perfusion with VIP immediately before the induction of ischemia significantly limits the effects of ischemia on myocardium as evidenced by a decrease in the release of creatine kinase, reduced formation of hydroxyl radicals, limitation of calcium overload in ventricular myocytes and an enhancement of myocardial contractile performance. These results indicate that VIP is released from neurons in the coronary vasculature and myocardium after acute coronary occlusion and acts as a vasodilator and free-radical scavenger to reduce myocardial ischemia and reperfusion injury. In patients with acute myocardial infarction, VIP plasma concentration in serum increases by 33% within 6 h after the onset of symptoms, from normal values of 12.2–16.2 fmol ml^{-1}, but then abruptly decreases below normal values to 8.9 fmol ml^{-1} after 24 h.[21] This abrupt decrease may be due to depletion of VIP from nerve endings and impairment of neurogenic synthesis of VIP in the heart. In patients

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who die from acute myocardial infarction, the VIP plasma concentration is significantly lower than in those who survive after myocardial infarction. The normal plasma concentrations of VIP vary with a circadian rhythm which peaks at 2000 h in adults aged 25–50 years and at 1800 h in older adults aged 65–75 years.[28] Moreover, the VIP plasma concentration in older adults of 3.3–4.4 fmol ml^{-1} is significantly less than plasma concentration in younger adults of 12.7–17.0 fmol ml^{-1}. Recently VIP has been postulated to have an important role in the circadian regulation of heart rate. In this regard, VPAC2 or VIP deficient mice lose the circadian pattern in heart rate during constant darkness or constant light.[12,13,30] These studies suggest that VIP/VPAC2 signaling is necessary for circadian and light regulated heart rate control. Patients with heart transplants for treatment of primary dilated cardiomyopathy also lack a circadian pattern in heart rate.[28]

In animal models of heart failure and in patients with cardiomyopathy secondary to coronary artery disease, the left ventricular concentration of VIP can decrease as much as 85%.[33] Further, in patients with severe heart failure, the VIP receptor density in the left ventricle and the contractile response to VIP in right ventricle can decrease by as much as 62%.[17] These decreases in myocardial VIP concentration, receptor density and function may impair myocardial contractility and enhance coronary vascular resistance, thereby perpetuating heart failure. In addition, a decrease in myocardial VIP concentration can lead to myocardial fibrosis. In animal models of myocardial fibrosis, the myocardial concentration of VIP is inversely correlated with the degree of fibrosis present in the heart.[35] Normotensive Wistar Kyoto and spontaneously hypertensive rats fed a high salt diet have lower myocardial VIP concentrations and greater myocardial interstitial and perivascular fibrosis than rats fed a normal or low salt diet. In normotensive Wistar Kyoto rats fed a high salt diet and treated with the nitric oxide synthase inhibitor L-nitro-ω-methylarginine (L-NAME) to induce hypertension, the vasopeptidase inhibitor omapatrilat increases the myocardial VIP concentration and reduces the degree of interstitial and perivascular fibrosis.[35] These findings suggest that enhancing the myocardial VIP concentration may reduce myocardial fibrosis possibly by reducing the synthesis of one or more profibrotic mediators, such as angiotensin II, TNF-α and TGF-β.[7,35] Consequently, myocardial dysfunction and heart failure may result, at least in part, from myocardial VIP depletion.

Our understanding of the cardiovascular effects of exogenously administered and endogenously released VIP has evolved since the initial discovery of VIP in 1970 and suggests a role for VIP in the regulation of blood flow and cardiac performance. In addition, myocardial VIP depletion seems to be important in the development of cardiomyopathy and myocardial fibrosis. Additional studies are needed to determine the importance of endogenous VIP in the physiological regulation of blood flow, heart rate, and myocardial contractility. Studies are also needed to determine the roles of VPAC1, VPAC2, cyclic AMP, and other receptors and signaling mechanisms in the cardiovascular effects of VIP. These studies will be instrumental in the further development of VIP analogs, endopeptidase inhibitors and other novel VIP-related therapies for the treatment of patients with coronary artery disease and cardiomyopathy.

ACKNOWLEDGMENTS

This work was supported, in part, by grants from the Bugher Foundation, the Muscular Dystrophy Association, and by facilities of the Department of Veterans' Affairs. The author thanks Darrell Sawmiller, PhD, and Lillybeth Feliciano, PhD, who have made significant contributions to the investigations of VIP at our Center for Cardiovascular Research.

REFERENCES

1. Anderson FL, Wynn JR, Kimball J, Hanson GR, Hammond E, Hershberger R, et al. Vasoactive intestinal peptide in canine hearts: effect of total cardiac denervation. *Am J Physiol* 1992;**262**:H598–602.
2. Bell D, McDermott BJ. Secretin and vasoactive intestinal peptide are potent stimulants of cellular contraction and accumulation of cyclic AMP in rat ventricular cardiomyocytes. *J Cardiovasc Pharmacol* 1994;**23**:959–69.
3. Beny JL, Brunet PC, Huggel H. Effect of mechanical stimulation, substance P and vasoactive intestinal polypeptide on the electrical and mechanical activities of circular smooth muscles from pig coronary arteries contracted with acetylcholine: role of endothelium. *Pharmacol* 1986;**33**:61–8.
4. Brum JM, Bove AA, Sufan Q, Reilly W, Go VLW. Action and localization of vasoactive intestinal peptide in the coronary circulation: evidence for nonadrenergic, noncholinergic coronary regulation. *J Am Coll Cardiol* 1986;**7**:406–13.
5. Christophe J, Waelbroeck M, Chatelain P, Robberecht P. Heart receptors for VIP, PHI and secretin are able to activate adenylate cyclase and to mediate inotropic and chronotropic effects. Species variations and physiopathology. *Peptides* 1984;**5**:341–53.
6. Davis RE, Shelley S, MacDonald GJ, Duggan KA. The effects of a high sodium diet on the metabolism and secretion of vasoactive intestinal peptide in the rabbit. *J Physiol* 1992;**451**:17–23.
7. Delgado M, Pozo D, Martinez C, Leceta J, Calvo JR, Ganea D, et al. Vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide inhibit endotoxin-induced TNF-α production by macrophages: in vitro and in vivo studies. *J Immunol* 1999;**162**:2358–67.
8. Duggan KA, Jones DM, Ye VZC, Davis RE, MacDonald GJ. Effects of endopeptidase 24.11 inhibition on plasma and tissue concentrations of vasoactive intestinal peptide. *Clin Sci* 1995;**89**:267–71.
9. Feliciano L, Henning RJ. Vagal nerve stimulation during muscarinic and beta-adrenergic blockade causes significant coronary artery dilation. *J Autonomic Nervous Syst* 1998;**68**:78–88.
10. Feliciano L, Henning RJ. Vagal nerve stimulation releases vasoactive intestinal peptide which significantly increases coronary artery blood flow. *Cardiovasc Res* 1998;**40**:45–55.

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11. Forssmann WG, Triepel J, Daffner C, Heym CH, Cuevas P, Noble MIM, et al. Vasoactive intestinal peptide in the heart. *Ann NY Acad Sci* 1988;**527**:405–20.
12. Hannibal J, Hsiung H, Fahrenkrug J. Temporal phasing of locomotor activity, heart rate rhythmicity and core body temperature is disrupted in VIP receptor 2-deficient mice. *Am J Physiol Regul Integr Comp Physiol* 2011;**300**:R519–30.
13. Harmer A, Fahrenkrug J, Gozes I, Laburthe M, May V, Pisegna J, et al. Pharmacology and functions of receptors for vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide: IUPHAR Review. 1. *Brit J Pharmacol* 2012;**166**:4–17.
14. Henning RJ. Vagal stimulation during muscarinic and β-adrenergic blockade increases atrial contractility and heart rate. *J. Autonomic Nervous Syst* 1992;**40**:121–30.
15. Henning RJ, Feliciano L, Coers CM. Vagal nerve stimulation increases right ventricular contraction and relaxation and heart rate. *Cardiovasc Res* 1996;**32**:846–53.
16. Henning RJ, Sawmiller DR. Vasoactive intestinal peptide: cardiovascular effects. *Cardiovasc Res* 2001;**49**:27–37.
17. Hershberger RE, Anderson FL, Bristow MR. Vasoactive intestinal peptide receptor in failing human ventricular myocardium exhibits increased affinity and decreased density. *Circ Res* 1989;**65**:283–94.
18. Humphrey CS, Murray P, Ebeid AM, Fisher JE. Hepatic and pulmonary clearance of exogenous vasoactive intestinal peptide in the rat. *Gastroenterology* 1979;**77**:55–60.
19. Ignarro LJ, Byrns RE, Buga GM, Wood KS. Mechanisms of endothelium-dependent vascular smooth muscle relaxation elicited by bradykinin and VIP. *Am J Physiol* 1987;**253**:H1074–82.
20. Kawasaki J, Kobayashi S, Miyagi Y, Nishimura J, Fujishima M, Kanaide H. The mechanisms of the relaxation induced by vasoactive intestinal peptide in the porcine coronary artery. *Br J Pharmacol* 1997;**121**:977–85.
21. Lucia P, Caiola S, Coppola A, Maroccia E, Belli M, De Martinis C, et al. Effect of age and relation to mortality on serial changes of vasoactive intestinal peptide in acute myocardial infarction. *Am J Cardiol* 1996;**77**:644–6.
22. Markos F, Snow HM. The potentiation of sinus arrhythmia by vasoactive intestinal polypeptide (VIP) in the anesthetized dog. *Neuropeptides* 2001;**35**:238–43.
23. Pinter A, Nadeau R, Danden N, Page PL. Effect of vasoactive intestinal peptide on pacemaker location and heart rate in the dog atrium. *Can J Physiol Pharmacol* 1998;**76**:457–62.
24. Popma JJ, Smitherman TC, Bedotto JB, Eichhorn EJ, Said SI, Dehmer GJ. Direct coronary vasodilaton induced by intracoronary vasoactive intestinal peptide. *J Cardiovasc Pharmacol* 1990;**16**:1000–6.
25. Rigel DF, Lathrop DA. Vasoactive intestinal polypeptide facilitates atrioventricular nodal conduction and shortens atrial and ventricular refractory periods in conscious and anesthetized dogs. *Circ Res* 1990;**67**:1323–33.
26. Robberecht P, Gillet L, Chatelain P, De Neef P, Camus JC, Vincent M, et al. Specific decrease of secretin/VIP-stimulated adenylate cyclase in the heart from the Lyon strain of hypertensive rats. *Peptides* 1984;**5**:355–8.
27. Roques BP, Noble F, Dauge V, Fournie-Zaluski MC, Beaumont A. Neutral endopeptidase 24.11: structure, inhibition, and experimental and clinical pharmacology. *Pharmacol Rev* 1993;**45**:87–146.
28. Sawmiller DR, Henning RJ. Vasoactive intestinal peptide. In: Kastin AJ, editor. *Handbook of biologically active peptides*. 1st ed. San Diego, CA: Elsevier Academic Press; 2006. p. 1215–22.
29. Sawmiller DR, Henning RJ, Cuevas J, DeHaven WI, Vesely DL. Coronary vascular effects of vasoactive intestinal peptide in the isolated perfused rat heart. *Neuropeptides* 2004;**38**:289–97.
30. Schroeder A, Loh D, Jordan M, Roos K, Colwell C. Circardian regulation of cardiovascular function: a role for vasoactive intestinal peptide. *Am J Physiol Heart Circ Physiol* 2011;**300**:H241–50.
31. Smitherman TC, Popma JJ, Said SI, Krejs GJ, Dehmer GJ. Coronary hemodynamic effects of intravenous vasoactive intestinal peptide in humans. *Am J Physiol* 1989;**257**:H1254–62.
32. Tiaho F, Nerbonne JM. VIP and secretin augment cardiac L-type calcium channel currents in isolated adult rat ventricular myocytes. *Eur J Physiol* 1996;**432**:821–30.
33. Unverferth DV, O'Dorisio TM, Miller MM, Uretsky BF, Magorien RD, Leier CV, et al. Human and canine ventricular vasoactive intestinal polypeptide: decrease with heart failure. *J Lab Clin Med* 1986;**108**:11–6.
34. Wei Y, Mosjov S. Tissue specific expression of different human receptor types for pituitary adenylate cyclase activating polypeptide and vasoactive intestinal polypeptide: implications for their role in human physiology. *J Neuroendocrinol* 1996;**8**:811–7.
35. Ye VZC, Hodge G, Yong JLC, Duggan KA. Vasopeptidase inhibition reverses myocardial vasoactive intestinal peptide depletion and decreases fibrosis in salt sensitive hypertension. *Eur J Pharmacol* 2004;**485**:235–42

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Chapter 197

VP/OT

Jolanta Gutkowska and Marek Jankowski

ABSTRACT

Two structurally similar neurohypophysial hormones, arginine vasopressin (AVP) and oxytocin (OT) have different actions in the cardiovascular system. The major cardiovascular function of AVP is to protect the blood volume and osmolality through influences on renal handling of free water and vasoconstriction. AVP acts as an important neurohormonal mediator of heart failure syndrome, septic shock, and hyponatremic states in humans. AVP agonists and antagonists are emerging as therapeutic agents for these disorders. OT stimulation of atrial natriuretic peptide release from the heart is associated with cardioprotection. Other OT cardiovascular effects include natriuresis and lowering of blood pressure; negative cardiac inotropy, and chronotropy; the NO pathway stimulation involved in vasodilatation and endothelial cell growth; anti-inflammatory activity and wound healing; antioxidant activity; and metabolic effects, including stimulation of glucose uptake in the cardiac muscle. In addition, both OT and AVP have the capacity to generate cardiomyocytes from stem cells.

DISCOVERY

Interest in the cardiovascular action of pituitary extracts dates back more than a hundred years. In 1895, the vasoconstrictive and hypertensive effects of pituitary extracts were demonstrated by Oliver and Schafer. Two years later, Howell concluded that glycerine and saline extracts of the pituitary anterior lobe produced little or no change in blood pressure and heart rate, and that Oliver and Schafer's results were explained by substance(s) residing in the gland's posterior lobe.

In 1928, Kamm et al. and Gaddum observed a wide diversity of physiological effects by extracts of the neurohypophysis and provided conclusive evidence using factional precipitation for the presence of the two active "principle," one, which produces an increase in blood pressure in dogs, another that stimulates the contraction of uterine muscles in guinea pigs; the two hormones were later recognized as arginine–vasopressin (AVP) and oxytocin (OT). In the 1940s, Woodbury et al. reported that uterine-active fractions of pituitary extracts are associated with the cardiovascular system and its depressor activity in various species, including humans. The results of Verney's pioneering studies in dogs, published in 1947, led to the concept of "osmoreceptors" and the hypothesis of antidiuretic hormone (ADH) present in the neurohypophyseal extract. The structures of OT and AVP were elucidated by Du Vigneaud et al. in the 1950s. Since these early studies, it has become widely accepted that AVP and OT, known as neurohypophysial hormones, are not only important to the central nervous system (CNS) but they also have physiological actions in the peripheral organs, including the kidneys, heart, and vascular beds.

PEPTIDE STRUCTURE

Both AVP and OT have a simple structure, being nonapeptides and consisting of a six-amino acid ring closed by a disulfide bridge with three amino acids in a side chain. The two hormones differ only by two amino acids (Fig. 1) and are encoded by distinct genes, but they lie on the same chromosome at 20 p, separated by a segment of DNA only 12 kb long. The similarities in structure, and the close localization, are suggestive of recent gene duplication. The peptides are synthesized in the distinct populations of magnocellular and parvocellular neurons of the hypothalamic paraventricular and supraoptic nuclei. They are transcribed as larger prohormones with the carrier protein neurophysin and are cleaved into their final forms while in transit by the supraoptic-hypophyseal tract to the secretory granules in the posterior pituitary gland. On appropriate stimulation, AVP and OT are released from the storage granules and rapidly enter

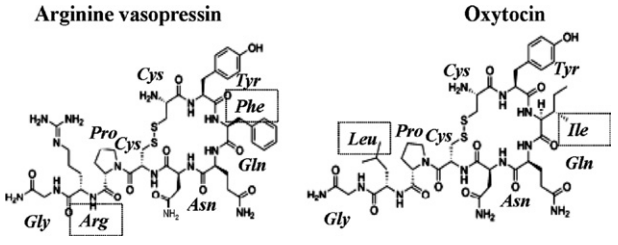

FIGURE 1 Structures of the oxytocin and arginine–vasopressin. The different amino acids are marked in boxes.

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00197-4
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the bloodstream by the well-vascularized posterior pituitary capillary bed, which is devoid of a blood–brain barrier.

BIOLOGICAL ACTIVITY

The primary function of AVP in the body is to regulate the extracellular fluid volume by affecting renal handling of water, although it is also a vasoconstrictor and pressor agent (hence, the name "vasopressin"). The major stimuli to produce AVP in the supraoptic nuclei and paraventricular nuclei of the hypothalamus are low blood volume and hypernatremia (low blood sodium levels). Angiotensin II enhances and atrial natriuretic peptide (ANP) inhibits osmotically stimulated AVP release. AVP has long been suspected of playing a role in blood pressure control resulting from its vasoconstrictive effects, which have been well demonstrated *in vitro*. However, AVP elevation above normal concentrations in patients with the syndrome of inappropriate antidiuretic hormone secretion (SIADH) does not result in hypertension, but it renders patients hypervolemic and hyponatremic. Disorders of the AVP system also include neurogenic and nephrogenic diabetes insipidus (DI); when the hormone is absent or unable to activate its antidiuretic receptors, diuresis and consequently water needs are enhanced about tenfold.

OT represents the endocrine component of a neuroendocrine reflex that mediates suckling-induced lactation (the milk "let-down" reflex). It is also a potent stimulant of uterine contractions, especially in an estrogen-primed uterus. This latter effect is exploited in its major pharmacological application—induction or augmentation of labor—the the precise contribution of endogenous OT to normal labor is still controversial (i.e. parturition can occur in its absence). However, recent studies have shown that OT is a ubiquitous hormone, synthesized at many loci, and a wide array of physiological activities has been attributed to this peptide. Similar numbers of oxytocinergic neurons have been found in the male and female hypothalamus, and the same stimuli induce OT release in both genders, suggesting other physiological functions than its role in female reproduction. Recent evidence indicates that there are some unknown effects of OT. It has been shown that this peptide is involved in the regulation of memory, trust, food and water consumption, respiratory function, metabolic processes (regulation of carbohydrate and fat metabolism), and immune homeostasis. OT regulates the functions of the cardiovascular system. Systemic administration of OT has significant effects on blood pressure, vascular tone, and heart contractility.

RECEPTORS

The cellular effects of AVP and OT are mediated by the stimulation of tissue-specific G protein-coupled receptors with different kinases through specific motifs that are present in the carboxyl termini of the receptor. The signals are mediated through Gs and Gq protein subtypes. A single receptor can activate multiple second-messenger pathways by interacting with different G-proteins. OTs act on only one type of OT receptor (OTR), an integral membrane protein that is a member of the rhodopsin-type (class I) G protein-coupled receptor family, which includes AVP receptor subtypes (V1aR, V1bR or V3R and V2R). OTR can be considered a "nonselective" AVP receptor. OTR has equal affinity for AVP and OT, whereas V1aR has a 30-fold higher affinity for vasopressin than for OT.[29]

V1aR-stimulated Gq leads to the mobilization of endoplasmic reticulum calcium stores and generation of inositol 3 phosphate (IP3). The biological effects mediated through V1aR include vasoconstriction, myocardial hypertrophy, platelet aggregation, and growth of vascular smooth muscle cells. V1aR, which is abundant in vascular smooth muscles, causes vasoconstriction and a positive inotropic effect in cardiac muscle. Prolonged V1aR stimulation leads to the synthesis of proteins involved in cellular hypertrophy in vascular and myocardial tissues. V3R, predominantly found in the anterior pituitary gland, is linked to IP3 with intracellular calcium as a second messenger. The physiologic role of the V3R is not completely understood, and it is presumed to be involved in corticotropin and glucagon release, in addition to cellular proliferation and differentiation effects. V2R, however, couples to a Gs protein, which, by a cAMP (cyclic adenosine 3′,5′-monophosphate) dependent mechanism, allows for the active transport of water and sodium regulation and is found primarily in the principal cells of the renal-collecting duct. Consequently, the activation of cyclic AMP-dependent protein kinase (PKA) mediates the hydroosmotic effects of AVP. Through protein phosphorylation, AVP promotes trafficking of aquaporin 2 (AQP2) water channels, allowing free-water reabsorption back into the circulation. V2R has long been thought to be exclusively expressed in renal tubules, but there is increasing evidence of extrarenal V2R expression.[11]

OT has peripheral actions and actions in the brain. In addition to the mammary glands, OTR may be also found in the heart, vascular endothelium, kidney epithelium of mammals, hypothalamus, anterior pituitary, some areas of the cortex, and in the regions of the CNS that are not innervated by oxytocinergic fibers.[8] The activation of this receptor stimulates Gq and increases phospholipase-C with an increase in IP3 and diacylglycerol (DAG). IP3 triggers calcium release from intracellular stores, whereas DAG stimulates protein kinase C, which phosphorylates unidentified target proteins. A variety of cellular events are initiated in response to an increase in intracellular calcium. For example, forming calcium–calmodulin complexes trigger the activation of neuronal and endothelial isoforms of nitric oxide synthase (NOS). NO in turn stimulates the soluble

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guanylate cyclase to produce cGMP, leading to vasodilation. Activation of cardiac OTR stimulates the release of atrial naturetic peptide (ANP), which also regulates diuresis and blood pressure using cGMP as a second messenger.[9] In smooth muscle cells, the calcium–calmodulin system triggers the activation of myosin light chain kinase activity, which initiates smooth muscle contractions (e.g. in myometrial or mammary myoepithelial cells)[8]

PATHOPHYSIOLOGICAL IMPLICATIONS IN THE CARDIOVASCULAR SYSTEM

Stimulation of AVP Release

A change in osmolality of 1–2% of the extracellular fluid compartment stimulates the release of AVP. An increase in plasma osmolality above approximately 280 mOsm/kg is the main stimulus for AVP release.[31] Hypothalamic osmoreceptors sense extracellular osmolarity and stimulate AVP release when osmolarity rises, as occurs with dehydration. Peripheral osmoreceptors are found in the portal veins. Baroreceptors located in the left atrium, carotid sinus, and aortic arch detect arterial underfilling, which stimulates the neurons in the supraoptic nucleus and paraventricular nucleus to produce AVP. Unlike osmoreceptors, baroreceptors must be suppressed in order to stimulate the release of AVP; this suppression in turn comes after a fall in blood pressure. In severe hypovolemic shock, when AVP release is very high, AVP contributes to the compensatory increase in systemic vascular resistance. Skin, muscle, and splanchnic arterial beds are highly sensitive to the vasoconstrictor actions of AVP, an effect that may be important for shunting blood to more vital organs. Exceptionally, rat mesenteric arteries were found to be particularly sensitive to AVP and constrict in response to concentrations of AVP as low as 1 pM.[13] The measurement of endogenous AVP in plasma is a useful tool for guiding therapy in heart failure and in different states of shock; however, serious concerns exist about the methodologic reliability of plasma AVP measurements because AVP is known to be unstable, largely attached to platelets, and rapidly cleared (half-life: 5–15 min). By contrast, the C-terminal portion of provasopressin (copeptin) is stable for several days after blood withdrawal and can be quickly measured, being secreted in equimolar amounts to AVP.[23] Recent studies have shown that copeptin is a strong prognostic marker in patients after an acute myocardial infarction and in patients with chronic heart failure.[34]

AVP in Septic Shock

AVP concentrations are inappropriately low in septic shock, although the reason for this is not clear. Loss of sensitivity to or downregulation of the AVP receptors may also be an important factor in the loss of pressor effects in some shock situations. However, in these conditions, which are caused by a bacterial infection in the blood, the infusion of AVP increases systemic vascular resistance and thereby elevates arterial pressure. Besides deficiency of AVP, there are two fundamental physiologic upsets: increased synthesis of NO and activation of ATP-sensitive potassium channels in vascular smooth muscle. The plasma concentration of NO is markedly increased in septic shock. The production of this endogenous vasodilator seems to occur because of the expression of inducible NOS by cytokines. Tissue hypoxemia, acidosis, and NO cause the activation of ATP-sensitive potassium channels, which facilitates the entry of potassium into smooth muscle cells and causes hyperpolarization of the membrane: the cells become resistant to depolarization and contraction. AVP agonists could provide an additive pressor to maintain cardiovascular dynamics in shock states. They reverse hypotension and restore vascular tone by activation of V1aR, modulation of ATP-sensitive potassium channels, interference with NO action, and potentiating of norepinephrine and other vasoconstrictor agents.[20]

AVP in Heart Failure

Heart failure commonly manifests as a syndrome of salt and water retention. The decrease in left ventricular (LV) systolic and diastolic function results in reduced cardiac output, stroke volume, and intraarterial blood volume. Arterial underfilling, sensed by arterial baroreceptors in the aortic arch and intrarenal sensors, leads to the subsequent activation of the sympatho-adrenal system and release of a cascade of neurohormones. Serum AVP concentrations are chronically elevated in several animal models of cardiac failure, and patients with heart failure consistently show elevated circulating levels of AVP in proportion to disease severity.[32] The increase in AVP observed in heart failure appears to be independent of osmotic stimuli. AVP worsens heart failure by causing vasoconstriction of arteries and veins, potentially contributing to remodeling of the LV and causing fluid retention and worsening of hyponatremia (Fig. 2). V2R receptor stimulation by AVP may contribute to volume expansion and therefore to increased cardiac preload. Increased preload exacerbates diastolic wall stress, in turn aggravating eccentric remodellng. This increase in AVP potentiates other pathways, including the renin–angiotensin–aldosterone system (RAAS), to promote retention of sodium and water and also the release of norepinephrine from the adrenal gland to increase inotropy. Moreover, AVP production and secretion by the heart has been suggested, especially in buffer perfused rat hearts.[14] The chronic elevation of AVP levels seen in patients with heart failure may ultimately have detrimental myocardial

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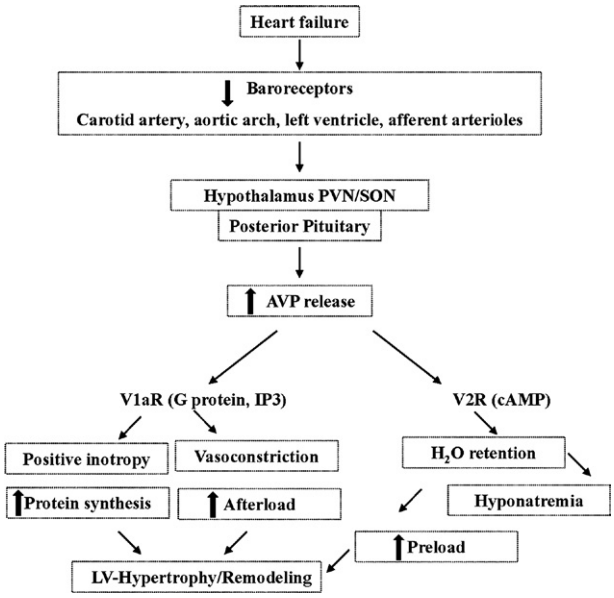

FIGURE 2 Mechanisms illustrating how the increased levels of circulating AVP worsen heart failure symptoms and function of left ventricle via activation cardiac V1AR linked to inositol 3 phosphate (IP3) and V2R in the kidney signaling cyclic adenosine 3′,5′-monophosphate (cAMP).

effects. The study of ratcardiomyocytes cultured in the presence of AVP has shown protein synthesis stimulation with concomitant increases in calcium concentration and activation of mitogen-activated protein kinase. Although it is not known whether AVP contributes to cardiomyocyte hypertrophy in humans, these mechanisms of cellular hypertrophy share many of the features seen in the typical hypertrophic response of the adult heart that is associated with other agonists, such as angiotensin II and endothelin-1.

AVP Antagonists

Because AVP regulates vascular tone and free-water reabsorption via V1aR and V2R, respectively, these receptors have become pharmacological targets for the treatment of congestive heart failure, and receptor antagonists are currently being tested in clinical trials.[21,27]. The problem with the treatment of congestive heart failure is to find drugs affecting both cardiac dysfunction and renal water balance function. Despite the recognized antagonistic effects of these molecules in animals, their use in the therapy of edematous disease has been hampered by marked species differences, partial agonist actions, poor bioavailability, and short half-life. As a result of subsequent developmental research, several studies have shown that AVP receptor antagonism increases urine volume, reduces body weight in patients with moderate to severe heart failure, and increases serum sodium concentrations, over hours to days, in the subset of patients with baseline hyponatremia. Tolvaptan (OPC-41061) is a selective competitive V2R antagonist approved by the Federal Drug Administration (FDA) for the treatment of hyponatremia associated with congestive heart failure, cirrhosis, and the syndrome of inappropriate antidiuretic hormone. Tolvaptan, when administered with traditional diuretics, increases excretion of excess fluids and improves blood sodium levels in patients with heart failure without producing side effects such as hypotension or hypokalemia (decreased blood levels of potassium) or having an adverse effect on kidney function. Lixivaptan and satavaptan are other highly specific antagonists of V2R, successfully investigated in clinical trials. Conivaptan is currently the only available dual V1aR/V2R nonpeptide antagonist approved for hypervolemic hyponatremia. Because AVP acts on both the receptors to cause water retention and vasoconstriction in heart failure, respectively, conivaptan represents an excellent treatment option. Decreased intracellular free calcium and mitogen-activated protein kinase activity have been observed to occur in a dose-dependent manner, suggesting a reduction in the intracellular protein synthesis and possibly cardiomyocyte hypertrophy.[4] However, conivaptan is yet to be approved for the treatment of heart failure in humans.

In summary, AVP antagonists are effective in treating volume overload by increasing diuresis in patients with heart failure, but there has been little evidence of associated symptomatic improvement. AVP antagonists have a favorable renal profile. Unlike other neurohormonal antagonists (β-blockers and RAAS blockers), inhibition of AVP receptors has no documented beneficial effects on ventricular remodeling .

OT in the Heart

The discovery of the cardiac OT system emerged from long-time investigations into the role of the brain in the control of cardiorenal homeostasis. These experiments led to the observations that OT and its OTR are synthesized in human and rat hearts and that OT exerts cardioprotection either directly or via stimulation of mediators, such as ANP and NO. OTR in the rat heart seems to be identical to those in the uterus and other organs. The presence of OTR in rat and human hearts was detected by RT-PCR and by in situ hybridization, autoradiography of atrial and ventricular sections, and confirmed by competitive binding assay with iodinated OT antagonist.[9,10] The functionality of heart OTR was shown by the ability of OT to release ANP from the isolated perfused rat heart.[9] OT injected intraperitoneally or intravenously induces dose-related increases in plasma ANP that correlate with increases in sodium and potassium excretion.[10] Blood volume expansion with isotonic and hypertonic saline stimulates ANP and OT release. Suckling, a physiological stimulus for OT release, is also associated

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with significant increases in plasma ANP. OT antagonist inhibits the stimulated and also basal ANP release from perfused rat hearts, suggesting the presence of cardiac OT.[17] Indeed, the presence of OT in the heart has been detected in rodent and human hearts.

OT in the Vasculature

Because we discovered that OT is produced and released by the heart and acts on its cardiac receptors to decrease the cardiac rate and force of contraction, it was expected that OT might be generated in the vasculature and dilate vessels. Indeed, studies showed that an intrinsic OT system, sensitive to estrogenic regulation, exists in the vasculature.[18,35] Immunoreactive OT has been found in rat, sheep, and dog vessels by specific radioimmunoassay, and characterized by high-performance liquid chromatography. RT-PCR analysis has identified transcripts containing the coding sequence of OT and OTR genes in the aorta and vena cava.[18] Further studies have confirmed that OTR is present in several major cell types that are important in the progression of vascular pathologies, including vascular smooth muscle cells, endothelial cells, macrophages, and adipocytes.

Cardiovascular Effects of OT

Systemic administration of OT has significant effects on blood pressure, vascular tone, and cardiovascular regulation. Conversely, the absence of either OT or its receptors in knockout mice has not been reported to produce cardiac insufficiencies. Although OT knockout mice have a normal heart structure, experiments have shown increased intrinsic heart rates in these animals, indicating that an intracardiac OT system controls cardiac electrical activity.[2]

Accumulating evidence indicates multiple beneficial effects in the heart and vasculature (Fig. 3). To date, the cardiovascular properties of OTs include i. natriuresis and decreased blood pressure, possibly secondary to ANP release;[9,10] ii. negative inotropic and chronotropic effects[9] and parasympathetic neuromodulation;[24] iii. vasodilatation via the OTR-induced NO pathway and endothelial cell growth;[35] iv. wound healing and anti-inflammatory action.[15]

Both increases and decreases in blood pressure in response to OT have been reported, depending on the species, route of administration, dose, etc. Experiments with central administration of OT or OT antagonists have not provided unequivocal results. In some studies, OT has elicited a significant increase of blood pressure or cardioacceleration, whereas other investigations have provided evidence that OT enhances baroreflex bradycardia and reduces resting arterial blood pressure and exercise-induced tachycardia.[33] Peripheral administration of OT, on the contrary, lowers the average arterial pressure in rats and does not affect the heart rate.[30] So far, OT is the only known factor that protects the cardiovascular system from the excessive increases in blood pressure and heart rate during stress. However, in the absence of a central regulatory influence, OT can bring down the heart rate and reduce the strength of contractions of isolated atria during perfusion of rat hearts.[24] The negative chronotropic action of OTs has recently been associated with attenuation of cardiac damage evidenced by ischemia reperfusion.[25]

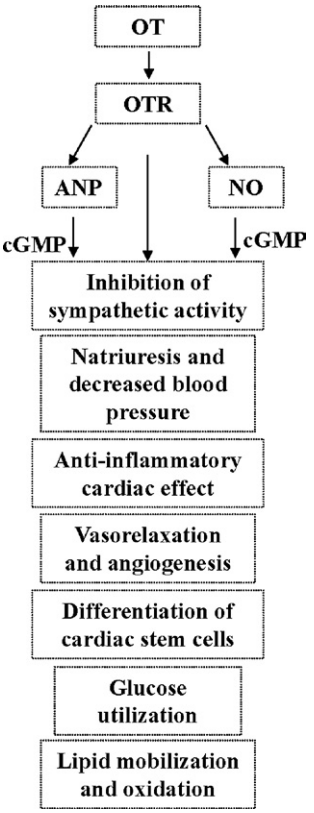

FIGURE 3 Cardiac OTR activation can induce several effects in the heart, including inhibition of sympathetic activity, stem cell differentiation, vasorelaxation, and angiogenesis, improved glucose utilization, lipid oxidation as well as anti-inflammatory outcomes. These actions of OT are mediated by atrial natriuretic peptide (ANP) or nitric oxide (NO), known to reduce oxidative stress and fibrosis, and result in improved intracellular Ca^{++} handling in pathological conditions.

Positive cardiac effects can be attributed to the fact that OT stimulates ANP release by improving hydromineral homeostasis, and cardiac hypertrophy and regulating and maintaining a balance of anti-inflammatory and proinflammatory cytokines within the injured heart in rats. The different cardioprotective mechanisms of OT have recently been demonstrated in animal models of myocardial infarction. In rat and rabbit models of ischemic heart disease, OT treatment significantly reduced infarct size and improved parameters of heart function.[15,19]

OT has an impact on metabolism.[8] OTR knockout in mice results in obesity. In fact, OT has insulin-like effects

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and properties, that is, it stimulates glucose oxidation and lipogenesis in adipocytes, inhibits lipolysis, decreases the blood level of free fatty acids, and increases pyruvate dehydrogenase activity. Cardiovascular abnormalities in OTR-deficient mice are yet to be found. However, in the db/db mouse model of obesity and diabetes, a deficiency in the leptin receptor leads to increased cardiac apoptosis and fibrosis, as well as downregulation of OTR and natriuretic peptides expression in the heart.

OT signaling in Cardiac Cells

OT increases glucose uptake in cardiomyocytes via phosphoinositide-3-kinase (PI3 K) and potentiates the glucose uptake effect of 2,4-dinitrophenol, an uncoupler of oxidative phosphorylation targetting the mitochondria.[5] PI3 K pathways are considered beneficial during myocardial injuries. The calcium–calmodulin kinase kinase (Ca-CAMKK) and AMP-activated protein kinase (AMPK) pathways are also involved in OT-mediated glucose uptake in cardiomyocytes.[5] AMPK activation in the heart after ischemia and reperfusion is recognized as cardioprotective because it limits both apoptosis and cell damage.

Studies conducted *in vitro* have revealed that OT modulates processes that are critical to early lesion formation within vascular and immune tissues.[35] Specifically, OT has antioxidant effects on vascular smooth muscle cells, aortic endothelial cells, and macrophages through attenuation of NADPH-oxidase-dependent superoxide production.[10,15,19] *In vivo*, peripheral OT administration inhibits atherosclerotic lesions in the thoracic aorta. In addition, OT promotes the migration of human dermal endothelial cells (EC), breast-derived EC and human umbilical vein EC (HUVEC). The promigratory effect of OT requires OTR activation of the PI3 K/AKT/eNOS pathway. Moreover, OT increases the proliferation of EC and alters gene expression for adhesion molecules and matrix metalloproteinases, contributing to improved cell motility and growth. An angiogenic and antiapoptotic OT effect is indicated by increased cardiac $CD31^+$ microvessels. In this way, OT can control the flow of blood to the heart.

OT AND AVP IN CARDIOMYOGENESIS

Effect of OT in Embryonic stem (ES) Cells

OT possesses proliferative properties depending on the cell type and the subcellular localization of OTR. Furthermore, OT can induce mammalian stem cells into a special cell type that retains the ability to self-renew (i.e. undergo cell division in an undifferentiated state) indefinitely and to differentiate into specialized cells. The OT system activation in fetal and newborn hearts at a stage of intense cardiac hyperplasia[16] suggests a role for OT in cardiomyocyte differentiation. Initial experiments have shown that OT induces beating cell colonies in the murine embryonal carcinoma P19 cell line, an established cell model for studying early heart differentiation.[28] Since that study, several other reports have confirmed OT-stimulated cardiomyogenesis in different lines of embryonic stem cells. In embryonic D3 stem cells, spontaneously producing beating cell colonies on aggregation, the mesodermal derivatives formed in embryoid bodies include subtypes of cardiac cells (atrial cardiomyocytes, ventricular cardiomyocytes, and pacemaker cells) that are potently enhanced by treatment with OT as identified by histological, molecular, and electrophysiological criteria.[6] The OTR-NO-cGMP pathway that is essential for OT-elicited differentiation of P19 stem cells into cardiomyocytess (Fig. 4) is associated with elevation of GATA-4 and myocyte enhancer factor 2c (MEF2c).[3] GATA-4 regulates the expression of genes that are critical for cardiomyocyte differentiation. MEF2c is a member of the MEF2 family, which is involved in cardiac, skeletal and smooth muscle development. GATA-4 has also been implicated in intercellular crosstalk by inducing hypertrophy-associated angiogenesis via vascular endothelial growth factor (VEGF) release and targeting the endothelium.[12] Stimulation of cardiomyocyte differentiation can be concomitant with neovascularization because OT stimulates endothelial cell growth and angiogenesis.[35] The upstream sequence of OTR contains putative binding sites for GATA-4 and Nkx2.5 and GATA-4 also serves as a key transcriptional regulator of numerous cardiac peptides, including ANP, brain natriuretic peptide (BNP), and OTR. GATA-4 has also been identified in stem and progenitor cells of the heart in combination with OT-mediated cardiomyocyte differentiation.[3,22,26] Undifferentiated murine ES cells express BNP and its receptors, with its signaling being essential for cell survival and clonal growth.[1] This observation suggests possible interaction of the OT and natriuretic peptide systems in ES cells during cardiomyogenesis.

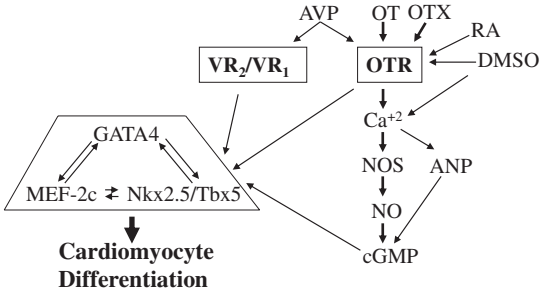

FIGURE 4 Major steps during OT-mediated cardiomyocyte differentiation. ANP, atrial natriuretic peptide; AVP, arginine vasopressin; cGMP, cyclic guanosine monophosphate; DMSO, dimethylsulfoxide; NO, nitric oxide; NOS, nitric oxide synthase; OTR, oxytocin receptor; OTX, carboxy-extended form of OT; RA, retinoic acid.

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Effect of AVP in Cardiomyogenesis

Similarly to OT, AVP also induces the expression of early and late myogenic differentiation markers in cultured myogenic cell lines. As reported recently, AVP modulates spontaneous cardiomyogenesis in embryonic D3 cells by augmenting cardiomyocyte yield and promoting the selection of the ventricular electrophenotype.7 The AVP-mediated increase in endothelial NO synthase expression also seems to be partly regulated by OTR, inasmuch as lower endothelial NO synthase mRNA and protein levels have been observed in D3 cells treated with OTR antagonist.[7] There is considerable diversity of OT-mediated signaling pathways, including cAMP activation and cGMP-mediated growth stimulation with calcium increases, which corroborates AVP signaling mediated by V1aR and V2R. The specific pathway that activates cardiomyogenesis in response to OT and AVP is unknown. However, enhanced GATA-4 expression, consistently observed throughout cardiac differentiation upon AVP stimulation[7] and after OT exposure of stem cells,[3,11] may contribute significantly to the cardiomyogenic actions of both peptides.

OT and Cell Therapy of Cardiac Injuries

OT can be considered an established myogenic morphogen. The idea that OT has cardioregenerative capacities derives from the observation that this hormone induces the differentiation of cultured mice[22] and rats[26] resident cardiac stem cells (CSCs). In the adult heart, CSCs maintain a balance of survival, proliferation and self-renewal to replace mature cells that are lost during injury or turnover. Matsuura et al.[22] revealed the presence of a Sca-1$^+$ stem cell population in adult mouse hearts expressing telomerase reverse transcriptase, which has been associated with self-renewal potential. These cells, lacking hematopoietic markers, are easily distinguished from hematopoietic stem cells of bone marrow origin, and when treated with OT, differentiate into functional cardiomyocytes. Although the cells present the early cardiac markers GATA-4 and MEF2, they do not express Nkx-2 or genes encoding cardiac sarcomeric proteins. When exposed to OT, a small population of Sca-1$^+$ cells manifests sarcomeric structures and forms spontaneously-beating cardiomyocytes. In addition, after intravenous delivery, Sca-1+ cardiac stem cells can be recruited to the myocardium injured by ischemia/reperfusion and can functionally differentiate in situ.[22]

In pathological conditions such as cardiac ischemia and diabetes, OT can be used to stimulate the production of lost cardiac cells. The advantage of this therapy is supported by the fact that OT is endogenously produced in the organism, and does not have significant side effects when used in clinics. Moreover, it is now possible to transplant one's own stem cells after previous stimulation with OT inducers, as in the case of a myocardial infarct. Alternatively, direct treatment with OT molecules could promote cardiomyogenesis in situ and regeneration of a damaged heart.

REFERENCES

1. Abdelalim EM, Tooyama I. BNP signaling is crucial for embryonic stem cell proliferation. *PLoS ONE* 2009;**4**:e5341.
2. Bernatova I, Rigatto KV, Key MP, Morris M. Stress-induced pressor and corticosterone responses in oxytocin-deficient mice. *Exp Physiol* 2004;**89**:549–57.
3. Danalache BA, Paquin J, Donghao W, Grygorczyk R, Moore JC, Mummery CL, et al. Nitric oxide signaling in oxytocin-mediated cardiomyogenesis. *Stem Cells* 2007;**25**:679–88.
4. Finley JJT, Konstam MA, Udelson JE. Arginine vasopressin antagonists for the treatment of heart failure and hyponatremia. *Circulation* 2008;**118**:410–21.
5. Florian M, Jankowski M, Gutkowska J. Oxytocin increases glucose uptake in neonatal rat cardiomyocytes. *Endocrinology* 2010;**151**: 482–91.
6. Gassanov N, Devost D, Danalache B, Noiseux N, Jankowski M, Zingg HH, et al. Functional activity of the carboxyl-terminally extended oxytocin precursor peptide during cardiac differentiation of embryonic stem cells. *Stem Cells* 2008;**26**:45–54.
7. Gassanov N, Jankowski M, Danalache B, Wang D, Grygorczyk R, Hoppe UC, et al. Arginine vasopressin-mediated cardiac differentiation: insights into the role of its receptors and nitric oxide signaling. *J Biol Chem* 2007;**282**:11255–65.
8. Gimpl G, Fahrenholz F. The oxytocin receptor system: structure, function, and regulation. *Physiol Rev* 2001;**81**:629–83.
9. Gutkowska J, Jankowski M, Lambert C, Mukaddam-Daher S, Zingg HH, McCann SM. Oxytocin releases atrial natriuretic peptide by combining with oxytocin receptors in the heart. *Proc Natl Acad Sci U S A* 1997;**94**:11704–9.
10. Gutkowska J, Jankowski M. Oxytocin revisited: it is also cardiovascular hormone. *J Am Soc Hypertens* 2008;**2**:318–25.
11. Gutkowska J, Miszkurka M, Danalache B, Gassanov N, Wang D, Jankowski M. Functional arginine vasopressin system in early heart maturation. *Am J Physiol Heart Circ Physiol* 2000;**293**:H2262–2270.
12. Heineke J, Auger-Messier M, Xu J, Oka T, Sargent MA, York A, et al. Cardiomyocyte GATA4 functions as a stress-responsive regulator of angiogenesis in the murine heart. *J Clin Invest* 2007;**117**:3198–210.
13. Henderson KK, Byron KL. Vasopressin-induced vasoconstriction: two concentration-dependent signaling pathways. *J Appl Physiol* 2007;**102**:1402–9.
14. Hupf H, Grimm D, Riegger GA, Schunkert H. Evidence for a vasopressin system in the rat heart. *Circ Res* 1999;**84**:365–70.
15. Jankowski M, Bissonauth V, Gao L, Gangal M, Wang D, Danalache B, et al. Anti-inflammatory effect of oxytocin in rat myocardial infarction. *Basic Res Cardiol* 2010;**105**:205–18.
16. Jankowski M, Danalache B, Wang D, Bhat P, Hajjar F, Marcinkiewicz M, et al. Oxytocin in cardiac ontogeny. *Proc Natl Acad Sci U S A* 2004;**101**:13074–9.
17. Jankowski M, Hajjar F, Kawas SA, Mukaddam-Daher S, Hoffman G, McCann SM, et al. Rat heart: a site of oxytocin production and action. *Proc Natl Acad Sci U S A* 1998;**95**:14558–63.
18. Jankowski M, Wang D, Hajjar F, Mukaddam-Daher S, McCann SM, Gutkowska J. Oxytocin and its receptors are synthesized in the rat vasculature. *Proc Natl Acad Sci U S A* 2000;**97**:6207–11.

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19. Kobayashi H, Yasuda S, Bao N, Iwasa M, Kawamura I, Yamada Y, et al. Postinfarct treatment with oxytocin improves cardiac function and remodeling via activating cell-survival signals and angiogenesis. *J Cardiovasc Pharmacol* 2009;**54**:510–9.
20. Landry DW, Oliver JA. The pathogenesis of vasodilatory shock. *N Engl J Med* 2001;**345**:588–95.
21. Lehrich RW, Greenberg A. Hyponatremia and the use of vasopressin receptor antagonists in critically ill patients. *J Intensive Care Med* 2011 May 13:[Epub ahead of print].
22. Matsuura K, Nagai T, Nishigaki N, Oyama T, Nishi J, Wada H, et al. Adult cardiac Sca-1-positive cells differentiate into beating cardiomyocytes. *J Biol Chem* 2004;**279**:11384–91.
23. Morgenthaler NG, Struck J, Alonso C, Bergmann A. Assay for the measurement of copeptin, a stable peptide derived from the precursor of vasopressin. *Clin Chem* 2006;**52**:112–9.
24. Mukaddam-Daher S, Yin YL, Roy J, Gutkowska J, Cardinal R. Negative inotropic and chronotropic effects of oxytocin. *Hypertension* 2001;**38**:292–6.
25. Ondrejcakova M, Ravingerova T, Bakos J, Pancza D, Jezova D. Oxytocin exerts protective effects on in vitro myocardial injury induced by ischemia and reperfusion. *Can J Physiol Pharmacol* 2009;**87**:137–42.
26. Oyama T, Nagai T, Wada H, Naito AT, Matsuura K, Iwanaga K, et al. Cardiac side population cells have a potential to migrate and differentiate into cardiomyocytes in vitro and in vivo. *J Cell Biol* 2007;**176**:329–41.
27. Pang PS, Gheorghiade M, Dihu J, Swedberg K, Khan S, Maggioni AP, et al. Effects of tolvaptan on physician-assessed symptoms and signs in patients hospitalized with acute heart failure syndromes: analysis from the efficacy of vasopressin antagonism in heart failure outcome study with tolvaptan (EVEREST) trials. *Am Heart J* 2011;**161**:1067–72.
28. Paquin J, Danalache BA, Jankowski M, McCann SM, Gutkowska J. Oxytocin induces differentiation of P19 embryonic stem cells to cardiomyocytes. *Proc Natl Acad Sci U S A* 2002;**99**:9550–5.
29. Peter J, Burbach H, Adan RA, Lolait SJ, van Leeuwen FW, Mezey E, et al. Molecular neurobiology and pharmacology of the vasopressin/oxytocin receptor family. *Cell Mol Neurobiol* 1995;**15**:573–95.
30. Petersson M. Cardiovascular effects of oxytocin. *Prog Brain Res* 2002;**139**:281–8.
31. Robertson GL. The use of vasopressin assays in physiology and pathophysiology. *Semin Nephrol* 1994;**14**:368–83.
32. Rosner MH, Ronco C. Hyponatremia in heart failure: the role of arginine vasopressin and its antagonism. *Congest Heart Fail* 2010;**16**(Suppl. 1):S7–14.
33. Szczepanska-Sadowska E. Role of neuropeptides in central control of cardiovascular responses to stress. *J Physiol Pharmacol* 2008;**59**(Suppl. 8):61–89.
34. Tentzeris I, Jarai R, Farhan S, Perkmann T, Schwarz MA, Jakl G, et al. Complementary role of copeptin and high-sensitivity troponin in predicting outcome in patients with stable chronic heart failure. *Eur J Heart Fail* 2011;**13**:726–33.
35. Thibonnier M, Conarty DM, Preston JA, Plesnicher CL, Dweik RA, Erzurum SC. Human vascular endothelial cells express oxytocin receptors. *Endocrinology* 1999;**140**:1301–9.

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Chapter 198

Adrenomedullin 2/ Intermedin

Yoshio Takei

ABSTRACT

Adrenomedullin 2 (AM2), or intermedin (IMD), is a new member of the calcitonin-gene-related peptide (CGRP) family that was formerly believed to consist of CGRP, AM, and amylin in mammals. Five AM peptides (AM1–5) form an independent subfamily in teleost fish, of which AM1 is an ortholog of mammalian AM. Comparative genomic analyses reveal that that AM2 and AM5 are also present in mammals. The AM2/IMD gene is expressed in various tissues, including the kidney, digestive tract, heart, brain, and pituitary, and the peptide exerts spectra of actions in these tissues such as diuresis/natriuresis, hypotension, and regulations of appetite and other hormone secretion. AM2/IMD also has cytoprotective functions against oxidative stress. Unlike other members of the CGRP family that have specific receptors, AM2/IMD binds almost all types of CGRP family receptors with lower affinity. The physiological and pharmacological results indicate that unknown AM2-specific receptor(s) may exist.

DISCOVERY

Because of the difference in the habitat, aquatic fishes and terrestrial tetrapods have developed distinct mechanisms for cardiovascular and body fluid regulation, which leads to the difference in major regulatory hormones, vasopressor and antidiuretic hormones in tetrapods and vasodepressor and natriuretic hormones in fishes. The CGRP family is one of the vasodepressor/natriuretic hormone groups, of which AM is most potent for these actions.[15] We expected that AM is an important cardiovascular and osmoregulatory hormone in fishes and searched for the AM ortholog in the genome database of tiger pufferfish, *Takifugu rubripes*, using the human AM sequence as query, which resulted in the identification of five AM-like peptides.[21] Molecular phylogenetic analyses reveal that five AM-like peptides form an independent subfamily in the CGRP family. Thus, we named them AM1–5 (Fig. 1). Fish AM1 is an ortholog of mammalian AM as determined by the synteny analysis and the presence of proadrenomedullin N-terminal 20 peptide (PAMP)-like sequence in the N-terminal region of the prohormone. Chromosomal localization of the five teleost AMs shows that duplication of AM1/AM4 and AM2/AM3 occurs at the third-round whole-genome duplication (3R) early in the teleost lineage, whereas the counterpart of the duplicated AM5 gene has disappeared[22] (Fig. 2). This shows that AM1/4, AM2/3, and AM5 already existed at the divergence of ray-finned fishes and lobe-finned fishes (tetrapods). Thus, we hypothesized that the AM2 and AM5 genes are still retained in mammalian genomes. The extensive search for AM2 and AM5 in the genome and EST databases of mammals, birds, and amphibians could identify probable candidates in most species.

Based on the molecular phylogenetic analysis and synteny analysis, we determined that the novel peptides are mammalian orthologs of teleost AM2 and AM5[22,30] (Fig. 3). Teddy Hsu's group[24] also found AM2 by comparative genomic analysis in fishes and mammals and named it intermedin (IMD) because of its dense localization in the intermediate lobe of the pituitary. The name "intermedin" has also been used for α-melanocyte stimulating hormone that is processed from the proopiomelanocortin in the intermediate lobe.[28] As many published articles have already used both names, the name AM2/IMD is used in this chapter to avoid confusion.

AM-like peptides were also identified in cartilaginous fish (sharks and rays) and cyclostomes (lampreys and hagfishes) that are most primitive extant vertebrates without jaw.[15] The cyclostome AM-like peptides seem to be hybrids of AM1 and AM2 because the mature sequence in the C-terminus is highly similar to AM2 but the PAMP-like sequence exists in the N-terminal region of the lamprey AM-like prohormone. The CGRP and amylin genes are not identifiable in the genome database of sea lamprey, *Petromyzon marinus*. CGRP/AM(1) and AM2/amylin exist on the same chromosome of teleost fish (Fig. 2). As CGRP/amylin and AM(1)/AM2 seem to be duplicated counterparts, CGRP or AM is an ancestral molecule of the CGRP family, and after

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00198-6
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Adrenomedullin 1
Takifugu          SKNLVNQSRKNGCSLGTCTVHDLAFRLHQLGFQYKIDIAPVDKISPQGY-NH2
Medaka            SKISNSQSRRQGCSLGTCTVHDLAHRLHELN--LRIGSAPADKISPKGY-NH2
Zebrafish         SKNSINQSRRSGCSLGTCTVHVLAHRLHDLNNKLKIGNAPVDKINPYGY-NH2

Adrenomedullin 2
Takifugu     HANNGGGRSHGQLMRVACVLGTCQVQNLSHRLYQLIGQSGKEDSSPMNPHSPHSY-NH2
Medaka       HANGSNGRGH--MMRVGCVLGTCQVQNLSHRLYQLIGQSGKEDSSPINPRSPHSY-NH2
Zebrafish    HAFRGS-RGHPQLMRVGCVLGTCQVQNLSHRLYQLNSQSRRQE-SPINPRSPHSY-NH2

Adrenomedullin 3
Takifugu    HIHSRGHHYPHPNQLIRAGCALGTCQVQNLSHRLYQLIGQSGRDDSSPINPKSPHSY-NH2
Medaka      HPRGV-HQYPHNAQLMRVGCFLGTCQVQNLSHRLYQLVGQKGREESSPFNPKSPHSY-NH2
Zebrafish   HVHSRGHHSHHHPQLMRVGCVLGTCQVQNLSHRLYQLVGQSGREDS-PINPRSPHSY-NH2

Adrenomedullin 4
Takifugu                     AGCALFMCAYHDLLQRLNHIYNKQKEVTAPKNKILSTGY-NH2
Medaka                       SGCALITCLYHDLVHLLHETNNKQREPCAPLKKMGINGY-NH2
Zebrafish                    AGCNLATCSVHELAHLLNIMHAKTN--NAPTDKIGSNGY-NH2

Adrenomedullin 5
Takifugu                   APQRGCQVGTCQVHNLANKLYQIG-RQGKDESTKVNDPQGY-NH2
Medaka                     ALQRGCQLGTCQLHNLANTLYHINKTNGKEESTKAHDPQGY-NH2
Zebrafish                  AAQRGCQLGTCQVHNLVNKLYRMGQSNGKDESKKANDPTGY-NH2
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FIGURE 1 Amino acid sequences of the adrenomedullin family in fishes. Sequences conserved in more than half of the species are shaded. AM2 and AM3 duplicated at the third-round whole-genome duplication are more conserved and more potent than AM1 and AM4 (Fig. 2). Brackets show disulfide bridges.

tandem duplication of amylin or another AM, the two tandem genes are duplicated together probably at the 2R whole-genome duplication that seems to have occurred during the transition from agnathans (jawless fishes) to gnathostomes (jawed fishes). It should be determined whether an AM type or a CGRP type is an ancestral molecule of the family (Fig. 2). Other fish-derived hormones including AM2/IMD are also described in Chapter 211 of this volume.

STRUCTURE OF THE PRECURSOR mRNA AND GENE

The AM2/IMD gene consists of three exons and is located on chromosome 22 in the human, chromosome 15 in the mouse and chromosome 7 in the rat, chromosome 6 in medaka and chromosome 4 in zebrafish.[22] The AM2/IMD precursors deduced from the mRNA sequences have 147–151 amino acid residues including the signal sequence of 24–25 amino acids in mammals and 169 amino acids with 20-amino-acid signal peptide in the pufferfish (Fig. 3). Mature AM2/IMD is at the C-terminus of the prohormone and the C-terminal glycine residue is followed by a stop codon. This precursor sequence is characteristic only for AM2 and AM3 of fish and for AM2/IMD of mammals, as AM1 and AM4 precursors are followed by a C-terminal peptide connected by 4–5 consecutive arginine residues as observed by mammalian AM.[21] Molecular phylogenetic analysis using mammalian and fish AM peptides shows that AM2/IMD is in the same cluster within the AM subfamily and distinct from the clusters of CGRP and amylin[21,29] (Fig. 4).

DISTRIBUTION OF mRNA

The tissue distribution of mRNA shows that the pufferfish AM2 gene is expressed almost exclusively in the brain.[21] In mice, however, AM2/IMD transcripts are detected in various tissues; high expression is observed in the kidney, submaxillary gland, stomach, and mesentery, followed by the pituitary, lung, pancreas, intestines, spleen, and thymus.[29] High expression is also detected in the digestive tract, especially in the stomach and colon in humans.[24] Radioimmunoassay for AM2/IMD measures high concentrations in the kidney, stomach, and plasma of rats (39.3 ± 11.4 fmol/ml, $n = 5$).[31]

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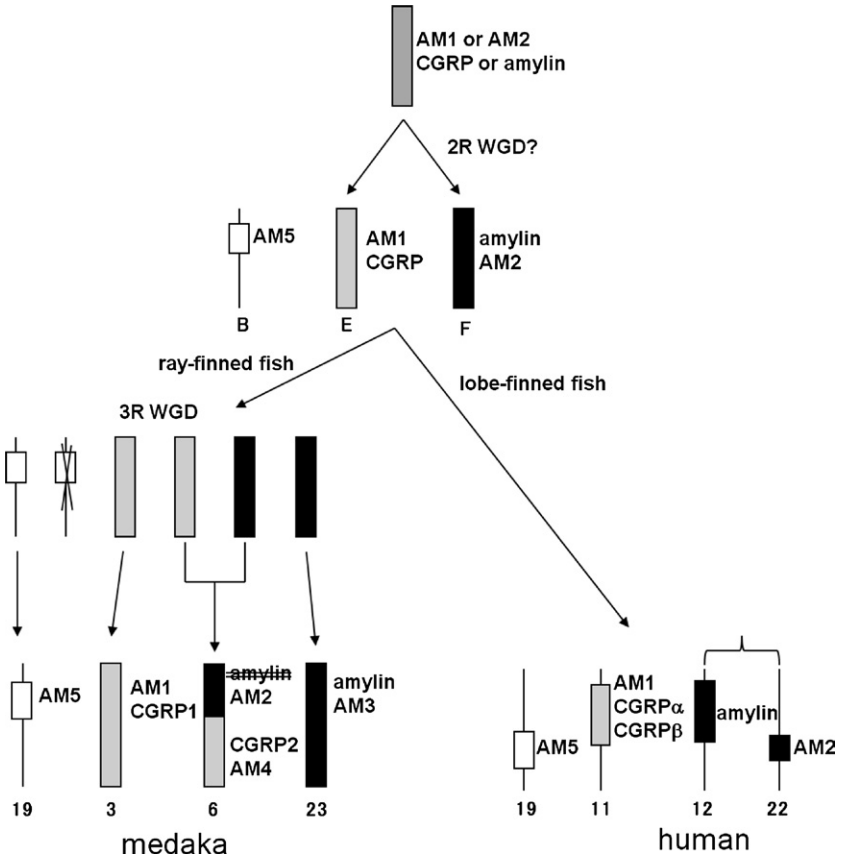

FIGURE 2 History of diversification of the calcitonin-gene-related peptide (CGRP) family. Adrenomedullin (AM) 1/AM2 and CGRP/amylin existed on the same chromosome before second-round whole-genome duplication (2R WGD) and AM5 may be generated from AM1 or AM2 by gene duplication after 2R WGD. AM1/CGRP, AM2/amylin, and AM5 existed before the divergence of ray-finned fish and lobe-finned fish, and all are retained in mammals, in which amylin and AM2 are separated after chromosomal reorganization and CGRPs are duplicated in tandem. In teleost fish, all genes are duplicated by third-round (3R) WGD but duplicated amylin and AM5 have disappeared. Duplicated AM2, AM4 (from AM1), and CGRP are on the same chromosome by chromosomal fusion. Chromosome name and number are shown below each chromosome.

AM2/IMD can be measured in human brain, pituitary, heart, and kidney, and its plasma concentration is 24.7 ± 1.8 fmol/ml ($n = 3$).[18] Immunoreactive AM2/IMD is localized in various brain areas including paraventricular and supraoptic nuclei in rats and humans.[27] AM2/IMD-positive cells increased profoundly after salt loading in rats. Immunoreactive AM2/IMD in the magnocellular neurons colocalizes with oxytocin in rats.[13] In the kidney, immunoreactive AM2/IMD is localized in glomerulus and vasa recta in mice[28] but only in the renal tubules in humans.[27] The heart also contains a moderate amount of AM2/IMD, where only endothelial cells of the coronary vessels exhibit positive staining in mice[28] but in the myocardial cells in humans.[27] Staining for immunoreactive AM2/IMD is localized in the intermediate lobe of pituitary in a few species,[24] though the pituitary concentration is lower than in the rat hypothalamus where the peptide is concentrated compared within other brain regions.[31] In addition, the AM2/IMD gene is expressed in the ovary but not in the testis, indicating some specific role for the female reproductive system.[29]

PROCESSING OF PRECURSOR

The inferred mature sequence of rat AM2/IMD consists of 47 amino acid residues (Fig. 3), assuming that proAM2/IMD is cleaved after two consecutive arginine residues by prohormone convertases, and that the C-terminal glycine residue is used for amidation. AM2/IMD-47 is more potent than N-terminally truncated AM2/IMD-42 for biological actions including vasodepressor and chronotropic effects as well as suppressive effects on food intake and gastric emptying.[24] However, the shorter AM2/IMD-42 is more potent for cAMP accumulation in cells expressing calcitonin-receptor-like receptor (CLR) and receptor-activity-modifying proteins (RAMPs), the combination of which produces AM receptors (see below). There are additional dibasic amino acids in the prosegment of rat and mouse AM2/IMD, and the cleavage at this site produces longer molecule consisting of 53 amino acid residues. This longer peptide seems to have a comparable cardioprotective activity.[33] Mature fish AM2 may be cleaved by a processing enzyme, furin,

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(A)

```
Human        1: MARIP--TAALGCISLLCLQLPGSLSRSLGGDPRPVKPREPPARSPSSSLQPRHPAPRPV 58
Rat          1: MAQLLMVTVTFGCISLLYL-LPGTLSGSLGKGLR---PREPPAKIPSSGPQPGHPSLRPV 56
Mouse        1: MAQLLMVTVTLGCISLLYL-LPGTLSGSLGKGLRHSRPREPPAKIPSSNLQPGHPSLQPV 59
Pufferfish   1: MRVLLPVWLLLSLLPLEVQARALSQQN-LGLPHRFSLLRTL--KIPKSSFIVIGPAASDP 57

Human       59: VWKLH-RALQAQRG--------------------AGLAPVMGQPLRDGGRQHSGPRRHSG 97
Rat         57: VWKPP-HALQPQGR--------------------GNPALATVHLPQGGGSRHPGPQRHVG 95
Mouse       60: VWKSRRHAPQPQGR--------------------GNRALAMVHLPQGGGSRHPGPQRPTG 99
Pufferfish  58: PEVTYHHVAQGDGRVTWMAWLRGKPLLGSSDPPLGQTDRVLRDTPAWRGRSRGRRHANNG 117

Human       98: PRRTQAQLLRVGCVLGTCQVQNLSHRLWQLMGPAGRQDSAPVDPSSPHSYG*        149
Rat         96: SRRPHAQLLRVGCVLGTCQVQNLSHRLWQLVRPSGRRDSAPVDPSSPHSYG*        147
Mouse      100: SRRPHAQLLRVGCVLGTCQVQNLSHRLWQLVRPAGRRDSAPVDPSSPHSYG*        151
Pufferfish 118: GGRSHGQLMRVACVLGTCQVQNLSHRLYQLIGQSGKEDSSPMNPHSPHSYG*        169
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(B)

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Human       TQAQLLRVGCVLGTCQVQNLSHRLWQLMGPAGRQDSAPVDPSSPHSY-NH2
Chimpanzee  TQAQLLRVGCVLGTCQVQNLSHRLWQLMGPAGRQDSAPVDPSSPHSY-NH2
Monkey      TQAQLLRVGCVLGTCQVQNLSHRLWQLMGPAGRQDSAPVDPSSPHSY-NH2
Ox          PRAQLLRVGCALGTCQVQNLSHRLWQLVGSAGPRDSAPVDPSSPHSY-NH2
Elephant    PRAQLLRVGCMLGTCQVQNLSHRLWQIVEQAGQRDLAPVDPRSPHSY-NH2
Horse       PRAQLLRVGCVLGTCQVQNLSHRLWQLVGSAGPRDSAPVDPSSPHSY-NH2
Dog         SRAQLLRVGCVLGTCQVQNLSHRLWQLVGSAGPRNAAPMDPSSPYSY-NH2
Rabbit      TQAQLLRVGCVLGTCQVQNLSHRLWQLVRPAGRQDSAPIDPSSPHSY-NH2
Rat         PHAQLLRVGCVLGTCQVQNLSHRLWQLVRPSGRRDSAPVDPSSPHSY-NH2
Mouse       PHAQLLRVGCVLGTCQVQNLSHRLWQLVRPAGRRDSAPVDPSSPHSY-NH2
Squirrel    PHAQLLRVGCMLGTCQVQNLSHRLWKLVGPAGRRDLAPVDPSSPHSY-NH2
Pika        PHAQLLRVGCALGTCQVQNLSHRLWQLVRPTGRQDSAPIDPSSPHSY-NH2
Hedgehog    PRAQLLRVGCALG*CQVQNLSHRLWQLFGSAGPRDSVPVDPSSPHSY-NH2
Opossum     HGAKLMRVGCVLSTCQVQNLSHRLRQLMGQSGRQDSAPMNPKSPHSY-NH2
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(C)

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Human       RGHVCYLGVCRTHRLAEIIYWIR*VSTKEPSGKASHEPQDPYSY-NH2
Chimpanzee  RGHVCYLGVCRTHRLAEIIYWIR*VSTKEPSGKASHEPQDPYSY-NH2
Gorilla     RGHVCYLGVCRTHRLAEIIYWIR*VSTKEPSGKASHEPQDPYSY-NH2
Orangutan   RGQVCHLGVCWTHRLAEIIYWIRSVSTKEPSGKASREPQDPYSY-NH2
Monkey      RGHVCYLGVCRTHRLAEIIQWIRSASTKEPTGKASREPQNPYSY-NH2
Lemur       RGRLCSLGTCQTHRLPEMIYWLRSASTKEPSGKASRKPQDPHGY-NH2
Tupai       RGRLCSLGTCQTHRLPQIIYWLRSASTKEPSGKAGREPQDPHSY-NH2
Pig         SGRLCSLGTCQTHRLPEIIYWLRFASTKELSGKAGRKPQDPYSY-NH2
Ox          RGRLCSLGTCQTHRLPEIIYWLRSASTKEPSGKAGRKPQDPHSY-NH2
Sheep       RGRLCSLGTCQTHRLPEIIYWLRSASTKEPSGKAGRKPQDPHSY-NH2
Horse       RGRPCSLGTCQAHRLPDILHWLRSASTKEPSAKAGREPQDPRSY-NH2
Dog         RGRLCSLGTCQTHRLPEMIYWLRSASTKELSGKAGREPQDPHSY-NH2
Cat         RGRLCSLGTCQTHRLPEIIYWLSSASTKELSGKAGREPQDPHSY-NH2
Guinea pig  RGRSCSLGTCQTHLLPHRLAWVRALSAKEPSGKAAREPQDPRSY-NH2
```

FIGURE 3 Amino acid sequences of adrenomedullin (AM) 2/intermedin precursors from mammals and pufferfish, *Takifugu rubripes* (A) and mature amino acid sequences of AM2/IMD (B) and AM5 (C) in mammals. Amino acid sequences conserved in more than half species are shaded. Brackets show disulfide bridges. Lines and arrows in (A) are the signal sequence and processing site, respectively. Asterisks in (B) and (C) show the occurrence of nucleotide modification at the site, resulting in translation of different proteins.

because its processing signal (Arg–X–X–Arg) exists in the prosegment of all fish AMs. The truncation at this site produces mature AM2 of 55 amino acid residues in the tiger pufferfish.

The processed, mature AM2/IMD possesses an intramolecular ring structure of six amino acid residues flanked by a single disulfide bond in the N-terminal region and an amidated C-terminus (Figs. 1 and 3). The length of the N-terminal sequence extended from the intramolecular ring varies among species and has relatively minor effects on its biological activity. However, the amidated C-terminus may be essential for the activity as is the case for AM. With respect to AM, the C-terminally nonamidated (inactive) molecule circulates in the blood in amounts much larger than that of the amidated, mature AM. The AM2/IMD precursor terminates with a glycine residue, but the AM precursor is followed by 4 arginine residues and a C-terminal peptide. Thus, a complex processing is required before C-terminal amidation of AM with endopeptidase and carboxypeptidase. These differences may influence the efficiency of amidation.

RECEPTORS AND SIGNALING CASCADE

The CGRP family members bind the calcitonin receptor (CTR) or CLR associated with one of the three types of RAMPs and use the cAMP signaling cascade for biological action.[15] Cyclic AMP accumulation is increased by CGRP when given to cells coexpressing CLR and RAMP1, by AM in cells coexpressing CLR and RAMP2/3, and by amylin in cells coexpressing CTR and RAMP1/3. Although the potency of RAMP for cAMP accumulation is different among culture cell types used for the expression, AM2/IMD is ubiquitously effective in cells coexpressing CLR/CTR and various RAMPs, but in no case is it more potent than other CGRP family members.[15,24,28] Among

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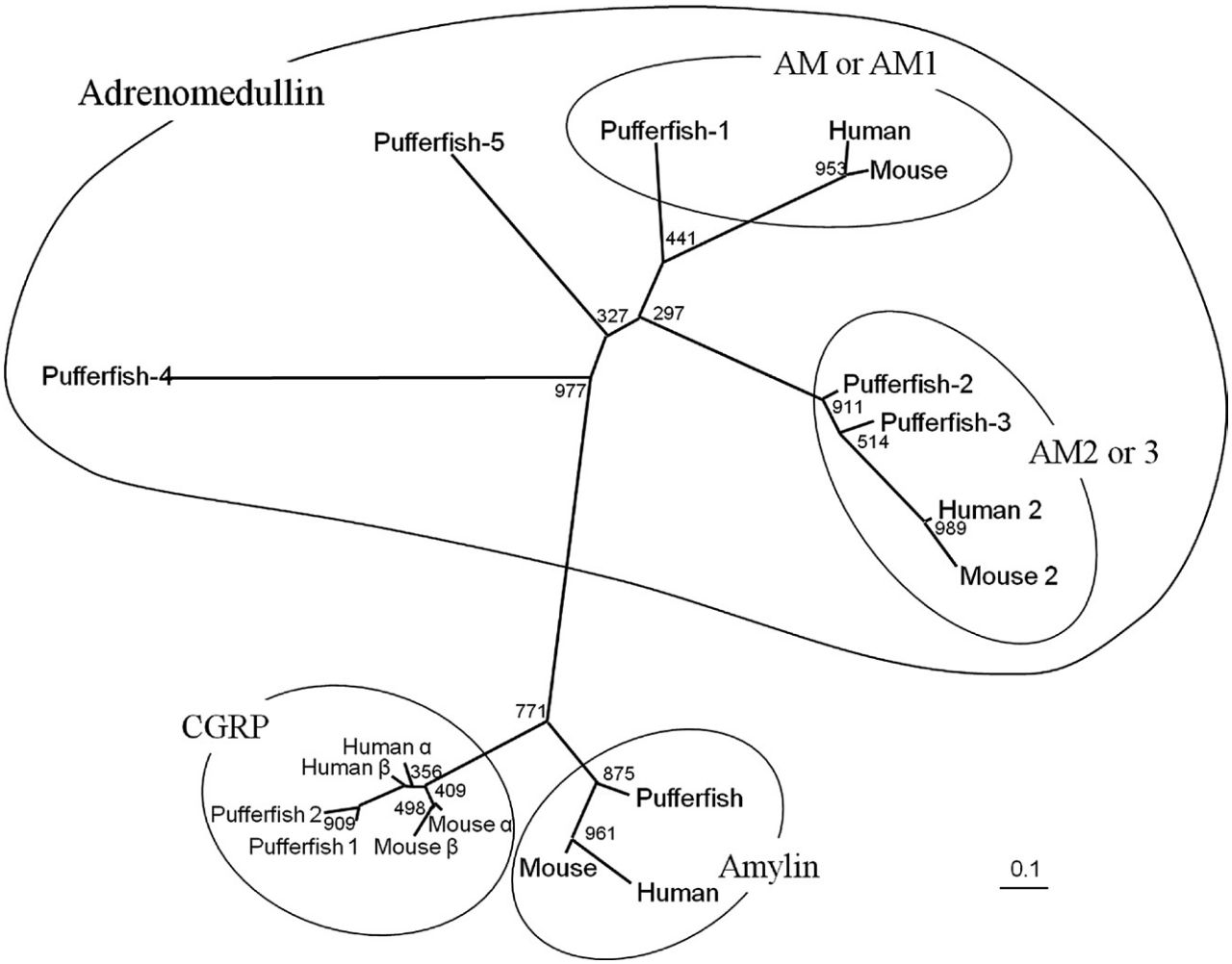

FIGURE 4 Molecular phylogenetic analyses of adrenomedullins (AMs), calcitonin-gene-related peptides (CGRPs), and amylin from mammals and pufferfish, *Takifugu rubripes*. Precursor sequences are analyzed by the Neighbor-Joining method. Numbers on the tree represent bootstrap values.

the combinations, CLR + RAMP3 and CTR + RAMP1 are most effective at10^{-9} M of AM2/IMD, but less effective than AM and amylin respectively for each receptor, suggesting the presence of AM2/IMD-specific receptors other than CTR/CLR associated with RAMP.

In fish, genes coding for two types of CLRs (CLR1 and CLR2), and five types of RAMPs (RAMP1–5) are identified in the pufferfish.[19] Molecular phylogenetic analyses and linkage analyses indicate that RAMP1/4, RAMP2/5, and RAMP3 are akin to RAMP1, RAMP2, and RAMP3 of mammals, respectively. Coexpression of CLR and RAMP shows that pufferfish AM2 increased cAMP production in cells expressing CLR1 and RAMP3, whereas pufferfish AM1, an ortholog of mammalian AM, is more widely effective in cells coexpressing CLR1 and RAMP2/3/5 and CLR2 and RAMP2.[19] Thus, the AM2-specific combination of CLR and RAMP is not found even in fishes having such diversified CLRs and RAMPs.

BIOLOGICAL ACTIONS

AM2/IMD exerts spectra of peripheral actions similar to AM in mammals. The cardiovascular and renal actions of AM2/IMD have been reviewed.[1] Intravenous (iv) infusion of AM2/IMD into the renal artery of rats at nondepressor doses is diuretic and natriuretic.[9] Because glomerular filtration rate does not change during diuretic/natriuretic dose of AM2/IMD infusion in rats, a direct tubular action is probable. Despite a high expression in the kidney of rodents and humans, the renal effects of AM2/IMD are generally less efficacious than those of AM in rats[9] and sheep.[3] Intravascular administration of AM2/IMD decreased arterial pressure and increased heart rate in rats,[10,24] mice,[29] and sheep.[3] The hypotension results from the dilatation of resistance vessels mediated by a local production of nitric oxide.[17] The cardiovascular effects of AM2/IMD are comparable with or somewhat more potent and shorter-lasting than

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AM.[9] When administered into the cerebral ventricle (icv injection), AM2/IMD causes immediate hypertension and delayed tachycardia in rats through activation of the sympathetic nervous system.[14,31] The site of the cardiovascular action appears to be the nucleus tractus solitarius as icv injection of AM2/IMD increases *c-fos* gene expression in the nucleus.[13] The central effect of AM2/IMD is usually more potent than that of AM.

The icv injection of AM2/IMD stimulates prolactin and ACTH secretion and inhibits growth hormone secretion from the anterior pituitary of rats *in vivo* or *in vitro*.[2,27,32] The AM2/IMD gene expresses abundantly in the intermediate lobe and ACTH cells of rat anterior lobe of pituitary, and AM2/IMD administered to the cultured rat anterior pituitary cells stimulates prolactin secretion but not ACTH, GH, and FSH secretion.[2] These results are consistent with the brisk expression of CLR and RAMPs in the pituitary. The effect on ACTH secretion seems to be mediated by CRH secreted from the hypothalamus as evidenced by the use of CRH antagonist. AM2/IMD is synthesized in the adrenal medulla and its candidate receptors (CLR+RAMP1–3) are localized in the human adrenal tissue,[18] but there is no evidence for the direct action of AM2/IMD on glucocorticoid secretion. In the sheep, AM2/IMD infusion activates the renin–angiotensin system, and plasma angiotensin II stimulates aldosterone secretion (but not cortisol). The direct action of AM2/IMD on the adrenal glomerulosa cells for aldosterone secretion is unlikely in sheep.[4] The iv AM2/IMD infusion increases plasma ANP and BNP concentrations in sheep.[3]

The icv injection of AM2/IMD augments *c-fos* gene expression of the neurons in the supraoptic nucleus, paraventricular nucleus, and arcuate nucleus of rats as shown by *in situ* hybridization.[13] Immunohistochemical staining of the same section with antiserum specific to vasopressin or oxytocin revealed that most *c-fos*-positive cells are oxytocin-producing cells, and only few are vasopressin-producing cells. Consistent with this observation, plasma oxytocin concentrations are profoundly increased after icv administration of AM2/IMD but only slightly for vasopressin. In addition, the icv injection of AM2/IMD inhibits water and food intake in both satiated rats and those deprived of water and food overnight.[25,31] AM2/IMD also inhibits food intake and stomach motility in rats when given by intraperitoneal injection.[24] The effect on stomach may be mediated by locally produced AM2/IMD as the tissue abundantly synthesizes and stores this peptide.

AM2/IMD seems to have cytoprotective effects in various organs. AM2/IMD protects cardiac myocytes against ischemia/reperfusion injury.[33] In rats with chronic inhibition of nitric oxide, the expression of the AM2/IMD gene is induced solely by the oxidative stress, not by accompanying hypertension,[1] and AM2/IMD seems to protect the hypertrophied cardiomyocytes from oxidative stress in a paracrine/autocrine fashion. AM2/IMD protects endothelial cells in an autocrine manner from oxidative stress in the rat cerebral circulation.[7] Consistent with the high production in the kidney, AM2/IMD seems to function as a renoprotective peptide against oxidative stress,[12] but its expression was downregulated in the kidney of spontaneously hypertensive rats and with artificial injury.[16] AM2/IMD also has angiogenic effects in the ischemic rat hindlimb caused by femoral artery ligation as shown by the AM2/IMD gene delivery.[26]

The AM, CLR, and RAMP2 heterozygote females mated to wild-type males have reduced fertility, reduced litter size and fetal growth compared with that of wild-type females mated to the heterozygote males.[6] Although AM2/IMD gene knockout has not yet been successful, the AM2/IMD gene is expressed in the murine uterus and ovary and plasma immunoreactive AM2/IMD increases during pregnancy in rats. Thus, AM2/IMD may be involved in the female reproduction. Infusion of AM2 antagonist AM2/$IMD_{17–47}$ into the pregnant rat causes fetoplacental growth restriction through the activation of mitochondrial apoptotic pathways.[5] Apart from the direct effect, the maintenance of pregnancy by AM2/IMD results from the relaxant effect on mesenteric arteries to ensure sufficient blood supply to the fetoplacenta, as the vascular effect becomes more sensitive during pregnancy. The AM2/IMD gene is expressed in human placenta and seems to be involved in the regulation of trophoblast invasion and migration. In the rat uterus, the AM2/IMD gene is expressed in the implantation site and inhibition of AM2/IMD by its antagonist impairs the expression of factors responsible for implantation and growth of embryos such as MMP and VEGF.[6] The consensus sequence of estrogen response element exists in the AM2/IMD gene promoter, and injection of 17β-estradiol into the ovariectomized female rat restores AM2/IMD expression and prolactin secretion.[2]

The biological actions of AM2/IMD are basically similar to those of AM (see the Brain, Cancern, Endocrine, Gastrointestinal, Cardiovascular, and Respiratory Peptides sections of this volume), but some actions are apparently different from those of AM in mammals and fishes.[1,20] The difference in the response to receptor blockers suggests the presence of AM2/IMD-specific receptor that is different from CLR associated with RAMP. The AM2/IMD stimulates oxytocin secretion with higher potency than AM although affinity of AM2/IMD to CLR+RAMPs is lower than that of AM, and the AM2/IMD effect is blocked only slightly by pretreatment with $CGRP_{8–37}$ (CGRP/AM receptor antagonist) and $AM_{22–52}$ (AM receptor antagonist).[14] The inhibition of GHRH-induced GH release from the anterior pituitary cells occurs only by AM2/IMD and not by other CGRP peptides, and the effect is mediated at least in part via inhibition of intracellular cAMP production.[32] Further, AM2/IMD antagonist, AM2/$IMD_{17–47}$, inhibits peripheral cardiovascular actions, but it fails to inhibit orexigenic and dipsogenic effect of icv AM2/IMD *in vivo* and inhibitory effect on GHRH-induced GH

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secretion from the dispersed anterior pituitary cells *in vitro*.[35] Likewise, the failure to displace ^{125}I-AM and ^{125}I-CGRP binding by AM2/IMD in dispersed rat spinal cord cells suggests the presence of distinct class of receptor for AM2/IMD.[23] In teleost fish, AM2 is >100-fold more potent than AM(1) in terms of cardiovascular and osmoregulatory actions in eels.[20] but AM2 exhibits less affinity to CLRs associated with various RAMPs in pufferfish.[19] Therefore, fish may be an ideal material to pursue novel non-CLR + RAMP receptor(s) specific for AM2/IMD in vertebrates.

PATHOPHYSIOLOGICAL IMPLICATIONS

AM2/IMD mRNA was initially registered in the EST database (accession number: AK024788) as a mRNA from the primary vascular smooth muscle cells of human coronary artery. In fact, immunoreactive AM2/IMD is detectable in the endothelial cells or smooth muscle cells of coronary vessels of mammals.[18,28] Consistently, AM2/IMD relaxes precontracted coronary of pigs more potently than AM.[11] Further, AM2/IMD protects the heart from ischemic injury.[33] These results suggest that AM2/IMD may be secreted from the coronary artery to protect the heart as a local paracrine system at the onset of ischemic heart failure as suggested by AM.[1] The expression of the AM2/IMD, CLR and RAMP genes increases in cardiomyocytes of rats with congestive heart failure and in spontaneously hypertensive rats. AM2/IMD and RAMP1 are also upregulated in the ventricular myocytes of hypertensive rats with inhibition of nitric oxide synthesis. In isolated, paced murine ventricular myocytes, AM2/IMD administration enhances contractility via protein kinase C and A activation.[8] Further, angiotensin II induces cardiac hypertrophy and increases AM2/IMD gene expression in the ventricular myocytes of rats, and the AM2/IMD treatment inhibited angiotensin II-induced hypertrophy.[34] AM2/IMD also inhibits fibroblast activation in angiotensin II-induced hypertrophic heart. Thus AM2/IMD may ameliorate the deterioration of dilated cardiomyopathy. The AM2/IMD knockout mice will delineate the cardioprotective action of this intriguing peptide more clearly.

REFERENCES

1. Bell D, McDermott BJ. Intermedin (adrenomedullin-2): a novel counter-regulatory peptide in the cardiovascular and renal systems. *Br J Pharmacol* 2008;**153**:S247–62.
2. Chang CL, Roh J, Park JI, Klein C, Cushman N, Haberberger RV, et al. Intermedin functions as a pituitary paracrine factor regulating prolactin release. *Mol Endocrinol* 2005;**19**:2824–38.
3. Charles CJ, Rademaker MT, Richards AM. Hemodynamic, hormonal, and renal actions of adrenomedullin-2 in normal conscious sheep. *Endocrinology* 2006;**147**:1871–7.
4. Charles CJ, Rademaker MT, Nicolls MG, Richards AM. Adrenomedullin 2 attenuates the pressor but not adrenal responses to angiotensin II in conscious sheep. *Peptides* 2010;**31**:878–82.
5. Chauhan M, Yallampalli U, Reed L, Yallampalli C. Adrenomedullin 2 antagonist infusion to rats during midgestation causes fetoplacental growth restriction through apoptosis. *Biol Reprod* 2006;**75**:940–7.
6. Chauhan M, Etkins R, Balakrishnan M, Yallampalli C. Potential role of intermedin/adrenomedullin 2 in early embryonic development in rats. *Regul Pept* 2011;**170**:65–71.
7. Chen L, Kis B, Hashimoto H, Busija DW, Takei Y, Yamashita H, et al. Adrenomedullin 2 protects rat cerebral endothelial cells from oxidative damage in vitro. *Brain Res* 2006;**1086**:42–6.
8. Dong F, Taylor MM, Samson WK, Ren J. Intermedin (adrenomedullin-2) enhances cardiac contractile function via a protein kinase C- and protein kinase A-dependent pathway in murine ventricular myocytes. *J Appl Physiol* 2006;**101**:778–84.
9. Fujisawa Y, Nagai Y, Miyatake A, Takei Y, Miura K, Shoukouji T, et al. Renal effects of a new member of adrenomedullin family, adrenomedullin 2, in rats. *Eur J Pharmacol* 2004;**497**:75–80.
10. Fujisawa Y, Nagai Y, Miyatake A, Miura K, Shoukoji T, Nishiyama A, et al. Roles of adrenomedullin 2 in regulating the cardiovascular and sympathetic nervous system in conscious rats. *Am J Physiol* 2006:H1120–7.
11. Grossini E, Molinari C, Mary DASG, Uberti F, Caimmi PP, Vacca G. Intracoronary intermedin 1–47 augments cardiac perfusion and function in anesthetized pigs: role of calcitonin receptors and β-adrenoreceptor-mediated nitric oxide release. *J Appl Physiol* 2009;**107**:1037–50.
12. Hagiwara M, Bledsoe G, Yang Z-R, Smith Jr RS, Chao L, Chao J. Intermedin ameliorates vascular and renal injury by inhibition of oxidative stress. *Am J Physiol* 2008:F1735–43.
13. Hashimoto F, Hyodo S, Kawasaki M, Mera T, Chen L, Soya A, et al. Centrally administered adrenomedullin 2 activates hypothalamic oxytocin-secreting neurons causing elevated plasma oxytocin level in rats. *Am J Physiol* 2005;**289**:E753–61.
14. Hashimoto H, Hyodo S, Kawasaki M, Shibata M, Saito T, Suzuki H, et al. Adrenomedullin 2 (AM2)/intermedin is a more potent activator of hypothalamic oxytocin-secreting neurons than AM possibly through an unidentified receptor in rats. *Peptides* 2007;**28**:1104–12.
15. Hay DL, Dickerson IM, editors. *The calcitonin gene-related peptide family. Form, function and future perspectives*. Springer; 2010. p. 1–236.
16. Hirose T, Totsune K, Mori N, Mori T, Morimoto R, Metoki H, et al. Expression of adrenomedullin 2/intermedin, a possible reno-protective peptide, is decreased in the kidneys of rats with hypertension or renal failure. *Am J Physiol* 2010;**299**:F128–34.
17. Kandilci HB, Gumusei B, Lippton H. Intermedin/adrenomedullin-2 (IMD/AM2) relaxes rat main pulmonary arterial rings via cGMP-dependent pathway: role of nitric oxide and large conductance calcium-activated potassium channel (BK_{Ca}). *Peptides* 2008:1321–8.
18. Morimoto R, Satoh F, Murakami O, Totsune K, Suzuki T, Sasano H, et al. Expression of adrenomedullin2/intermedin in human brain, heart and kidney. *Peptides* 2007;**28**:1095–103.
19. Nag K, Kato A, Nakada T, Hoshijima K, Mistry AC, Takei Y, et al. Molecular and functional characterization of adrenomedullin receptors in pufferfish. *Am J Physiol* 2006;**190**:R467–78.
20. Nobata S, Ogoshi M, Takei Y. Potent cardiovascular actions of homologous adrenomedullins in eels. *Am J Physiol* 2008;**294**:R1544–53.
21. Ogoshi M, Inoue K, Takei Y. Identification of a novel adrenomedullin gene family in teleost fish. *Biochem Biophys Res Commun* 2003;**311**:1072–7.

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22. Ogoshi M, Inoue K, Naruse K, Takei Y. Evolutionary history of the calcitonin gene-related peptide family in vertebrates revealed by comparative genomic analyses. *Peptides* 2006;**27**:3154–64.
23. Owji AA, Chabot J-G, Dumont Y, Quirion R. Adrenomedullin-2/intermedin induces cAMP accumulation in dissociated rat spinal cord cells: evidence for the existence of a distinct class of sites of action. *J Mol Neurosci* 2008;**35**:355–61.
24. Roh J, Chang CL, Bhalla A, Klein C, Hsu SY. Intermedin is a calcitonin/calcitonin gene-related peptide family peptide acting through the calcitonin receptor-like receptor/receptor activity-modifying protein receptor complexes. *J Biol Chem* 2004;**279**:7264–74.
25. Samson WK. Adrenomedullin and the control of fluid and electrolyte homeostasis. *Annu Rev Physiol* 1999;**61**:363–89.
26. Smith RS, Gao L, Bledsoe G, Chao L, Chao J. Intermedin is a new angiogenic growth factor. *Am J Physiol* 2009;**297**:H1040–7.
27. Takahashi K, Morimoto R, Hirose T, Satoh F, Totsune K. Adrenomedullin 2/intermedin in the hypothalamo–pituitary–adrenal axis. *J Mol Neurosci* 2011;**43**:182–92.
28. Takei Y, Hyodo S, Katafuchi T, Minamino N. Novel fish-derived adrenomedullin in mammals: structure and possible function. *Peptides* 2004;**25**:1643–56.
29. Takei Y, Inoue K, Ogoshi M, Kawahara T, Bannai H, Miyano S. Identification of novel adrenomedullin in mammals: a potent cardiovascular and renal regulator. *FEBS Lett* 2004;**556**:53–8.
30. Takei Y, Hashimoto H, Inoue K, Osaki T, Yoshizawa-Kumagaye K, Watanabe TX, et al. Central and peripheral cardiovascular actions of adrenomedullin 5, a novel member of the calcitonin gene-related peptide family, in mammals. *J Endocrinol* 2008;**197**:391–400.
31. Taylor MM, Bagley SL, Samson WK. Intermedin/adrenomedullin-2 acts within central nervous system to elevate blood pressure and inhibit food and water intake. *Am J Physiol* 2005;**288**:919–27.
32. Taylor MM, Bagley SL, Samson WK. Intermedin/adrenomedullin-2 inhibits growth hormone release from cultured, primary anterior pituitary cells. *Endocrinology* 2006;**147**:859–64.
33. Yang J-H, Qi Y-F, Jia Y-X, Pan C-S, Zhao J, Yang J, et al. Protective effects of intermedin/adrenomedullin 2 on ischemia/reperfusion injury in isolated rat hearts. *Peptides* 2005;**26**:501–7.
34. Yang J-H, Cai Y, Duan XH, Ma CG, Wang X, Tang CS, et al. Intermedin1-53inhibitsratcardiacfibroblastactivationinducedbyangiotensin II. *Regul Pept* 2009;**158**:19–25.
35. White MM, Samson WK. Intermedin 17–47 does not function as a full intermedin antagonist within the central nervous system or pituitary. *Peptides* 2007;**28**:2171–8.

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Chapter 199

ANP

Jochen R. Hirsch and Wolf-Georg Forssmann

ABSTRACT

In the kidney, the main task of the atrial natriuretic peptide (ANP) is the induction of natriuresis and diuresis, but the mechanism behind these effects is far from being clarified. Although the involvement of ANP in the regulation of the cardiovascular system is well established, its regulatory role in transport processes in the nephron is still unresolved. Here, we take a closer look at the regulation of a variety of transport proteins in the renal nephron by ANP from the proximal tubule to the cortical and inner medullary collecting duct.

DISCOVERY OF ATRIAL NATRIURETIC PEPTIDE ACTIONS IN THE KIDNEY

For nearly half a century, an involvement of the heart in renal functions has been postulated. In 1969, a hormonal influence of the heart on the kidney was mentioned for the first time, whereas in 1974, it was observed that the granular index of myoendocrine cells is affected during experiments, altering the body fluid and electrolyte homeostasis. Application of atrial extracts led to a previously unknown strong natriuresis and diuresis. A further biological effect of atrial extracts, relaxation of vascular smooth muscle, was discovered by several research groups. The extract had a strong vasorelaxant effect on cortical arterial blood vessels. Based on these two main biological effects, the first isolation of atrial natriuretic peptides (ANPs) was performed. Depending on the bioassay used for isolation, the peptides were designated cardiodilatin (from the pig) or cardionatrin (from the rat). The first atrial natriuretic peptide isolated from the human heart was named a-human atrial natriuretic peptide (a-hANP). Since then, remarkable advances have been made regarding the characterization of its gene and protein nature, synthesis, intracellular processing, release, metabolism, second-messenger systems, and receptors. An explanation for ANP-induced natriuresis and diuresis has not yet been found, but most research groups favor a combination of hemodynamic and tubular effects.[9] Because the hemodynamic effects are well established, the following paragraphs focus on the tubular effects of ANP.

EFFECTS OF ANP ON TUBULAR TRANSPORT SYSTEMS

Effects of ANP on Proximal Tubules

Early investigations on proximal tubules regarding the possible actions and effects of ANP resulted in controversial results. Many *in vivo* and *in vitro* microperfusion studies of proximal convoluted, and straight tubules in the rat or rabbit have failed to find any effects of ANP on Na^+, bicarbonate, and Cl^- transport, and only a few managed to show ANP-induced transport changes. Solute delivery out of the proximal tubule increases by approximately 30–35%.[5] Furthermore, Harris et al.[10] reported that the stimulatory effect of angiotensin II on fluid reabsorption is inhibited by peritubular ANP at physiological concentrations and eliminated by higher concentrations of ANP. In contrast to the results of Cogan et al.,[5] the authors concluded that, at physiological concentrations, ANP acts within the kidney to decrease proximal reabsorption by the inhibition of angiotensin II-stimulated Na^+ and water transport.

With the introduction of modern techniques such as patch clamping, molecular biology, and protein chemistry, more reports showed direct effects of ANP and related natriuretic peptides (Fig. 1) such as urodilatin, B natriuretic peptide (BNP), and C natriuretic peptide (CNP) on various transport systems, ion channels, and pumps in the nephron and its various tubular structures.

ANP inhibits several Na^+-dependent transport systems. In proximal tubular brush border membrane vesicles, the Na^+/H^+-antiporter, which is responsible for approximately 50% of the Na^+ intake in this segment, is inhibited, as well as the Na^+-coupled PO_4^{3-} transport. Na^+-coupled PO_4^{3-}-transport has been extensively researched by Murer and his group, and in 2001, they reported that the inhibitory effect of ANP results from membrane retrieval containing the Na-Pi type IIa cotransporter.[2] Low-affinity high-capacity Na^+-glucose cotransporter (SGLT2) activity is drastically reduced when ANP is administered together with endothelin 3.[18] This transport system is the most important retrieval pathway for sugar in the kidney and accounts for 90% of the Na^+-coupled

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00199-8
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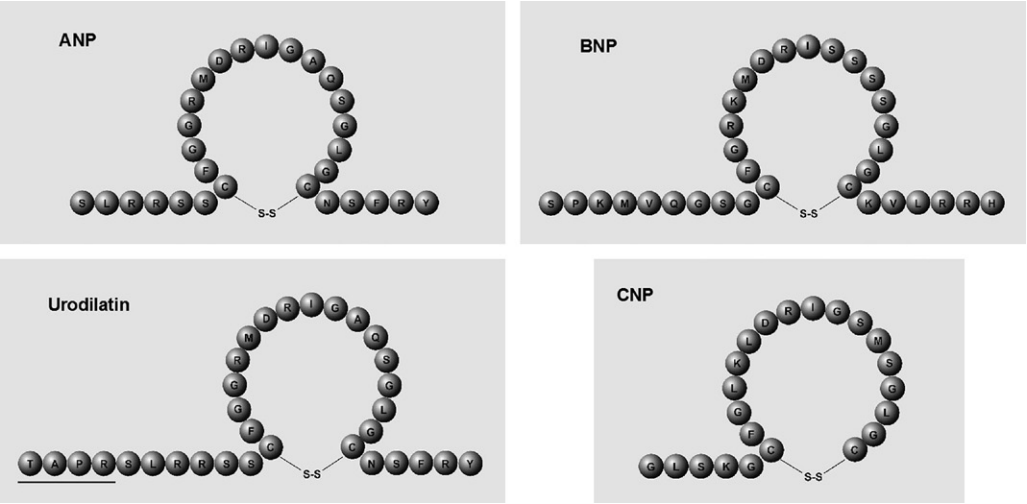

FIGURE 1 Amino acid sequence of circulating ANP (95–126), BNP, urodilatin (99–126), and CNP. Urodilatin differs from ANP by an N-terminal extension of four amino acids (indicated by black bar).

sugar intake. Na^+/K^+-ATPase, essential for all secondary active transport processes, is deactivated in the proximal tubule by phosphorylation/dephosphorylation result from ANP-induced cGMP increase.[1] Deactivation of this enzyme's activity also occurs in isolated basolateral membranes and LLC-PK1 cells. Low concentrations of ANP and urodilatin (10 pM) are responsible for a 50% decrease in activity of the Na^+/K^+-ATPase through cGMP-dependent guanylate cyclase activation.[31] However, not just Na^+-dependent processes are involved in the regulatory pattern of ANP.

The organic cation transporter of the basolateral membrane of isolated human proximal tubules shows a 20–30% increase in transport after being stimulated by ANP or 8-Br-cGMP.[25] In the meantime, Cl^- as well as K^+ channels are also the target of regulation by natriuretic peptides. In cultured rat proximal convoluted tubule (PCT) cells, a DIDS-inhibitable Cl^- conductance is activated by ANP, urodilatin or their second-messenger cGMP,[7] and in the human proximal tubule cell line RPTEC, an inwardly rectifying K^+ channel is phosphorylated by a cGMP-dependent protein kinase (PKG) and stimulated by the application of ANP.[21]

Several inwardly rectifying K^+ channels have been identified in the proximal tubule.[11,19,21] Earlier, the authors described a K^+ conductance that is inhibited by various natriuretic peptides such as ANP, urodilatin, BNP, and CNP in a cGMP-dependent and -independent fashion in IHKE-1 cells, another human proximal tubule cell line. This K^+ conductance is the first directly cGMP-dependent K^+ channel described in humans. Interestingly, in IHKE-1 cells, this K^+ channel is regulated by cGMP, not only intracellular but also from the outside, showing that ANP and cGMP can also display regulatory effects from the luminal side of the tubule system.[11]

Therefore, ANP, urodilatin, and other cGMP-generating factors have three possible ways to regulate transport in the tubular system: (1) binding to glomerular cells and subsequently releasing cGMP into the proximal lumen, (2) binding to its specific receptor (NPR-A/GC-A) and increasing intracellular cGMP, and (3) binding to its specific receptor, increasing intracellular cGMP with the consecutive extrusion of cGMP via a specific transport system[30] into the lumen, and acting from the outside on the target transmembrane protein. An overview of transport proteins involved in the regulatory pathway of ANP in the proximal tubule cell is given in Fig. 2.

Effects of ANP on Henle's Loop

The functional responses of ANP in Henle's loop are unknown. It has been inferred that ANP may alter salt transport in this segment. However, most studies have shown little effect of ANP on the loop of Henle. Peterson et al.[24] investigated the effects of these hormones on thick ascending limb Na^+Cl^- reabsorption *in vivo*. With the microstop-flow conductivity technique, despite the presence of a dramatic natriuretic response, ANP does not significantly increase the minimum Na^+Cl^- concentration reached by the thick ascending limb. By contrast, vasopressin increased the rate at which luminal Na^+ concentration declined by 23%. Up to now, the only direct effects of ANP on Na^+Cl^- transport are a report on a decrease in Cl^- reabsorption[3] and an inhibitory effect of ANP on Na^+/K^+-ATPase.[29] An explanation might be the low response of the cells of Henle's loop in cGMP formation after incubation with ANP.[4]

Comparable to other segments of the nephron where ANP is able to modulate intracellular Ca^{2+} levels, a putative physiological importance of ANP could also be shown in

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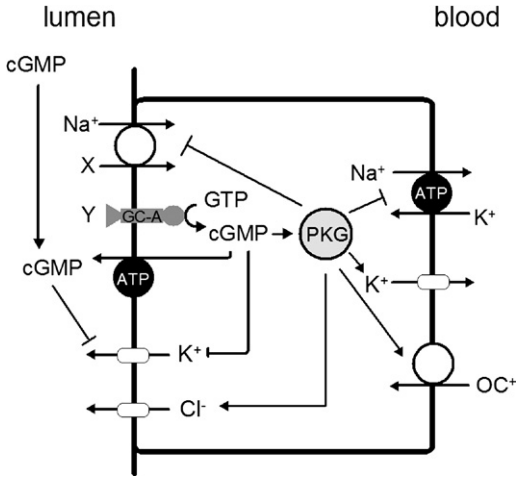

FIGURE 2 Cell model of the proximal tubule. Shown are the transport systems that are regulated via ANP and its second-messenger cGMP. In the luminal membrane, the guanylate cyclase receptor (GC-A) for ANP, urodilatin, and BNP (Y) is located and, after being activated, generates cGMP. This second-messenger can activate a cGMP-dependent kinase (PKG), which phosphorylates several Na^+ coupled transporters, such as the basolateral Na^+/K^+-ATPase and the Na^+/H^+ exchanger, Na^+-glucose and Na^+-PO_4^{3-} cotransporter (X) of the luminal membrane but also the Na^+ independent organic cation transporter (OC) of the basolateral membrane. Furthermore, PKG activates a Cl^--channel in the luminal membrane and an inwardly rectifying K^+ channel (location is not verified and the basolateral membrane was chosen accidentally). cGMP can also act as an inhibitor on a K^+ channel from the tubular lumen. When transported to the lumen by a specific pump, it can inhibit this channel from the outside. This mechanism also works when cGMP is released from glomerular cells because of stimulation by ANP or related natriuretic peptides.

this portion of the nephron.[6] In primary cell cultures, this group reported an ANP-induced receptor-mediated Ca^{2+} transient in cortical thick ascending limb cells resulting from internal release and influx across the plasma membrane. The authors discuss whether this receptor is most probably the clearance receptor that initiates Ca^{2+} signals. They conclude that these ANP-induced Ca^{2+} transients mediated by the putative clearance receptor may have significant physiological actions within Henle's loop.

The rather moderate effects of ANP, urodilatin, or cGMP on Henle's loop do not necessarily mean that natriuretic peptides are not involved in the regulatory processes of this tubular segment. A splice variant of the CNP-activated NPR-B/GC-B receptor, NPR-Bi, is highly expressed in human thick ascending limb and the cortical collecting duct. This receptor lacks guanylate cyclase activity and probably acts as a tyrosine kinase.[13]

Effects of ANP on the Collecting Duct System

There is evidence that ANP affects natriuresis and diuresis in part by inhibiting net Na^+ and water reabsorption in the collecting duct. The first review of the day-to-day regulation of fluid and electrolyte balance using the micro catheterization technique and the micropuncture method was in 1979, and the first ANP effects on the tubular system were reported in 1982. Experiments were performed in anesthetized rats to collect tubular fluid from the proximal and distal tubules and from the outer medullary collecting duct. Samples were taken from the same sites before and after intravenous (iv) administration of atrial tissue extract. Na^+ excretion increase 17-fold after injection of atrial extracts, whereas the clearances of insulin and single nephron filtration rates do not change significantly. The infusions of ANP do not alter the delivery of Na^+ and water to Henle's loop but lead to increases in Na^+ and water delivery out of the outer medullary duct. Because there is a large increment of fractional Na^+ excretion between the outer medullary collecting duct and the final urine, it is probable that the atrial extracts interfere with the capacity of solute load reabsorption in the inner medullary collecting duct (IMCD). Furthermore, the infusion of ANP is associated with increased Na^+ delivery and reduced fractional reabsorption in the duct, whereas K^+Cl^- infusion has no effect on fractional reabsorption, suggesting that ANP has a highly specific inhibitory effect on net Na^+ transport in this part of the nephron.[28] Even if the *in vivo* studies strongly indicate the actions of ANP by inhibition of net Na^+ and water reabsorption in the collecting duct, it cannot be excluded that these actions could also be caused by alterations of transepithelial driving forces. ANP can preferentially increase blood flow to juxtamedullary nephrons and augment *vasa recta* blood flow. ANP-induced alterations in transepithelial driving forces in the renal medulla cause unfavorable hydraulic gradients for net Na^+ reabsorption in the IMCDe.[20] These findings suggest that ANP can increase the hydraulic gradient from the *vasa recta* to papillary collecting duct, favoring reduced net volume and Na^+ reabsorption in the IMCD.

Effects of ANP on the Late Distal Tubule

HEK-293 cells display a variety of features of the late distal tubule. Furthermore, they have the ability to release urodilatin, the structural analog to ANP. These human distal tubule cells contain specific mRNA for the ANP/urodilatin receptor NPR-A/GC-A, whereas the receptors for other members of the natriuretic family, GC-B and GC-C, were not detectable. In these cells, ANP inhibits K^+ conductance via PKG that is rather inactive, but the effect becomes apparent when genistein activates a different K^+ conductance and thus, potentiates ANP-induced inhibition.[12] Novel observations show that ANP and urodilatin, besides activating and inhibiting K^+-channels, counterregulate the effects of bradykinin on Ca^{2+}-dependent Cl^- channels via PKG. However, natriuretic peptides do not interact directly with the target channels but with the bradykinin-activated Ca^{2+} pathway (unpublished results).

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Effects of ANP on the Cortical Collecting Duct

Water reabsorption takes place in the cortical collecting duct (CCD) cells, induced by the peptide hormone vasopressin. An increase in 3'5'-cyclic adenosine monophosphate (cAMP) levels in these cells leads to an increasing permeability of the CCD apical membrane to water. The ANP-induced diuresis probably results from the direct inhibition of renal tubular epithelial water transport.[8] The results that ANP leads to an inhibition of the hydraulic conductivity response to the hormone vasopressin but not to either cAMP or forskolin suggests that ANP acts proximally to cAMP formation. A similar result was found for aquaporin (AQP-1),[23] which is also localized in the kidney but predominantly in the proximal tubule, descending limb of Henle's loop, and *vasa recta*. AQP-1 is upregulated when expressed in *Xenopus laevis* oocytes and treated with AVP. Water permeability increases and could be inhibited by ANP or incubation with 8-Br-cGMP.[23] A competing phosphorylation mechanism between protein kinase A (PKA) and PKG has been shown previously for dopamine and ANP in regulating the Na^+/K^+-ATPase of the proximal tubule[1] and might be a mechanism in the CCD as well. However, in several cell types, natriuretic peptides decrease cAMP concentration via the activation of the NPR-C receptor to which all natriuretic peptides can bind. This effect can either be mediated by the activation of the receptor's coupling to G-protein or by regular cGMP increase and subsequent stimulation of cAMP-sensitive phosphodiesterases. What is the effect of ANP on ion transport processes at this nephron site? A few groups were able to convincingly show that ANP neither has an effect on Na^+ transport in the CCD[26] nor does it regulate electrogenic electrolyte transport in principal cells of rat CCD.[27] With the localization of the NPR-A/GC-A receptor in the luminal membrane of intercalated cells, the authors show that ANP, urodilatin, and its membrane-permeable second-messenger 8-Br-cGMP inhibit Na^+/H^+ exchange activity,[11] thus regulating the acid-base equilibrium of these cells (Fig. 3).

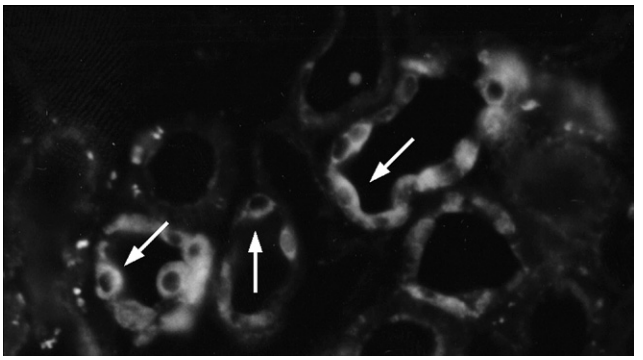

FIGURE 3 Immunohistochemistry of NPR-A/GC-A in the cortical collecting duct of rat. The white arrows show typical intercalated cells protruding into the lumen. Note that the staining is prominent in the luminal membrane.

Effects of ANP on the Inner Medullary Collecting Duct

There is strong evidence that most of the natriuretic effects of ANP contribute to the inhibition of Na^+ reabsorption in the IMCD. Great efforts have been made to clarify the mechanisms by which ANP acts and induces the natriuretic response in the IMCD. ANP inhibits transport-dependent oxygen consumption (QO_2) in the IMCD, yet has no effect on the outer medullary collecting duct. Furthermore, studies investigating the interaction of ANP with amiloride, ouabain, and amphotericin with QO_2 measurements suggest that ANP inhibits Na^+ entry into these cells.[34] ANP reduces isotopic Na^+ uptake in suspensions of IMCD cells by two-thirds at concentrations identical to those found effective in oxygen consumption studies.[33] To precisely characterize the ANP-induced effect on Na^+ reabsorption in IMCD, the patch-clamp technique shows that ANP and cGMP inhibit an apically located cation channel in cell-attached patches. Thus, it seems probable that the natriuretic action of ANP is related partly to the cGMP-mediated inhibition of electrogenic Na^+ absorption by the IMCD. This finding is also supported by Nonoguchi et al.[22] ANP and NO inhibit water transport in IMCD cells via activation of a guanylate cyclase when pretreated with vasopressin. Incubation of these cells with ANP leads to a retrieval of AQP-2 water channels from the plasma membrane into the cytosol.[17]

For some points, the contradictory results of several groups and some open questions may reflect the uncertainties and difficulties in precisely characterizing and defining the role of ANP in Na^+ and water homeostasis. Some authors even question the functional role of ANP in the kidney and favor a regulatory role primarily in the cardiovascular system. However, this view is highly unlikely, because the application of an NPR-A/GC-A antagonist counterregulates natriuresis induced by acute volume expansion.[32] Mice lacking the ANP gene show salt-sensitive arterial hypertension and elevated extracellular fluid volume.[15] Furthermore, ANP knockout mice are unable to inhibit Na^+ reabsorption in the collecting duct when kept on a high-salt diet and intravenous salt loading,[14] and the natriuretic effect is virtually absent in NPR-A knockout mice.[16]

In addition to the established hemodynamic effects of ANP, there are a growing number of reports showing direct inhibitory regulatory effects on transport proteins throughout the nephron. Among these are several Na^+-dependent transport systems such as the Na^+/H^+ exchanger, which accounts for 50% of the Na^+ intake in this segment; the Na^+-glucose and Na^+-phosphate transporters from the proximal

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tubule; and the Na^+/K^+-ATPase from the proximal tubule and the thick ascending limb of Henle's loop. In the collecting duct system, ANP inhibits a nonselective cation channel and the Na^+/H^+-exchanger responsible for cellular pH regulation. Furthermore, ANP reduces the number of AQP-2 channels in the plasma membrane of IMCD cells, thus, leading to diuresis.

Taken together, these direct effects on tubular transport proteins add to the natriuretic and diuretic effect of ANP because all mentioned Na^+-coupled processes are inhibited by ANP and a diuretic effect is coupled with an increased Na^+ loss. Therefore, a combination of hemodynamic and direct tubular effects is the most probably the explanation for the natriuretic and diuretic effects of ANP. Whether ANP is solely responsible for these effects or the renal natriuretic peptide urodilatin with a higher stability is the key regulator in the kidney remains to be answered.

REFERENCES

1. Aperia A, Holtbäck U, Syrén M-L, Svensson L-B, Fryckstedt J, Greengard P. Activation/deactivation of renal Na^+, K^+-ATPase: a final common pathway for regulation of natriuresis. *FASEB J* 1994;**8**: 436–9.
2. Bacic D, Hernando N, Traebert M, Lederer E, Völkl H, Biber J, et al. Regulation of the renal type IIa Na/Pi cotransporter by cGMP. *Pflügers Arch* 2001;**443**:306–13.
3. Bailly C. Effect of luminal atrial natriuretic peptide on chloride reabsorption in mouse cortical thick ascending limb: inhibition by endothelin. *J Am Soc Nephrol* 2000;**11**:1791–7.
4. Charbardès D, Montégut M, Mistaoui M, Butlen D, Morel F. Atrial natriuretic peptide effects on cGMP and cAMP contents in microdissected glomeruli and segments of the rat and rabbit nephrons. *Pflügers Arch* 1987;**408**:366–72.
5. Cogan MG. Atrial natriuretic factor can increase renal solute excretion primarily by raising glomerular filtration. *Am J Physiol* 1986;**250**: F710–4.
6. Dai L-J, Quamme GA. Atrial natriuretic peptide initiates Ca^{2+} transients in isolated renal cortical thick ascending limb cells. *Am J Physiol* 1993;**265**:F592–7.
7. Darvish N, Winaver J, Dagan D. A novel cGMP-activated Cl- channel in renal proximal tubule. *Am J Physiol* 1995;**268**:F323–9.
8. Dillingham MA, Anderson RJ. Inhibition of vasopressin action by atrial natriuretic factor. *Science* 1986;**231**:1572–3.
9. Forssmann W-G, Richter R, Meyer M. The endocrine heart and natriuretic peptides: histochemistry, cell biology, and functional aspects of the renal urodilatin system. *Histochem Cell Biol* 1998;**110**: 335–57.
10. Harris PJ, Thomas D, Morgan TO. Atrial natriuretic peptide inhibits angiotensin-stimulated proximal tubular sodium and water reabsorption. *Nature* 1987;**326**:697–8.
11. Hirsch JR. Renal K^+-transport regulated by cGMP. In: Pandalai SG, editor. *Recent research developments in physiology*. Tivandrum: Research Signpost; 2003. p. 465–88.
12. Hirsch JR, Schlatter E. Genistein potentiates the ANP effect on a K^+-conductance in HEK-293 cells. *Cell Physiol Biochem* 2003;**13**:223–8.
13. Hirsch JR, Skutta N, Schlatter E. Signaling and distribution of NPR-Bi, the human splice form of the natriuretic peptide receptor type B. *Am J Physiol Renal Physiol* 2003;**285**:F370–4.
14. Honrath U, Chong CK, Melo LG, Sonnenberg H. Effect of saline infusion on kidney and collecting duct function in atrial natriuretic peptide (ANP) gene "knockout" mice. *Can J Physiol Pharmacol* 1999;**77**:454–7.
15. John SW, Veress AT, Honrath U, Chong CK, Peng L, Smithies O, et al. Blood pressure and fluid–electrolyte balance in mice with reduced or absent ANP. *Am J Physiol* 1996;**271**:R109–14.
16. Kishimoto I, Dubois SK, Garbers DL. The heart communicates with the kidney exclusively through the guanylyl cyclase-A receptor: acute handling of sodium and water in response to volume expansion. *Proc Natl Acad Sci USA* 1996;**93**:6215–9.
17. Klokkers J, Langehanenberg P, Kemper B, Kosmeier B, von Bally G, Riethmüller C, et al. Atrial natriuretic peptide and nitric oxide signaling antagonizes vasopressin-mediated water permeability in inner medullary collecting duct cells. *Am J Physiol Renal Physiol* 2009;**297**:F693–703.
18. Majowicz MP, Gonzalez Bosc LV, Albertoni Borghese MF, Delgado MF, Ortiz MC, Sterin Speciale N, et al. Atrial natriuretic peptide and endothelin-3 target renal sodium–glucose cotransporter. *Peptides* 2003; **24**:1971–6.
19. Mauerer UR, Boulpaep EL, Segal AS. Properties of an inwardly rectifying ATP-sensitive K^+ channel in the basolateral membrane of renal proximal tubule. *J Gen Physiol* 1998;**111**:139–60.
20. Mendez RE, Dunn BR, Troy JL, Brenner BM. Modulation of the natriuretic response to atrial natriuretic peptide by alterations in peritubular Starling forces in the rat. *Circ Res* 1986;**59**:605–11.
21. Nakamura K, Hirano J, Itazawa S-I, Kubokawa M. Protein kinase G activates inwardly rectifying K^+ channel in cultured human proximal tubule cells. *Am J Physiol Renal Physiol* 2002;**283**: F784–91.
22. Nonoguchi H, Sands JM, Knepper MA. ANF inhibits NaCl and fluid absorption in cortical collecting duct of rat kidney. *Am J Physiol* 1989;**256**:F179–86.
23. Patil RV, Han Z, Wax MB. Regulation of water channel activity of aquaporin 1 by arginine vasopressin and atrial natriuretic peptide. *Biochem Biophys Res Commun* 1997;**238**:392–6.
24. Peterson LN, De Rouffignac C, Sonnenberg H, Levine RZ. Thick ascending limb response to dDAVP and atrial natriuretic factor in vivo. *Am J Physiol* 1987;**252**:F374–81.
25. Pietig G, Mehrens T, Hirsch JR, Çetinkaya I, Piechota H, Schlatter E. Properties and regulation of organic cation transport in freshly isolated human proximal tubules. *J Biol Chem* 2001;**276**:33741–6.
26. Rouch AJ, Chen L, Troutman SL, Schafer JA. Na^+ transport in isolated rat CCD: effects of bradykinin, ANP, clonidine, and hydrochlorothiazide. *Am J Physiol* 1991;**260**:F86–95.
27. Schlatter E, Cermak R, Forssmann W-G, Hirsch J, Kleta R, Kuhn M, et al. cGMP activating peptides do not regulate electrogenic electrolyte transport in principal cells of rat CCD. *Am J Physiol* 1996;**271**: F1158–65.
28. Schulz-Knappe P, Honrath U, Forssmann W-G, Sonnenberg H. Endogenous natriuretic peptides: effect on collecting duct function in rat kidney. *Am J Physiol* 1990;**259**:F415–8.
29. Syrén M-L. Effect of atrial natriuretic factor and fate of cyclic-guanosine-monophosphate in the rat kidney. *Acta Physiol Scand* 1997;**160**:1–7.

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30. van Aubel RAMH, Smeets PHE, Peters JGP, Bindels RJM, Russel FGM. The MRP4/ABCC4 gene encodes a novel apical organic anion transporter in human kidney proximal tubules: putative efflux pump for urinary cAMP and cGMP. *J Am Soc Nephrol* 2002;**13**:595–603.
31. Vives D, Farage S, Motta R, Lopes AG, Caruso-Neves C. Atrial natriuretic peptides and urodilatin modulate proximal tubule Na^+-ATPase activity through activation of the NPR-A/cGMP/PKG pathway. *Peptides* 2010;**31**:903–8.
32. Yokota N, Bruneau BG, de Bold MLK, de Bold AJ. Atrial natriuretic factor significantly contributes to the mineralocorticoid escape phenomenon. *J Clin Invest* 1994;**94**:1938–46.
33. Zeidel ML, Kikeri D, Silva P, Burrowes M, Brenner BM. Atrial natriuretic peptides inhibit conductive sodium uptake by rabbit inner medullary collecting duct cells. *J Clin Invest* 1988;**82**:1067–74.
34. Zeidel ML, Seifter JL, Lear S, Brenner BM, Silva P. Atrial peptides inhibit oxygen consumption in kidney medullary collecting duct cells. *Am J Physiol* 1986;**251**:F379–83.

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Chapter 200

Endothelin

David M. Pollock

ABSTRACT

Thanks to the development of highly specific receptor antagonists and cell-specific knockout mice over the past decade, we now know that endothelin-1 (ET-1) plays a critical role in the management of salt and water excretion, and, thus, in blood pressure regulation. In addition, an imbalance to the ET system, either through excessive ET-1 production with increased ET_A receptor activation or reduced ET_B receptor function, contributes to the development of hypertension, renal inflammation, and diabetic renal injury. The kidney is a major site of ET-1 synthesis, and ET is involved in the regulation of tubular transport and fluid–electrolyte balance. Progress in our understanding has been slowed down by the opposing functions of ET_A and ET_B receptors along with the autocrine and paracrine nature of ET-1. Nonetheless, recent studies have helped to clarify the physiological role of this peptide system, especially within the kidney.

BASIC COMPONENTS OF THE ENDOTHELIN SYSTEM

Other chapters in the Cardiovascular, Respiratory, and Cancer/Anticancer Peptides sections of this book provide more extensive overviews of the history of the endothelin system and so this will not be reviewed here in detail. The endothelin system consists of three distinct ET peptides, ET-1, ET-2, and ET-3 that are synthesized through a series of proteolytic cleavages of the original gene product to result in a 21-amino-acid biologically active peptide. ET-1 is the peptide primarily released from vascular endothelium along with its immediate precursor, big ET-1. Conversion of big ET-1 to ET-1 is primarily achieved by a unique serine protease referred to as the endothelin-converting enzyme (ECE) that has several intracellular and extracellular isoforms. A variety of different cell types in the kidney synthesize all three ET peptides, but little is known about specific ET-2 or ET-3 isopeptide function.

ET_A and ET_B receptors were originally classified by their binding affinity to the peptide isoforms. ET_A receptors bind ET-1 and ET-2 with high affinity, whereas the ET_B receptor binds all three ET isopeptides with equal affinity.[18] ET_A receptors located primarily on vascular smooth muscle cause constriction as well as promote cellular growth and proliferation. However, ET_B receptors are primarily located on endothelial cells to stimulate vasodilation through release of nitric oxide and other endothelial-derived vasodilators. However, there are some vascular beds, including the kidney, that also have ET_B receptors located on vascular smooth muscle to produce vasoconstriction. For reasons yet to be discerned, ET_B-dependent constriction appears only at pharmacological concentrations of ET-1. Endothelial and vascular ET_B receptors seem to be derived from the same gene, yet they can be distinguished pharmacologically. Some have referred to these as functional ET_B receptor subtypes and have been called ET_{B1} and ET_{B2} located on endothelium and vascular smooth muscle, respectively. The general function of the ET system in the vascular wall is depicted in Figure 1.

LOCALIZATION OF THE ET-1 SYSTEM IN THE KIDNEY

Physiological regulation of ET-1 synthesis within the kidney seems to occur primarily in response to dietary salt intake. Under healthy conditions, increases in dietary salt increase intrarenal expression of preproET-1 mRNA, ET-1 peptide content, and urinary ET-1 excretion.[18] There are many additional factors that have been shown to increase endothelial and epithelial cell ET-1 synthesis. These include hormonal factors, such as angiotensin II and aldosterone. Under pathophysiological conditions, factors such as hyperglycemia, inflammation, shear stress, oxidative stress, and hypoxia, are among the reported stimulators. Within the renal cortex, ET-1 can be synthesized not only by vascular endothelium but also by vascular smooth muscle, mesangial cells, podocytes, and tubular epithelial cells.[18] Within the renal medulla, ET-1 synthesis has been reported within vasa recta, thick ascending limb, and collecting duct cells.[18] Similar to the immunoreactivity studies, gene expression experiments confirmed the idea that sites of ET-1 mRNA expression include glomeruli, proximal tubules, thick ascending

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00200-1
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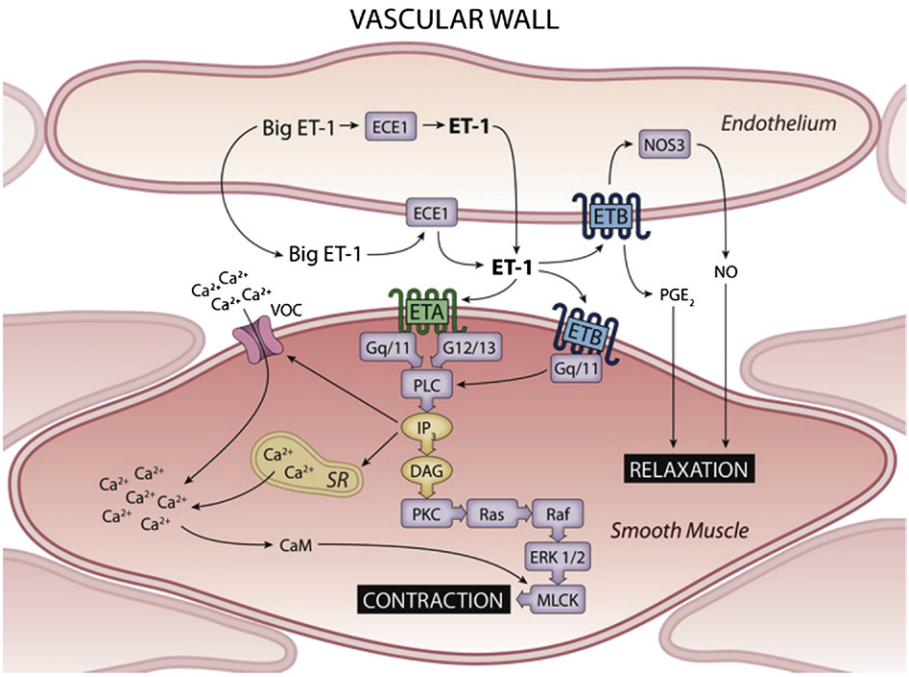

FIGURE 1 Basic function of the endothelin (ET-1) system in the vascular wall. (Used with permission from the American Physiological Society.[18])

limbs, vasa recta, and outer/inner medullary collecting ducts and that relatively higher amounts of ET-1 are synthesized within the renal medulla.[18]

Similar to ET-1 peptide, animal studies have reported that endothelin-converting enzyme mRNA and protein expression can be identified in glomeruli, proximal straight tubule, cortical/medullary thick ascending limb, and inner medullary collecting duct.[18] ECE-1 immunoreactivity in the human kidneys is observed in vascular and glomerular endothelial cells and vasa recta capillaries. The tubular epithelium of the outer/inner medullary collecting ducts also contains ECE-1.

Localization of ET receptors was first identified in autoradiography studies. Both ET_A and ET_B receptors are present in the kidney as described above for the ET-1 peptide. The common localization has confirmed the belief that the peptide serves as an autocrine and as a paracrine function rather than any sort of classic hormonal role. The net result of the binding studies has revealed the presence of ET_A receptors in the renal vascular smooth muscle, mesangial cells, and some tubular cells. The majority of specific binding sites in the renal cortex are localized in what appear to be endothelial cells of glomeruli and peritubular capillaries. However, the largest number of binding sites has been localized to the renal medulla, and in particular, to the inner medullary collecting duct.

RENAL HEMODYNAMIC EFFECTS OF ET-1

Intravenous infusion of exogenous ET-1 peptide in several species has resulted in a profound decreased renal blood flow and GFR.[18] However, direct infusion into the renal artery of dogs results in a short-lived vasodilation followed by a prolonged vasoconstriction. Vasodilatory actions of ET-1 have been difficult to discern in rodent species although blockade of ET_B receptors increases the vasoconstrictor response to ET-1. ET_B receptors in the kidney are also believed to be important in producing vasoconstrictor actions of ET-1, but when examining lower doses of ET-1, all of the vasoconstrictor actions can be blocked by an ET_A selective antagonist, thus suggesting that the ET_B constriction in the kidney is of only pharmacological significance.

When considering the renal hemodynamic effects of ET-1, there are significant differences within the renal cortex versus medulla. Gurbanov et al. were the first to show that an intravenous bolus of ET-1 actually increases medullary blood flow while at the same time decreasing cortical blood flow by using implanted single optical fibers for measuring blood flow velocity.[15] Subsequently, studies in our laboratory showed that a continuous intravenous infusion of the precursor, big ET-1, into the rat produces a decrease in renal cortical blood flow without a change in medullary blood flow.[31] However, when rats are maintained on a high salt diet, medullary blood flow actually increases, whereas the cortical blood flow decreases as before.

The difficulty with *in vivo* hemodynamic studies using exogenous peptide is that delivery of the peptide may not be the same as that seen with endogenous production. ET-1 is released preferentially to the basal side of the endothelial cell and binds irreversibly to its receptor.[18] Therefore, intravenous or intraarterial infusion may not always mimic endogenous activity.

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Studies attempting to localize ET-1-receptor-dependent action in the preglomerular and postglomerular vessels have resulted in inconsistent results [18] In isolated rabbit renal arterioles, ET-1 has been shown to be equipotent at constricting afferent and efferent arterioles. By contrast, another study conducted in isolated rat vessels suggested that ET-1 is more potent in efferent arterioles compared with in afferent arterioles. ET-3 also constricts isolated renal arterioles, but at much lower concentrations, and so one would conclude that the primary function in the renal microcirculation is mediated by the ET_A receptor. In the hydronephrotic kidney, Endlich et al. reported that ET-1 induced preglomerular constriction largely results from ET_A receptor activation, whereas ET_B receptor dependent vasoconstriction is more evident in postglomerular vessels.

ET-1, and presumably ET_A receptor activation, increases cytosolic Ca^{2+} in vascular smooth muscle by activating T- and L-type channels.[18] However, there may be some Ca^{2+} independent effects of ET-1 to produce the renal vasoconstriction. Several studies have shown that ET-1 increases intracellular Ca^{2+} concentrations in isolated afferent arterioles or isolated preglomerular vascular smooth muscle cells.[29] In the hydronephrotic kidney, Ca^{2+} channel antagonism prevents ET-1 induced renal vasoconstriction. However, blockade of L-type Ca^{2+} channels *in vivo* has only minor effects, if any, on the renal blood flow response to ET-1 or sarafotoxin 6c, an ET_B receptor-selective agonist. Although it is apparent that ET-1 increases intracellular Ca^{2+} levels in vascular smooth muscle, it seems as though L-type Ca^{2+} channel activation does not play a major role in the renal vasoconstriction produced by ET-1 via either the ET_A or ET_B receptor.

Much of the known facts about the actions of endogenous ET-1 has been discerned from the use of receptor-selective antagonists. In a euvolemic rat, blockade of both receptors with a nonselective ET_A/ET_B receptor antagonist, has little effect on arterial pressure or renal blood flow, but produces a small decrease in GFR.[25] This is also associated with a decrease in glomerular blood flow caused by a significant increase in preglomerular arteriole resistance. However, selective blockade of the ET_A receptor produces the same effect on arterial pressure and renal blood flow as combined ET_A and ET_B blockade but does not change vascular resistance. These results support the idea that the physiological role of ET-1 in the renal vasculature is primarily vasodilation as a means of regulating GFR, presumably via the ET_B receptor. This effect is most probably a result of the release of vasodilator agents such as NO or prostacyclin, but little is specifically known about the endothelial-derived mediators that stimulate ET_B-dependent vasodilation in the kidney.

ET-1 CONTROL OF EXCRETORY FUNCTION

Historically, factors that increase blood pressure, such as angiotensin, norepinephrine and vasopressin, were all considered to also enhance retention of sodium and water in the kidney. Thus, it made sense when initial studies investigating the influence of ET-1 on renal excretion of salt and water suggested that ET-1 induced increases in renal perfusion pressure account for the natriuretic and diuretic effects of ET-1. However, systemic administration of ET-1 at relatively low doses that does not increase blood pressure or decrease the filtered load of salt and water were reported to have potent diuretic and natriuretic effects.[18] Consistent with this effect being via the ET_B receptor, the diuretic and natriuretic response to big ET-1 is not influenced by ET_A receptor blockade despite significant attenuation of the blood pressure response.[23] Increases in medullary blood flow have the potential for also facilitating natriuresis and diuresis. The medullary hemodynamic actions produced by ET-1 have also been shown to be mediated by ET_B receptors.[15,31] As discussed below, a series of elegant studies from the Kohan laboratory have established that direct renal tubular actions of ET-1 via the ET_B receptor on collecting duct cells account for the reduced reabsorption and increased renal excretion of sodium and water. Although the primary actions of ET-1 may be within the collecting duct, other tubular segments appear to have a functional ET-1/ ET_B receptor system.

Proximal Tubule

ET-1 seems to have a biphasic effect on ion transport in the proximal tubule by stimulating influx at low concentrations and inhibiting at high concentrations, yet both effects are protein-kinase-C-dependent (PKC).[18] There is additional evidence supporting both effects. The stimulatory effect of ET-1 on fluid transport at low concentration can be attributed to the stimulation of the Na^+/H^+ exchanger. This seems to result from the activation of ET_B receptors since Na^+/H^+ exchanger activity is increased in opossum kidney cells transfected with ET_B receptors but not ET_A receptors. In these same cells, ET-1 increases intracellular Ca^{2+} levels, decreases adenylyl cyclase activity, and increases tyrosine phosphorylation of several proteins, suggesting that the fluid transport effect is independent of these distinct pathways. Further, ET-1 has been reported to inhibit Na^+/K^+-ATPase activity along with increasing cGMP concentration in cultured proximal tubule cells. Consistent with most tubular actions of ET-1, inhibition of Na^+/K^+-ATPase activity probably results from ET_B receptor activation because ET-1 and ET-3 are equally potent. Other studies have shown that ET-1 inhibits proximal tubule fluid reabsorption by augmenting the production of arachidonic acid metabolites. At high concentrations, ET-1 not only stimulates PKC but also enhances phospholipase A_2 metabolite generation.

There is also evidence that 20-hydroxyeicosa-5,8,11, 14-tetraenoic acid (20-HETE), a cytochrome P450 metabolite, contributes to ET-1-mediated natriuretic responses. Inhibition of 20-HETE formation in isolated proximal tubules

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prevented the blockade of ion transport produced by ET-1 providing fairly clear evidence that 20-HETE acts as a second messenger of ET-1 in this nephron segment.[20] In addition, blockade of the enzyme responsible for 20-HETE synthesis (CYP4A) prevents the increase in urinary sodium excretion produced by ET-1.[20] An additional link between the ET and cytochrome P450 systems comes from the observation that chronic ET_B receptor blockade reduces CYP4A expression in the kidney, although secondary actions of increased blood pressure and/or increased ET_A receptor activation could also play a role.

Thick Ascending Limb of Henle's Loop

Isolated segments of thick ascending limb express ET-1 mRNA and secrete ET-1.[18] ET_B receptor activation decreases chloride reabsorption in isolated mouse medullary and cortical thick ascending limbs via a cAMP-independent and Ca^{2+}-insensitive diacylglycerol-responsive PKC pathway. Plato et al. extended these observations to determine that activation of ET_B receptors in the medullary thick ascending limbs inhibits chloride influx via a NO-dependent mechanism.[21] The NO synthase inhibitor, L-NAME completely blocks the ET-1 induced decrease in chloride reabsorption. Blockade of ET_B receptors with BQ-788 also prevents the effects of exogenous ET-1 on chloride flux.

Collecting Duct

The renal collecting duct, more specifically, the principal cell, is a major site of ET-1 synthesis, receptor binding, and action.[18] ET-1 receptor expression and production increase along the length of the collecting duct from the cortex to the inner medulla. The inner medullary collecting duct produces up to ten times as much ET-1 than any other cell type in the nephron does, and most probably more than any cell type in the body.

Very little is known about factors that regulate ET-1 synthesis in the collecting duct. A fairly convincing case has been made that dietary salt intake has a major influence on ET-1 production within the kidney as reflected by increased urinary ET-1 excretion.[18] Work from Garvin's laboratory has provided evidence that a high salt diet produces a significant increase in renal medullary interstitial sodium concentration and that ET-1 production in the medullary thick ascending limb is directly correlated to extracellular sodium concentration. Further, there is evidence that increases in fluid shear rate within the renal tubular system, similar to that observed during increased salt and water intake, can stimulate ET-1 production. A wide range of other factors can influence ET-1 gene expression in the collecting duct including hypoxia. However, most other factors that work in this region of the kidney have relatively modest effects on ET-1 production, for example, atrial natriuretic peptide and nitric oxide. However, this is not to suggest that these other factors do not modulate ET-1 action, but there is relatively little known about mechanisms that control production.

Endothelin seems to be released from the basolateral side of the collecting duct similar to what has been reported for endothelial cell production and is consistent with an autocrine function given the preponderance of ET_B receptors on these same cells.[18] ET-1 effects on sodium versus water handling seem to be somewhat distinct, that is, the effects on water are not simply a response to changing sodium movement. ET-1 blocks water transport by inhibiting AVP-stimulated cAMP accumulation and osmotic water permeability in the inner medullary collecting duct. The ability of ET-1 to inhibit water reabsorption involves the inhibition adenylyl cyclase activity because administration of a cAMP mimetic to increase water reabsorption is unaffected by ET-1. Unfortunately, it has been very difficult to measure actual sodium transport in the inner medullary collecting duct where most of the ET-1 is produced because of technical limitations.

Soon after the discovery of ET-1, Zeidel et al. reported that ET-1 reduces sodium transport in freshly isolated inner medullary collecting duct cells by inhibiting the Na^+/K^+-ATPase on the basolateral side of the membrane, an effect dependent upon prostaglandin E_2 production stimulated by ET_B receptor activation.[35] ET-1 is also believed to decrease sodium transport by negatively regulating epithelial sodium channel (ENaC) activity. Several laboratories have reported that ET-1 decreases ENaC activity through activation of Src kinase and can be inhibited by a specific ET_B receptor antagonist, but not ET_A receptor blockade.[4] Stockand's laboratory has reported that ENaC activity measured by patch-clamping methods is absent in response to ET-1 in isolated, split open collecting ducts from collecting-duct-specific ET_B receptor knockout mice.[3]

Assessment of tissue homogenates from inner medullary tissue clearly show that the ET_B receptor is expressed at a much higher level compared with the ET_A receptor, although the precise ratio within the principal cell of the collecting duct itself is not clear because of the presence of receptors in other cell types including vascular tissue where the ET_A receptor is expressed at higher levels. Interestingly, the fact that infusion of ET-1 into the renal medulla can increase medullary blood flow indicates that many of the ET_B receptors could be on vascular elements.[15,31]

Activation of ET_B receptors inhibits vasopressin (AVP) stimulated water transport by decreasing cAMP levels through the activation of PKC.[30] Mineralocorticoid and AVP-stimulated sodium and chloride transport is diminished by ET-1 via inhibition of apical sodium and chloride entry.[9] *In vitro* studies have shown that ET-1 inhibits sodium transport by decreasing amiloride-sensitive sodium channel activity and increasing channel mean closed time.[9]

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Similar to the proximal tubule, ET-1 seems to have a biphasic response in the cortical collecting duct. A concentration of ET-1 in the picomolar range will decrease sodium channel activity through ET_B activation while a concentration in the nanomolar range can increase sodium channel activity through ET_A activation; however, this mechanism is not well understood.

Lessons from Genetically Modified Animals

A series of studies from the Kohan laboratory has provided more compelling evidence that ET-1 produced by the collecting duct plays a key role in fluid-volume balance and blood pressure regulation. Collecting-duct-specific ET-1 knockout (CD ET-1 KO) mice have altered renal sodium and water handling suggesting a physiological role of collecting-duct-derived ET-1 in regulating tubular fluid reabsorption.[1,14] Similar to CD ET-1 KO mice, the same laboratory generated collecting-duct-specific ET_B receptor knockouts that have a very similar phenotype although the increase in blood pressure at baseline and in response to a high salt diet are roughly half that observed in CD ET-1 KO mice.[12]

The low level of ET_A receptor expression in the collecting duct suggests a less important function compared with the ET_B receptor although there have been some reports that ET_A receptors regulate tubular transport by increasing endothelial nitric oxide synthase expression.[34] Ge et al. observed that collecting duct-specific ET_A receptor knockout (CD ET_A KO) mice have similar arterial pressure, sodium excretion, and water excretion as their control counterparts when challenged with normal and high salt diets.[14] However, CD ET_A KO mice are unable to excrete an acute water load appropriately and have an increased sensitivity to AVP-induced cAMP accumulation.[14] These results suggest that ET_A receptors in the collecting duct are not involved in the physiological regulation of arterial pressure or sodium excretion but more specifically help to regulate water reabsorption. Findings from the collecting duct knockout mice provide insight into the possibility that there may be an interaction between ET_A and ET_B receptors to function in a cooperative manner. As mentioned, CD ET-1 KO mice have a significant level of salt-sensitive changes in blood pressure that are roughly twice as much as CD ET_B KO mice while CD ET_A KO mice have similar blood pressures as control mice regardless of salt intake. When both ET_A and ET_B receptors are simultaneously knocked out of the collecting duct, the salt sensitivity is identical to CD ET-1 KO mice.[13] These findings suggest a cooperative, although not independent, role for the ET_A receptor in regulating blood pressure sensitivity to salt.

Additional evidence that ET-1 may contribute to fluid-electrolyte balance, and, thus, blood pressure regulation arises from increased renal ET-1 activity associated with increased dietary salt intake. Investigators generally agree that urinary ET-1 is exclusively derived from intrarenal sources. Because a high salt diet increases urinary ET-1 excretion and intrarenal mRNA expression, ET-1 production is clearly increased in the kidney in response to a high salt diet. It can be presumed that this increase in intrarenal ET-1 in response to high salt intake is a physiological mechanism designed to reduce sodium and water reabsorption and elimination of the increased salt ingested. There is little conclusive information on whether renal ET_A or ET_B receptor expression is changed in response to dietary salt intake. Increased ET_B receptor function in rats on a high salt diet is also evidenced by the observation that increases in medullary blood flow produced by big ET-1 are more pronounced in rats on a high salt diet.[31]

As discussed above, gene-targeting strategies have provided more specific evidence that ET-1 regulates salt and water excretion. The initial findings arise from the spotting lethal rat that carries a naturally occurring deletion of the ET_B receptor gene resulting in deadly megacolon.[11] Gariepy et al. rescued these rats with a dopamine-β-hydroxylase promoter that directed tissue-specific ET_B transgene expression to support normal enteric nervous system development yet remain without ET_B receptors on endothelial or renal tubular epithelial cells.[10] Rats homozygous for the ET_B receptor deletion have a higher basal arterial pressure compared to their control counterparts. Similar to pharmacological ET_B receptor blockade, ET_B deficient rats become hypertensive when challenged with a high salt diet.[10] Figure 2 summarizes the general mechanisms within the renal medulla whereby both vascular and tubular actions of both ET_A and ET_B receptors can influence sodium excretion.

RENAL ET-1 IN HYPERTENSION

The influence of ET-1 on sodium and water balance along with potent vascular actions suggests that the intrarenal ET system may contribute to the development of hypertension. This could occur by inappropriate or excessive ET_A receptor activation or a loss of ET_B function (Figure 3). Initial evidence that renal ET-1 may contribute to the development of hypertension came from a series of reports that administration of both ET_A selective and ET_A/ET_B antagonists reduce blood pressure in salt-dependent models' hypertension, but not in other models. These include DOCA-salt, chronic Ang II infusion, SHR-SP, and Dahl salt-sensitive rats. ET antagonists are not effective in all models such as the SHR and the 2-kidney, 1-clip Goldblatt hypertensive rat that have been reported to be unresponsive to ET receptor blockade. The blood-pressure-lowering effects appear to exclusively result from ET_A receptor blockade because selective inhibition of ET_B receptors exacerbates the elevated arterial pressure in models such as the DOCA-salt hypertensive rat. Further, in

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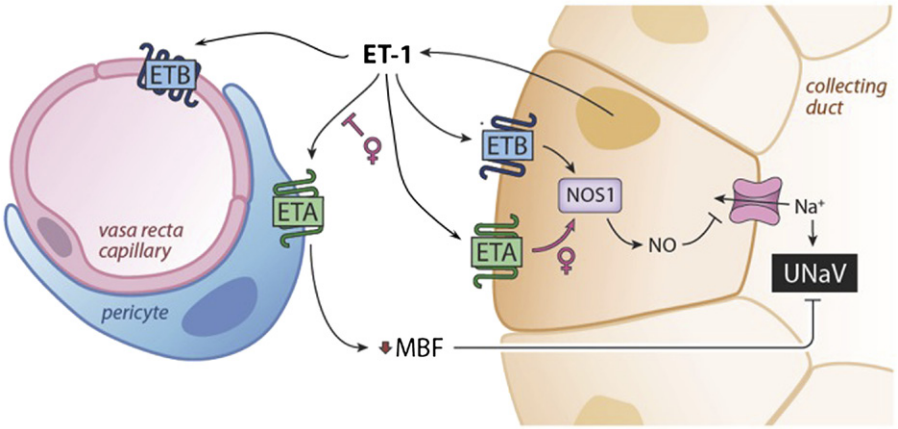

FIGURE 2 Basic function of the endothelin (ET-1) system in the renal medulla. (Used with permission from Morgan & Claypool Life Sciences.[22]) See color plate 53.

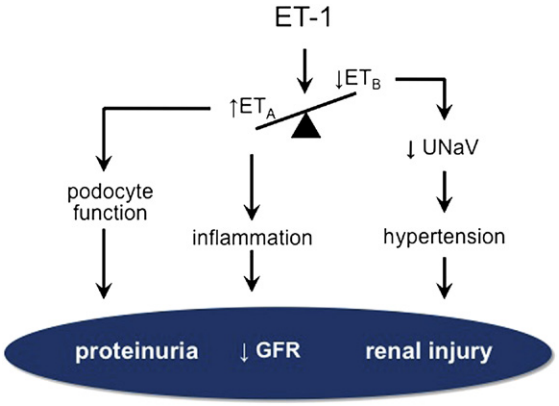

FIGURE 3 Hypothetical scheme for ET-1 contribution to renal injury associated with hypertension and diabetes.

normotensive rats, chronic ET_B receptor blockade elevates blood pressure in a salt-dependent manner.[24]

To date, there is no clear explanation for what triggers the imbalance of the ET system to contribute to hypertension. This could occur through inappropriately excessive ET-1 production that overwhelms the ability of ET_B receptors to protect against ET_A activation. However, inappropriate ET_A receptor activation could occur in response to a loss of ET_B receptor function without any change in ET-1 production.

A new hypothesis perhaps should be considered to explain why and/or how ET-1 causes hypertension, that is, the need to increase renal perfusion pressure and facilitate pressure natriuresis. Given the clear function of ET-1 to increase salt and water excretion, a concomitant elevation in systemic blood pressure in association with reduced renal tubular reabsorption can work synergistically to increase excretory function. This idea fits well with Guyton's view of renal control of blood pressure.

In human hypertension, early reports suggested that plasma concentrations of ET-1 are elevated.[28] However, this has not been a universal finding. There are many factors for this inconsistency including variety in specificity of many of the initial assays, the heterogeneity of patient populations, and the fact that plasma ET concentrations are more a factor of ET_B receptor function (clearance of circulating ET-1) as much as they are an indication of production. There has been one report also that the stress of venipuncture can increase plasma ET-1 acutely, and so the method of blood drawing could contribute to this variability as well (see Treiber et al., 2000, in *Hypertension*).

Some investigators have suggested that urinary ET-1 may be a marker for renal injury associated with chronic kidney disease.[6] Although ET-1 gene expression is elevated by many factors associated with CKD such as hypoxia and inflammation, ET-1 as a biomarker is questionable given the strong association between renal ET-1 production and dietary salt intake. In hypertension, there have been reports of elevated urinary ET excretion compared with subjects who are normotensive, but this has not always been observed. There have been no real attempts to use urinary ET-1 as a predictor of hypertension-dependent renal injury.

The use of ET receptor antagonists for the treatment of human hypertension and other cardiovascular diseases has received considerable attention.[5] Most results would suggest that ET antagonists are effective at reducing blood pressure in systemic human hypertension. A phase III trial for resistant hypertension provided evidence that darusentan, an ET antagonist with fairly high selectivity for the ET_A receptor, may be effective at producing significant reductions in blood pressure even in subjects already taking at least three different drugs for hypertension including angiotensin converting enzyme inhibitors and angiotensin receptor blockers.[32] In a follow-up to this promising study, a similar effective blood pressure reduction was observed when measured by ambulatory methods; however, acute blood pressure measurements did not reach the efficacy end point and so the company decided to discontinue

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its efforts to develop this drug.[2] At present, there do not seem to be any major efforts to develop ET antagonists for hypertension despite the clear evidence for ET involvement.

ET AND KIDNEY DISEASE

A role for ET in the pathogenesis of a wide range of organ failure diseases has received considerable attention including acute and chronic renal disease. Animal model studies first suggested that ET-1 contributes to reduced renal function after ischemia/reperfusion or administration of nephrotoxic agents.[8] Hypoxia and a wide range of inflammatory agents can stimulate ET-1 production and so targeting the ET system became the focus of many drug discovery efforts. Treatment with ET antagonists can attenuate development of renal failure and end organ damage in many of these models although most studies have used preventative protocols. These observations also are valid for models of chronic kidney disease such as 5/6 nephrectomy.[19] However, clinical trials were disappointing for reasons that are not clear. It is possible that the mechanistic pathways whereby ET-1 is stimulated are well established and difficult to reverse by the time therapy is initiated.

In recent years, there has been a series of very promising developments for the use of endothelin-receptor blockade in the treatment of proteinuric renal disease including diabetic nephropathy (Figure 4). A large multicenter trial showed that avosentan, a modestly selective ET_A antagonist, produced an average of 50% reduction in proteinuria in patients with diabetic nephropathy, the vast majority of whom were already taking ACE and ARB therapy.[33] However, this trial had to be stopped because of widespread development of edema in roughly 40% of the subjects enrolled. Kohan et al. used a more selective ET_A antagonist, atrasentan, at very low doses and observed similar reductions in proteinuria without the high degree of edema.[17]

The rationale for targeting proteinuria with an endothelin antagonist has also been supported by mechanistic animal model studies. In glomeruli isolated from streptozotocin-induced diabetic rats, Saleh et al. showed that *in vitro* treatment with an ET_A antagonist acutely reduces glomerular permeability to albumin.[27] These authors also showed that chronic treatment of STZ-diabetic rats reduces proteinuria and renal inflammation. In fact, increased glomerular permeability to albumin can be seen in rats given a chronic infusion of ET-1 for 2 weeks without the development of hypertension, an effect that can be reversed by ET_A antagonist treatment.[26] These studies suggest a direct, dynamic influence of ET-1 on glomerular permeability characteristics that seem to be related to direct effects on glomerular podocytes. This idea is further supported by studies in humans with nondiabetic proteinuric renal disease showing that acute administration of an ET_A antagonist reduces proteinuria within one hour.[7]

CONCLUSIONS

There is little question now that the renal endothelin system plays a critically important role in the control of fluid–electrolyte balance, and therefore, in blood pressure regulation. The localized action of the renal ET system explains how what has been considered the most potent vasoconstrictor can also produce vasodilation and facilitate the excretion of salt and water. Although human kidneys have similar ET-1 production and distribution of ET receptors, there have been no studies to determine the intrarenal actions of endogenous ET-1. In one study examining the influence of ET-1 infusion on renal function, however, the dose used produced a significant degree of renal vasoconstriction and reduced GFR, so any potential influence of ET_B receptors to promote natriuresis would have been prevented.[16] Clearly, the factors that regulate the production of ET-1, receptor expression,

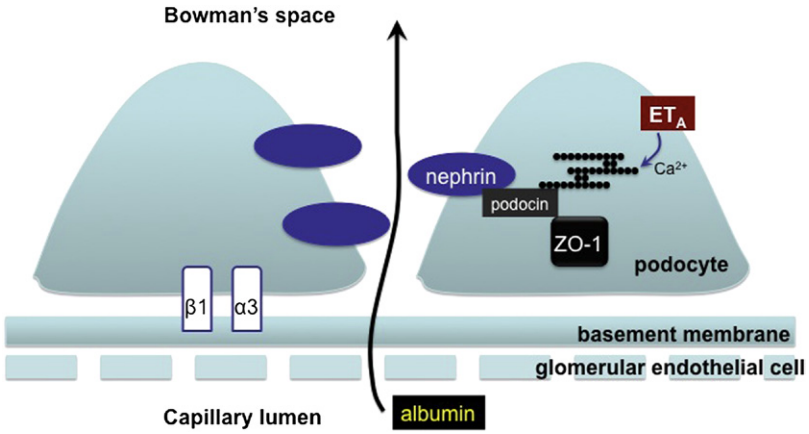

FIGURE 4 Theoretical role of ET_A receptors in influencing the glomerular filtration barrier and filtration of albumin. (Used with permission from the Morgan & Claypool Life Sciences.[22])

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and activity in the human kidney remain to be elucidated, especially in the setting of high dietary salt intake typical of the Western diet.

REFERENCES

1. Ahn D, Ge Y, Stricklett PK, Gill P, Taylor D, Hughes AK, et al. Collecting duct-specific knockout of endothelin-1 causes hypertension and sodium retention. *J Clin Invest* 2004;**114**:504–11.
2. Bakris GL, Lindholm LH, Black HR, Krum H, Linas S, Linseman JV, et al. Divergent results using clinic and ambulatory blood pressures: report of a darusentan-resistant hypertension trial. *Hypertension* 2010;**56**:824–30.
3. Bugaj V, Mironova E, Kohan DE, Stockand JD. Collecting duct-specific endothelin B receptor knockout increases ENaC activity. *Am J Physiol Cell Physiol* 2012;**302**:C188–94.
4. Bugaj V, Pochynyuk O, Mironova E, Vandewalle A, Medina JL, Stockand JD. Regulation of the epithelial Na+ channel by endothelin-1 in rat collecting duct. *Am J Physiol Renal Physiol* 2008;**295**:F1063–70.
5. Dhaun N, Goddard J, Kohan DE, Pollock DM, Schiffrin EL, Webb DJ. Role of endothelin-1 in clinical hypertension: 20 years on. *Hypertension* 2008;**52**:452–9.
6. Dhaun N, Lilitkarntakul P, Macintyre I, Muilwijk E, Johnston NR, Kluth DC, et al. Urinary endothelin-1 in chronic kidney disease and as a marker of disease activity in lupus nephritis. *Am J Physiol Renal Physiol* 2009;**296**:F1477–83.
7. Dhaun N, MacIntyre IM, Kerr D, Melville V, Johnston NR, Haughie S, et al. Selective endothelin-A receptor antagonism reduces proteinuria, blood pressure, and arterial stiffness in chronic proteinuric kidney disease. *Hypertension* 2011;**57**:772–9.
8. Gagliardini E, Buelli S, Benigni A. Endothelin in chronic proteinuric kidney disease. *Contrib Nephrol* 2011;**172**:171–84.
9. Gallego MS, Ling BN. Regulation of amiloride-sensitive sodium channels by endothelin-1 in distal nephron cells. *Am J Physiol* 1996;**271**:F451–60.
10. Gariepy CE, Ohuchi T, Williams SC, Richardson JA, Yanagisawa M. Salt-sensitive hypertension in endothelin-B receptor-deficient rats. *J Clin Invest* 2000;**105**:925–33.
11. Gariepy CE, Williams SC, Richardson JA, Hammer RE, Yanagisawa H. Transgenic expression of the endothelin-B receptor prevents congenital intestinal aganglionosis in a rat model of Hirschsprung disease. *J Clin Invest* 1998;**102**:1092–101.
12. Ge Y, Bagnall A, Stricklett PK, Strait K, Webb DJ, Kotelevtsev Y, et al. Collecting duct-specific knockout of the endothelin B receptor causes hypertension and sodium retention. *Am J Physiol Renal Physiol* 2006;**291**:F1274–80.
13. Ge Y, Bagnall A, Stricklett PK, Webb D, Kotelevtsev Y, Kohan DE. Combined knockout of collecting duct endothelin A and B receptors causes hypertension and sodium retention. *Am J Physiol Renal Physiol* 2008;**295**:F1635–40.
14. Ge Y, Stricklett PK, Hughes AK, Yanagisawa M, Kohan DE. Collecting duct-specific knockout of the endothelin A receptor alters renal vasopressin responsiveness, but not sodium excretion or blood pressure. *Am J Physiol Renal Physiol* 2005;**289**:F692–8.
15. Gurbanov K, Rubinstein I, Hoffman A, Abassi Z, Better O, Winaver J. Differential regulation of renal regional blood flow by endothelin-1. *Am J Physiol* 1996;**271**:F1166–72.
16. Kaasjager KA, Koomans HA, Rabelink TJ. Endothelin-1-induced vasopressor responses in essential hypertension. *Hypertension* 1997;**30**:15–21.
17. Kohan DE, Pritchett Y, Molitch M, Wen S, Garimella T, Audhya P, et al. Addition of atrasentan to renin-angiotensin system blockade reduces albuminuria in diabetic nephropathy. *J Am Soc Nephrol* 2011;**22**:763–72.
18. Kohan DE, Rossi NF, Inscho EW, Pollock DM. Regulation of blood pressure and salt homeostasis by endothelin. *Physiol Rev* 2011;**91**:1–77.
19. Longaretti L, Benigni A. Endothelin receptor selectivity in chronic renal failure. *Eur J Clin Invest* 2009;**39**(Suppl. 2):32–7.
20. Oyekan AO, McAward K, McGiff JC. Renal functional effects of endothelins: dependency on cytochrome P450-derived arachidonate metabolites. *Biol Res* 1998;**31**:209–15.
21. Plato CF, Pollock DM, Garvin JL. Endothelin inhibits thick ascending limb chloride flux via ET(B) receptor-mediated NO release. *Am J Physiol Renal Physiol* 2000;**279**:F326–33.
22. Pollock DM, Boesen EI. *Endothelin in the kidney*. : Morgan & Claypool Life Sciences; 2012.
23. Pollock DM, Opgenorth TJ. ET_A receptor-mediated responses to endothelin-1 and big endothelin-1 in the rat kidney. *Br J Pharmacol* 1994;**111**:729–32.
24. Pollock DM, Pollock JS. Evidence for endothelin involvement in the response to high salt. *Am J Physiol Renal Physiol* 2001;**281**:F144–50.
25. Qiu C, Samsell L, Baylis C. Actions of endogenous endothelin on glomerular hemodynamics in the rat. *Am J Physiol* 1995;**269**:R469–73.
26. Saleh MA, Boesen EI, Pollock JS, Savin VJ, Pollock DM. Endothelin-1 increases glomerular permeability and inflammation independent of blood pressure in the rat. *Hypertension* 2010;**56**:942–9.
27. Saleh MA, Boesen EI, Pollock JS, Savin VJ, Pollock DM. Endothelin receptor A-specific stimulation of glomerular inflammation and injury in a streptozotocin-induced rat model of diabetes. *Diabetologia* 2011;**54**:979–88.
28. Schiffrin EL. Role of endothelin-1 in hypertension and vascular disease. *Am J Hypertens* 2001;**14**:83S–9S.
29. Schroeder AC, Imig JD, LeBlanc EA, Pham BT, Pollock DM, Inscho EW. Endothelin-mediated calcium signaling in preglomerular smooth muscle cells. *Hypertension* 2000;**35**:280–6.
30. Tomita K, Nonguchi H, Terada Y, Marumo F. Effects of ET-1 on water and chloride transport in cortical collecting ducts of the rat. *Am J Physiol* 1993;**264**:F690–6.
31. Vassileva I, Mountain C, Pollock DM. Functional role of ETB receptors in the renal medulla. *Hypertension* 2003;**41**:1359–63.
32. Weber MA, Black H, Bakris G, Krum H, Linas S, Weiss R, et al. A selective endothelin-receptor antagonist to reduce blood pressure in patients with treatment-resistant hypertension: a randomised, double-blind, placebo-controlled trial. *Lancet* 2009;**374**:1423–31.
33. Wenzel RR, Littke T, Kuranoff S, Jurgens C, Bruck H, Ritz E, et al. Avosentan reduces albumin excretion in diabetics with macroalbuminuria. *J Am Soc Nephrol* 2009;**20**:655–64.
34. Ye Q, Chen S, Gardner DG. Endothelin inhibits NPR-A and stimulates eNOS gene expression in rat IMCD cells. *Hypertension* 2003;**41**:675–81.
35. Zeidel ML, Brady HR, Kone BC, Gullans SR, Brenner BM. Endothelin, a peptide inhibitor of Na^+–K^+-ATPase in intact tubular epithelial cells. *Am J Physiol* 1989;**257**:C1101–7.

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Chapter 201

Neurohypophyseal Peptides

Joseph G. Verbalis

ABSTRACT

The most prominent physiological function of the neurohypophyseal peptides vasopressin (VP) and oxytocin (OT) is maintenance of water homeostasis. The VP V2 receptor subtype (V2R) is responsible for VP-induced antidiuresis via changes in collecting duct water permeability. This process is initiated by V2R ligand binding in collecting duct principal cells, leading to increased intracellular cAMP levels and fusion of aquaporin-2 (AQP2)-containing intracytoplasmic vesicles with the apical plasma membranes of the principal cells, a process that increases water permeability by increasing the number of water-conducting pores in the apical membrane. Data in animals and man have implicated renal effects of OT in pathological antidiuresis, primarily via crossreactivity at V2R at high plasma OT concentrations. In contrast, the natriuretic effects of OT seem to occur at physiological plasma concentrations of OT and can be blocked by OT-specific antagonists, likely as a result of OT receptor (OTR)-ligand binding in the macula densa.

BRIEF HISTORY AND OVERVIEW OF THE NEUROHYPOPHYSEAL PEPTIDES

As described in the Brain Peptides section of this handbook, the neurohypophyseal hormones vasopressin (VP) and oxytocin (OT) are nonapeptides consisting of a 6-amino-acid ring with a cysteine to cysteine bridge, and a 3-amino-acid tail. Some forms of these nonapeptides have existed throughout the evolutionary species from amphibia and reptiles to birds and mammals, and in all species their most prominent physiological function is participation in the maintenance of water homeostasis (although the target organ may vary, from skin to bladder to kidney[2]). It is assumed that OT and VP derive from a single ancestral gene that is expressed in the lamprey and hag fish as only a single gene and hormone, vasotocin, which consists of a ring identical to oxytocin and a tail identical to vasopressin.[2] All mammals synthesize and secrete both OT and arginine VP with the exception of the pig; in the pig a lysine is substituted for arginine in position 8 of vasopressin, producing lysine VP.

Similar to other peptide hormones, VP is synthesized as part of a prohormone, including a midmolecule neurophysin and a carboxy-terminal glycopeptide, named *copeptin*. For OT, the peptide products are the nonapeptide and a neurophysin but not glycopeptide. At the physiologic pH of plasma, there is no binding of either OT or AVP to their respective neurophysins, so each peptide circulates independently in the blood stream. Although each neurophysin and copeptin circulate in plasma, no biological functions have ever been demonstrated for these peptides. Therefore, this chapter will focus exclusively on the actions of VP and OT in the kidney; effects at other organs, and specifically the reproductive functions of OT and the vascular and metabolic actions of VP, are covered elsewhere in this handbook.

BODY FLUID HOMEOSTASIS

Because the most important homeostatic function of VP is the maintenance of normal body water stores, the renal effects of VP that serve to accomplish this function can best be appreciated and understood within the context of the overall body mechanisms that regulate body fluid homeostasis. The maintenance of normal water balance in humans is achieved principally through an integration of thirst, VP secretion, and renal responsiveness to VP. Plasma osmolality is maintained within narrow limits by osmotically regulated VP secretion and thirst. Despite large variations of water intake, the osmolality of body fluids in healthy individuals is maintained within a relatively narrow range (275–295 mOsm/kg H_2O). To maintain plasma osmolality at such a constant level, VP secretion must vary in response to relatively small changes in plasma osmolality, which is achieved through the activation of the osmoreceptors located in the anterior hypothalamus.

The primary stimulus for VP release is an increase in plasma osmolality. There are individual variations in the osmotic threshold, or set point, for VP release, probably reflecting variations in the sensitivity of individual

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00201-3
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osmoreceptor cells. At all plasma osmolalities below this set point, plasma VP is suppressed to low or undetectable levels. Above this set point, secretion of the hormone increases in direct proportion to the increasing plasma osmolality. Therefore, with plasma hypoosomolality, VP is suppressed and urine osmolality is maximally dilute (*diuresis*), while with plasma hyperosmolality, VP levels increase and the urine is progressively more concentrated (*antidiuresis*). The sensitivity of this response can be appreciated by noting that an increase in plasma osmolality of only 1% leads to an increase in plasma VP of 0.4–0.8 pg/mL, a concentration sufficient to significantly increase urinary concentration and decrease urine flow, whereas maximum antidiuresis occurs at a plasma VP concentration of only 5 pg/mL.[27] In general, the osmotic threshold for thirst is approximately 5–10 mOsm/kg H_2O above that for VP release. This allows the kidney to primarily regulate body water homeostasis in response to very small changes in plasma osmolality, whereas thirst is only activated by larger and more threatening osmotic perturbations to maintain total fluid balance by the ingestion of additional fluid.

In addition to changes in plasma osmolality, VP secretion is also stimulated by hypotension and volume depletion, mediated largely by baroreceptors located in the cardiac atria and large arteries. By contrast to the extremely sensitive osmotic regulation of VP secretion, a reduction in effective circulating volume of approximately 10–15% is required before a significant VP response occurs. VP is also released in response to other nonosmotic stimuli such as nausea, pain, hypoxia, hypercapnia, hypoglycemia, physical exercise, and various medications. Although nonosmotic secretion of VP is not involved with normal body fluid homeostasis, such secretion is of importance because the renal effects of VP are the same regardless of the stimulus that caused secretion of the hormone, leading to potential disturbances in water homeostasis from inappropriate antidiuresis.[4]

VASOPRESSIN AND OXYTOCIN RECEPTORS

Three known receptor subtypes mediate the peripheral actions of VP. They are classified according to the second messenger system to which they are coupled. The vasopressin V1a (V1aR) and V1b (V1bR) receptors are linked to the phosphoinositol signaling pathway, with intracellular calcium acting as the second messenger. In contrast, the vasopressin V2 receptors (V2R) are linked to the adenylate cyclase signaling pathway with intracellular cAMP acting as the second messenger.[33] The V1aR subtype is ubiquitous and is present on vascular smooth muscle cells, hepatocytes, and platelets where it mediates vascular constriction, glycogenolysis, and platelet aggregation, respectively. V1aR also seems to be the major AVP receptor subtype found within the brain, where it presumably mediates central effects of this peptide. The V1bR subtype is found predominately in the anterior pituitary, where it mediates adrenocorticotropin release in concert with corticotropin releasing hormone. The V2R subtype is present predominately in the kidney, where it mediates free water reabsorption. In addition, there is now evidence indicating the presence of extrarenal V2R in the endothelium that are involved in the secretion of von Willebrand factor and factor VIIIc.[17] In contrast to vasopressin, only one oxytocin receptor (OTR) has been identified. Similar to the V1aR and V1bR, the OTR is coupled to the phosphoinositol signaling pathway, with intracellular calcium acting as the second messenger.[13] The OTR subtype is present predominantly in breast and uterine tissue, with lesser expression in the brain, kidney, and heart.[13]

LOCALIZATION OF VASOPRESSIN AND OXYTOCIN RECEPTORS IN THE KIDNEY

V2R Expression

V2R is the predominant neurohypophyseal receptor expressed in the kidney, apropos its crucial role in the urinary concentrating process. Prominent expression has been found by autoradiography,[19] immunohistochemistry,[12] and receptor mRNA expression[21] throughout all portions of the collecting duct (cortical, outer, and inner medullary), the connecting segment, the distal convoluted tubule, and the thick ascending limb of the loop of Henle.

V1aR Expression

High levels of V1aR expression have also been found in the renal medulla by autoradiography,[31] Western analysis,[24] and receptor mRNA in situ hybridization histochemistry.[21] However, in contrast to the V2R, the V1aR is localized predominantly among the medullary interstitial cells and vascular elements of the vasa recta rather than the collecting ducts; sparser labeling is found in the renal cortex, but not in the glomeruli.

OTR Expression

In the adult rat, OTR expression by receptor autoradiography occurs predominantly in the macula densa cells of the juxtaglomerular apparatus, with a lesser degree of expression in the medulla on the loops of Henle of juxtamedullary nephrons.[30] The cortical OT binding sites express higher selectivity for OT than AVP compared with the medullary sites, which may be due to binding of OT to medullary V2R. Estrogen treatment of ovariectomized rats induced OTR mRNA expression in the outer stripe of the outer medulla and increased expression in macula densa cells.[22] The induction of outer stripe OTR mRNA expression by

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estradiol was blocked by the antiestrogen, tamoxifen, but was not significantly affected by high levels of circulating oxytocin.[22]

RENAL EFFECTS OF VASOPRESSIN

The primary renal response to increased circulating VP is an increase in water permeability of the kidney collecting tubules, leading to antidiuresis. Although an increase in solute reabsorption (primarily urea, but also sodium) occurs as well, the total solute reabsorption is proportionally much less than water. Consequently, a decrease in urine flow and an increase in urine osmolality occur as secondary responses to the increased net water reabsorption. With refinement of radioimmunoassays for VP, the unique sensitivity of this hormone to small changes in osmolality, as well as the corresponding sensitivity of the kidney to small changes in plasma VP concentrations, have become apparent.[27] Although some debate still exists with regard to the exact pattern of osmotically stimulated AVP secretion, most studies to date have supported the concept of a discrete *osmotic threshold for VP secretion* above which a linear relationship between plasma osmolality and VP concentrations occurs. The slope of the regression line relating VP to plasma osmolality can vary significantly across individual human subjects, in part because of genetic factors. In general, each 1 mOsm/kg H_2O increase in plasma osmolality causes an increase in plasma VP from 0.4 to 0.8 pg/ml. The renal response to circulating VP is similarly linear, with urinary concentration that is directly proportional to VP levels from 0.5 to 4–5 pg/ml, after which urinary osmolality is maximal and cannot increase further despite additional increases in VP levels. Thus, changes of ≥1% in plasma osmolality are sufficient to cause significant increases in plasma VP levels with proportional increases in urine concentration, and maximal antidiuresis is achieved after increases in plasma osmolality of only 5–10 mOsm/kg H_2O (2–4%) above the threshold for VP secretion.

However, even this analysis underestimates the sensitivity of this system to regulate free water excretion for the following reason: Urinary osmolality is directly proportional to plasma VP levels as a consequence of the fall in urine flow induced by the VP, but urine volume is inversely related to urine osmolality. Thus, an increase in plasma VP concentration from 0.5 to 2 pg/ml has a much greater relative effect to decrease urine flow than does a subsequent increase in VP concentration from 2 to 5 pg/ml, thereby further magnifying the physiological effects of small initial changes in plasma VP levels [28] (Fig. 1). The net result of these relations is a finely tuned regulatory system that adjusts the rate of free water excretion accurately to the ambient plasma osmolality via small changes in pituitary VP secretion. Further, the rapid response of pituitary VP secretion to changes in plasma osmolality coupled with the short half-life of VP in human plasma (10–20 minutes) enables this regulatory system to adjust the renal water excretion to changes in plasma osmolality on a minute-to-minute basis.

Over the last decade, the cellular mechanisms responsible for this exquisitely sensitive mechanism for renal water conservation have been elucidated after identification of the aquaporin family of water channels by Agre et al.[7] Renal concentrating ability is determined primarily by proximal water reabsorption, the corticopapillary osmotic gradient, and VP-mediated changes in collecting duct water permeability, all of which involve molecular water channels of the aquaporin family. Four major aquaporin water channels are expressed in the renal tubules.[18] Aquaporin-1 (AQP1) is primarily responsible for the high constitutive water permeability of the proximal tubules and thin descending limbs of the loop of Henle. Aquaporin-3 (AQP3) and aquaporin-4 (AQP4) are expressed in the basolateral membrane of collecting duct principal cells, and confer this epithelial membrane with high water permeability. The most unique water channel is aquaporin-2 (AQP2), which is expressed exclusively in renal collecting duct principal cells and whose activity is largely regulated by VP. VP induces changes in collecting duct water permeability via both short-term and long-term effects that regulate renal aquaporin synthesis and function.[18]

Short-term regulation of renal aquaporins is associated with increases in water permeability within a few minutes of VP exposure, an effect that is rapidly reversible. VP triggers this response by binding to the V2R in the collecting duct principal cells and increasing intracellular cAMP levels by activating adenylyl cyclase. The increase in collecting duct water permeability is a consequence of fusion of AQP2-containing intracytoplasmic vesicles with the apical plasma membranes of the principal cells, a process that increases apical water permeability by markedly increasing the number of water-conducting pores in the apical plasma membrane.[18] Dissociation of VP from the V2R allows intracellular cAMP levels to decrease, and the water channels are reinternalized into the intracytoplasmic vesicles thereby terminating the increased water permeability. By virtue of the subapical membrane localization of the AQP2-containing vesicles, they can be quickly "shuttled" into and out of the membrane in response to changes in intracellular cAMP levels. This mechanism therefore allows minute-to-minute regulation of renal water excretion through changes in ambient plasma VP concentrations. Long-term regulation of collecting duct water permeability represents a sustained increase in collecting duct water permeability in response to prolonged high levels of circulating VP. This response requires at least 24 hours to elicit and is not as rapidly reversible. This conditioning effect results largely from the ability of vasopressin to induce large increases in the abundance of AQP2 and AQP3 water channels in the collecting duct

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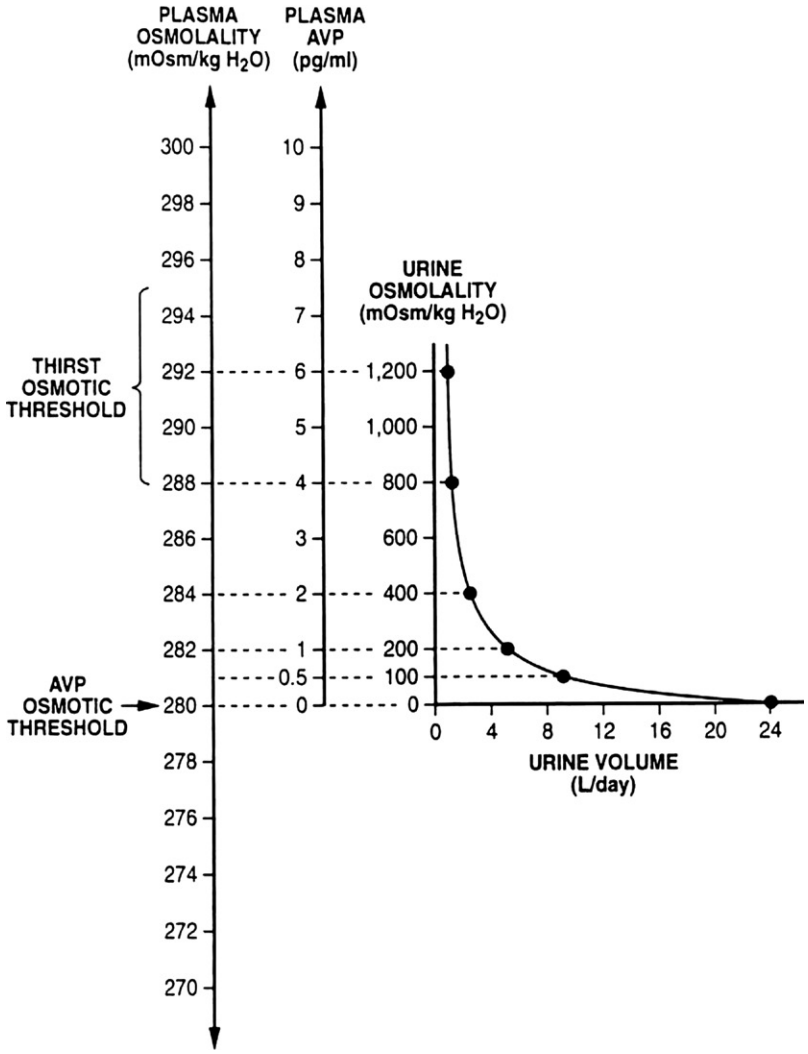

FIGURE 1 Relationship of plasma osmolality, plasma AVP concentrations, urine osmolality, and urine volume in humans. Note that the osmotic threshold for AVP secretion defines the point at which urine concentration begins to increase, but the osmotic threshold for thirst is significantly higher and approximates the point at which maximal urine concentration has already been achieved. Note also that because of the inverse relation between urine osmolality and urine volume, changes in plasma AVP concentrations have much larger effects on urine volume at low plasma AVP concentrations than at high plasma AVP concentrations.

principal cells.[9] Greater total expression of the number of AQP2 and AQP3 water channels, when combined with the short-term effect of vasopressin to shift AQP2 into the apical plasma membrane, allows the collecting ducts to achieve higher water permeabilities during conditions of prolonged dehydration, thereby further enhancing urine concentrating capacity in response to equivalent levels of VP.

In addition to these effects on water permeability of the collecting duct cells to regulate the degree of antidiuresis in response to plasma VP levels, VP also acts to enhance the other major factor regulating renal concentrating ability, namely, establishment and maintenance of the corticopapillary osmotic gradient that is the determinant of maximal urine concentration. VP-dependent reabsorption of sodium into the renal interstitium in the thick ascending limb of the loop of Henle loop is regulated by the V2R in this region of the nephron via upregulation of the Na–K-2Cl cotransporter expression in the outer medulla,[16] and also V2R-mediated increased expression of the epithelial sodium channel (ENaC) in the distal convoluted tubule.[3] Similarly, VP causes increased expression of the UT-A1 urea transporter in rats, either directly or indirectly via effects at V2R in the inner medulla of the kidney.[29] Thus, each of these factors in addition to AQP2 regulation can contribute to the achievement of maximal urinary concentration in response to chronic VP stimulation via long-term upregulation of the countercurrent multiplication system that is responsible for the generation of the corticopapillary osmotic gradient.

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In contrast to the well-understood effects of VP acting at V2R receptors in the kidney, much less is understood about the effects of the V1aR that are also clearly expressed in the kidney. Early studies suggested that V1aR acted at the collecting duct principal cells to antagonize the V2R effects of VP by virtue of increasing intracellular Ca^{2+} by phosphoinositide hydrolysis[5] and/or stimulating synthesis and release of prostaglandins such as PGE_2 and nitric oxide. However, subsequent results have implicated V2R in many of these effects,[10,15] and although some *in vitro* studies have suggested the presence of functional V1aR activity in isolated collecting tubules, no studies to date have conclusively shown expression of V1aR in collecting duct principal cells. Alternatively, in view of the localization of V1aR in the renal cortical and medullary microcirculation combined with results indicating that VP reduces renal medullary blood flow,[8] it is reasonable to postulate an intrarenal hemodynamic effect for VP actions at kidney V1aR. Studies using reverse transcription-polymerase chain reaction and Western blot analyses confirm that V1aR mRNA and protein, but not V2R mRNA and protein, are present in isolated cortical and medullary vasculature.[24] Thus, VP effects at V1aR are probably responsible for the vasoconstrictor actions on renal medullary blood flow.[8]

The physiological ramifications of this effect are multiple, but it has been suggested that regulation of the descending vasa recta microcirculation is a determinant of Na^+ and water excretion, and possibly also the development of hypertension.[8,23] Results support the idea that regulation of descending vasa recta blood flow occurs via the actions of multiple vasoactive substances on the contractile pericytes surrounding these vessels.[23] Substances known to reduce descending vasa recta blood flow via vasoconstriction include angiotensin II, vasopressin, endothelins, norepinephrine, acetylcholine, and adenosine (via adenosine A1R); substances known to increase descending vasa recta blood flow through vasodilatation include nitric oxide, prostaglandin E_2, bradykinin, acetylcholine, and adenosine (via adenosine A2R).[23] The complexity of this regulation is illustrated by the observation that some stimuli activate opposing systems. For example, both angiotensin II and VP have been shown to stimulate nitric oxide production in the kidney,[20,25] which may serve to counterbalance the otherwise unopposed vasoconstrictive effects of these hormones. Decreases in vasa recta blood flow would have the effect of maximizing the corticopapillary concentration gradient, whereas increases in vasa recta flow would enhance a washout of the solutes that establish this gradient. Thus, VP acting at V1aR in the vasa recta and/or the intramedullary pericytes would complement the effects of VP acting at V2R in the nephron to produce maximally concentrated urine by limiting blood flow into the inner medulla.

RENAL EFFECTS OF OXYTOCIN

Because OT has been associated primarily with reproductive functions, studies of the renal effects of OT have been much more limited. However, abundant results in animals and man have implicated renal effects of OT in pathological antidiuresis, and in rats in physiological natriuresis.

Antidiuretic effects of OT were first suggested by cases of hyponatremia in females infused with OT for induction of labor or induced abortion. Studies of OT infusion to normal subjects confirmed the antidiuretic effects of OT infusions,[1] and other studies suggested an antidiuretic potency of OT approximately 1/200 that of VP. Subsequent studies in Brattleboro rats indicated that the limited urinary concentrating abilities in this animal with genetic absence of VP synthesis result from OT effects in the kidney.[11] Early studies did not differentiate whether these effects on urinary concentration resulted from the effects of OT at the OTR, or from crossreactivity of OT at V2R. Subsequent *in vitro* studies using isolated collecting ducts from rats indicate that the antidiuretic effects of OT could be blocked by administration of V2R-specific antagonists, consistent with OT crossreactivity at V2R to explain the antidiuretic effects of high concentrations of plasma OT.[6]

In contrast to the antidiuretic effects of OT, the natriuretic effects of this peptide seem to occur at physiological concentrations of plasma OT,[34] and are blocked by OT-specific antagonists.[35] Initial studies suggested that OT-induced natriuresis may result from stimulation of atrial natriuretic peptide (ANP) secretion form the cardiac atria.[14] However, subsequent studies have indicated that the natriuresis produced by low doses of OT involves a nitric oxide mechanism, unlike ANP-induced natriuresis, and therefore probably represents a direct effect of OT at the OTR on cells of the macula densa,[32] presumably by altering the set point of glomerular–tubular feedback mechanism. However, similar natriuretic effects have not been observed in human studies, suggesting that this represents a species-specific effect of OT.[1,26]

REFERENCES

1. Abdul-Karim R, Assalins NS. Renal function in human pregnancy. V. Effects of oxytocin on renal hemodynamics and water and electrolyte excretion. *J Lab Clin Med* 1961;**57**:522–32.
2. Acher R. Evolution of neurohypophysial control of water homeostasis: integrative biology of molecular, cellular and organismal aspects. In: Saito T, Kurakawa K, Yoshida S, editors. *Neurohypophysis: recent progress of vasopressin and oxytocin research*. Amsterdam: Elsevier; 1995. p. 7–16.
3. Bankir L. Antidiuretic action of vasopressin: quantitative aspects and interaction between V1a and V2 receptor-mediated effects. *Cardiovasc Res* 2001;**51**:372–90.
4. Bartter FC, Schwartz WB. The syndrome of inappropriate secretion of antidiuretic hormone. *Am J Med* 1967;**42**:790–806.

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5. Burnatowska-Hledin MA, Spielman WS. Vasopressin V1 receptors on the principal cells of the rabbit cortical collecting tubule. Stimulation of cytosolic free calcium and inositol phosphate production via coupling to a pertussis toxin substrate. *J Clin Invest* 1989;**83**:84–9.
6. Chou CL, Di Giovanni SR, Luther A, et al. Oxytocin as an antidiuretic hormone. ii. role of v2 vasopressin receptor. *Am J Physiol* 1995;**269**:F78–85.
7. Chrispeels MJ, Agre P. Aquaporins: water channel proteins of plant and animal cells. *Trends Biochem Sci* 1994;**19**:421–5.
8. Cowley Jr AW. Control of the renal medullary circulation by vasopressin V1 and V2 receptors in the rat. *Exp Physiol* 2000;**85**:Spec No:223S-231S.
9. DiGiovanni SR, Nielsen S, Christensen EI, Knepper MA. Regulation of collecting duct water channel expression by vasopressin in Brattleboro rat. *Proc Natl Acad Sci U S A* 1994;**91**:8984–8.
10. Ecelbarger CA, Chou CL, Lolait SJ, et al. Evidence for dual signaling pathways for v2 vasopressin receptor in rat inner medullary collecting duct. *Am J Physiol* 1996;**270**:F623–33.
11. Edwards BR, LaRochelle Jr FT. Antidiuretic effect of endogenous oxytocin in dehydrated Brattleboro homozygous rats. *Am J Physiol* 1984;**247**:F453–65.
12. Fahrenholz F, Jurzak M, Gerstberger R, Haase W. Renal and central vasopressin receptors: immunocytochemical localization. *Ann N Y Acad Sci* 1993;**689**:194–206.
13. Gimpl G, Fahrenholz F. The oxytocin receptor system: structure, function, and regulation. *Physiol Rev* 2001;**81**:629–83.
14. Haanwinckel MA, Elias LK, Favaretto AL, et al. Oxytocin mediates atrial natriuretic peptide release and natriuresis after volume expansion in the rat. *Proc Natl Acad Sci U S A* 1995;**92**:7902–6.
15. Kaufmann JE, Iezzi M, Vischer UM. Desmopressin (DDAVP) induces NO production in human endothelial cells via V2 receptor- and cAMP-mediated signaling. *J Thromb Haemost* 2003;**1**:821–8.
16. Kim GH, Ecelbarger CA, Mitchell C, et al. Vasopressin increases Na-K-2Cl cotransporter expression in thick ascending limb of Henle's loop. *Am J Physiol* 1999;**276**:F96–103.
17. Kinter LB, McConnell I, Goodwin BT, et al. Vasopressin antagonist inhibition of clotting factor release in the rhesus monkey (*Macaca mulatta*). *J Pharmacol Exp Ther* 1992;**261**:462–9.
18. Knepper MA. Molecular physiology of urinary concentrating mechanism: regulation of aquaporin water channels by vasopressin. *Am J Physiol* 1997;**272**:F3–12.
19. Mimura Y, Ogura T, Hayakawa N, et al. In vitro macro- and micro-autoradiographic localization of v1 and v2 receptors in the rat kidney using opc-21268 and opc-31260. *Nephron* 1997;**76**:331–6.
20. Navar LG, Ichihara A, Chin SY, Imig JD. Nitric oxide-angiotensin II interactions in angiotensin II-dependent hypertension. *Acta Physiol Scand* 2000;**168**:139–47.
21. Ostrowski NL, Lolait SJ, Bradley DJ, et al. Distribution of v1a and v2 vasopressin receptor messenger ribonucleic acids in rat liver, kidney, pituitary and brain. *Endocrinology* 1992;**131**:533–5.
22. Ostrowski NL, Young WS3, Lolait SJ. Estrogen increases renal oxytocin receptor gene expression. *Endocrinology* 1995;**136**:1801–4.
23. Pallone TL, Zhang Z, Rhinehart K. Physiology of the renal medullary microcirculation. *Am J Physiol Renal Physiol* 2003;**284**:F253–66.
24. Park F, Mattson DL, Skelton MM, Cowley Jr AW. Localization of the vasopressin v1a and v2 receptors within the renal cortical and medullary circulation. *Am J Physiol* 1997;**273**:R243–51.
25. Park F, Zou AP, Cowley Jr AW. Arginine vasopressin-mediated stimulation of nitric oxide within the rat renal medulla. *Hypertension* 1998;**32**:896–901.
26. Rasmussen MS, Simonsen JA, Sandgaard NC, et al. Effects of oxytocin in normal man during low and high sodium diets. *Acta Physiol Scand* 2004;**181**:247–57.
27. Robertson GL. The regulation of vasopressin function in health and disease. *Rec Prog Horm Res* 1976;**33**:333–85.
28. Robinson AG. Disorders of antidiuretic hormone secretion. *Clin Endocrinol Metab* 1985;**14**:55–88.
29. Sands JM. Renal urea transporters. *Curr Opin Nephrol Hypertens* 2004;**13**:525–32.
30. Schmidt A, Jard S, Dreifuss JJ, Tribollet E. Oxytocin receptors in rat kidney during development. *Am J Physiol* 1990;**259**:F872–81.
31. Serradeil-Le Gal C, Raufaste D, Marty E, et al. Autoradiographic localization of vasopressin V1a receptors in the rat kidney using [3H]-SR 49059. *Kidney Int* 1996;**50**:499–505.
32. Soares TJ, Coimbra TM, Martins AR, et al. Atrial natriuretic peptide and oxytocin induce natriuresis by release of cGMP. *Proc Natl Acad Sci U S A* 1999;**96**:278–83.
33. Thibonnier M, Conarty DM, Preston JA, et al. Molecular pharmacology of human vasopressin receptors. *Adv Exp Med Biol* 1998;**449**:251–76.
34. Verbalis JG, Mangione MP, Stricker EM. Oxytocin produces natriuresis in rats at physiological plasma concentrations. *Endocrinology* 1991;**128**:1317–22.
35. Windle RJ, Judah JM, Forsling ML. Effect of oxytocin receptor antagonists on the renal actions of oxytocin and vasopressin in the rat. *J Endocrinol* 1997;**152**:257–64.

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Chapter 202

Peptides, Sex, Diabetes, and the Kidney

Michaele B. Manigrasso and Christine Maric-Bilkan

ABSTRACT

Diabetic nephropathy is characterized by a complex interaction between metabolic and hemodynamic factors that activate common second messenger signaling pathways leading to the production of several locally active growth factors and peptides that contribute to the development of renal injury. Unlike nondiabetic renal diseases that predominantly affect men, diabetic renal disease affects women and men similarly. This loss of the female sex as a protective factor against renal disease in the setting of diabetes has been attributed to the reduction in estrogen levels. Further, men with diabetes also exhibit an imbalance in sex hormone levels, mainly characterized by the loss of testosterone. Experimental studies have shown that estrogens and androgens, via interacting with locally active peptides, such as angiotensin II, contribute to the development and progression of renal disease. These observations show that estrogens and androgens have a role beyond reproduction and are important contributors to diabetic nephropathy.

INTRODUCTION

Diabetes is the leading cause of end-stage renal disease (ESRD) in the Western world and is associated with increased cardiovascular risk, high morbidity, and mortality.[30] With the increasing prevalence of diabetes mellitus worldwide, the prevalence of ESRD is also likely to steadily increase despite the advancements in the clinical management and treatment of the disease. Thus, ESRD resulting from diabetes, that is, diabetic nephropathy, remains a significant economic and health burden.

Diabetic nephropathy is characterized by a complex interaction between metabolic and hemodynamic factors that activate common second messenger signaling pathways leading to the production of a number of locally active growth factors and peptides that contribute to the development of renal injury. One of the most important vascoative peptides, and certainly the most studied in the context of diabetic nephropathy is angiotensin II (Ang II). However, other peptides and growth factors, such as endothelin, also play an important role in the development and progression of diabetic renal injury. This review will focus on the contribution of these peptides, especially via their interaction with estrogens and androgens in mediating cellular processes that contribute to diabetic nephropathy.

Although ESRD affects both men and women, there is an intriguing gender disparity in the prevalence of ESRD with respect to its cause. Specifically, the incidence of ESRD resulting from causes other than diabetes (e.g., hypertension) is significantly lower in premenopausal women compared with that in age-matched men.[24] After the onset of menopause, this gender disparity is largely diminished and the incidence of ESRD due to hypertension dramatically increases in women. These observations lead to the belief that it is the loss of estrogens associated with menopause that contributes to this increase in the incidence of ESRD in postmenopausal women. But in contrast to ESRD resulting from hypertension in which premenopausal women are relatively protected, women with either type 1 or type 2 diabetes are just as likely to develop ESRD as men regardless of their age and menopausal status.[26] Studies from this and other laboratories have gone on to show that women with type 1 or type 2 diabetes are characterized by reduced levels of circulating estradiol,[20] which could possibly explain why women with diabetes seem to be less protected from developing renal disease compared with nondiabetic women. Experimental studies using rodent models of diabetic renal disease have shown that treatment with 17-β estradiol (E_2) attenuates the disease development and progression,[21] suggesting that estrogens may regulate biological processes that are adversely regulated and contribute to diabetic renal disease. Indeed, several studies have examined some of the mechanisms by which estrogens may regulate cell proliferation, apoptosis, and extracellular matrix (ECM) metabolism,[10] all of which are important contributors to diabetic renal disease. Further, studies have shown that estrogens exert their actions in the kidney by interacting with the locally active vasoactive peptides, including Ang II.[19]

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00202-5
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Similar to women, men with either type 1 or type 2 diabetes exhibit an imbalance in sex hormones levels, in particular low testosterone, which correlates with the decline in renal function.[22] Experimental studies have shown that supplementation with testosterone, to levels of those observed in nondiabetic animals, protects the kidney.[34] Specifically, testosterone obviates ECM metabolism and promotes apoptosis in the diabetic kidney contributing to the development of glomerulosclerosis and tubulointerstitial fibrosis.[11] Similar to estrogens, studies show that the effects of testosterone are mediated via interaction with vasoactive peptides such as Ang II and endothelin.[19] This review will summarize our current understanding of the contribution of sex hormones, both estrogens and androgens, via their interaction with locally active peptides and growth factors, in the pathophysiology of diabetic nephropathy.

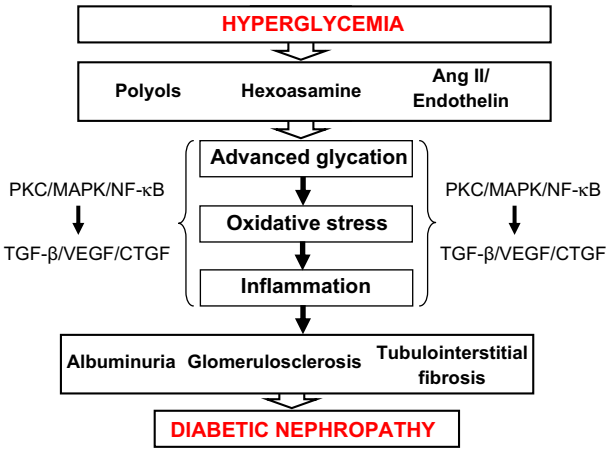

FIGURE 1 Interaction between vasoactive peptides, estrogens, and androgens in modulating metabolic and hemodynamic pathways in the pathophysiology of diabetic nephropathy.

ETIOLOGY OF DIABETIC NEPHROPATHY

Diabetic nephropathy is manifested clinically in a sequence of stages beginning with initial glomerular hyperfiltration, glomerular hypertrophy, and microalbuminuria. Elevated mean arterial pressure (MAP), which is common in patients with diabetic nephropathy, further exacerbates the disease progression to proteinuria, nodular glomerulosclerosis and tubulointerstitial injury and a decline in glomerular filtration rate (GFR), eventually leading to ESRD.[30]

Diabetic nephropathy develops as a result of an interaction between several metabolic and hemodynamic factors. These factors all activate common intracellular signaling pathways which, in turn, trigger the production of numerous growth factors, peptides and cytokines that contribute to the development of renal injury. In particular, chronic hyperglycemia, which is characteristic of diabetes mellitus, activates nonenzymatic glycation and oxidative stress, polyol pathway, and hexosamine flux.[6] Chronic hyperglycemia also dysregulates renal hemodynamics via activating a number of vasoactive peptides including members of the renin–angiotensin system (RAS) and endothelin.[15] These vasoactive peptides then activate common second messenger signaling pathways including protein kinase C (PKC) and MAP kinase (MAPK), and transcription factors such as nuclear factor-κB (NF-κB). These second messenger pathways are involved in regulating the expression of genes of a number of growth factors and cytokines including transforming growth factor-beta (TGF-β), vascular endothelial growth factor (VEGF), connective tissue growth factor (CTGF), etc., all of which have been implicated in the development of albuminuria, glomerulosclerosis, and tubulointerstitial fibrosis associated with diabetic nephropathy.[4] This complex interaction between metabolic and hemodynamic pathways in the etiology of diabetic nephropathy is summarized in Figure 1.

PEPTIDE REGULATION OF RENAL FUNCTION IN THE DIABETIC KIDNEY

The Role of the Ang II in Diabetic Nephropathy

The initial understanding of the role of the RAS in the pathophysiology of diabetic nephropathy was complicated by the observation that circulating levels of Ang II, one of the main effector molecules of the RAS, were unaltered in both type 1 and type 2 diabetes.[33] However, angiotensin-converting enzyme (ACE) inhibitors (which inhibit the conversion of ANG I into the biologically active peptide Ang II) such as enalapril and lisinopril, clearly offer renoprotection in both experimental models of diabetic nephropathy and in humans.[28] After the discovery of specific tissue RASs, including the kidney, it was clear that despite no differences in circulating Ang II concentrations, the renal Ang II levels are elevated in the setting of diabetes[18] and that blocking its formation may be an effective treatment of diabetic nephropathy. Until about a decade ago, most of the known biological actions of Ang II were believed to be mediated via the Ang II AT_1 receptor subtype. Thus, in addition to ACE inhibition, AT_1 receptor subtype blockade (ARB) has become a common and effective treatment for diabetic nephropathy. Further, in recent years, both clinical trials and experimental studies have reported the superiority of the combination therapy of ACE inhibition and ARB over either treatment alone in diabetic nephropathy.[23]

As indicated above, for decades, the AT_1 receptor subtype has been believed to mediate most, if not all, of the known

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biological effects of Ang II, despite the observation that another receptor, namely, AT_2, has also been identified. However, in the past 15 years, emerging evidence suggests that the AT_2 receptor has just as important role in the diabetic kidney as does the AT_1 receptor. It is now believed that the AT_1 and AT_2 receptors mediate opposing effects of Ang II in the kidney. Specifically, activation of AT_1 receptors leads to renal vasoconstriction, sodium retention, cell proliferation, and ECM synthesis,[35] whereas activation of AT_2 receptors leads to vasodilatation, natriuresis, and inhibition of cell proliferation.[14] Other actions of Ang II in the diabetic kidney include stimulation of differentiation and proliferation of monocytes and macrophages via increasing monocyte chemoattractant protein-1 (MCP-1), ostepontin, and RANTES expression. These actions of Ang II have mainly been attributed to stimulation of a number of profibrotic cytokines such as TGF-β, VEGF and CTGF as well as activation of downstream signaling pathways involving PKC and NF-κB leading to the development of glomerulosclerosis and tubulointerstitial fibrosis.[13]

The Role of the Endothelin in Diabetic Nephropathy

Several studies show that the endothelin system is important in the pathophysiology of chronic kidney disease, including diabetes, and that endothelin levels correlate with serum creatinine and the degree of albuminuria in patients with diabetic nephropathy. Multiple factors have been reported to increase renal endothelin-1 levels, including cytokines, growth factors, and sex hormones.[3] As discussed in other chapters of this book, endothelins are potent vasoconstrictors with proinflammatory, promitogenic, and profibrotic properties in many target organs, including the kidney. In the context of diabetic nephropathy, most of the biological actions of endothelins are mediated by endothelin-1, which exerts its renal actions through its two receptor subtypes, the ET_A and ET_B. Studies show that selective ET_A receptor antagonists and combined ET_A/ET_B receptor antagonists attenuate hypertension, renal vasoconstriction, inflammation, proteinuria, glomerulosclerosis, and tubulointerstitial fibrosis in experimental models of diabetic nephropathy.[9] Although hyperglycemia-associated inflammation and fibrosis are mediated via both ET_A and ET_B receptors, vasoconstrictor actions of endothelin-1 seem to be mediated via ET_A receptors. In addition to increased synthesis of the ligand itself, diabetic nephropathy is associated with increased ET_A receptor expression. Some of the actions of endothelin-1 in the diabetic kidney include promoting ECM matrix protein synthesis involving the MAPK and PKC signaling pathways, mesangial cell proliferation, constriction of cortical and medulary vessels, and inhibition of sodium and water reabsorption.[25] Collectively, these observations suggest that inhibiting the expression and actions of endothelins may be a beneficial treatment of diabetic nephropathy.

ESTROGEN–PEPTIDE INTERACTIONS IN THE DIABETIC KIDNEY

Although estrogen is generally thought of in the context of reproduction, studies, especially in the past several years, have shown that estrogens play an important role in mediating biological functions beyond that of the reproductive system. With respect to the kidney, several studies show that estrogens, E_2 in particular, modulate cellular processes that are involved in the pathophysiology of diabetic nephropathy. Experimental studies using the streptozotocin (STZ)-induced diabetic rat show that E_2 attenuates glomerulosclerosis and tubulointerstitial fibrosis via reducing collagen type I and type IV synthesis, increasing expression of matrix metalloproteinases (MMPs), and by inhibiting apoptosis of mesangial cells and podocytes involving the PI3 K-AKT signaling cascade.[12] Further, E_2 decreases the expression of growth factors and cytokines, such as TGF-β, VEGF, and CTGF, all of which stimulate abnormal cell growth, ECM metabolism and contribute to vascular dysfunction associated with renal disease. At least in the kidney, all of these functions have mainly been ascribed to activation of the estrogen ERα receptor. Until recently, all the biological effects of estrogens have been thought to be mediated via binding to the classical estrogen nuclear ERα and ERβ receptors. More recently, a novel seven-transmembrane, G-protein-coupled estrogen receptor (GPR30) has been identified;[31] however, the importance of activation of the GPR30 receptor by estrogens in the kidney is currently unclear.

Estrogens tonically downregulate renal AT_1R in the Dahl salt-sensitive rat kidney,[16] increase the expression of renal AT_2Rs,[2] and reduce renal ACE and Ang II levels.[5] Studies in the rats containing the mouse DBA/Ren-2 transgene show that E_2 attenuates the hypertensive response to infusion with Ang II via shifting the vasoconstrictor/vasodilator balance.[5] These effects add to the protective effects of estrogens via suppressing the activity of the renal RAS. Although the direct interaction between estrogens and the RAS in the pathophysiology of diabetic nephropathy has not been fully explored, studies suggest that ACE inhibition in women with type 1 diabetes is associated not only with the decline in blood pressure, but also a reduction in GFR. Further, the renal blood flow responses to Ang II infusion are negatively correlated with estrogen levels, with the higher levels predicting a blunted response.[8] These observations support the notion that estrogens may regulate renal function both directly and via modulating the expression and activity of the RAS. Figure 2 summarizes the known interactions between estrogens and

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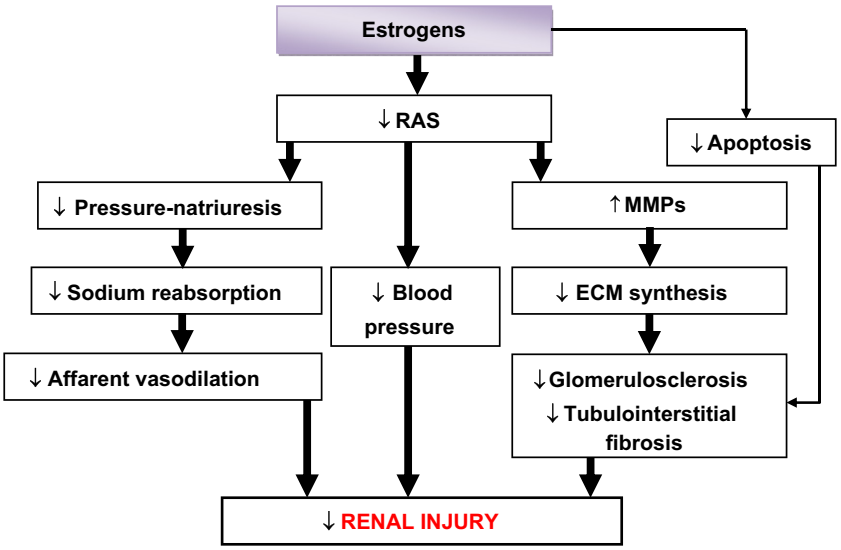

FIGURE 2 Interaction between estrogens and the RAS in modulating metabolic and hemodynamic pathways in the pathophysiology of nondiabetic and diabetic renal injury.

the RAS in the kidney: please note that the current data indicate that the effects of estrogens in the nondiabetic and diabetic kidney are similar.

There is a growing body of evidence suggesting that metabolites of E_2, such as 2-methoxyestradiol (2-ME) and 2-hydroxyestradiol (2-OHE), mediate the beneficial effects of E_2 but without the feminizing side effects. Treatment with 2-OHE attenuates proteinuria in chronic puromycin aminonucleoside nephropathy[32] and attenuates the development of obesity, metabolic syndrome, and vascular and renal dysfunction in obese ZSF1 rats. In mesangial cells, 2-ME and 2-OHE inhibit mitogenesis and ECM synthesis via estrogen-receptor-independent mechanisms. However, no study to date has examined the effects of 2-OHE in diabetic nephropathy and the mechanisms of their action in the diabetic kidney remain largely unexplored. Preliminary data from our laboratory show that 2-OHE is renoprotective in an experimental model of hypertension-accelerated diabetic nephropathy via reducing albuminuria and tubulointerstitial fibrosis (unpublished observations). Further, we have evidence to show that these effects of 2-OHE in the diabetic kidney may be mediated via regulation of the expression of the components of the RAS (unpublished observations); however, further studies are needed to fully understand the importance of the interaction between estrogen metabolites and the RAS in the pathophysiology of diabetic nephropathy.

Collectively, these studies clearly indicate that estrogens have a beneficial effect in the kidney; however, it should be pointed out that all these described effects of estrogens are observed only in the females. Although no study to date has examined the effect of estrogens in the male diabetic kidney, there is reason to believe that these effects may differ in the two sexes, but why? As mentioned above, diabetes is associated with an imbalance in sex hormone levels. Specifically, in the females, diabetes is characterized by decreased estrogen levels alongside increased testosterone levels. In contrast, in the males, diabetes is associated with decreased testosterone and increased circulating estradiol levels. Thus, it seems that in the females, the loss of estrogen may contribute to the loss of the female sex as a protective factor against renal disease and that estrogen supplementation is renoprotective. However, given that estrogen levels are elevated in males with diabetes, it is conceivable that, in the males, estrogens may contribute to the development and progression of diabetic nephropathy. Indeed, experimental studies from our laboratory show that inhibition of estrogen synthesis in diabetic males may be renoprotective, suggesting that estrogens may promote renal injury in the setting of diabetes. Future studies are warranted to fully examine the renal effects of estrogens in both males and females with diabetic renal disease.

ANDROGEN–PEPTIDE INTERACTIONS IN THE DIABETIC KIDNEY

Similar to estrogens, the actions of testosterone are best understood in the context of reproduction; however, studies over the past decade have shown that these classic actions of testosterone may be involved in modulating functions in peripheral organs, including the kidney. It is well known that testosterone provides a negative biofeedback mechanism on the hypothamlamus-pituitary axis to inhibit gonadotropin secretion. Other pituitary hormones such as growth hormone (GH) and insulin-like growth factor-I (IGF-I) may promote diabetic renal disease. Studies have

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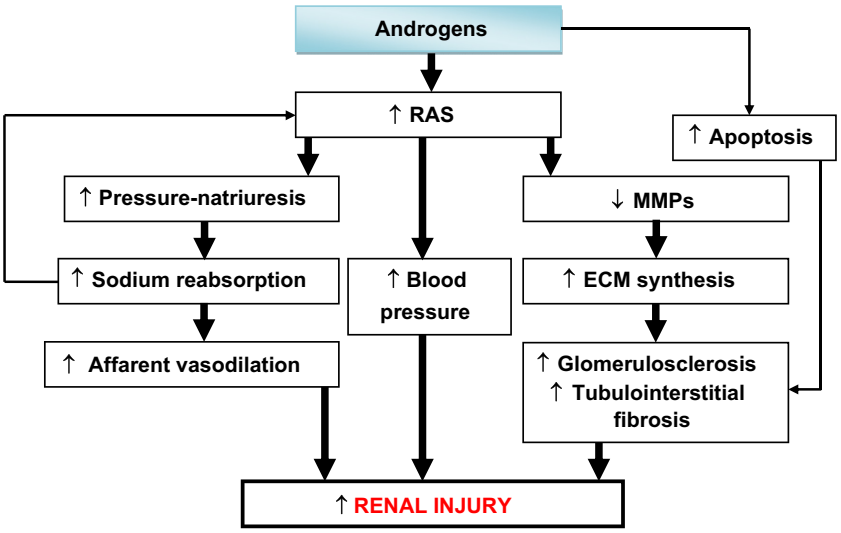

FIGURE 3 Interaction between androgens and the RAS in modulating metabolic and hemodynamic pathways in the pathophysiology of nondiabetic renal injury.

shown that there is a direct correlation between GH and IGF-I activity and an increase in renal hypertrophy and glomerulosclerosis in experimental models of diabetic renal disease.[7] The peak in testosterone, GH and IGF-I levels during puberty suggests that these hormones may promote the development of diabetic renal disease, as diabetic end-organ complications, including diabetic nephropathy, rarely develop before puberty. Therefore, alterations observed in sex hormone levels that occur with puberty may be a contributing factor to the onset and increased rate of progression of diabetic nephropathy. Corroborating this evidence is the observation that GH concentrations are elevated in adolescents with type 1 diabetes compared with that of healthy non-diabetic individuals and there is a correlation between GH and microalbuminuria along with decreased GFR.[27] In contrast to plasma levels of IGH-I, which are elevated in diabetic patients, renal levels of IGF-I are decreased, suggesting that local production and biological metabolism of IGF-I may possibly be more important in determining the renal effects of this peptide.

The effects of testosterone in the kidney are best understood in the context of blood pressure regulation. It has been documented that androgens can directly stimulate the expression and activity of the components of the renal RAS in experimental models of hypertension, leading to increases in blood pressure and sodium retention. Namely, plasma renin activity is lowered after castration in the male spontaneously hypertensive (SHR) rat and elevated in ovariectomized SHR females after supplementation with testosterone.[29] Similarly, renal angiotensinogen mRNA levels decrease after castration and increase after supplementation with exogenous testosterone, suggesting androgens also directly regulate angiotensinogen levels. Further, this increase in angiotensinogen substrate may partially explain the increase in renin observed in males. In the hypertensive mRen(2) Lewis rat, ACE activity is higher in males than in females and androgens increase ACE activity. Treatment with an ACE inhibitor, enalapril, reduces blood pressure in SHR male and testosterone-supplemented ovariectomized females, supporting the notion that androgens play a role in the development of hypertension by stimulating RAS expression and activity. Although the exact mechanisms underlying androgen-driven hypertension are not completely understood, the currently accepted hypothesis is that testosterone causes a rightward shift in the pressure-natriuresis curve via enhancing proximal tubular reabsorption leading to decreased sodium delivery to the macula densa. This consequently stimulates renin release and tubuloglomerular feedback-mediated afferent vasodilation, which, in combination with the increase in arterial pressure, results in glomerular hypertension and renal injury. With respect to the interaction between androgens and the RAS in the context of diabetic nephropathy, very few studies have examined this relationship either in experimental models or humans (Fig. 3). What is known is that ACE inhibition in men with type 1 diabetes reduces arterial pressure, but only females show a reduction in GFR.[8] This suggests that ACE inhibition is more effective in reducing renal injury associated with early diabetes in women than men, possibly because of the testosterone-stimulated increase in other components of the RAS. Clinical trials examining the efficacy of inhibitors of the RAS in patients with diabetic nephropathy have yielded conflicting findings with respect to the differences in men and women, with some studies showing that ARBs and ACE inhibitors are more effective in females while others reporting the opposite. However, very few studies

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have examined the direct interaction between the inhibitors of the RAS and sex hormones in diabetic nephropathy, and drawing any conclusions from these observations would be risky. Thus, the summary of the known interactions between androgens and the RAS depicted in Figure 3 only applies to the nondiabetic kidney.

In addition to the interactions between androgens and the RAS, testosterone inhibits endothelial production and vasodilator action of nitric oxide in obese fa/fa Zucker diabetic rats [1] and enhances the platelet aggregation and the TXA2-endothelin-1 receptor crosstalk in this experimental model of type 2 diabetes.[1] Further, endogenous androgens regulate the systemic and renal microvascular response to endothelin-1. In addition to growth factors and sex hormones, insulin also stimulates both the production and action of endothelin-1 in both experimental animals and in humans. Furthermore, clinical studies have shown that testosterone reduces insulin sensitivity, whereas testosterone replacement reduces insulin resistance and improves glycemic control in hypogonadal men with type 2 diabetes.[17] The mechanisms by which testosterone improves insulin sensitivity in men is likely due to a combination of effects on multiple organs and may involve reduction of the production of inflammatory cytokines (such as TNF-α and IL-6) which promote insulin resistance and diabetic renal injury.

Interestingly, although most of the above studies examining the effects of androgens on renal function have been performed in experimental models of nondiabetic renal disease, in the setting of diabetes, testosterone seems to exert opposite effects. Specifically, castration in experimental models of hypertension-associated renal injury improves renal structure and function, in the male STZ-induced diabetic rat, castration actually contributes to the progression of diabetic renal disease.[34] Further, supplementation of testosterone to physiological levels observed in nondiabetic improves albuminuria, glomerulosclerosis, tubulointerstitial fibrosis, and TGF-β protein expression associated with diabetes.[34] These observations suggest that the mechanisms by which androgens exert their actions in the kidney differ under normo- and hyperglycemic conditions.

Collectively, these observations suggest that androgens play an integral role in the development and progression of hypertension and renal injury, in both nondiabetic and diabetic conditions. Although most of the studies investigating the role of androgens in pathological conditions have been directed at males, the role androgens play in the development of renal disease in females warrants further investigation. This statement is based on the observation that diabetic females exhibit increase in testosterone levels that correlate with the decline in renal function, suggesting that testosterone may contribute to the development of diabetic nephropathy in females. Further, the interplay between androgens and locally active peptides, such as angiotensin, endothelin-1, etc., also requires further investigation to better understand the mechanisms involved in the development and progression of diabetic renal disease.

SUMMARY AND PERSPECTIVES

Clinical and experimental studies show that diabetes is a state of imbalance in sex hormone levels. Specifically, males exhibit low testosterone and elevated estradiol levels, whereas females exhibit low estradiol and elevated testosterone levels. Both estrogens and androgens, despite being considered sex hormones, play an active role in mediating renal function in the setting of diabetes. Although the mechanisms by which sex hormones exert their actions in the kidney need to elucidated, studies to date point to estrogens and androgens modulating the expression and activity of locally active peptides, such as Ang II and endothelin. We conclude that therapies that include sex hormones in conjunction with blocking of the actions of the classical pathways involved in the development and progression of renal injury may be an effective, sex-specific treatment for diabetic nephropathy.

ACKNOWLEDGMENTS

The authors acknowledge the financial support of NIH/NIDDK (RO1DK075832 to C. Maric-Bilkan).

REFERENCES

1. Ajayi AA, Ogungbade GO, Okorodudu AO. Sex hormone regulation of systemic endothelial and renal microvascular reactivity in type-2 diabetes: studies in gonadectomized and sham-operated Zucker diabetic rats. *Eur J Clin Invest* 2004;**34**:349–57.
2. Baiardi G, Macova M, Armando I, Ando H, Tyurmin D, Saavedra JM. Estrogen upregulates renal angiotensin II AT1 and AT2 receptors in the rat. *Regul Pept* 2005;**124**:7–17.
3. Barton M. Reversal of proteinuric renal disease and the emerging role of endothelin. *Nat Clin Pract Nephrol* 2008;**4**:490–501.
4. Brosius FC, Khoury CC, Buller CL, Chen S. Abnormalities in signaling pathways in diabetic nephropathy. *Expert Rev Endocrinol Metab* 2010;**5**:51–64.
5. Brosnihan KB, Senanayake PS, Li P, Ferrario CM. Bi-directional actions of estrogen on the renin–angiotensin system. *Braz J Med Biol Res* 1999;**32**:373–81.
6. Brownlee M. Biochemistry and molecular cell biology of diabetic complications. *Nature* 2001;**414**:813–20.
7. Chen NY, Chen WY, Bellush L, Yang CW, Striker LJ, Striker GE, et al. Effects of streptozotocin treatment in growth hormone (GH) and GH antagonist transgenic mice. *Endocrinology* 1995;**136**:660–7.
8. Cherney DZ, Sochett EB, Miller JA. Gender differences in renal responses to hyperglycemia and angiotensin-converting enzyme inhibition in diabetes. *Kidney Int* 2005;**68**:1722–8.

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9. Ding SS, Qiu C, Hess P, Xi JF, Zheng N, Clozel M. Chronic endothelin receptor blockade prevents both early hyperfiltration and late overt diabetic nephropathy in the rat. *J Cardiovasc Pharmacol* 2003;**42**:48–54.
10. Dixon A, Maric C. 17beta-Estradiol attenuates diabetic kidney disease by regulating extracellular matrix and transforming growth factor-beta protein expression and signaling. *Am J Physiol Renal Physiol* 2007;**293**:F1678–90.
11. Doublier S, Lupia E, Catanuto P, Periera-Simon S, Xia X, Korach K, et al. Testosterone and 17beta-estradiol have opposite effects on podocyte apoptosis that precedes glomerulosclerosis in female estrogen receptor knockout mice. *Kidney Int* 2011;**79**:404–13.
12. Elliot SJ, Karl M, Berho M, Potier M, Zheng F, Leclercq B, et al. Estrogen deficiency accelerates progression of glomerulosclerosis in susceptible mice. *Am J Pathol* 2003;**162**:1441–8.
13. Flyvbjerg A. Putative pathophysiological role of growth factors and cytokines in experimental diabetic kidney disease. *Diabetologia* 2000; **43**:1205–23.
14. Hakam AC, Siddiqui AH, Hussain T. Renal angiotensin II AT2 receptors promote natriuresis in streptozotocin-induced diabetic rats. *Am J Physiol Renal Physiol* 2006;**290**:F503–8.
15. Hanes DS, Nahar A, Weir MR. The tissue renin-angiotensin-aldosterone system in diabetes mellitus. *Curr Hypertens Rep* 2004;**6**:98–105.
16. Hinojosa-Laborde C, Craig T, Zheng W, Ji H, Haywood JR, Sandberg K. Ovariectomy augments hypertension in aging female Dahl salt-sensitive rats. *Hypertension* 2004;**44**:405–9.
17. Kapoor D, Goodwin E, Channer KS, Jones TH. Testosterone replacement therapy improves insulin resistance, glycaemic control, visceral adiposity and hypercholesterolaemia in hypogonadal men with type 2 diabetes. *Eur J Endocrinol* 2006;**154**:899–906.
18. Kennefick TM, Oyama TT, Thompson MM, Vora JP, Anderson S. Enhanced renal sensitivity to angiotensin actions in diabetes mellitus in the rat. *Am J Physiol* 1996;**271**:F595–602.
19. Komukai K, Mochizuki S, Yoshimura M. Gender and the renin-angiotensin-aldosterone system. *Fundam Clin Pharmacol* 2010;**24**: 687–98.
20. Lamon-Fava S, et al. Differences in serum sex hormone and plasma lipid levels in Caucasian and African-American premenopausal women. *J Clin Endocrinol Metab* 2005;**90**:4516–20.
21. Mankhey RW, Bhatti F, Maric C. 17beta-Estradiol replacement improves renal function and pathology associated with diabetic nephropathy. *Am J Physiol Renal Physiol* 2005;**288**:F399–405.
22. Maric C, Forsblom C, Thorn L, Waden J, Groop PH. Association between testosterone, estradiol and sex hormone binding globulin levels in men with type 1 diabetes with nephropathy. *Steroids* 2010;**75**:772–8.
23. Mogensen CE, Neldam S, Tikkanen I, Oren S, Viskoper R, Watts RW, et al. Randomised controlled trial of dual blockade of renin-angiotensin system in patients with hypertension, microalbuminuria, and non-insulin dependent diabetes: the candesartan and lisinopril microalbuminuria (CALM) study. *BMJ* 2000;**321**:1440–4.
24. Neugarten J. Gender and the progression of renal disease. *J Am Soc Nephrol* 2002;**13**:2807–9.
25. Neuhofer W, Pittrow D. Role of endothelin and endothelin receptor antagonists in renal disease. *Eur J Clin Invest* 2006;**36**(Suppl. 3): 78–88.
26. Orchard TJ, Dorman JS, Maser RE, Becker DJ, Drash AL, Ellis D, et al. Prevalence of complications in IDDM by sex and duration. *Pittsburgh Epidemiol Diabetes Complications Study II Diabetes* 1990;**39**: 1116–24.
27. Pan FP, Stevenson JL, Donaldson DL, Levy J, Wiegmann T, Moore WV. Correlation of urinary albumin and beta-2-microglobulin and growth hormone excretion in patients with diabetes mellitus and short stature. *J Clin Endocrinol Metab* 1990;**71**:611–7.
28. Parving HH. Effects of ACE inhibitors on renal function in incipient and overt diabetic nephropathy. *J Diabetes Complications* 1996; **10**:133–5.
29. Reckelhoff JF, Zhang H, Srivastava K. Gender differences in development of hypertension in spontaneously hypertensive rats: role of the renin–angiotensin system. *Hypertension* 2000;**35**:480–3.
30. Remuzzi G, Macia M, Ruggenenti P. Prevention and treatment of diabetic renal disease in type 2 diabetes: the BENEDICT study. *J Am Soc Nephrol* 2006;**17**:S90–7.
31. Revankar CM, Cimino DF, Sklar LA, Arterburn JB, Prossnitz ER. A transmembrane intracellular estrogen receptor mediates rapid cell signaling. *Science* 2005;**307**:1625–30.
32. Tofovic SP, Dubey R, Salah EM, Jackson EK. 2-Hydroxyestradiol attenuates renal disease in chronic puromycin aminonucleoside nephropathy. *J Am Soc Nephrol* 2002;**13**:2737–47.
33. Wolf G, Ziyadeh FN. The role of angiotensin II in diabetic nephropathy: emphasis on nonhemodynamic mechanisms. *Am J Kidney Dis* 1997; **29**:153–63.
34. Xu Q, Prabhu A, Xu S, Manigrasso MB, Maric C. Dose-dependent effects of dihydrotestosterone in the streptozotocin-induced diabetic rat kidney. *Am J Physiol Renal Physiol* 2009;**297**:F307–15.
35. Zhuo JL, Li XC. Novel roles of intracrine angiotensin II and signalling mechanisms in kidney cells. *J Renin Angiotensin Aldosterone Syst* 2007;**8**:23–33.

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Chapter 203

Prolactin

Willis K. Samson

ABSTRACT

Prolactin, a large peptide produced in and secreted from lactotrophs of the anterior pituitary gland, traditionally has been considered a reproductive hormone in man. In submammalian species, however, it plays an important role in osmoregulation. Recently, prolactin receptors have been described in mammalian kidney and the peptide seems to be produced there as well. Prolactin has been shown to exert natriuretic, diuretic, and kaliuretic actions by decreasing Na+, K+-ATPase activity in the proximal convoluted tubule. This effect requires interaction with renal dopamine D-1 receptors. Thus, it may be that instead of acting as an endocrine hormone (of pituitary origin), locally produced prolactin exerts direct, paracrine actions in the nephron leading to important changes in sodium and potassium excretion.

Because prolactin (PRL) was considered by most endocrinologists to be primarily a hormone associated with reproductive function, for years the prevailing editorial commentary obstructed attempts to understand the physiologic relevance of the consistent findings that receptors for the peptide were localized to nonreproductive tissues, including kidney. Yet in submammalian species, a clear role was indicated for the peptide in fluid and electrolyte homeostasis. What has emerged from years of sporadic interest in the possible renotropic actions of PRL is a body of evidence that suggests little significance in humans of pituitary-derived peptide; however, much promise still exists for renal actions of endogenous PRL, in particular PRL produced within the kidney acting in a local, paracrine, fashion to control tubular function. These effects would not have been observed in the human studies published to date, but were in fact predicted by not only the comparative studies in lower species but also some groundbreaking work in unique animal models.[12,19,26,31,33] Numerous excellent reviews of the roles played by PRL in fishes, birds, and other species have been published,[5,21,23] and those reviews will not be recapitulated here. Instead, the evidence for actions of PRL in the mammalian kidney will be highlighted.

CLASSICAL PRODUCTION SITE AND ACTIONS OF PROLACTIN

As reviewed by several recognized experts in the field,[3,4] PRL is produced primarily in the anterior pituitary gland where it is both stored and released as the mature 199 amino acid polypeptide after removal from the prohormone of a 28 amino acid signal peptide. It circulates as both a 23-kDa protein and, after enzymatic cleavage and reduction of one of its disulfide bonds in a variety of tissues sites, a 16-kDa, biologically active fragment.[2,9] In fact, several of the nontraditional actions of PRL have been ascribed to this 16-kDa fragment.[1,3,8,10] In the pituitary gland, PRL is produced in a unique population of cells, termed lactotrophs, because of the primary action of the protein in the mammary gland to stimulate milk production.[28] This "hallmark" action of PRL, together with its effects on corpus luteum function in the ovary,[3,28] formed the basis of the predominant view that PRL was a "reproductive" hormone, one associated primarily with female reproductive function. Why then do males make, store, and release PRL from the anterior pituitary gland? Why is the PRL gene transcribed and protein produced in extrapituitary sites? Why then are receptors for the protein found in numerous tissues sites other than the ovary and mammary gland?

PROLACTIN RECEPTORS

Prolactin receptors belong to the class 1 cytokine receptor superfamily that includes receptors for a wide variety of peptides (e.g., interleukins, erythropoietin, leukemia inhibiting factor, and the hormone leptin). As reviewed by Kelly et al.,[4] the PRL receptor itself exists in at least three major isoforms, all splice variants of the same gene product. They share homology in the extracellular, amino-terminal domain, and in the single chain transmembrane portion, but differ in the length of their cytoplasmic C-terminal tails. The extracellular domain establishes the homology to the cytokine receptors and is approximately 200 amino acids in length. The intracellular domains of the receptor contain not only the sequences necessary for appropriate signal transduction but also those responsible for receptor internalization.

Ligand binding to the PRL receptor results in stimulation of the tyrosine kinase activity of JAK2 (Janus kinase 2) and, except in the case of the short form isoform, receptor phosphorylation and subsequent activation of Stat proteins

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00203-7
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(signal transducer and activator of transcription proteins), in particular Stat5.[4] Interactions of the activated Stat proteins with DNA binding sites account for the specificity of the genomic effects of PRL. Signaling via the mitogen activated protein kinase (MAPK) pathway has also been identified and an interaction with the epidermal growth factor (EGF) family of receptors suggested. Within the kidney, evidence for an interaction of the PRL receptor with the dopamine, D-1 receptor has been shown,[12] an effect that determines the renal actions of the hormone.

As stated above, the primary actions of PRL were for a long time considered to be in mammary gland and ovary, tissues containing abundant receptors for the protein. However, a much broader distribution of PRL receptors has been cataloged in what were largely unexpected sites including brain, adrenal gland, bone, adipocytes, liver, pancreas, gastrointestinal tract, a variety of lymphoid tissues, and kidney.[4] Not surprisingly, the pharmacologic effects of PRL have now been described in those tissues as well, opening the possibility that PRL is not just a hormone of reproduction but instead one with diverse biologic potential. Best characterized to date are the actions of PRL in immune cells, bone, brain, and pancreas.[3,4] Some of those actions may be a reflection of circulating PRL, while others may require local production of the peptide, as in the case of the potential renal actions.[33]

PROLACTIN AND OSMOREGULATION

PRL plays a significant role in osmoregulation in species exposed to harsh environments.[4,21,28] Indeed, the protein is an important protective factor in fish in both the fresh and sea-water environments and in the transition between those two osmotic conditions. The importance of PRL in osmoregulation in fish has been reviewed by Manzon[23] and its role in birds and amphibians described.[5,28,31] Evidence for a role in osmoregulation in mammals has also accumulated and indeed the presence of PRL receptors in the mammalian kidney detailed.[16,27,33] However, most clinically based studies have failed to present a clear picture of PRL effects in humans and only some of the animal trials have been able to at least partially explain the possible similarity of PRL actions in mammals with those observed in submammalian species. What has been missing perhaps has been an appreciation for the importance of locally produced PRL as opposed to circulating, pituitary-derived peptide.

PROLACTIN RECEPTORS IN THE MAMMALIAN KIDNEY

Already in the 1980s[27] it was known that high affinity PRL binding could be seen in rat kidney membrane preparations (medullary and cortical fragments) and that maximal binding was present in epithelial cells of the glomerulus and proximal tubule as identified by autoradiography. Faint but detectable labeling was observed in tubule cells of the cortical and medullary nephron as well. That work extended the original observations of Marshall et al.[24] that showed by Scatchard analysis the presence of PRL binding activity in rat kidney. The proximal tubule site of autoradiographic PRL labeling suggested an action of the peptide on sodium handling in the kidney; however, the original article[24] failed to show a significant effect of uninephrectomy or salt loading on PRL binding in the kidney. Similarly, serum PRL levels failed to be significantly altered by manipulation of osmolality in several studies.[25]

With the cloning of the mammalian PRL receptors much more was learned about the potential roles played by circulating or locally produced PRL in renal function. *In situ* hybridization histochemistry reveals the presence of both the short and long forms of the PRL receptor in rat kidney. Unlike in the other tissues (e.g., adrenal, spleen, thymus, skin, and heart) where the long receptor isoform predominates, the short form is more abundantly detected in kidney with highest signal found in the glomeruli and in the tubules of the outer medulla.[29] This was later confirmed by Freemark et al.[16] by reverse transcription-polymerase chain reaction, which also showed the relative increased levels of short versus long isoform of the PRL receptor in fetal rat kidney. The same group of investigators[32] extended those initial studies with a combination of *in situ* hybridization histochemistry, immunhistochemistry, and radioligand binding studies that showed the presence of PRL receptors in fetal rat kidney by day 19–20 of gestation. On day 20, PRL binding is best observed in cortical and subcortical renal tubules. In human fetal kidney, PRL receptor immunoreactivity is detected at embryonic day 52 in tubules and at gestational day 96 in tubules and collecting ducts.[15] Staining is particularly intense on the luminal surfaces of the tubules and ducts. No immunoreactivity was observed in glomeruli. The luminal localization of PRL receptors was confirmed in murine kidney,[33] in particular in the proximal tubule and the parietal epithelium of Bowman's capsule, where mRNA encoding PRL itself also is detected raising the possibility of local actions of endogenously produced PRL within the kidney. This is further implied by the failure of fetal growth restriction to alter renal PRL receptor message in the face of suppressed synthesis and secretion of PRL in the pituitary gland.[30]

RENAL PRODUCTION OF PROLACTIN

How would PRL access its apparent binding sites on the luminal epithelium of the tubule? Certainly, circulating PRL of pituitary origin may be filtered through the glomerulus. An arteriovenous gradient of PRL across the human kidney[11] has been described suggesting either removal by filtration and metabolism by the kidney;[13] however, micropuncture

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studies describing the concentrations of PRL in tubular urine are lacking. It is clear that at least in the mouse[33] PRL mRNA and protein can be detected in luminal epithelial cells of the proximal tubule. In addition, those cells also express the message for Pit-1, a transcription factor known to be important in the regulation of PRL gene expression,[4] further suggesting significant, local production of the peptide.

RENAL ACTIONS OF PROLACTIN

Although there is clear consensus that PRL receptors exist in kidney, there is little agreement in the literature concerning the potential renal actions of the peptide. This may be a result of differing experimental protocols or the use of heterologous peptide preparations. The lack of agreement may also be a result of the commonly held belief that all PRL effects in kidney would be a result of peptide delivered by the general circulation. This may not be the case and a unique isoform of PRL may in fact be the effective agent.

Horrobin et al.[17] injected semipurified sheep PRL intramuscularly into five male volunteers. In addition to a long-lasting malaise, they observed a decrease in urine volume and urinary sodium and potassium excretion that was matched by increased plasma sodium and osmolality. The volunteers also expressed a sensation of thirst. The absolute purity of the peptide preparation could not at the time be assured and thus contamination with other anterior lobe hormones, as well as the neurohypophysial peptides, oxytocin and vasopressin, could not be ruled out. That human study was designed to mirror work by Lockett et al.[20] in which antinatriuretic, antidiuretic, and antikaliuretic effects of purified PRL preparations were observed in conscious rats, in the absence of demonstrable actions on glomerular filtration rate. Thus tubular effects to increase sodium reabsorption were the first suggested actions of PRL.

The decreased urine volume observed in human volunteers led many to speculate that PRL is a unique antidiuretic agent. This was further suggested by the observation in rats that ovine PRL infusions reduce the diuresis and natriuresis of an intravenous saline load.[22] However, that same year, a report appeared, which suggested that the antidiuretic effect of PRL resulted from occupancy of the vasopressin (AVP) receptors in kidney.[14] Indeed, Clapp and colleagues have elegantly shown that prolactin administered into the brain or systemically promotes AVP, and oxytocin, release.[35]

In the absence of AVP (Brattleboro rats or Sprague Dawley rats fluid loaded to decrease endogenous AVP secretion), recombinant mouse PRL exerts antinatriuetic and antidiuretic effects. However, when AVP is present, the action is diuretic. These excellent studies show several important shortcomings of earlier work: the use of recombinant material obviating the potential for contamination with other renotropic factors, the use of species-specific peptide, and the separation of the potentiative effects of PRL on AVP actions. The study[26] does not, however, isolate the problem of identifying the actions of circulating (pituitary origin) versus locally produced peptide.

It also does not address the possibility that prolactin of renal origin may exert actions in differing nephron segments than that administered systemically. In fact, more recent convincing evidence for a natriuretic action[12] in proximal convolutes tubule (PCT) is opposed by earlier work[6] indicating an antinatriuretic effect in medullary ascending limb (MAL) and distal convoluted tubule (DCT). Indeed, although prolactin decreases Na+, K+-ATPase activity in PCT,[19] it seems to increase the activity in MAL and DCT.[6]

The physiologic relevance of a PCT site of action of prolactin produced within the kidney is beginning to gain support. Not only does prolactin decrease Na+, K+-ATPase activity there, it signals through activation of protein kinase A, protein kinase C, and phosphorylation of cellular proteins (including Na+, K+-ATPase) and via heterologous recruitment of the dopamine, D-1 receptor.[12] Dopamine, acting via the D-1 receptor, is a potent natriuretic agent and interactions between dopamine and other natriuretic agents (e.g., atrial natriuretic factor) resulting from heterologous recruitment of the D-1 receptor. Crambert et al.[12] take their argument one step further by showing that prolactin fails to alter Na+, K+-ATPase activity in SHR rats, a strain that displays a blunted natriuretic response to dopamine.

THE JURY IS STILL OUT ON THE RENAL ACTIONS OF PROLACTIN

Despite abundant suggestive evidence for renotropic actions of PRL, these still have not been conclusively shown to exist. Perhaps the approach taken has led to the confusion. Almost all the studies described in the literature focus on PRL of pituitary origin as the potential renotropic agent. Now that it is known that the gene for PRL is expressed in kidney[33] and that PRL receptors are expressed on the luminal epithelium of the renal tubule,[15] attention should be focused on the potential for PRL of renal origin to act in the tubule to alter sodium and water handling. Micropuncture studies using homologous, recombinant peptide should be conducted to include infusion of PRL into the tubular fluid.

Continued investigation of the functional significance of the prevalence of the short form of the prolactin receptor in kidney[16] is needed. Is the presence of this form of the receptor in medullary nephron segments the reason why the action of PRL in MCT and DCT[6] differs from that in PCT?[12,19] Clearly, the heterodimerization of the long form of the PRL receptor with the dopamine D-1 receptor is an important interaction leading to changes in Na+, K+-ATPase activity[12,19] Is it possible that the predominance of the short form of the PRL receptor in more distal nephron segments

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is acting as a dominant negative, blocking the dimerization observed in more proximal elements? Indeed, Tan and Walker[34] present convincing evidence in HEK-293 cells that increased short form to long form ratios interrupt signaling of PRL, by prevention of homodimerization (of the long form). Increased expression of the short form of the receptor also reduces expression of the long form. Thus, in distal tubule, the apparent dominant negative action of the receptor short form may interrupt dopamine signaling and lead to the opposite effect on sodium handling as that observed in the proximal tubule. This hypothesis deserves further study.

The potential actions of PRL variants in the kidney also need to be further examined. For example, the effect of glycosylated PRL or the smaller 16-kDa isoform should be examined. In particular, the 16-kDa form may hold promise. This is the posttranslationally modified form of PRL that is created by enzymatic cleavage followed by reduction of the disulfide bridge between amino acids 58 and 174. The resultant 16-kDa isoform is poorly recognized by the classical PRL receptors[9] but does express unique biologic activities, including antiangiogenesis and antitumorigenesis.[8,10,16] Clearly, this modified form of PRL may be produced in kidney, where the necessary cathepsin-D-like enzymes and reductases are present,[2] and it also may play a role in the local regulation of sodium and water transport.

HAVE TRANSGENE METHODS CLARIFIED THE ISSUE?

Two excellent models for the study of the potential renotropic actions of PRL have been developed, the PRL[18] and the PRL receptor[4] knockout mice. Recognizing that embryonic gene deletion may not uncover the physiologic relevance of a protein that plays, along with other unique proteins that may compensate for its deletion, a role in renal function, these models if properly studied may provide insight into the tubular actions of 23- or 16-kDa PRL. Likewise, the availability of a modified PRL homolog with antagonistic activity[7] may well explain the confusion of the natiuretic/antinatriuretic or diuretic/antinatriuretic effects of PRL, and may clarify the potential role played by endogenous PRL in mammals that seems to be predicted by the important osmoregulatory actions of the peptide in submammalian species.

REFERENCES

1. Aranda J, Rivera JC, Jeziorski MC, Riesgo-Escovar J, Nava G, López-Barrera F, et al. Prolactins are natural inhibitors of angiogenesis in the retina. *Invest Ophthalmol Vis Sci* 2005;**46**:2947–53.
2. Baldocci RA, Tan L, Nicoll CS. Processing of rat prolactin by rat tissue explants and serum in vitro. *Endocrinology* 1992;**130**:1653–9.
3. Ben-Jonathan N, Mershon JL, Allen DL, Steinmetz RM. Extrapituitary prolactin: distribution, regulation, functions and clinical aspects. *Endocr Rev* 1996;**17**:639–69.
4. Bole-Feysot C, Goffin V, Edery M, Binart N, Kelly PA. Prolactin (PRL) and its receptor: actions, signal transduction pathways and phenotypes observed in PRL receptor knockout mice. *Endocr Rev* 1998;**19**:225–68.
5. Brown SC, Horgan EA, Savage LM, Brown PS. Changes in body water and plasma constituents during bullfrog development: effects of temperature and hormones. *J Exp Zool* 1986;**237**:25–33.
6. Bussieres L, Laborde K, Dechaux M, Sachs C. Effects of prolactin on Na–K-ATPase activity along the rat nephron. *Pfluegers Arch* 1987;**409**:182–7.
7. Chen TJ, Kuo CB, Tsai KF, Liu JW, Chen DY, Walker AM. Development of recombinant human prolactin receptor antagonists by molecular mimicry of the phosphorylated hormone. *Endocrinology* 1998;**139**:609–15.
8. Clapp C, Martial J, Guzman RC, Rentier-Delrue F, Weiner RI. The 16-kilodalton N-terminal fragment of human prolactin is a potent inhibitor of angiogenesis. *Endocrinology* 1993;**133**:1292–9.
9. Clapp C, Sears PS, Nicoll CS. Binding studies with intact rat prolactin and a 16K fragment of the hormone. *Endocrinology* 1989;**125**:1054–9.
10. Corbacho A, Martinez de la Escalera G, Clapp C. Roles of prolactin and related members of the prolactin/growth hormone/placental lactogen family in angiogenesis. *J Endocrinol* 2002;**173**:219–38.
11. Cowden EA, Ratcliffe WA, Ratcliffe JG, Dobbie JW, Kennedy AC. Hyperprolactinemia in renal disease. *Clin Endocrinol* 1978;**9**:241–8.
12. Crambert S, Sjoeberg A, Ekloef AC, Ibarra F, Holbaeck U. Prolactin and dopamine 1-like receptor interaction in renal proximal tubular cells. *Am J Physiol* 2010;**299**:F49–54.
13. Emmanouel D, Fang VS, Katz AI. Prolactin metabolism in the rat: role of the kidney in degradation of the hormone. *Am J Physiol* 1981;**240**:F437–45.
14. Evan AP, Palmer GC, Lucci MS, Solomon S. Prolactin-induced stimulation of rat renal adenylate cyclase and autoradiographic localization to the distal nephron. *Nephron* 1977;**18**:266–76.
15. Freemark M, Driscoll P, Maaskant R, Petryk A, Kelly PA. Ontogenesis of prolactin receptors in the human fetus in early gestation. *J Clin Invest* 1997;**99**:1107–17.
16. Freemark M, Nagano M, Edery E, Kelly PA. Prolactin receptor gene expression in the fetal rat. *J Endocr* 1995;**144**:285–92.
17. Horrobin DF, Bursyn PG, Lloyd IJ, Durkin N, Lipton A, Muiruri KL. Actions of prolactin on human renal function. *Lancet* 1971;**2**:352–4.
18. Horseman ND, Zhao W, Montecino-Rodriguez E, Tanaka M, Nakashima K, Engle SJ, et al. Defective mammopoiesis, but normal hematopoiesis, in mice with a targeted disruption of the prolactin gene. *EMBO J* 1997;**16**:6926–35.
19. Ibarra F, Crambert S, Ekloef AC, Lundquist A, Hansell P, Holtbaeck U. Prolactin, a natriuretic hormone, interacting with the renal dopamine system. *Kidney Int* 2005;**68**:1700–7.
20. Lockett MF, Nail B. A comparative study of the renal actions of growth and lactogenic hormones in rats. *J Physiol* 1965;**180**:147–56.
21. Loretz CA, Bern HA. Prolactin and osmoregulation in vertebrates. *Neuroendocrinology* 1987;**35**:632–7.
22. Lucci MS, Bengele HH, Solomon S. Suppressive action of prolactin on renal response to volume expansion. *Am J Physiol* 1975;**229**:81–5.
23. Manzon LA. The role of prolactin in fish osmoregulation: a review. *Gen Comp Endocrinol* 2002;**125**:291–310.
24. Marshall S, Gelato M, Meites J. Serum prolactin levels and prolactin binding activity in adrenals and kidneys of male rats after dehydration, salt loading, and unilateral nephrectomy. *Proc Soc Exp Biol Med* 1975;**149**:185–8.

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25. Mattheij JAM. Evidence against a role for prolactin in osmoregulation in the rat: water balance studies. *Endo Res Commun* 1977;**4**:1–9.
26. Morrissey SE, Newth T, Rees R, Barr A, Shora F, Laycock JF. Renal effects of recombinant prolactin in anesthetized rats. *Eur J Endocrinol* 2001;**145**:65–71.
27. Mountjoy K, Cowden EA, Dobbie JW, Ratcliffe JG. Prolactin receptors in the rat kidney. *J Endocr* 1980;**87**:47–54.
28. Nicoll CS. Role of prolactin in water and electrolyte balance in vertebrates. In: Jaffe RB, editor. *Current endocrinology—prolactin*. Amsterdam: Elsevier; 1981. p. 127–66.
29. Ouhtit A, Morel G, Kelly PA. Visualizaion of gene expression of short and long forms of prolactin receptor in the rat. *Endocrinology* 1993;**133**:135–44.
30. Phillips ID, Anthony RV, Simonetta G, Owens JA, Robinson J, McMillan IC. Restriction of fetal growth has a differential impact on fetal prolactin and prolactin receptor mRNA expression. *J Neuroendocrinol* 2001;**13**:175–81.
31. Roberts JR, Danzler WH. Micropuncture study of avian kidney: effect of prolactin. *Am J Physiol* 1992;**262**:933–7.
32. Royster M, Driscoll P, Kelly PA, Freemark M. The prolactin receptor in the fetal rat: cellular localization of messenger ribonucleic acid, immunoreactive protein, and ligand-binding activity and induction of expression in late gestation. *Endocrinology* 1995;**136**:3892–900.
33. Sakai Y, Hiraoka Y, Ogawa M, Yakeuchi Y, Aiso S. The prolactin gene is expressed in mouse kidney. *Kid Int* 1999:833–40.
34. Tan D, Walker AM. Short form 1b human prolactin receptor downregulates expression of the long form. *J Mol Endocrinol* 2010;**44**: 187–94.
35. Vega C, Moreno-Carranza B, Zamorano M, Quintanar-Stephano A, Mendez I, Thebault S, et al. Prolactin promotes oxytocin and vasopressin release by activating neuronal nitric oxide synthase in the supraoptic and paraventricular nuclei. *Am J Physiol* 2010;**299**:R1701–8.

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Chapter 204

Renin–Angiotensin System

Minolfa C. Prieto, Alexis A Gonzalez, Ryousuke Satou, Hiroyuki Kobori and L. Gabriel Navar

ABSTRACT

Renal angiotensin (Ang) II is regulated independently of the systemic renin–angiotensin system. Intrarenal Ang II concentrations are higher in renal interstitium and proximal tubular compartments than in the circulation. The transcript for angiotensinogen (AGT) is in proximal tubule cells and for renin in juxtaglomerular and collecting duct cells. Secretion of their proteins increases intratubular formation of angiotensin peptides. Ang II is internalized via AT_1R into renal endosomes. When inappropriately stimulated, Ang II augments synthesis and secretion of AGT in proximal tubules and renin in collecting ducts, which increase further intrarenal Ang II and cause hypertension. Ang II has pleiotropic actions on cortical and medullary blood flow, vascular tone of afferent and efferent arterioles, mesangial cell function, and sensitivity of the tubuloglomerular feedback mechanism via the activation of AT_1R. AT_2R and Ang III partially oppose these actions. Ang II also regulates sodium transport in proximal and distal nephrons via the activation of luminal and basolateral AT_1R.

INTRODUCTION

The contribution of the intrarenal renin–angiotensin system (RAS) to the regulation of renal function is unique because renal functional, and transport alterations caused by angiotensin II (Ang II) influence overall sodium balance and cardiovascular status and thus contribute to blood pressure regulation. When inappropriately stimulated, an enhanced intrarenal RAS markedly influences renal function by acting on vascular and tubular components causing the development and maintenance of hypertension and associated cardiovascular dysfunction.[12,18–20,33]

This chapter covers the intrarenal RAS, the formation of several angiotensin peptides by various enzymes, and the characterization of angiotensin receptors including AT_{1a}, AT_{1b}, and AT_2 for Ang II and Ang III, and the Mas receptor for Ang 1–7. In addition, we describe some newly discovered aspects of the intrarenal RAS including the regulation of proximal tubule angiotensinogen (AGT), collecting duct renin, and the renin/(pro)renin receptor.

DISTRIBUTION OF ANGIOTENSINOGEN, RENIN, (PRO)RENIN RECEPTORS, ANGIOTENSIN CONVERTING ENZYMES, AND ANG TYPE 1 RECEPTORS IN THE KIDNEY

All components of the RAS are present in the kidney and are compartmentalized to the tubular and interstitial networks. Because kidney Ang II tissue concentrations are greater than can be explained by the concentrations delivered in the arterial blood flow, it is generally acknowledged that most of the Ang II in the intrarenal components is generated locally from AGT delivered to the kidney as well as that locally produced by proximal tubule cells. The presence of abundant sources of renin, AGT, and angiotensin-converting enzyme (ACE) in the kidney provides an environment conducive to high levels of Ang II synthesis.[19,21]

Angiotensinogen

AGT, the only known substrate for renin, is a 60-kD glycoprotein synthesized and released from the liver, and cleaved in the circulation to form the biologically inactive, Ang I (Fig. 1). In addition, renal AGT mRNA and protein are localized to proximal tubule cells indicating that much of the intratubular Ang II is derived from locally formed and secreted AGT. AGT is regulated by an amplification mechanism such that AGT mRNA and protein are stimulated by Ang II, which maintains or increases the production of Ang II in Ang II-dependent hypertension. Producing its metabolites intracellularly and secreting them into the tubule lumen, AGT is taken up by megalin-dependent endocytosis and is then secreted into the tubular lumen.[12,19,24] More recent studies have evaluated the molecular mechanisms responsible for the regulation of AGT expression and synthesis in proximal tubular cells.[21] Activation of AT1 receptors stimulates intrarenal NF-κB, which is a key factor in the augmentation of AGT expression in proximal tubule cells.[1,20] The activation of NF-κB by Ang II leads to the stimulation

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00204-9
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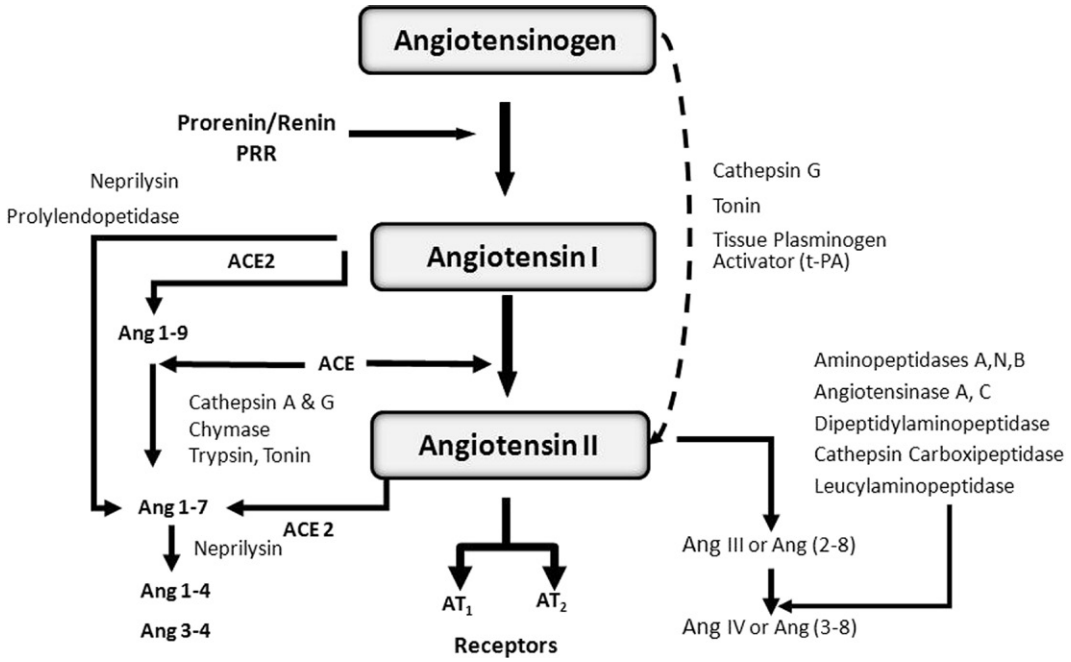

FIGURE 1 Intrarenal renin–angiotensin system cascade. Known and postulated enzymatic pathways responsible for formation and metabolism of angiotensin peptides.

of intrarenal proinflammatory cytokine expressions such as interleukin-6, contributing to the increase in AGT expression via activation of a JAK-STAT pathway.[29] Therefore, Ang II increases intrarenal AGT levels via both direct and indirect mechanisms mediated by activated NF-κB and the JAK-STAT pathway. By contrast, tumor necrosis factor α, which is also an Ang II-induced proinflammatory factor in the kidney, suppresses AGT expression by increasing p50/p50 complex in proximal tubular cells.[30] This inhibitory action on NF-κB provides a counterregulatory influence limiting the magnitude of Ang II-induced AGT augmentation in renal proximal tubular cells, which may explain why higher doses of Ang II infusion in mice fail to augment intrarenal AGT levels.[20]

Proximal tubule AGT concentrations in anesthetized rats are in the range of 300 nM, which greatly exceeds the free Ang I and Ang II tubular fluid concentrations. Because of its molecular size, it is unlikely that much plasma AGT filters across the glomerular membrane, further supporting the concept that proximal tubule cells secrete AGT directly into the tubule.[12] In response to chronic Ang II infusions there is an augmentation of AGT mRNA leading to increased synthesis and secretion with some of the secreted AGT spilling over to the distal nephron segments as reflected by increased urinary AGT excretion.[13] Under conditions of elevated salt intake combined with chronic Ang II infusion, the urinary AGT excretion response is elevated to much greater levels than those seen in Ang II-infused rats fed a normal salt diet.[14] By contrast, normal rats fed a high salt diet exhibit urinary AGT excretion rates similar to those observed in rats fed a normal salt diet. Thus, during chronic Ang II infusion, salt-sensitive hypertension develops and high salt paradoxically stimulates intrarenal RAS activity, as reflected by exacerbation of the urinary AGT despite inhibition of plasma renin activity.[14] Formation of Ang I and II in the tubular lumen subsequent to AGT secretion is probable because some renin is filtered and/or secreted from juxtaglomerular apparatus (JGA) cells.[12] Once Ang I is formed, conversion readily occurs because there is abundant ACE associated with the proximal tubule brush border.[32] Intact AGT in urine reflects its presence throughout the nephron and, to the extent that renin and ACE are available along the nephron, substrate availability supports continued Ang I generation and Ang II conversion in distal segments.[19,25]

Renin and (Pro)renin Receptor

Renin, synthesized by the JGA cells, is the primary source of both circulating and intrarenal renin levels. Juxtaglomerular apparatus cells secrete renin into the renal interstitium, thus providing a stimulus for the local generation of Ang I, and this secreted active form contains 339–343 amino acid residues after proteolytic removal of the 43 amino acid residue at the N-terminus of prorenin. Circulating active renin and prorenin are derived mainly from the kidney; and circulating prorenin is taken up by some tissues where it may contribute to the local synthesis of angiotensin peptides. The only well-established role of

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renin is to cleave Ang I from AGT. However, the recently cloned (pro)renin receptor ((P)RR) binds renin or prorenin, thus enhancing renin activity and fully activating the biologically inactive prorenin peptide, which leads to an increase in the catalytic efficiency of Ang I formation from AGT. Activation of the (P)RR by renin or prorenin also directly elicits intracellular signals that stimulate ERK1/ERK2 and downstream pathways.[22]

There is growing awareness of the possible roles of renin in other intrarenal structures where renin has been localized. Renin producing cells have a unique origin that allows their abundance along the afferent arteriole to be regulated by various stimuli. ACE inhibition induces a recruitment of cells in afferent arterioles beyond the JGA even though they were not expressing the renin gene in the basal state.[31] Positive renin immunoreactivity has also been observed in the cells of glomeruli and in proximal and distal tubular segments. More recently, increased attention has been focused on the presence of renin mRNA and protein expression in distal nephron segments, specifically in the principal cells of connecting tubules and collecting ducts, suggesting local formation and secretion into the distal tubular fluid. Renin synthesis in principal cells of collecting ducts is further increased by chronic infusions of Ang II.[25,26] Rats chronically infused with Ang II for 13 days show a suppression of plasma renin activity but increases in urinary prorenin content and renin excretion.[16] In addition, the urinary renin and prorenin protein levels, examined by Western blot, are augmented about 10-fold in Ang II-infused rats. Concomitant AT1R blockade with candesartan prevents increases in both urinary and tissue renin and in prorenin levels. Although there is marked suppression of plasma renin activity, increased renin synthesis by CD cells and secretion into the luminal fluid lead to increased urinary levels of renin and prorenin.[16]

The presence of renin in distal nephron segments provides the requisite pathway for Ang I generation from proximally delivered AGT.[25,26] Renin synthesis is augmented in the principal cells of connecting tubules and cortical and medullary collecting ducts of chronic Ang II-infused rats and mice, Cyp1a1Ren2 transgenic rats, and in both kidneys of two-kidney one clip Goldblatt hypertensive rats.[9,25,26] The AT1R via a PKC pathway directly mediates the stimulation of renin synthesis in rat inner medullary collecting duct because inhibition of PKC with calphostin C prevents the Ang II-mediated increases in renin transcript and prorenin protein levels in rat inner medullary collecting duct (IMCD) cells while activation of PKC with phorbol 12-myristate 13-acetate increases renin expression to the same extent as Ang II.[7] Further, renin activity and renin and prorenin protein levels are increased in the urine of these rats[6] indicating that both renin and prorenin are secreted by the collecting duct cells into the lumen of distal nephron segments and are also augmented in chronic Ang II-infused hypertensive rats. The discovery of the (P)RR provides new insights regarding its possible role in a setting of activated intrarenal RAS. The (P)RR is expressed in the kidneys[6] particularly in mesangial cells, podocytes, and intercalated type A cells of distal nephron segments.[2,6,11] Chronic infusion of Ang II for 14 days in Sprague–Dawley rats increases renal (P)RR transcript levels and augments the soluble form of the (P)RR, s(P)RR.[6] The augmentation of s(P)RR in the urine of Ang II hypertensive rats suggests that s(P)RR can bind renin and prorenin secreted by the principal collecting duct cells and can catalyze additional formation of Ang I. The existence of an inducible and functional form of s(P)RR in the renal medulla and in the urine of chronic Ang II-infused rats reflects increased s(P)RR in collecting duct tubular fluid and further supports the potential role of this soluble form to enhance intratubular renin activity. Thus, the interaction between the (P)RR and renin in the lumen of the collecting ducts may contribute substantially to facilitate intratubular formation of Ang I in hypertension.

Angiotensin-Converting Enzymes

As in other organs, ACE is located on endothelial cells throughout the vasculature. ACE is also on membranes of both proximal and distal nephron segments with the greatest abundance found on the brush border of proximal tubules, particularly in the S3 segment.[21,32] Marked upregulation of ACE in the brush border occurs in kidneys from Goldblatt hypertensive rats and Ang II infused hypertensive rats.[32] Humans predominantly express ACE in the brush border of proximal tubular segments with very little ACE expression on vascular endothelial cells or in the vasculature of the glomerular tuft. The lower renal vascular endothelial expression in humans helps explain the much lower Ang I to Ang II conversion rates that have been reported for human kidneys as compared with that of other species. In rats, ACE activity is present in tubular fluid throughout the nephron except in the distal tubule, with activity higher at the initial portion of the proximal tubule, decreasing toward the distal nephron, and increasing again in the collecting ducts and urine. Therefore, intratubular Ang II formation may occur not only in the proximal tubule but also beyond the distal convoluted tubule. Renal tissue ACE activity is critical to maintain the steady-state Ang II levels in the kidney. The presence of ACE only in the kidney is sufficient to increase kidney Ang II levels and promote the development of hypertension in response to chronic Ang I infusions.[8] Tissue-ACE knockout mice exhibit 80% lower intrarenal Ang II levels compared with wild-type mice.[3]

ACE2, which acts on Ang II to form Ang 1–7 or on Ang I to form Ang 1–9 (Fig. 1), is a membrane-associated and secreted enzyme expressed predominantly on endothelium,

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but highly restricted in humans to heart, kidneys, and testes.[21] Ang 1–7 may serve as an endogenous antagonist of the Ang II induced actions mediated via AT_1 receptors and changes in ACE2 activity could affect the balance of Ang peptides found in the kidney. This probably explains the elevated Ang II levels in the ACE2 knockout mice.[28] Collectrin, a novel homolog of ACE2 has been identified in mouse, rat, and human. Both ACE2 and collectrin have tissue-restricted expression in the kidney. Collectrin is localized on the luminal surface and in the cytoplasm of collecting duct cells, and its mRNA is expressed in the renal collecting ducts cells while ACE2 is present throughout the endothelium, in proximal tubular epithelial cells and in medullary tissue.[21] In Ang-II-dependent hypertension, intrarenal ACE is increased, but ACE2 levels are decreased leading to another mechanism maintaining increased Ang II levels.[26]

Intrarenal Angiotensin II Receptors

Ang II receptors are widely distributed in various regions and cell types of the kidney. Two major categories of Ang II receptors, AT_1 and AT_2, have been described, pharmacologically characterized and cloned.[18,21] As depicted in Figure 2, most of the Ang-II-mediated hypertensinogenic actions are generally attributed to the AT_1 receptor.[5,18] In rodents, there are two AT_1 receptor subtypes, with the AT_{1a} being the predominant subtype in all nephron segments, while the AT_{1b} is more abundant in the glomerulus.[18,21] In mature kidneys, AT_{1a} receptors have been localized to the renal microvasculature in cortex and medulla, smooth muscle cells of afferent and efferent arterioles, thick ascending limb of the loop of Henle, proximal tubular apical and basolateral membranes, mesangial cells, distal tubules, collecting ducts, and macula densa cells. The afferent arteriole has both AT_{1a} and AT_{1b} receptors, whereas the efferent arteriole primarily expresses AT_{1a} receptors.[10] The essential role of the AT_{1a} receptor in mice is apparent from studies showing that 2-kidney, 1-clip (2K1C) Goldblatt hypertension does not develop in AT_{1a} knockout mice.[4]

Vascular and tubular receptors respond differently during high Ang II states.[18] High Ang II levels associated with a low salt diet decrease glomerular AT_1 receptor expression but increase proximal tubular AT_1 receptor levels. Glomerular AT_1 receptors and AT_{1a} receptor protein are reduced in both clipped and contralateral kidneys of 2K1C Goldblatt and of 2 kidney-1-wrap hypertensive models and in kidneys of Ang II infused rats.[18] In the TGR(mRen2) harboring the mouse renin2 gene, AT_1 receptor binding is increased in vascular smooth muscle of afferent and efferent arterioles, JGA, glomerular mesangial cells, proximal tubular cells, and renomedullary interstitial cells suggesting that the upregulation of AT_1 receptors contributes to the pathogenesis of hypertension in these rats. In Ang II infused rats studied with *in vitro* autoradiography, there are differential responses with significant decreases in glomeruli and inner stripe but not in proximal tubules.[18]

The AT_2 receptor is highly expressed in human and rodent kidney mesenchyme during fetal life and decreases dramatically after birth. AT_2 receptors have been localized to the glomerular epithelial cells, proximal tubules, collecting ducts, and parts of the renal vasculature of the adult rat. AT_2 receptor activation is believed to counteract AT_1 receptor

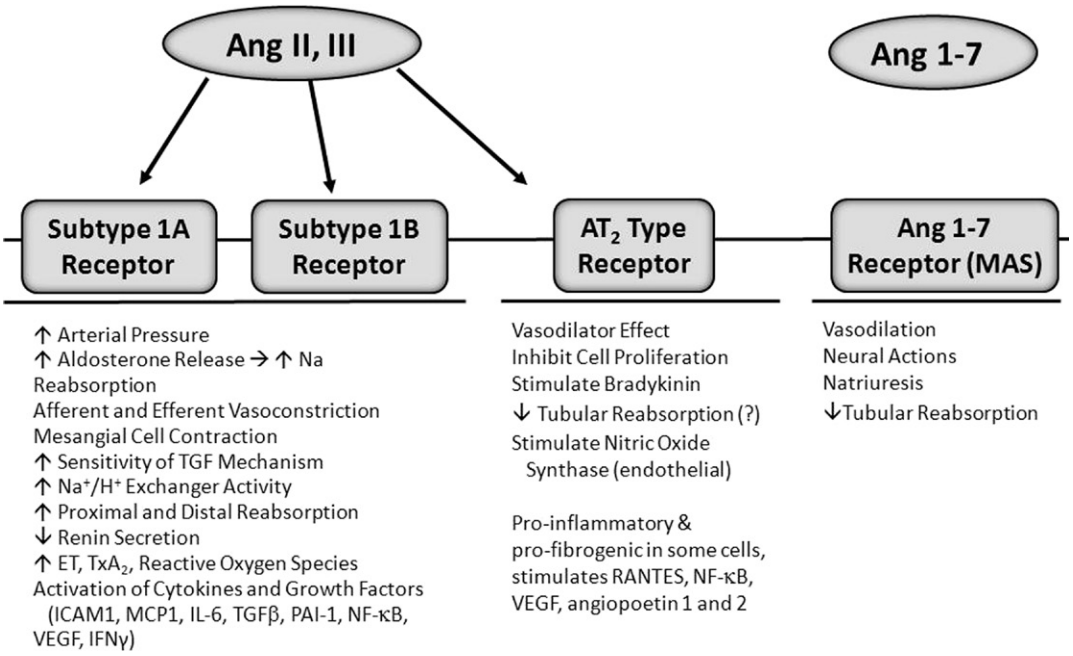

FIGURE 2 Major Ang II receptor subtypes and their renal actions.

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effects by stimulating the formation of bradykinin and nitric oxide leading to increases in interstitial fluid concentration of cyclic guanosine monophosphate (cGMP). AT_2 receptor activation influences proximal tubule sodium reabsorption either by a cell-membrane-receptor-mediated mechanism or via an interstitial nitric-oxide-cGMP pathway.[17,18]

INTRARENAL LEVELS OF ANGIOTENSIN II

Interstitial Angiotensin II

Intrarenal Ang II is not distributed in a homogeneous fashion but is compartmentalized in both a regional and segmental manner.[19] Medullary Ang II levels are higher than cortical levels in normal rats and increase further in Ang II infused hypertensive rats.[18] Within the cortex, there is distribution of Ang II in the interstitial fluid, tubular fluid, and intracellular compartments. The interstitial and intratubular compartments contribute to the disproportionately high intrarenal Ang II levels. Ang II concentrations in interstitial fluid are much higher than the plasma concentrations with values in the range of 3–5 pmoles/mL.[19] Renal interstitial fluid Ang II levels increase further in hypertensive rats infused with Ang II for 2 weeks. The high renal interstitial values indicate local regulation of Ang II formation in the renal interstitial compartment and an enhancement of interstitial Ang II production in Ang II dependent hypertension.[12]

Tubular Ang II

As shown in Figure 3, proximal tubule fluid concentrations of Ang I and Ang II are also much greater than the plasma concentrations and have been found to be in the range of 5–10 pmoles/ml.[19] In perfused tubules, where the contribution resulting from glomerular filtration is prevented, Ang II concentrations are similar to those in nonperfused tubules indicating that the proximal tubule secretes Ang II or a precursor into the proximal tubule fluid. In addition to AGT, proximal tubule cells have renin that can act on AGT to generate Ang I. Evidence that renin is augmented in the urine of chronic Ang II-infused rats also indicates secretion of renin by the collecting duct cell into the tubular lumen[16] and provides a pathway for Ang I generation from proximally delivered AGT, leading to concentrations of Ang II in distal tubular fluid in the picomolar range.[34]

Intracellular Angiotensin II

Ang II is internalized via AT_1–receptor-mediated endocytosis and endosomal accumulation of Ang II in intermicrovillar clefts and endosomes is increased further in Ang II infused hypertensive rats.[35] AT_1 receptor blockade prevents the endosomal accumulation even though plasma Ang II increases. The presence of Ang II in renal endosomes indicates that some of the internalized Ang II remains intact and contributes to the total Ang II content measured in tissue homogenates.[35] Endocytosis of the Ang II-AT_1 receptor complex is required for the full expression of functional responses coupled to the activation of signal transduction pathways, but the full extent of the functions exerted by intracellular Ang II has not been determined.[15] In Ang-II-dependent hypertension, a higher fraction of the total kidney Ang II is internalized into intracellular endosomes consisting of both light endosomes and intermicrovillar clefts via an AT_1-receptor-mediated process. AT_1 receptor blockade prevents the augmentation of intracellular Ang II.[35]

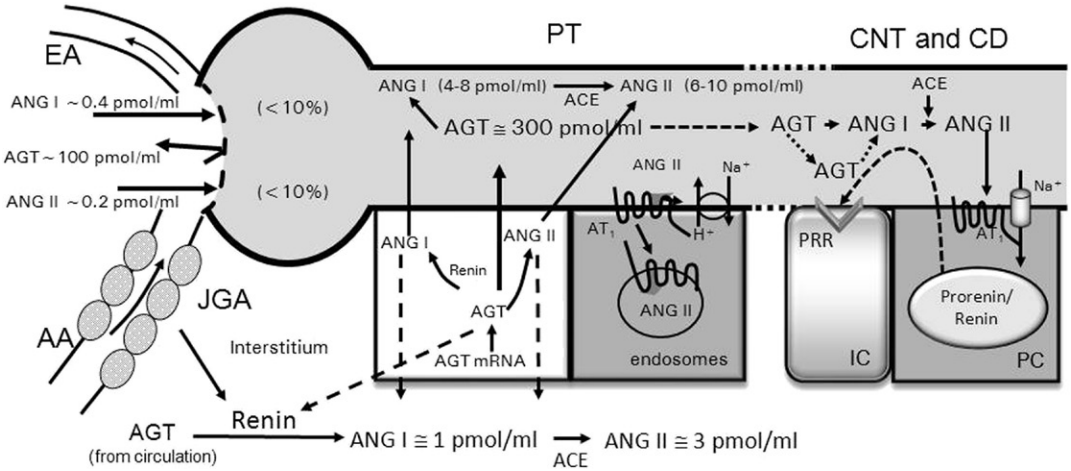

FIGURE 3 Intratubular processing of the renin–angiotensin system. All components of the renin–angiotensin system are present in the kidney providing multiple pathways for enhanced intrarenal Ang II formation. PT—proximal tubule; DT—distal tubule; CD—collecting duct; AGT—angiotensinogen; JGA—juxtaglomerular apparatus; AA—afferent arteriole; EA—efferent arteriole; ACE—angiotensin converting enzyme; PRR—(pro)renin receptor (modified from Ref. 19).

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Our understanding of the functions of internalized Ang II remains incomplete. Ang II may be recycled and secreted to exert further actions by binding to Ang II receptors on the cell membranes. Ang II may also act on cytosolic receptors to activate intracellular signaling mechanisms.[15] Ang II may also migrate to the nucleus to exert genomic effects.[21] Nuclear binding sites of the AT_1 subtype in renal cells have been reported. Because Ang II exerts a positive stimulation on AGT mRNA and protein production, intracellular Ang II may have genomic actions to regulate AGT or renin mRNA expression in proximal tubule cells either directly or via NF-κB.[1,21,30]

BIOLOGICAL ACTIONS OF INTRARENAL ANG II

In view of the near ubiquitous distribution of Ang II receptors in the various structures of the kidney, it is not surprising that Ang II exerts pleiotropic effects at multiple levels within the kidney. As shown in Figure 2, the actions of Ang II span both cortical and medullary, vascular and tubular and can be stimulatory or inhibitory.[18] At the level of the vasculature, Ang II exerts important direct and indirect effects to regulate vascular tone of the afferent and efferent arterioles and of the mesangial cells.[17] Ang II also regulates afferent arteriolar tone through its influence on the tubuloglomerular feedback mechanism with elevated Ang II levels increasing the sensitivity of the feedback mechanism, thus regulating the amount of filtrate that escapes the proximal nephron and enters the distal nephron segments. In the postglomerular circulation, Ang II regulates both the cortical and medullary capillary blood flow and helps to regulate the relative blood flowing to the medullary tissues through its control of the tone of the pericytes.[17]

Interestingly, although Ang II constricts both afferent and efferent arterioles, the mechanisms are different. The afferent arteriolar constrictor responses to Ang II involve activation of L-type Ca^{++} channels to provide sustained Ca^{++} entry and vasoconstriction.[17] By contrast, efferent arterioles of normal rats do not vasodilate or vasodilate much less in response to L-type Ca^{++} channel blockers and the vasoconstrictor effects of Ang II are not blocked by L-type Ca^{++} channel blockers. Rather, T-type channels have greater functional significance in regulating the efferent arteriolar constrictor responses to Ang II.[17] T-type Ca^{++} channel blockers markedly attenuate the efferent vasoconstrictor responses to Ang II. Storage operated Ca^{++} channels in efferent and afferent arterioles are also activated by Ang II. Thus, although both afferent and efferent arterioles respond to Ang II, the mechanisms for Ca^{++} entry and the activation pathways are different. These differences may be attributable to the differential abundance of AT_{1A} and AT_{1B} receptors on the afferent and efferent arterioles.[10]

Virtually, all aspects of the filtration and tubular reabsorptive processes are influenced by Ang II. Ang II directly regulates the glomerular filtration coefficient with high Ang II concentrations reducing and low levels or blockade of AT_1 receptors increasing the filtration coefficient.[17,18] At the level of the proximal tubule, Ang II binds to receptors at both the apical and basolateral membranes, stimulating the activity of the sodium/hydrogen exchanger and the sodium bicarbonate co-transporter, thereby exerting an important influence on net proximal reabsorption rate.[18] Transport mechanisms in the proximal tubule, loop of Henle, and distal nephron segments are also influenced by Ang II.[18,27,34]

Under conditions in which the activity of the RAS is stimulated and greater amounts of AGT are secreted from the proximal tubule, the locus of influence shifts to include the distal nephron segments where the interaction between the (P)RR and renin/prorenin may contribute substantially to increase intrarenal and intratubular formation of Ang II (Fig. 4).[7,20] The *in vivo* demonstration that increased urinary Ang II concentrations in mice infused chronically with Ang II enhance distal sodium reabsorption[34] emphasizes further the importance that renin and the (P)RR may have in the distal nephron segments contributing to the increases in intratubular Ang II formation. Ang II stimulates the sodium/hydrogen exchanger and the sodium chloride cotransporter in distal tubules, and the amiloride sensitive sodium channel in principal cells of connecting tubules and collecting duct segments.[20,23] This enhanced stimulatory effect allows the kidney to have maximum capability to conserve sodium by markedly decreasing fractional sodium reabsorption.[34] At the whole kidney level, this is manifested as a marked suppression of the pressure natriuresis relationship. Ang II also regulates acid base balance through its effects on the Na^+/H^+ exchanger as well as by influencing the activity of the H^+ ATPase in intercalated cells.[2] Collectively, the actions of Ang II allow the kidney to maximize sodium conservation without compromising its ability to regulate its many other functions.

CONCLUDING COMMENTS AND PERSPECTIVE

Recent findings have stimulated interest in the molecular mechanisms regulating the various components of the intrarenal RAS and particularly the interstitial, intracellular, and intratubular concentrations of Ang II and related peptides. Intratubular and interstitial Ang II levels are regulated independently of the circulating Ang II and exert powerful actions via the stimulation of AT1 receptors on the vascular, glomerular, and tubular structures to provide a synchronous cascade of effects contributing to the ability of the kidney to retain >99% of the filtered sodium. From a functional perspective, the effects of Ang II on proximal nephron reabsorption as

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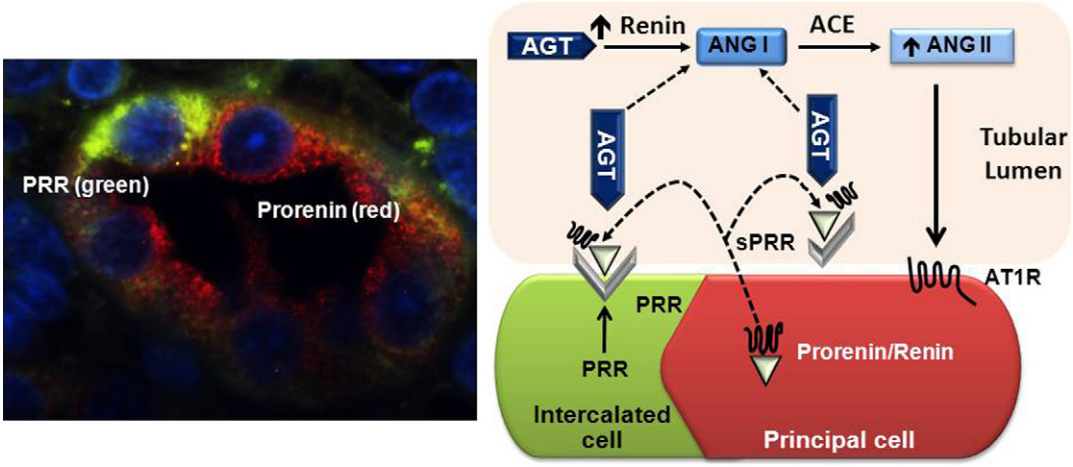

FIGURE 4 Renin and (pro)renin receptor interaction in the collecting duct. Prorenin and renin stimulation in the principal cells of the collecting ducts may increase Ang I formation in the tubular lumen and increase Ang II generation. The presence of (pro)renin receptor at the surface of the intercalated cells along with its soluble form in the tubular lumen increases the catalytic efficiency for renin to form Ang I. In addition, the pro(renin) receptor bound to the membrane of the intercalated cells may anchor prorenin secreted by the principal cells to reduce the washout of prorenin or renin into the urine. ANG—angiotensin; AGT—angiotensinogen; AT1R—angiotensin II type 1 receptor; PRR—(pro)renin receptor; sPRR—soluble form of the (pro)renin receptor; ACE—angiotensin converting enzyme. See color plate 54.

well as on distal nephron transport function, coupled with the associated actions of elevated aldosterone levels, markedly increase the sodium retaining capability of the kidney. When activated in a physiologically appropriate setting under conditions of volume contraction or salt deficient states, these actions can be life saving. When inappropriately maintained or augmented, however, these effects contribute to the development and maintenance of hypertension. Further, the sustained increases in intrarenal Ang II in a setting of hypertension can lead to progressive renal injury, cell proliferation, and fibrosis associated with activation of several major cytokines and growth factors.[12,33]

ACKNOWLEDGMENTS

The authors thank Debbie Olavarrieta for the preparation of the manuscript and figures. The authors' research related to this review has been supported by research grants from NHLBI, NIDDK, NCRR, and AHA.

REFERENCES

1. Acres OW, Satou R, Navar LG, Kobori H. Contribution of a Nuclear Factor-{kappa}B binding site to human angiotensinogen Promoter activity in renal proximal tubular cells. *Hypertension* 2011;**57**: 608–13.
2. Advani A, Kelly DJ, Cox AJ, White KE, Advani SL, Thai K, et al. The (Pro)renin receptor: site-specific and functional linkage to the vacuolar H+-ATPase in the kidney. *Hypertension* 2009;**54**:261–9.
3. Bernstein KE, Xiao HD, Adams JW, Frenzel K, Li P, Shen XZ, et al. Establishing the role of angiotensin-converting enzyme in renal function and blood pressure control through the analysis of genetically modified mice. *J Am Soc Nephrol* 2005;**16**:583–91.
4. Cervenka L, Vaneckova I, Huskova Z, Vanourkova Z, Erbanova M, Thumova M, et al. Pivotal role of angiotensin II receptor subtype 1A in the development of two-kidney, one-clip hypertension: study in angiotensin II receptor subtype 1A knockout mice. *J Hypertens* 2008;**26**:1379–89.
5. Crowley SD, Gurley SB, Herrera MJ, Ruiz P, Griffiths R, Kumar AP, et al. Angiotensin II causes hypertension and cardiac hypertrophy through its receptors in the kidney. *Proc Natl Acad Sci U S A* 2006;**103**:17985–90.
6. Gonzalez AA, Lara LS, Luffman C, Seth DM, Prieto MC. Soluble form of the (pro)renin receptor is augmented in the collecting duct and urine of chronic angiotensin II-dependent hypertensive rats. *Hypertension* 2011;**57**:859–64.
7. Gonzalez AA, Liu L, Lara LS, Seth DM, Navar LG, Prieto MC. Angiotensin II stimulates renin in inner medullary collecting duct cells via protein Kinase C and Independent of epithelial sodium channel and Mineralocorticoid receptor activity. *Hypertension* 2011;**57**:594–9.
8. Gonzalez-Villalobos RA, Billet S, Kim C, Satou R, Fuchs S, Bernstein KE, et al. Intrarenal angiotensin-converting enzyme induces hypertension in response to Angiotensin I infusion. *J Am Soc Nephrol* 2011;**22**:449–59.
9. Gonzalez-Villalobos RA, Satou R, Ohashi N, Semprun-Prieto LC, Katsurada A, Kim C, et al. Intrarenal mouse renin-angiotensin system during ANG II-induced hypertension and ACE inhibition. *Am J Physiol Ren Physiol* 2010;**298**:F150–7.
10. Harrison-Bernard LM, Cook AK, Oliverio MI, Coffman TM. Renal segmental microvascular responses to ANG II in AT1A receptor null mice. *Am J Physiol Ren Physiol* 2003;**284**:F538–45.
11. Ichihara A, Sakoda M, Kurauchi-Mito A, Kaneshiro Y, Itoh H. Involvement of (pro)renin receptor in the glomerular filtration barrier. *J Mol Med* 2008;**86**:629–35.
12. Kobori H, Nangaku M, Navar LG, Nishiyama A. The intrarenal renin–angiotensin system: from physiology to the pathobiology of hypertension and kidney disease. *Pharmacol Rev* 2007;**59**:251–87.

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13. Kobori H, Prieto-Carrasquero MC, Ozawa Y, Navar LG. AT1 receptor mediated augmentation of intrarenal angiotensinogen in angiotensin II-dependent hypertension. *Hypertension* 2004;**43**:1126–32.
14. Lara LS, McCormack M, Semprum-Prieto LC, Shenouda S, Majid DS, Kobori H, et al. AT1 receptor-mediated augmentation of angiotensinogen, oxidative stress, and inflammation in ANG II-salt hypertension. *Am J Physiol Ren Physiol* 2012;**302**:F85–94.
15. Li XC, Hopfer U, Zhuo JL. AT1 receptor-mediated uptake of angiotensin II and NHE-3 expression in proximal tubule cells through a microtubule-dependent endocytic pathway. *Am J Physiol Ren Physiol* 2009;**297**:F1342–52.
16. Liu L, Gonzalez AA, McCormack M, Seth DM, Kobori H, Navar LG, et al. Increased renin excretion associated with augmented urinary angiotensin (Ang) II levels in chronic angiotensin II-infused hypertensive rats. *Am J Physiol Ren Physiol* 2011;**301**: F1195–201.
17. Navar LG, Arendshorst WJ, Pallone TL, Inscho EW, Imig JD, Bell PD. The renal Microcirculation. In: Tuma RF, Duran WN, Ley K, editors. *Handbook of physiology: microcirculation*. Academic Press; 2008. p. 550–683.
18. Navar LG, Harrison-Bernard LM, Imig JD, Mitchell KD. Renal actions of angiotensin II and AT1 receptor blockers. In: Epstein M, Brunner HR, editors. *Angiotensin II receptor antagonists*. Philadelphia: Hanley & Belfus, Inc.; 2000. p. 189–214.
19. Navar LG, Kobori H, Prieto MC, Gonzalez-Villalobos RA. Intratubular renin-angiotensin system in hypertension. *Hypertension* 2011;**57**:355–62.
20. Navar LG, Prieto MC, Satou R, Kobori H. Intrarenal angiotensin II and its contribution to the genesis of chronic hypertension. *Curr Opin Pharmacol* 2011;**11**:180–6.
21. Navar LG, Prieto-Carrasquero MC, Kobori H. Molecular aspects of the renal renin–angiotensin system. In: Re R, DiPette DJ, Schiffrin EL, Sowers JR, editors. *Molecular mechanisms in hypertension*. Taylor & Francis Group; 2006. p. 3–14.
22. Nguyen G, Muller DN. The biology of the (pro)renin receptor. *J Am Soc Nephrol* 2010;**21**:18–23.
23. Peti-Peterdi J, Warnock DG, Bell PD. Angiotensin II directly stimulates ENaC activity in the cortical collecting duct via AT(1) receptors. *J Am Soc Nephrol* 2002;**13**:1131–5.
24. Pohl M, Kaminski H, Castrop H, Bader M, Himmerkus N, Bleich M, et al. Intrarenal renin angiotensin system Revisited: role of megalin-dependent endocytosis along the proximal nephron. *J Biol Chem* 2010;**285**:41935–46.
25. Prieto-Carrasquero MC, Botros FT, Kobori H, Navar LG. Collecting duct renin: a major player in angiotensin II-dependent hypertension. *J Am Soc Hypertens* 2009;**3**:96–104.
26. Prieto-Carrasquero MC, Botros FT, Pagan J, Kobori H, Seth DM, Casarini DE, et al. Collecting duct renin is upregulated in both kidneys of 2-kidney, 1-clip goldblatt hypertensive rats. *Hypertension* 2008;**51**:1590–6.
27. Riquier-Brison AD, Leong PK, Pihakaski-Maunsbach K, Mcdonough AA. Angiotensin II stimulates trafficking of NHE3, NaPi2, and associated proteins into the proximal tubule microvilli. *Am J Physiol Ren Physiol* 2010;**298**:F177–86.
28. Santos RA, Simoes e Silva AC, Maric C, Silva DM, Machado RP, de B I, et al. Angiotensin-(1-7) is an endogenous ligand for the G protein-coupled receptor Mas. *Proc Natl Acad Sci U S A* 2003;**100**:8258–63.
29. Satou R, Gonzalez-Villalobos RA, Miyata K, Ohashi N, Urushihara M, Acres OW, et al. IL-6 augments angiotensinogen in primary cultured renal proximal tubular cells. *Mol Cell Endocrinol* 2009;**311**:24–31.
30. Satou R, Miyata K, Katsurada A, Navar LG, Kobori H. Tumor necrosis factor-{alpha} suppresses angiotensinogen expression through formation of a p50/p50 homodimer in human renal proximal tubular cells. *Am J Physiol Cell Physiol* 2010;**299**:C750–9.
31. Sequeira-Lopez ML, Weatherford ET, Borges GR, Monteagudo MC, Pentz ES, Harfe BD, et al. The microRNA-processing enzyme dicer maintains juxtaglomerular cells. *J Am Soc Nephrol* 2010;**21**:460–7.
32. Vio CP, Jeanneret VA. Local induction of angiotensin-converting enzyme in the kidney as a mechanism of progressive renal diseases. *Kidney Int Suppl* 2003;**86**:S57–63.
33. Wolf G. *The renin–angiotensin system and progression of renal diseases*. Hamburg: Karger; 2002.
34. Zhao D, Seth DM, Navar LG. Enhanced distal nephron sodium reabsorption in chronic angiotensin II-Infused mice. *Hypertension* 2009;**54**:120–6.
35. Zhuo JL, Imig JD, Hammond TG, Orengo S, Benes E, Navar LG. Ang II accumulation in rat renal endosomes during Ang II-induced hypertension: role of AT(1) receptor. *Hypertension* 2002;**39**:116–21.

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Chapter 205

Adrenomedullin

Stefan Hippenstiel, Andreas C. Hocke, Norbert Suttorp
and Bettina Temmesfeld-Wollbrück

ABSTRACT

Pulmonary diseases or involvement of the lung during severe systemic illness significantly contribute to mortality worldwide. In particular, acute or chronic inflammation of the lung impairs vital gas exchange thereby causing life-threating diseases. Adrenomedullin (AM) is a 52-amino-acid peptide expressed throughout the body and induced at sites of inflammation. AM may possess anti-inflammatory and protective effects in the lung, with extensive binding in the lung vasculature. *In vivo* studies provide considerable evidence that AM may improve acute lung injury (ALI), a severe condition with extensive mortality. Studies in humans and animal models indicate that the peptide reduces pulmonary arterial hypertension. Further, in chronic lung disease models of lung fibrosis or emphysema, AM application improved critical markers of the diseases. Overall, AM seems to be an innovative and promising pharmaceutical molecule for treatment of important lung diseases causing serious human illness.

INTRODUCTION

Exchange of gas in vertebrates takes place in the lung. In a human being, via the tracheobronchial tree, >10.0001 of air each day reach the alveolus, a filigree structure essentially consisting of lung epithelial type I and type II cells as well as endothelial cells. Passive diffusion of oxygen or carbon dioxide over the alveolar barrier guarantees blood gas homeostasis.

Several acute or chronic lung diseases are characterized by the perturbation of the alveolar structure and function finally leading to impaired vital gas exchange in the lung. Acute lung inflammation in pneumonia or severe acute lung injury (ALI) is accompanied by the perturbation of the alveolar barrier resulting, for example, from lung edema formation and the infiltration of inflammatory cells during the host pathogen defense.[19] Subsequent proinflammatory mediator production and release of toxic metabolites and proteases by invading polymorphonuclear leukocytes finally cause damage to the alveolar wall with lung edema formation. A critical step is the failure of endothelial barrier function allowing for interstitial and subsequent intraalveolar fluid flux.[19]

In more chronic inflammation of the lung, rarefication of the alveolar structure and/or thickening of the alveolar wall with fibrosis of lung tissue takes place.[27] Finally, independently of the initial cause of the disease, reduced gas diffusion will lead to deleterious oxygenation failure. Further, these processes may be accompanied by diseases such as pulmonary artery hypertension or pulmonary perfusion mismatch, which furthermore aggravate the pathology. These chronic changes not only disturb lung function, they also may induce remote organ dysfunction. In pulmonary arterial hypertension, for example, the blood pressure increase subsequently causes right ventricular heart dysfunction.

Because despite extensive research, all these diseases show high mortality worldwide, it is of outmost importance to find innovative interventional strategies targeting the central pathophysiological processes. This chapter will show that AM, referred to as the amidated mature form of the peptide, is an attractive pharmacological candidate for the treatment of inflammatory pulmonary diseases. AM is a 52-amino-acid peptide belonging to the calcitonin gene-related peptide family, which was first isolated by Kitamura et al. from a human pheochromocytoma in 1993.[16] For details regarding its discovery, gene structure, and processing please see the AM chapters in several sections of the book.

AM EXPRESSION AND CLEARANCE IN THE LUNG

AM is expressed in human lung cells including lung vascular endothelial cells.[17] Intraperitoneal exposure of rats to lipopolysaccharide (LPS) of gram-negative bacteria increases the immunoreactivity of AM in the lung.[2] The authors showed increased AM expression in ciliated epithelium, endothelium, and alveolar type I cells, but they did not prove the specificity of the staining and identified cells based solely on their morphological characteristics.

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00205-0
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In another study, AM expression was analyzed in a rat model of combined LPS exposure and acute normobaric hypoxia (9% O_2).[1] The combination of hypoxia and LPS treatments overcomes AM expression observed after each treatment alone. Using *in situ* techniques, AM expression was documented in inflammatory cells within blood vessels, in lung parenchyma, and bronchiolar epithelial cells.[1]

The report of Nishikimi et al.[25] was the first indicating that the lung may be a target for AM in humans. The authors presented results derived from human subjects in which they show reduced AM plasma concentrations in the aorta compared with that of the pulmonary artery. This observation in humans was verified by Hirayama et al.[11] Further, application of the AM receptor antagonist hAM(22–52) in rats causes an increase of AM liberated by lungs, which could not be attributed to the alterations of AM transcription.[4] High expression of AM receptors, consisting of calcitonin-receptor-like receptor (CRLR) and receptor activity modifying protein (RAMP) 2 was noted in the lungs of normal mice and human lung cells including lung vascular endothelial cells.[17] Notably, the high capture of AM in the lung vasculature is highlighted by the suggestion to use AM derivatives as innovative molecules for molecular imaging of lung microcirculation.[9]

AM IN MODELS OF ACUTE LUNG INFLAMMATION

The most severe forms of lung inflammation are ALI and acute respiratory distress syndrome (ARDS).[19] ARDS is the more severe form of the disease, defined by acute onset, bilateral infiltrates on chest radiographs, pulmonary artery occlusion pressure <18 mmHg (if measured or absence of clinical signs of left atrial hypertension), and <200 mmHg ratio of partial pressure of oxygen in arterial blood (PaO_2) to the fraction of inspired oxygen (FiO_2).[19]

The acute inflammation of the lung in ALI/ARDS is caused by primary pulmonary activation (e.g., bacterial pneumonia, inhalation trauma) or systemic inflammatory syndromes (e.g., sepsis). Despite the primary cause, the initial phase of ALI/ARDS is characterized by a severe oxygenation defect, reduced lung compliance, bilateral pulmonary infiltrates, endothelial and epithelial cell injury, subsequent edema formation in interstitium and airspaces, inactivation of surfactant, and neutrophil sequestration and migration in lung.[19] Effective mechanical ventilation with low tidal volume is the standard therapy. However, despite high mortality ranging from 30 to 80%, currently there is no pharmaceutical therapy for ALI/ARDS available.[19] Because there are many different causes, such as bacteria-related pneumonia, massive blood transfusion, peripheral organ reperfusion, or thermal trauma that induce ALI/ARDS, a potential effective pharmaceutical treatment has to show its efficiency in various models of injury.[19]

Intratracheal instillation of the cell wall component lipopolysaccharide (LPS) of Gram-negative bacteria is a widely used experimental model of ALI/ARDS simulating direct injury of the lung. Itoh et al. continuously infused AM in the left jugular vein via implanted osmotic minipumps and induced ARDS 2 h later by the intratracheal application of 1 mg/kg LPS.[13] AM reduced the total protein and albumin levels in bronchoalveolar lavage (BALF) and normalized histological findings. Reduced cleavage of caspase-3 was noted in alveolar wall cells in AM-treated animals suggesting that AM reduces apoptosis of lung cells in this model. Further, the authors noted decreased numbers of total cells and neutrophils accompanied by lower levels of TNF-α and CINC in the BALF.[13] AM also reduces total protein and albumin levels in BALF.

The massive recruitment of polymorphonuclear leukocytes into the alveolar space is an integral part of the acute inflammation of the lung in pneumonia and ALI/ARDS.[19] In the lung, these cells liberate highly reactive oxygen radicals which directly injure the alveolar compartment. Hydrogen-peroxide infusion into blood-free perfused and ventilated rabbit lungs was used to mimic a granulocyte attack of the lung.[10] The application of a single bolus of 0.1 nmol/L ADM 15 min before infusion of hydrogen peroxide reduces the hydrogen-peroxide-related increase in lung capillary filtration coefficient, indicating endothelial barrier stabilization in AM-treated lungs.[10]

Besides leukocyte-derived radicals, hyperoxia is known to induce oxidative stress-related lung injury. In a rat model of hyperoxia-induced ALI/ARDS, Tao et al. tested the effect of AM administered by osmotic minipumps.[28] After the start of AM infusion, the animals were exposed to >95% oxygen for 72 h. Histological examination revealed that AM remarkably improves lung injury score, reduces the infiltration of inflammatory cells, as well as edema formation. The AM infusion increases glutathione peroxidase and superoxide dismutase activities suggesting that AM strengthened the anti-inflammatory capacity of the lung. Along those lines, the authors showed reduced lipid hydroperoxide formation, nuclear factor-kappa B activity, and less inflammatory mediator release (IL-6, KC, MMP-9).[28]

Severe systemic infection (sepsis) is a leading cause of ALI/ARDS and severe lung pneumonia may cause sepsis.[19] The combination of sepsis and ALI/ARDS is characterized by high mortality in man. The pathogen *Staphylococcus aureus* is frequently isolated in septic patients. These bacteria express the highly potent pore-forming α-toxin. To study septic shock related ALI/ARDS in rats, we used central venous infusion of α-toxin.[29] In this model, α-toxin infusion induces a mortality of 50% within 5 h, accompanied by depression of arterial blood pressure, cardiac index, renal failure, and massive systemic endothelial hyperpermeability. In the lung, ALI/ARDS was indicated by pronounced edema formation and albumin extravasation.[29] One hour

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after the start of central venous α-toxin infusion, therapeutic AM (24 μg/kg per hour) was applied via the peripheral tail vein.[29] After 6 h, AM reduced mortality from 53% to 7%, stabilized arterial blood pressure, increased cardiac index, reduced renal failure, and normalized endothelial barrier function in the lung and other affected organs.[29]

Remote organ damage of the lung is a frequently observed clinical problem in, for example, severe burn or ischemia-reperfusion injury. In severe burn, remote oxidant-induced organ injury with increased lipid peroxidation and generalized tissue inflammation in uninjured organs occurs within hours of injury. The effect of AM administration on burn-induced remote organ damage was investigated in rats.[12] The authors treated rats with subcutaneous application of AM 10 min before burn insult covering 30% of total body surface area. Rats were sacrificed 24 h after the burn insult. AM treatment reduced lipid peroxidation, tissue neutrophil infiltration, oxidant generation in analyzed organs, including the lung. In particular, malondialdehyde and myeloperoxidase activities were reduced in AM-exposed animals compared with that in -treated controls.[12]

Like massive burns, ischemic bowel disease is known to induce remote organ damage including the lung associated with high mortality, Dwivedi et al. hypothesized that the administration of AM and its binding protein (AMBP-1) would have protective effects on intestinal ischemia-reperfusion-related ALI/ARDS in rats.[5] They induced intestinal ischemia-reperfusion by clipping the superior mesenteric artery for 90 min. On release of the clamp, the animals were treated with AM/AMBP-1 for 30 min via a femoral vein catheter. After 4 h, the authors examined lung water content, morphologic changes, and granulocyte myeloperoxidase activity as well as TNF-α and IL-6 expression. In this model, AM/AMBP-1 reduces lung injury, including edema formation, histopathologic changes, myeloperoxidase activity, and cytokine levels in the lung.[5] Comparable results regarding reduced lung tissue injury were presented by the same group in models of obstructive jaundice[32] and gut ischemia and reperfusion injury[34] in rats.

In ALI/ARDS, mechanical ventilation (MV) is a life-saving treatment without alternatives.[19] However, although ventilation strategies have been optimized over the years, even minimal MV-induced physical forces on lung tissue may evoke ventilator-induced lung injury (VILI).[6] VILI is characterized by pulmonary inflammation with liberation of cytokines, recruitment of leucocytes to the lung, increased lung permeability, and surfactant dysfunction finally resulting in the deterioration of pulmonary gas exchange. We tested the hypothesis that AM application would be an adjuvant pharmacological strategy to reduce VILI.[20] Mice were treated with AM, and lung permeability, local and systemic inflammation and markers of distal organ function were assessed after 2 or 6 h of mechanical ventilation with 100% oxygen and protective or moderately injurious ventilator settings, respectively. In this experimental system, AM dramatically reduces lung permeability in VILI in mice, leading to improved oxygenation. AM attenuates leucocyte infiltration in the lung and prevents the increase in lactate and creatinine levels in mice ventilated with high tidal volumes. Importantly, AM protects against VILI even when treatment is initiated 2 h after the beginning of mechanical ventilation in the 6-h VILI mouse model.[20]

AM AND PULMONARY ARTERIAL HYPERTENSION

Pulmonary arterial hypertension (PAH) is characterized by a progressive rise in pulmonary artery pressure and pulmonary vascular resistance with limited dysfunction of the left ventricle.[7] Conditions that result in PAH are heterogeneous and seemingly unrelated and the pathogenesis is poorly understood. Nevertheless, there is an imbalance between vasoconstrictive or proliferative and vasodilatory or antiproliferative mediators in the lung vasculature leading to the proliferation of endothelial and smooth muscle cells, as well as increased extracellular matrix deposition. Finally, the end result is a reduction and/or obliteration of luminal space in the pulmonary vasculature.[7]

Two studies in humans suffering from PAH published by Nagaya et al. provide direct evidence that AM may be an attractive pharmaceutical agent to reduce pulmonary arterial pressure. Intravenous infusion of 0.05 μg/kg/min AM was tested in 13 patients with precapillary pulmonary hypertension.[24] Besides a strong increase in cardiac index, a 32% decrease in pulmonary vascular resistance and a 4% reduction in mean pulmonary arterial pressure were noted.[24] In a second study, the effect of inhaled AM on pulmonary hemodynamics and exercise capacity was determined in 11 patients with idiopathic pulmonary arterial hypertension.[22] Inhalation of 10 μg/kg body weight aerosolized AM produced a 13% decrease in mean pulmonary arterial pressure and reduced pulmonary vascular resistance about 22% without affecting systemic arterial pressure or heart rate. This was accompanied by an increase in peak oxygen consumption.[22] Overall, these pilot studies indicate that AM may have beneficial effects on pulmonary hemodynamics and exercise capacity in patients with pulmonary arterial hypertension.

These results obtained in humans are supported by several animal studies. In three studies, administration of monocrotaline in rats was used as a model.[8,23,33] Monocrotaline induces vascular endothelial cell damage, medial thickening of the muscular pulmonary arteries, and neomuscularization of the nonmuscular distal arteries, leading to pulmonary hypertension and right ventricular hypertrophy. Continuous infusion of monocrotaline-exposed rats with AM via an osmotic minipump for 21 days reduces

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the increase in right ventricular systolic pressure and the ratio of right ventricular weight to body weight observed after monocrotaline treatment.[33] Further, medial thickening of the pulmonary artery is reduced in AM-treated rats. Harada-Shiba et al. tested the effect of a nonviral gene delivery system of intratracheal administration of polyplex nanomicelles formulated with a therapeutic plasmid of the human AM gene in monocrotaline-exposed rats.[8] Gene transfer of AM by this approach notably increases pulmonary human AM mRNA levels and significantly reduces right ventricular pressure.[8] In a third study with the monocrotaline model, isolated circulating endothelial progenitor cells (EPC) were nonvirally transfected with plasmid DNA of AM and intravenously administered.[23] Transplantation of AM DNA-transduced EPC reduces pulmonary hypertension in monocrotaline-exposed rats and these animals show an increased survival rate.[23]

A severe increase of pulmonary arterial pressure could also be induced by surfactant depletion in newborn piglets. In these animals, Kandler et al. analyzed the effect of aerosolized AM on pulmonary arterial pressure in the presence of the nitric-oxide synthase inhibitor L-NAME.[14] Aerosolized AM reduces mean pulmonary artery pressure, increases oxygenation of the animals without influencing mean systemic arterial pressure.

Finally, experiments in mice with hypoxia-associated pulmonary vascular remodeling also indicate the protective effect of AM on PAH.[18] In AM+/− mice, hypoxia induces significantly greater media wall thickness of the pulmonary arteries and shows enhanced production of pulmonary reactive oxygen species compared with AM+/+ mice. Interestingly, the administration of both exogenous AM and of a superoxide dismutase mimetic normalizes pulmonary vascular media wall thickness and oxidative stress in the lung.[18]

AM AND CHRONIC LUNG DISEASE

Idiopathic pulmonary fibrosis (IPF) is a fatal interstitial lung disease characterized by inexorable, chronic-progressive fibrosis with limited treatment options. A mouse model of bleomycin-induced lung injury was used to test the effect of daily intraperitoneal AM injection.[3] Compared with solvent-exposed animals, AM-treated mice show reduced lung damage and inflammation. In particular, myeloperoxidase activity, the expression of cytokines, of transforming growth factor beta, as well as of nitric-oxide synthase is reduced in AM-treated mice. In addition, reduced activity of nuclear enzyme poly (ADP-ribose) polymerase is indicated by lower poly (ADP-ribose) formation.[3]

In contrast to fibrosis, pulmonary emphysema is defined as abnormal permanent enlargement of airspaces distal to terminal bronchioles. As for IPF, there is no effective treatment that prevents the progression of this disease or even restores normal lung function. In a mouse model, elastase was injected to induce destruction of the alveolar wall. AM administration by osmotic minipumps inhibits the increase in lung volume, static lung compliance, and mean linear intercept in mice exposed to elastase.[21] Importantly, even when AM administration is started 25 days after elastase injection, AM slightly, but significantly attenuates the increase in mean linear intercept, in lung volume and in static lung compliance. By using chimeric mice in which the bone marrow is reconstituted with GFP-positive cells, the authors further show that AM infusion increases the number of GFP-positive cells in the alveolar walls and of GFP/cytokeratin double-positive cells.[21] Hence, it is hypothesized that AM would promote lung regeneration by increasing the number of bone-marrow-derived alveolar epithelial cells.

In addition to emphysema, bronchopulmonary dysplasia (BPD) is characterized by alveolar simplification and abnormal distal airspace enlargement. After intranasal administration of the AM antagonist AM22–52 in rat pubs, decreased lung capillary density, impaired alveolar development and reduced septal counts are detected.[31] Using a model for arrested lung vascular and alveolar growth (oxygen-induced BPD), the study shows that AM attenuates arrested lung angiogenesis and alveolar development. Interestingly, reduced pulmonary hypertension was noted, indicated by decreased right ventricular hypertrophy and pulmonary artery medial wall thickness.[31]

AM—MODE OF ACTION IN THE LUNG

The mechanisms by which AM act in the lung may be as diverse as the diseases mentioned above and can only be briefly highlighted in this chapter.

In acute inflammation of the lung, protection of lung alveolar barrier is a critical point. AM reduces actin–myosin-based endothelial cell contraction and stabilizes endothelial cell–cell contacts thus preventing deleterious edema formation.[10,29,30] Protective lung vascular effects are also mediated by reducing cell apoptosis and the influence of AM on the liberation of vasoactive mediators such as nitric oxide.[5,12,13,18,20,24,33] Increased expression and activity of antioxidative enzymes such as gamma–glutamate–cysteine ligase, glutathione peroxidase, and reductase in AM-exposed lung cells may protect against oxygen radicals-related injury.[15] AM suppresses increase of reactive oxygen species by inhibiting the reduction of glutathione levels in hypoxia/reoxygenation injury. Further, the peptide maintains total and active reduced thioredoxin levels under such conditions in lung cells.

Taking into account that surfactant malfunction is observed in severe lung inflammation, the AM-related increase in the secretion of phosphatidylcholine, the predominant component of pulmonary surfactant, by type II lung alveolar epithelial cells may be protective, too.[26] Besides these effects, numerous

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reports show that AM reduces the expression of proinflammatory mediators and influences the liberation of growth factors. How bone-marrow-derived alveolar epithelial cells contribute to the lung protective effects needs further investigation in the different models of disease discussed.[21]

CONCLUSIONS

Acute and severe lung inflammation, pulmonary hypertension, emphysema, and lung fibrosis cause tremendous mortality worldwide. In some of the diseases mentioned little or no effective pharmaceutical therapy is available. Thus, there is high medical need for innovative therapies for these important lung diseases. The human endogenous peptide AM has beneficial effects on key aspects of these ailments. Because the available results show a low-risk profile for the use of AM in humans and AM is already given to humans by several application modes, clinical proof-of-concept studies using this peptide in appropriate lung diseases are highly warranted. Nevertheless, there are still large gaps in knowledge regarding AM. For example, signaling mechanisms of AM are still obscure, and most of the complex pathways positively affected by AM in general and even in a particular lung disease are unknown. There are only limited results available about the long-term use of AM and it would be of great interest to understand mechanisms of its beneficial effects. Results from existing *in vitro* and animal studies should be applied wherever possible in human material to substantiate the rationale for the use of AM in humans. Overall, AM seems to be a promising candidate for pharmaceutical therapy of lung diseases.

REFERENCES

1. Agorreta J, Zulueta JJ, Montuenga LM, Garayoa M. Adrenomedullin expression in a rat model of acute lung injury induced by hypoxia and LPS. *Am J Physiol Lung Cell Mol Physiol* 2005;**288**:L536–45.
2. Cheung BM, Hwang IS, Li CY, WS O, Tsang KW, Leung RY, et al. Increased adrenomedullin expression in lungs in endotoxaemia. *J Endocrinol* 2004;**181**:339–45.
3. Di Paola R, Talero E, Galuppo M, Mazzon E, Bramanti P, Motilva V, et al. Adrenomedullin in inflammatory process associated with experimental pulmonary fibrosis. *Respir Res* 2011;**12**:41.
4. Dschietzig T, Azad HA, Asswad L, Bohme C, Bartsch C, Baumann G, et al. The adrenomedullin receptor acts as clearance receptor in pulmonary circulation. *Biochem Biophys Res Commun* 2002;**294**:315–8.
5. Dwivedi AJ, Wu R, Nguyen E, Higuchi S, Wang H, Krishnasastry K, et al. Adrenomedullin and adrenomedullin binding protein-1 prevent acute lung injury after gut ischemia-reperfusion. *J Am College Surg* 2007;**205**:284–93.
6. Gattinoni L, Protti A, Caironi P, Carlesso E. Ventilator-induced lung injury: the anatomical and physiological framework. *Crit Care Med* 2010;**38**:S539–48.
7. Gladwin MT, Ghofrani HA. Update on pulmonary hypertension 2009. *Am J Respir Crit Care Med* 2010;**181**:1020–6.
8. Harada-Shiba M, Takamisawa I, Miyata K, Ishii T, Nishiyama N, Itaka K, et al. Intratracheal gene transfer of adrenomedullin using polyplex nanomicelles attenuates monocrotaline-induced pulmonary hypertension in rats. *Mol Ther* 2009;**17**:1180–6.
9. Harel F, Fu Y, Nguyen QT, Letourneau M, Perrault LP, Caron A, et al. Use of adrenomedullin derivatives for molecular imaging of pulmonary circulation. *J Nucl Med* 2008;**49**:1869–74.
10. Hippenstiel S, Witzenrath M, Schmeck B, Hocke A, Krisp M, Krull M, et al. Adrenomedullin reduces endothelial hyperpermeability. *Circ Res* 2002;**91**:618–25.
11. Hirayama N, Kitamura K, Imamura T, Kato J, Koiwaya Y, Eto T. Secretion and clearance of the mature form of adrenomedullin in humans. *Life Sci* 1999;**64**:2505–9.
12. İseri SÖ, Ersoy Y, Gedik N, Ercan F. Alican İ. Protective role of adrenomedullin in burn-induced remote organ damage in the rat. *Regulat Pept* 2008;**146**:99–105.
13. Itoh T, Obata H, Murakami S, Hamada K, Kangawa K, Kimura H, et al. Adrenomedullin ameliorates lipopolysaccharide-induced acute lung injury in rats. *Am J Physiol Lung Cell Mol Physiol* 2007;**293**:L446–52.
14. Kandler MA, Von Der Hardt K, Mahfoud S, Chada M, Schoof E, Papadopoulos T, et al. Pilot intervention: aerosolized adrenomedullin reduces pulmonary hypertension. *J Pharmacol Exp Ther* 2003;**306**:1021–6.
15. Kim JY, Yim JH, Cho JH, Kim JH, Ko JH, Kim SM, et al. Adrenomedullin regulates cellular glutathione content via modulation of gamma–glutamate–cysteine ligase catalytic subunit expression. *Endocrinology* 2006;**147**:1357–64.
16. Kitamura K, Kangawa K, Kawamoto M, Ichiki Y, Nakamura S, Matsuo H, et al. Adrenomedullin: a novel hypotensive peptide isolated from human pheochromocytoma. *Biochem Biophys Res Commun* 1993;**192**:553–60.
17. Martinez A, Miller MJ, Catt KJ, Cuttitta F. Adrenomedullin receptor expression in human lung and in pulmonary tumors. *J Histochem Cytochem* 1997;**45**:159–64.
18. Matsui H. Adrenomedullin can protect against pulmonary vascular remodeling induced by hypoxia. *Circulation* 2004;**109**:2246–51.
19. Matthay MA, Zemans RL. The acute respiratory distress syndrome: pathogenesis and treatment. *Annu Rev Pathol* 2011;**6**:147–63.
20. Muller HC, Witzenrath M, Tschernig T, Gutbier B, Hippenstiel S, Santel A, et al. Adrenomedullin attenuates ventilator-induced lung injury in mice. *Thorax* 2010;**65**:1077–84.
21. Murakami S. Adrenomedullin regenerates alveoli and vasculature in elastase-induced pulmonary emphysema in mice. *Am J Respir Crit Care Med* 2005;**172**:581–9.
22. Nagaya N. Effects of adrenomedullin inhalation on hemodynamics and exercise capacity in patients with idiopathic pulmonary arterial hypertension. *Circulation* 2004;**109**:351–6.
23. Nagaya N, Kangawa K, Kanda M, Uematsu M, Horio T, Fukuyama N, et al. Hybrid cell-gene therapy for pulmonary hypertension based on phagocytosing action of endothelial progenitor cells. *Circulation* 2003;**108**:889–95.
24. Nagaya N, Nishikimi T, Uematsu M, Satoh T, Oya H, Kyotani S, et al. Haemodynamic and hormonal effects of adrenomedullin in patients with pulmonary hypertension. *Heart* 2000;**84**:653–8.
25. Nishikimi T, Kitamura K, Saito Y, Shimada K, Ishimitsu T, Takamiya M, et al. Clinical studies on the sites of production and clearance of circulating adrenomedullin in human subjects. *Hypertension* 1994;**24**:600–4.

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26. Okumura M, Kai H, Arimori K, Iwakiri T, Hidaka M, Shiramoto S, et al. Adrenomedullin increases phosphatidylcholine secretion in rat type II pneumocytes. *Eur J Pharmacol* 2000;**403**:189–94.
27. Strieter RM, Mehrad B. New mechanisms of pulmonary fibrosis. *Chest* 2009;**136**:1364–70.
28. Tao W, Shu Y-S, Miao Q-B, Zhu Y- B. Attenuation of hyperoxia-induced lung injury in rats by adrenomedullin. *Inflammation* 2011.
29. Temmesfeld-Wollbruck B, Brell B, David I, Dorenberg M, Adolphs J, Schmeck B, et al. Adrenomedullin reduces vascular hyperpermeability and improves survival in rat septic shock. *Intensive Care Med* 2007;**33**:703–10.
30. Temmesfeld-Wollbruck B, Hocke AC, Suttorp N, Hippenstiel S. Adrenomedullin and endothelial barrier function. *Thromb Haemost* 2007;**98**:944–51.
31. Vadivel A, Abozaid S, van Haaften T, Sawicka M, Eaton F, Chen M, et al. Adrenomedullin promotes lung angiogenesis, alveolar development, and repair. *Am J Respir Cell Mol Biol* 2009;**43**:152–60.
32. Yang J, Wu R, Zhou M, Wang P. Human adrenomedullin and its binding protein ameliorate sepsis-induced organ injury and mortality in jaundiced rats. *Peptides* 2010;**31**:872–7.
33. Yoshihara F, Nishikimi T, Horio T, Yutani C, Takishita S, Matsuo H, et al. Chronic infusion of adrenomedullin reduces pulmonary hypertension and lessens right ventricular hypertrophy in rats administered monocrotaline. *Eur J Pharmacol* 1998;**355**:33–9.
34. Zhang F, Wu R, Zhou M, Blau SA, Wang P. Human adrenomedullin combined with human adrenomedullin binding protein-1 is protective in gut ischemia and reperfusion injury in the rat. *Regul Pept* 2009;**152**:82–7.

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Chapter 206

Endothelin-1

Karen A. Fagan, Natalie N. Bauer and Ivan F. McMurtry

ABSTRACT

Endothelin-1 is a 21-amino acid peptide with diverse biological activity that has been implicated in numerous lung diseases. ET-1 is a potent regulator of smooth muscle tone, a mitogen, and an inflammatory mediator that may play a key role in diseases of the airways, pulmonary vasculature, and inflammatory lung diseases, both acute and chronic. This chapter will focus on the biology of ET-1 and its role in lung disease.

INTRODUCTION

Endothelins 1, 2, and 3 (ET-1, -2, and -3) are peptides of much interest in the last several decades. Our laboratory and that of Highsmith's identified an endothelial cell-derived peptide vasoconstrictor, which was later sequenced and cloned by Yanagisawa in 1988.[15,17,23,33] Since then, endothelins have been implicated in both normal lung homeostatic pathways and acute and chronic lung diseases. This chapter focuses on the role of endothelins (especially ET-1) in a wide variety of lung disorders, including diseases of the airways and pulmonary vasculature, lung tumors, the acute respiratory distress syndrome, and pulmonary fibrosis.

ENDOTHELIN GENES, CELLULAR DISTRIBUTION, SYNTHESIS, RECEPTORS, AND REGULATION

The endothelins are a family of three distinct isoforms 21 amino acid peptides long (ET-1, -2, and -3). The ET-2 and -3 isoforms differ from ET-1 by 2 and 6 amino acids, respectively, and share significant homology especially at the carboxy terminus with sarafotoxins a–e. The gene for ET-1 is located on chromosome 6, ET-2 on chromosome 1, and ET-3 on chromosome 20.[3] Endothelin-1 is the most abundant isoform. In the normal lung, ET-1 primarily localizes to vascular endothelium, airway and vascular smooth muscle cells, and, to a lesser degree, the epithelium. ET-1 can also be measured in the plasma. ET-2 has similar functions as ET-1 but is found in the myocardium, kidney, and placental tissues primarily. ET-3 circulates in plasma and is also found in the CNS, gastrointestinal tract, lung, and kidney.

All three endothelins are synthesized as preprohormones and then posttranslationally processed to active peptides. For ET-1, the 212-amino acid protein (preproET-1 or ppET-1) is proteolytically cleaved by endopeptidases to big ET-1 (39 amino acids called proET-1 or pET-1), which has 1% the biologic activity of ET-1. pET-1 is then cleaved by the metalloendoprotease endothelin converting enzyme (ECE) to the 21-amino acid peptide. ECE is found in many cell types in the lung, including endothelium, epithelium, and alveolar macrophages. ET-1 is not stored in the cell but is processed and transported through the cell in vesicles resulting in directional secretion of ET-1 toward the interstitium and smooth muscle (80%) and away from the luminal surface of the airway or vessel. This directional secretion allows ET-1 to act in a paracrine or autocrine manner on neighboring cells while secretion into the circulation allows ET-1 to act as a hormone.[10]

There are two primary human endothelin receptors known. Endothelin A (ETA) and B (ETB) receptors are members of the seven transmembrane, G protein-coupled rhodopsin superfamily. The ETA and ETB receptor genes are located on chromosomes 4 and 13, respectively.[2,26] ETA has the highest affinity for ET-1, but all three have equal affinity for ETB receptor. In the lung, ETA receptors are found on vascular and airway smooth muscle cells, whereas ETB receptors are found on the endothelium.[5,21] The ETB receptor mediates clearance of ET-1 from the circulation primarily in the lung and also in the kidney and liver.[9]

ETA and ETB receptor activation on smooth muscle cells leads to vasoconstriction, whereas ETB receptor activation causes bronchoconstriction. Endothelial cell activation of ETB receptors may lead to vasodilation by increasing nitric oxide (NO) production. ETA receptor activity primarily mediates the mitogenic and inflammatory modulator functions of ET-1. Binding of ET-1 to its receptor results in coupling of cell-specific G proteins that activate or inhibit multiple intracellular signaling events. The varied effects of ET-1 receptor activation depend on the G protein and signal

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00206-2
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transduction pathways active in the cell of interest. A growing number of receptor antagonists exist with variable selectivity for one or both receptor subtypes.[22]

ET-1 is primarily transcriptionally regulated by stimuli including shear stress, hypoxia, cytokines lipopolysaccharides, and multiple growth factors (TGFβ, PDGF, EGF, etc) that induce transcription of ET-1 mRNA and secretion of protein. ET-1 itself may also increase ET-1 expression through autocrine activity. Additionally, some stimuli may enhance preproET-1 mRNA stability, leading to increased and sustained ET-1 expression. The number of ETA and B receptors is also cell specific and regulated by a variety of growth factors. The complex regulation of ET-1 expression, cell-specific receptor expression, and receptor-mediated cell signaling contribute to lung disease in a variety of ways.[11]

AIRWAY DISEASES

In the airway, ET-1 is localized primarily to the bronchial smooth muscle with low expression in the epithelium. However, multiple cellular subsets of the epithelium secrete ET-1 including mucous cells, serous cells, and Clara cells with ET-1-binding sites are found on bronchial smooth muscle, alveolar septae, endothelial cells, and parasympathetic ganglia. ET-1 expression in the airways is in large part regulated by inflammatory mediators.

All three endothelins cause bronchoconstriction in intact airways, with ET-1 being the most potent, while denuded bronchi constrict equally to all three endothelins suggesting considerable modulation of ET-1 effects by the epithelium. The majority of ET-1 binding sites on bronchial smooth muscle are ETB receptors. ETA antagonists fail to inhibit bronchoconstriction, whereas ETB agonists augment this activity. Cultured airway epithelium secretes equal amounts of ET-1 and -3, which have equivalent affinity for the ETB receptor, and thus, bronchoconstriction could be mediated by both endothelins.[10]

Inhibition of cyclooxygenase or blockade of histamine or leukotriene receptors has no effect on bronchoconstriction suggesting that, despite the ET-1-stimulated release of these bronchoconstrictors, ET-1-mediated bronchoconstriction is a direct effect of activation of the ET receptors (ETB most importantly). ETA-mediated bronchoconstriction may also be important after ETB receptor desensitization or denudation of the airway epithelium, as may occur during airway inflammation and the late, sustained airway response to inhaled antigens. Interestingly, heterozygous ET-1 knockout mice, with a 50% reduction in ET-1 peptide, have airway hyperresponsiveness but not remodeling, suggesting that the decrease in ET-1 modulates bronchoconstriction activity by a functional mechanism, possibly by decreasing basal NO production.[10]

Asthma is characterized by bronchoconstriction and hyperreactivity with influx of inflammatory cells, mucous production, edema, and airway thickening and ET-1 may have important roles in each of these processes. Although ET-1 causes immediate bronchoconstriction, it also increases bronchial reactivity to inhaled antigens and influx of inflammatory cells, increased cytokine production, airway edema, and airway remodeling. Inhibition of ETA receptors or combined ETA and B receptor blockade leads to decreased airway inflammation in antigen-challenged animals, suggesting that the proinflammatory effects of ET-1 in the airway are mediated by ETA receptors.[11] Inflammation-induced ET-1 secretion may also act in an autocrine or paracrine fashion (or as a hormone released from peripheral mononuclear cells), via the ETA receptor, to increase transepithelial potential difference and ciliary beat frequency, and to exert mitogenic effects on airway epithelium and smooth muscle cells contributing to airway remodeling.[24,10]

ET-1 immunostaining and in situ hybridization for ET-1 in asthmatic airways is increased and correlates with asthma symptoms. In asthmatic patients, there is also a relative increase in ETB vs. ETA receptor expression in the airway, which may contribute to increased bronchoconstriction. Children with asthma have increased circulating levels of ET-1, whereas adults have normal levels between attacks but during acute exacerbations, elevated serum ET-1 correlate inversely with airflow measurements and decrease with treatment. Similarly, bronchoalveolar lavage (BAL) ET-1 in asthmatics is increased to concentrations that may cause bronchoconstriction and inversely correlates with FEV1.[25] Importantly, treatment of asthma with steroids and/or beta-adrenergic agonists decreases BAL ET-1.[10] Polymorphisms in the ET-1 and ETB receptor genes have been associated with asthma.[24,32,35]

Smoking is associated with increased ET-1 concentrations, but chronic obstructive pulmonary disease (COPD), in the absence of hypoxemia or pulmonary hypertension, is not associated with increased circulating ET-1. Also, smokers have impaired ET-1-mediated vasodilation, which correlates with bronchial hyperresponsiveness and may contribute to pulmonary hypertension (and the systemic vascular effects of smoking).[10] In cystic fibrosis (CF), sputum ET-1 levels are increased and may promote neutrophil activation and chemotaxis. *Pseduomonas* infection in non-CF-related bronchiectasis is associated with increased ET-1.[34]

ET-1 has also been implicated in the pathogenesis of *bronchiolitis obliterans* (BO) characterized by dysregulated repair and formation of proliferative, collagen-rich tissue obliterating the normal small airway anatomy. The proinflammatory and mitogenic properties of ET-1 in the airways has led to speculation that ET-1 may be involved in the formation of the lesion. *In vivo* gene transfer of ET-1 to the airway epithelium using the hemagglutinating virus of Japan (HVJ) in rats results in pathologic changes in the distal airways identical to those seen in human BO specimens.

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These changes do not result from nonspecific effects of the HVJ itself but can be attributed to the presence of the ET-1 gene, which is localized to the airway epithelium, hyperplastic lesions, and alveolar cells.[31]

PULMONARY VASCULAR DISEASE

Pulmonary arterial hypertension (PAH) is a fatal disease characterized by vasoconstriction, pulmonary vascular remodeling, and right heart failure. Human PAH is characterized by progressive increases in pulmonary vascular resistance and pulmonary vascular remodeling including formation of the complex plexiform lesion. PAH is classified as idiopathic, heritable, or associated with a variety of other conditions such as connective tissue diseases, human immunodeficiency virus, portal hypertension, hemolytic anemias, schistosomiasis, and congenital heart disease. Pulmonary hypertension (PH) can result from cardiac disease, chronic lung disease, recurrent pulmonary thromboemboli, and a variety of miscellaneous conditions. PH is distinguished from PAH by the arteriopathy and the clinical course. In humans, circulating ET-1 is increased in PAH or PH related to other cardiopulmonary disease. Concentrations are highest in patients with idiopathic PAH. Because the lung is the major source for clearance of ET-1 from the circulation, increased arteriovenous ratios as seen in PAH suggest either decreased clearance or increased production in the lung. ET-1 is also increased in the small resistance arteries and the plexiform lesions in lung tissue of patients with PAH.[10]

ET-1 has been implicated as a mediator in many of the pulmonary vascular changes seen in PAH. In the pulmonary circulation, ET-1 is found in endothelial cells and to a lesser extent the vascular smooth muscle cells. The endothelium secretes ET-1 primarily to the basolateral surface of the cell. ET-1 secretion may be increased by a variety of stimuli including cytokines, catacholamines, and physical forces such as shear stress, and decreased by NO, prostaglandins, and oxidant stress.

The distribution of receptors has important implications in understanding ET-1 regulation of vascular tone as activation of ET-1 receptors leads to both vasodilation and vasoconstriction depending on cell type and receptor. In the whole lung, ETA receptors are the most abundant and are localized to the medial layer of the arteries, decreasing in intensity in the peripheral circulation. ETB receptors are also found in the media of the pulmonary vessels, increasing in intensity in the distal circulation.[29]

In the pulmonary circulation, ET-1 can act as both a vasodilator and vasoconstrictor. The overall effect of ET-1 on vascular tone depends on both the dose and the preexisting tone in the lung. Low dose ET-1 administration during acute hypoxic vasoconstriction will result in transient pulmonary vasodilation while higher or repetitive doses cause vasoconstriction after an initial, brief vasodilation. The vasodilating activities of ET-1 are the result of ETB-mediated nitric oxide production in endothelial cells. Both ETA and ETB receptors acting on vascular smooth muscle mediate ET-1-induced vasoconstriction. In the normal lung, ET-1 causes vasoconstriction by the activation of ETA receptors in the large, conducting vessels of the lung. In the smaller, resistance vessels of the lung, ETB receptors in the media predominate and are responsible for the ET-1-induced vasoconstriction, especially when tone is increased.[27]

The importance of ET-1 in the pathogenesis of PH has also been studied in several models of PH. In the chronic hypoxic PH models, plasma and lung tissue ET-1 is increased after exposure to hypoxia.[29] Treatment with either ETA or ETA and B combined receptor antagonists attenuates the development of hypoxic PH by decreasing vasoconstriction and vascular remodeling.[6] ET-1 is increased in fawn-hooded rats that develop severe PH when raised under conditions of mild hypoxia and in monocrotaline-treated rats that develop PH.[13,30] In both these forms of pulmonary hypertension, the increase in ET may be contributing to increases in vascular tone and to vascular remodeling. Rats that are deficient in the ETB receptor have increased susceptibility to hypoxic PH.[19] Interestingly, transgenic mice overexpressing the human preproET-1 gene, with modestly increased lung ET-1 levels (35–50%), do not develop pulmonary hypertension under normoxic conditions or an exaggerated response to chronic hypoxia.[18]

Several ET-1 receptor antagonists have demonstrated clinical benefit in patients with pulmonary arterial hypertension. The mixed ETA and B receptor antagonist, bosentan, was the first receptor antagonist to demonstrate improvement in patient exercise tolerance, leading to approval of this drug for the treatment of pulmonary arterial hypertension in many parts of the world. ETA selective antagonists, sitaxsentan and ambrisentan, have also shown clinical effectiveness. Ambrisentan is approved for clinical use in many parts of the world. Sitaxsentan, which was approved in many countries (excluding the United States), was withdrawn from the market owing to hepatotoxicity concerns. Long-term follow-up of patients treated with endothelin receptor antagonists suggests an overall improvement in survival compared with historical controls.

LUNG TRANSPLANTATION AND REJECTION

ET-1 has been implicated as an important factor in ischemia–reperfusion injury at the time of transplant and in acute and chronic rejection of the allograft.[16] ET-1 concentration is after perfusion of the donor allograft in the recipient, increasing 3-fold within minutes and remains high for 12 hours after transplantation, declining to near normal levels within 24 hours. Circulating ET-1 correlates with increased

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pulmonary vascular resistance occurring about 6 hours after transplantation, suggesting that ET-1 may be responsible for the vasoconstriction. This activity may also be associated with the ischemia–reperfusion injury that is the leading cause of early postoperative graft failure and death. BAL ET-1 in lung allografts is also increased several fold and remains elevated up to 2 years posttransplant. Importantly, increased BAL ET-1 is found in the transplanted lung as compared with the native lung in patients receiving single lung transplants. However, ET-1 in BAL fluid does not correlate with episodes of infection or rejection. The source of ET-1 in lung allografts is uncertain with several reports suggesting that it is derived from alveolar inflammatory cells and/or from *de novo* expression in the airway epithelium.[10]

Rejection of the allograft is an important clinical problem. Acute rejection is manifested by diffuse infiltrates, hypoxia, airflow limitation and may lead to respiratory insufficiency and death. In some species, BAL ET-1 levels correlate with acute rejection, whereas in humans, acute episodes of rejection are not associated with further increases in BAL ET-1. Chronic, long-term rejection of allografts, manifested as bronchiolitis obliterans, is the major cause of morbidity and mortality in long-term lung transplant survivors. The etiology of BO is unclear but may be related to repeated episodes of acute rejection, chronic low-grade rejection, or organizing pneumonia. As discussed earlier, an increase in allograft ET-1 may contribute to BO. Importantly, in single lung transplant recipients, BAL ET-1 is increased in the transplanted lung but not the native lung.[10]

PULMONARY MALIGNANCIES

ET-1 may play a role in the development of lung cancer and metastasis to the lung. ET-1 has been implicated in a wide variety of human cancers including prostatic, colorectal, breast, lung, bladder, etc. The importance of ET-1 on tumor growth and survival may lie in its effects on cell survival, mitogenic effects, angiogenesis, and metastatic potentiation. ET-1 stimulates cell proliferation in several tumor cells including prostate, ovarian, and cervical. This proliferation can be inhibited by blockade of the ETA but not ETB receptors suggesting an autocrine function of ET-1 in promoting tumor growth. Several signaling pathways have been implicated most notably the activation of epidermal growth factor receptors (EGFRs). Inhibition of ETA decreased EGFR activation, whereas inhibition of EGFR with gefitinib decreased ET-1 activation of downstream targets. ET-1 has also been implicated in promoting cell survival through the activation of the ETA receptor. ETA activation increased antiapoptotic pathways such as AKT and indeed may contribute to chemotherapeutic resistance observed in several cancer cell lines. These observations suggest the potential of inhibitors of ET-1 receptors, notably the ETA receptor, as a therapeutic tool in the treatment of cancer.[4]

Tumor angiogenesis is necessary for continued growth of the tumor beyond the limits of oxygen diffusion. The growth of vessels into the tumor is also important to metastatic potential of the tumor. ET-1 may play an important role in angiogenesis and tumor growth and survival through induction of VEGF expression and sprouting of new vessels into the tumor and surrounding tissue. This effect is further enhanced by hypoxia (as may be seen in tumors that are expanding rapidly) by ET-1 potentiation of HIF1alpha potentiation of VEGF expression. Inhibition of the ETA receptor inhibits the upregulation of VEGF. Because metastasis is dependent on neovascularization, ET-1 may also be an important mediator of this phenomenon. Tumor invasion is also frequently accompanied by epithelial-to-mesenchymal transition (EMT). ET-1 has been implicated in EMT possibly through downregulation of E-cadherin and beta-catenin as well as upregulation of metastasis-related proteinases (MMPs).[4,7,8]

ET-1 is increased in lung tumors compared to surrounding normal lung tissue. Immunoreactivity was found in pulmonary adenocarcinomas and squamous cell tumors and much less in small cell and carcinoid tumors. In situ hybridization also shows a similar pattern of ET-1 mRNA expression in nonneuroendocrine tumors.[14] ETA receptors are increased in small cell tumors, adenocarcinomas, and large cell tumors, while ETB receptors are expressed primarily in adenocarcinomas and small cell tumors although to a level below normal surrounding lung. ET-1 and ETA receptors are notably found in the cells that comprise the tumor vasculature as well as in the cancer cells derived from epithelium.[4,10] Additionally, ECE, which converts big ET-1 to ET-1, the committed step in ET-1 biosynthesis, is also found in human lung tumors but not in adjacent normal lung. ET-1 expression in lung tumors is also correlated with VEGF levels and a poorer prognosis in non-small-cell lung tumors. Exhaled ET-1 levels may also serve as a biomarker of non-small-cell cancer and may be an early noninvasive test for lung cancer.[4]

Inhibition of ET-1 in cancer is a promising therapeutic approach. Inhibition of ET-1 production through inhibition of ECE is one approach. Targeted, selective inhibition of ETA vs. ETB receptors is another. ETA selective receptor inhibitors are of particular interest despite the clinical trial of zibotentan in prostate cancer that was terminated for lack of efficacy (www.clinicaltrials.gov).

ACUTE RESPIRATORY DISTRESS SYNDROME (ARDS)

Lung injury results in increased circulating ET-1, BAL ET-1, and lung tissue ET-1. In humans, ET-1 levels are increased in sepsis, burns, disseminated intravascular coagulation, acute lung injury, and ARDS and correlate with a poorer

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outcome with multiple organ failure, increased pulmonary arterial pressure, increased airway pressure and decreased PaO_2/FiO_2, while clinical improvement correlates with decreased in ET-1 levels. The arteriovenous ratio for ET-1 is increased in patients with ARDS but it is not clear if this results from increased secretion of ET-1 in the lungs or decreased clearance.[20] Additionally, in patients who succumb to ARDS, there is a marked increase in tissue ET-1 immunostaining in vascular endothelium, alveolar macrophages, smooth muscle, and airway epithelium, compared with lungs of patients who died without ARDS.[1]

ARDS is characterized by the presence of inflammatory cells in the lung. Since ET-1 may act as an immune modulator, an increase in ET-1 may contribute to lung injury by inducing expression of cytokines including TNF and IL-6 and 8. These cytokines may in turn stimulate the production of many inflammatory mediators leading to lung injury. Additionally, ET-1 activates neutrophils and increases neutrophil migration and trapping in the lung.

Another hallmark of ARDS is disruption and dysfunction of the pulmonary vascular endothelium leading to accumulation of lung water. Infusion of ET-1 raises pulmonary vascular pressure, but it is uncertain if ET-1 by itself increased pulmonary protein or fluid transport in the lung.[28] Impaired alveolar fluid clearance may result from ET-1 activation of the endothelial ETB receptor raising the potential therapeutic utility of mixed ETA and ETB receptor antagonists in patients with ARDS.

PULMONARY FIBROSIS

Pulmonary fibrosis is the final outcome for a variety of lung injuries. After injury to the alveolar wall, inflammatory cells are recruited, inflammatory mediators are released, and then decreased with resolution. Occasionally, the reparative phase becomes disordered resulting in progressive fibrosis.

ET-1 in the lung may be important in the initial events in lung injury by activating neutrophils to aggregate and release elastase and oxygen radicals, increasing neutrophil adherence, activating mast cells, and inducing cytokine production from monocytes. Among the many cytokines induced by ET-1 that are important in mediating pulmonary fibrosis are CCN2, TGFβ, and TNFα. ET-1 is also profibrotic by stimulating fibroblast replication, migration, contraction, and collagen synthesis and secretion while decreasing collagen degradation. Additionally, ET-1 enhances the conversion of fibroblasts into contractile myelofibroblasts. ET-1 also increases fibronectin production by bronchial epithelial cells. Lastly, ET-1 has mitogenic effects on vascular and airway smooth muscle. Thus, ET-1 may play an important role in the initial injury and eventual fibrotic reparative process of many inflammatory events in the lung.[10,12]

Several lines of evidence regarding the importance of ET-1 in human pulmonary fibrosis are available. Plasma and BAL ET-1 levels are increased in idiopathic pulmonary fibrosis. Additionally, lung biopsies from patients with idiopathic pulmonary fibrosis have increased ET-1 immunostaining in airway epithelial cells and type II pneumocytes, which correlates with disease activity. ECE is also increased. Scleroderma is commonly associated with pulmonary hypertension and pulmonary fibrosis. Plasma and BAL ET-1 is increased in these patients, but it is unclear if the presence of either pulmonary hypertension or pulmonary fibrosis increases these levels further. BAL fluid from patients with scleroderma increased the proliferation of cultured lung fibroblasts, which was inhibited by ETA receptor antagonist. This suggests that the ET-1 in the airspace may be contributing significantly to the fibrotic response. Additionally, an increase in ET-1 binding has been reported in lung tissue from patients with scleroderma associated with pulmonary fibrosis. Pulmonary inflammatory cells also seem to be primed for ET-1 production because cultured alveolar macrophages from patients with scleroderma and lung involvement secrete increased amounts of ET-1 in response to stimulation with LPS. Collectively, these observations suggest that augmented ET-1 release may contribute to and perpetuate the inflammatory process.[10,12]

Bleomycin-induced pulmonary fibrosis in animals is associated with increased ET-1 expression in alveolar macrophages and epithelium. The increase in ET-1 precedes the development of pulmonary fibrosis. The use of ET-1 receptor antagonists has had mixed results in limiting the development of bleomycin-induced fibrosis. Pulmonary fibrosis was reported in mice that overexpress human ET-1.[18] These mice are known to develop progressive nephrosclerosis in the absence of systemic hypertension. The transgene is localized throughout the lung with the strongest expression in the bronchial wall. In the lung, the mice develop age-dependent accumulation of collagen and accumulation of CD4+ lymphocytes in the perivascular space. This observation suggests that an increase in lung ET-1 alone may play a causative role in the development of pulmonary fibrosis.

Several clinical trials of ET-1 receptor antagonists in patients with pulmonary fibrosis have yielded mixed results with some suggesting a decrease in disease progression with others failing to show a clinical effect.

CONCLUSIONS

Since its discovery, much evidence has accumulated regarding the biologic activity of ET-1 and its potential role in lung disease. The development of ET receptor antagonists has helped elucidate the role of ET-1 in lung disease and future studies will continue to clarify the role of endothelins in health and disease.

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REFERENCES

1. Albertine KH, Wana ZM, Michael JR. Expression of endothelial nitric oxide synthase, inducible nitric oxide synthase and endothelin-1 in lungs of subjects who died with ARDS. *Chest* 1999;**116**:1015–25.
2. Arai H, Hori S, Aramori I, Ohkubo H, Nakanishi S. Cloning and expression of a cDNA encoding an endothelin receptor. *Nature* 1990;**348**:730–2.
3. Arinami T, Ishikawa M, Inoue A, Yanagisawa M, Masaki T, Yoshida MC, et al. Chromosomal assignments of the human endothelin family genes: the endothelin-1 gene (EDN-1) to 6p23-p24, the endothelin-2 gene (EDN-2) to 1p34, and the endothelin-3 gene (EDN-3) to 20q13.2-q13.3. *Am J Human Genetics* 1991;**48**:990–6.
4. Bagnati A, Spinella F, Rosano L. The endothelin axis in cancer: the promise and challenges of molecularly targeted therapy. *Can J Physiol Pharmacol* 2008;**86**:473–84.
5. Battistini B, Dussault P. Biosynthesis, distribution and metabolism of endothelins in the pulmonary system. *Pulm Pharmacol Therap* 1998;**11**:79–88.
6. Bonvallet ST, Zamora MR, Hasunuma K, Sato K, Hanasato N, Anderson D, et al. BQ-123, and ETa-receptor antagonist, attenuates hypoxic pulmonary hypertension in rats. *Am J Physiol* 1994;**266**: H1327–31.
7. Cohen AJ, Yanagisawa M, Zamora M, Gilman L, Bunn P, Franklin W, et al. Evidence for an endothelin-1 autocrine loop in lung cancer. *Lung Cancer* 1997;**18**(Suppl. 574).
8. Dawas K, Loizidou M, Shankar A, Ali H, Taylor I. Angiogenesis in cancer: the role of endothelin-1. *Ann Royal Coll Surg Eng* 1999;**81**:306–10.
9. Dupuis J, Stewart DJ, Cernacek P, Gosselin G. Human pulmonary circulation is an important site for both clearance and production of endothelin-1. *Circulation* 1996;**94**:1578–84.
10. Fagan KA, McMurtry IF, Rodman DM. Role of endothelin-1 in lung disease. *Respir Res* 2001;**2**:90–101.
11. Finsnes F, Skjonsberg OH, Tonnessen T, Naess O, Lyberg T, Christensen G. Endothelin production and effects of endothelin antagonism during experimental airway inflammation. *Am J Resp Crit Care Med* 1997;**155**:1404–12.
12. Fonseca C, Abraham D, Renzoin EA. Endothelin in pulmonary fibrosis. *Am J Resp Cell Molec Biol* 2011;**44**:1–10.
13. Frasch HF, Marshall C, Marshall BE. Endothelin-1 is elevated in monocrotaline pulmonary hypertension. *Am J Physiol* 1999;**276**:L304–10.
14. Giaid A, Hamid QA, Springall DR, Yanagisawa M, Shinmi O, Sawamura T, et al. Detection of endothelin immunoreactivity and mRNA in pulmonary tumors. *J Pathol* 1990;**162**:15–22.
15. Gillespie MN, Owasoyo JO, McMurtry IF, O'Brien RF. Sustained coronary vasoconstriction provoked by a peptidergic substance released from endothelial cells in culture. *J Pharmacol Exp Ther* 1986;**236**:339–43.
16. Göttmann U, van der Woude FJ, Braun C. Endothelin receptor antagonists: a new therapeutic option for improving the outcome after solid organ transplantation? *Curr Vasc Pharmacol* 2003;**1**:281–99.
17. Hickey KA, Rubani GB, Paul RJ, Highsmith RF. Characterization of a coronary vasoconstrictor produced by cultured endothelial cells. *Am J Physiol* 1985;**248**:C550–6.
18. Hocher B, Schwarz A, Fagan KA, Thone-Reineke C, El-Hag K, Kusserow H, et al. Pulmonary fibrosis and chronic lung inflammation in ET-1 transgenic mice. *Am J Respir Cell Mol Biol* 2000;**23**:19–26.
19. Ivy DD, McMurtry IF, Colvin K, Imamura M, Oka M, Lee DS, et al. Development of occlusive neointimal lesions in distal pulmonary arteries of endothelin B receptor-deficient rats: a new model of severe pulmonary arterial hypertension. *Circulation* 2005;**111**:2988–96.
20. Langleben D, DeMarchie M, Laporta D, Spanier AH, Schlesinger RD, Stewart DJ. Endothelin-1 in acute lung injury and the adult respiratory distress syndrome. *Am Rev Respir Dis* 1993;**148**:1646–50.
21. Lippton HL, Hauth TA, Cohen GA, Hyman AL. Functional evidence for different endothelin receptors in the lung. *J Appl Physiol* 1993;**75**:38–48.
22. Michael JR, Markewitz BA. Endothelins and the lung. *Am J Respir Crit Care Med* 1996;**154**:555–81.
23. O'Brien RF, Robbing RJ, McMurtry IF. Endothelial cells in culture produce a vasoconstrictor substance. *J Cell Physiol* 1987;**132**:263–70.
24. Polikepahad S, Moore RM, Venugopal CS. Endothelins and airways–a short review. *Res Commun Mol Pathol Pharmacol* 2006;**119**:3–51.
25. Redington AE, Springall DR, Ghatei MA, Lau LCK, Bloom SR, Holgate ST, et al. Endothelin in bronchoalveolar lavage fluid and its relation to airflow obstruction in asthma. *Am J Respir Crit Care Med* 1995;**151**:1034–9.
26. Sakurai T, Yanagisawa M, Takuwa Y, Miyazaki H, Kimura S, Goto K, et al. Cloning of a cDNA encoding a non-isopeptide selective subtype of the endothelin receptor. *Nature* 1990;**348**:732–5.
27. Schmeck J, Gluth H, Mihaljevic N, Born M, Wendel-Wellner M, Krafft P. ET-1 induced vasoconstriction shifts from ETa- to ETB-receptor mediated reaction after preconstriction. *J Appl Physiol* 1999; **87**:2284–9.
28. Schmeck J, Heller A, Groschler A, Recker A, Neuhof H, Urbascheck R, et al. Impact of endothelin-1 in endotoxin-induced pulmonary vascular reactions. *Crit Care Med* 2000;**28**:2851–7.
29. Soma S, Takahashi H, Muramatsu M, Oka M, Fukudu Y. Localization and distribution of endothelin receptor subtypes in pulmonary vasculature of normal and hypoxia exposed rats. *Am J Respir Cell Mol Biol* 1999;**20**:620–30.
30. Stelzner TJ, O'Brien RF, Yanigisawa M, Sakurai T, Sato K, Webb S, et al. Increased lung endothelin-1 production in rats with idiopathic pulmonary hypertension. *Am J Physiol* 1992;**262**:L614–20.
31. Takeda S, Sawa Y, Minami M, Kaneda Y, Fujii Y, Shirakura R, et al. Experimental *bronchiolitis obliterans* induced by HVJ-liposome mediated endothelin-1 gene transfer. *Ann Thorac Surg* 1997;**63**:1562–7.
32. Taillé C, Guénégou A, Almolki A, Piperaud M, Leynaert B, Vuillaumier S, et al. ETB receptor polymorphism is associated with airway obstruction. *BMC Pulm Med* 2007;**7**:5.
33. Yanagisawa M, Kurihara H, Kimura S, Tomobe Y, Kobayashi M, Mitsui Y, et al. A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature* 1999;**332**:411–5.
34. Zheng L, Tipoe G, Lam W-K, Ho JCM, Shum I, Ooi GC, et al. Endothelin-1 in stable bronchiectasis. *Eur Resp J* 2000;**16**:146–9.
35. Zhu G, Carlsen K, Carlsen KH, Lenney W, Silverman M, Whyte MK, et al. Polymorphisms in the endothelin-1 (EDN1) are associated with asthma in two populations. *Genes Immun* 2008;**9**:23–9.

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Chapter 207

Kinin Receptors

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ABSTRACT

Kinin B1 and B2 receptors (B1R and B2R) are involved in lung inflammation and pulmonary diseases. An emerging role for kinin B1R is proposed in the onset and chronicity of cigarette smoke (CS)-induced lung inflammation and interstitial diseases. B1R can be an IL-1β downstream emphysematous mediator expressed on lung fibroblasts, neutrophils, and parenchymal epithelial cell types I and II. Oxidative stress is another factor operating through the transcriptional nuclear factor NF-kB pathway to enhance B1R expression induced by CS. Through a positive feedback loop, B1R may amplify and perpetuate oxidative stress by enhancing NADPH oxidase activity and thereby promoting chronic lung inflammation and diseases. The detrimental effects of B1R activation include several interactive mechanisms, notably neutrophil infiltration and activation, chemokine and cytokine expression, release of matrix metalloproteinases, and excessive extracellular matrix and collagen formation that lead to airway tissue remodeling and lung fibrosis. Hence, kinin receptor antagonists are expected to become therapeutic in lung inflammation and pulmonary diseases.

INTRODUCTION

Inflammation is a common pathological feature of chronic lung diseases such as lung fibrosis and chronic obstructive pulmonary disease (COPD). Lung fibrosis is characterized by overgrowth of active lung fibroblasts and excessive deposition of extracellular matrix (ECM) within the alveolar septa, which appear to be thicker than the normal ones. The pathological descriptions for COPD include bronchitis, narrowed small airways resulting from airway wall remodeling and corrupted alveoli, that is, emphysema. For both diseases, excessive inflammatory cells appear in the airway lumen and in the disorganized parenchyma. They are involved in a complex inflammatory cascade by releasing cytokines and matrix metalloproteinases,[6,30] which in turn contribute to chronic disease development.[3] Animals exposed to lung inflammation inducers, such as cadmium and cigarette smoke (CS), develop both lung fibrosis and emphysema, two diseases of opposite pathological outcomes.

Acute CS exposure increases the quantity of inflammatory cells and inflammatory mediators in bronchoalveolar lavage fluid (BALF) and pulmonary parenchyma.[31] The expression of inflammatory mediators is associated with ECM breakdown products,[7] which can become an endogenous source of inflammatory inducers leading to chronic inflammation.[21] Thus, CS exposure complicates the networks between inflammatory mediators, inflammatory cells, and parenchymal tissues, which can disturb the physiological role of inflammation in the process of tissue repair. Because of the involvement of such complicated networks, it has been difficult to highlight the inflammatory or pathological effects of CS on pulmonary parenchyma, where fibrosis and emphysema actually occur. We have developed and validated a model using rat lung slices, to study the parenchymal responses to acute CS exposure.[19] In this model, blood supply is absent and the effect of inflammatory cells can be reduced to minimum. We found that kinin B1 receptor (B1R) but not kinin B2 receptor (B2) can be induced in the parenchymal cells by CS exposure, which strongly suggests that B1R might be involved in the CS-associated pulmonary inflammation and interstitial diseases.

KININS

Kinins belong to a group of 9–11 amino acid peptides, including bradykinin (BK), kallidin (KD or Lys-BK), T-kinin (only in rat) and their active metabolites, des-Arg^9-kinins.[11] BK and KD are generated after the proteolytic cleavage of their respective precursors, high-molecular-weight kininogen (HMWK), and low-molecular-weight kininogen (LMWK), by plasma and tissues serine proteases named kallikreins. These peptides undergo rapid metabolic degradation by amino-, carboxy-, and endopeptidases found in tissues and biological fluids.[18] The most physiologically relevant enzymes are kininase I (carboxypeptidases N and M), which remove arginine^9 from kinins to produce active metabolites, neutral endopeptidase 24.11 (enkephalinase), which cleaves the C-terminal dipeptide (Phe–Arg) and pentapeptide (Phe–Ser–Pro–Phe–Arg) from BK, and kininase II (also named angiotensin-I-converting

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00207-4
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enzyme or ACE), which acts as dipeptidyl carboxypeptidase to remove the C-terminal dipeptide, Phe–Arg. Further, ACE cleaves the C-terminal dipeptide Ser–Pro of BK (1–7) to produce BK (1–5), which is the final metabolite of BK and des-Arg^9-BK. T-kinin and KD are also subjected to transformation into BK by aminopeptidases M and P (Figure 1). (See the Peptide Biosynthesis/Processing section of the book.)

KININ RECEPTORS

Kinin receptors denoted as B1 (B1R) and B2 (B2R) have been cloned in several species, belong to the superfamily of G-protein-coupled receptors (GPCR), and preferentially signal through the Gαq/11 and Gαi subunit.[18] The preferential agonists of B2R are BK and Lys-BK (KD), while their metabolites, des-Arg^9-BK and Lys-des-Arg^9-BK, are the preferred endogenous ligands for B1R. Pharmacological sensitivities of kinin receptors vary among species. For instance, human and rabbit B1R show a higher sensitivity to ligands with N-terminal Lysine residue (i.e., Lys-des-Arg^9-BK) contrary to the mouse/rat receptor (i.e., des-Arg^9-BK). Kinin receptors are primarily linked to phospholipase C, phosphoinositide and mitogen-activated protein kinase (MAPK) pathways.[18] B2R can also activate protein tyrosine kinases, phosphatases, phospholipase A_2, and phospholipase D in some cellular systems.[10,18]

KININ RECEPTORS EXPRESSION

The B2R is widely and constitutively expressed in the whole body and mediates most of the physiological responses to kinins. In contrast, the B1R is weakly expressed in healthy tissues,[10] and its expression is enhanced after exposure to proinflammatory cytokines (IL-1β, TNF-α), bacterial endotoxins, and oxidative stress.[10] B1R expression is regulated by MAPK and the transcriptional nuclear factor κappa B (NF-κB).[18,25] A transcriptional regulatory site for NF-κB is found in the promoter region of human and rat B1R gene and specific NF-kB blockers prevent B1R upregulation *in vitro* and *in vivo*.[18]

As a general rule, the constitutive kinin B2R has been associated with acute inflammation, while the inducible B1R seems to play a greater role in the chronic phase of inflammation.[11,27] Consistently, on agonist stimulation, the B2R shows much faster rates of internalization and desensitization than B1R.[18]

ROLE OF KININ RECEPTORS IN AIRWAYS

Thus far, it is known that the stimulation of B2R with BK in airways promotes acute inflammation, bronchoconstriction, cholinergic and sensory nerve stimulation, mucus secretion, cough, and microvascular leakage leading to edema.[16,17,19] B2R-induced bronchoconstriction is mediated by increased intracellular calcium influx, PKC and PLC activation, the

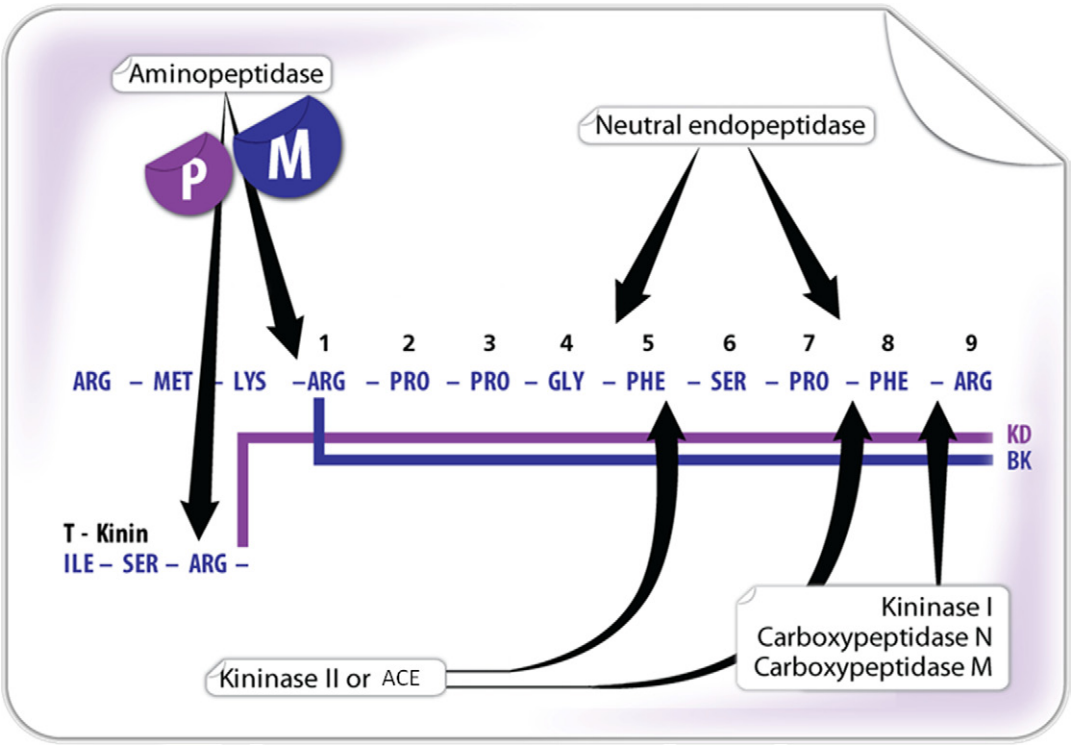

FIGURE 1 Kinins and their metabolites under the action of amino- endo- and carboxy-peptidases.

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release of prostanoids, and activation of sensory C-fibers in normal or inflammatory conditions. Kinins generated during the allergic response cause an activation of sensory nerves to release tachykinins in guinea-pig airways contributing to antigen-induced bronchoconstriction. NO release accounts for B2R-induced vasodilatation in airways microcirculation. Under noninflamed conditions, B1R agonists have no effect on airways in human and animal species both *in vitro* and *in vivo*.

CONTRIBUTION OF IMMUNE CELLS TO AIRWAY INFLAMMATION INDUCED BY KININ RECEPTORS

Peripheral immune cells including neutrophils, eosinophils, leukocytes, and mast cells exert a critical role in airway diseases and chronic lung inflammation.[3] The group of P. Sirois at Sherbrooke University has reported two salient findings: (1) untreated and ovalbumin-sensitized rat BALF cells express functional B2R, yet functional B1R is expressed only in BALF cells of ovalbumin-sensitized and challenged mice; (2) sephadex bead-induced guinea-pig lung inflammation increases BALF cells (neutrophil, eosinophil, and macrophage), an effect prevented by B1R and B2R antagonists. In 1996, the group of E. Whalley reported that B1R and B2R agonists cause human PMN elastase release favoring endothelial cell monolayer permeability. This suggests a role for kinin receptors in inflammation-induced lung permeability and edema. These findings suggest that both B1R and B2R may enhance airway leukocyte infiltration in inflammatory states.

KININ RECEPTORS IN LUNG FIBROSIS AND AIRWAY TISSUE REMODELING

Evidence suggests that BK may play a role in airway tissue remodeling and lung fibrosis. BK enhances alpha-smooth muscle actin expression, fibroblast proliferation, and collagen production through MAPK (p-ERK 1/2) in human lung fibroblasts.[32] Also, BK stimulates the release of the matrix metalloproteinase-2 via B2R and endogenous prostaglandins in guinea-pig tracheal smooth muscle cells. The stimulation of the constitutively expressed B1R in human embryonic lung fibroblasts increases mRNA levels of connective tissue growth factor (4 h) and alpha1 (1) collagen (20 h), suggesting the involvement of B1R in fibrogenesis. Interestingly, expression of B1R is found in the fibrous tissue of patients with pulmonary sarcoidosis, whereas it is nondetectable in normal human lungs. B1R is also found on the basement membranes of bronchopulmonary cells and the surrounding fibrous stroma in transbronchial biopsies taken from patients with interstitial lung disease associated with progressive systemic sclerosis. Moreover, B1R activation induces chemotaxis of human neutrophils and enhances the release of neutrophil metalloproteinase, which leads to lung extracellular matrix (ECM) destruction and accumulation.[6]

KININ RECEPTORS IN LUNG INFLAMMATION INDUCED BY CIGARETTE SMOKE EXPOSURE

Herein, we summarize the current knowledge regarding the expression and pathological function of kinin receptors in lung inflammation induced by CS exposure. Acute (24 h) exposure to cigarette smoke condensate (CSC) increases the expression of B1R (but not that of B2R)

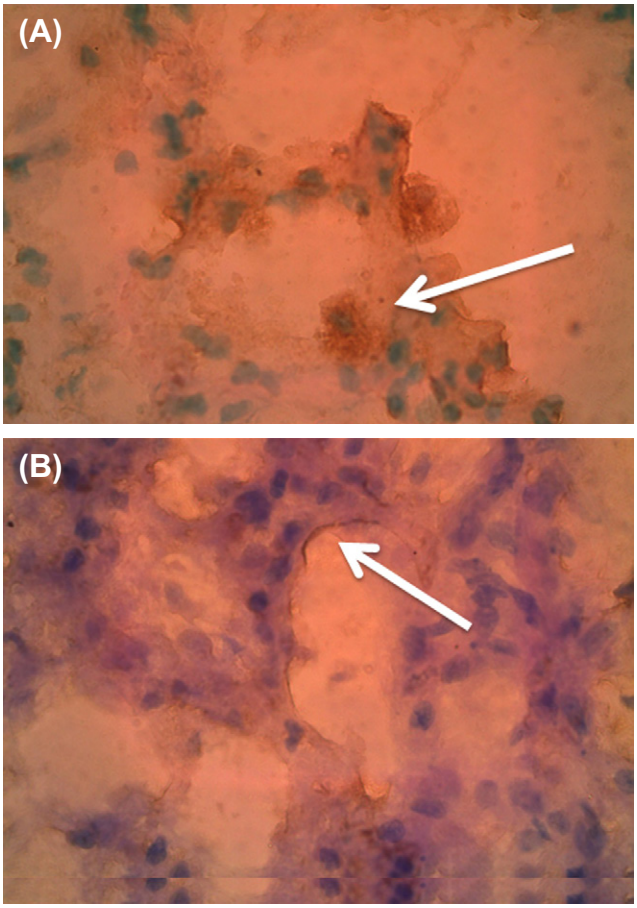

FIGURE 2 Cultured rat lung slices freshly isolated were exposed for 24 h to CSC (5 μg/ml), as described elsewhere.[19] After fixation in 4% paraformaldehyde for 30 min at room temperature, lung slices (20 μm thick coronal sections) were permeabilized in PBS containing 0.2% Triton X-100 (30 min) and exposed to rabbit anti-rat B_1R antibody (1:500; 120 min),[19] washed, incubated with biotin-conjugated antirabbit secondary antibody (1:300; 60 min), washed and incubated with horseradish peroxidase (HRP)-conjugated streptavidin antibody (1:300; 60 min). After final washing, sections were incubated with PBS 0.05% 3,3'-Diaminobenzidine (DAB) + 0.015% H_2O_2 (5 min) for revelation. Nucleus (blue) was counterstained by incubating slides in Mayer's hematoxilin solution (5 min). Slides were coverslipped using DPX mountant (Fluka) and examined under an inverted microscope. Positive B1R staining (brown) is seen on (A) type II pneumocytes (great alveolar cells), according to the large/cuboidal (arrowed) morphology of stained cells, and (B) type I pneumocytes (simple squamous alveolar cells), according to the thin/stretched (arrowed) morphology of stained cells. Magnification = 400×. Pictures are representative of a minimum three slices per rat from three different rats. See color plate 55.

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through a mechanism involving oxidative stress, IL-1R and the NF-κB pathway in cultured human alveolar epithelial cell line (A549)[28] and in *ex vivo* rat lung slices.[19] Moreover, rats exposed to inhaled CS for a period of 6 weeks show increased expression of kinin B1R in the trachea.[19] We have further identified that the particulate phase but not the vapor phase of CS is more responsible for B1R induction (see also Lin et al. (2012) *Toxicology*). Interestingly, nicotine enhances kinin-receptor-mediated tracheal contraction in mice through inducing the expression of B1R and B2R.[33] The stimulation of B1R, after its induction by CSC, enhances the production of superoxide anion through NADPH oxidase in human alveolar epithelial cells (A549).[28] Although the exact significance of this finding remains to be confirmed in preclinical studies, it is suggested that B1R can amplify and perpetuate the oxidative stress and promote inflammation and human lung diseases associated with CS.[28]

A more accurate localization of kinin B1R is mandatory to further determine its pathological role in human lung diseases associated with CS. *Ex vivo* rat lung slices represent a unique model to address this issue by immunohistochemistry, considering that lung slices maintain not only the 3-dimensional lung parenchyma structure but also cell–cell and cell–matrix interactions. This *ex vivo* model has been considered a standard tool for approaching lung inflammation and disease.[24] Using such a model, we found specific B1R immunostaining in the alveolar type I and type II pneumocytes of the rat lung slices treated with CSC (Figure 2). Using confocal microscopy and a selective B1R fluorescent agonist,[29] the presence of B1R in lung slices exposed to CSC has been confirmed on lung epithelial cells (Figure 3) and infiltrating neutrophils (Figure 4).

The expression of B1R on neutrophils and the absence of neutrophil migration to inflamed tissues in B1R knockout mice[2] suggest a role for B1R in infiltration of neutrophils in lung tissues after CS exposure. The expression of B1R by parenchymal epithelium after exposure to CS is also relevant as alveolar epithelial cells play a critical role in the generation of lung diseases.[1] CS is a major risk factor in the development of various lung diseases, including pulmonary emphysema and pulmonary fibrosis,[14] COPD, and lung cancer.[34] The mechanisms of these diseases include alterations in alveolar epithelial cells, which are essential in the maintenance of normal alveolar architecture and function. After exposure to CS, alterations in alveolar epithelial cells increase epithelial permeability, decrease surfactant production, and cause an inappropriate production of inflammatory cytokines and growth factors. Ultimately, CS causes the death of alveolar epithelial cells by oxidative stress.[1,31,34] CS contains and generates various ROS and reactive nitrogen species that damage alveolar epithelial cells. Apoptosis of alveolar epithelial and endothelial cells contributes to the pathogenesis of pulmonary emphysema.[12]

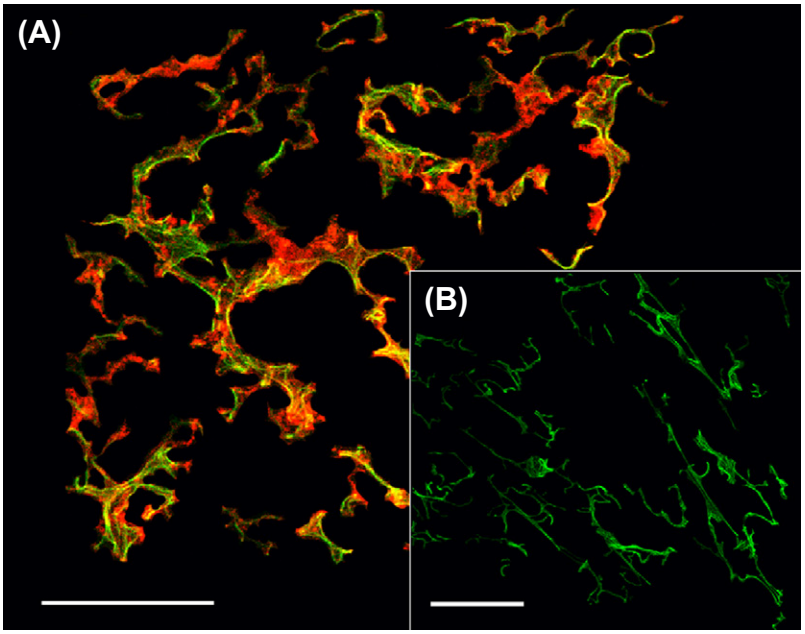

FIGURE 3 Rat lung slices treated with 5 μg/ml CSC (A) or vehicle (B) for 24 h were exposed to [N^{α}-Bodipy]-des-Arg^9-BK, a specific fluorescent ligand for B1R[29] and to Anti-EpCAM (1:1000), a specific antibody against epithelial cells (Abcam, Cambridge, MA; stock number: AB68892). The experimental protocol for confocal microscopy is described elsewhere.[29] The B1R fluorescent dye is red (ext: 550 nm/em: 570 nm) and Anti-EpCAM dye is green (ext: 505 nm/em: 515 nm). Colocalization between B1R and epithelial cells is shown in yellow. Scale bar = 100 μm, magnification 20×. Confocal pictures are representative of a minimum four slices per rat from three different rats. See color plate 56.

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The inducible nitric oxide synthase and peroxynitrite are closely related to CS-induced emphysema and pulmonary hypertension.[26] Knowing that the B1R is induced in lung alveolar epithelial cells by CSC and that B1R activation enhances oxidative stress, it is tempting to suggest that B1R may contribute to CS-induced alveolar dysfunction, remodeling, and cellular death.

To further define the downstream mechanism of B1R activation in lung inflammation, we performed a series of PCR arrays to assess changes in expression levels of 86 chemokines and cytokines genes in the presence of des-Arg^9-BK (10 μM, 30 min) in rat lung slices pre-exposed to CSC (5 μg/ml, 4 h). The mRNA expression ratio of selected genes was evaluated against the mean of five housekeeping genes (Rplp1, Hprt1, Rpl13a, Ldha, and β-actin). Des-Arg^9-BK increased mRNA levels of TNF-α (4.6-fold), IL-3 (35.4-fold), Cx3CL1 (31.3-fold), and IL-11 (14.5-fold), but decreased mRNA levels of CCL11 (−21.4-fold), CCL25 (−9.4-fold), Pf4 (−8.1-fold), CCL6 (−4.5-fold), Mif (−1.7-fold), IL-15 (−1.2-fold), IL-4 (−10.7-fold), and CCL22 (−10.2-fold) without affecting the expression of IL-2rg (0.4-fold) and that of the 74 others. Results of the qPCR array were confirmed with a standard qPCR on two target genes, which are among the mostly affected by CSC. Results reveal that the B1R agonist reduces by 76% IL-4 mRNA expression level, while it increases by 930% Cx3CL1 mRNA expression. Moreover, the B1R agonist enhances mRNA expression of its own receptor by 310%, as previously described in human lung fibroblasts[25] (Figure 5).

An important feature of pulmonary inflammation implies the adhesion and transmigration of polymorphonuclear (PMN) cells toward the lung alveolar epithelial cells. The contact is generally mediated by inflammatory mediators. NO and mast cell mediators serve to modulate PMN responses within the microcirculation and interstitium. In addition, many cytokines can act uniquely to alter epithelial adhesive interactions with PMN. Potent inhibitors of PMN-epithelial interactions include interferon-γ (IFN-γ) and IL-4,

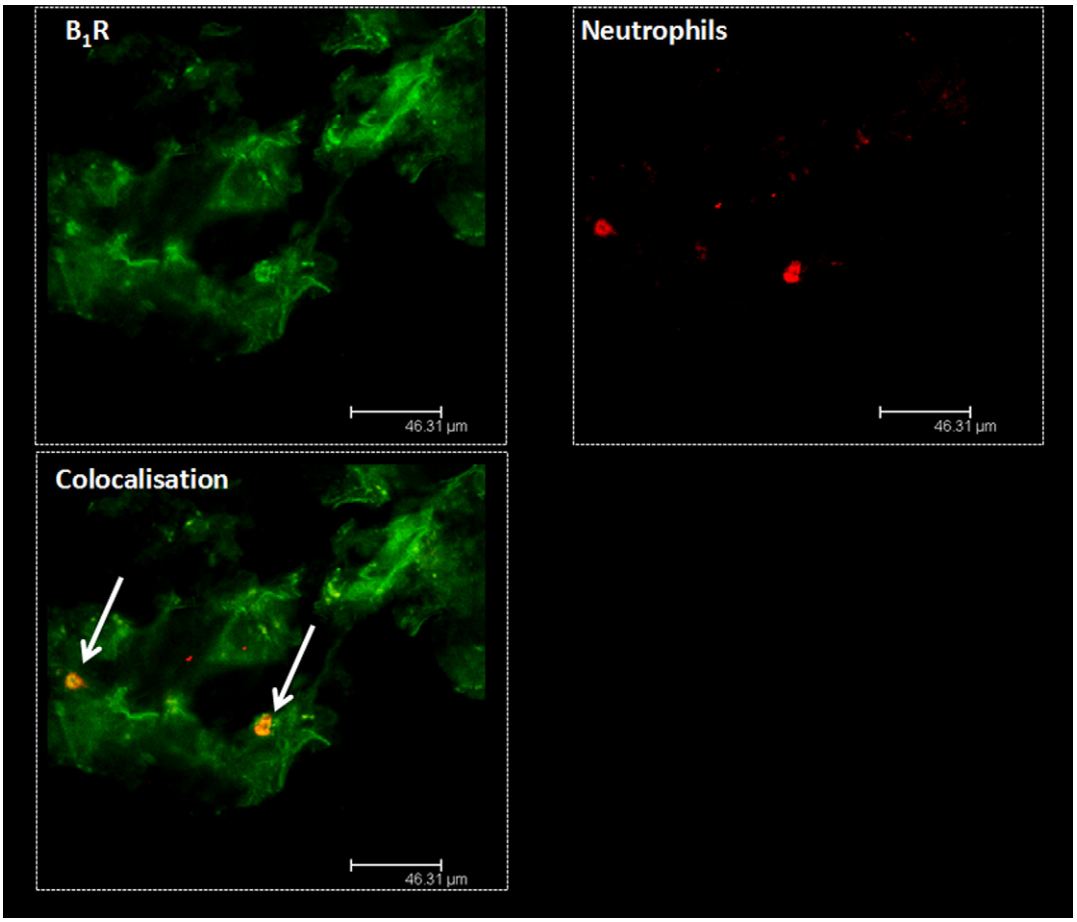

FIGURE 4 Rat lung slices treated with 5 μg/ml CSC for 24 h were exposed to [N$^{\alpha}$-Bodipy]-des-Arg^9-BK, a specific fluorescent ligand for B1R[29] and to Antineutrophil elastase, a specific antibody against neutrophil (1:1000) (Abcam, Cambridge, MA; stock number: AB21595). Colocalization between B1R and infiltrating neutrophils in yellow is indicated by two arrows in the bottom panel. The fluorescent ligand for B1R is green (ext: 505 nm/em: 515 nm) and the Antineutrophil elastase dye is red (ext: 550 nm/em: 570 nm). Scale bar = 46.31 μm. Confocal pictures are representative of a minimum four slices per rat from three different rats. See color plate 57.

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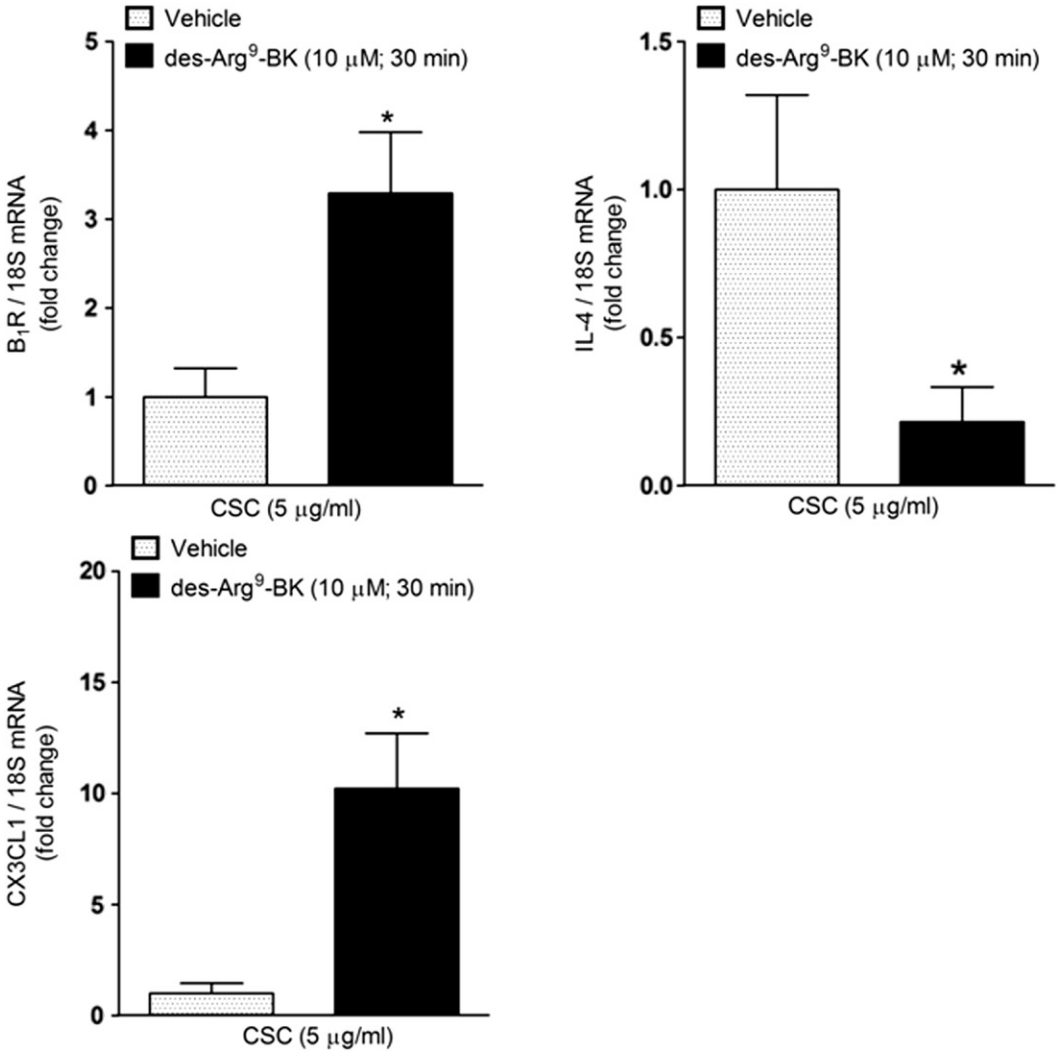

FIGURE 5 Rat lung slices were exposed to CSC (5 μg/ml, 4 h)[19] and then to des-Arg^9-BK (10 μM, 30 min) or its vehicle. The mRNA expression ratios between B1R, IL-4, CX3CL1, and 18S were evaluated using qRT-PCR as described elsewhere[19] and presented as the mean ± S.E.M from six slices/group of two different experiments. Significant comparison to vehicle is indicated by $*P<0.05$.

whereas IL-1, LPS, and TNF-α strongly modulate PMN endothelial interactions.[3] In both cases, modulation at least in part result from expression of new surface proteins. Generally, enhanced PMN adhesion/activation is followed by tissue destruction as observed in COPD and emphysema.[3]

However, the chemokine CX3CL1 (Fractalkine) is a membrane-bound adhesion molecule or a circulating glycoprotein acting through the CX3CR1 receptor. To be liberated in an active form, Fractalkine has to be cleaved by the tumor necrosis factor-[alpha]-converting enzyme to exhibit chemotactic activity for monocytes, natural killer cells and T cells.[5] CX3CR1−/− mice display impairment in the recruitment of immune cells to the inflamed lung.[15] The duo CX3CR1-CX3CL1 may play a role in the sustained recruitment of immune cells in the pathogenesis of CS-induced emphysema.[20] As described above, immune cells and particularly neutrophil migration/activation is an important feature of emphysema and COPD related to CS. Consequently, the enhanced expression of CX3CL1 and the reduction of cytokine expression (e.g., IL-4) by B1R agonist suggest that B1R may contribute to disease onset and disease chronicity in CS-induced lung inflammation.

CONCLUSIONS

1. Growing evidence gathered mainly in animal models suggest a strategic role for kinin receptors in pulmonary and airway inflammatory diseases. An emerging role for B1R is proposed in the onset and chronicity of CS-induced lung inflammatory diseases. IL-1β and TNF-α are pro-inflammatory cytokines involved in CS-induced lung

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inflammation.[4,7,13] Both are also critical mediators in the pathogenesis of CS-induced emphysema.[8,9] Our results suggest that B1R can be an IL-1β downstream emphysematous mediator expressed in the parenchyma. CS is a potent oxidative stress inducer,[1,23,31] and the oxidative stress is confirmed to be another factor for CSC-induced B1R expression in addition to IL-1β.[28]

2. CS-associated lung inflammatory diseases can be mediated by neutrophils through B1R activation. In addition to neutrophils, activating B1R expressed in type II pneumocyte and lung fibroblasts can stimulate the release of chemotactic cytokines and chemokines for neutrophil infiltration.[16,17] Our new findings also suggest that type I pneumocyte can express B1R and therefore be another cell type mediating neutrophil infiltration.
3. Activation of B1R in human epithelial cells (A549) enhances ROS production through NADPH oxidase.[28] Genetic deletion of NADPH oxidase enhances susceptibility to CS-induced lung inflammation and emphysema in mice.[35] However, sustained CS exposure may result in persistent inflammatory cells infiltration through B1R activation leading to ECM breakdown, which in turn can cause further chronic lung inflammation.[22] Even though the inflammatory and pathological influence of B1R activation in parenchymal epithelial cells has not yet been fully characterized, it is clear that B1R involves neutrophil infiltration and activation. We therefore propose B1R as a biomarker of CS-associated lung diseases.

ACKNOWLEDGMENTS

S. Talbot holds a PhD Studentship Award from the Canadian Institutes of Health Research (Frederick Banting and Charles Best Canada Graduate Scholarships-Doctoral Award).

REFERENCES

1. Aoshiba K, Nagai A. Oxidative stress, cell death, and other damage to alveolar epithelial cells induced by cigarette smoke. *Tob Induc Dis* 2003;**1**:219–26.
2. Araujo RC, Kettritz R, Fichtner I, Paiva AC, Pesquero JB, Bader M. Altered neutrophil homeostasis in kinin B1 receptor-deficient mice. *Biol Chem* 2001;**382**:91–5.
3. Barnes PJ. Cytokine-directed therapies for the treatment of chronic airway diseases. *Cytokine Growth Factor Rev* 2003;**14**:511–22.
4. Bhalla DK, Hirata F, Rishi AK, Gairola CG. Cigarette smoke, inflammation, and lung injury: a mechanistic perspective. *J Toxicol Environ Health B Crit Rev* 2009;**12**:45–64.
5. Bisset LR, Schmid-Grendelmeier P. Chemokines and their receptors in the pathogenesis of allergic asthma: progress and perspective. *Curr Opin Pulm Med* 2005;**11**:35–42.
6. Chua F, Laurent GJ. Neutrophil elastase: mediator of extracellular matrix destruction and accumulation. *Proc Am Thorac Soc* 2006;**3**:424–7.
7. Churg A, Dai J, Tai H, Xie C, Wright JL. Tumor necrosis factor-{alpha} is central to acute cigarette smoke-induced inflammation and connective tissue breakdown. *Am J Respir Crit Care Med* 2002;**166**:849–54.
8. Churg A, Wang RD, Tai H, Wang X, Xie C, Wright JL. Tumor necrosis factor-alpha drives 70% of cigarette smoke-induced emphysema in the mouse. *Am J Respir Crit Care Med* 2004;**170**:492–8.
9. Churg A, Zhou S, Wang X, Wang R, Wright JL. The role of interleukin-1{beta} in murine cigarette smoke-induced emphysema and small airway remodeling. *Am J Respir Cell Mol Biol* 2009;**40**:482–90.
10. Couture R, Girolami JP. Putative roles of kinin receptors in the therapeutic effects of angiotensin 1-converting enzyme inhibitors in diabetes mellitus. *Eur J Pharmacol* 2004;**500**:467–85.
11. Couture R, Harrisson M, Vianna RM, Cloutier F. Kinin receptors in pain and inflammation. *Eur J Pharmacol* 2001;**429**:161–76.
12. Domej W, Foldes-Papp Z, Flogel E, Haditsch B. Chronic obstructive pulmonary disease and oxidative stress. *Curr Pharm Biotechnol* 2006;**7**:117–23.
13. Doz E, Noulin N, Boichot E, Guenon I, Fick L, Le Bert M, et al. Cigarette smoke-induced pulmonary inflammation is TLR4/MyD88 and IL-1R1/MyD88 signaling dependent. *J Immunol* 2008;**180**:1169–78.
14. Galvin JR, Franks TJ. Smoking-related lung disease. *J Thorac Imaging* 2009;**24**:274–84.
15. Hall JD, Kurtz SL, Rigel NW, Gunn BM, Taft-Benz S, Morrison JP, et al. The impact of chemokine receptor CX3CR1 deficiency during respiratory infections with *Mycobacterium tuberculosis* or *Francisella tularensis*. *Clin Exp Immunol* 2009;**156**:278–84.
16. Koyama S, Sato E, Nomura H, Kubo K, Miura M, Yamashita T, et al. Bradykinin stimulates type II alveolar cells to release neutrophil and monocyte chemotactic activity and inflammatory cytokines. *Am J Pathol* 1998;**153**:1885–93.
17. Koyama S, Sato E, Numanami H, Kubo K, Nagai S, Izumi T. Bradykinin stimulates lung fibroblasts to release neutrophil and monocyte chemotactic activity. *Am J Respir Cell Mol Biol* 2000;**22**:75–84.
18. Leeb-Lundberg LM, Marceau F, Muller-Esterl W, Pettibone DJ, Zuraw BL. International union of pharmacology. XLV. Classification of the kinin receptor family: from molecular mechanisms to pathophysiological consequences. *Pharmacol Rev* 2005;**57**:27–77.
19. Lin JC, Talbot S, Lahjouji K, Roy JP, Senecal J, Couture R, et al. Mechanism of cigarette smoke-induced kinin B(1) receptor expression in rat airways. *Peptides* 2010;**31**:1940–5.
20. McComb JG, Ranganathan M, Liu XH, Pilewski JM, Ray P, Watkins SC, et al. CX3CL1 up-regulation is associated with recruitment of CX3CR1+ mononuclear phagocytes and T lymphocytes in the lungs during cigarette smoke-induced emphysema. *Am J Pathol* 2008;**173**:949–61.
21. Medzhitov R. Origin and physiological roles of inflammation. *Nature* 2008;**454**:428–35.
22. O'Reilly PJ, Gaggar A, Blalock JE. Interfering with extracellular matrix degradation to blunt inflammation. *Curr Opin Pharmacol* 2008;**8**:242–8.
23. Rangasamy T, Misra V, Zhen LJ, Tankersley CG, Tuder RM, Biswal S. Cigarette smoke-induced emphysema in A/J mice is associated with pulmonary oxidative stress, apoptosis of lung cells, and global alterations in gene expression. *Am J Physiol Lung Cell Mol Physiol* 2009;**296**:L888–900.

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24. Sanderson MJ. Exploring lung physiology in health and disease with lung slices. *Pulm Pharmacol Ther* 2011;**24**:452–65.
25. Schanstra JP, Bataille E, Marin Castano ME, Barascud Y, Hirtz C, Pesquero JB, et al. The B1-agonist [des-Arg10]-kallidin activates transcription factor NF-kappaB and induces homologous upregulation of the bradykinin B1-receptor in cultured human lung fibroblasts. *J Clin Invest* 1998;**101**:2080–91.
26. Seimetz M, Parajuli N, Pichl A, Veit F, Kwapiszewska G, Weisel Friederike C, et al. Inducible NOS inhibition reverses tobacco-smoke-induced emphysema and pulmonary hypertension in mice. *Cell* 2011;**147**:293–305.
27. Talbot S, Couture R. Emerging role of microglial kinin B1 receptor in diabetic pain neuropathy. *Exp Neurol* 2012;**234**:373–81.
28. Talbot S, Lin JC, Lahjouji K, Roy JP, Senecal J, Morin A, et al. Cigarette smoke-induced kinin B1 receptor promotes NADPH oxidase activity in cultured human alveolar epithelial cells. *Peptides* 2011;**32**:1447–56.
29. Talbot S, Theberge-Turmel P, Liazoghli D, Senecal J, Gaudreau P, Couture R. Cellular localization of kinin B1 receptor in the spinal cord of streptozotocin-diabetic rats with a fluorescent [Nalpha-Bodipy]-des-Arg9-bradykinin. *J Neuroinflammation* 2009;**6**:11.
30. Vaday GG, Franitza S, Schor H, Hecht I, Brill A, Cahalon L, et al. Combinatorial signals by inflammatory cytokines and chemokines mediate leukocyte interactions with extracellular matrix. *J Leukocyte Biol* 2001;**69**:885–92.
31. van der Vaart H, Postma DS, Timens W, Ten Hacken NHT. Acute effects of cigarette smoke on inflammation and oxidative stress: a review. *Thorax* 2004;**59**:713–21.
32. Vancheri C, Gili E, Failla M, Mastruzzo C, Salinaro ET, Lofurno D, et al. Bradykinin differentiates human lung fibroblasts to a myofibroblast phenotype via the B2 receptor. *J Allergy Clin Immunol* 2005;**116**:1242–8.
33. Xu Y, Zhang Y, Cardell LO. Nicotine enhances murine airway contractile responses to kinin receptor agonists via activation of JNK- and PDE4-related intracellular pathways. *Respir Res* 2010;**11**:13.
34. Yao H, Rahman I. Current concepts on the role of inflammation in COPD and lung cancer. *Curr Opin Pharmacol* 2009;**9**:375–83.
35. Yao HW, Edirisinghe I, Yang SR, Rajendrasozhan S, Kode A, Caito S, et al. Genetic ablation of NADPH oxidase enhances susceptibility to cigarette smoke-induced lung inflammation and emphysema in mice. *Am J Pathol* 2008;**172**:1222–37.

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Chapter 208

PACAP

Mario Delgado

ABSTRACT

In this chapter, I focus on the expression and functions of the pituitary adenylate cyclase-activating polypeptide (PACAP) and its receptors in different areas and structures of the respiratory system. Numerous studies indicate that PACAP is an endogenous regulator of the airway and lung functions, with predominant roles in bronchodilation, pulmonary vascular relaxation, airway inflammation, and mucus secretion. PACAP emerges as a promising candidate for the development of therapies for the treatment of human respiratory pathological conditions such as asthma, primary pulmonary hypertension, bronchitis, and emphysema.

DISCOVERY, STRUCTURE, AND SYNTHESIS OF PACAP

As its name indicates, the pituitary adenylate cyclase-activating polypeptide (PACAP) was originally isolated from the ovine hypothalamus by Dr. Arimura's group in 1989 based on its capacity to stimulate cAMP production in cultures of rat anterior pituitary cells.[19] After the initial isolation of the peptide, its sequencing revealed a C-terminally-amidated polypeptide of 38 amino acids, named PACAP38, which contained an internal cleave-amidation site GKR at position 28. One year later, the same group isolated from the same hypothalamus extracts another peptide able to stimulate adenylate cyclase activity in cells of adenohypophysis, which corresponded to the N-terminal 27 residues of PACAP38, which they called PACAP27.

Based on their sequence and structure, both forms of PACAP belong to a family of structurally related peptide hormones that include secretin, glucagon, growth hormone releasing factor, glucagon-like peptides-1 and -2, helodermin, gastric inhibitory peptide, and vasoactive intestinal peptide (VIP, which shows ~70% identity with PACAP27). The amino acid sequences of the various members of the PACAP family are depicted in Fig. 1A. It is assumed that this superfamily of peptides resulted from the duplication of a common ancestral gene, which then diverged extensively. The structure and sequence of PACAP27 have been highly conserved during the evolution. PACAP27 sequence (the biologically active region of PACAP) is identical in mammals, lizards, frogs, and fishes, and it only changes in one residue in chick and tunicates, suggesting that PACAP must play important physiological roles.

PACAP, like other members of this family, has a secondary structure characterized by long α-helical structure at the C terminus. PACAP27 is proposed to have an initial N terminus sequence of eight amino acid residues, probably with disordered structure, followed by two helical segments at residues 9–20 and 22–26, connected by a region of undefined structure that confers mobility to the molecule.[31] The conformation of PACAP38 mirrors that of PACAP27 and the C-terminal (28–38) extension exhibits a short helix connected by a flexible hinge to the 1–27 region.

PACAP27 and PACAP38 are synthesized from the same precursor molecule (preproPACAP), which also contains a PACAP-related peptide called PRP (Fig. 1B). PACAP38 is the predominant molecular form in the brain and almost all the peripheral tissues (for instance, PACAP27 represents 10% of total PACAP in the brain and 30% in the colon). The occurrence of different proportions of the two peptides in various tissues could be due to the existence of different processing enzymes of the PACAP precursor. The preferential expression of PACAP38 in most organs could be because of the higher metabolic stability of PACAP38 versus PACAP27. The discovery of PACAP and PRP sequences on the same gene suggests that both peptides are cosynthesized in the same tissues. However, PACAP and PRP are not always found in the same cell, suggesting that alternative processing of the nuclear precursor RNA or differential protein processing may occur, resulting in cells expressing either PACAP or PRP.

After its isolation, PACAP was rapidly identified in the central and peripheral nervous system and has since been recognized as a widely distributed neuropeptide [reviewed in Ref. 31]. PACAP was initially localized in hypothalamic neurons that project toward the median eminence and terminate in the capillary loops of the hypothalamo–pituitary

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00208-6
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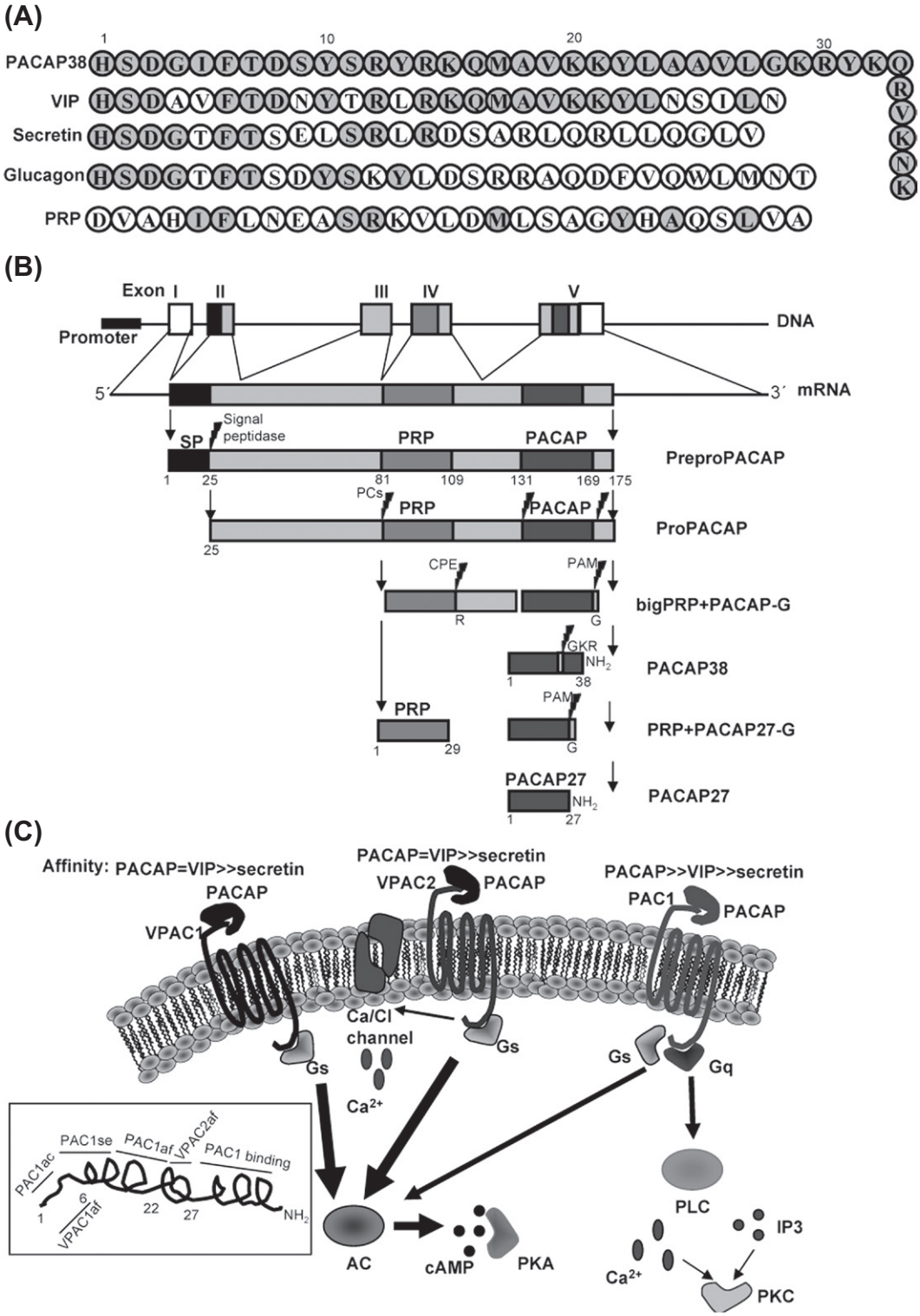

FIGURE 1 PACAP sequence, gene, and receptors. A. Sequence of PACAP and related peptides of the family. PACAP27 shows 68% identity with VIP. B. PACAP gene structure (DNA and RNA): the human PACAP gene, located on chromosomal region P11 of the chromosome 18, contains five exons, each encoding a distinct functional domain on the protein precursor or its mRNA. Investigation of the promoter activity has revealed that PACAP is constitutively expressed and that transcription of the gene can be enhanced by cAMP, phorbol diester, thyroid-specific transcription factor-1, glucocorticoids, progesterone, and PACAP itself. Both forms of PACAP are synthesized from a precursor molecule (preproPACAP), which also contains PACAP-related peptide (PRP). The 175-amino acid preproPACAP is metabolized by a signal peptidase in the endoplasmic reticulum to yield the 151-amino acid pro-PACAP. Pro-PACAP is cleaved by various prohormone convertases (PCs) to a glycine-extended PACAP-G ($PACAP_{125–155}$) and a large intermediate precursor of PRP

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portal system. Moreover, PACAP is substantially expressed by neurons in several extrahypothalamic brain areas, including cerebral cortex, thalamus, hippocampus, striatum, substancia nigra, nucleus accumbens, locus ceruleus, and pineal gland.[31] PACAP-containing fibers are also found in the periphery in many organs and tissues, including the heart, genital organs, eye, pancreas, adrenal gland, urinary and gastrointestinal tracts and lymphoid tissues (thymus, spleen, and duodenal mucosa), frequently associated with sensory and parasympathetic neurons.[31] Besides neurons, many endocrine, immune, and germ cells also produce PACAP under different physiological and pathological situations.[31]

Within the lung, PACAP-containing nerve fibers have been detected in the respiratory tract of different mammalian species, including man.[11,27,29,32] In the human upper-respiratory tract, PACAP immunoreactivity is found in nerve fibers in close association with blood vessels and glandular structures.[11] In other mammals, nerve fibers with PACAP are found close to small bronchioles and in bronchi beneath the epithelium, among bundles of smooth muscle cells of the bronchial wall, around blood vessels, penetrating the pulmonary arterial smooth muscle bundles, and in seromucous glands.[3,20,27,29,32] As is observed in other peripheral organs, PACAP immunoreactivity mainly colocalizes with VIP in cholinergic nerve fibers in the lung.[29]

PACAP RECEPTORS AND SIGNALING

PACAP27 and PACAP38 elicit their biological actions through binding to a subset of specific membrane receptors belonging to the large family B of G protein-coupled receptors (GPCRs) [reviewed in Ref. 31]. These receptors share a common molecular architecture, consisting of seven transmembrane-spanning domains, which are linked to one another by three extracellular (EC1, EC2, and EC3) and three intracellular (IC1, IC2, and IC3) loops, a long amino-terminal extracellular domain, and an intracellular carboxyl terminus. To date, three types of receptors that can interact with PACAP have been cloned (Fig. 1C). Due to the high homology that exists between PACAP and VIP, these receptors are shared by the two peptides. According to the International Union of Pharmacology nomenclature the VIP/PACAP receptors have been classified as follows: VPAC1 (also known as VIP1, VIP/PACAP type II, or PVR 2) and VPAC2 (also known as VIP2, VIP/PACAP type III, or PVR 3). Both receptors bind VIP and PACAP with equal affinity ($K_d \approx 1$ nM) and activate primarily the adenylate cyclase pathway. The PAC1 (also known as VIP/PACAP type I receptor, or PVR 1) shows a high affinity for PACAP-27 and PACAP-38 ($K_d \approx 0.5$ nM), but a much lower affinity for VIP ($K_d > 500$ nM). This PACAP-preferring receptor activates both adenylate cyclase and phospholipase C and exists in at least 8 different variants that result from alternative splicing of a single transcript and inclusion or exclusion of one or two cassettes, the hip and hop cassettes, which are involved in the differential coupling to second messengers.

VPAC1 is expressed in the brain (especially in the cerebral cortex and hippocampus) and in peripheral tissues such as liver, lung (see below), and intestine, and immune cells. VPAC2 has been localized in brain (especially the thalamus and suprachiasmic nucleus, and at lower levels in the hippocampus, brainstem, spinal cord, and dorsal root ganglia) and in a number of peripheral tissues, including the pancreas, skeletal muscle, lung, heart, kidney, adipose tissue, testis, stomach, and in smooth muscle of blood vessels and of the gastrointestinal and reproductive tracts. PAC1 has been detected predominantly in the brain (olfactory bulb, thalamus, hypothalamus, dentate gyrus of the hippocampus and cerebellum), the adrenal medulla, the anterior pituitary, testis and macrophages.[31]

In general, PACAP receptors in the respiratory system share the same molecular basis of ligand–receptor interaction as in other cells and tissues. Analysis of binding studies at different levels of the respiratory tract indicates the presence of a single class of type II binding sites that show the same affinity for VIP as for both forms of PACAP, and the absence of type I binding sites (selective for PACAP).[14,31] Type II binding sites are localized in acini and excretory ducts of submucosal glands of the trachea and in the lung.[9,14,28] Most of the studies of expression confirmed these initial findings. VPAC1 and VPAC2 receptors have been previously detected in rat and human lung by Northern blot, RT-PCR, Western blot and in situ hybridization

(big-$PRP_{81–110}$). Big-PRP is then cleaved by carboxypeptidase-E like enzymes (CPE) to PRP, and PACAP38G is metabolized and amidated in C-terminus by peptidyl glycine α-amidating monooxygenase (PAM) enzymes to PACAP38. Finally, the internal GRK motif in PACAP38 can be cleaved and amidated to generate PACAP27. C. Three receptors for PACAP, belonging to family B1 of the G protein–coupled receptors, have been described: $VPAC_1$ and $VPAC_2$, which show high affinity for both forms of PACAP and VIP, and PAC_1 that shows selective affinity for PACAP. The three receptors are coupled to Gαs proteins, increase the levels of cAMP through the activation of adenylate cyclase (AC), and activate the PKA pathway. In addition, PAC1 is coupled to the activation of phospholipase C (PLC), elevation of intracellular calcium and phosphoinositides (In3P), and subsequent activation of PKC. Finally, VPAC2 is also coupled to a membrane calcium/chloride channel. The inset represents the schematic secondary structure of PACAP38 with indications of the domains responsible for recognition (PAC1af), activation (PAC1ac), and selectivity (PAC1se) for the different PACAP receptors.

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histochemistry. VPAC1 mRNA is highly expressed in the epithelium of large bronchi, whereas VPAC2-R mRNA is present in small terminal bronchioles.[2,30] VPAC1 have been also located by immunohistochemistry in smooth muscle of pulmonary veins, airway epithelium from the trachea to the respiratory bronchioles of rat and human lungs.[2] Moreover, immunohistochemical and gene expression methods indicate that VPAC1 and VPAC2 are present in resident and infiltrating immune cells, including macrophages, lymphocytes and dendritic cells, in the lungs of rodents and humans, especially in inflammatory situations.[2,15] Although PAC1 has been detected in human and rat lungs at mRNA (by RT-PCR) and protein (by immunoblotting of lung membranes), the immunohistochemical methods have failed to identify PAC1 in the respiratory system and the autoradiographycal studies of PACAP27 binding indicate that the putative PACAP receptors were not the PAC1 receptor subtype.[30] This apparent contradiction can be understood in terms of a low density of the PAC1 in lung that makes it difficult to visualize immunostaining because of the relatively low sensitivity of the immunohistochemical method. Alternatively, the expression of PAC1 in the lung could be solely due to the PAC1 expressed by monocytes, macrophages and dendritic cells present in lung in normal and pathological situations.[15,31]

In summary, until the tissue localization of PAC1 is confirmed, VPAC1 and VPAC2 seem to be the major PACAP receptors expressed in the respiratory system. Because these receptors show similar affinities and selectivity for PACAP and VIP, one could assume that most of the effects described for VIP in the respiratory system (see the chapter written by Dr. S. Said in this book section) may be also attributed to PACAP.

BIOLOGICAL ACTIONS AND PATHOLOGICAL IMPLICATIONS

The widespread distribution of PACAP and its receptors is correlated with its involvement in a large variety of biological activities (see Ref. 31 and other chapters in this book for references), especially acting as a highly potent vasorelaxant factor in several tissues, including the gut, eye, brain, and testis. PACAP also acts as a neuromodulator in the brain and has been proposed to be associated with the "visceral forebrain system" that influences and temporarily overrides brainstem control of cardiovascular, respiratory, and gastrointestinal functions. Thus, PACAP increases cardiac output, induces bronchodilatation and smooth muscle relaxation, and regulates secretory processes (saliva and gastric acid) in the gastrointestinal tract and gastric motility. In the endocrine system, PACAP releases prolactin, luteinizing hormone, adrenocorticotrophin, somatostatin, and growth hormone from the pituitary and regulates the release of insulin and glucagon in the pancreas. It also acts on the exocrine pancreas to increase the bicarbonate output. PACAP also promotes analgesia, hyperthermia, learning, and behavior; has neurotrophic effects (modulating cell proliferation and migration and promoting cell survival and differentiation); and regulates bone metabolism, circadian rhythms, embryonic development, and the inflammatory and immune responses.

Regarding the respiratory system, the pattern of expression of PACAP within lungs and respiratory airways suggests a clear role for this peptide in secretory activities and in the regulation of smooth muscle tone of bronchi and pulmonary blood vessels. Thus, different studies have demonstrated that PACAP acts as a potent bronchodilator, a relaxant of tracheal smooth muscle and of pulmonary arteries, a stimulator of airway mucus secretion, and a modulator of the inflammatory response in airways (Fig. 2).

PACAP as a Bronchodilator and a Tracheal Relaxant

Strong evidence in various species indicates that PACAP causes relaxation of tracheal smooth muscle,[1,4,10,35] promotes bronchodilation,[16] and increases nasal airway resistance.[12] Depending on the experimental conditions, different nonexcluding mechanisms have been proposed for the relaxant activities of PACAP on airway smooth muscle, involving direct and indirect actions of smooth muscle cells. Most of the studies implicate the activation of the cAMP/PKA pathway[1,10] and the cAMP-mediated activation of calcium-dependent potassium channels. Others have demonstrated the involvement of nitric oxide/cGMP[26] and carbon monoxide as indirect mediators in the relaxant activities of PACAP.[13] However, another study showed in tracheal strips that most of the effect of PACAP depends on the presence of the airway epithelium and is independent of nitric oxide production. Shigyo and collaborators[27] demonstrated that suppression of smooth muscle contraction by PACAP occurs at a prejunctional level, by decreasing the release of acetylcholine from vagal nerve termini innervating the smooth muscle bundles of the trachea and by blocking the excitatory nonadrenergic noncholinergic component of the vagal nerve in the main bronchi. The bronchodilatatory effect of PACAP was confirmed *in vivo* in a model of histamine-induced pulmonary resistance in anesthetized guinea pigs.[25] Interestingly, the bronchodilatory effect of inhaled PACAP38 lasted much longer than that of VIP or PACAP27,[25] presumably because of a greater resistance to endopeptidase-mediated degradation of PACAP38 versus VIP or PACAP27, a hypothesis that was sustained by *in vitro* studies with precontracted bronchial smooth muscle segments from humans or precontracted tracheal rings from guinea pigs. In fact, more stable PACAP27 analogs, generated by substitutions of various amino acids of naive peptide, showed long-lasting dilatory effects on

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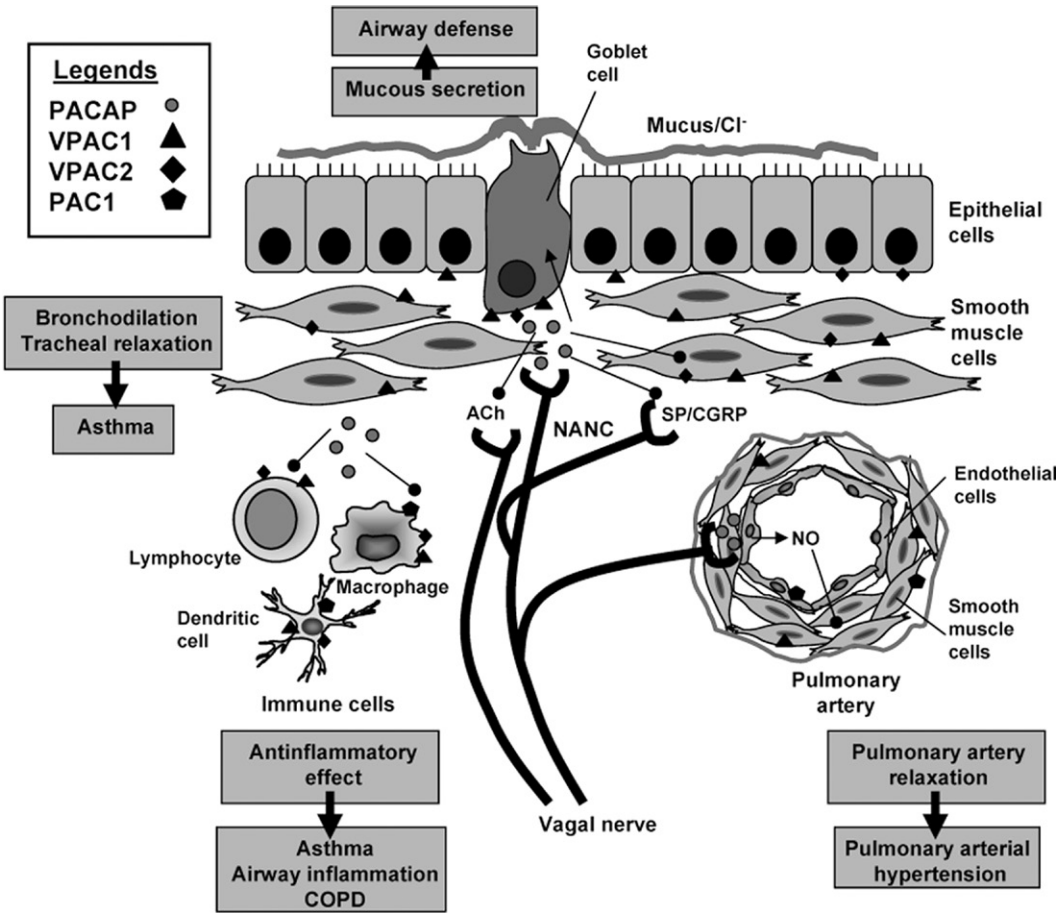

FIGURE 2 Functions and expression of PACAP and its receptors in the respiratory system. ACh, acetylcholine; COPD, chronic obstructive pulmonary disease; NANC, nonadrenergic noncholinergic terminal nerves; NO, nitric oxide.

human bronchi.[35] Based in their bronchorelaxant properties, various works have described the beneficial effects of VIP and stable VPAC1/VPAC2 agonists in models of asthma and chronic obstructive pulmonary disease (see the chapter written by Dr. Said in this book section). Although PACAP could exert similar actions to VIP in these conditions, through VPAC1 and VPAC2, the effect of PACAP through its selective receptor PAC1 is not clear. Although mice deficient for PACAP and PAC1 show higher bronchial hyperreactivity in various models of chronic asthma, this phenotype seems to be mainly dependent on the antinflammatory action of the PACAP–PAC1 signaling on immune cells (see below).

PACAP as a Relaxant on Pulmonary Artery

Similar to its effect on other tissues, PACAP has shown potent relaxant activities on pulmonary arteries from human, primates and guinea pigs *in vitro* and in anesthetized cats *in vivo* (reviewed in Ref. 16). This effect seems to be mediated through the induction of nitric oxide in the endothelial cells, because PACAP failed to induce vasorelaxation in the presence of inhibitors of endothelial nitric oxide synthase or in pulmonary arteries denudated of endothelium. This is an interesting finding because PACAP acts as vasodilator in an endothelial-independent manner in other arteries (i.e., aorta or coronary arteries) (reviewed by Ref. 31), and VIP induced vasodilatation in pulmonary artery segments acting on smooth muscle cells independently of the presence of intact endothelium. Although it needs further investigation, these findings hypothesize that PACAP and VIP act in pulmonary vasorelaxation through different receptors localized in different cells/localization. VIP has been proven to be efficient in the treatment of primary pulmonary hypertension in humans.[24] Moreover, patients suffering from pulmonary hypertension exhibit reduced levels of VIP in serum and lung and increased expression of VPAC1 and VPAC2. Similarly, in piglets, overcirculation-induced pulmonary hypertension decreased VIP and increased VPAC1 expressions in pulmonary arterial smooth muscle, although neither PACAP nor PAC1 expression significantly changed.[32] On the other hand, mice deficient in VIP developed features of pulmonary arterial hypertension in normoxia, with right ventricular hypertrophy and pulmonary vascular remodeling (see the chapter of Dr. Said in this book section). As far as I

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know, no data exist about the phenotype of PACAP-deficient mice regarding pulmonary hypertension, although deficiency of PACAP leads to breathing defects and sudden neonatal death,[34] because PACAP may control breathing by acting on carotid bodies and via the respiratory centers in the central nervous system.[34] However, PAC1-deficient mice suffer from pulmonary hypertension leading to severe right heart failure and death,[23] suggesting that PACAP signaling through PAC1 seems to play a crucial role in the maintenance of normal pulmonary vascular tone during early postnatal life, despite the scarcity of PAC1 expression on the respiratory system.

Effects of PACAP on Airway Mucous Secretion

Although some controversy exists, some evidence indicates that PACAP could contribute to the natural airway defense against pathogens by regulating mucous and electrolyte secretion by submucosal glands and epithelial goblet cells. Wagner et al.[33] clearly demonstrated that PACAP and VIP are potent stimulators of mucous secretion in isolated rat trachea, with an order of potency of PACAP27 > VIP > PACAP38. In contrast, Liu et al.[17] described in ferret trachea that VIP caused a modest increase in mucus secretion, whereas both forms of PACAP failed to significantly increase secretion. Discrepancies between both studies could be due to the different experimental designs used or to the fact that the main source of mucus in rat trachea is the goblet cells and in ferret trachea is the submucosal glands. Interestingly, VIP, PACAP38 and PACAP27 (in this order of potency) suppressed cholinergic mucous secretion in ferret trachea, although they did not affect acetylcholine-induced mucous secretion.[17] This suggests a prejunctional regulation of cholinergic neuronal secretion by the three neuropeptides, as previously described for the relaxant activity of PACAP on tracheal smooth muscle cells.[27] However, VIP and PACAP27, but not PACAP38, increased the secretion of chloride by human bronchial epithelial cells by inducing the chloride channel CFTR (cystic fibrosis transmembrane conductance regulator).[6] This effect seems to be mediated by VPAC1, although this finding does not explain the lack of effect by PACAP38. In conclusion, PACAP seems to play an important role in airway secretion and the clearance of pathogens, a hypothesis that is in agreement with the antimicrobial activities recently described for VIP and PACAP.

Effects of PACAP on Airway Inflammatory Responses

PACAP has lately emerged as a potent antinflammatory and immunomodulatory factor that acts at various levels (reviewed in Ref. 5). PACAP is produced by immune cells, mainly T lymphocytes, especially under inflammatory conditions, and different immunocompetent cells, including macrophages, monocytes, lymphocytes, and dendritic cells express VPAC1, VPAC2, and PAC1.[5] Through interaction with these receptors, PACAP deactivates macrophages and dendritic cells, inhibiting the production of a plethora of inflammatory and cytotoxic mediators, including tumor necrosis factor-α, IL-6, IL-12, IL-1β, prostaglandin E2, nitric oxide, and various chemokines.[5] A growing body of literature describes the protective effect of VIP in airway inflammatory disorders (see the chapter written by Dr. Said in this book section), although few works, especially *in vitro*, have addressed the effect of PACAP in this scenario. Because PACAP seems to exert its immunomodulatory actions in other tissues/organs mainly through VPAC1,[5] mimicking the effects of VIP, one could assume that PACAP must share with VIP the same prominent role in lung protection under inflammatory conditions. In a model of septic shock, where the lung is one of the major affected organs, systemic injection of PACAP significantly reduced leukocyte infiltration and disseminated coagulation in the lung, and consequently decreased mortality due to sepsis.[5] In agreement, PAC1-deficient mice were more susceptible to suffer sepsis, with the lung especially affected.[18] Similarly, in a model of allergic chronic asthma induced by ovoalbumine infusion, treatment with a PAC1-specific agonist resulted in significant amelioration of airway inflammation, and PAC1-deficient mice showed markedly increased lung inflammatory markers.[8] However, lung function, measured by airway hyperresponsiveness, was affected neither by the PAC1-agonist nor in the PAC1-deficient mice.[8,23] This suggests that PACAP signaling through PAC1 plays a major role in the regulation of the inflammatory response in the respiratory system but not against bronchoconstriction. In agreement with this hypothesis, PACAP-deficient mice showed the same bronchial hyperreactivity to carbacol inhalation than did wild-type mice, but they were more susceptible to bronchoconstriction in conditions of lung inflammation.[8] Moreover, PACAP-deficient mice showed increased inflammatory markers in airways after exposure to bacterial endotoxin.[8]

Effects of PACAP on Proliferation and Apoptosis

Numerous studies indicate that PACAP is a cytoprotective factor in various cells and tissues subjected to different injuries, acting at multiple levels on proliferation, differentiation and survival (discussed in various sections of this book). In the respiratory system, the effect of PACAP on cell proliferation and survival is diverse. As in other cell types, PACAP inhibits, in a VPAC2-dependent manner, apoptotic death of alveolar cells induced by chronic exposure to cigarette-smoke extracts, which is attractive for the treatment of conditions such chronic obstructive pulmonary disease and other lung inflammatory disorders.[22] Similarly, PACAP acts as a protective factor against glutamate

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cytotoxicity in the lung by reducing oxidant and apoptotic activities.[31] On the other hand, PACAP inhibits the proliferation of pulmonary smooth muscle cells, an effect that could contribute to the amelioration of asthma and other pulmonary obstructive disorders.[31] Contrary to its effects on the proliferative response of smooth muscle cells and other cell types, PACAP induces the growth of human lung tumor cells (small lung cancer and non-small lung cancer), through a mechanism that depends of VPAC1 and PAC1, induction of cAMP and calcium and activation of mitogen-activated protein kinase and the oncongenes fos, myc and jun.[21] Contrarily, VIP inhibited the proliferation of both types of lung cancer cells (see the other chapters of this book). Interestingly, a VIP hybrid with capacity to block VPAC1, VPAC2 and PAC1 inhibited the growth of several human lung cancer lines,[21] whereas treatment with this VIP hybrid or with VIP inhibited proliferation of human lung cancer cells in a xenograft system and reduced the number of blood vessels in tumors (see the chapter written by S. Said in this book section). However, PACAP27 induced the production of vascular endothelial growth factor by human lung cancer lines and the potential of promoting neovascularity in tumors.[21]

CONCLUSIONS

The findings reviewed here clearly indicate that PACAP is an endogenous regulator of airway and lung functions, with predominant roles in bronchodilation, pulmonary vascular relaxation, inflammation, and mucus secretion. Because PACAP38 has higher stability and improved pharmacokinetics than VIP, PACAP38 emerges as an attractive candidate for the treatment of pulmonary disorders where VIP has shown effectiveness such as pulmonary arterial hypertension and sarcoidosis. Although inhalation of PACAP in healthy humans was shown to be safe,[7] caution should be exercised in its use in patients, in view of the growth-promoting activities showed by PACAP on lung cancer cells. In any case, elucidation of the role of PAC1 as a preferring PACAP receptor in the normal physiology and pathology of airways and lung should increase the interest of pharmaceutical companies in the development of nonpeptide analogs of PAC1 for the treatment of asthma, pulmonary hypertension, bronchitis, and emphysema.

REFERENCES

1. Araki N, Takagi K. Relaxant effect of pituitary adenylate cyclase activating polypeptide on guinea-pig tracheal smooth muscle. *Eur J Pharmacol* 1999;**216**:113–7.
2. Busto R, Carrero I, Guijarro LG, Solano RM, Zapatero J, Noguerales F, et al. Expression, pharmacological, and functional evidence for PACAP/VIP receptors in human lung. *Am J Physiol* 1999;**277**:L42–8.
3. Cardell LO, Uddman R, Luts A, Sundler F. Pituitary adenylate cyclase activating peptide (PACAP) in guinea-pig lung: distribution and dilatory effects. *Regul Pept* 1991;**36**:379–90.
4. Conroy DM, St-Pierre S, Sirois P. Relaxant effects of pituitary adenylate cyclase activating polypeptide (PACAP) on epithelium-intact and -denuded guinea-pig trachea: a comparison with vasoactive intestinal peptide (VIP). *Neuropeptides* 1995;**29**:121–7.
5. Delgado M, Pozo D, Ganea D. The significance of vasoactive intestinal peptide in immunomodulation. *Pharmacol Rev* 2004;**56**:249–90.
6. Derand R, Montoni A, Bulteau-Pignoux L, Janet T, Moreau B, Muller JM, et al. Activation of VPACl receptors by VIP and PACAP-27 in human bronchial epithelial cells induces CFTR-dependent chloride secretion. *Br J Pharmacol* 2004;**141**:698–708.
7. Doberer D, Gschwandtner M, Mosgoeller W, Bieglmayer C, Heinzl H, Petkov V. Pulmonary and systemic effects of inhaled PACAP38 in healthy male subjects. *Eur J Clin Invest* 2007;**37**:665–72.
8. Elekes K, Sandor K, Moricz A, Kereskai L, Kemeny A, Szoke E, et al. Pituitary adenylate cyclase-activating polypeptide plays an anti-inflammatory role in endotoxin-induced airway inflammation: in vivo study with gene-deleted mice. *Peptides* 2011;**32**:1439–46.
9. Fischer A, Kummer W, Couraud JY, Adler D, Branscheid D, Heym C. Immunohistochemical localization of receptors for vasoactive intestinal peptide and substance P in human trachea. *Lab Invest* 1992;**67**:387–93.
10. Foda HD, Sharaf HH, Absood A, Said SI. Pituitary adenylate cyclase-activating peptide (PACAP), a VIP-like peptide, has prolonged airway smooth muscle relaxant activity. *Peptides* 1995;**16**:1057–61.
11. Hauser-Kronberger C, Hacker GW, Albegger K, Muss WH, Sundler F, Arimura A, et al. Distribution of two VIP-related peptides, helospectin and pituitary adenylate cyclase activating peptide (PACAP), in the human upper respiratory system. *Regul Pept* 1996;**65**:203–9.
12. Kinhult J, Adner M, Uddman R, Cardell LO. Pituitary adenylate cyclase activating polypeptide, effects in the human nose. *Clin Exp Allergy* 2003;**33**:942–9.
13. Kinhult J, Uddman R, Cardell LO. The induction of carbon monoxide-mediated airway relaxation by PACAP 38 in isolated guinea pig airways. *Lung* 2001;**179**:1–8.
14. Lam HC, Takahashi K, Ghatei MA, Kanse SM, Polak JM, Bloom SR. Binding sites of a novel neuropeptide pituitary-adenylate-cyclase-activating polypeptide in the rat brain and lung. *Eur J Biochem* 1990;**193**:725–9.
15. Lauenstein HD, Quarcoo D, Plappert L, Schleh C, Nassimi M, Pilzner C, et al. Pituitary adenylate cyclase-activating peptide receptor 1 mediates anti-inflammatory effects in allergic airway inflammation in mice. *Clin Exp Allergy* 2011;**41**:592–601.
16. Linde'n A, Cardell LO, Yoshihara S, Nadel JA. Bronchodilation by pituitary adenylate cyclase-activating peptide and related peptides. *Eur Respir J* 1999;**14**:443–51.
17. Liu YC, Khawaja AM, Rogers DF. Effect of vasoactive intestinal peptide (VIP)-related peptides on cholinergic neurogenic and direct mucus secretion in ferret trachea in vitro. *Br J Pharmacol* 1999;**128**:1353–9.
18. Martinez C, Abad C, Delgado M, Arranz A, Juarranz MG, Rodriguez-Henche N, et al. Anti-inflammatory role in septic shock of pituitary adenylate cyclase-activating polypeptide receptor. *Proc Natl Acad Sci U S A* 2002;**99**:1053–8.

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19. Miyata A, Arimura A, Dahl RR, Minamino N, Uehara A, Jiang L, et al. Isolation of a novel 38 residue-hypothalamic polypeptide which stimulates adenylate cyclase in pituitary cells. *Biochem Biophys Res Commun* 1989;**164**:567–74.
20. Moller K, Zhang YZ, Håkanson R, Luts A, Sjo¨lund B, Uddman R, et al. Pituitary adenylate cyclase activating peptide is a sensory neuropeptide: immunocytochemical and immunochemical evidence. *Neuroscience* 1993;**57**:725–32.
21. Moody TW, Hill JM, Jensen RT. VIP as a trophic factor in CNS and cancer cells. *Peptides* 2003;**24**:163–77.
22. Onoue S, Ohmori Y, Endo K, Yamada S, Kimura R, Yajima T. Vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide attenuate the cigarette smoke extract-induced apoptotic death of rat alveolar L2 cells. *Eur J Biochem* 2004;**271**:1757–67.
23. Otto C, Hein L, Brede M, Jahns R, Engelhardt S, Grone HJ, et al. Pulmonary hypertension and right heart failure in pituitary adenylate cyclase-activating polypeptide type I receptor-deficient mice. *Circulation* 2004;**110**:3245–51.
24. Petkov V, Mosgoeller W, Ziesche R, Raderer M, Stiebellehner L, Vonbank K, et al. Vasoactive intestinal peptide as a new drug for treatment of primary pulmonary hypertension. *J Clin Invest* 2003;**111**:1339–46.
25. Saguchi Y, Ando T, Watanabe T, Yamaki K, Suzuki R, Takagi K. Inhibitory effects of pituitary adenylate cyclase activating polypeptide on histamine-induced respiratory resistance in anesthetized guinea pigs. *Regul Pept* 1997;**70**:9–13.
26. Saotome M, Uchida Y, Nomura A, Endo T, Hasegawa S. Pituitary adenylate cyclase activating peptide induces cGMP-mediated relaxation in guinea-pig airways. *Pulm Pharmacol Ther* 1998;**11**:281–5.
27. Shigyo M, Aizawa H, Inoue H, Matsumoto K, Takata S, Hara N. Pituitary adenylate cyclase activating peptide regulates neurally mediated airway responses. *Eur Respir J* 1998;**12**:64–70.
28. Shivers BD, Görcs TJ, Gottschall PE, Arimura A. Two high affinity binding sites for pituitary adenylate cyclase-activating polypeptide have different tissue distributions. *Endocrinology* 1991;**128**:3055–65.
29. Uddman R, Luts A, Arimura A, Sundler F. Pituitary adenylate cyclase-activating peptide (PACAP), a new vasoactive (VIP)-like peptide in the respiratory tract. *Cell Tissue Res* 1991;**265**:197–201.
30. Usdin TB, Bonner TI, Mezey E. Two receptors for vasoactive intestinal polypeptide with similar specificity and complementary distributions. *Endocrinology* 1994;**135**:2662–80.
31. Vaudry D, Falluel-Morel A, Bourgault S, Basille M, Burel D, Wurtz O, et al. Pituitary adenylate cyclase-activating polypeptide and its receptors: 20 years after the discovery. *Pharmacol Rev* 2009;**61**:283–357.
32. Vuckovic A, Rondelet B, Brion JP, Naeije R. Expression of vasoactive intestinal peptide and related receptors in overcirculation-induced pulmonary hypertension in piglets. *Pediatr Res* 2009;**66**:395–9.
33. Wagner U, Bredenbroker D, Storm B, Tackenberg B, Fehman HC, Von Wichert P. Effects of VIP and related peptides on airway mucus secretion from isolated rat trachea. *Peptides* 1998;**19**:241–5.
34. Wilson RJ, Cumming KJ. Pituitary adenylate cyclase-activating polypeptide is vital for neonatal survival and the neuronal control of breathing. *Respir Physiol Neurobiol* 2008;**164**:168–78.
35. Yoshihara S, Yamada Y, Abe T, Kashimoto K, Linden A, Arisaka O. Long-lasting smooth-muscle relaxation by a novel PACAP analogue in human bronchi. *Regul Pept* 2004;**123**:161–5.

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Chapter 209

VIP

Sami I. Said, Valerie Chappe and Sayyed A. Hamidi

ABSTRACT

The neuropeptide vasoactive intestinal peptide (VIP) is widely distributed in the central and peripheral nervous systems and is expressed in all pulmonary structures, including airway epithelial cells, airway and pulmonary vascular smooth muscle cells, secretory glands, and immune and inflammatory cells. VIP actions in the lung, mediated predominantly by the adenylyl cyclase–cAMP pathway and by receptors shared with PACAP, include relaxation of airway and vascular smooth muscle and inhibition of their proliferation, modulation of inflammatory and immune responses, protection against cell injury, and stimulation of water and electrolyte transport. Strong evidence suggests that physiological roles of VIP in the lung include cotransmission of airway and pulmonary vascular smooth muscle relaxation, modulation of immune and inflammatory responses, and guarding against the pathogenesis of bronchial asthma, pulmonary hypertension, and CFTR dysfunction in cystic fibrosis. Therefore, it seems probable that VIP has strong therapeutic potential in a variety of clinical disorders.

HOW WAS VIP DISCOVERED?

In 1967, as a pulmonary physician in Richmond, Virginia, I (S.I.S.) wondered why serious acute events in the lung, such as severe pneumonia and the Acute Respiratory Distress Syndrome (ARDS), are often complicated by systemic hypotension. One possibility that appealed to me was that injured lung tissue releases into the circulation one or more vasodilator compounds. Preliminary experiments confirmed that supernatants of lung extracts, when injected into other animals, reduced systemic arterial blood pressure. This hypotension was not fully explained by the presence in lung extracts of the then-known vasodilators histamine, prostaglandins, and kallikreins, suggesting that one or more vasodilator peptides contributed to the phenomenon.[16]

To seek validation of this explanation, and identification of such a peptide, I decided to spend a sabbatical year at the Karolinska Institute, in Stockholm, Sweden, with Professor Viktor Mutt, a leading peptide biochemist, as my guide and mentor.

Although we soon confirmed the presence of strong vasodilator activity in extracts of porcine lung, isolation of a pure peptide with this property was progressing too slowly, because of the difficulty in securing sufficient quantities of fresh lungs.[22] Although lung extracts were in short supply, extracts of upper small intestine were plentiful in Viktor's laboratory, because he was preparing the gastrointestinal hormones secretin and cholecystokinin on a large scale. One day, as I wished we were searching for our peptide in the intestine instead of in the lung, I recalled that both upper intestine and lungs originated from the same embryonic bud, the foregut, and decided to look for vasodilator activity in intestinal extracts. The search proved fruitful, culminating in the isolation of the "vasoactive intestinal peptide," so named because, at that point, we did not know whether it also existed outside the intestine or whether it had any other biological activity. During its purification, the dominant biological activity of VIP was closely associated with that of secretin.[23,24] Reading the original articles on the discovery of secretin, I noted that Bayliss and Starling observed some "vasodepressor" activity in the secretin-containing preparations, which they attributed to the presence of an unidentified compound that, in all likelihood, was VIP!

Soon after VIP became available in pure form, its amino acid composition and sequence were identified,[12] and it was successfully synthesized, by Miklos Bodanszky.[1] After the development of a radioimmunoassay for the measurement of VIP content in plasma and tissues, and reports that the "watery diarrhea" or "pancreatic cholera" syndrome was attributable to excessive production of VIP by certain tumors, we began to receive biological samples from around the country and abroad, from patients suspected of having that disorder. Those assays revealed that the highest levels of VIP were associated with neurogenic tumors, especially neuroblastomas.[21] In addition, analysis of neuroblastoma cell lines showed them to be rich in immunoreactive VIP. As I looked into the biology of these cells, I learned that they share many features with normal neurons, so I wondered whether VIP could also be produced by normal neurons.

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00209-8
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With neurologist colleagues, we searched for the peptide in the human brain and peripheral nerves and found it to be widely and selectively present. VIP was thus rediscovered as a neuropeptide and a probable neurotransmitter.[27]

VIP IS A MEMBER OF A FAMILY OF PEPTIDES[24]

VIP is a 28-amino acid residue peptide (Fig. 1) that belongs to a family of structurally and functionally related peptides, including secretin, glucagon, gastric inhibitory peptide or glucose-dependent insulinotropic peptide (GIP), the peptide with N-terminal histidine and C-terminal isoleucine amide (PHI) and its counterpart in human tissues, the peptide with N-terminal histidine and C-terminal methionine amide (PHM), corticotropin-releasing hormone (CRH), growth hormone-releasing hormone (GHRH), sauvagine, urotensin I, helodermin, and the pituitary adenylate cyclase-activating polypeptides (PACAP), with which VIP shares the greatest degree of homology (Fig. 2).

TISSUE DISTRIBUTION AND CELLULAR LOCALIZATION

The expression of VIP in different cells and organs has been defined by immunofluorescence, radioimmunoassay, and more recently, by measurement of VIP mRNA. In the brain, the peptide is found in the highest concentrations in the cerebral cortex, suprachiasmatic nucleus, hippocampus, amygdala, and striatum, but it is also present in the midbrain periaqueductal gray and the sacral spinal cord. In the peripheral nervous system, VIP is localized in the sympathetic ganglia, the vagus nerve, predominantly motor nerves such as the sciatic nerve, and nerves supplying exocrine glands, blood vessels, and nonvascular smooth muscle.[3–5,25]

In the lungs, VIP-containing nerve fibers and nerve terminals are principally localized in the smooth muscle layer of the airways from the trachea through the small bronchioles, around submucosal mucous and serous glands, and in the walls of both pulmonary and bronchial arteries. Immunoreactive VIP is also present in neuronal cell bodies forming microganglia that provide a source of intrinsic innervation of pulmonary structures. In addition to its neuronal localization, VIP is richly present in the immune system, including inflammatory cells such as mast cells, eosinophils, and B and T lymphocytes.

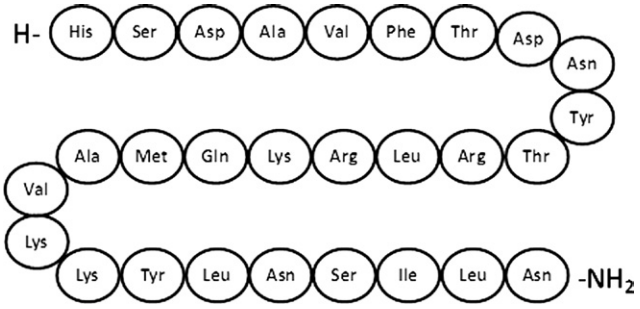

FIGURE 1 Amino acid sequence of VIP.

COLOCALIZATION WITH OTHER PEPTIDES AND NEUROTRANSMITTERS

VIP coexists with acetylcholine in most cholinergic neurons, both central and peripheral. In exocrine glands, this colocalization serves the dual functional advantages of promoting both blood flow and glandular secretion. VIP may also be colocalized with other peptides, including PHI (or PHM, its human counterpart), peptides that are synthesized from the same precursor molecule, and substance P. Other neurotransmitters that may coexist with VIP include nitric oxide (nitric oxide synthase) and the excitatory transmitter glutamic acid.

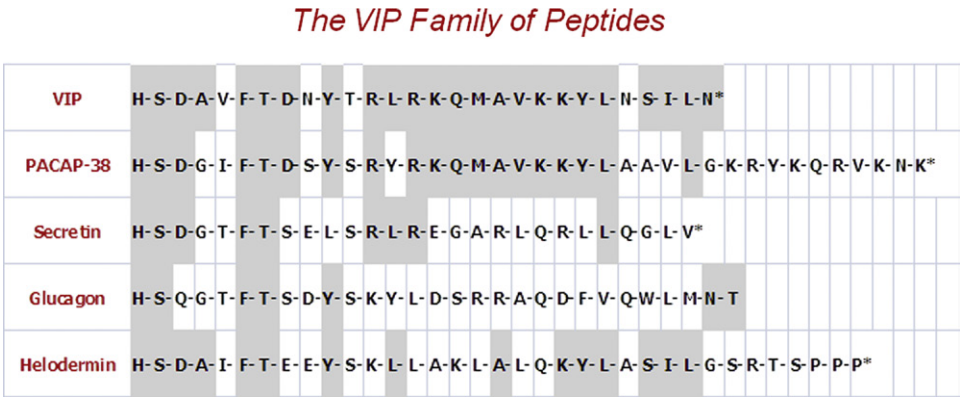

FIGURE 2 Some members of the VIP family of peptides. Amino acid homologies are noted by shading.

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BIOLOGICAL ACTIONS IN THE LUNG

Airways

Airway Smooth Muscle Tone[5,6,11,15,17]

VIP relaxes airway smooth muscle, both *in vitro* and *in vivo*, in guinea pigs, rats, mice, rabbits, dogs, and humans. VIP also counteracts airway smooth muscle contraction induced by a variety of bronchoconstrictor agents, including histamine, prostaglandin F2α, kallikrein, leukotriene D4, neurokinin A, serotonin, and endothelin (Fig. 3). Relative to most other bronchodilators, VIP-induced airway relaxation is longer lasting and is unaffected by blockade of adrenergic or cholinergic receptors or of cyclooxygenase activity. In limited trials in human asthmatics, however, VIP, given by inhalation, is generally less effective than expected in relieving bronchoconstriction, probably because of its degradation by proteases in airway secretions.

Airway Smooth Muscle Proliferation

VIP inhibits human airway smooth muscle proliferation,[9] an action that is potentially beneficial in reducing airway remodeling, which contributes to increased airway resistance in bronchial asthma.

Tracheobronchial Secretion

VIP stimulates water and ion transport in canine tracheal epithelium and may also promote macromolecular secretion. The coexistence of VIP and acetylcholine in cholinergic neurons brings together the blood flow-promoting and secretion-promoting effects of VIP and acetylcholine, respectively.

Pulmonary Circulation[6,13,15,20]

VIP dilates the vessels supplying the nose, upper airways, trachea, and bronchi, as well as pulmonary vessels. As a pulmonary vasodilator, VIP is more potent than isoproterenol, acetylcholine, or prostacyclin, and its action is independent of endothelium. Given intravenously in sufficient concentrations, VIP reduces both pulmonary and systemic vascular resistance. Delivered as an aerosol, however, it acts as a selective pulmonary vasodilator. As with airway smooth muscle, VIP suppresses human pulmonary vascular smooth muscle proliferation, a major feature of pulmonary hypertensive disease.

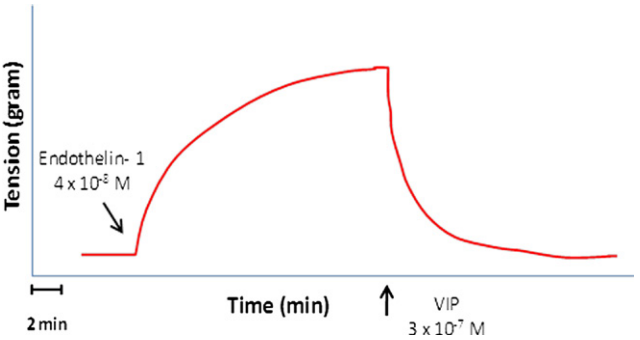

FIGURE 3 VIP as a tracheal smooth muscle relaxant. Isolated strip of guinea pig trachea was strongly contracted in response to endothelin 1. Application of VIP promptly and completely restored tracheal tension to resting level.

Immune and Inflammatory Responses[2,28,30]

VIP modulates several aspects of the immune and inflammatory responses: (1) It has a moderate inhibitory effect on antigen-induced release of histamine from guinea pig lung. Because the peptide is normally present in mast cells, the peptide may act as a natural modulator of mast-cell degranulation, an important mechanism underlying acute asthmatic and anaphylactic reactions. (2) VIP also modulates T-cell activation and inhibits the expression of proinflammatory cytokines (TNF-α, IL-6, IL-12, IL-18) and chemokines (e.g. RANTES); inducible nitric oxide synthase (*i* NOS), and proteases (MMP-2 gelatinase), while upregulating the expression of anti-inflammatory cytokine IL-10 and promoting the generation of tolerogenic dendritic and regulatory T-cells.

Anti-Injury and Cell-Protective Effects[18,19,30]

Acting by a number of actions, VIP prevents or attenuates acute lung injury in a variety of experimental models. These actions include antiapoptotic, prosurvival effects that are mediated by 3 complementary and synergistic mechanisms: Inhibition of the activation of caspases, key effectors of apoptosis; upregulation of the antiapoptotic protein bcl2; and suppression of cytoplasmic translocation of cytochrome c, a critical step in mitochondrial-mediated apoptosis.

RECEPTORS AND SIGNAL TRANSDUCTION PATHWAYS

Specific, high-affinity receptors for VIP have been identified in membrane preparations of normal lungs and human lung tumor cells. These receptors, localized immunocytochemically and by the increased cyclic (c)AMP levels resulting from activation by VIP, exist in a variety of pulmonary cells. Molecular and pharmacologic studies have led to the identification, characterization, and cloning of at least three receptors for VIP and the related PACAP peptides (see the chapter in this section of the book by Mario Delgado). These receptors, known as VPAC receptors, belong to a distinct family of seven transmembrane-domain receptors coupled to G proteins. The three VPAC receptors are VPAC1 and VPAC2, both of which respond to VIP and PACAP with

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comparable affinity, and PAC1, which has greater affinity for PACAP than for VIP.

The actions of VIP are mediated principally by adenylyl cyclase stimulation and cAMP production. VIP biosynthesis itself is promoted by higher cAMP levels, and thus, the peptide is potentially capable of stimulating its own generation. In some VIP actions, additional second messengers and signal transduction pathways are involved, for example, phospholipase C activation in the case of the PACAP-preferring PAC 1 receptor.

Some actions of VIP, such as the relaxation of airway and vascular smooth muscle cells, are mediated partly by NO, released from neurons or smooth muscle cells. Through its activation of a constitutive NOS in neural elements and smooth muscle, leading to activation of cytosolic guanylyl cyclase, VIP can stimulate intracellular cyclic GMP formation. The contribution of neuronal NOS to VIP-induced relaxation of the trachea was examined in neuronal NOS-knockout mice and estimated to account for a significant proportion of the relaxation. It is now believed that both NO and carbon monoxide (CO) mediate VIP-induced relaxation of airway, gastrointestinal, and other smooth muscle. The combined activation of cyclic AMP and GMP pathways ensures greater smooth muscle relaxation, inhibition of mitogenesis, and smooth muscle cell proliferation.[26]

PHYSIOLOGICAL ROLES

The physiological significance of VIP is suggested by 3 lines of evidence:

1. It is widely present, in a conserved structure, throughout the animal kingdom.
2. Deletion of the VIP gene alters the expression of almost 27% of all other genes.
3. VIP KO mice express the phenotypes of at least 4 disorders, which are correctable with VIP.[7]

Deletion of the VIP gene results in (i) bronchial asthma, manifested by airway hyperresponsiveness and airway inflammation; (ii) pulmonary arterial hypertension, with vascular remodeling, right ventricular hypertrophy, and perivascular inflammation; (iii) cystic fibrosis (CF)-like features, including dysfunctional CFTR, associated with structural abnormalities in the lung and gastrointestinal tract; and (iv) increased susceptibility to death from endotoxemia.

Several lines of evidence suggest the following physiological roles for VIP, with special reference to the lungs:

1. Mediation of vascular and nonvascular smooth muscle relaxation: Together with NO and CO, VIP is a cotransmitter of neurogenic, noncholinergic, nonadrenergic smooth muscle relaxation.
2. Modulation of smooth muscle cell proliferation.
3. Modulation of inflammatory responses.
4. Modulation of immune responses and promotion of immune tolerance.
5. Enhancement of cell survival, and defense against injury and apoptosis: VIP exerts these effects by attenuating inflammation and reducing apoptotic cell death. For example, VIP protects lung cells against oxidant stress resulting from: xanthine + xanthine oxidase, paraquat, excitotoxicity (glutamate toxicity), and ischemia-reperfusion injury; as well as platelet-activating factor and endotoxin lipopolysaccharide.
   In addition, VIP also protects neuronal cells against injury, as by oxidants, for example, H_2O_2 and glutamate; as well as by envelope protein of HIV, nutrient or nerve growth factor deprivation, tetrodotoxin, and β-amyloid (Aβ). Further, as discussed in the chapter by Paul Gressens, VIP promotes embryonic brain growth and differentiation.
6. Stimulation of water and electrolyte transport: Among the earliest biological actions of VIP to be recognized is its ability to promote water and electrolyte transport. This is true for Cl^- in bronchial and intestinal epithelium, and for HCO_3^- in pancreatic secretion.

As earlier mentioned, mice lacking the VIP gene express CF-like pathophysiological features, and the role of VIP in the pathogenesis and possible modulation of the human disease is further discussed later. Finally, excessive stimulation of H_2O/Cl^- transport, in the presence of VIP-secreting tumors (VIPomas), results in the "watery diarrhea syndrome."

VIP IN THE PATHOGENESIS AND MODULATION OF PULMONARY DISEASE

Bronchial Asthma[17,31]

A close link between VIP deficiency and the pathogenesis of asthma is suggested by 3 sets of findings:

1. VIP-immunoreactive nerves are absent in airways of a group of severely asthmatic subjects who died accidentally.
2. Mice with targeted deletion of the VIP gene exhibit airway hyperresponsiveness and histopathologic features of airway inflammation.
3. As an airway smooth muscle cell relaxant, with anti-inflammatory and antiproliferative activities, VIP is well qualified to counteract the pathophysiological features of bronchial asthma, including airway constriction, hyperresponsiveness, airway inflammation, and, in severe cases, smooth muscle proliferation.

Despite its demonstrated effectiveness in animal models *in vivo*, however, the results of several clinical trials in asthmatics are less impressive than anticipated. The inhalation

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route would seem to be ideally suited for delivering VIP to asthmatics. Unfortunately, success of this approach seems to be limited by degradation of the inhaled peptide by peptidases in airway secretions. This problem may be solved by either of 2 alternate solutions, neither of which has been put to the test in clinical trials: (a) Instead of VIP itself, use a VIP-like analog that is more resistant to enzymatic degradation, such as the C-terminally extended helodermin (Fig. 2) or (b) combining VIP with one or more enzyme inhibitors that are particularly effective against VIP-selective peptidases, notably dipeptidyl peptidase (DPP IV) and neutral endopeptidase (enkephalinase).

Pulmonary Arterial Hypertension (PAH)

Strong experimental evidence and 2 clinical studies suggest a useful therapeutic potential for VIP in PAH:

1. As noted above, in the absence of the VIP gene, mice express a PAH phenotype, with pulmonary vascular remodeling, inflammation, and right ventricular hypertrophy.[29] RT-PCR arrays in these mice show the upregulated expression of pathways that promote vasoconstriction, smooth muscle and collagen proliferation, and inflammation, together with the downregulation of key pathways that promote vasodilation, and suppress vascular proliferation, including endothelial NOS (eNOS) and prostacyclin (PGI_2).
2. VIP relaxes pulmonary vascular smooth muscle cells *in vitro* and *in vivo*.[26] Further, it suppresses pulmonary vascular smooth muscle cell proliferation, and corrects pulmonary vascular remodeling, inflammation, and RV pathology in experimental PAH induced by (a) VIP gene deletion in mice, (b) monocrotaline in rats (Fig. 4), and (c) chronic hypoxia in mice.
3. In patients with Idiopathic Pulmonary Hypertension (IPAH), serum VIP concentrations are below normal, and VIP-containing nerves are absent in pulmonary arterial walls, whereas VIP receptors VPAC1 and VPAC2 are upregulated in pulmonary arterial smooth muscle cells.[13]

Lung Cancer

VIP suppresses the growth of small-cell lung cancer cells in culture and in athymic nude mice *in vivo*.[10] These results suggest that VIP may offer an effective and less toxic alternative to conventional chemotherapy in the management of this highly malignant form of cancer, but no clinical trials have been conducted to validate this possibility.

Acute Lung Injury (ALI) and Related Conditions[19,30]

1. VIP protects the lungs and airways in at least 13 experimental models (Table 1). This protection is attributable to a combination of anti-inflammatory, antioxidant, and antiapoptotic properties.
2. Based on these encouraging data, a phase I clinical trial of VIP, funded by the Orphan Drug Program of the FDA, was conducted in 8 patients with relatively severe ARDS, with or without Multiorgan Dysfunction Syndrome (MODS), in the setting of sepsis. VIP was given by continuous iv infusion, for 6 or 12 h. The study confirmed the safety of the peptide under these conditions: of the 8 patients, only 2 died, with neither death being drug related. A follow-up, multicenter clinical trial to evaluate efficacy is being planned.

Inflammatory and Autoimmune Diseases[2]

Based on its anti-inflammatory and immune-modulating properties, VIP has undergone early trials in a number of clinical disorders and experimental models of such disorders, including pulmonary sarcoidosis, COPD, rheumatoid arthritis, experimental autoimmune encephalomyelitis, inflammatory bowel disease, and type I diabetes. It is still too early to draw firm conclusions from these studies.

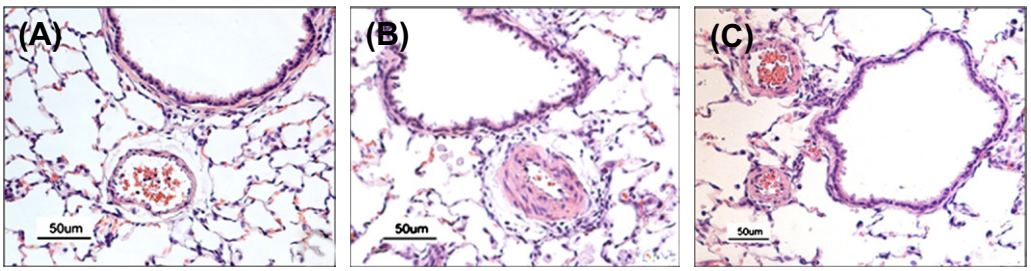

FIGURE 4 VIP corrects pulmonary vascular and RV pathology in monocrotaline (MCT)-induced PAH in rats: (A) H&E section of normal control lung, showing alveoli, bronchus, and small PA. No evidence of inflammation or vascular thickening. (B) Lung section 3 weeks after injection of MCT, showing vascular thickening and inflammatory cell infiltrates. (C) 3 weeks after VIP treatment, almost complete reversal of MCT-induced pathology. See color plate 58.

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TABLE 1 Experimental Models of Acute Lung and Airway Injury Prevented by VIP

(A) In isolated lung

1. HCl intratracheally
2. Platelet-activating factor into the PA
3. Xanthine + xanthine oxidase into the PA
4. Phospholipase C into the PA
5. Prolonged perfusion ex vivo
6. Paraquat into the PA
7. Capsaicin into the airways
8. Anaphylaxis
9. Eosinophil-mediated injury of cultured bronchial epithelial cells

(B) In vivo:

10. Platelet-activating factor i.v.
11. Cobra venom factor
12. Endotoxemia
13. Excitotoxicity (NMDA receptor activation)

VIP and the NFAT Transcription Factor

In considering the role of VIP in disease, it is important to keep in mind the relationship of VIP to transcription factor nuclear factor of activated T-cells (NFAT). Activation of calcineurin–NFAT signaling is triggered by increased concentration of intracellular Ca^{2+}, which leads to dephosphorylation (i.e. activation) of the phosphatase enzyme calcineurin (Fig. 5). In its resting, inactive state, this enzyme is located in the cytosol; on activation, it enters the nucleus to act on its target genes.

In addition to T-cell activation, activation of this pathway results in inflammation, smooth muscle cell contraction and proliferation, and autoimmune responses. Inhibition of calcineurin-mediated effects, as by cyclosporine A and the peptide VIVIT, can prevent organ transplant rejection. With special reference to VIP, mice lacking the VIP gene show activation of NFAT, and strong evidence suggests that VIP is a physiological modulator of this pathway.

CYSTIC FIBROSIS (CF)

CF, a fatal autosomal recessive disease, is characterized by abnormal ion transport across epithelia, viscous secretions, chronic bacterial infections, and airway inflammation. Over 25 years ago, we first observed that normal skin had a rich network of VIP-immunoreactive nerves around sweat gland acini and ducts, in contrast to absent or minimal VIP innervation of CF sweat glands. We wondered (1) whether deficient VIP innervation is responsible for the chloride ion abnormality in CF; and (2) to what extent is VIP involved in the pathogenesis, or possible correction, of CF pathology?[8]

We now know that the basic molecular defect in CF is a deficiency or absence of the cystic fibrosis transmembrane conductance regulator (CFTR) from the apical membrane of epithelial cells, due to mutations in the CFTR gene.

CFTR and Its Mutations

The most common (>90%) mutation in CF, a deletion of phenylalanine 508 (ΔF508), causes improper folding of the CFTR protein, resulting in its retention in the endoplasmic reticulum and proteosomal degradation. Major CF research efforts are devoted to the attempt to rescue ΔF508-CFTR defective trafficking to restore normal epithelial function. We have investigated the potential rescue and stability at the cell membrane of ΔF508-CFTR, by VIP treatment, in human nasal epithelial cells JME/CF15, derived from a patient with this homozygous mutation. When the cells, maintained at 37 °C, are treated with VIP for 1 or 2 h, mature ΔF508-CFTR proteins are observed: Immunostaining confirms localization at the cell membrane, and functional assays confirm that CFTR-dependent chloride secretion has been restored after VIP treatment. In these cells, which express the VPAC1 receptor, we found that VIP-dependent rescue of ΔF508-CFTR trafficking is mediated by the PKA-dependent signaling pathway, whereas membrane stability by prolonged VIP treatment involves the Gαq and PKCε signaling cascade. Evidence from airway cells thus shows that the VIP mechanisms of regulating CFTR activity involve both PKA and PKC signaling in a synergistic manner to rescue defective trafficking of mutant CFTR, activate CFTR gating through direct phosphorylation of its regulatory domain, and most importantly, stabilize CFTR channels at the cell surface by reducing their internalization rate, thus *optimizing CFTR-dependent secretions.*

In Vivo Confirmation of the Role of VIP[14]

As earlier mentioned above, VIP knockout mice express several features of the absence of the VIP gene, including an asthma-like phenotype, a PAH phenotype, and increased vulnerability to endotoxemia. In addition, they also show CF-like pathological changes: the lungs show significant lymphocyte accumulation, increased airway secretion, alveolar thickening, and edema; the pancreas also shows increased secretion, and inflammatory cell infiltrates around the ducts. These pathological changes could be reversed, close to a wild-type phenotype, by treatment with VIP (15 μg) intraperitoneally, every other day, for 3 weeks. In this model, whereas normal control tissues show CFTR predominantly at the apical membrane of epithelial cells, CFTR distribution in VIP KO tissues is mainly observed intracellularly. VIP treatment restores strong CFTR membrane localization, confirming the important role of chronic

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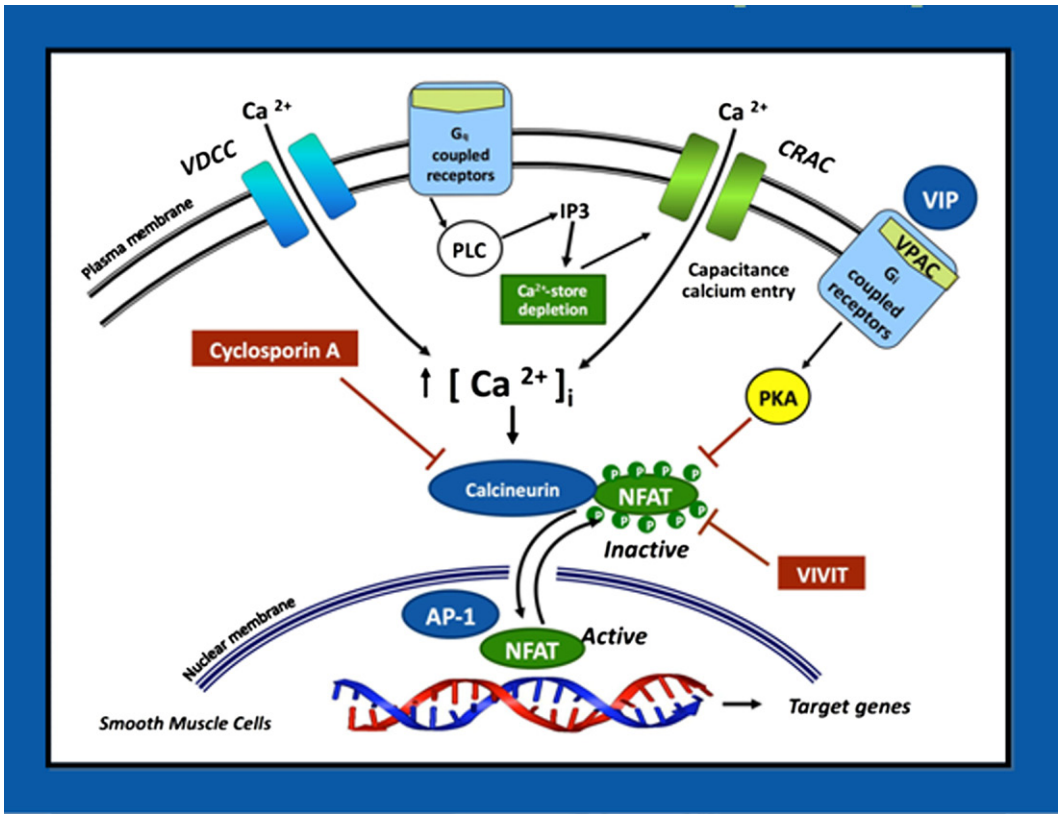

FIGURE 5 Calcineurin–NFAT signaling and its inhibition by VIP. In resting cells, NFAT resides in the cytoplasm, where it is maintained in a phosphorylated, inactive state. Stimulation of cell-surface receptors coupled to phospholipase C (PLC) results in calcium mobilization, initially from intracellular stores, followed by influx of Ca^{2+} through specialized voltage-dependent Ca^{2+} channels (VDCC) and Ca^{2+} release-activated Ca^{2+} channels (CRAC). On activation by Ca^{2+}, the phosphatase calcineurin dephosphorylates NFAT, triggering its activation and nuclear translocation. In the nucleus, NFAT interacts with other transcription factors, including activator protein (AP)-1, to stimulate gene expression. When NFAT is rephosphorylated, it is exported back to the cytoplasm. The immunosuppressive drug cyclosporin A inhibits calcineurin interactions with all its substrates, whereas VIVIT selectively inhibits NFAT activation. VIP seems to inhibit this pathway, but its mechanism of action is yet to be determined. IP3: inositol-1,4,5-triphosphate. See color plate 59.

VIP exposure tin maintaining CFTR channels at the membrane, and for exocrine epithelial tissues integrity. We conclude that inflammation and damage observed in VIP KO tissues can be attributed, at least in part, to the lack of CFTR-dependent secretions that ultimately depend on VIP stimulation, both for acute and long-term regulation of CFTR function. These observations also provide evidence of the molecular link between early observations of deficient VIP-containing fibers innervation of epithelial layers of exocrine organs in CF tissues and the absence of CFTR-dependent secretions.

The results set VIP, or its analogs, as a potential candidate for the treatment of CF as it corrects many features of this disease including its molecular basis. Ivacaftor, an investigational drug targeting the second most common CFTR genetic mutation, G551D, found in 4–5% of CF patients, boosts lung function in patients with CF with that mutation. If the ongoing studies with VIP are equally successful, the result could be an even bigger prize.

REFERENCES

1. Bodanszky M, Klausner YS, Lin CY, Mutt V, Said SI. Synthesis of the vasoactive intestinal peptide (VIP). *J Am Chem Soc* 1974;**96**:4973–8.
2. Delgado M, Pozo D, Ganea D. The significance of vasoactive intestinal peptide in immunomodulation. *Pharmacol Rev* 2004;**56**:249–90.
3. Fuxe K, Hökfelt T, Said SI, Mutt V. Vasoactive intestinal polypeptide and the nervous system: immunohistochemical evidence of localization in central and peripheral neurons, particularly intracortical neurons of the cerebral cortex. *Neurosci Lett* 1977;**5**:241–6.
4. Giachetti A, Said SI, Reynolds RC, Koniges FC. Vasoactive intestinal polypeptide (VIP) in brain: localization in, and release from, isolated nerve terminals. *Proc Natl Acad Sci U S A* 1977;**74**:3424–8.
5. Goyal RK, Rattan S, Said SI. VIP as a possible neurotransmitter of non-cholinergic non-adrenergic inhibitory neurones. *Nature* 1980;**288**:378–80.
6. Hamasaki Y, Mojarad M, Said SI. Relaxant action of VIP on cat pulmonary artery: comparison with acetylcholine, isoproterenol and PGE_1. *J Appl Physiol Respir Environ Exerc Physiol* 1983;**54**:1607–11.
7. Hamidi SA, Szema AM, Lyubsky S, Dickman KG, Degene A, Mathew SM, Waschek JA, Said SI. Clues to VIP function from knockout mice. *Ann NY Acad Sci* 2006;**1070**:5–9.

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8. Heinz-Erian P, Flux M, Dey RD, Said SI. Deficient vasoactive intestinal peptide innervation in sweat glands of cystic fibrosis patients. *Science* 1985;**229**:1407–8.
9. Maruno K, Absood A, Said SI. VIP inhibits basal and histamine-stimulated proliferation of human airway smooth muscle cells. *Am J Physiol* 1995;**268**:L1047–51:(Lung Cell Mol Physiol 12).
10. Maruno K, Absood A, Said SI. Vasoactive intestinal peptide inhibits human small-cell lung cancer proliferation *in vitro* and *in vivo*. *Proc Natl Acad Sci U S A* 1998;**95**:14373–8.
11. Matsuzaki Y, Hamasaki Y, Said SI. Vasoactive intestinal peptide: a possible transmitter of non-adrenergic relaxation of guinea pig airways. *Science* 1980;**210**:1252–3.
12. Mutt V, Said SI. Structure of the porcine vasoactive intestinal octacosapeptide: the amino acid sequence. Use of kallikrein in its determination. *Eur J Biochem* 1974;**42**:581–9.
13. Petkov V, Mosgoeller W, Ziesche R, Raderer M, Stiebellehner L, Vonbank K, et al. Vasoactive intestinal peptide as a new drug for treatment of primary pulmonary hypertension. *J Clin Invest* 2003;**111**:1339–46.
14. Rafferty S, Alcolado N, Norez C, Chappe F, Pelzer S, Becq F, et al. Rescue of functional F508del CFTR by VIP in the human nasal epithelial cell line JME/CF15. *J Pharmacol Exp Ther* 2009;**331**:2–13.
15. Saga T, Said SI. Vasoactive intestinal peptide relaxes isolated strips of human bronchus, pulmonary artery, and lung parenchyma. *Trans Assoc Am Physicians* 1984;**97**:304–10.
16. Said SI. Vasoactive substances in the lung. In: *Proceedings of Tenth Aspen Emphysema Conference*, Aspen, CO, June 7–10, 1967. *U.S. Public Health Service Publication*. 1967;**1787**:223–8.
17. Said SI. Vasoactive intestinal polypeptide and asthma (Editorial). *New Engl J Med* 1989;**320**:1271–3.
18. Said SI. Molecules that protect: the defense of neurons and other cells (Editorial). *J Clin Invest* 1996;**97**:2163–4.
19. Said SI. The Viktor Mutt Memorial Lecture: protection by VIP and related peptides against cell death and tissue injury. *Ann NY Acad Sci* 2000;**921**:264–74.
20. Said SI. The VIP gene is a key modulator of pulmonary vascular remodeling and inflammation. *Ann N Y Acad Sci* 2008;**1144**:148–53.
21. Said SI, Faloona GR. Elevated plasma and tissue levels of vasoactive intestinal polypeptide in the watery diarrhea syndrome due to pancreatic, bronchogenic and other tumors. *N Engl J Med* 1975;**293**:155–60.
22. Said SI, Mutt V. Long acting vasodilator peptide from lung tissue. *Nature* 1969;**224**:699–700.
23. Said SI, Mutt V. Polypeptide with broad biological activity: isolation from small intestine. *Science* 1970;**169**:1217–8.
24. Said SI, Mutt V. Isolation from porcine intestinal wall of a vasoactive octacosapeptide related to secretin and to glucagon. *Eur J Biochem* 1972;**28**:199–204.
25. Said SI, Porter JC. Vasoactive intestinal polypeptide: release into hypophyseal portal blood. *Life Sci* 1979;**24**:227–30.
26. Said SI, Rattan S. The multiple mediators of neurogenic smooth muscle relaxation. *Trends Endocrinol Metab* 2004;**15**:189–91.
27. Said SI, Rosenberg RN. Vasoactive intestinal polypeptide: abundant immunoreactivity in neural cell lines and normal nervous tissues. *Science* 1976;**192**:907–8.
28. Said SI, Berisha HI, Pakbaz H. Excitotoxicity in lung: *N*-methyl-D-aspartate-induced, nitric oxide-dependent, pulmonary edema is attenuated by vasoactive intestinal peptide and by inhibitors of poly (ADP-ribose) polymerase. *Proc Natl Acad Sci U S A* 1996;**93**:4688–92.
29. Said SI, Hamidi SA, Dickman KG, Szema AM, Lyubski S, Lin RZ, et al. Moderate pulmonary arterial hypertension in male mice lacking the vasoactive intestinal peptide gene. *Circulation* 2007;**115**:1260–8.
30. Said SI, Dickman KG. Pathways of inflammation and cell death in the lung: modulation by vasoactive intestinal peptide. *Regul Pept* 2000;**93**:21–9.
31. Szema AM, Hamidi SA, Lyubsky S, Dickman KG, Mathew S, Abdel-Razek T, et al. Mice lacking the VIP gene show airway hyperresponsiveness and airway inflammation, partially reversible by VIP. *Am J Physiol Lung Cell Mol Physiol* 2006;**291**:L880–6.

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Chapter 210

Antiopioid Peptides

Ji-Sheng Han

ABSTRACT

The first demonstration in 1979 of an antiopioid peptide and prediction of the existence of other endogenous antiopioid peptides in the central nervous system have prompted extensive research endeavors exploring and identifying these peptides during the past 3 decades. Several peptides that fall into this category include cholecystokinin, neuropeptide FF, Tyr-MIF-1-related peptides, nociceptin/orphanin FQ, and several others. Hypothetically, they are collectively viewed as a negative feedback system to antagonize the excessive activation of the opioid receptors induced by the repeated administration of opiates or excessive release of opioid peptides. From a practical point of view, the blockade of the antiopioid peptides may have clinical implications for the treatment of intractable pain.

INTRODUCTION

Numerous pharmacological and behavioral studies have demonstrated a potent antagonizing effect of the antiopioid peptides on various forms of opioid analgesia after systemic, intracerebroventricular (ICV), or intrathecal (IT) injection. Most of these antiopioid peptides also contribute to the development of tolerance to opioid analgesia. At the cellular level, cholecystokinin (CCK) receptors may colocalize with opioid receptors in the same neurons,[16] which provides an anatomical basis for direct interactions between CCK and opioid systems at the receptor and postreceptor levels.

Although MIF-1 was the first peptide described to have antiopioid activity,[14] CCK has been the most thoroughly studied. This peptide, therefore, is mainly reviewed in this chapter regarding its antiopioid action, followed by a summation of three other antiopioid peptides (neuropeptide FF, NPFF); nociceptin/orphanin FQ (N/OFQ); and the Tyr-MIF-1 (Tyr-Pro-Leu-Gly-NH_2)-family of peptides, as well as mention of some more recently described ones.

REGULATION OF OPIOID ANALGESIA BY CCK

CCK, a member of the gastrin family of peptides, is present in the central nervous system (CNS) mainly in the form of a carboxy-terminal octapeptide (CCK-8). Faris et al.[6] in 1983 first reported that CCK, when administered systemically or perispinally, antagonized opiate analgesia. The antiopioid action of CCK was afterward confirmed and characterized in a large number of pharmacological and behavioral studies. For instance, the ICV or IT administration of CCK-8 suppressed morphine analgesia in rats.[8,10] Apparently, CCK-8 possesses the ability to antagonize morphine analgesia at both the supraspinal and spinal levels. Acupuncture and electroacupuncture (EA) have been documented to release endogenous opioid peptides in the CNS and thus produce strong analgesia.[9] Like morphine analgesia, EA-induced analgesia was substantially attenuated by ICV or IT injection of CCK-8. Thus, CCK-8 exerts its influence over analgesia induced by both exogenous and endogenous opioids in an inhibitory fashion.

The antagonizing effect of exogenous CCK on morphine suggests that endogenous CCK may act physiologically as an opioid antagonist to dose dependently suppress opioid analgesia. Indeed, previous studies[28] have shown that the CCK receptor antagonist proglumide or MK329 (L364,718) when given by the ICV, IT, or systemic route potentiated various forms of analgesia produced by morphine and endogenous opioids and seemed to reverse tolerance to morphine analgesia. Active immunization against CCK also resulted in a potentiation of morphine analgesia. In addition, the antiserum against CCK could reverse tolerance to EA analgesia. Even clinical placebo-induced analgesia, which may be opioid in nature, was potentiated by proglumide. These results suggest that in response to upregulated opioidergic activities the endogenous CCK system can act as a negative feedback control to return pain sensitivity to its basal level. Because neither proglumide nor CCK-8 antiserum produced measurable analgesia in the absence of morphine, the endogenous CCK system does not seem to be tonically active in affecting basal pain perception.

Two subtypes of CCK receptors have been identified: CCKA (A for alimentary) receptors, predominantly located in the periphery, and CCKB (B for brain) receptors, in the CNS. These 2 receptors are now designated as CCK1 and CCK2. Results from a large number of pharmacological

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00210-4
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studies support the concept that the central type CCKB receptor, rather than the peripheral type CCKA receptor, is primarily responsible for the antiopioid effect of CCK. The CCKB subtype-selective antagonist L365,260, L740,093, or CI988 readily mimicked the effect of the non-subtype-selective CCK receptor antagonists and CCK antiserum in augmenting morphine- and EA-induced analgesia and preventing tolerance to morphine analgesia.[3,28] In mice lacking CCKB receptors, morphine induced a significantly stronger antinociceptive effect as compared with that in wild-type animals.[27] Noticeably, the role of CCKB receptor in modulating opioid analgesia varies with different opioid receptor subtypes. Wang XJ et al. have shown that the CCKB receptor plays a significant role in antagonizing the analgesia mediated by μ- and κ-opioid receptors, with a minimal influence over the δ-opioid receptor-mediated analgesia.[28]

CCK GENE EXPRESSION IN RESPONSE TO OPIOID ANALGESIA

CCK mRNAs and CCK-like immunoreactive materials are broadly distributed in the CNS. Generally, substantial overlap in the anatomical distribution of CCK, endogenous opioid peptides and their receptors exists in the brain and spinal cord, providing an anatomical basis for the interaction between the two systems. Alterations in CCK peptide gene expression in the CNS were investigated in rodents exposed to opioids or that had developed tolerance to opioid or EA analgesia. Zhou et al. reported that the basal levels of CCK mRNAs were elevated in the whole brain of rats rendered tolerant to repeated injections of morphine.[28] The increased levels of CCK mRNAs suggest an accelerated synthesis of CCK peptides in the brain at the transcription level, probably leading to enhanced CCK activity. As a compensatory response, enhanced CCK activity could then engage in counteracting the analgesic effect of opioids. The results from the pharmacological studies previously described support this notion. In line with this, ICV injection of CCK antisense RNA to the rat to knock down the biosynthesis of CCK in the CNS has been shown to increase the analgesic effect induced by EA or low dose morphine, and changed low responder rats to morphine and EA analgesia into high responders.[23]

The region-specific increase in CCK mRNAs was also investigated by in situ hybridization. Pu et al. found that CCK mRNA levels were elevated in the amygdala in morphine-tolerant rats. Moreover, the increase in CCK mRNA levels in the amygdala kinetically corresponded well with the development of tolerance to morphine. Thus, CCK in the amygdala may play a functional role in regulating morphine tolerance. This was substantiated by the finding that the CCKB receptor antagonist locally injected into the amygdala augmented the analgesic effect of systemic morphine.[28]

The periaqueductal gray (PAG) of the midbrain represents another strategic site in the CNS where CCK-8 can exert its antiopioid action.[26] The reduction of cellular levels of CCK-8 through an antisense approach converted low responder rats into high responders to morphine and EA analgesia, and delayed the development of analgesic tolerance to prolonged EA stimulation or repeated morphine administrations.[23] Similarly, the CCK receptor antagonist injected into the PAG reversed tolerance to morphine analgesia.[26] These results demonstrate a strong antagonizing effect of endogenous CCK-8 in the PAG on opioid analgesia.

Ding and Bayer revealed that chronic treatment with morphine increased CCK mRNA levels and CCK immunoreactive peptides in the hypothalamus, the spinal cord, and the brainstem, areas known to be involved in opioid analgesia.[28]

THE ROLE OF CCK IN THE DEVELOPMENT AND MAINTENANCE OF CHRONIC PAIN

The analgesic effect of morphine varies in different pain states. Patients or experimental animals with neuropathic pain after injury to the nervous system usually respond poorly to opioids. This poor response may be related to the antiopioid action of CCK. Hokfelt and his group have conducted a series of studies showing that axotomy caused an upregulation of CCK and CCKB receptor mRNAs in rat dorsal root ganglion neurons. The CCKB receptor antagonist CI988 was able to reverse the reduction of morphine analgesia in axotomized rats. CI988 could also reverse the lack of effect of IT morphine in alleviating neuropathic pain like symptoms in rats after complete or partial peripheral nerve injury. Thus, insensitivity to opioid analgesia in rats with nerve injury may result from enhanced CCK and CCK receptor expression.[28]

In a rat experiment, ligation of the sciatic nerve or microinjection of CCK into the brainstem rostral ventral medulla (RVM) results in the production of hyperalgesia of the hind paw, an indication of the neuropathic pain. This neuropathic pain seems to require the activity of a subset of RVM neurons that are distinguished by coexpression of the mu opioid receptor (MOR) and cholecystokinin type 2 receptor (CCK2). Lesion of these RVM neurons significantly shortened the duration of the hyperalgesia, suggesting that RVM CCK is involved in the maintenance of neuropathic pain.[34]

In addition to the opioid/CCK interaction in the CNS, it may also occur in the periphery. In diabetic rats, the antinociceptive effect of local injection of morphine in the second phase of the formalin test was about twofold less than that in nondiabetic rats. Pretreatment with proglumide

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enhanced the antinociceptive effect of morphine, suggesting that in diabetic rats, CCK exerts a tonic antagonistic role on peripheral morphine antinociception.[25]

The psychic component plays an important role in the maintenance of chronic pain. It is well recognized that placebo analgesia is mediated by opioid mechanisms. Little is known on the mechanisms of nocebo hyperalgesia. Benedetti et al. studied verbally induced nocebo effects using experimental ischemic arm pain in healthy volunteers. They found that the nocebo hyperalgesia can be completely abolished by proglumide, suggesting a CCK-ergic mechanism.[1]

CCK RELEASE IN RESPONSE TO OPIOID ANALGESIA

The extracellular level of CCK under different conditions determines its potency of modulating opioid action. Through *in vivo* microdialysis technique in freely moving and conscious animals combined with radioimmunoassay, Gustafsson et al. found that the basal CCK level was usually below or close to the detection limit of the radioimmunoassay in the normal rat spinal cord. The normal extracellular CCK levels could be markedly elevated by depolarization caused by perfusion of the microdialysis probe with Krebs–Ringer solution containing a high concentration of potassium. In addition to depolarization, morphine is a potent stimulator for facilitating CCK release. When injected intravenously or applied topically on the spinal cord, morphine significantly increased CCK in the dialysate. Because the release of CCK by morphine was sensitive to naloxone blockade, opioid receptors are believed to mediate the morphine-induced CCK release. CCK-8 was also detectable in the cerebrospinal fluid (CSF) of rats with a normal range of 14.4–15.1 fmol/ml. EA stimulation resulted in a naloxone-sensitive increase in CCK-8 levels in the CSF. This increase lasted for at least 6 h. Such a long-lasting increase in CCK-8 release may require a parallel increase in biosynthesis of the peptides as already noted.[28]

The mechanism(s) underlying the opioid facilitation of CCK release are unclear. There is evidence that opioid receptors may facilitate the formation of inositol 1,4,5-phosphate (IP3) through their coupling to phospholipase C. Because IP3 is a strong stimulator of intracellular Ca^{2+} release, the activation of opioid receptors may lead to a Ca^{2+}-dependent CCK release, although it needs to be proven experimentally. It can be argued that the CCK release may not be mediated by a direct action of opioids because generally opioids exert an inhibitory modulation for a given cellular activity. The ability of opioids to facilitate CCK release can therefore be explained by a disinhibitory mechanism. It is possible that both direct and indirect mechanisms mediate CCK release by opioids.

Using an electrophysiological approach, Heinricher et al. showed that, although morphine activated the OFF cell and inhibited the ON cell simultaneously in the RVM, microinjection of CCK within the RVM selectively prevented opioid activation of OFF cells, without affecting the suppression of spontaneous ON-cell firing by morphine.[12]

CCK–OPIOID INTERACTIONS IN PHYSIOLOGICAL FUNCTIONS OTHER THAN PAIN

The extensive presence of CCK in the CNS implies that its function may not be limited to the perception and the control of pain. A role for CCK in the induction and persistence of anxiety and major depression seems to be conspicuous. Hebb et al. summarized that there appears to be an intricate balance between the anxiety-provoking and memory-enhancing effects of CCK on the one hand, and the anxiolytic and amnesic effects of opioid peptides on the other hand.[11]

Gospic et al. tried to observe whether CCK and opioids can modulate the processing of other external signals, for example, visual stimuli-induced emotional experience. The results showed that the CCKB receptor agonist pentagastrin (0.1 μg/kg) increased the rating of unpleasantness for both neutral and unpleasant pictures, whereas the mu opioid receptor agonist remifentanil (0.0625 μ/kg) increased the pleasantness rating for the neutral pictures.[7]

The CCK/opioid interaction seems critical for maintaining maternal behavior during lactation. In contrast to the general expectation, morphine inhibits maternal behavior, whereas CCK restores it. The PAG was found to be the critical brain site responsible for this interaction.[17]

CELLULAR AND MOLECULAR MECHANISMS FOR CCK–OPIOID INTERACTIONS

The cellular and molecular mechanisms underlying the CCK antiopioid action are poorly understood. At the receptor level, Wang et al. have observed that CCK-8 reduced the ligand binding affinity to μ- and κ-opioid receptors. Considerable colocalization of CCK receptors in the dorsal horn neurons of the spinal cord with μ-opioid receptors seems to provide a morphological basis for this receptor–receptor interaction in the same neuron.[33] At the postreceptor level, crosstalk between CCK and opioid-receptor-associated signaling pathways may exist, and the activation of CCK receptor-derived signaling cascades has been found to affect the intracellular calcium availability downstream to opioid receptor activation assessed by electrophysiological[16] and neurochemical approach.[28]

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CCK AS MEDIATOR OF OTHER PEPTIDES WITH ANTIOPIOID EFFECT

Yoshikawa et al. have recently identified two peptides with antiopioid activities. Enterostatin (APGPR)[22] was found in the gastrointestinal tract and brain as an anorectic pentapeptide. ICV injection to the mice inhibited morphine-induced analgesia. This effect could be antagonized by lorglumide and LY225910, antagonists for CCK1 and CCK2 receptors, respectively. Because APGPR did not have affinity for CCK receptors, it was postulated that the antianalgesic effect of APGPR may be mediated by CCK release. Another peptide was rapakinin, Arg-Ile-Tyr.[30] It is a vasorelaxing, antihypertensive, and anorexigenic peptide derived from rapeseed napin. ICV administration of rapakinin to mice inhibited the analgesic effect of morphine. This antiopioid activity seemed to be mediated by the CCK2 receptor, because it was blocked by LY225910, an antagonist of the cholecystokinin CCK2 receptor, but not by lorglumide, an antagonist of the CCK1 receptor. Moreover, the antiopioid activity of rapakinin was also found to be blocked by CAY10441, an antagonist of the prostaglandin (PG) IP receptor. Further studies revealed that rapakinin showed antiopioid activity via the activation of the PGI(2)-IP receptor system followed by the CCK2 receptor system.

These two examples can be taken to show the complicated interaction between biologically active peptides in modulating the opioid/antiopioid interaction, modulating body functions related with pain and pleasure.

REGULATION OF OPIOID ANALGESIA BY NPFF

NPFF (FLFQPQRFamide, F8Famide, Phe-Leu-Phe-Gln-Pro-Gln-Arg-Phe-NH_2) is another important endogenous octapeptide that possesses CCK-like antiopioid action.[34] NPFF and NPFF receptors are densely distributed in the CNS, especially in the spinal dorsal horn and the PAG, areas important in opioid-mediated pain modulation.

ICV injection of NPFF reduced the analgesic effect of systemic morphine, whereas NPFF antisense oligonucleotides (ICV) decreased tolerance to morphine analgesia. Electrophysiological studies also showed an antagonizing effect of NPFF on μ-opioid receptor-mediated inhibition of the dorsal horn neuronal activity evoked by C-fiber stimulation.[28] These results indicate a significant role played by NPFF at the supraspinal level in antagonizing opioid analgesia.

Several brain areas have been identified as important sites for the NPFF-opioid interaction.[28] Local infusion of NPFF into the hippocampus reduced the electrophysiological response of hippocampal neurons to opioids. The NPFF analogs when injected into the nucleus raphe dorsalis also suppressed the antinociceptive effect of morphine. Other central sites where application of NPFF or NPFF analogs produced antiopioid action include the ventral tegmental area (VTA) of the midbrain and the parafascicular nucleus. In contrast to the supraspinal antiopioid effect, IT injection of NPFF produced antinociceptive effect and/or a potentiation of morphine analgesia.

The receptor of NPFF can be divided into two types: NPFF(1) and NPFF(2). The NPFF(2) receptor is mainly distributed in the CNS, especially in brain areas related to pain perception, such as the dorsal horn of the spinal cord, the parafascicular and the dorsolateral nuclei of the thalamus nucleus. However, recent studies have shown that ICV injection of specific agonists for type 1 (NPVF) and type 2 (dNPA), respectively, produced the same antiopioid actions on the antinociceptive effect of systemic morphine as the authentic peptide NPFF, suggesting that the two receptors have a similar effect in this action.[5] Kersanté et al. also studied whether NPFF antagonizes the rewarding effect of opiates. Results showed that systemic morphine-induced elevation of dopamine and serotonin metabolites within the nucleus accumbens (NAc) can be inhibited by NPFF administered into the VTA, suggesting that NPFF in the VTA can also antagonize the rewarding effect of opiates in the mesolimbic dopamine system.[15]

Develliers et al. noted that remarkable similarities in several key antiopioid actions are noted between CCK and NPFF. First, the two peptides when applied alone cause no significant pronociceptive effect; rather they exert a dose-dependent antagonism of opioid-induced analgesia. Second, like CCK and CCK receptors, NPFF and NPFF receptors strategically overlap with the opioid receptor distribution in many brain regions related to pain perception and modulation. Finally, opioids can increase NPFF gene expression and NPFF release. Thus, the NPFF system, like the CCK system, can be upregulated and mobilized to exert its antiopioid action in response to enhanced opioidergic transmission.[28]

REGULATION OF OPIOID ANALGESIA BY NOCICEPTIN/ORPHANIN FQ

N/OFQ and its regulatory role in pain modulation are discussed by Calo in another chapter in this section of the book. The available results show that nociceptin has a complex pharmacology in rodents, eliciting either an antiopioid/hyperalgesic action or analgesic effect depending on the dose, testing paradigm, and brain regions surveyed.

ICV injection of nociceptin blocked the supraspinal antinociception produced by the injection of all three opioid receptor subtypes (μ, δ, and κ)-selective agonists administered via the same route.[18] Nociceptinergic terminals are located in the PAG, and intra-PAG injection of nociceptin

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attenuates morphine analgesia, indicating the PAG as a site for nociceptin to antagonize opioid actions. Recent studies showed that nociceptin also antagonizes analgesia induced by nonopioid substances such as paracetamol[21] and serotonin (5-HT). Therefore, it is not only an antiopioid peptide but may also be an antianalgesic peptide.

In contrast to its supraspinal effect, IT nociceptin showed no antagonistic effect against antinociception induced by IT or systemic injection of morphine. In fact, nociceptin *per se* produced an antinociceptive effect in the spinal cord.[24] Recent findings revealed that IT injection of a specific nociceptin receptor agonist UFP-112 produced long-lasting antinociceptive effects that could be reversed by an NOP receptor antagonist J-113397 but not by a classic opioid receptor antagonist naltrexone. IT injection of inactive doses of UFP-112 in combination with a small dose of morphine produced significant and long-lasting antinociceptive effects without increasing the adverse effects such as scratching responses.[13]

Yuan et al. showed that the continuous use of high doses of morphine accelerated the release and biosynthesis of nociceptin in rat brain, which antagonizes the analgesic effect of opioids and promotes the development of morphine tolerance. Besides, administration of the nociceptin or synthetic agonist Ro 64–6198 can block the rewarding effects of cocaine, morphine, amphetamines, and alcohol in various behavioral models of drug reward and reinforcement, such as conditioned place preference and drug self-administration. Addiction to multiple abused drugs (polydrug addiction) is now very common, and no satisfactory pharmacotherapies are yet available. In this sense, the NOP receptor may serve as a target for drug abuse medications.[31]

The mechanisms underlying the antiopioid effect of nociceptin are not clear. Rady and Fujimoto[20] pointed out that the antianalgesic action of nociceptin is mediated by spinal PGE2 and attenuated by IT PGD2 or indomethacin. At the cellular level, μ-opioid receptor-mediated inhibition of calcium currents can be antagonized by nociceptin applied at the same neuron.[32] At the circuitry level, nociceptin, which profoundly inhibits all three classes of RVM neurons (ON, OFF, and neutral), blocks the opioid activation of OFF cells and interferes with opioid antinociception.[12]

REGULATION OF μ-OPIOID SYSTEMS BY MIF-1, TYR-MIF-1, AND TYR-W-MIF-1

The first endogenous peptide to show antiopioid properties was reported by Kastin et al. in 1979 with MIF-1 (Pro-Leu-Gly-NH_2).[14] Two more related endogenous brain peptides, Tyr-MIF-1 and Tyr-W-MIF-1 (Tyr-Pro-Trp-Gly-NH_2), were later also shown to have antiopioid activity. These peptides show inhibitory actions not only against endogenous opioid activity but also against several pharmacological effects of morphine, including antinociception,[14] hypothermia, and inhibition of guinea pig ileum contractions. Their antiopioid properties seem to be mediated by μ-opioid receptors because these peptides show low, but significant, affinity and very high selectivity for μ-opioid receptors. Correlating with their affinity to μ-opioid receptors, their inhibitory effects against morphine are relatively weaker than those of traditional μ-opioid receptor antagonists, such as naloxone or CTOP.[28]

Yehuda et al. revealed that these peptides show an inverted U-shaped dose–response curve for the inhibition of morphine-induced pharmacological effects. As expected, most of these peptides later were reported to have agonistic properties for μ-opioid receptors in several experimental paradigms, including analgesia[29] and electrically stimulated guinea pig ileum contraction. Their agonistic effects are completely attenuated by μ-opioid, but not δ- and κ-opioids, receptor antagonists. Moreover, the potencies of their agonistic effects are relatively lower than those of morphine. The evidence clearly suggests that these peptides are partial agonists for μ-opioid receptors with relatively low intrinsic activity and affinity, although effects at the Tyr-MIF-1 receptor itself may be involved. Physiologically, these peptides may biphasically, both negatively and positively, control the μ-opioid system as a partial agonist. Bocheva et al. reported that IP injection of Tyr-MIF-1 peptides (all in a dose of 1 mg/kg i.p.) in the male Wistar rats reduced morphine-induced analgesia using the paw-pressure (PP) and the tail-flick (TF) tests, and also the immobilization stress-induced antinociception using the PP test. Doses as low as 0.01 mg/kg have also be effective in studies of the antiopioid effects of these small peptides in rodents, and MIF-1 significantly reduced the effects of morphine in a clinical study in men. The results suggest that overall, Tyr-MIF-1 peptides can manifest themselves as functional antiopioid peptides.[2]

CONCLUSIONS

Mollereau et al.[34] noted that CCK and NPFF produce an antiopoid effect via cellular mechanisms, whereas N/OFQ, showing antiopioid effects in the brain and proopioid effects in the spinal cord, seems to act via circuitry-induced mechanism. Antiopioid effects have also been reported for MSH (melanocortin—see the chapter in Brain Peptides section), TRH, complement 3a, angiotensin II, enterostatin, and rapakinin, the effects of last two being mediated through CCK. Although there has been much progress since the first description of antiopioid effects of peptides in 1979 with a member of the Tyr-MIF-1 family of brain peptides,

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elucidation of the opioid-modulating effects of peptides such as the ones described here should stimulate novel means to alleviate pain and treat opioid abuse.

ACKNOWLEDGMENTS

This study was supported by a grant (2007CB512500) from the Ministry of Science and Technology, PR China to the author. Sincere thanks are due to Dr. A. Kastin for his detailed corrections and Dr. M. Yoshikawa for help in collection of information.

REFERENCES

1. Benedetti F, Amanzio M, Vighetti S, Asteggiano G. The biochemical and neuroendocrine bases of the hyperalgesic nocebo effect. *J Neurosci* 2006;**26**:12014–22.
2. Bocheva A, Dzambazova-Maximova E. Antiopioid properties of the TYR-MIF-1 family. *Methods Find Exp Clin Pharmacol* 2004;**26**:673–7.
3. Dourish CT, O'Neill MF, Coughlan J, Kitchener SJ, Hawley D, Iversen SD. The selective CCK-B receptor antagonist L-365,260 enhances morphine analgesia and prevents morphine tolerance in the rat. *Eur J Pharmacol* 1990;**176**:35–44.
4. Erchegyi J, Kastin AJ, Zadina JE. Isolation of a novel tetrapeptide with opiate and antiopiate activity from human brain cortex: Tyr-Pro-Trp-Gly-NH2 (Tyr-W-MIF-1). *Peptides* 1992;**13**:623–31.
5. Fang Q, Jiang TN, Li N, Han ZL, Wang R. Central administration of neuropeptide FF and related peptides attenuate systemic morphine analgesia in mice. *Protein Pept Lett* 2011;**18**:403–9.
6. Faris PL, Komisaruk BR, Watkins LR, Mayer DJ. Evidence for the neuropeptide cholecystokinin as an antagonist of opiate analgesia. *Science* 1983;**219**:310–2.
7. Gospic K, Gunnarsson T, Fransson P, Ingvar M, Lindefors N, Petrovic P. Emotional perception modulated by an opioid and a cholecystokinin agonist. *Psychopharmacology (Berl)* 2008;**197**: 295–307.
8. Han JS. Cholecystokinin octapeptide (CCK-8): a negative feedback control mechanism for opioid analgesia. *Prog Brain Res* 1995;**105**:263–71.
9. Han JS, Terenius L. Neurochemical basis of acupuncture analgesia. *Ann Rev Pharmacol Toxicol* 1982;**22**:193–220.
10. Han NL, Luo F, Bian ZP, Han JS. Synergistic effect of cholecystokinin octapeptide and angiotensin II in reversal of morphine induced analgesia in rats. *Pain* 2000;**85**:465–9.
11. Hebb AL, Poulin JF, Roach SP, Roach SP, Zacharko RM, Drolet G. Cholecystokinin and endogenous opioid peptides: interactive influence on pain, cognition, and emotion. *Prog Neuropsychopharmacol Biol Psychiat* 2005;**29**:1225–38.
12. Heinricher MM, McGaraughty S, Tortorici V. Circuitry underlying antiopioid actions of cholecystokinin within the rostral ventromedial medulla. *J Neurophysiol* 2001;**85**:280–6.
13. Hu E, Calò G, Guerrini R, Ko MC. Long-lasting antinociceptive spinal effects in primates of the novel nociceptin/orphanin FQ receptor agonist UFP-112. *Pain* 2010;**148**:107–13.
14. Kastin AJ, Olson RD, Ehrensing RH, Berzas MC, Schally AV, Coy DH. MIF-1's differential actions as an opiate antagonist. *Pharmacol Biochem Behav* 1979;**11**:721–3.
15. Kersanté F, Wang JY, Chen JC, Mollereau C, Zajac JM. Anti-opioid effects of neuropeptide FF receptors in the ventral tegmental area. *Neurosci Lett* 2011;**488**:305–9.
16. Liu NJ, Xu T, Xu C, Li CQ, Yu YX, Kang HG, et al. Cholecystokinin octapeptide reverses mu-opioid-receptor-mediated inhibition of calcium current in rat dorsal root ganglion neurons. *J Pharmacol Exp Ther* 1995;**275**:1293–9.
17. Miranda-Paiva CM, Canteras NS, Sukikara MH, Nasello AG, Mackowiak II, Felicio LF. Periaqueductal gray cholecystokinin infusions block morphine-induced disruption of maternal behavior. *Peptides* 2007;**28**:657–62.
18. Mogil JS, Grisel JE, Zhangs G, Belknap JK, Grandy DK. Functional antagonism of mu-, delta- and kappa-opioid antinociception by orphanin FQ. *Neurosci Lett* 1996;**214**:131–4.
19. Mollereau C, Roumy M, Zajac JM. Opioid-modulating peptides: mechanisms of action. *Curr Top Med Chem* 2005;**5**:341–55.
20. Rady JJ, Fujimoto JM. Nociceptin and dynorphin A(1–17) produce antianalgesia through independent systems in mice. *Life Sci* 2002;**70**:1085–93.
21. Sandrini M, Vitale G, Pini LA, Lopetuso G, Romualdi P, Candeletti S. Nociceptin/orphanin FQ prevents the antinociceptive action of paracetamol on the rat hot plate test. *Eur J Pharmacol* 2005;**507**:43–8.
22. Takenaka Y, Shimano T, Yamada Y, Yoshida M, Ohinata K, Yoshikawa M. Enterostatin (APGPR) suppresses the analgesic activity of morphine by a CCK-dependent mechanism. *Peptides* 2008;**29**(4):559–63.
23. Tang NM, Dong HW, Wang XM, Tsui ZC, Han JS. Cholecystokinin antisense RNA increases the analgesic effect induced by electroacupuncture or low dose morphine: conversion of low responder rats into high responders. *Pain* 1997;**71**:71–80.
24. Tian JH, Xu W, Fang Y, Mogil JS, Grisel JE, Grandy DK, et al. Bidirectional modulatory effect of orphanin FQ on morphine-induced analgesia: antagonism in brain and potentiation in spinal cord of the rat. *Br J Pharmacol* 1997;**120**:676–80.
25. Torres-López JE, Juárez-Rojop IE, Granados-Soto V, Diaz-Zagoya JC, Flores-Murrieta FJ, Ortíz-López JU, et al. Peripheral participation of cholecystokinin in the morphine-induced peripheral antinociceptive effect in non-diabetic and diabetic rats. *Neuropharmacology* 2007;**52**:788–95.
26. Tortorici V, Nogueira L, Salas R, Vanegas H. Involvement of local cholecystokinin in the tolerance induced by morphine microinjections into the periaqueductal gray of rats. *Pain* 2003;**102**:9–16.
27. Veraksits A, Runkorg K, Kurrikoff K, Raud S, Abramov U, Matsui T, et al. Altered pain sensitivity and morphine-induced anti-nociception in mice lacking CCK2 receptors. *Psychopharmacology* 2003;**166**:168–75.
28. Wang JQ, Fibuch EE, Sakurada S, Han JS. Anti-opioid peptides. In: *Biologically active peptides*. Elsevier; 2006 [Chapter 187], 1345–50.
29. Watanabe H, Nakayama D, Ito K, Watanabe C, Mizoguchi H, Fujimura T, et al. A Tyr-W-MIF-1 analog containing D-Pro2 acts as a selective μ2-opioid receptor antagonist in the mouse. *J Pharmacol Exp Ther* 2005;**312**:1075–81.
30. Yamada Y, Ohinata K, Lipkowski AW, Yoshikawa M. Rapakinin, Arg-Ile-Tyr, derived from rapeseed napin, shows anti-opioid activity via the prostaglandin IP receptor followed by the cholecystokinin CCK(2) receptor in mice. *Peptides* 2011;**32**:281–5.

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31. Zaveri NT. The Nociceptin/Orphanin FQ Receptor (NOP) as a target for drug abuse medications. *Curr Top Med Chem* 2011;**11**:1151–6.
32. Zhang M, Wang XM, Zhang DB, Xu GH, Dong HW, Yu YX, et al. Orphanin FQ antagonizes the inhibition of Ca2 currents induced by μ-opioid receptors. *J Mol Neurosci* 2005;**25**:21–8.
33. Zhang X, Lucas GA, Elde R, Wiesenfeld-Hallin Z, Hokfelt T. Effect of morphine on cholecystokinin and μ-opioid receptor-like immunoreactivities in rat spinal dorsal horn neurons after peripheral axotomy and inflammation. *Neuroscience* 2000;**95**:197–207.
34. Zhang W, Gardell S, Zhang D, Xie JY, Agnes RS, Badghisi H, et al. Neuropathic pain is maintained by brainstem neurons co-expressing opioid and cholecystokinin receptors. *Brain* 2009;**132**(Pt 3):778–87.

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Chapter 211

Casomorphins/Hemorphins

Fred Nyberg, Anna Carlsson and Mathias Hallberg

ABSTRACT

In addition to classical opioid compounds with their N-terminal Tyr-Gly-Gly-Phe sequence, atypical opioid peptides typified by their N-Terminal Tyr-Pro are derived by the partial hydrolysis of endogenous existing functional proteins and are mainly produced in peripheral tissues and body fluids. The β-casomorphins and the hemorphins are formed through the hydrolysis of the milk protein β-casein and hemoglobin, respectively. They are less potent compared with the classical opioid peptides but share most of their typical effects. Among the atypical endogenously produced opioid active peptides are the β-casomorphins and hemorphins. Their concentrations in body fluids greatly exceed those of β-endorphin and the enkephalins. This chapter gives a brief review about past and current research on the β-casomorphins and hemorphins regarding the mechanisms for their release, their distribution, their biological actions, and their pathophysiological roles.

INTRODUCTION

The endogenous opioids are derived from at least three genetically distinct systems giving rise to several active peptides, including β-endorphin, the dynorphins, and the enkephalins. The active structures in these peptides are all typified by their N-terminal Tyr-Gly-Gly-Phe tetrapeptide sequence. However, atypical opioid active peptides containing the Tyr-Pro dipeptide residues in their N-terminal sequence have been identified and characterized. These so-called atypical endogenously produced opioid active peptides share most of their typical effects with the classical opioid peptides. Originally, the β-casomorphins and the hemorphins were isolated from enzymatically treated bovine milk and blood, respectively. Later they were identified as naturally occurring compounds present in high concentrations in human plasma and cerebrospinal fluid.

DISCOVERY OF THE β-CASOMORPHINS AND THE HEMORPHINS

Soon after the discovery of opioid receptors in mammalian brain, the presence of endogenous compounds acting on these receptors were identified and found to be present in the central nervous system (CNS) and also in peripheral tissues and in a variety of body fluids. Interestingly, most opioid peptides, which are present in the CNS, have also been identified in the cerebrospinal fluid (CSF).[22] The CNS distribution of the well-known β-endorphin, enkephalins, dynorphins, and endomorphins are described in other chapters in this section of this book.

The presence of opioid active material in bovine milk was originally reported by Brantl et al.[1] They isolated a peptide fragment from an enzymatic digest of the milk protein β-casein. This fragment exhibited binding affinity for opioid receptors and was shown to produce opioid effects in the guinea-pig ileum assay (GPI assay). Because of this and its origin, the isolated peptide was named β-casomorphin. Sequence analysis of β-casomorphin revealed that the structure of this compound diverges from that of the classical endogenous opioids, in that the milk-derived peptide contains a Pro residue next to the N-terminal Tyr (Table 1). In subsequent studies, β-casomorphin was found to exist in bovine plasma[34] as a naturally occurring compound in both truncated and extended forms of the first isolated entity and also in human body fluids. For instance, β-casomorphin-8-like immunoreactivity was measured in human plasma,[15,23] in human CSF,[22,23] and in human milk.[29]

The β-casomorphins are fragments of the bovine β-casein sequence 60–68 and in sheep, water buffalo, and human whey, they are found in analogous positions. In human β-casein the casomorphin unit also resides in the sequence 60–68. Moreover, the human β-casomorphin differs from its bovine analog by having a Val-Glu instead of Pro-Gly in positions 4–5 (Table 1). An analog, β-casomorphin-4 amide (named morphiceptin, Table 1) isolated from a commercial hydrolysate of bovine casein, appears as a highly potent and μ-opioid receptor (MOP) receptor selective peptide.[3] Further, opioid active peptides in milk lacking the Tyr-Pro sequence in their N-terminal have also been found. Among these are the Arg-Tyr-Leu-Gly-Tyr-Leu-Glu sequence from bovine α-casein, the whey protein-derived α-lactorphin (Tyr-Gly-Leu-Phe), and β-lactorphin (Tyr-Leu-Leu-Phe). These last peptides behave as MOP receptor agonists but exhibit much weaker potency than that of the β-casomorphins.

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00211-6
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TABLE 1 The Amino Acid Sequence of β-Casomorphins, Hemorphins, and Related Peptides

Peptide	Amino acid sequence
β-Casomorphin-5 (bovine)	H-Tyr-Pro-Phe-Pro-Gly-OH
β-Casomorphin-7 (bovine)	H-Tyr-Pro-Phe-Pro-Gly-Pro-Ile-OH
β-Casomorphin-8 (bovine)	H-Tyr-Pro-Phe-Pro-Gly-Pro-Ile-Pro-OH
β-Casomorphin-5 (human)	H-Tyr-Pro-Phe-Val-Glu-OH
β-Casomorphin-7 (human)	H-Tyr-Pro-Phe-Val-Glu-Pro-Ile-OH
β-Casomorphin-8 (human)	H-Tyr-Pro-Phe-Val-Glu-Pro-Ile-Pro-OH
Morphiceptin	H-Tyr-Pro-Phe-Pro-NH_2
Hemorphin-4 (human)	H-Tyr-Pro-Trp-Thr-OH
Hemorphin-7 (human)	H-Tyr-Pro-Trp-Thr-Gln-Arg-Phe-OH
LVV-Hemorphin-4	H-Leu-Val-Val-Tyr-Pro-Trp-Thr-OH
LVV-Hemorphin-6	H-Leu-Val-Val-Tyr-Pro-Trp-Thr-Gln-Arg-OH
LVV-Hemorphin-7	H-Leu-Val-Val-Tyr-Pro-Trp-Thr-Gln-Arg-Phe-OH
Valorphin	H-Val-Val-Tyr-Pro-Trp-Thr-Gln-OH
Spinorphin	H- Leu-Val-Val-Tyr-Pro-Trp-Thr-OH
Endomorphin-1	H-Tyr-Pro-Trp-Phe-NH_2
Endomorphin-2	H-Tyr-Pro-Phe-Phe-NH_2

The first report on the hemorphins also came from Brantl and coworkers.[2] This time they isolated and determined the sequence of an opioid active tetrapeptide (Tyr-Pro-Trp-Thr) from bovine blood treated with gastrointestinal enzymes. From a computer search, this peptide was identified as fragment 34–37 in the β-chain of bovine hemoglobin. In analogy with β-casomorphin, this tetrapeptide was named hemorphin-4, and it was also found to reside in positions 35–38 of the β-, k-, ∂-, and ε-chains of human hemoglobin. During the past decades, many opioid active peptides containing the Tyr-Pro-Trp-Thr core have been identified and characterized (Table 1). The existence of naturally occurring peptides containing the hemorphin-4 sequence in humans was reported in 1991.[10] In subsequent studies, similar structures in human CSF[11] and plasma[12] were found. All these hemorphin structures (LVV-hemorphin-6, LVV-hemorphin-7, and hemorphin-7) shown to retain biological activity were found to exhibit affinity for opioid receptors. Among other fragments containing the hemorphin-4 sequence shown to be released from the β-chain of hemoglobin are peptides given the names valorphin[7] and spinorphin (Table 1).

THE FORMATION OF β-CASOMORPHINS AND HEMORPHINS

In contrast to the classical opioid peptides, the pathways for the formation of the atypical opioids β-casomorphins and hemorphins are not yet fully clarified. For instance, although the enkephalins or dynorphins are formed within and released from neurons in which their precursors are processed the atypical opioids are not necessarily formed at the site of their precursor production. Prestages or intermediately processed entities of both β-casomorphins and hemorphins have been identified in the circulation[12,23] and CSF.[11,23] Extended forms of β-casomorphin-8 were found both in the CSF[23] and in the blood.[15,23] Therefore, the release of β-casomorphins from β-casein was suggested to occur stepwise. In CSF, a radioimmunoassay (RIA)-active component chromatographically characterized as β-casomorphin-8 was identified in addition to a larger sized RIA-active congener.[22,23] In plasma and milk, the β-casomorphin-8-like RIA activity was mainly derived from a structure that is of a larger size than the octapeptide.[23] However, in a later study, the presence of β-casomorphin-8 in milk from a patient with postpartum psychosis was confirmed.[29]

The presence of large fragments of the β-chain of hemoglobin containing the hemorphin sequence in human pituitaries[10] and plasma[12] was believed to indicate that the blood protein subunit is processed or degraded in sequential steps. It was thus suggested that a chymotryptic-like cleavage at the Phe^{41}–Phe^{42} bond in the β-chain was followed by a similar cleavage to generate LVV-hemorphin-7,[24] which can be further hydrolyzed by exopeptidases to yield smaller hemorphin peptides.[17] Further, the role of macrophage proteolytic complexes in the hemorphin generation has been investigated. When hemoglobin was exposed to rat liver lysosomes a rapid release of hemorphin-related peptides including hemorphin-7, LVV-hemorphin-7, and VV-hemorphin-7 was observed.[9]

RECEPTOR SELECTIVITY AND ITS MODULATION

Like most of the atypical opioid peptides containing the Tyr-Pro dipeptide in their N-terminal sequence, the β-casomorphins and the hemorphins show binding preference for the MOP receptor,[1,2,10] The peptides seem to act agonistically on this opioid receptor subunit as shown by means of the GTPγS assay. Their opioid activity was also confirmed in the GPI –bioassay.[1,8,10] Although these peptides are found

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endogenously in quite high amounts, their agonistic actions on opioid receptors are comparatively weak. For instance, compared with β-endorphin, the affinity of the hemorphins for the MOP receptor is about 500-fold lower. Interestingly, two more recently discovered opioid peptides, endomorphin-1 and endomorphin-2,[35] with a structure (Table 1) similar to that of hemorphin and β-casomorphin, respectively, exhibit a very high potency to stimulate the MOP receptor.

Studies have also indicated that the β-casomorphins may act on non-opioid sites. For example, the bovine pentapeptide was shown to induce behavioral effects in the rat, which was not completely reversed by appropriate doses of naloxone.[14]

In recent years, studies have revealed that LVV-hemorphin-7 may act as an endogenous ligand for the insulin-regulated aminopeptidase (IRAP)/AT4 receptor in the brain.[20] The AT4 receptor was originally defined as the specific, high-affinity binding site for angiotensin (ang) IV (Val-Tyr-Ile-His-Pro-Phe), a bioactive fragment derived from Ang II. Ang IV is known to be released from both Ang I and Ang II. In addition to Ang IV, the hemoglobin fragment LVV-hemorphin-7 exhibits high binding affinity to the IRAP/AT4 receptor.[25] Both LVV-hemorphin-7 and Ang IV are competitive inhibitors of this enzyme activity and do not serve as substrates of the IRAP.[13] Moreover, central administration of LVV-hemorphin-7 enhances learning and memory capabilities in normal rodents and reverses memory deficits observed in animal models of amnesia.[13] The presence of high-affinity binding sites for this peptide was observed in the brain, including areas such as the hippocampus that are involved in memory processing.

BIOLOGICAL ACTIONS AND FUNCTIONAL RELEVANCE OF THE β-CASOMORPHINS AND HEMORPHINS

Like other endogenous opioid peptides, the β-casomorphins and hemorphins retain several effects related to opiates. These include analgesic effects and effects on respiration, constipation, sedative effects as well as effects on various behaviors. Analgesic effects of the β-casomorphins have been demonstrated in animals subjected to intracerebral injections of the peptides.[9] Likewise, in an animal model for phasic nociception (tail-flick assay), both hemorphin-4 and hemorphin-5 produce a dose-related and naloxone-reversible antinociceptive effect in the mice.[5]

Specific Actions and Functions of the β-Casomorphins

The β-casomorphins have been shown to produce effects in the gastrointestinal tract. For instance, β-casomorphin-7 markedly induces mucus release in the rat jeujenum,[33] an effect leading to the protection of the mucosa. The effect, which is dose dependent, is reversible by naloxone. It was suggested that the peptides from dairy products provide dietary prospects for improving gastrointestinal protection.[33] Given orally, the β-casomorphins influence postprandial metabolism by stimulating the secretion of insulin and somatostatin and take part in the regulation of postprandial gastrointestinal endocrine function.[30] Also, the β-casomorphins and their derivatives prolong gastrointestinal transit time and are useful in the treatment of diarrhea.[6] The β-casomorphins also affect hypoxia. Studies on the effects of β-casomorphins on immediate and delayed changes in electrocardiography (ECG) in female rats subjected to acute hypobaric hypoxia during pregnancy were performed.[19] Hypoxia induces alterations in the structure of the EKG that reflect development of arrhythmias and conduction disturbances. Intranasal administration of β-casomorphin-7 to pregnant female rats promotes recovery from acute hypoxia and normalized the EKG in the posthypoxic period.

Studies examining the ability of β-casomorphins to induce various behaviors have been reported.[14,25] The effect on rotational behavior in rat of β-casomorphins (bovine and human origin) was compared with that produced by three reference compounds, morphine, D-ala^2-D-leu^5-enkephalin, and U50,488H, ligands for MOP, DOP, and KOP types of opioid receptors, respectively. In this assay, the bovine β-casomorphin-5 seems to be more potent than a human analog (β-casomorphin-4).[14] The effects of both morphine and bovine β-casomorphin-5 in producing rotational behavior are partly antagonized by naloxone.

With respect to their functional relevance, it has been speculated that the β-casomorphins may be involved in the interplay between mother and child during lactation. Studies dealing with these milk-derived peptides have been directed to both mothers and infants. At term pregnancy, β-casomorphin-8 is elevated in both plasma and CSF, and this elevation further increases six months later in the puerperal period.[23] Interestingly, there is a positive correlation between plasma and CSF concentrations and CSF levels of the peptide. This indicates that β-casomorphin crosses the breast parenchyma–blood barrier into plasma and subsequently penetrate the blood–brain barrier to reach the CNS. It was suggested that the peptide may have a function in maternal behavior.[23] Further, the studies suggested that the milk-derived opioid also may reach the brain of the infant. Using a peptide extraction procedure combined with high-performance liquid chromatography and RIA, Pasi et al. [26] detected rostrocaudally increasing levels of immunoreactive β-casomorphin-8 in functionally relevant brain areas including mesencephalon, pons cerebri, and medulla oblongata. Indeed, it has been discussed that the β-casomorphins also may affect the child during lactation and it has been assumed that the milk opioids may induce sedation.

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Specific Actions and Functions of the Hemorphins

Several actions have been studied with regard to the hemorphins. They affect blood pressure,[21] produce anti-inflammatory effects,[24] and affect the immune system.[22] The effect of hemorphin-7 on inflammatory processes is probably mediated through its action on the MOP receptor. Using a blister model of inflammation in the rat hind footpad local administration of the heptapeptide inhibits the acute but not chronic peripheral inflammatory response.[24] The effects are reversible by naloxone. The vasoconstrictive effect may result from the ability of the peptides to inhibit angiotensin converting enzyme (ACE). A potent inhibitory effect on ACE has been shown for hemorphin-7 and its N-terminal extensions LVV-hemorphin-7 but also for LVV-hemorphin-4 and –6.[17] Thus, the hemorphins not only interact with ACE but also with the previously mentioned IRAP/AT4 receptor, again showing a coupling to the angiotensin system.

The hemorphins induce the release of β-endorphins in the pituitary or hypothalamus,[24] and in humans a concomitant enhancement of hemorphin-7 and β-endorphin was observed after a completed marathon run[12] and in patients subjected to open-heart surgery.[24] In the case of marathon runners, a significant positive correlation between the plasma concentrations of hemorphin-7 and β-endorphin was observed. This relationship suggests a link between the heptapeptide and β-endorphin as later found in studies of patients undergoing open-heart surgery using a heart-lung machine.[24] During this treatment, which continues for several hours, the red blood cells are lysed as a result of their mechanical contact with the instrument and hemoglobin is released into the arterial blood flow and degraded by enzymes to generate hemorphins. Indeed, RIA-detectable hemorphin-7 is increased in the arterial blood flow with time but not in the jugular venous bulb. By contrast, the concentration of RIA-active β-endorphin in the arterial blood did not change during surgery but was significantly increased over time in blood collected from the venous bulb.[24] It is known that patients subjected to cardiopulmonary bypass generally have a sense of well-being during the first postoperative day and this could be explained by the released peptides, which may reach brain nuclei associated with reward and produce euphoria through their actions on opioid receptors.

IMPLICATION OF β-CASOMORPHINS AND HEMORPHINS IN PATHOPHYSIOLGY

There are still limited studies on the β-casomorphins and hemorphins in relation to pathophysiology. The β-casomorphins have been suggested to be involved in the mechanism underlying childhood autism[25] and apnea in relation to sudden infant death (SID).[28] A review article highlighted the possible link between β-casomorphins and SIDs in the context of the passage of these peptides through the gastrointestinal tract and the blood–brain barrier.[32] Thus, it was suggested that in infants predisposed to respiratory apnea because of abnormal autonomic nervous system development and respiratory control mechanisms, milk-derived opioid peptides such as the β-casomorphins might be one of the etiological factors for the SID syndrome.[28] Another aspect studied in relation to β-casomorphins is their possible role in psychomotor development in infants. It has been suggested that breast feeding may have an advantage over feeding with cow milk for the development of infants during their first year of life.[16] Thus, increased plasma concentrations of both bovine and human immunoreactive β-casomorphin were observed mainly during the first 3 months of life. High levels of basal peptide activity were observed in breast-fed infants with a normal muscle tone and psychomotor development. Contrarily, increased basal levels of bovine β-casomorphin were detected in infants fed with cow milk, and these individuals showed a heightened muscle tone and a delayed psychomotor development.[16] This finding was considered to give support for the idea that bovine β-casomorphin may influence the infant's psychomotor development.[16]

The β-casomorphins have also been implicated in allergic reactions. In a study of breast-feeding women having a history and clinical manifestation of food allergy, the levels of β-casomorphin-5 and -7 were measured in their colostrum and milk and compared with those assessed in healthy age-matched breast-feeding controls.[31] The results from this study show that the peptide concentrations in colostrum and milk of these groups of women exhibit a diverse profile with highest levels of the opioid peptides in colostrums of healthy women followed by a rapid decrease in their mature milk, whereas the levels in women with food allergy remained constant during lactation. It was suggested that the content of the β-casomorphins in their milk could serve as an indicator of allergic conditions in the lactating women.[31]

Further, studies have implicated β-casomorphin-like peptides in postpartum psychosis.[18] In fact, the presence of high levels of β-casomorphin-8 in milk from a patient with postpartum psychosis has been reported.[29] A more recent study has indicated that the levels of β-casomorphin in plasma and milk samples are elevated in women with mastitis and that there is a relationship between this complication and the prevalence of postpartum psychosis (Righard, Carlsson, and Nyberg, unpublished data).

As mentioned above, the hemorphins have been found at high levels within the central and peripheral nervous systems. In addition to opioid-receptor binding, hemorphins have many effects on the renin–angiotensin system, including the inhibition of ACE[17] and IRAP/AT4 receptor binding.[20]

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However, only a few studies have examined the role of hemorphins in pathophysiology. A study[27] of hemorphins in Alzheimer's disease (AD) and control brains measured the levels of both LVV- and VV-hemorphin-6 and -7 by quantitative MALDI-TOF mass spectrometry. The authors found that LVV-hemorphin-6 and total hemorphin levels are elevated in AD temporal neocortex but not in the hippocampus, occipital lobe, or frontal lobe. They suggested that the observed elevation of hemorphins could reflect a vascular abnormality resulting from cerebral amyloid angiopathy associated with both neurodegenerative disease and aging.[27] Increased concentrations of hemorphins in the circulatory system, also mentioned above, have been seen under certain physiological[4] and clinical conditions, such as hemodialysis and during open-heart surgery.[24] Finally, during the past few years it has been reported that the serum concentrations of hemorphins in patients with diabetes are reduced.[8] Some studies have attempted to investigate the biochemical mechanisms that may underlie this anomaly through studying the influence of hemoglobin glycation on hemorphin generation.[8] Another aspect of the involvement of hemorphins in pathophysiology arises from the observations that their N-terminal extensions LVV-hemorphin-7 and VV-hemorphin-7 may interact with IRAP/AT4 receptors. Effects mediated through these sites are involved in cognitive function. Thus, the extended hemorphins may constitute a group of compounds suitable for the development of new therapeutic agents, which may be used for various cognitive disorders.[13]

CONCLUDING REMARKS

Although the past decades have seen much progress in opioid peptide research, there are comparatively few studies directed to the atypical opioid peptides, like β-casomorphins and hemorphins, and their role in physiology or pathophysiology. They are produced in relatively high amounts, but their weak activity on opioid receptors may have resulted in less attention. However, currently, the β-casomorphins seem to be receiving new interest regarding their role in allergic reactions[31] and in apnea related to SID.[32] The hemorphins have become very attractive compounds since their link to the renin–angiotensin system was discovered.[13] Therefore, it is probable that both these peptide systems will receive renewed interest in the years to come.

ACKNOWLEDGMENTS

This work was supported by the Swedish Medical Research Council (Grant 9459).

REFERENCES

1. Brantl V, Teschemacher H, Blasig J, Henschen A, Lottspeich F. Opioid activities of beta-casomorphins. *Life Sci* 1981;**28**:1903–9.
2. Brantl V, Gramsch C, Lottspeich F, Mertz R, Jaeger KH, Herz A. Novel opioid peptides derived from hemoglobin: hemorphins. *Eur J Pharmacol* 1986;**125**:309–10.
3. Chang KJ, Killian A, Hazum E, Cuatrecasas P. Morphiceptin: a potent and specific agonist for morphine (m) receptors. *Science* 1981;**212**:75–7.
4. Collinder E, Nyberg F, Sanderson-Nydahl K, Gottlieb-Vedi M, Lindholm A. The opioid haemorphin-7 in horses during low-speed and high-speed treadmill exercise to fatigue. *J Vet Med* 2005;**52**:162–5.
5. Davis TP, Gillespie TJ, Porreca F. Peptide fragments derived from the beta-chain of hemoglobin (hemorphins) are centrally active in vivo. *Peptides* 1989;**10**:747–51.
6. Daniel H, Vohwinkel M, Rehner G. Effect of casein and b-casomorphins on gastrointestinal motility in rats. *J Nutr* 1990;**120**:252–7.
7. Erchegyi J, Kastin AJ, Zadina JE, Qiu XD. Isolation of a heptapeptide Val-Val-Tyr-Pro-Trp-Thr-Gln (valorphin) with some opiate activity. *Int J Pept Protein Res* 1992;**39**:477–84.
8. Feron D, Piot JM, Fruitier-Arnaudin I. Proteolytic degradation by cathepsin D of glycated hemoglobin from diabetes patients gives rise to hemorphin-7 peptides. *Peptides* 2010;**31**:956–61.
9. Fruitier I, Garreau I, Lacroix A, Cupo A, Piot JM. Proteolytic degradation by endogenous lysosomal proteases gives rise to bioactive peptides: hemorphins. *FEBS Lett* 1999;**447**:81–6.
10. Glamsta EL, Marklund A, Hellman U, Wernstedt C, Terenius L, Nyberg F. Isolation and characterization of a hemoglobin-derived opioid peptide from the human pituitary gland. *Regul Pept* 1991;**34**:169–79.
11. Glamsta EL, Nyberg F, Silberring J. Application of fast-atom bombardment mass spectrometry for sequencing of a hemoglobin fragment, naturally occurring in human cerebrospinal fluid. *Rapid Commun Mass Spectrom* 1992;**6**:777–80.
12. Glamsta EL, Morkrid L, Lantz I, Nyberg F. Concomitant increase in blood plasma levels of immunoreactive hemorphin-7 and beta-endorphin following long distance running. *Regul Pept* 1993;**49**:9–18.
13. Hallberg M. Targeting the insulin-regulated aminopeptidase/AT4 receptor for cognitive disorders. *Drug News Perspect* 2009 Apr;**22**(3): 133–9:Review.
14. Herrera-Marschitz M, Terenius L, Grehn L, Ungerstedt U. Rotational behaviour produced by intranigral injections of bovine and human beta-casomorphins in rats. *Psychopharmacology (Berl)* 1989;**99**:357–61.
15. Koch G, Wiedemann K, Drebes E, Zimmermann W, Link G, Teschemacher H. Human beta-casomorphin-8 immunoreactive material in the plasma of women during pregnancy and after delivery. *Regul Pept* 1988;**20**:107–17.
16. Kost NV, Sokolov OY, Kurasova OB, Dmitriev AD, Tarakanova JN, Gabaeva MV, et al. Beta-casomorphins-7 in infants on different type of feeding and different levels of psychomotor development. *Peptides* 2009;**30**:1854–60.
17. Lantz I, Glamsta EL, Talback L, Nyberg F. Hemorphins derived from hemoglobin have an inhibitory action on angiotensin converting enzyme activity. *FEBS Lett* 1991;**287**:39–41.

This book belongs to Central Library BADJI Mokhtar-ANNABA UNIVERSITY Copyright Elsevier 2024

18. Lindstrom LH, Nyberg F, Terenius L, Bauer K, Besev G, Gunne LM, et al. CSF and plasma beta-casomorphin-like opioid peptides in postpartum psychosis. *Am J Psychiatry* 1984;**141**:1059–66.
19. Maslova MV, Graf AV, Samoilenkova NS, Maklakova AS, Sokolova NA, Andreeva LA. Consequences of progestational hypoxia in pregnant rats and its peptide correction (EEG data). *Bull Exp Biol Med* 2003;**135**:526–9.
20. Moeller I, Lew RA, Mendelsohn FA, Smith AI, Brennan ME, Tetaz TJ, et al. The globin fragment LVV-hemorphin-7 is an endogenous ligand for the AT4 receptor in the brain. *J Neurochem* 1997;**68**:2530–7.
21. Moisan S, Harvey N, Beaudry G, Forzani P, Burhop KE, Drapeau G, et al. Structural requirements and mechanism of the pressor activity of Leu-Val-Val- hemorphin-7, a fragment of hemoglobin beta-chain in rats. *Peptides* 1998;**19**:119–31.
22. Nyberg F. Opoid peptides in cerebrospinal fluid — methods for analysis and their significance in the clinical perspective. *Front Biosci* 2004;**9**:3510–25.
23. Nyberg F, Lieberman H, Lindstrom LH, Lyrenas S, Koch G, Terenius L. immunoreactive beta-casomorphin-8 in cerebrospinal fluid from pregnant and lactating women: correlation with plasma levels. *J Clin Endocrinol Metab* 1989;**68**:283–9.
24. Nyberg F, Sanderson K, Glamsta EL. The hemorphins: a new class of opioid peptides derived from the blood protein hemoglobin. *Biopolymers* 1997;**43**:147–56.
25. Panksepp J, Normansell L, Siviy S, Rossi J, Zolovick AJ. Casomorphins reduce separation distress in chicks. *Peptides* 1984;**5**:829–31.
26. Pasi A, Mahler H, Lansel N, Bernasconi C, Messiha FS. Beta-Casomorphin — immunoreactivity in the brain stem of the human infant. *Res Commun Chem Pathol Pharmacol* 1993;**80**:305–22.
27. Poljak A, McLean CA, Sachdev P, Brodaty H, Smythe GA. Quantification of hemorphins in Alzheimer's disease brains. *J Neurosci Res* 2004;**75**:704–14.
28. Ramabadran K, Bansinath M. Opioid peptides from milk as a possible cause of sudden infant death syndrome. *Med Hypotheses* 1988;**27**:181–7.
29. Renlund S, Erlandsson I, Hellman U, Silberring J, Wernstedt C, Lindstrom L, et al. Micropurification and amino acid sequence of beta-casomorphin-8 in milk from a woman with postpartum psychosis. *Peptides* 1993;**14**:1125–32.
30. Schusdziarra V, Schick R, de la Fuente A, Specht J, Klier M, Brantl V, et al. Effect of b-casomorphins and analogs on insulin release in dogs. *Endocrinology* 1983;**112**:885–9.
31. Sidor K, Jarmołowska B, Kaczmarski M, Kostyra E, Iwan M, Kostyra H. Content of beta-casomorphins in milk of women with a history of allergy. *Pediatr Allergy Immunol* 2008 Nov;**19**(7):587–91.
32. Sun Z, Zhang Z, Wang X, Cade R, Elmir Z, Fregly M. Relation of beta-casomorphin to apnea in sudden infant death syndrome. *Peptides* 2003;**24**:937–43.
33. Trompette A, Claustre J, Caillon F, Jourdan G, Chayvialle JA, Plaisancie P. Milk bioactive peptides and ß-casomorphins induce mucus release in rat jejunum. *J Nutr* 2003;**133**:3499–503.
34. Umbach M, Teschemacher H, Praetorius K, Hirschhauser R, Bostedt H. Demonstration of a beta-casomorphin immunoreactive material in the plasma of newborn calves after milk intake. *Regul Pept* 1985;**12**:223–30.
35. Zadina JE, Hackler L, Ge LJ, Kastin AJ. A potent and selective endogenous agonist for the μ-opiate receptor. *Nature* 1997;**386**:499–502.

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Chapter 212

Endomorphins

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ABSTRACT

Endomorphins are highly selective endogenous μ-opioid peptides discovered in mammalian brain in 1997. Endomorphins have several identical pharmacological and physiological characters, as well as identical sequence structures, with other endogenous opioid peptides and μ-opioid receptor agonists. Interestingly, endomorphin-1 and endomorphin-2, which have only one difference in the amino acid residue at position 3, show some characteristic differences from each other in their pharmacological and physiological effects. In this chapter, we describe the pharmacological and physiological characteristics of endomorphins, with emphasis on their differences.

DISCOVERY OF ENDOMORPHINS

Since the discovery of opioid receptors, neuroscientists have searched for their endogenous ligands. The search led to the discovery of enkephalins, endorphins, and dynorphins in the 1970s. The three families of opioid peptides contain a common N-terminal amino acid sequence, Tyr-Gly-Gly-Phe. The enkepkalins ([Met^5]enkephalin and [Leu^5]enkephalin) are the endogenous ligands for δ-opioid receptors and dynorphins (dynorphin A, dynorphin B, α-neoendorphin, and β-neoendorphin) are the endogenous ligands for κ-opioid receptors. β-Endorphin binds μ-opioid receptors and δ-opioid receptors equally. These compounds can also bind μ-opioid receptors, but they display relatively low selectivity and efficacy at the μ-opioid receptors. Before 1997, no mammalian opioid peptide that exhibited both high affinity and selectivity for μ-opioid receptors was identified. As a peptide with opioid-related activity in the central nervous system, Tyr-Pro-Trp-Gly-NH_2 (Tyr-W-MIF-1), which was isolated from bovine and human brain and contains the same structure at the N-terminus (Tyr-Pro-aromatic amino acid) with casomorphin, shows high selectivity for μ-opioid receptors, but its affinity for μ-opioid receptors is relatively low.[34] Based on this information, the peptide with the highest affinity and selectivity for μ-opioid receptors was searched from the 20 peptides that have a common N-terminal sequence Tyr-Pro-Trp-X and whose amino acid at the fourth position was substituted by the possible natural amino acids. Among the 20 peptides tested (Tyr-Pro-Trp-X-NH_2, X: Phe, Leu, Ile, Met, Val, Pro, Gln, Trp, Cys, Thr, Tyr, Asn, Ser, Ala, Gly, Arg, Lys, His, Asp, Glu), Tyr-Pro-Trp-Phe-NH_2 showed a highest affinity and selectivity for μ-opioid receptors. This peptide showed very potent analgesia after intracerebroventricular and intrathecal administration.[33] Moreover, specific μ-opioid receptor antagonists reversed the pharmacological effects of this peptide in both *in vitro* and *in vivo* experiments. Zadina and colleagues generated a specific antibody against this peptide that showed no crossreactivity for >40 opioid or nonopioid peptides and used it in combination with HPLC to screen extracts of bovine brain for immunoreactivity. After multiple purification steps, the immunoreactive fraction was applied to automated Edman degradation, and this yielded two peptide sequences, Tyr-Pro-Trp-Phe-NH_2 and Tyr-Pro-Phe-Phe-NH_2. Both peptides showed a high affinity and selectivity for μ-opioid receptors and pharmacological effects as μ-opioid receptor agonists. Because these peptides are the first endogenous mammalian peptides to have a high affinity and a clear specificity for μ-opioid receptors (see below), they were named endomorphin-1 (Tyr-Pro-Trp-Phe-NH_2) and endomorphin-2 (Tyr-Pro-Phe-Phe-NH_2).[33] Interestingly, the structure of endomorphins is different from those of traditional endogenous opioid peptides, which have a common N-terminal sequence, Tyr-Gly-Gly-Phe. Unlike other endogenous opioid peptides, however, the precursors or its cording DNAs for endomorphins have not yet been identified, although it has been already been 15 years since their discovery. Therefore, the biosynthesis for endomorphins is still unknown, although Ronai and colleagues have provided evidence of de novo biosynthesis of endomorphin.[10,11,19]

DISTRIBUTION OF THE ENDOMORPHINS IN THE CENTRAL NERVOUS SYSTEM

Soon after the discovery of endomorphins, the distribution of the endomorphin-2-like immunoreactive neurons was shown in the central nervous system. The endomorphin-2-like

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00212-8
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immunoreactive fibers are present in the superficial layers of spinal dorsal horn and medulla oblongata.[12,13] The dorsal horn of the spinal cord is an important site for nociceptive transmission. It constitutes the first relay station for incoming somatosensory information and contains many neurotransmitters, such as substance P and glutamate. This region contains the highest densities of both μ-opioid receptors and endomorphin-2-like immunoreactivity. Mechanical disruption of the primary afferent neurons by dorsal rhizotomy dramatically diminished the endomorphin-2-like immunoreactivity.[12] Similarly, chemical disruption of primary sensory afferent neurons by the neurotoxin capsaicin eliminated its staining in the dorsal horn. These results provide direct evidence that endomorphin-2-like immunoreactive fibers may originate from the dorsal root ganglion, which contains the endomorphin-2-like immunoreactive neuronal perikarya.

The μ-opioid receptors are expressed in the primary afferent neurons that terminate in the superficial layers of the spinal dorsal horn.[1] Based on the distribution and physiological function of μ-opioid receptors, two major mechanisms for producing μ-opioid receptor-mediated analgesia have been proposed: presynaptic inhibition of neurotransmitter release from primary afferent neurons and postsynaptic hyperpolarization of excitatory neurons. μ-Opioid receptor agonists are known to regulate the release of glutamate, substance P, and calcitonin gene-related peptide from primary afferent neurons. Endomorphin-2 also modulates release of these excitatory neurotransmitters presynaptically.[29] Endomorphin-2-like immunoreactivity is present in dense-core vesicles in the terminals of primary afferent neurons at the spinal dorsal horn[25] and endomrphin-2 may be released from the primary afferent neurons by stimulation of the dorsal roots. The morphological demonstration and synaptic relationship provides evidence that endomorphin-2 may functionally work as a neurotransmitter and/or neuromodulator regulating pain processes.

Although both endomorphin-1- and endomorphin-2-like immunoreactivity are present in most areas of the central nervous system, several differences in their distribution and density were observed. Endomorphin-2-like immunoreactivity is more prevalent in the spinal cord and lower brain stem, whereas endomorphin-1-like immunoreactivity is more widely and densely distributed throughout the brain than endomorphin-2-like immunoreactivity.[13] However, very limited regions in the central nervous system contain endomorphin-1-like immunoreactive cell bodies. Like the endomorphin-2-like immunoreactive neuronal perikarya, the endomorphin-1-like immunoreactive neuronal perikarya are found in the dorsomedial hypothalamic nucleus and arcuate hypothalamic nucleus. The endomorphin-1-like immunoreactive neuronal perikarya are also present in ventromedial hypothalamic nucleus and the nucleus of solitary tract.

In the telencephalon, a dense plexus of endomorphin-1- and endomorphin-2-like immunoreactive nerve fibers are seen in the nucleus accumbens, septum, substantia innominata and the bed nucleus of the stria terminalis.[13,25] Endomorphin-1-like immunoreactivity is also very evident in the cortex, the dorsal lateral septal nucleus, and nucleus of the vertical limb of the diagonal band of Broca. The distribution of endomorphin-2-like immunoreactivity closely parallels that of endomorphin-1-like immunoreactivity, but its contribution to the telencephalic structures is much smaller.

In general, endomorphin-1-like immunoreactivity is extensively present in hypothalamic structures. Endomorphin-1-like immunoreactive fibers are detected in the organum vasculosum lamina terminalis, the median preoptic nuclei, and posterior hypothalamic nuclei. Other regions containing relatively large numbers of endomorphin-1-like immunoreactive fibers include the lateral preoptic area, anterior hypothalamic area, lateral hypothalamic area, ventromedial hypothalamic nucleus, dorsal hypothalamic area, dorsomedial hypothalamic nucleus, arcuate nucleus, and ventral premammillary and supramammillary nuclei. Endomorphin-2-like immunoreactivity is also widely distributed in hypothalamic structures. However, unlike endomorphin-1-like immunoreactive fibers, endomorphin-2-like immunoreactive fibers are infrequently detected in hypothalamic regions outside the regions containing endomorphin-2-like immunoreactive cell bodies.

In the midbrain, endomorphin-1-like immunoreactive fibers, and endomorphin-2-like immunoreactive fibers, are detected in the superior and inferior colliculii, periaqueductal gray, dorsal raphe nucleus, laterodorsal tegmental nucleus, ventral tegmental area, and the substantia nigra pars reticularis. However, in the lower brain stem, most of the endomorphin-1-like immunoreactive fibers are spread over different parts of the locus coeruleus and the parabrachial nucleus, in which the endomorphin-2-like immunoreactive fibers are intensely observed. The principal sensory nucleus of the trigeminal, the nucleus of the spinal trigeminal tract, and the nucleus of the solitary tract also contain some endomorphin-1-like immunoreactive fibers but at lower density than the endomorphin-2-like immunoreactivity.

RECEPTOR SELECTIVITY AND ITS MODULATION

The receptor selectivities of endomorphins have been well established. As Zadina and his colleagues described when they firstly reported the existence of endomorphins in mammalian brain, endomorphins show very high affinity and selectivity for μ-opioid receptors.[33] The affinity of endomorphin-1 to μ-opioid receptors mimics that of the selective μ-opioid receptors agonist [D-Ala^2,N-MePhe^4,Gly-ol^5]enkephalin (DAMGO), and its selectivity for μ-opioid receptors is >4000 times and 15,000 times

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higher than those for δ-opioid receptors and κ-opioid receptors, respectively. However, the affinity of endomorphin-2 to μ-opioid receptors is approximately 2 times lower than that of DAMGO, but its selectivity for μ-opioid receptors is still >13,000 times and 7000 times higher than those for δ-opioid receptors and κ-opioid receptors, respectively. The extremely high affinity and selectivity of endomorphins for μ-opioid receptors have been confirmed in the membrane preparations obtained from several brain regions and spinal cord in mice and rats.[8] With high affinity and selectivity to the μ-opioid receptors, in addition to some biological action mimicking traditional μ-opioid receptor agonists, endomorphins were predicted to be the long-sought endogenous μ-opioid peptides.[33] Interestingly, endomorphins are the only endogenous opioid peptides to show high receptor selectivity, whereas other endogenous opioid peptides show selectivity to multiple opioid receptors.

Because opioid receptors are coupled with Gi/Go protein, the intrinsic activity of opioid receptor agonists has been preferentially evaluated in the [^{35}S]GTPγS binding assay. Like other μ-opioid receptor agonists, both endomorphin-1 and endomorphin-2 produce marked increase in the [^{35}S]GTPγS binding in membrane preparations obtained from several brain regions and spinal cord in mice and rats.[8] The endomorphin-induced increase in [^{35}S]GTPγS binding is completely eliminated by the co–incubation with selective μ-opioid receptor antagonists but not δ-opioid receptor and κ-opioid receptor antagonists.[17] Consistent with their selectivity to opioid receptors, G-protein activation induced by endomorphins is selectively mediated by μ-opioid receptors. In fact, the endomorphin-induced increase in [^{35}S]GTPγS binding is completely abolished in the membrane preparation obtained from μ-opioid receptor knockout mice.[15] The maximal increase in [^{35}S]GTPγS binding by endomorphins (the intrinsic activities of endomorphins) is approximately half that of DAMGO, an μ-opioid receptor full agonist.[8,17] Moreover, the maximal increase in [^{35}S]GTPγS binding by the μ-opioid receptor full agonists, DAMGO and β-endorphin, is partially attenuated by coincubation with high concentrations of endomorphins.[2,16] The evidence clearly suggests that endomorphins, as well as morphine and fentanyl, are partial agonists for μ-opioid receptors. It is of interest to note that endomorphins are the only endogenous opioid peptides to have a partial agonistic property, whereas other endogenous opioid peptides are full agonists for their respective opioid receptors.[2,35] The physiological role of endomorphins may be somewhat different from that of other endogenous opioid peptides.

BIOLOGICAL ACTIONS OF ENDOMORPHINS

Like other endogenous opioid peptides or selective μ-opioid receptor agonists, endomorphins have a variety of biological actions. As expected, a major biological action of endomorphins is their remarkable antinociceptive effect. After intrathecal and intracerebroventricular injections, endomorphins show as potent antinociception as the other endogenous opioid peptides [Met^5]enkephalin, [Leu^5]enkephalin, and dynorphin A.[24] The antinociception induced by both endomorphin-1 and endomorphin-2 is mediated by μ-opioid receptors. However, their antinociceptive effects are characteristically somehow different. The antinociception induced by endomorphin-2, but not endomorphin-1, is attenuated by the μ_1-opioid receptor antagonist naloxonazine,[21,24] whereas the antinociception induced by endomorphin-1, but not endomorphin-2, is attenuated by the recently identified μ_2-opioid receptor antagonist D-Pro^2-Tyr-W-MIF-1.[31] These results clearly suggest that the antinociception induced by endomorphin-1 and endomorphin-2 is characteristically distinct from each other, and selectively mediated through μ_2- and μ_1-opioid receptors, respectively. This theory is additionally supported by the evidence that the antinociception induced by endomorphin-1 and endomorphin-2 is selectively attenuated by the μ_2-opioid receptor antagonist D-Pro^2-endomorphin-1 and the μ_1-opioid receptor antagonist D-Pro^2-endomorphin-2, respectively, but not vice versa.[23] Moreover, endomorphin-2-induced antinociception is attenuated by 3-methoxynaltrexone, which is a heroin-sensitive and morphine-insensitive antagonist, suggesting that endomorphin-2 has selectivity to multiple μ-opioid receptors, including the μ_1-opioid receptor and other μ-opioid receptor as a third party of the μ-opioid receptor, but not to the μ_2-opioid receptor.[21] More interestingly, the antinociceptive effect of endomorphin-2, but not endomorphin-1, is mediated by δ-opioid and κ-opioid receptors in addition to the μ-opioid receptor,[22] even though endomorphin-2-induced antinociception is completely abolished in the μ-opioid receptor knockout mice.[15] This controversial result is shown by the ability of endomorphin-2 to lead to the release of other endogenous opioid peptides, [Met^5]enkephalin and dynorphin A, by its stimulation of μ-opioid receptors.[22] β-Endorphin is another endogenous opioid peptide known to release [Met^5]enkephalin.[30] However, at present, endomorphin-2 is the only endogenous opioid peptide, which releases endogenous κ-opioid peptides. Like other endogenous opioid peptides, the duration of endomorphin-induced antinociception is short-lived, and completely disappears within half an hour.[15,24] Unfortunately, enzymatic degradation of endomorphins cannot explain this phenomenon. The process of enzymatic degradation of endomorphins by enzymes identified in the cerebrospinal fluid and central nervous systems has already been described.[20,27] The enzymatic degradation of endomorphins in cerebrospinal fluid, like that described earlier for the related peptide Tyr-MIF-1 (Tyr-Pro-Leu-Gly-NH_2), proceeds more slowly than that of other endogenous opioid peptides, and it requires >2 h to degrade endomorphins.[20,27]

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The identical character of endomorphins on acute tolerance may help to explain their short-lived effects. As is well known, a single injection of opioid receptor agonists leads to a short-lived desensitization to the same agonists, the so-called acute tolerance effect, and the time course of acute tolerance is identical to the receptor to which the agonist binds. Unlike the traditional μ-opioid receptor agonist, DAMGO, whose acute antinociceptive tolerance shows up 3 h after the treatment and lasts for 6 h, the acute antinociceptive tolerance to endomorphins rapidly develops within half an hour and disappears by 3 h.[32] The rapid development of the acute antinociceptive tolerance to endomorphins may be involved in its short-lived antinociceptive effect.

The other traditional biological activities of μ-opioid receptor agonists, including rewarding effect, locomotor enhancement, inhibition of gastrointestinal transit, cardiovascular depression, and respiratory depression, have also been described for endomorphins. Like other μ-opioid receptor agonists, both endomorphin-1 and endomorphin-2 produce potent inhibition of gastrointestinal transit[7] and cardiovascular depression[8] with similar efficacy. Both endomorphin-1 and endomorphin-2 also produce respiratory depression; however, an approximately 12 times higher dose of endomorphin-1 is required to produce the respiratory depression in comparison with endomorphin-2.[5] The difference in the sensitivity for respiratory depression may be caused by the different distribution of endomorphin-1 and endomorphin-2-containing neurons. Although endomorphin-1-like immunoreactivity and endomorphin-2-like immunoreactivity both densely distribute in areas associated with respiratory function such as the nucleus tractus solitarius and the parabrachial nucleus, endomorphin-2-like immunoreactivity is more prominent than endomorphin-1-like immunoreactivity in the ventrolateral nucleus tractus solitarius and external medial parabrachial nucleus, which are the most crucial regions for respiratory regulation.[13] Regarding the rewarding effect and locomotor enhancement, endomorphin-1 and endomorphin-2 show more significant differences from each other. Endomorphin-1, and other traditional μ-opioid receptor agonists, produces a remarkable rewarding and locomotor-enhancing effect in a dose-dependent manner.[3,18] By contrast, endomorphin-2 shows a bell-shaped dose-response curve for the rewarding and locomotor-enhancing effects, and prominently produces an aversive rather than rewarding effect.[4,18] As is well known, the rewarding and locomotor-enhancing effects of μ-opioid receptor agonists are mediated by the disinhibition of mesolimbic and nigrostriatal dopaminergic neurons via the activation of μ-opioid receptors located on the GABAergic neurons in the ventral tegmental area and substantia nigra, respectively.[18] In the terminal of mesolimbic and nigrostriatal dopaminergic neurons on the nucleus accumbens and striatum, respectively, dynorphinergic neurons are localized to inhibit the release of dopamine. Unlike endomorphin-1 and other traditional μ-opioid receptor agonists, endomorphin-2 has a characteristic pharmacological ability to release dynorphin A.[22] The release of dynorphin A in the nucleus accumbens and striatum may be involved in the lack of the remarkable rewarding effect and locomotor-enhancing effect of endomorphin-2. In fact, endomorphin-2 shows a remarkable rewarding effect when dynorphin A is blocked by the use of its antiserum.[18]

PATHOPHYSIOLOGICAL IMPLICATIONS OF ENDOMORPHINS

The pathophysiological roles of endomorphins still have not been well characterized. However, the functional changes induced by endomorphins in several pathological conditions may be helpful. In a neuropathic pain state induced by sciatic nerve ligation, endomorphin-2-like immunoreactivity is dramatically decreased in the superficial layer of dorsal horn.[26] This evidence strongly suggests that endomorphin-2 may regulate the transmission of nociceptive stimuli in the spinal cord, and the reduction of endomorphin-2-containing neurons may be associated with the nerve ligation-induced neuropathic pain. By contrast, endomorphin-2-like immunoreactivity in the periaqueductal gray matter is greatly increased by spinal nerve ligation.[28] Taken together with the evidence that endomorphin-2 is particularly more effective than morphine in nerve ligation-induced neuropathic pain, endomorphin-2 in the periaqueductal gray matter may be involved in the regulation of the pain sensation induced by nerve injury via activation of the descending pain control system.

In peripheral inflammation, especially knee joint inflammation induced by adjuvant, endomorphin-1-like immunoreactivity is greatly increased in the synovium of the knee joint.[14] The increase of endomorphin-1-like immunoreactivity is also observed in the synovium of adjuvant-inflamed paws.[9] At present, the increased endomorphin-1-like immunoreactivity is limited to the peripheral tissues. Unlike endomorphin-2, which seems to control pain sensation in the central nervous system, endomorphin-1 may control painful inflammation in the peripheral tissues.

The endomorphins are the only endogenous opioid peptides identified as partial agonists.[16] The other endogenous opioid peptides are all full agonists for their respective receptors. Theoretically, a high dose of partial agonists can partially attenuate the effect of full agonists working on the same receptors. As expected, endomorphins partially attenuate the effects of β-endorphin, another endogenous μ-opioid peptide.[16] In the specific physiological condition in which the μ-opioid system is overstimulated, endomorphins may prevent excessive stimulation of the μ-opioid receptor by β-endorphin by a negative feedback mechanism in

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tissues in which endomorphins and β-endorphin are colocalized.

CONCLUDING REMARKS

A growing body of evidence indicates some differences between endomorphin-1 and endomorphin-2. Overall, the pharmacological characteristics of endomorphin-1 are relatively close to those of the traditional μ-opioid receptor agonists, whereas endomorphin-2 has several differences in its pharmacological characteristics from other μ-opioid receptor agonists. There are >30 subclasses of μ-opioid receptors in mice based on the splice variants of μ-opioid receptor mRNA.[6] The differences in their pharmacological and physiological character may be revealed by the different selectivity of endomorphins for μ-opioid receptor subclasses and by the different colocalization of endomorphins and their preferred μ-opioid receptor subclasses.

ACKNOWLEDGMENTS

Research in the author's laboratory was supported by a Grant-in-Aid for Scientific Research (C) [KAKENHI 21600013 and 22600009] from the Japan Society for the Promotion of Science and a Matching Fund Subsidy for Private Universities from the Ministry of Education, Culture, Sports, Science, and Technology Japan (2010–2014).

REFERENCES

1. Aicher SA, Sharma S, Cheng PY, Liu-Chen LY, Pickel VM. Dual ultrastructural localization of μ-opiate receptors and substance P in the dorsal horn. *Synapse* 2000;**36**:12–20.
2. Alt A, Mansour A, Akil H, Medzihradsky F, Traynor JR, Woods JH. Stimulation of guanosine-5′-O-(3- [^{35}S]thio)triphosphate binding by endogenous opioids acting at a cloned mu receptor. *J Pharmacol Exp Ther* 1998;**286**:282–8.
3. Bujdoso E, Jaszberenyi M, Tomboly C, Toth G, Telegdy G. Effects of endomorphin-1 on open-field behavior and on the hypothalamic-pituitary-adrenal system. *Endocrine* 2001;**14**:221–4.
4. Bujdoso E, Jaszberenyi M, Tomboly C, Toth G, Telegdy G. Behavioral and neuroendocrine actions of endomorphin-2. *Peptides* 2001;**22**:1459–63.
5. Czapla MA, Gozal D, Alea OA, Beckerman RC, Zadina JE. Differential cardiorespiratory effects of endomorphin 1, endomorphin 2, DAMGO, and morphine. *Am J Respir Crit Care Med* 2000;**162**:994–9.
6. Doyle GA, Sheng XR, Lin SS, Press DM, Grice DE, Buono RJ, et al. Identification of five mouse μ-opioid receptor (MOR) gene (Oprm1) splice variants containing a newly identified alternatively spliced exon. *Gene* 2007;**395**:98–107.
7. Goldberg IE, Rossi GC, Letchworth SR, Mathis JP, Ryan-Moro J, Leventhal L, et al. Pharmacological characterization of endomorphin-1 and endomorphin-2 in mouse brain. *J Pharmacol Exp Ther* 1998;**286**:1007–13.
8. Horvath G. Endomorphin-1 and endomorphin-2: pharmacology of the selective endogenous μ-opioid receptor agonists. *Pharmacol Ther* 2000;**88**:437–63.
9. Jessop DS, Richards LJ, Harbuz MS. Opioid peptides endomorphin-1 and endomorphin-2 in the immune system in humans and in a rodent model of inflammation. *Ann N Y Acad Sci* 2002;**966**:456–63.
10. Király K, Lambeir AM, Szalai J, Szentirmay A, Luyten W, Barna I, et al. The dipeptidyl peptidase IV (CD26, EC 3.4.14.5) inhibitor vildagliptin is a potent antihyperalgesic in rats by promoting endomorphin-2 generation in the spinal cord. *Eur J Pharmacol* 2011;**650**:195–9.
11. Király K, Szalay B, Szalai J, Barna I, Gyires K, Verbeken M, et al. Intrathecally injected Ile-Pro-Ile, an inhibitor of membrane ectoenzyme dipeptidyl peptidase IV, is antihyperalgesic in rats by switching the enzyme from hydrolase to synthase functional mode to generate endomorphin 2. *Eur J Pharmacol* 2009;**620**:21–6.
12. Martin-Schild S, Gerall AA, Kastin AJ, Zadina JE. Endomorphin-2 is an endogenous opioid in primary sensory afferent fibers. *Peptides* 1998;**19**:1783–9.
13. Martin-Schild S, Gerall AA, Kastin AJ, Zadina JE. Differential distribution of endomorphin 1- and endomorphin 2-like immunoreactivities in the CNS of the rodent. *J Comp Neurol* 1999;**405**:450–71.
14. McDougall JJ, Barin AK, McDougall CM. Loss of vasomotor responsiveness to the μ-opioid receptor ligand endomorphin-1 in adjuvant monoarthritic rat knee joints. *Am J Physiol Regul Integr Comp Physiol* 2004;**286**:R634–41.
15. Mizoguchi H, Narita M, Oji DE, Suganuma C, Nagase H, Sora I, et al. The μ-opioid receptor gene-dose dependent reduction in G-protein activation in the pons/medulla and antinociception induced by endomorphins in μ-opioid receptor knockout mice. *Neuroscience* 1999;**94**:203–7.
16. Mizoguchi H, Wu H-E, Narita M. Partial agonistic action of endomorphins in the mouse spinal cord. *Neurosci Lett* 2001;**310**:66–8.
17. Narita M, Mizoguchi H, Oji GS, Tseng EL, Suganuma C, Nagase H, et al. Characterization of endomorphin-1 and -2 on [^{35}S]GTPγS binding in the mouse spinal cord. *Eur J Pharmacol* 1998;**351**:383–7.
18. Narita M, Ozaki S, Ioka M, Mizoguchi H, Nagase H, Tseng LF, et al. Different motivational effects induced by the endogenous μ-opioid receptor ligands endomorphin-1 and -2 in the mouse. *Neuroscience* 2001;**105**:213–8.
19. Rónai AZ, Király K, Szebeni A, Szemenyei E, Prohászka Z, Darula Z, et al. Immunoreactive endomorphin 2 is generated extracellularly in rat isolated L4,5 dorsal root ganglia by DPP-IV. *Regul Pept* 2009;**157**:1–2.
20. Sakurada C, Sakurada S, Hayashi T, Katsuyama S, Tan-No K, Sakurada T. Degradation of endomorphin-2 at the supraspinal level in mice is initiated by dipeptidyl peptidase IV: an in vitro and in vivo study. *Biochem Pharmacol* 2003;**66**:653–61.
21. Sakurada S, Hayashi T, Yuhki M, Fujimura T, Murayama K, Yonezawa A, et al. Differential antagonism of endomorphin-1 and endomorphin-2 spinal antinociception by naloxonazine and 3-methoxynaltrexone. *Brain Res* 2000;**881**:1–8.
22. Sakurada S, Hayashi T, Yuhki M, Orito T, Zadina JE, Kastin AJ, et al. Differential antinociceptive effects induced by intrathecally administered endomorphin-1 and endomorphin-2 in the mouse. *Eur J Pharmacol* 2001;**427**:203–10.
23. Sakurada S, Watanabe H, Hayashi T, Yuhki M, Fujimura T, Murayama K, et al. Endomorphin analogues containing D-Pro^2 discriminate different μ-opioid receptor mediated antinociception in mice. *Br J Pharmacol* 2002;**137**:1143–6.
24. Sakurada S, Zadina JE, Kastin AJ, Katsuyama S, Fujimura T, Murayama K, et al. Differential involvement of μ-opioid receptor subtypes in endomorphin-1- and -2-induced antinociception. *Eur J Pharmacol* 1999;**372**:25–30.

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25. Schreff M, Schulz S, Wiborny D, Hollt V. Immunofluorescent identification of endomorphin-2-containing nerve fibers and terminals in the rat brain and spinal cord. *Neuroreport* 1998;**9**:1031–4.
26. Smith RR, Martin-Schild S, Kastin AJ, Zadina JE. Decreases in endomorphin-2-like immunoreactivity concomitant with chronic pain after nerve injury. *Neuroscience* 2001;**105**:773–8.
27. Sugimoto-Watanabe A, Kubota K, Fujibayashi K, Saito K. Antinociceptive effect and enzymatic degradation of endomorphin-1 in newborn rat spinal cord. *Jpn J Pharmacol* 1999;**81**:264–70.
28. Sun R-Q, Wang Y, Zhao C-S, Chang J-K, Han J-S. Changes in brain content of nociceptin/orphanin FQ and endomorphin-2 in a rat model of neuropathic pain. *Neurosci Lett* 2001;**311**:13–6.
29. Tao PL, Lai YS, Chow LH, Huang EY. Effects of morphine and endomorphins on the polysynaptic reflex in the isolated rat spinal cord. *Naunyn Schmiedebergs Arch Pharmacol* 2005;**371**:72–80.
30. Tseng LF, Higgins MJ, Hong JS, Hudson PM, Fujimoto JM. Release of immunoreactive Met-enkephalin from the spinal cord by intraventricular β-endorphin but not morphine in anesthetized rats. *Brain Res* 1985;**343**:60–9.
31. Watanabe H, Nakayama D, Ito K, Watanabe C, Mizoguchi H, Fujimura T, et al. A Tyr-W-MIF-1 analog containing D-Pro^2 acts as a selective μ_2-opioid receptor antagonist in the mouse. *J Pharmacol Exp Ther* 2005;**312**:1075–81.
32. Wu HE, Hung KC, Mizoguchi H, Fujimoto JM, Tseng LF. Acute antinociceptive tolerance and asymmetric cross-tolerance between endomorphin-1 and endomorphin-2 given intracerebroventricularly in the mouse. *J Pharmacol Exp Ther* 2001;**299**:1120–5.
33. Zadina JE, Hackler L, Ge LJ, Kastin AJ. A potent and selective endogenous agonist for the μ-opiate receptor. *Nature* 1997;**386**:499–502.
34. Zadina JE, Kastin AJ, Ge LJ, Hackler L. Mu, delta, and kappa opiate receptor binding of Tyr-MIF-1 and of Tyr-W-MIF-1, its active fragments, and two potent analogs. *Life Sci* 1994;**55**:PL461–466.
35. Zhu J, Luo L-Y, Li J-G, Chen C, Liu-Chen L-Y. Activation of the cloned human kappa opioid receptor by agonists enhances [^{35}S]GTPγS binding to membranes: determination of potencies and efficacies of ligands. *J Pharmacol Exp Ther* 1997;**282**:676–84.

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Chapter 213

Evolution of the Opioid System

Dan Larhammar, Görel Sundström and Robert M. Dores

ABSTRACT

Evolutionary analyses suggest that the vertebrate ancestor had a single opioid peptide precursor gene and a single opioid receptor gene. The two genome doublings before the origin of gnathostomes generated two more peptide precursor genes. A fourth copy probably arose by a local gene duplication. The sequences diverged and became the precursors for endorphin (POMC), enkephalin, dynorphin, and nociceptin/orphanin. The ancestral receptor gene was quadrupled in the same events to give the receptors called delta, kappa, and mu and the nociceptin/orphanin receptor. Subsequently, certain ligand–receptor preferences have evolved. A third genome doubling in the ancestor of teleost fishes generated additional copies. For instance, the zebrafish has one additional delta receptor gene and three additional peptide precursor genes. Thus, the opioid system had already considerable complexity at an early stage in vertebrate evolution and expanded further in the teleost fish lineage but remained constant in most tetrapods.

PEPTIDE FAMILY

Soon after the seminal discoveries in the 1970s that subsets of neurons in the central nervous system of mammals have receptors that bind opiates,[26] peptides with the ability to bind to these receptors were purified from brain tissue, namely Met-enkephalin and Leu-enkephalin.[13] Subsequently, additional opiate receptor-binding peptides (opioids) were identified, including β-endorphin, α-neoendorphin, and dynorphin (for a review see Ref. 9). A unifying feature of these peptides is the presence of the N-terminal motif YGGF called the opioid core sequence. Later, an opioid-like peptide named nociceptin or orphanin was identified,[18,28] which has the modified core motif FGGF in mammals and chicken, while in teleost fishes, it still has the original YGGF motif.[30] In this chapter, nociceptin will from an evolutionary point of view be considered a member of the opioid peptide family. Beyond this core motif, each peptide has a distinct C-terminal sequence giving sizes that range from five for the enkephalins to >30 amino acid residues for β-endorphin.[9] Unlike many other biologically active peptides, the opioid peptides do not have a C-terminal amide.

Cloning of the opioid peptides revealed that they are generated from large and complex precursors (Fig. 1). The peptide β-endorphin was found in the same precursor as the one encoding the pituitary hormone ACTH and additional nonopioid peptides.[20] This precursor has been named POMC for proopiomelanocortin. Enkephalin and dynorphin were found to be generated from separate precursors, each of which encodes multiple opioid peptides.[9] The nociceptin precursor has 1–2 opioid-like peptides depending on species.[30]

In the precursors, the opioid sequences are flanked by sets of paired basic amino acids (i.e. KR, RR, RK, KK), except for a few of the sequences in some species.[9,30] Each precursor has a cleavable signal peptide for transfer into the endoplasmic reticulum during synthesis, followed by an N-terminal part containing a cysteine-rich region with 4 cysteines in POMC and 6 cysteines in the other three precursors at conserved positions. Apart from these shared features, the precursor proteins share only limited and patchy amino acid sequence identity.

Four Propeptide Genes

Proenkephalin is the common precursor for the pentapeptides Met-enkephalin and Leu-enkephalin and two C-terminally extended forms of Met-enkephalin, as well as three additional opioid core motifs (Figs 1 and 2).[22] The seven opioid sequences in this precursor are designated A through G, and the positions of some of these sequences coincide with the positions in the other precursors. In addition to mammals (for review, see Ref. 11), proenkephalin has been cloned and characterized or predicted from genome sequences in several nonmammalian tetrapods and bony fishes[9,30] and in a cartilaginous fish.[17]

Prodynorphin in mammals encodes three opioid core motifs, named α-neoendorphin, dynorphin A, and dynorphin B.[15] Amphibians and teleost fishes have two additional

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00213-X
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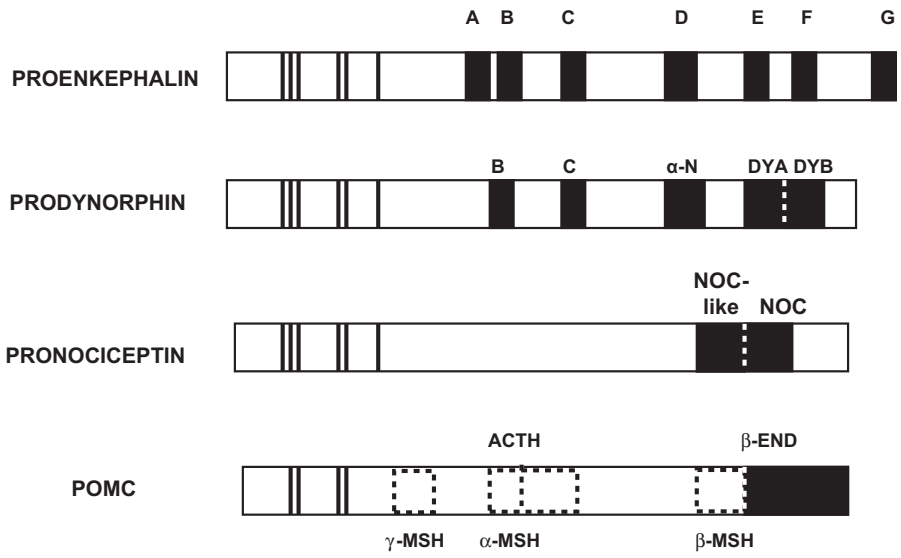

FIGURE 1 Outline of the four gnathostome opioid peptide precursors. Vertical bars in the amino terminal part show the positions of the 4–6 cysteine residues. The filled boxes marked with letters or abbreviations show the locations of the opioid peptides. The number of peptides encoded by each precursor differs between species. The dashed boxes show the locations ACTH and MSH peptides in POMC. See text for description of the peptides.

```
Enkephalins: YGGFM
             YGGFL
             YGGFMRF
             YGGFMRGL
Dynorphins:  YGGFLRRIRPKLKWDNQ
             YGGFLRRQFVVT
             YGGFLRKYPK
Endorphin:   YGGFMTSEKSQTPLVTLKNAIIKNAYKKGE
Nociceptin:  FGGFTGARKSARKLANQ
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FIGURE 2 Amino acid sequence alignment for the opioid peptides in placental mammals.

opioid peptides corresponding to positions B and C in proenkephalin[1,9] (Fig. 1).

The POMC precursor contains β-endorphin at its C-terminus that seems to correspond to opioid site E or F in Fig. 1. Shorter cleavage products containing part of the β-endorphin sequence have been purified and named α- and γ-endorphin, but the β form is the predominant peptide that can bind to opioid receptors. The melanocortin-related peptides ACTH, α-MSH (which constitutes the beginning of ACTH), β-MSH, and γ-MSH,[20] and in cartilaginous fish δ-MSH,[2] are encoded between the cysteine-rich region and β-endorphin. The melanocortin peptides all have the core amino acid motif HFRW, and these ligands activate the melanocortin receptors, a distinct family of G protein-coupled receptors (GPCR).[4] POMC has been characterized in representatives of all the major classes of vertebrates.[8]

The nociceptin precursor in mammals contains only a single opioid-like peptide, nociceptin itself,[12,23,24] whereas in teleost fishes, this is preceded by another peptide with a slightly modified core sequence, FGGF (as in the nociceptin peptide in mammals but not in fish).[30] Possibly, the two fish peptides correspond to positions E and F with the latter one being the nociceptin present in mammals. It has also been proposed that a relic YGGF motif is present C-terminally to the nociceptin peptide; this sequence is YLGM in chicken and some fishes, YLVL in placental mammals.[30]

Propeptide Comparisons

The vertebrates that possess all four propeptides span the groups of Actinopterygians (ray-finned fishes) and Sarcopterygians (flesh- or lobe-finned fishes) (Fig. 3). Among Chondrichthyes (sharks, rays, and chimaeras), it has so far been possible to identify proenkephalin, prodynorphin and POMC. In lampreys, two POMC homologs exist, but no additional propeptides have yet been published.

The number of opioid core motifs that were present in the ancestral precursor at the time of its quadruplication in early vertebrate evolution is difficult to deduce because, as for the various MSH peptides, some copies of the core motif may have been added and others may have been lost[7] in the different genes and in different lineages. With the exception of POMC, the other three precursors contain two or more opioid core motifs, indicating that the ancestral precursor may have had at least two, possibly more. POMC probably lost all opioid core motifs except the one in β-endorphin coincident with the insertion of the region encoding one or more MSH motifs. A single intron in the corresponding position and in the same reading frame of all four opioid precursor genes further emphasizes their shared evolutionary ancestry.

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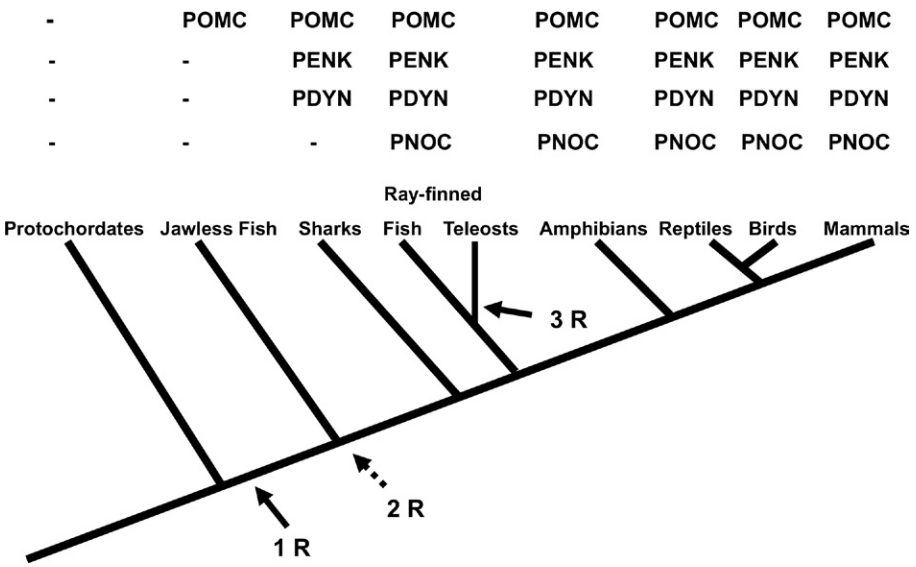

FIGURE 3 Phylogenetic distribution of the opioid peptide precursors. The three arrows mark the three major genome doublings. The time point for 2R (dashed arrow) relative to the divergence of the jawless fishes is still uncertain. The ray-finned fish branch is divided into teleosts (that underwent 3R) and other ray-finned fishes. Some teleost fishes have duplicates of some of the opioid precursor genes due to 3R. The protochordates consist of urochordates (tunicates) and cephalochordates (lancelets).

Interestingly, the propeptides contain a number of potential dibasic cleavage sites that are not used for proteolytic cleavage, resulting in the presence of dibasic motifs in both mature dynorphins and mature nociceptin (and ACTH). It may be speculated that the absence of cleavage at dibasic sites could have resulted from either amino acid changes elsewhere in the propeptide that reduce the efficiency of cleavage at the dibasic sites, or from altered specificity or expression of the processing enzymes, primarily prohormone convertases 1 and 2.

The genome doubling that took place in the ancestor of teleost fishes[14] has resulted in additional duplicates of some of the propeptide genes.[30] For instance, zebrafish has duplicates of both proenkephalin, pronocicpetin and POMC. The latter two are duplicated also in medaka and three-spined stickleback, two other teleost fish species whose genomes have been sequenced. The duplicates generally display high sequence conservation, but β-endorphin has diverged considerably in the POMCb gene in medaka and stickleback where the core opioid motif YGGF has changed to NGSF and NGST, respectively, but has remained intact in zebrafish. Also the ACTHb peptide has changed considerably in the three fishes, whereas α-MSHb differs in only a single position. The barfin flounder *Verasper moseri* has a third POMC gene[31] that probably resulted from a recent duplication. Sturgeons and paddlefish have two copies of POMC, presumably as a result of their independent tetraploidizations. In lampreys, duplicates of the POMC gene have been identified, both of which display partial degeneration[32]: one has been named POC and can generate ACTH and a variant of β-endorphin but has lost the ability to make α-MSH and β-MSH. The other copy is called POM and can give rise to the MSH peptides and a distinct version of β-endorphin. POC and POM are expressed in distinct parts of the hypothalamus. The frog *Xenopus laevis* has undergone a recent tetraploidization that probably explains its duplicate genes for POMC, proenkephalin, and prodynorphin.

Anatomical studies have shown that in mammals an individual neuron will express only one precursor-coding gene.[16] As a result, distinct enkephalinergic, dynorphinergic, and endorphinergic circuits can be identified in the mammalian CNS. Regarding the additional duplicates of the propeptide genes that exist in teleost fishes, it is still largely unknown to what extent the duplicates differ from each other with regard to anatomical and temporal expression, as well as propeptide processing and receptor affinity and efficacy of the mature peptides.

Given the sequence similarity between the opioid peptides, it is not surprising that each one may bind to more than one of the different receptor types but with different affinities (Fig. 4). In mammals, the enkephalins have the highest affinity for the δ receptor (OPRD), the dynorphins for the K receptor (OPRK), and β-endorphin for the μ receptor (OPRM), but each category displays some affinity also for the other receptor types. Nociceptin, by contrast, with its FGGF motif in mammals and chicken, is selective for the nociceptin receptor. Even zebrafish nociceptin with the ancestral YGGF motif does not bind appreciably to the other opioid receptors.

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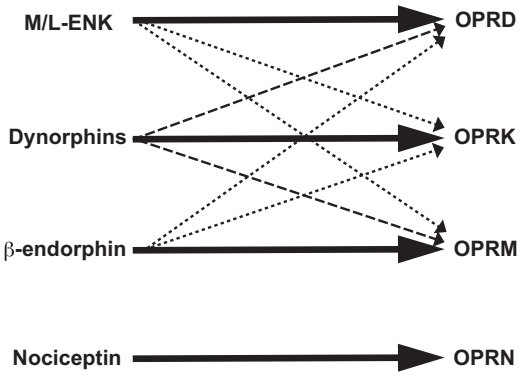

FIGURE 4 Opioid peptide–receptor selectivity in human. Ligand–receptor preferences may differ in other species.

Invertebrate Opioid System?

Several studies published in the 1980s reported opioid-like immunoreactivity in invertebrates as detected by antisera raised to the mammalian opioid peptides. Some of the reports described opioid-like peptide sequences and even two complete POMC sequences for invertebrates have been published, one of them also cloned as cDNA. However, there was no proof with appropriate control experiments that the POMC gene was present in the genome of this invertebrate. Also an opioid receptor sequence has been reported for an invertebrate, but its nucleotide sequence was almost identical to the human sequence and therefore probably resulted from contamination. No opioid propeptides or receptors have yet been identified in any invertebrate genome project, not even deuterostome invertebrates such as the tunicate *Ciona intestinalis*, the lancelet *Branchiostoma floridae*, or the sea urchin *Strongylocentratus purpuratus*.[30] Also melanocortin receptors are still unknown outside the vertebrates.

RECEPTORS

The opioid receptors were discovered by Pert and Snyder in 1973[26] before the first endogenous opioid peptides were characterized,[13] and it soon became apparent that distinct receptor types exist in mammals. The receptors were named using the initial Greek letter for certain characteristic features, namely, μ (mu) for the morphine-preferring receptor, K (kappa) for the ketocyclazocine-binding receptor, and δ (delta) for the receptor found in vas deferens. Clones for all three receptors were reported in mammals in 1992–1993, and a fourth receptor was identified by sequence similarity and therefore called opioid receptor-like.[19] Subsequently, a peptide was discovered that bound to this receptor, nociceptin/orphanin, hence leading to the name NOP for nociceptin receptor belonging to the opioid receptor family. The genes encoding the four receptors in mammals have been named OPRD1, OPRK1, OPRM1, and OPRL1. All the four genes have two introns in the corresponding positions, supporting origin from the same opioid receptor ancestor. The overall identity among the three opioid-binding receptor types in mammals is in the range 55–58%, while the NOP receptor is slightly more divergent displaying 48–49% identity to the other three. The transmembrane regions of the receptors are more highly conserved than the loops and the termini, as is usually the case for GPCRs.

Subtypes of some of the receptors have been reported in mammals and probably result from alternative splicing and possibly also posttranslational modifications or interactions with other proteins or dimerization.[6] Detailed studies of the mouse gene for the μ receptor have shown that in addition to the three major coding exons that are conserved between the four receptor types, numerous splice variants also can be detected.[25] Many of these seem to result in prematurely truncated receptors that probably fail to be expressed at the cell surface, but several variants exist that differ in the length of the cytoplasmic tail and result in different coupling efficiency.[25] The physiological importance of these splice variants and their conservation between species are still largely unexplored.

The four opioid receptor genes that have been identified in mammals have also been found in other tetrapods including chicken,[10] the rough-skinned newt *Taricha granulosa*[3] and the frog *Silurana/Xenopus tropicalis*[10] (Figs 5 and 6). In teleost fishes, homologs of all four receptors are present in all species whose genomes have been sequenced.[10] Furthermore, duplicates have arisen in the teleost-specific tetraploidization like for some of the propeptide genes as shown in the phylogenetic tree in Fig. 6: an extra copy of the δ receptor gene was identified in zebrafish and three-spined stickleback and a duplicate K receptor gene was found in medaka, three-spined stickleback and the green spotted

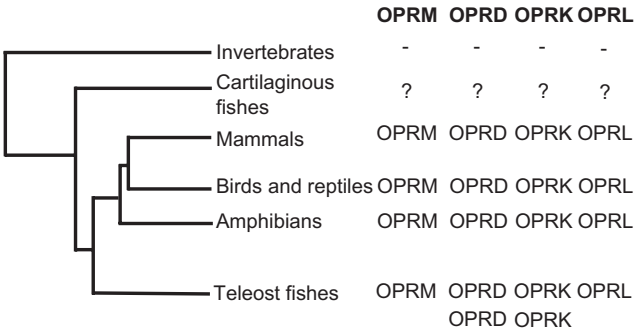

FIGURE 5 Phylogenetic distribution of opioid receptors mu (OPRM), delta (OPRD), kappa (OPRK), and the nociceptin receptor (OPRL). The question marks indicate that four partial receptor sequences have been found in a cartilaginous fish (the elephant shark), but it is still unclear whether they are orthologs (species homologs) of the four subtypes found in tetrapods. Note that duplicates of delta and kappa receptors have been found in some teleosts.

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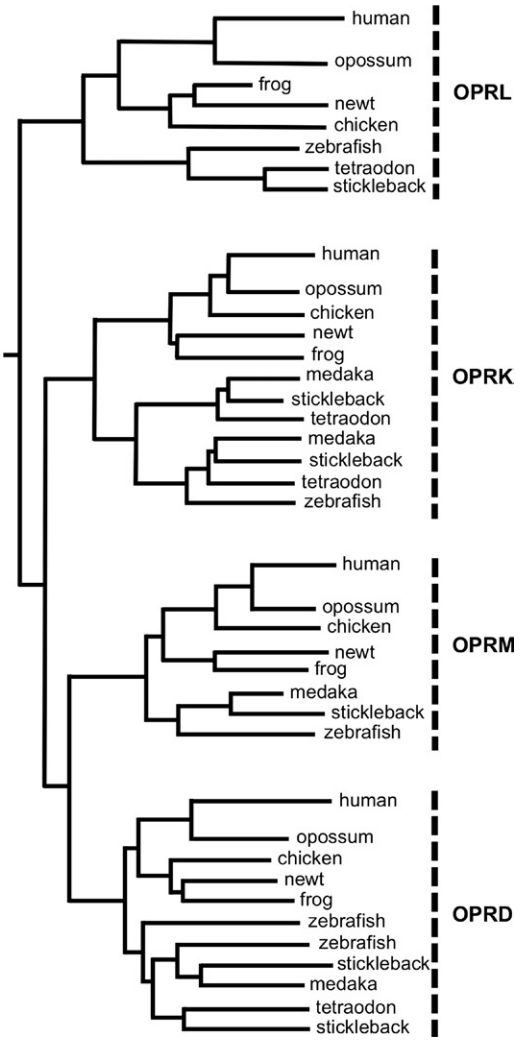

FIGURE 6 Phylogenetic tree for opioid receptors in human, opossum, chicken, the rough-skinned newt *Taricha granulosa*, the frog *Silurana (Xenopus) tropicalis*, and four teleost fish species. Note that duplicates of delta and kappa receptors have been found in some teleosts. The tree was rooted with somatostatin receptor sequences.[10]

pufferfish (*Tetraodon nigroviridis*). However, no duplicates were found for the μ receptor or the NOP receptor in any of these species.

Opioid receptor-like sequences have also been identified in the sequenced genome of a holocephalan cartilaginous fish, the elephant shark *Callorhinchus milii*.[10] However, it was not possible to say if these partial sequences correspond to the four opioid receptor types found in mammals.

Comparison of the receptor sequences between tetrapods and teleost fishes show that no striking insertions or deletions have taken place, indicating strong conservative evolutionary pressure. Binding studies with opioid peptides have been performed to cloned receptors in the rough-skinned newt[3] and in zebrafish.[5,29] It was noted in these studies that the newt receptors seem to have less stringent ligand selectivity than the mammalian receptors.

EVOLUTION OF THE OPIOID SYSTEM

The four propeptide precursors have arisen from a single ancestral gene in early vertebrate evolution as shown by similarities in precursor organization (Fig. 1), the conserved cysteine cluster, and a single conserved intron. The species distribution of the propeptides shows that the duplications took place before the origin of gnathostomes (Fig. 3).

Likewise, the four receptor types originated from a single ancestral receptor as supported by two conserved introns in the coding region of all four genes. The phylogenetic tree based on sequence comparisons (Figs 5 and 6) supports duplications in early vertebrate evolution, as for the propeptide genes.

An additional type of evidence that can be used to deduce duplication mechanisms and time points is the chromosomal locations of genes. Gene families that expanded in early vertebrate evolution often have their members on different chromosomes, and each chromosomal region contains members from the same set of gene families. Usually, the genomes of tetrapods have four related such chromosomal regions with similar repertoires of genes. This is a result of the two tetraploidizations or genome doublings that took place in early vertebrate evolution before the radiation of the gnathostomes.[21,27] Subsequently, a third tetraploidization happened in the ancestor of teleost fishes.[14]

The four opioid propeptide genes are located on three chromosomes that share members from several other gene families that display similar sequence-based phylogenetic trees. This suggests that all of these families were duplicated at the same time as part of a large block of genes or an entire chromosome. The exception is the fourth gene, POMC, which is located in the same chromosomal region as the pronociceptin gene. This may be the result of a local gene duplication as suggested in Fig. 7A.[30] More complicated (less parsimonious) evolutionary scenarios are also conceivable.

The four opioid receptor genes display an even better match with the double tetraploidization scheme, because all four share neighboring gene families that have similar phylogenetic relationships. Thus the opioid receptor expansion coincided with the opioid propeptide duplications.

One important aspect of duplication by chromosome doubling is that large gene regions including regulatory elements such as promoters and even distant enhancers are included in the duplication. This means that the daughter genes will initially share the same regulation. Gradually, the regulatory elements and the coding regions may diverge, leading to distinct roles of the gene duplicates. They may evolve either novel functions (neofunctionalization) or more specialized functions (subfunctionalization). This mode of

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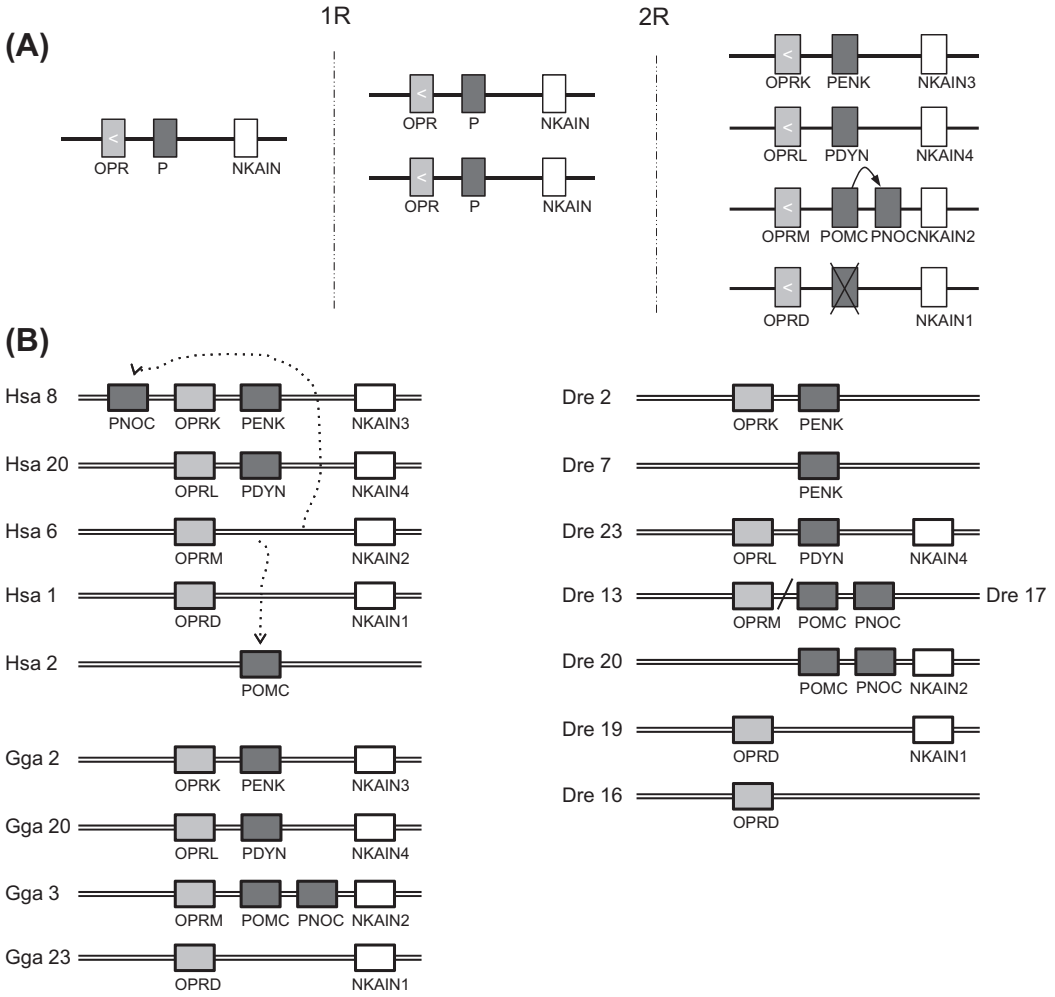

FIGURE 7 (A) A parsimonious duplication scheme for the opioid precursor and receptor genes found in the vertebrates, as explained by the two rounds of chromosome duplication in early vertebrate evolution. PENK stands for proenkephalin, PDYN for prodynorphin, and PNOC for pronociceptin. This chromosomal segment also contains numerous other gene families that were duplicated simultaneously, one of which is included in the figure, namely, NKAIN (Na+/K+ transporting ATPase interacting). The cross marks a gene that was lost. More complicated schemes are conceivable. (B) Chromosomal locations of the opioid precursor and receptor genes in chicken (Gga for *Gallus gallus*), human, and zebrafish (Dre for *Danio rerio*). The dashed arrows show translocations that have taken place in the lineage leading to *Homo sapiens*, as deduced from comparisons with several other vertebrates. The zebrafish genome has a duplicate set of chromosomal regions as a result of the teleost fish genome doubling. The figure was modified from Ref. 30.

duplication explains why considerable functional overlap may exist for the duplicates. Duplications that happen locally on the same chromosome, by contrast, do not necessarily include all of the flanking regulatory elements of a gene and may immediately result in distinct expression pattern of the duplicated gene.

Probably, the duplicated propeptides and receptors specialized rather quickly because all duplicates seem to have been retained in all vertebrate lineages investigated. Not only do the opioid propeptides and receptors seem to have duplicated concomitantly twice in early vertebrate evolution and again in the teleost ancestor, it also seems that the ancestral propeptide gene and the ancestral receptor gene were located in the same chromosomal region (Fig. 7). This suggests that their gene expression may once have been coregulated. However, after the tetraploidizations, there have been several translocations so any such coregulation seems to have faded. Furthermore, the peptide-receptor preferences that exist today in mammals do not agree with colocalization of the genes. For instance, proenkephalin and the K receptor are encoded on the same chromosome in both human, chicken, and zebrafish, but the preferred ligand of this receptor is dynorphin, which is encoded on a different chromosome (Fig. 7).

Because long periods of time have passed since the vertebrate classes diverged from one another, it might be expected that peptide-receptor preferences may differ between evolutionary lineages. Therefore it is probably important to investigate this in each species that is being studied. It is also possible that the physiological roles of the various

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opioid peptides and receptors may have drifted differently in the evolutionary lineages. It will be very interesting to learn how the different components contribute differentially in distantly related vertebrates to pain regulation, reward mechanisms, metabolism, and the many other functions of the opioid system.

CONCLUDING REMARKS

Thus, both the opioid peptides and the opioid receptors can be traced back to a single ancestral gene in early vertebrate evolution that underwent genome quadruplication. However, beyond this point there is still no firm evidence for an opioid system. Likewise, the melanocortin system seems to be a vertebrate innovation with the insertion of melanocortin peptide(s) in POMC and the emergence of MC receptors. The genomes of representatives for the two major cyclostome lineages, lampreys and hagfishes, will hopefully shed further light on the origin of the opioid and melanocortin systems.

The expansion of the opioid system in early vertebrate evolution probably resulted in rapid specialization of the genes, because four propeptide genes and four receptor genes are still expressed in all tetrapod classes. Teleost fishes have received additional copies of both propeptide and receptor genes and thus display even greater opioid complexity.

REFERENCES

1. Alrubaian J, Lecaude S, Barba J, Szynskie L, Jacobs N, Bauer D, et al. Trends in the evolution of the prodynorphin gene in teleosts: cloning of eel and tilapia prodynorphin cDNAs. *Peptides* 2006;**27**:797–804.
2. Amemiya Y, Takahashi A, Suzuki N, Sasayama Y, Kawauchi H. Molecular cloning of proopiomelanocortin cDNA from an elasmobranch, the stingray, Dasyatis akajei. *Gen Comp Endocrinol* 2000; **118**:105–12.
3. Bradford CS, Walthers EA, Stanley DJ, Baugh MM, Moore FL. Delta and mu opioid receptors from the brain of a urodele amphibian, the rough-skinned newt Taricha granulosa: cloning, heterologous expression, and pharmacological characterization. *Gen Comp Endocrinol* 2006;**146**:275–90.
4. Cone RD. Studies on the physiological functions of the melanocortin system. *Endocr Rev* 2006;**27**:736–49.
5. de Velasco EM, Law PY, Rodriguez RE. Mu opioid receptor from the zebrafish exhibits functional characteristics as those of mammalian mu opioid receptor. *Zebrafish* 2009;**6**:259–68.
6. Dietis N, Rowbotham DJ, Lambert DG. Opioid receptor subtypes: fact or artifact? *Br J Anaesth* 2011;**107**:8–18.
7. Dores RM, Baron AJ. Evolution of POMC: origin, phylogeny, post-translational processing, and the melanocortins. *Ann N Y Acad Sci* 2011;**1220**:34–48.
8. Dores RM, Lecaude S. Trends in the evolution of the proopiomelanocortin gene. *Gen Comp Endocrinol* 2005;**142**:81–93.
9. Dores RM, Lecaude S, Bauer D, Danielson PB. Analyzing the evolution of the opioid/orphanin gene family. *Mass Spectrom Rev* 2002; **21**:220–43.
10. Dreborg S, Sundström G, Larsson TA, Larhammar D. Evolution of vertebrate opioid receptors. *Proc Natl Acad Sci USA* 2008;**105**: 15487–92.
11. Eiden LE. The enkephalin-containing cell: strategies for polypeptide synthesis and secretion throughout the neuroendocrine system. *Cell Mol Neurobiol* 1987;**7**:339–52.
12. Houtani T, Nishi M, Takeshima H, Nukada T, Sugimoto T. Structure and regional distribution of nociceptin/orphanin FQ precursor. *Biochem Biophys Res Commun* 1996;**219**:714–9.
13. Hughes J, Smith TW, Kosterlitz HW, Fothergill LA, Morgan BA, Morris HR. Identification of two related pentapeptides from the brain with potent opiate agonist activity. *Nature* 1975;**258**:577–80.
14. Jaillon O, Aury JM, Brunet F, Petit JL, Stange-Thomann N, Mauceli E, et al. Genome duplication in the teleost fish Tetraodon nigroviridis reveals the early vertebrate proto-karyotype. *Nature* 2004;**431**:946–57.
15. Kakidani H, Furutani Y, Takahashi H, Noda M, Morimoto Y, Hirose T, et al. Cloning and sequence analysis of cDNA for porcine beta-neo-endorphin/dynorphin precursor. *Nature* 1982;**298**:245–9.
16. Khachaturian H, Lewis ME, Alessi NE, Watson SJ. Time of origin of opioid peptide-containing neurons in the rat hypothalamus. *J Comp Neurol* 1985;**236**:538–46.
17. Komorowski LK, Lecaude SG, Westring CG, Danielson PB, Dores RM. Evolution of gnathostome prodynorphin and proenkephalin: characterization of a shark proenkephalin and prodynorphin cDNAs. *Gen Comp Endocrinol* 2012;**177**:353–64.
18. Meunier JC, Mollereau C, Toll L, Suaudeau C, Moisand C, Alvinerie P, et al. Isolation and structure of the endogenous agonist of opioid receptor-like ORL1 receptor. *Nature* 1995;**377**:532–5.
19. Mollereau C, Parmentier M, Mailleux P, Butour JL, Moisand C, Chalon P, et al. ORL1, a novel member of the opioid receptor family. Cloning, functional expression and localization. *FEBS Lett* 1994;**341**:33–8.
20. Nakanishi S, Inoue A, Kita T, Nakamura M, Chang AC, Cohen SN, et al. Nucleotide sequence of cloned cDNA for bovine corticotropin-beta-lipotropin precursor. *Nature* 1979;**278**:423–7.
21. Nakatani Y, Takeda H, Kohara Y, Morishita S. Reconstruction of the vertebrate ancestral genome reveals dynamic genome reorganization in early vertebrates. *Genome Res* 2007;**17**:1254–65.
22. Noda M, Furutani Y, Takahashi H, Toyosato M, Hirose T, Inayama S, et al. Cloning and sequence analysis of cDNA for bovine adrenal preproenkephalin. *Nature* 1982;**295**:202–6.
23. Nothacker HP, Reinscheid RK, Mansour A, Henningsen RA, Ardati A, Monsma Jr FJ, et al. Primary structure and tissue distribution of the orphanin FQ precursor. *Proc Natl Acad Sci U S A* 1996;**93**: 8677–82.
24. Pan YX, Xu J, Pasternak GW. Cloning and expression of a cDNA encoding a mouse brain orphanin FQ/nociceptin precursor. *Biochem J* 1996;**315**(Pt 1):11–3.
25. Pasternak GW. Multiple opiate receptors: deja vu all over again. *Neuropharmacology* 2004;**47**(1):312–23.
26. Pert CB, Snyder SH. Opiate receptor: demonstration in nervous tissue. *Science* 1973;**179**:1011–4.
27. Putnam NH, Butts T, Ferrier DE, Furlong RF, Hellsten U, Kawashima T, et al. The amphioxus genome and the evolution of the chordate karyotype. *Nature* 2008;**453**:1064–71.

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28. Reinscheid RK, Nothacker HP, Bourson A, Ardati A, Henningsen RA, Bunzow JR, et al. Orphanin FQ: a neuropeptide that activates an opioidlike G protein-coupled receptor. *Science* 1995;**270**:792–4.
29. Rivas-Boyero AA, Herrero-Turrion MJ, Gonzalez-Nunez V, Sanchez-Simon FM, Barreto-Valer K, Rodriguez RE. Pharmacological characterization of a nociceptin receptor from zebrafish. (*Danio rerio*) *J Mol Endocrinol* 2011;**46**:111–23.
30. Sundstrom G, Dreborg S, Larhammar D. Concomitant duplications of opioid peptide and receptor genes before the origin of jawed vertebrates. *PLoS One* 2010;**5**:e10512.
31. Takahashi A, Kobayashi Y, Amano M, Yamanome T. Structural and functional diversity of proopiomelanocortin in fish with special reference to barfin flounder. *Peptides* 2009;**30**:1374–82.
32. Takahashi A, Nakata O, Moriyama S, Nozaki M, Joss JM, Sower SA, et al. Occurrence of two functionally distinct proopiomelanocortin genes in all modern lampreys. *Gen Comp Endocrinol* 2006;**148**: 72–8.

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Chapter 214

Exorphins

Masaaki Yoshikawa

ABSTRACT

Exorphin is a term suggested by Zoiudrou for opioid peptides derived from proteins of exogenous origin such as milk or plant. Many peptides having opioid activities have been shown to be released from proteins other than the classical precursor proteins for endogenous opioid peptides. Most of these exogenous opioid peptides have a Tyr residue at their amino termini. However, they differ from the typical endogenous ones by the absence of the enkephalin sequence. Such a typical opioid peptides are released from various proteins of animal and plant origin. Exorphins in the true sense may be those from plant proteins since those from animal proteins may function as endogenous peptides under certain conditions. Nevertheless, in this chapter, a typical opioid peptides of both plant and animal origin are described. Exogenous opioid peptides are classified into 2 groups based on whether they contain Tyr-Pro sequence, and they are subdivided into 4 groups.

OPIOID PEPTIDES DERIVED FROM PLANT PROTEINS

Gluten Exorphins

By using the mouse vas deferens (MVD) assay, Zioudou found opioid activity in the enzymatic digests of wheat gluten.[35] We isolated four opioid peptides, Gly-Tyr-Tyr-Pro-Thr (gluten exorphin-A5), Gly-Tyr-Tyr-Pro (gluten exorphin-A4), Tyr-Gly-Gly-Trp-Leu (gluten exorphin-B5), and Tyr-Gly-Gly-Trp (gluten exorphin-B4), from the pepsin–thermolysin digest of wheat gluten.[6] All of these were rather selective for the δ-receptor. Their IC_{50} values in the MVD assay were 60, 70, 0.017, and 3.4 μM, respectively. Gluten exorphins-A5, -B5, and -B4 were also released by the action of pepsin and pancreatic elastase.[3] Large amounts of des-Gly[1]-gluten exorphin-A5 (Tyr-Tyr-Pro-Thr) showing marginal δ-opioid activity in MVD assay: (IC_{50} = 800 μM) were also found in the same digest. Gluten exorphin-A5 sequence was found 15 times in the primary structure of high-molecular-weight glutenin. However, gluten exorphin-A5 is released from only one of these sites (-SGYYPTS-) by the action of pepsin and pancreatic elastase. The Tyr[3] in gluten exorphins-A5 is absolutely essential for the activity, whereas Tyr[2] can be replaced with Phe. Therefore, gluten exorphin-A could be classified as opioid peptide containing Tyr-Pro.

Among gluten exorphins, gluten exorphin-B5, which corresponds to [Trp[4]]-[Leu]-enkephalin, showed the most potent δ-opioid activity in the MVD and δ-receptor binding assays. However, in most *in vivo* assays, gluten exorphin-A is weaker than gluten exorphin-B probably because of susceptibility to peptidases. In a single trial passive avoidance experiment with step-through cages, gluten exorphin-A5 stimulated acquisition of memory after oral administration at doses of 300 mg/kg.[19] However, gluten exorphin-B5 was ineffective at the same dose. Gluten exorphins-A5 and -B5 stimulated postprandial insulin secretion after oral administration at a dose of 30 and 300 mg/kg, respectively, in rats.[3] In contrast, gluten exorphin-B5 is reported to stimulate prolactin secretion after intravenous administration at a dose of 3 mg/kg in rats.[2]

Another opioid peptide Tyr-Pro-Ile-Ser-Leu was isolated from the pepsin–chymotrypsin–trypsin digest of wheat gluten. This peptide was named gluten exorphin-C.[5] Gluten exorphin C was slightly selective for δ-receptor. Its IC_{50} value in the MVD assay was 13.5 μM. It should be noted that the Tyr-Pro sequence in gluten exorphin C was not followed by an aromatic amino acid, which has been believed to be essential for the opioid activity but by an aliphatic amino acid.

We found the Tyr-Pro-Leu-Gly-Gln sequence in the primary structure of wheat gliadin as an analog of Tyr-Pro-Leu-Gly-NH_2 (Tyr-MIF-I), and synthesized it. The peptide was rather selective for the μ-receptor because it exhibited opioid activity in the guinea-pig ileum (GPI) assay (IC_{50} = 7 μM) as did Tyr-MIF-I, an antiopioid peptide.[7] We named it gluten exorphin D.

Based on homology to β–casomorphin-7, Tyr-Pro-Gln-Pro-Gln-Pro-Phe sequence found in α-gliadin was synthesized and named gliadorphin.[8] Although the peptide did not exhibit opioid activity in the MVD assay, its binding to human peripheral lymphocytes was blocked by naloxone and an enkephalin derivative.

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00214-1
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Rubiscolins

Ribulose bisphosphate carboxylase/oxygenase (Rubisco) is a key enzyme in photosynthesis catalyzing corbondioxide fixation. Rubisco is ubiquitus for photosynthetic organisms and is regarded as the most abundant protein on earth., From a nutritional point of view, the large subunit of Rubisco has an exceptionally ideal composition of essential amino acids among plant proteins. Therefore, plant Rubisco is expected to be a large source of food protein in the future. We found the Tyr-Pro-Leu sequence, which satisfies the requirements for opioid activity as described above, in the conserved region in the large subunit of plant Rubisco. We then synthesized the corresponding sequences, Tyr-Pro-Leu-Asp-Leu-Phe and Tyr-Pro-Leu-Asp-Leu. Both peptides were active in the MVD assay; IC_{50} values were 24.4 and 50 μM, respectively.[26] We named them Rubiscolins-6 and -5, respectively. Their opioid activities were very weak in the GPI and μ receptor binding assays. Results from the receptor binding assay also suggested that rubiscolins are δ-selective opioid peptides.

We examined the conditions for the enzymatic release of rubiscolins from spinach Rubisco by using a model peptide and partially purified protein. Rubiscolins-6 and -5 were released by the action of pepsin, pancreatic elastase, and leucine aminopeptidase. The yield of rubiscolin-5 was higher than that of rubiscolin-6.

In certain algae species, the residue corresponding to Leu^3 of rubiscolin-6 is replaced by Ile. Tyr-Pro-Ile-Asp-Leu-Phe is about 4 times more active than Tyr-Pro-Leu-Asp-Leu-Phe in the MVD assay.[28] The Asp residue in Rubiscolin is essential for δ-opioid activity; it could not be replaced with Glu. Replacement of Leu^3 or Ile^3 with Phe or Trp also results in a reduction of δ-opioid activity. The rubiscolin sequence has been present in Rubisco far longer than the appearance of δ-receptors in animals. The sequence might be essential for enzymatic activity. It may be attractive to speculate that the δ-receptor in animals has evolved to fit the rubiscolin sequence in Rubisco. However, this possibility might be remote. We think that the rubiscolin sequence might have affinity for the δ-receptor in animals just by chance. This might be the case for most plant-derived peptide sequences acting on animal receptors. In this sense, these bioactive peptides could be regarded as those obtained from a sort of random library.

Rubiscolin-6 showed antinociceptive activity in mice after oral administration at a dose of 300 mg/kg. Rubiscolin-6 stimulated acquisition of memory after oral administration at doses of 100/mgkg in a passive avoidance experiment.[27] In the elevated-plus maze test in mice, rubisolin-6 showed anxiolytic activity after oral administration at a dose of 30 mg/kg. Thus, rubiscolin-6 is about 3 times more potent than gluten exorphin-A5 *in vivo*.

Both μ and δ opioids have been reported to inhibit memory consolidation, while κ opioid stimulated it. It is an interesting observation that food-derived δ opioid such as gluten exorphin and rubiscolin stimulate memory consolidation after oral administration.

Rubiscolin-6 stimulated food intake on a normal diet after oral administration in mice at a dose of 0.3 mg/kg in a δ-receptor-dependent manner, followed by activation of prostaglandin D_2 and neuropeptide Y systems (see Kanako et al., 2012, *Mol Nutr Food Res*). However, it suppressed food intake on a high fat diet via a different mechanism. Recently, rubiscolin-6 was reported to suppress the escape reaction of the American cockroach, which might suggest the protective role of the peptide for plants against insects.[9]

Soymorphin

A μ-selective opioid peptide soymorphin-5 (YPFVV), which has a homology to human β-casomorphin, is released from soybean β-conglycinin β subunit by the action of pancreatic elastase and leucine aminopeptidase.[18] It exhibits anxiolytic-like activity after oral administration in mice. Soymorphin-7 (YPFVVN) suppresses food intake and intestinal transit after oral administration in mice at a dose of 10 mg/kg.[15] Soymorphin-5 decreases glucose and triglyceride levels through activating adiponectin and PPARα systems in diabetic KK-Ay mice (see Yamada et al., 2012, *Am J Physiol Endocrinol Metab*). It has been reported that impairment of glucose and lipid metabolism associated with diabetes is improved by ingestion of soy protein. Soymorphin might be one of the active components responsible for such effects.

OPIOID PEPTIDES DERIVED FROM ANIMAL PROTEINS (SEE THE HEMORPHINS/CASOMORPHINS CHAPTER IN THIS SECTION OF THE BOOK)

β-Casomorphin, Cytochrophin, and Hemorphin

β-Casomorphin-7 Tyr-Pro-Phe-Pro-Gly-Pro-Ile, which is derived from amino acid residues 60–66 of bovine β-casein, was isolated by Brantl et al. from commercial casein peptone as the first example of exogenous opioid peptide.[24] β-Casomorphin-5 showed higher activity than β-Casomorphin-7. β-Casomorphin-4 amide Tyr-Pro-Phe-Pro-N_{H2}, which was synthesized first and then isolated from *in vivo* digests, was the most potent among the derivatives. It was the first peptide ligand selective for μ receptor and named morphiceptin. Elongated forms such as β-casomorphins-9, -11, -13, and -21 have been isolated from *in vitro* or *in vivo* digests of β-casein.[12] Opioid activities of these elongated forms were all weaker than

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β-Casomorphin-7. It should be noted that β-casomorphin-7 is released from β-casein A1, B, and C, minor genetic variants in which Pro^{67} is replaced with His, by the action of pepsin, pancreatic elastase, and leucine aminopeptidase.[12] In human β-casein, β-casomorphin with modified sequence Tyr-Pro-Phe-Val-Glu-Pro-Ile and weaker activity is conserved.[24,34] However, similar opioid peptides will not be released from murine β-casein because Tyr residue essential for opioid activity is not conserved.

From enzymatic digest of bovine blood, two opioid peptides Tyr-Pro-Phe-Thr, and Tyr-Pro-Trp-Thr were isolated by Brantl et al.[24] They were derived from cytochrome b and hemoglobin β and named cytochrophin and hemorphin, respectively. These peptides might work as endogenous opioid peptides under certain conditions. Hemorphin-5, Tyr-Pro-Trp-Thr-Gln, is released by the action of pancreatic elastase from hemoglobin. It should be noted that pancreatic elastase is engaged in the release of β-casomorphin, hemorphin, gluten exorphin, rubiscolin, and soymorphin[3,12,13,18].

Thus, β-casomorphin, cytochrophin, and hemorphin have a Tyr-Pro-aromatic sequence in common, which is also found in endomorphins 1 and 2 (Tyr-Pro-Trp-Phe-NH_2 and Tyr-Pro-Phe-Phe-NH_2)

α-Casein Exorphin

Loukas et al. isolated two opioid peptides RYLGYLE, and RYLGYL from pepsin digest of α-casein.[17] These are derived from the amino acid residues 90–96 and 90–95 of the protein. These peptides are selective for the δ-receptor, and their IC_{50} values in the MVD assay are 30 and 70 μM, respectively.

αs1-Casomorphin

Kampa et al. found that synthetic human αs1-casein(158–162), Tyr-Val-Pro-Phe-Pro, had affinity for $\kappa_1$and κ_2 receptors and named it αs1-casomorphin.[14] αs1-Casomorphin inhibited growth of T47D breast cancer cells.[10] The αs1-casomorphin sequence is found in the structure of casoxin D, Tyr-Val-Pro-Phe-Pro-Pro-Phe, which has been isolated from the pepsin–trypsin–chymotrypsin digest of human casein as an antiopioid and ileum-contracting peptide. As will be described later, Casoxin D has affinity for bradykinin B_1 receptor[29,32] (see the chapter on antiopioid peptides in this section of the book).

Neocasomorphin and Other Casein-Derived Peptides

We isolated an opioid peptide Tyr-Pro-Val-Glu-Pro-Phe, which corresponds to β-casein(194–119), from a pepsin–pancreatin digest of bovine β-casein and named it neocasomorphin.[12] The peptide showed weak opioid activity in the GPI assay (IC_{50}=59 μM). This peptide is classified as an opioid containing the Tyr-Pro-aliphatic residue.

We synthesized Tyr-Pro-Ser-Phe-NH_2 and Tyr-Gly-Phe-Leu-Pro, which are found in the primary structure of human β-casein, residue 59–63, and 41–44, respectively. Their IC_{50} values in the GPI assay were 30 and 270 μM, respectively. They were almost inactive in the MVD assay. We named the latter β-casorphin.[33]

Other Opioid Peptides Derived from Other Animal Proteins

To look for possible opioid peptide sequences in whey proteins, we synthesized Tyr-Gly-Leu-Phe-NH_2 and Tye-Leu-Leu-Phe-NH_2, which correspond to bovine α-lactalbumin(50–53) and β-lactoglobulin(102-105), respectively, and tested them in the GPI assay system. Both peptides inhibited electrically induced twitches of the ileum preparation with IC_{50} values 50 μM and 160 μM, respectively.[33] We named them α-lactorphin and β-lactorphin, respectively. Although inhibition of the twiches by α-lactorphin was blocked by naloxone, those by β-lactorphin was unaffected. Nonamidated α-lactorphin is released by the action of pepsin.

We isolated an opioid peptide Tyr-Gly-Phe-Gln-Asn-Ala from a pepsin digest of bovine serum albumin and named it serorphin.[23] This peptide is derived from amino acid residues 399–404 of the protein. Serorphin is selective for δ-receptor because its IC_{50} values in the MVD and GPI assays were 8.5 and 230 μM, respectively.

As for similar opioid peptide containing Tyr-Gly-Phe sequence, Tyr-Gly-Phe-Gly-Gly and Tyr-Gly-Phe-Ile-Leu have been synthesized by Khachenko et al. These are found in the primary structure of histone H4 and carboxypeptidases A and B, and named historphin and valentorphin, respectively. Historphin showed opioid activity in mouse vas deferens assay (IC_{50}=2.5 μM). They also synthesized similar peptide Tyr-Ser-Phe-Gly-Gly found in the primary structure of immunoglobulin κ–chain and named it kapporphin.[23]

INTRAPROTEIN OPIOID SEQUENCE

Most endogenous and opioid peptides have a Tyr residue at the amino terminus, and a few of them such as gluten exorphin-A have Tyr residue in the second position from the terminus. This is because a positive charge near the phonol group of Tyr is essential for the opioid activity. Lipkowski et al. found that Tyr residue in the midst of peptides could bind to μ-receptor if it is followed by basic residues.[16] They found that this is the case in the GAG protein of HIV virus and speculated that HIV might infect immuno- and neurocells via μ-receptor expressed on them.

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CLASSIFICATION OF EXORPHINS ACCORDING TO THEIR STRUCTURE

In Table 1, a typical opioid peptides derived from proteins are classified into 2 groups, those containing Tyr-Pro sequence and others. Then, the Tyr-Pro group is further classified into two groups, those containing Tyr-Pro-aromatic amino acid residues, and Tyr-Pro-non-aromatic amino acid residues. The non-Tyr-Pro group is also subdivided into two groups according to the distance between Tyr and the aromatic amino acid residues. In *in vitro* assay systems, potency of exorphins are usually 1/100–1/100 that of endogenous opioid peptides. However, some exogenous opioid peptides are active after oral administration in which none of the endogenous opioid peptides are active. One of the reason for this may be that Tyr-Pro-X sequence is more resistant to peptidases than enkaphalin sequence.

TABLE 1 Opioid Peptides Derived from Natural Proteins

Structute	Peptides
Tyr-Pro type	
Tyr-Pro-Aromatic type	
H-Tyr-Pro-Phe-Pro-Gly-Pro-Ile-OH	β-Casomorphin
H-Tyr-Pro-Phe-Val-Glu-Pro-Ile-OH	*h*β-Casomorphin
H-Tyr-Pro-Phe-Thr-OH	Cytochrophin
H-Tyr-Pro-Trp-Thr-OH	Hemorphin
H-Tyr-Pro-Phe-Val-Val-OH	Soymorphin-5
Tyr-Pro-nonaromatic type	
H-Gly-Tyr-Tyr-Pro-Thr-OH	Gluten exorphin A5
H-Tyr-Pro-Ile-Ser-Leu-OH	Gluten exorphin C
H-Tyr-Pro-Leu-Gly-Gln-OH	Gluten exorphin D
H-Tyr-Pro-Leu-Asp-Leu-Phe-OH	Rubiscolin-6
H-Tyr-Pro-Val-Glu-Pro-Phe-OH	Neocasomorphin
non-Tyr-Pro type	
Tyr-X_1-X_2-Aromatic type	
H-Arg-Tyr-Leu-Gly-Tyr-Leu-Asp-OH	α-Casein exorphin
H-Tyr-Gly-Gly-Trp-Leu-OH	Gluten exorphin B5
H-Tyr-Gly-Leu-Phe-NH_2	α-Lactorphin
H-Tyr-Leu-Leu-Phe-NH_2	β-Lactorphin
H-Tyr-Pro-Ser-Phe-NH_2	*h*Casorphin
H-Tyr-Val-Pro-Phe-Pro-OH	*h*αsl-Casomorphin
Tyr-X-Aromatic type	
H-Tyr-Gly-Phe-Gln-Asn-Ala-OH	Serorphin
H-Tyr-Gly-Phe-Leu-Pro-OH	*h*β-Casein(41-44)
H-Tyr-Gly-Phe-Gly-Gly-OH	Historphin
H-Tyr-Gly-Phe-Ile-Leu-OH	Valentorphin
H-Tyr-Ser-Phe-Gly-Gly-OH	Kapporphin
Intraprotein opioid sequence	
Ac-Asp-Ile-Tyr-Arg-Arg-Trp-Ile-Ile-Leu-NH_2	HIV GAG protein 24 fragment

Prefix ***h*** means human origin.

OPIOID ANTAGONIST AND ANTIOPIOID PEPTIDE DERIVED FROM PROTEINS

During the screening of opioid peptides in enzymatic digests of proteins, we found that some peptides showed antiopioid activity in the GPI and antinociceptive assays. These are classified into antagonists acting on opioid receptor, and antiopioid peptides acting at the postreceptor level (Table 2).

TABLE 2 Opioid Antagonist and Antiopioid Peptides Derived from Natural Proteins

Structure	Peptides
Opioid antagonists	
H-Tyr-Pro-Ser-Tyr-Gly-Leu-Asn-OH	Casoxin A
H-Pro-Ty-Pro-Tyr-Tyr-OH	Casoxin B
H-Ser-Ty-Pro-Tyr-Tyr-OH	*h*Casoxin B
Peptide methylester	
H-Ser-Arg-Tyr-Pro-Ser-Tyr-OCH_3	Casoxin-6
H- Arg-Tyr-Pro-Ser-Tyr-OCH_3	Casoxin-5
H-Tyr-Pro-Ser-Tyr-OCH_3	Casoxin-4
H-Tyr-Leu-Gly-Ser-Gly-Tyr-OCH_3	*h*Lactoforroxin A
H-Arg-Tyr-Tyr-Gly-Tyr-OCH_3	*h*Lactoforroxin B
H-Lys-Tyr-Leu-Gly-Pro-Gln-Tyr-OCH_3	*h*Lactoforroxin C
Antiopioid peptides	
H-Tyr-Ile-Pro-Ile-Gln-Tyr-Val-Leu-Ser-Arg-OH	Casoxin C
H-Tyr-Val-Pro-Phe-Pro-Pro-Phe-OH	*h*Casoxin D
H-Gly-Tyr-Pro-Met-Tyr-Pro-Leu-Pro-Arg-OH	Oryzatensin
H-Arg-Ile-Tyr-OH	Rapakinin

Prefix ***h*** means human origin.

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Opioid Antagonist Peptides

The first example of an endogenous opioid antagonist was described in 1979 for MIF-1 (see the Antiopioid chapter in this section of the book). The first exogenous antiopioid peptide is Ser-Arg-Tyr-Pro-Ser-Tyr-OCH_3, a peptide carrying Tyr-methyl ester at the carboxyl terminus. This peptide was obtained as an artificial product from a pepsin digest of bovine κ-casein, and named casoxin-6.[30,31] The esterification of Tyr occurred during the extraction of the lyophilized digest with anhydrous chloroform-methanol. The peptide showed weak affinity for μ and κ-receptors (IC_{50} = 5.5 and 10.1 μM, respectively), and antagonized opioid activities of DAMGO and dynorphin A at 20 μM in the guinea-pig ileum assay. Interestingly, a truncated peptide Tyr-Pro-Ser-Tyr-OCH_3 (casoxin-4) had no affinity for κ-receptor. From the observation that basic residues at the amino terminus region direct the antagonist for te κ–receptor, we speculated that casoxin-6 binds κ-receptor reversewise to κ agonist peptides such as dynorphin, in which basic residues in the carboxyl terminus region are essential for κ selectivity. Affinities of nonesterified peptides for opioid receptors are <1/10 those of the esterified from.

As for natural peptides showing opioid antagonist activitiy, we obtained Tyr-Pro-Ser-Tyr-Gly-Leu-Asn-Tyr from pepsin–trypsin digest of bovine κ-casein.[1] This peptide was named casoxin A, and antagonized opioid activity of DAMGO at 50 μM in the GPI assay. From the synthetic approach, we found that Pro-Tyr-Pro-Tyr-Tyr, and Ser-Tyr-Pro-Tyr-Tyr derived from bovine and human κ-casein, respectively, showed opioid antagonist activity under the same conditions. We named them bovine and human casoxin B, respectively.

As for opioid antagonists having Tyr-methyl ester, we obtained three peptides, Tyr-Leu-Gly-Ser-Gly-Tyr-OCH_3, Arg-Tyr-Tyr-Gly-Tyr-OCH_3, and Lys-Tyr-Leu-Gly-Pro-Gln-Tyr-OCH_3, from pepsin digest of human lactoferrin, and named them lactoferroxins A, B, and C.[22] Because of basic amino acid residues situated at their amino termini, lactoforroxins-B and C showed affinity for κ receptor in addition to μ receptor. Physiological significance of these peptides might be small because opioid antagonist activities of nonesterified forms are very weak.

Antiopioid Peptides

Using the guinea-pig ileum assay, we isolated an antiopioid peptide Tyr-Ile-Pro-Gln-Tyr-Val-Leu-Ser-Arg from a trypsin digest of bovine κ-casein.[1] This peptide was derived from the amino acid residue 25–34 of the protein, and named casoxin C. Casoxin-C showed weak affinity for μ receptor (Ki = 200 μM). Its antiopioid activity in the GPI was more potent than expected from receptor affinity. Further, casoxin-C was different from a typical opioid antagonists such as naloxone and casoxin-A in that inducing contraction of the ileum preparation in the absence of electrical stimuli. Casoxin-C proved to have affinity for receptor for complement C3a.[21] Complement C3a is made of 75 amino acid residues and released from complement C3 on activation of complement system to stimulate immune systems. It induces ileum contraction and inflammation and is called anaphylatoxin. The peptapeptide sequence at the carboxyl terminus of C3a, Leu-Gly-Leu-Ala-Arg, is the minimally essential structure required for C3a activity. This structural requirement could be generalized to hydrophobic residue-X_1-Leu-X_2-Arg. Casoxin C has affinity for complement C3a receptor (IC_{50} = 40 μM) because it meets this requirement. In the GPI, casoxin C and complement C3a antagonized opioid activities of DAMGO and dynorphin A at 10 μM.[21] However, they did not antagonize DPDEP in the MVD. In GPI, casoxin C and C3a induce biphasic contraction, rapid and slow ones. The rapid contraction is mediated by histamine release while the slow contraction is mediated by prostaglandin E_2 and acetylcholine. The antiopioid activities of casoxin C and C3a might be mediated by prostaglandin E_2 and acetylcholine because they are related to the slow contraction. After icv administration, casoxin C antagonized antinociceptive activity of morphin and dynorphin A, but DPDPE. Further, it reversed amnesia induced by scopolamine or cerebral ischemia in mice. Based on this observation, we also found for the first time that complement C3a itself also had antianalgesic and antiamnesic activites.[11] This suggests that C3a may work as an antiopioid peptide *in vivo*.

We obtained a peptide Gly-Tyr-Pro-Met-Tyr-Pro-Leu-Pro-Arg, which showed similar antiopioid activity in the GPI, from a trypsin digest of rice albumin and designated it oryzatensin.[20] Besides affinity for μ-opioid receptor (IC_{50} = 39 μM), oryzatensin has affinity for C3a receptors (IC_{50} = 44 μM) because it satisfies the structural requirements as described above.

A tripeptide Arg-Ile-Tyr isolated from a subtilisin digest of rapeseed protein based on inhibitory activity for Angiotensin I-converting enzyme (ACE) exhibited antihypertensive activity after oral administration in spontaneously hypertensive rats (SHR). The peptide was named rapakinin because it showed vasorelaxing activity in mesenteric artery isolated from SHR, which is mediated by the IP receptor for prostaglandin I_2 and CCK_A receptor downstream of the unidentified receptor of the peptide. The antihypertensive activity of rapakinin is mainly mediated by its vasorelaxation rather than ACE inhibition. Rapakinin antagonized the antinociceptive activity of morphine though it showed no affinity for opioid receptors.[25] This antiopioid activity is also by mediated IP receptor and CCK_B receptor.

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Another peptide Tyr-Val-Pro-Phe-Pro-Pro-Phe, which shows antiopioid activity in GPI and MVD assays, was obtained from a chymotrypsin digest of human casein.[32] This peptide is derived from human αs1-casein and named casoxin D. EC_{50} values for antiopioid activity in the GPI and MVD assays were 10^{-6}–10^{-5} M, respectively. However, affinities of casoxin D for μ and δ receptors were very weak (IC_{50} = 250 and 150 μM, respectively). Casoxin D has weak ileum-contracting activity (EC_{50} = 80 μM).[28] Casoxin D also exhibits relaxing activity in canine mesenteric artery (EC_{50} = 3 μM), which was blocked by des-Arg[9]-[Leu[8]]-bradykinin, an antagonist for bradykinin B_1 receptor, and indomethacin. In fact, casoxin D showed affinity for B_1 receptor. However, its antiopioid and ileum-contracting activities are not blocked by the B_1 antagonist. Thus, receptors mediating antiopioid and ileum-contracting activities of casoxin D are still unknown.

CONCLUDING REMARKS

Thus, exorphins and antiopioid peptides are released from various proteins. Stimulated by the concept of exorphin, many kinds of bioactive peptides acting through various types of receptors other than opioid have been also isolated from the enzymatic digest of animal and plant proteins. Those derived from animal proteins may have teleological functions. However, those of plant origin could be regarded as those derived from a sort of random library. Studies on physiological and pathological significance of those bioactive peptides will contribute to the design of foods and drugs to prevent diseases and promote our health.

REFERENCES

1. Chiba H, Tani F, Yoshikawa M. Opioid antagonist peptides derived from κ-casein. *J Dairy Res* 1989;**56**:363–6.
2. Fanciulli G, Dettori A, Fenude E, Demontis MP, Alberico E, Delitala G, et al. Intravenous administration of the food-derived opioid peptide gluten exorphin B5 stimulates prolactin secretion in rats. *Pharmacol Res* 2003;**47**:53–85.
3. Fukudome S, Jinsmaa Y, Matsukawa T, Sasaki R, Yoshikawa M. Release of opioid peptides, gluten exorphins by the action of pancreatic elastase. *FEBS Lett* 1997;**412**:475–9.
4. Fukudome S, Shimatsu A, Suganuma H, Yoshikawa M. Effect of gluten exorphins A5 and B5 on the postprandial plasma insulin level in conscious rats. *Life Sci* 1995;**57**:729–34.
5. Fukudome S, Yoshikawa M. Gluten exorphin C. A novel opioid peptide derived from wheat gluten. *FEBS Lett* 1993;**316**:17–9.
6. Fukudome S, Yoshikawa M. Opioid peptides derived from wheat gluten: their isolation and characterization. *FEBS Lett* 1992;**296**: 107–11.
7. Fukudome S, Yoshikawa M. The structure–activity relationships of gluten exorphins. In: Yanaihara N, editor. *Peptide chemistry 1992*. Leiden: ESCOM; 1993. p. 390–3.
8. Graf L, Horvath K, Walcz E, Berzetei I, Burnier J. Effect of two synthetic α-gliadin peptides on lymphocytes in celiac disease: identification of a novel class of opioid receptors. *Neuropeptides* 1987;**9**:113–22.
9. Gritsaĭ OB, Dubynin VA, Bespalova ZhD, Pilipenko VE. Effects of several exorphins and endorphins on the escape reaction of the cockroach *Periplaneta americana* under elevated temperature conditions]. *Zh Evol Biokhim Fiziol* 2009;**45**:391–7.
10. Hatzoglou A, Bakogeorgou E, Hatzoglou C, Martin PM, Castanas E. Antiproliferative and receptor binding properties of α- and β-casomorphins in the T47D human breast cancer cell line. *Eur J Pharmacol* 1996;**310**:217–23.
11. Jinsmaa Y, Takahashi M, Takahashi M, Yoshikawa M. Anti-analgesic and anti-amnesic effect of complement C3a. *Life Sci* 2000;**67**:2137–43.
12. Jinsmaa Y, Yoshikawa M. Enzymatic release of neocasomorphin and β-casomorphin from bovine β-casein. *Peptides* 1999;**20**:957–62.
13. Jinsmaa Y, Yoshikawa M. Release of hemorphin-5 from human hemoglobin by pancreatic elastase. *Biosci Biotechnol Biochem* 2002;**66**:1130–2.
14. Kampa M, Loukas S, Hatzoglou A, Martin P, Martin PM, Castanas E. Identification of a novel opioid peptide (Tyr–Val–Pro–Phe–Pro) derived from human αs1-casein (αs1-casomorphin, and αs1-casomorphin amide). *Biochem J* 1996;**319**:903–8.
15. Kaneko K, Iwasaki M, Yoshikawa M, Ohinata K. Orally administered soymorphins, soy-derived opioid peptides, suppress feeding and intestinal transit via gut, μ1-receptor coupled to 5-HT(1A), D(2), and GABA(B) systems. *Am J Physiol Gastrointest Liver Physiol* 2010;**299**:G799–805.
16. Lipkowsk AW, Kosson P, Misicka A, Carr DB, Hruby VJ, Yoshikawa M. Fragments of HIV GAG polyproteins expressing affinity to opioid receptors. *Biopolymers* 2003;**71**:294.
17. Loukas S, Varoucha D, Zioudrou C, Streaty RA, Klee WA. Opioid activities and structures of α-casein-derived exorphins. *Biochemistry* 1983;**22**:4567–73.
18. Ohinata K, Agui S, Yoshikawa M. Soymorphins, novel mu opioid peptides derived from soy beta-conglycinin beta-subunit, have anxiolytic activities. *Biosci Biotechnol Biochem* 2007;**71**:2618–21.
19. Takahashi M, Fukunaga H, Kaneto H, Fukudome S, Yoshikawa M. Behavioral and pharmacological studies on gluten exorphin A5, a newly isolated bioactive food protein fragment, in mice. *Jpn J Pharmacol* 2000;**84**:259–65.
20. Takahashi M, Moriguchi S, Ikeno M, Kono S, Ohata K, Usui H, et al. Studies on the ileum-contracting mechanisms and identification as a complement C3a receptor agonist of oryzatensin, a bioactive peptide derived from rice albumin. *Peptides* 1996;**17**:5–12.
21. Takahashi M, Moriguchi S, Suganuma H, Shiota A, Tani F, Usui H, et al. Identification of casoxin C, an ileum-contracting peptide derived from bovine κ-casein, as an agonist for C3a receptors. *Peptides* 1997;**18**:329–36.
22. Tani F, Iio K, Chiba H, Yoshikawa M. Isolation and characterization of opioid antagonist peptides derived from human lactoferrin. *Agric Biol Chem* 1990;**54**:1803–10.
23. Tani F, Shiota A, Chiba H & Yoshikawa M. Serorphin, an opioid peptide derived from bovine serum albumin. In β-casomorphins and related peptides: recent developments, pp. 49–53 [V Brantl and H Teschemacher, editors]. Weinheim: VCH.
24. Teschemacher H, Koch G, Brantl V. Milk protein-derived opioid receptor ligands. *Biopolymers* 1997;**43**:99–117.

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25. Yamada Y, Ohinata K, Lipkowski AW, Yoshikawa M. Rapakinin, Arg–Ile–Tyr, derived from rapeseed napin, shows anti-opioid activity via the prostaglandin IP receptor followed by the cholecystokinin CCK_2 receptor in mice. *Peptides* 2011;**32**:281–5.
26. Yang S, Jinsmaa Y, Sonoda S, Doyama N, Lipkowski AW, Kawamura Y, et al. Rubiscolin, a delta selective opioid peptide derived from plant Rubisco. *FEBS Lett* 2001;**509**:213–7.
27. Yang S, Kawamura Y, Yoshikawa M. Effect of rubiscolin, a delta opioid peptide derived from Rubisco, on memory consolidation. *Peptides* 2003;**24**:325–8.
28. Yang S, Sonoda S, Chen L, Yoshikawa M. Structure-activity relationship of rubiscolins as delta opioid peptides. *Peptides* 2003;**24**:503–8.
29. Yoshikawa M, Suganuma H, Shiota A, Tani F, Usui H, Kurahashi K, et al. Casoxin D: a bradykinin agonist peptide derived from human casein. In: Yanaihara N, editor. *Peptide chemistry 1992*. Leiden: ESCOM; 1993. p. 572–5.
30. Yoshikawa M, Tani F, Ashikaga T, Yoshimura T, Chiba H. Purification and characterization of an opioid antagonist from a peptic digest of bovine κ-casein. *Agric Biol Chem* 1986;**50**:2951–4.
31. Yoshikawa M, Tani F, Chiba H. Structure-activity relationship of opioid antagonist peptides derived from milk proteins. In: Shiba T, Sakakibara S, editors. *Peptide chemistry 1987*. Osaka: Protein Research Foundation; 1988. p. 473–6.
32. Yoshikawa M, Tani F, Shiota A, Suganuma H, Usui H, Kurahashi K, et al. Casoxin D: an opioid antagonist/ileum-contracting/vasorelaxing peptide derived from human αs1-casein. In: Brantl V, Teschemacher H, editors. β-*casomorphins and related peptides: recent developments*. Weinheim: VCH; 1994. p. 43–8.
33. Yoshikawa M, Tani F, Yoshimura T, Chiba H. Opioid peptides from milk proteins. *Agric Biol Chem* 1986;**50**:2419–21.
34. Yoshikawa M, Yoshimura T, Chiba H. Opioid peptides from human β-casein. *Agric Biol Chem* 1984;**48**:3185–7.
35. Zioudrou C, Streaty RA, Klee WA. Opioid peptides derived from food proteins. The exorphins. *J Biol Chem* 1979;**254**:2446–9.

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Chapter 215

Nociceptin/Orphanin FQ

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ABSTRACT

Nociceptin/orphanin FQ (N/OFQ; FGGFTGARKSARKLANQ) was identified as the endogenous ligand of a previously orphan GPCR now referred to as N/OFQ peptide (NOP) receptor. The peptide and its receptor are widely distributed in the nervous system where via inhibitory mechanisms they modulate several different biological functions including pain, stress and anxiety, locomotor activity, emotional responses, feeding, learning and memory, reward, and addiction. N/OFQ is also able to modulate peripheral functions of the cardiovascular, respiratory, intestinal, genitourinary, and immune systems. The pleiotropic actions of N/OFQ suggest NOP-selective ligands as potential therapeutics for a variety of different pathological conditions. In particular, based on preclinical evidence the following indications seem to be promising: depression and Parkinson's disease for NOP antagonists and anxiety and pain (after spinal administration) for NOP agonists. Finally clinical studies show that intravesical instillation of N/OFQ elicits beneficial effects in patients suffering from urinary incontinence resulting from an overactive bladder.

THE DISCOVERY OF NOCICEPTIN/ ORPHANIN FQ

In 1994, soon after the cloning of classical opioid receptors, several research groups identified a G protein–coupled receptor showing high homology with opioid receptors but not able to bind opioid ligands. On this basis, the protein was named opioid receptor–like-1 (ORL-1). This protein was used to fish for a natural ligand from brain extracts.[11] This process, called reverse pharmacology, was successful since one year after the cloning of ORL-1 receptor two groups reported the identification of the heptadecapeptide FGGFTGARKSARKLANQ, which was named nociceptin[17] for its ability to elicit hyperalgesia after supraspinal administration in mice and orphanin FQ[25] for its ability to recognize a previously orphan receptor and for its first and last amino acid residues (F and Q). Nociceptin/orphanin FQ (N/OFQ) displays a primary sequence very similar to that of opioid peptides. However, the presence of Phe in position 1 instead of Tyr makes this peptide highly selective for its receptor over classical opioid receptors. After the identification of N/OFQ as the naturally occurring ligand of ORL-1, the receptor was renamed, according to IUPHAR recommendations, N/OFQ peptide receptor and abbreviated as NOP. In this chapter, we review the biology of the peptide and its receptor, NOP-selective ligands, genetic tools such as NOP receptor (NOP(−/−)) and ppN/OFQ (ppN/OFQ(−/−)) knockout animals, and the biological functions controlled by the N/OFQ–NOP receptor system. Finally, we discuss the possible therapeutic indications of NOP-selective ligands.

THE N/OFQ PEPTIDE

N/OFQ derives from the peptide precursor preproN/OFQ (ppN/OFQ).[18] The ppN/OFQ gene comprises at least four exons separated by three introns. Exon I contains exclusively 5′ noncoding sequence, exons II and III share the open reading frame of the gene, and exon IV contains most of the 3′ noncoding region of the message. The sequence of murine and human ppN/OFQ genes displays organizational and structural features that are very similar to those of the genes encoding the precursors of the endogenous opioid peptides, namely, prepro-enkephalin, -dynorphin, and -opiomelanocortin, suggesting that the N/OFQ and opioid peptide genes have evolved from a common ancestor. The ppN/OFQ amino acid sequence is highly conserved across animal species, especially at the C-terminus where the sequence of the mature peptide is located between canonical Lys-Arg excision motifs. However, in addition to those framing N/OFQ, the ppN/OFQ sequence contains other cleavage sites, suggesting that ppNOP may generate other biologically active peptides. The peptide nocistatin has been shown to be biologically active[22]; in most cases, nocistatin does not produce any effect by itself but is able to counteract the actions of N/OFQ. Importantly, nocistatin does not bind to the NOP receptor. Despite considerable efforts put in by several groups, the receptor mediating the pharmacological actions of nocistatin is yet to be identified. Another peptide encoded by ppN/OFQ is known as N/OFQ II: this peptide stimulates locomotor activity in mice.

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00215-3
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ppN/OFQ mRNA and the N/OFQ peptide are broadly distributed in the central nervous system of the rat.[20] In most brain areas, peptide immunostaining and mRNA expression are very similar, suggesting that the peptide is mainly produced by interneurons. mRNA and peptide are particularly abundant in cortical and limbic structures (hippocampus, dentate gyrus, septal areas, and amygdala), a number of hypothalamic and brain stem nuclei, the dorsal and ventral horns of the spinal cord, and in cell bodies of the dorsal root ganglion. In several pain-related areas, including the superficial dorsal horn of spinal cord, sensory trigeminal complex, and periaqueductal gray, antisera to N/OFQ and opioid peptides label different fibers, consistent with the differential actions of N/OFQ and opioids on nociception.

This N/OFQ distribution in the central nervous system suggests that the peptide is potentially involved in the regulation of a variety of brain functions, including emotional processing, learning and memory, locomotion, reward, pain transmission, and autonomic regulation of peripheral organs and systems.

Little is known about the biosynthesis of N/OFQ, apart from the involvement of prohormone convertase 2 as shown by studies performed in mice knockout for this enzyme (see Peptide Biosynthesis/Processing section of the book). As far as N/OFQ metabolism is concerned, different studies show the involvement of aminopeptidase that generates [desPhe^1] N/OFQ a peptide lacking affinity for the NOP receptor. However, different endopeptidases are also involved in N/OFQ metabolism. Peptidase inhibitors are able to increase N/OFQ potency and, in some cases, maximal effects both *in vitro* and *in vivo*, thus showing an important role of peptidases in N/OFQ signaling. Indeed the inhibitory effect of the peptide in the human vas deferens can be detected in the presence of a cocktail of peptide inhibitors but not in their absence.

THE NOP RECEPTOR

The NOP receptor belongs to the superfamily of G protein–coupled receptors, characterized by seven transmembrane helices interconnected by alternate intracellular and extracellular loops. It displays high homology with opioid receptors, but it has no affinity for opioid ligands. The primary structure of the NOP receptor is highly conserved across mammalian species with mouse and human sequences being >95% identical. The NOP receptor gene is located in the distal region of mouse chromosome 2, and in the q13.2-13.3 region of human chromosome 20. Its coding sequence is organized into introns and exons in a manner similar to that of the opioid MOP, DOP, and KOP receptor genes, suggesting that the four genes have all evolved from a common ancestor, that is, they belong to the same family. Like other members of the opioid receptor family, the NOP receptor gene undergoes alternative splicing. The biological significance of these splice variants is unknown because pharmacological studies have not clearly established the existence of functionally distinct NOP receptor subtypes.

The NOP receptor mRNA is particularly abundant in the central nervous system, notably in the cortical and limbic areas, hypothalamus, brain stem, the dorsal and ventral horns of the spinal cord, and in the cell bodies of the dorsal root ganglion.[19] Extensive autoradiographic mapping of the NOP receptor protein shows similar distribution patterns to those of the mRNA, indicating that the receptor is expressed predominantly in local-circuit neurones. However, detailed studies on the expression of NOP protein by the different cell types have been limited to date by the lack of a specific and selective antibody.

The NOP receptor is coupled to Gi proteins that inhibit adenylate cyclase and calcium currents, and activate potassium channels. These cellular actions reduce synaptic efficacy, either by reducing transmitter release when the receptor is located presynaptically, or by reducing neuronal firing or excitability when the receptor is located postsynaptically. Indeed, many *in vitro* and *in vivo* studies have shown that NOP activation inhibits basal and/or stimulated release of various neurotransmitters, including acetylcholine, dopamine, serotonin, norepinephrine, GABA, glutamate, and substance P, in central as well as peripheral nerve tissues.[29] Similarly, when tested electrophysiologically on individual neurons, NOP activation produces an inhibition of basal and/or stimulated electrical activity.[7] These cellular effects produce inhibitory effects and can also be associated with circuit dependent disinhibitory actions, allowing an explanation of most, if not all of the biological actions elicited by N/OFQ.

There is evidence in the literature that NOP receptors can form heterodimers with classical opioid receptors particularly with MOP. This may have profound implication in terms of receptor signaling and trafficking. For instance NOP/MOP heterodimers were shown to associate with N-type calcium channels, with the activation of MOP receptors triggering N-type channel internalization, but only in the presence of NOP. Further, the formation of OP/NOP receptor heterodimers attenuated the NOP inhibition of N-type channels.[5] This kind of mechanism may be operative in primary sensory neurons and in brain areas relevant for nociceptive transmission and thus may have potentially profound effects on nociceptive processing.

PHARMACOLOGICAL AND GENETIC TOOLS

The first generation of NOP-selective ligands has been identified in the frame of structure activity studies on the N/OFQ peptide sequence.[2] N/OFQ(1-13)-NH_2 (but not N/OFQ(1-13)-OH) is the smallest fragment maintaining the same potency and efficacy of the natural peptide. C-terminal amidation is also useful in the peptide full sequence for increasing potency and reducing susceptibility to carboxypeptidases. A series of structure activity studies performed

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using N/OFQ(1-13)-NH_2 as chemical template allowed the identification of several interesting NOP-selective ligands such as (i) [Phe^{1}Ψ(CH_2-NH)Gly^2]N/OFQ(1-13)-NH_2, a partial agonist; (ii) [(pF)Phe^4]N/OFQ(1-13)-NH_2, a potent full agonist, (iii) [Nphe^1]N/OFQ(1-13)-NH_2 a low potency pure antagonist. Other chemical modifications such as the Aib substitution in positions 7 and 11 and the replacement of Leu-Ala with an extra couple of Arg-Lys residues in positions 14–15 were applied to the full peptide sequence and found to be useful for generating potent agonists. The above-mentioned chemical modifications applied to the N/OFQ sequence are shown in Fig. 1. In subsequent studies, these chemical modifications were combined into single molecules to identify the second generation of NOP-selective peptide ligands encompassing partial ([Phe^{1}Ψ(CH_2-NH) Gly^2(pF)Phe^{4}Aib^{7}Arg^{14}Lys^{15}]N/OFQ-NH_2, UFP-113) and full ([(pF)Phe^{4}Aib^{7}Arg^{14}Lys^{15}]N/OFQ-NH_2, UFP-112) agonist and pure antagonist ([Nphe^{1}Arg^{14}Lys^{15}]N/OFQ-NH_2, UFP-101) activities. These peptide ligands associated high potency with very high NOP selectivity and were instrumental in advancing our knowledge related to the N/OFQ–NOP receptor system.[1,3,11] Other NOP peptide ligands have been identified by screening of synthetic peptide combinatorial libraries. With this approach, the highly potent and NOP-selective partial agonists Ac-RYYRWK-NH_2 and Ac-RYYRIK-NH_2 were identified.[4] These peptide sequences were used in subsequent studies for generating other interesting NOP ligands including the peptide ZP120 that has been investigated and developed for its aquaretic activity. Another example of NOP ligand identified with combinatorial peptide chemistry approach is the nonselective NOP antagonist peptide III BTD.

The different biological functions controlled by the N/OFQ–NOP receptor system and the therapeutic potential of agents able to selectively activate or block the NOP receptor stimulated industrial interest in this field of research and several companies activated medicinal chemistry programs aimed at the identification of drug-like nonpeptide NOP ligands.[12] This led to the identification and characterization of interesting and useful molecules such as J-113397, a potent and selective antagonist identified by Banyu researchers and Ro 64-6198, a potent and selective agonist discovered at Roche.[30] These molecules are widely used for investigating the role played by the N/OFQ–NOP receptor system in physiological and pathological conditions and together with the peptides UFP-101[1] and UFP-112[3] still represent standard ligands for the NOP receptor. More recently, other nonpeptide NOP ligands were published including the highly potent and pure antagonists SB-612111 and Comp 24 and different series of full agonists identified by Pfizer and Schering-Plough researchers and characterized *in vivo* as innovative anxiolytics (see the chemical structure of nonpeptide NOP ligands in Fig. 2).

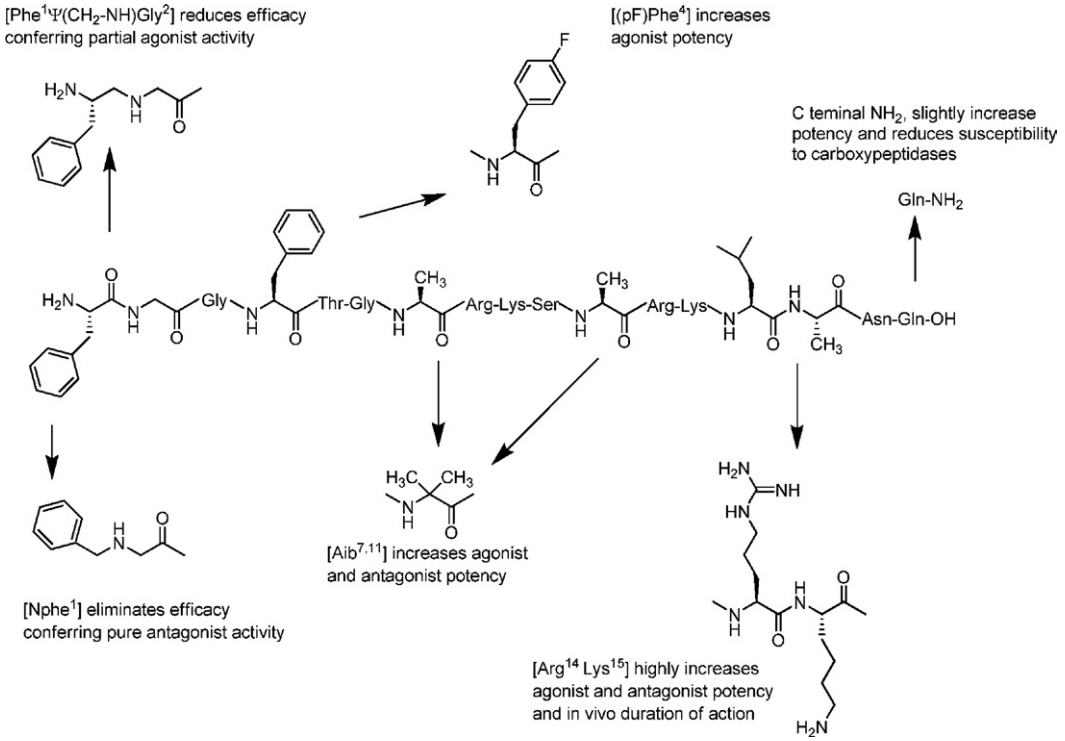

FIGURE 1 Overview of the most important chemical modifications applied to the N/OFQ sequence for modulating peptide efficacy, potency, and duration of action.

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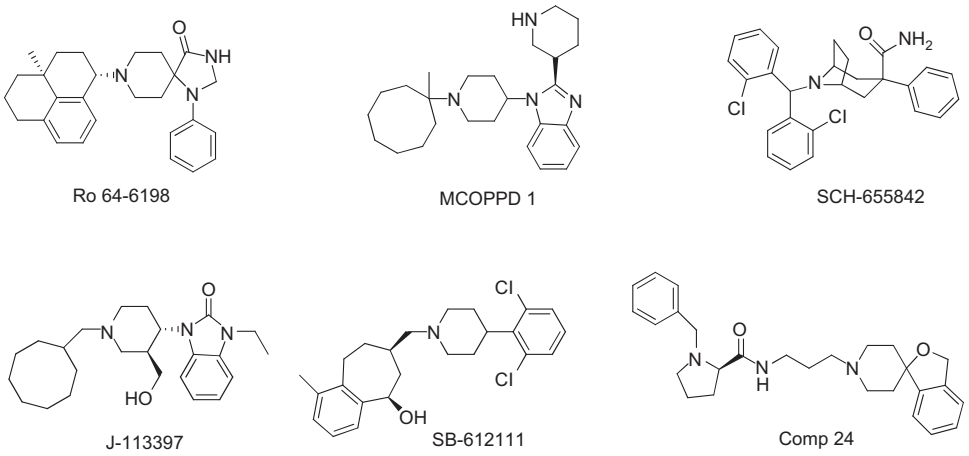

FIGURE 2 Chemical formulas of nonpeptide NOP receptor agonists (top) and antagonists (bottom).

Coupled with selective ligands transgenic animals, particularly knockouts, represent useful research tools in modern pharmacology. For instance, receptor knockout animals facilitate performing simple and meaningful experiments to investigate the *in vivo* selectivity of action of standard and novel ligands and the involvement of the receptor in the control of a given biological function. In most cases, the phenotype of knockout animals is similar to what was observed in normal animals after the administration of a selective receptor antagonist.

NOP(–/–) mice were first generated in 1997.[21] Autoradiography studies showed the complete loss of N/OFQ binding in the brain of these animals. Subsequent *in vitro* functional studies showed that in tissues taken from NOP(–/–) mice, N/OFQ no longer elicits any effect. This includes data from bioassay studies (contractile action in the colon, inhibitory effect in the electrically stimulated vas deferens, inhibition of capsaicin induced bronchoconstriction), to neurochemical investigations (inhibition of serotonin release from cerebral cortex synaptosomes), and to electrophysiological studies (inhibition of excitatory transmission in the spinal cord). In addition, *in vivo* studies on NOP(–/–) mice showed that the N/OFQ actions examined to date are solely mediated by the NOP receptor. Receptor knockout studies are available in the literature regarding the following biological actions of N/OFQ: supraspinal pronociceptive and spinal antinociceptive effects, induction of bradycardia, hypotension and diuresis, stimulation of food intake and inhibition of locomotor activity. NOP(–/–) mice were also used for investigating their phenotype. These animal show (i) normal response to acute pain while they display a pronociceptive phenotype in response to prolonged nociceptive stimulation (i.e. the formalin test), (ii) an antidepressant phenotype in the forced swimming and tail suspension test, (iii) increased anxiety-related behavior in the elevated plus-maze and light-dark box, whereas in other anxiety-related assays, no differences were found compared with that in normal animals, (iv) a better locomotor performance in the rotarod test. Recently, knockout rats for the NOP receptor gene were also generated.[8] In the brain of these animals, N/OFQ binding is not detectable, and the inhibitory effect elicited by the peptide in the electrically stimulated vas deferens is no longer present. *In vivo* studies performed in NOP(–/–) rats showed that these animals display phenotype differences superimposable to those described in mice in the formalin, rotarod, forced swimming, and elevated plus-maze assays[26]; this shows that the control exerted by the N/OFQ–NOP receptor system on these biological functions is very robust across animal species.

Knockout mice for the ppN/OFQ gene have been also generated.[10] However, these ppN/OFQ(–/–) animals were only used in a few studies. The limited information available suggests that these animals behave in a similar manner as NOP(–/–) mice in terms of pain transmission while they display differences from receptor knockout animals in terms of response to stress. However parallel experiments with NOP(–/–) and ppN/OFQ(–/–) mice should be performed before drawing firm conclusions on behavioral differences between the two mutant genotypes. Moreover, as mentioned before, the ppN/OFQ gene contains in addition to N/OFQ itself, other biologically active peptides. Because ppN/OFQ null mice do not express N/OFQ as well as the other peptides encoded by the same gene, caution should be exercised in interpreting behavioral differences between ppN/OFQ(–/–) and ppN/OFQ(+/+) mice as solely due to the lack of N/OFQ.

BIOLOGICAL ACTIONS

Pain

The abundant literature addressing the role of the N/OFQ–NOP receptor system in the control of pain transmission is highly contentious. N/OFQ has been variously reported

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to cause hyperalgesia, allodynia, analgesia, and even nocifensive behaviors.[11,32] One reason, among others, is that N/OFQ effects on nociception are dependent on the dose and route of administration. Therefore, the information available in this field is next summarized by the analysis of N/OFQ actions at supraspinal, spinal, and peripheral levels.

Supraspinal Effects

N/OFQ was initially reported to cause thermal hyperalgesia when injected supraspinally in mice. However, this effect was soon shown to reflect reversal of stress-induced analgesia, rather than a decrease of nociceptive threshold. Indeed, icv N/OFQ attenuates the analgesic action of opioid and nonopioid drugs. One mechanism whereby N/OFQ attenuates opioid-induced analgesia is by directly inhibiting a descending antinociceptive pathway, which itself is indirectly activated (disinhibited) by opioids. This mechanism might be activated during chronic opioid administration because NOP receptor knockout and antagonist studies show that blocking N/OFQ–NOP receptor signaling counteracts the development of morphine tolerance.

Spinal Effects

When administered intrathecally (i.t.) in rodents, very low doses (femtomole to picomole range) of N/OFQ are reported to cause pronociceptive effects, whereas higher doses (nanomole range) produce antinociceptive effects. Many studies have shown that intrathecal administration of nanomoles of N/OFQ produces robust antinociceptive effects, and this holds true in a wide variety of animal models of phasic (mechanical or thermal) and of tonic (inflammatory or neuropathic) pain. The spinal antinociceptive action of N/OFQ is consistent with the well-documented ability of the peptide to block both normal and sensitized excitatory (glutamate) transmission in the dorsal horn of the spinal cord.[32] Interestingly, spinal N/OFQ, in contrast to classical opioids, seems to be more efficacious in alleviating chronic neuropathic/inflammatory than acute pain states. Evidence first obtained with NOP(−/−) and ppN/OFQ(−/−) mice and then confirmed with receptor antagonists and rat NOP(−/−) studies shows that endogenous N/OFQ–NOP receptor signaling in the spinal cord is activated by prolonged (e.g. formalin, zymosan A, writhing tests, SBL response to i.t. substance P), but not acute (tail flick/immersion test) nociceptive stimulation and the consequence of its activation is the appearance of antinociceptive effects. Finally, in an elegant series of studies, the group of MC Ko showed that in nonhuman primates, spinal administration of N/OFQ or synthetic NOP ligands (i) does not elicit any effect at low doses, (ii) in the nanomolar range of doses, it induces a robust antinociceptive action that is sensitive to NOP antagonists but not naloxone, (iii) in contrast to morphine, it does not induce pruritus, and (iv) it elicits a synergistic antinociceptive effect when given in association with morphine. Thus, evidence coming from nonhuman primates strongly suggests NOP receptor agonists as innovative spinal analgesics. Interestingly, this same research group showed that the NOP-selective nonpeptide agonist Ro 64-6198 given systemically is able to induce dose-dependent antinociceptive effects in nonhuman primates while being inactive in rodents. In monkeys, antinociceptive doses of alfentanil (and in generally of opioids) are associated with respiratory depression, itch/scratching responses, and reinforcing effects under self-administration procedures, whereas in parallel experiments, antinociceptive doses of Ro 64-6198 do not produce these side effects. Further studies are however needed to draw firm conclusions on the therapeutic potential of systemic NOP agonists as analgesics.

Peripheral Effects

Similar to what has been seen at the spinal level in the periphery both pronociceptive and antinociceptive effects were reported for N/OFQ. For instance, intradermal administration of very low doses of N/OFQ stimulates the flexor reflex in mice. This effect involves the stimulation of the release of substance P from peripheral nerve endings. However, at higher doses, N/OFQ prevents the facilitatory effect of substance P. In addition, several groups reported the ability of N/OFQ to inhibit neuropeptide release from peripheral sensory neuron terminals in different organs including the airways, heart, and renal pelvis. Finally, in nonhuman primates, the coadministration of N/OFQ with capsaicin into the tail dose dependently inhibits thermal nociception suggesting that activation of peripheral NOP receptors produces antinociceptive effects.

Reward and Addiction

The rewarding properties of N/OFQ have been examined using the conditioned place preference test that pairs administration of the drug with a particular set of environmental cues. In rats, supraspinal injection of N/OFQ does not induce place preference or aversion, an indication that the peptide lacks intrinsic motivational properties. Most significantly, however, N/OFQ was shown to block morphine-induced place preference, an effect that was later extended to other drugs of abuse, all known to acutely stimulate the dopaminergic mesocorticolimbic pathway, such as alcohol, amphetamine, and cocaine.[31] One mechanism whereby N/OFQ attenuates reward elicited by drugs of abuse is by directly inhibiting dopaminergic mesocorticolimbic neurons, which express the NOP receptor. Of particular relevance to addiction are the observations that N/OFQ blocks reinstatement of alcohol-seeking behavior

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in alcohol-preferring rats and that the nonpeptide NOP receptor agonist Ro 64-6198 blocks reinstatement of morphine place preference in mice. The endogenous N/OFQ–NOP receptor system seems to control rewarding functions because pharmacological blockade or genetic knockout of the NOP receptor potentiates the rewarding effect of morphine in rats.[27]

Stress, Anxiety, and Depression

One of the most highly investigated actions of N/OFQ is its ability to counteract stress related behaviors and promote anxiolytic-like effects. The pivotal study in this field showed that N/OFQ acts as anxiolytic in several benzodiazepine-sensitive behavioral tests.[9] Similar results were reported in response to the systemic administration of Ro 64-6198, and these findings were confirmed in different assays, species, and laboratories.[30] Interestingly, the anxiolytic-like action of Ro 64-6198 does not show tolerance liability after daily administration for two weeks. In addition, anxiolytic-like effects were reported for several nonpeptide NOP agonists developed by Roche, Shering-Plough, and Pfizer laboratories. Knockout studies corroborate these findings. Indeed, in comparison with wildtype animals, ppN/OFQ(−/−) mice have a greater tendency toward anxiety-like behavior when exposed to a novel and threatening environment and have increased basal and stress-induced levels of plasma corticosterone. In addition NOP(−/−) mice display a mild anxiogenic phenotype in same anxiety-related assays. Superimposable results were obtained investigating the phenotype of receptor knockout rats. The mechanisms by which N/OFQ exerts its anxiolytic effects are not fully understood, but there is evidence that $GABA_A$ receptor signaling, CRF, and serotonin might be involved.[6]

As far as depression is concerned, the seminal study by Redrobe et al.[24] showed that NOP antagonists elicit antidepressant-like effects in the forced swimming test in mice. This initial finding was later confirmed and extended in subsequent studies (summarized in Ref. 6) showing that (i) antidepressant-like effects are measured in response to chemically different NOP antagonists including the peptides [$Nphe^1$]N/OFQ(1-13)-NH_2 and UFP-101 and the nonpeptides J-113397 and SB-612111, (ii) the antidepressant-like effect of NOP antagonists can be reversed by the supraspinal injection of N/OFQ, (iii) NOP(−/−) mice and rats display an antidepressant-like phenotype. Blockade of endogenous N/OFQ–NOP receptor signaling is associated with a facilitatory effect on exercise driven locomotor activity (see below), and this may possibly represent an important bias for the interpretation of findings obtained in the forced swimming test. However, evidence strongly supports the genuine nature of the antidepressant-like effects of NOP antagonists: (i) in rats subjected to chronic mild stress, three-week treatment with UFP-101 dose and time dependently reinstates sucrose consumption; this particular behavioral parameter is not influenced by locomotion, (ii) UFP-101 injected into the dorsal hippocampus produces antidepressant-like effects in the mouse forced swim and tail suspension tests without modifying locomotion, and (iii) there is no correlation between motor performance and phenotype in the forced swimming test in NOP(−/−) rats. Little is known about the possible mechanism(s) involved in the antidepressant effects of NOP antagonists. However, it has been reported that N/OFQ is able to inhibit noradrenaline and serotonin release from cerebral cortex as well as neuronal firing in the dorsal raphe and locus coeruleus. Therefore, assuming that chronic stress/despair conditions stimulate the release of N/OFQ, the peptide may reduce aminergic signaling acting at both presynaptic and postsynaptic sites. By preventing such effects of N/OFQ, NOP antagonists may restore normal levels of norepinephrine and serotonin at their respective synaptic clefts. Thus, NOP receptor antagonists, by acting at different levels and with different mechanisms, may achieve a similar endpoint to that of classical antidepressants, that is, an increase in cortical synaptic concentrations of monoamines.

Locomotor Activity

In the article reporting its identification as endogenous ligand of the NOP receptor, N/OFQ has been shown to inhibit spontaneous locomotor activity.[25] This effect has been confirmed in mice and rats by different laboratories and the involvement of the NOP receptor has been demonstrated by receptor antagonist and knockout studies. However, the endogenous N/OFQ–NOP receptor system does not tonically control spontaneous locomotion because NOP antagonists do not modify this behavior and knockout animals do not show any phenotype. However the seminal article by Marti et al.[15] shows that systemic administration of J-113397 or supraspinal injection of UFP-101 facilitates, in a dose-dependent manner, rat performance in the drag and rotarod tests. Moreover, NOP(−/−) outperform NOP(+/+) mice in the same assays (a finding later confirmed in NOP receptor knockout rats). These results suggest that endogenous N/OFQ may indeed exert an inhibitory influence over motor activity that becomes relevant during exercise rather than at rest. Consistent with these observations, systemic administration of J-113397 and its analogs Trap-101 and GF-4 increase motor performance in normal rats and in NOP(+/+) mice but are ineffective in NOP(−/−) mice. In addition, preliminary evidence suggests that J-113397 facilitates motor performance in nonhuman primates. Collectively, these findings reinforce the view that NOP receptor blockade may represent a new strategy for the control of hypokinetic disorders. Indeed, a series of elegant studies showed that NOP receptor antagonists attenuate motor deficits in rodent and nonhuman primate models (6-hydroxydopamine, haloperidol, reserpine, 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine)

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of parkinsonism. The finding that N/OFQ levels are 3.5-fold elevated in the cerebrospinal fluid of parkinsonian patients compared with controls further strengthens the rational for developing NOP receptor antagonists as drugs to treat Parkinson's disease.

Food Intake

icv N/OFQ dose dependently stimulates food intake in rodents. After the first report,[23] this effect of the peptide has been confirmed in several laboratories, and it has been shown that N/OFQ stimulation of food intake is mimicked by NOP-selective agonists, prevented by NOP antagonists, and no longer evident in NOP(−/−) mice. However, food intake induced by food deprivation is not reduced by NOP antagonists and is similar in NOP(+/+) and NOP(−/−) mice, thus suggesting that the endogenous peptide/receptor system is not activated under fasting conditions. Different neurotransmitter systems seem to be involved in the stimulant action of N/OFQ on food intake. N/OFQ acts as a functional antagonist against anorexia elicited by CRH, the primary mediator of stress, especially in the bed nucleus of stria terminalis. N/OFQ may also increase food intake by decreasing the release of the anorectic peptide CART (cocaine and amphetamine regulated transcript) and/or increasing the release of the orexigenic peptide agouti-related protein. In addition, there is evidence that corticosterone and central glucocorticoid receptors are involved in the orexigenic action of N/OFQ.

Learning and Memory

It was initially shown that microinjection of N/OFQ into the CA3 region of the dorsal hippocampus causes profound impairment of spatial learning in rats trained in the Morris water task.[28] Later, N/OFQ was reported to inhibit long-term potentiation in rat hippocampal slices. These observations received strong support from the observation that NOP(−/−) mice not only display greater learning ability and have better memory than NOP(+/+) animals but also show increased LTP in the hippocampal CA1 region. Improvement of cognitive abilities in mice lacking the NOP receptor gene has been linked to the hyperfunctioning of the NMDA receptor. However, systematic studies on the possible cognitive enhancing properties of selective NOP antagonists have not yet been performed.

N/OFQ Actions on Peripheral Organs

By acting both on the central and peripheral nervous systems, N/OFQ modulates the functioning of several organs and systems including the heart and vessels, airways, kidney, urinary bladder, and the gastrointestinal and immune systems. N/OFQ induces bradycardia and hypotension, possesses vasorelaxant properties, stimulates diuresis and in particular aquaresis, controls several gastrointestinal functions under physiological and pathological conditions, modulates immune function, produces proinflammatory effects, and inhibits some reflexes mediated by sensory fibers such as cough and micturition. Most of these N/OFQ actions have been reviewed in a special issue of the journal *Peptides*,[16] and recent reviews on some of these topics are available in the literature.

All the above-mentioned information regarding N/OFQ effects have been obtained in preclinical studies. Clinical results are only available for the inhibitory effect exerted by the peptide on the micturition reflex, and this makes this topic worthy of further description. In an elegant series of rodent studies,[14] Menarini researchers showed that intravenous administration of N/OFQ inhibits the micturition reflex, and this effect is no longer evident in capsaicin-pretreated rats indicating the involvement of capsaicin-sensitive nerve fibers innervating the urinary bladder. In support of this interpretation, N/OFQ also inhibits the reflex, but not the local bladder contraction, induced by topical capsaicin. Intrathecal N/OFQ produces urodynamic modifications similar to those induced by systemic administration. Intracerebroventricular administration of the peptide also inhibits the micturition reflex in a naloxone-resistant manner suggesting a direct effect on supraspinal sites controlling micturition. Beyond the inhibitory effects exerted by N/OFQ on the micturition reflex, there is a peripheral excitatory effect mediated by capsaicin-sensitive fibers. Application of the peptide onto the bladder serosa when the intravesical volume is subthreshold for triggering of the micturition reflex, activates the reflex, and this is associated with a local tonic-type contraction that is abolished by the coadministration of tachykinin NK_1 and NK_2 receptor antagonists. Collectively, these results indicate that in the rat, NOP receptors are present at several sites for the integration of the micturition reflex and that their activation mainly produces inhibitory effects. Based on these results, Lazzeri et al. performed the first clinical investigation with N/OFQ by testing the urodynamic effects of intravescical application of the peptide in normal subjects and in patients suffering from an overactive bladder.[13] In normal subjects, N/OFQ does not modify urodynamic parameters, whereas in patients, the peptide produces a statistically significant increase in mean bladder capacity and volume threshold for the appearance of detrusor hyperreflexia without affecting maximum bladder pressure. These results were confirmed in a randomized, placebo controlled, double-blind study in which [desPhe^1]N/OFQ (a N/OFQ metabolite that lacks affinity for the NOP receptor) was used as placebo, showing that N/OFQ, but not the placebo, elicits a robust acute inhibitory effect on the micturition reflex in patients with a neurogenic bladder. These promising results obtained after acute administration of N/OFQ allowed the investigation of the feasibility, safety and efficacy of daily intravesical instillation of 1 mg of N/OFQ for 10 days in patients who

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perform clean intermittent self-catheterization for neurogenic detrusor overactivity incontinence. Urodynamic parameters recorded during the study confirm previous findings. Moreover, during N/OFQ treatment, daily urine leakage episodes are approximately halved and bladder capacity increased by 172%. No significant problems related to feasibility of the procedure and significant side effects were reported by patients. This study shows the clinical efficacy of N/OFQ during 10 days of treatment supporting the use of NOP receptor agonists in the control of detrusor overactivity incontinence.

THERAPEUTIC POTENTIAL OF NOP RECEPTOR LIGANDS

The broad pharmacological spectrum of NOP actions points to a number of potential therapeutic applications of selective NOP receptor ligands. Thus, based on available evidence, NOP receptor agonists are claimed to be potentially useful for treating stress and anxiety, drug addiction, anorexia, and cough. One advantage of NOP receptor agonists over currently used drugs such as benzodiazepines or opioids would be a lack of abuse potential. However, given their broad spectrum of actions, NOP receptor agonists may also be anticipated to have many unwanted effects. This however might not be true for selected indications for which systemic administration is not needed such as urinary incontinence (intravescical route) and intractable pain (spinal route). On the other hand, NOP receptor antagonists could be useful for treating Pakinson's disease and depression, for enhancing cognitive function, and perhaps as novel antinflammatory agents or as adjuncts for preventing tolerance to morphine analgesia. Antagonists are expected to have fewer side effects than agonists, as suggested by the observation that NOP(−/−) mice and rats appear to be outwardly normal.

Finally, although the actions of N/OFQ on nociception have been most extensively studied, it is still highly contentious whether NOP receptor agonists or antagonists have a future as analgesics. The reported opposite pro- and antinociceptive actions of N/OFQ depending on dose and site of administration, raise the question as to the final effect of a ligand able to target simultaneously all NOP receptor sites. In addition, species specific responses to NOP ligands (e.g. systemic Ro 64-6198 elicits antinociceptive effects in non-human primates but not in rodents) further complicate the picture and make more difficult developments in this field.

Drug companies have been rather successful in developing NOP ligands, either agonists and antagonists,[12] but these need be modified in terms of NOP opioid receptor selectivity and oral bioavailability. Only the availability of highly potent, selective, and possibly orally active NOP receptor ligands will allow effective validation of the NOP receptor as a therapeutic target.

REFERENCES

1. Calo G, Guerrini R, Rizzi A, Salvadori S, Burmeister M, Kapusta DR, et al. UFP-101, a peptide antagonist selective for the nociceptin/orphanin FQ receptor. *CNS Drug Rev* 2005;**11**:97–112.
2. Calo G, Guerrini R, Rizzi A, Salvadori S, Regoli D. Pharmacology of nociceptin and its receptor: a novel therapeutic target. *Br J Pharmacol* 2000;**129**:1261–83.
3. Calo G, Rizzi A, Cifani C, Di Bonaventura MV, Regoli D, Massi M, et al. UFP-112 a potent and long-lasting agonist selective for the nociceptin/orphanin FQ receptor. *CNS Neurosci Ther* 2011;**17**:178–98.
4. Dooley CT, Spaeth CG, Berzetei-Gurske IP, Craymer K, Adapa ID, Brandt SR, et al. Binding and in vitro activities of peptides with high affinity for the nociceptin/orphanin FQ receptor, ORL1. *J Pharmacol Exp Ther* 1997;**283**:735–41.
5. Evans RM, You H, Hameed S, Altier C, Mezghrani A, Bourinet E, et al. Heterodimerization of ORL1 and opioid receptors and its consequences for N-type calcium channel regulation. *J Biol Chem* 2010;**285**:1032–40.
6. Gavioli EC, Calo G. Antidepressant- and anxiolytic-like effects of nociceptin/orphanin FQ receptor ligands. *Naunyn Schmiedebergs Arch Pharmacol* 2006;**372**:319–30.
7. Heinricher MM. Orphanin FQ/nociceptin: from neural circuitry to behavior. *Life Sci* 2003;**73**:813–22.
8. Homberg JR, Mul JD, de Wit E, Cuppen E. Complete knockout of the nociceptin/orphanin FQ receptor in the rat does not induce compensatory changes in mu, delta and kappa opioid receptors. *Neuroscience* 2009;**163**:308–15.
9. Jenck F, Moreau JL, Martin JR, Kilpatrick GJ, Reinscheid RK, Monsma Jr FJ, et al. Orphanin FQ acts as an anxiolytic to attenuate behavioral responses to stress. *Proc Natl Acad Sci U S A* 1997;**94**:14854–8.
10. Koster A, Montkowski A, Schulz S, Stube EM, Knaudt K, Jenck F, et al. Targeted disruption of the orphanin FQ/nociceptin gene increases stress susceptibility and impairs stress adaptation in mice. *Proc Natl Acad Sci U S A* 1999;**96**:10444–9.
11. Lambert DG. The nociceptin/orphanin FQ receptor: a target with broad therapeutic potential. *Nat Rev Drug Discov* 2008;**7**:694–710.
12. Largent-Milnes TM, Vanderah TW. Recently patented and promising ORL-1 ligands: where have we been and where are we going? *Expert Opin Ther Pat* 2010;**20**:291–305.
13. Lazzeri M, Calo G, Spinelli M, Guerrini R, Beneforti P, Sandri S, et al. Urodynamic and clinical evidence of acute inhibitory effects of intravesical nociceptin/orphanin FQ on detrusor overactivity in humans: a pilot study. *J Urol* 2001;**166**:2237–40.
14. Lecci A, Giuliani S, Meini S, Maggi CA. Nociceptin and the micturition reflex. *Peptides* 2000;**21**:1007–21.
15. Marti M, Mela F, Veronesi C, Guerrini R, Salvadori S, Federici M, et al. Blockade of nociceptin/orphanin FQ receptor signaling in rat substantia nigra pars reticulata stimulates nigrostriatal dopaminergic transmission and motor behavior. *J Neurosci* 2004;**24**:6659–66.
16. Massi M, Polidori C, Calo G, Regoli D. Nociceptin/orphanin FQ and its receptor. *Peptides* 2000;**21**.
17. Meunier JC, Mollereau C, Toll L, Suaudeau C, Moisand C, Alvinerie P, et al. Isolation and structure of the endogenous agonist of opioid receptor-like ORL1 receptor. *Nature* 1995;**377**:532–5.
18. Mollereau C, Simons MJ, Soularue P, Liners F, Vassart G, Meunier JC, et al. Structure, tissue distribution, and chromosomal localization of the prepronociceptin gene. *Proc Natl Acad Sci U S A* 1996;**93**:8666–70.

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19. Neal Jr CR, Mansour A, Reinscheid R, Nothacker HP, Civelli O, Akil H, et al. Opioid receptor-like (ORL1) receptor distribution in the rat central nervous system: comparison of ORL1 receptor mRNA expression with (125)I-[(14)Tyr]-orphanin FQ binding. *J Comp Neurol* 1999;**412**:563–605.
20. Neal Jr CR, Mansour A, Reinscheid R, Nothacker HP, Civelli O, Watson Jr SJ. Localization of orphanin FQ (nociceptin) peptide and messenger RNA in the central nervous system of the rat. *J Comp Neurol* 1999;**406**:503–47.
21. Nishi M, Houtani T, Noda Y, Mamiya T, Sato K, Doi T, et al. Unrestrained nociceptive response and dysregulation of hearing ability in mice lacking the nociceptin/orphanin FQ receptor. *EMBO J* 1997;**16**:1858–64.
22. Okuda-Ashitaka E, Minami T, Tachibana S, Yoshihara Y, Nishiuchi Y, Kimura T, et al. Nocistatin, a peptide that blocks nociceptin action in pain transmission. *Nature* 1998;**392**:286–9.
23. Pomonis JD, Billington CJ, Levine AS. Orphanin FQ, agonist of orphan opioid receptor ORL1, stimulates feeding in rats. *Neuroreport* 1996;**8**:369–71.
24. Redrobe JP, Calo G, Regoli D, Quirion R. Nociceptin receptor antagonists display antidepressant-like properties in the mouse forced swimming test. *Naunyn Schmiedebergs Arch Pharmacol* 2002;**365**:164–7.
25. Reinscheid RK, Nothacker HP, Bourson A, Ardati A, Henningsen RA, Bunzow JR, et al. Orphanin FQ: a neuropeptide that activates an opioidlike G protein-coupled receptor. *Science* 1995;**270**:792–4.
26. Rizzi A, Molinari S, Marti M, Marzola G, Calo' G. Nociceptin/orphanin FQ receptor knockout rats: in vitro and in vivo studies. *Neuropharmacology* 2011;**60**:572–9.
27. Rutten K, De Vry J, Bruckmann W, Tzschentke TM. Pharmacological blockade or genetic knockout of the NOP receptor potentiates the rewarding effect of morphine in rats. *Drug Alcohol Depend* 2011;**114**:253–6.
28. Sandin J, Georgieva J, Schott PA, Ogren SO, Terenius L. Nociceptin/orphanin FQ microinjected into hippocampus impairs spatial learning in rats. *Eur J Neurosci* 1997;**9**:194–7.
29. Schlicker E, Morari M. Nociceptin/orphanin FQ and neurotransmitter release in the central nervous system. *Peptides* 2000;**21**:1023–9.
30. Shoblock JR. The pharmacology of Ro 64-6198, a systemically active, nonpeptide NOP receptor (opiate receptor-like 1, ORL-1) agonist with diverse preclinical therapeutic activity. *CNS Drug Rev* 2007;**13**:107–36.
31. Zaveri NT. The nociceptin/orphanin FQ receptor (NOP) as a target for drug abuse medications. *Curr Top Med Chem* 2011;**11**:1151–6.
32. Zeilhofer HU, Calo G. Nociceptin/orphanin FQ and its receptor–potential targets for pain therapy? *J Pharmacol Exp Ther* 2003;**306**:423–9.

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Chapter 216

Opioid-Substance P Chimeric Peptides

Andrzej W. Lipkowski, Daniel B. Carr, Iwona Bonney and Piotr Kosson

ABSTRACT

Chimerization of tachykinin and opioid pharmacophores offers a new avenue for analgesic development. The complexities of such design are illustrated by the analgesic efficacy (via different mechanisms) of chimeras that combine pharmacophores with opioid activity and substance P activity, as well as those that combine opioid agonist and substance P antagonist moieties. Although the interaction between substance P and opioid neural systems is more complex than a simple one-way inhibition, the relative balance of activities between tachykinin and opioid pharmacophores will generally determine the net effect of the chimeric molecule as pronociceptive, antinociceptive, or neutral. Intriguingly, endomorphins— (μ-opioid receptor agonists with high intrinsic activity may owe some of this high activity to weak but significant antagonist properties at tachykinin NK2 receptors, implying that these native peptides are endogenous chimeric opioid agonist–tachykinin antagonist compounds.

INTRODUCTION

The discoveries of tachykinins and endogenous opioids were major milestones in the clarification of the role of neuropeptides in nociceptive signal formation, transmission, and perception. The undecapeptide substance P was the first recognized mammalian tachykinin; its amino acid sequence was successfully elucidated[6] in 1971. Subsequently, other members of the tachykinin family and superfamily, and their receptors and variants, were well characterized. Tachykinin peptides are widely distributed in neuronal central nervous system (CNS) and peripheral tissues. Tachykinins interact with specific membranę receptors belonging to the family of G-protein-coupled receptors. Currently, three distinct tachykinin receptors, NK1, NK2, and NK3, have been cloned in different species, including the human.[27] The three tachykinin receptors are recognized with moderate selectivity by endogenous tachykinins. Substance P, neurokinin A, and neurokinin B act as full agonists on the three tachykinin receptors, although they exhibit preferential binding to the NK1, NK2, and NK3 receptors, respectively. Within neuronal tissues, tachykinins act as excitatory neurotransmitters [see the tachykinin chapter in the Brain Peptides and other sections of this book]. However, substance P and other tachykinins are also widely distributed in nonneural tissues, where they mediate multiple homeostatic functions. The role of tachykinins in host defense systems has been extensively studied. Immune cells (T and B cells, endothelial cells, and macrophages) not only express NK1 receptors but also (in macrophages, eosinophils, and endothelial cells) synthesize substance P. The concentration of substance P increases in inflamed tissues,[26] suggesting its importance in the mammalian host defense system. Indeed, available results suggest a multifunctional role for substance P, in which it activates immunological responses, generates a pain signal alarm, and also may itself have direct antibacterial and antifungal properties.[15]

Numerous endogenous pathways are involved in neurological and other disease pathology. Modern therapies therefore commonly rely on multidrug therapies that supplant selective drug use. However, using combinations of drugs has disadvantages, including differences in pharmacodynamic and pharmacokinetic profiles of each component drug. Therefore, the combination of multiple active pharmacophores in one molecule is a promising approach. A good example of such a molecule, based on the sequence of substance P, is the anticancer peptide Arg-D-Trp-Phe(N-Me)-D-Trp-Leu-MetNH_2, which simultaneously blocks receptors for substance P, bombesin/gastrin-releasing peptide, vasopressin, cholecystokinin, and neurotensin.[12] Multitarget therapies have become popular in pain treatment as well (multimodal analgesia).[32] However, available analgesics in clinical practice are naturally, or have been crafted to be, maximally selective for one type of receptor. This chapter describes a new avenue for the development of new analgesics, the chimerization of tachykinin and opioid pharmacophores.

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00216-5
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CROSSINTERACTION OF TACHYKININ AND OPIOID SYSTEMS

A large body of experimental evidence indicates that regulatory neuropeptides play modulatory roles in the afferent transfer and postsynaptic processing of nociceptive information within the dorsal horn of the spinal cord.[5,22] The superficial dorsal horn of the spinal cord is an area that receives primary synaptic input from sensory fibers originating from dorsal root ganglion (DRG) neurons. This area is a potentially important site for functional regulation (by modulatory opioid peptides) of nociceptive input mediated by substance P[24] and excitatory amino acids released from primary afferent terminals. Anatomical studies indicate a similar distribution of substance P- and opioid-containing neural elements within this area.[1,29] Ligands of the μ receptor are highly effective in blocking or reducing nociceptive transmission at the spinal level, and this antinociception is believed to be caused at least partially by the inhibition of substance P release.[11] Substance P and opioid neuropeptides share a common metabolic system. The changes in proteolytic specificities and activities in pathological conditions may influence pain signal transmission. In addition, substance P and its metabolites express complicated multitargeted activities. C-terminal hepta- and hexapeptide amides are the fragments that express nociception properties comparable with intact undecapeptide substance P. However, N-terminal heptapeptide interacts with specific receptors, the activation of which results in antinociception and attenuation of opioid tolerance.[35] Endomorphins and, to some extent, enkephalins express affinities to these receptors as well.[4] Interestingly, the antinociceptive and analgesic fragments of substance P and endomorphins partially overlap, which means that changes of substance P metabolic system specificity on the spinal level may result in either nociceptive or antinociceptive effects. Therefore, substance P with a mosaic of receptors and metabolic enzymes forms a "yin-yang" system (Fig. 1) sensitive to pathological changes.[10]

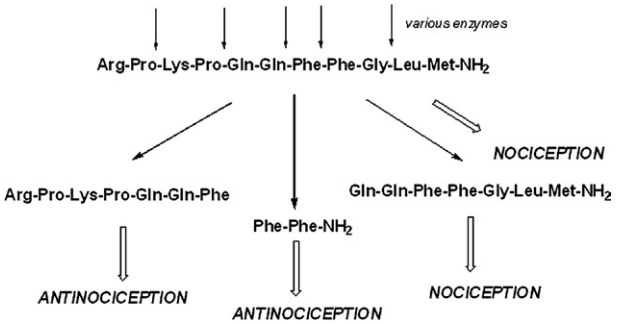

FIGURE 1 Substance P and its metabolites form a "yin-yang" nociception attenuating system (for simplicity, only three possible peptide metabolites are presented).

TACHYKININ ANTAGONIST–OPIOID AGONIST CHIMERIC COMPOUNDS

The majority of neurophysiological and pharmacological studies have indicated that substance P and opioid systems are functionally antagonistic in the mediation of nociception and antinociception *in vivo*.[22] Thus, the simultaneous blockade of substance P receptors and activation of opioid receptors seems to be quite a reasonable approach to the construction of chimeric peptides. Indeed, coinjection of a weak peptide substance P antagonist together with an opioid peptide strongly enhances opioid analgesia.[25] The conclusion obtained from this study indicates that incomplete blockade of substance P receptors enhances opioid analgesia.

Studies of the effects of mixtures of peptides prompted the design of compounds that in one molecule combine substance P antagonist and opioid agonist pharmacophores. The first of this series was AWL60, a peptide in which an endomorphin-like moiety is hybridized (head-to-tail) with a substance P antagonist (Table 1).[19] The resultant compound expresses properties of both hybridized components in an *in vitro* guinea pig ileum preparation. Although AWL60 expresses moderate affinity to mu-opioid receptors (median inhibitory concentration, IC_{50}, 1 μM), the *in vivo* intrathecal application of AWL produces intense, prolonged antinociception (Fig. 2).

Preinjection of naloxone strongly reduces this antinociceptive effect, but it does not abolish it completely. Further structure–activity relationship studies of chimeric morphiceptin–endomorphin molecules led to the conclusion that extension of the tetrapeptide with hydrophobic amino acids

TABLE 1 Examples of Primary Sequences of Opioid Agonist–Substance P Antagonist Chimeric Compounds

Compound*	Amino acid sequences†	Ref.
AWL60	Tyr-Pro-D-Phe-Phe-D-Phe-D-Trp-Met-NH_2	19
AA501	Tyr-D-Ala-Gly-Phe-NH-NH←Trp←Z	23
TY027	Dmt-D-Ala-Gly-Phe-Met-Pro-Leu-Trp-NH-[3′,5′-$(CF_3)_2$-Bzl]	34
6	Tyr-D-Ala-Gly-Phe-Nle-Pro-Leu-Ser(Glc)-Trp-NH-[3′,5′-$(CF_3)_2$-Bzl]	33
TY005	Tyr-DAla-Gly-Phe-Met-Pro-Leu-Trp-O-[3′,5′-$(CF_3)_2$-Bzl]	17
35	Dmt-D-Arg-Aba-Gly-NMe-[3′,5′-$(CF_3)_2$-Bzl]	2

*Compound symbols as in references.
†Three letter amino acid codes, in addition, Z-, benzyloxycarbonyl; Bzl-, benzyl; Aba, 4-amino-1,2,4,5-tetrahydro-2-benzazepin-3-one; Dmt, 3,5-dimethyl-Tyr; Ser(Glc), O-β-glucosylated serine.

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significantly diminishes its affinity for opioid mu receptors. Therefore, a second, different molecule of this type of chimera was constructed. Biphalin, which hybridizes two opioid tetrapeptides head-to-head, is one of the most potent opioid analgesics. Structure–activity studies of biphalin concluded that one opioid pharmacophore could be replaced by one of the hydrophobic amino acids or their derivatives or mimetics.[20] An independent search for substance P antagonists resulted in the development of small aromatic tryptophan derivatives.[20] The consolidation of information from these series of structure–activity studies allowed the development of a new compound, AA501, in which an opioid agonist–a substance P antagonist chimera was synthesized by head-to-head hybridization of a tetrapeptide with *N*-benzyloxycarbonyl-tryptophan.[23] In this chimeric compound, the opioid agonist and tachykinin antagonist pharmacophores partially overlap (Table 1). AA501 has high affinity for opioid receptors and moderate affinity for tachykinin receptors. *In vivo*, intrathecal injection of AA501 provides intense antinociception in acute as well as inflammatory and neuropathic pain models. Prolonged application of AA501 results in a slower rate of tolerance development than produced by the administration of a pure opioid. Introduction of a ditrifluoromethyl-benzyl group to substance P antagonist peptidomimetics greatly enhances antagonist properties at NK1 receptors. Therefore, recent development of analgesics with opioid agonist and substance P antagonist properties included this modification (Table 1) in peptidic[2,33,34] and nonpeptidic series of compounds.

The later discovered endomorphins possess unique analgesic potency and selectivity for a single opioid receptor type μ. Further studies reveal that these peptides also express weak but significant antagonist properties at tachykinin NK2 receptors, [14] implying that these peptides should be considered as endogenous chimeric opioid agonist–tachykinin antagonist compounds. The importance of the tachykinin antagonist component in their unique biological profile should be addressed in further studies.

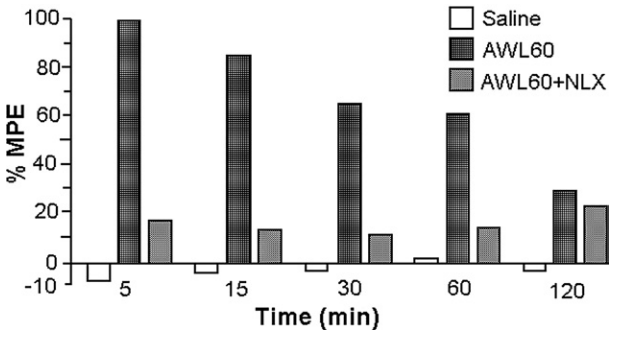

FIGURE 2 Antinociception in rats after intrathecal application of AWL60 (2.5 nmol).[19]

TACHYKININ AGONIST–OPIOID AGONIST CHIMERIC COMPOUNDS

The interaction between substance P and opioid neural systems is more complex than a simple one-way inhibition. In the spinal cord, these two systems are engaged in reciprocal regulation. Although the application of substance P in high doses is algesic, this molecule and its metabolites possess pain-alleviating effects when given in lower doses.[28,30] To some extent, the analgesic effects described for low doses of substance P seem to reflect an opioid-like action because naloxone neutralizes substance P analgesia.[9] The antinociceptive effects of substance P may also be explained by its activation of several self-regulatory mechanisms, including an influence on peptidase activities,[3] modulation of the binding of opioids to their receptors,[16] and influences on serotonin[13] or NMDA[31] pathways.

Selective pharmacological activation of the opioid system may perturb the complex interactions between tachykinin and opioid systems. Simultaneous activation of the opioid system along with partial compensatory supplementation of the tachykinin system is the rationale for the development of chimeras that combine an opioid agonist and a tachykinin agonist. Because the principal effects of substance P and opioids are opposite, the relative balance of activities between tachykinin and opioid pharmacophores will determine the net effect of the chimeric molecule as pronociceptive, antinociceptive, or neutral. In the first compound of this series, SPF[18,21] (Table 2), substance P activity predominates, and intrathecal injection of the compound induces nociceptive symptoms characteristic of substance P (Fig. 3). In that compound, the presence of an opioid component provides only partial antinociception, and coinjection of the opioid antagonist naltrexone further augments the nociceptive properties of the compound. In the second compound synthesized (ESP7), in which the endomorphin-2 and substance P C-terminal fragments were

TABLE 2 Examples of Primary Sequences of Opioid Agonist–Substance P Agonist Chimeric Compounds

Compound*	Amino acid sequences†	Ref.
SPF	Tyr-D-Ala-Gly-Phe-Phe-Gly-Leu-Met-NH_2	21
ESP7	Tyr-Pro-Phe-Phe-Gly-Leu- Met-NH_2	8
ESP6	Tyr-Pro-Phe-Phe-Pro-Leu- Met-NH_2	7
AWL3106	Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-D-Ala-Phe-Phe-Gly-Leu-Met-NH_2	

*Compound symbols as in references.
†Three letter amino acid codes.

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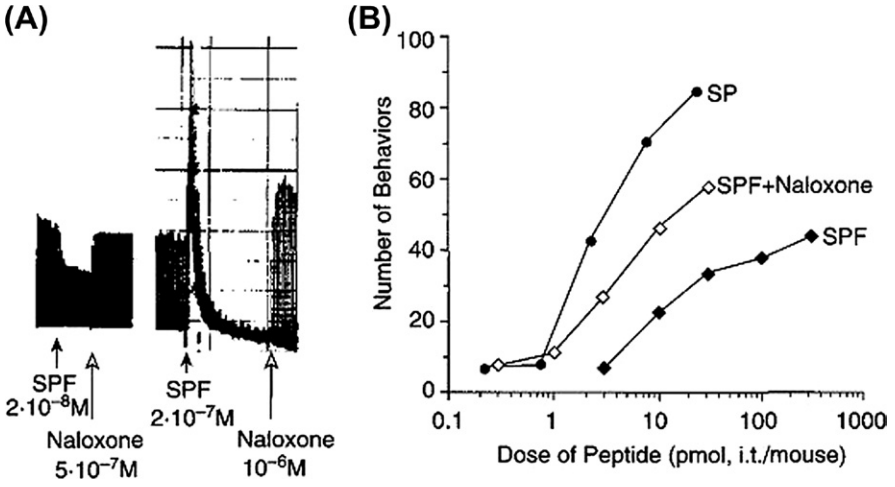

FIGURE 3 Examples of biological activities of SPF. (A) *In vitro* electrically stimulated mouse vas deference: Spontaneus contraction characteristic to substance P and inhibition of electrical stimulation characteristic to opioids, reversed with naloxone.[21] (B) Nociceptive behavior in mice after intrathecal application. Week nociception of SPF was potentiated by naloxone.[18]

hybridized, opioid activity predominates.[8] Although, ESP7 expresses moderate affinity for opioid ($K_i(\mu)$ 218 nM) and NK1 (K_i 289 nM) receptors, after intrathecal application in rats antinociception occurs at a very low dose (0.05 μg). Interestingly, because the molecule comprises two agonists at functionally opposite receptors, in rats, a ceiling effect was observed at 40% of the maximal possible effect (MPE). The application of ESP7 for several days does not produce any evident tolerance. In fact, the application of ESP7 produces analgesia in morphine-tolerant animals.

The C-terminal fragment of substance P is shared by all three endogenous tachykinins. Previous structure–activity studies of tachykinins resulted in the conclusion that substitution of glycine[12] with proline increases selectivity for NK1 receptors. Therefore, in subsequent analogs, the glycine of ESP7 was replaced with proline. The resultant peptide, ESP6, expresses higher affinity for opioid ($K_i(\mu)$ 92 nM) and similar affinity for NK1 (K_i 305 nM) receptors compared with ESP7.[7]

Intrathecal coadministration of morphine and ESP6 clearly slows down the development of tolerance to morphine. Although, ESP6 itself has low antinociceptive potency when it is administered during concurrent NK-1 receptor blockade, a decrease in analgesic potency results, similar to that seen with tolerance to morphine alone. It was thus concluded that ESP6 represents a novel tolerance-eliminating adjuvant for the maintenance of opioid analgesia over time.

Both ESP6 and ESP7 compounds were designed as chimeric compounds with minimal size. Because substance P and endomorphin pharmacophores overlap in these molecules, the size of chimeras was reduced to that of a hexapeptide. Nevertheless, the extension of the original tetrapeptide sequence of endomorphin with additional hydrophobic peptides results in significant decreases of affinity to opioid receptors. Therefore, in the next generation of analogs, two pharmacophores were hybridized in such a way that each pharmacophore was structurally independent and yet also connected by the spacer (Table 2). The resultant peptide, AWL3106, expresses high affinity for opioid ($K_i(\mu)$ 2.5 nM) and NK1 (K_i 7.5 nM) receptors and in addition is very potent when given intrathecally, with an analgesic ceiling at nearly 100% of MPE (Fig. 4). Chronic application of these peptides results in the very slow development of tolerance compared with that of morphine. Interestingly, this peptide is also very potent after intravenous application. It is highly probable that the hydrophobic tachykinin domain of the peptide plays an additional role as a vector that helps it to cross the blood–brain barrier. This latter observation allows the creation of still more templates for the further design of chimeric drugs.

CONCLUSIONS

The construction of opioid–tachykinin chimeric peptides seems to be a very promising avenue for the design of new analgesics. Hybridization of an opioid agonist with a substance P antagonist results in compounds with a synergistic antinociceptive interaction of both hybridized components. The modification of such compounds to provide a lesser degree of activation of the opioid system gives rise to the desirable property that the development of opioid tolerance during repeated administration is significantly slowed. The hybridization of opioid agonists with substance P agonists may also result in effective analgesics without apparent opioid tolerance during chronic application. The possible application of such compounds for the treatment of opioid

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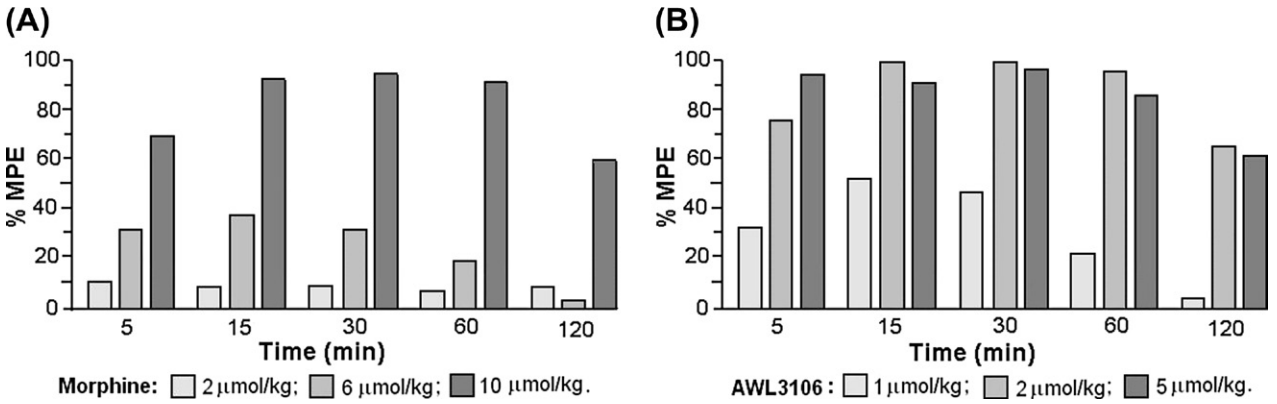

FIGURE 4 Comparison of antinociceptive effects of (A) morphine and (B) AWL3106 after intravenous application in rats.

tolerance and/or dependence is obvious, but further studies to evaluate their utility are needed.

REFERENCES

1. Aicher SA, Punnoose A, Goldberg A. Mu-opioid receptors often colocalize with the substance P receptor (NK1) in the trigeminal dorsal horn. *J Neurosci* 2000;**20**:4345–54.
2. Ballet S, Feytens D, Buysse K, Chung NN, Lemieux C, Tumati S, et al. Design of novel neurokinin 1 receptor antagonists based on conformationally constrained aromatic amino acids and discovery of a potent chimeric opioid agonist-neurokinin 1 receptor antagonist. *J Med Chem* 2011;**54**:2467–76.
3. Barcklay RK, Phillipps MA. Inhibition of enkephalin-degrad-ing aminopeptidase activity by certain peptides. *Biochem Biophys Res Commun* 1980;**96**:1732–8.
4. Botros M, Johansson T, Zhou Q, Lindeberg G, Tömböly C, Tóth G, et al. Endomorphins interact with the substance P (SP) aminoterminal SP(1–7) binding in the ventral tegmental area of the rat brain. *Peptides* 2008;**29**:1820–4.
5. Carr DB, Cousins MJ. Spinał route of analgesia: opioids and future options. In: Cousins MJ, Bridenbaugh PO, editors. *Neural blocade in clinical anesthesia and management of pain*. 3rd ed. Philadelphia: Lippincott Raven; 1998. p. 915–83.
6. Chang MM, Leeman SE, Niall HD. Amino-acid sequence of substance P. *Nature New Biol* 1971;**232**:86–7.
7. Foran SE, Carr DB, Lipkowski AW, Maszczynska I, Marchand JE, Misicka A, et al. Inhibition of morphine tolerance development by a substance Popioid peptide chimera. *J Pharm Exp Ther* 2000;**295**:1142–8.
8. Foran SE, Carr DB, Lipkowski AW, Maszczynska I, Marchand JE, Misicka A, et al. A substance P-opioid chimeric peptide as a unique non tolerance-forming analgesic. *Proc Natl Acad Sci USA* 2000;**97**:7621–6.
9. Hall M, Stewart JM. Substance P and antinociception. *Peptides* 1983; **4**:31–5.
10. Hyyppa MT, Alaranta H, Lahtela K, Nykvist F, Hurme M, Nyberg F, et al. Neuropeptide converting enzyme activities in CSF of low back pain patients. *Pain* 1990;**43**:163–8.
11. Jessel TM, Iversen LL. Opiate analgesics inhibit substance P release from rat trigeminal nucleus. *Nature* 1977;**268**:549–51.
12. Jones DA, Cummings J, Langdon SP, Smyth JF. Preclinical studies on the broad-spectrum neuropeptide growth factor antagonist. *Gen Pharmacol* 1997;**28**:183–9.
13. Klusa VE, Abissova NA, Muceniece RK, Sirskis SV, Bienert M, Lipkowski A. Comparative study of substance P and its fragments with special reference to the analgesic properties, influence on the behavior and monoaminergic processes. *Biull Ekspe Biol Med* 1981;**92**: 692–4.
14. Kosson P, Bonney I, Carr DB, Lipkowski AW. Endomorphins interact with tachykinin receptors. *Peptides* 2005;**26**:1667–9.
15. Kowalska K, Carr DB, Lipkowski AW. Direct antimicrobial properties of substance P. *Life Sci* 2002;**71**:747–50.
16. Krumnis SA, Kim DC, Igwe OJ, Larson AA. DAMGO binding to mouse brain membranes: influence of salts, guanine nucleotides, substance P, and substance P fragments. *Peptides* 1993;**14**:309–14.
17. Largent-Milnes YT, Nair P, Moulton JW, Hruby VJ, Lai J, Porreca F, et al. Spinal or systemic TY005, a peptidic opioid agonist/neurokinin 1 antagonist, attenuates pain with reduced tolerance. *Br J Pharmacol* 2010;**161**:986–1000.
18. Lei SZ, Lipkowski AW, Wilcox GL. Opioid and neurokinin activities of substance P fragments and their analogs. *Eur J Pharmacol* 1991;**193**:209–15.
19. Lipkowski AW, Carr DB, Misicka A, Misterek K. Biological activities of a peptide containing both casomorphin-like and substance P antagonist structural characteristics. In: Brantl V, Tes-chemacher H, editors. β-*Casomorphins and related peptides: recent developments*. Weinheim: VCH; 1994. p. 113–8.
20. Lipkowski AW, Misicka A, Carr DB, Ronsisvalle G, Kosson D, Maszczynska Bonney I. Neuropeptide mimetics for pain management. *Pure Appl Chem* 2005;**76**:941–50.
21. Lipkowski AW, Osipiak B, Gumulka WS. An approach to the self regulatory mechanism of substance P actions: II. Biological activity of new synthetic peptide analogs related both to enkeph-alin and substance P. *Life Sci* 1983;**33**(Suppl. 1):141–4.
22. Maszczynska I, Lipkowski AW, Carr DB, Kream RM. Dual functional interaction of substance P and opioids in nociceptive transmission: review and reconciliation. *Analgesia* 1998;**3**:259–68.
23. Maszczynska Bonney I, Foran SE, Marchand JE, Lipkowski AW, Carr DB. Spinal antinociceptive effects of AA501, a novel chimeric peptide with opioid receptor agonist and tachykinin receptor antagonist moieties. *Eur J Pharmacol* 2004;**488**:91–9.

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24. Matsumura H, Sakurada T, Hara A, Sakurada S, Kisara K. Characterization of the hyperalgesic effect induced by intrathecal injection of substance P. *Neuropharmacol* 1985;**24**:421–6.
25. Misterek K, Maszczynska I, Dorociak A, Gumulka SW, Carr DB, Szyfelbein SK, et al. Spinał co-administration of peptide substance P antagonist increases antinociceptive effect of the opioid peptide biphalin. *Life Sci* 1994;**54**:939–44.
26. O'Connor TM, O'Connell J, O'Brien DI, Goode T, Bredin CP, Shanahan F. The role of substance P in inflammatory disease. *Cell Physiol* 2004;**201**:167–80.
27. Pennefather JN, Lecci A, Candenas ML, Patak E, Pinto FM, Maggi CA. Tachykinins and tachykinin receptors: a growing family. *Life Sci* 2004;**74**:1445–63.
28. Sakurada G, Watanabe O, Sakurada T. Occurrence of substance P(l-4) in the metabolism of substance P and its antinociceptive activity at the mouse spinał cord level. *Met Find Exp Glin Pharmacol* 2004;**26**:171–6.
29. Sanderson Nydahl K, Skinner K, Julius D, Basbaum AI. Colocalization of endomorphin-2 and substance P in primary afferent nociceptors and effects of injury: a light and elektron microscopic study in the rat. *Eur J Neurosci* 2004;**19**:1789–99.
30. Stewart JM, Getto CJ, Neldner K, Reeve EB, Krivoy WA, Zimmermann E. Substance P and analgesia. *Nature* 1976;**262**:784–5.
31. Urban L, Nagy I. Is there a nociceptive carousel? *Trends Pharmacol Sci* 1997;**18**:223–4.
32. Walker SM, Goudas LC, Cousins MJ, Carr DB. Combination of spinal analgesic chemotherapy: a systemic review. *Anesth Analg* 2002;**95**:674–715.
33. Yamamoto T, Nair P, Jacobsen NE, Vagner J, Kulkarni V, Davis P, et al. Improving metabolic stability by glycosylation: bifunctional peptide derivatives that are opioid receptor agonists and neurokinin 1 receptor antagonists. *J Med Chem* 2009;**52**:5164–75.
34. Yamamoto T, Nair P, Largent-Milnes TM, Jacobsen NE, Davis P, Ma S-W, et al. Discovery of a potent and efficacious peptide derivative for δ/μ opioid agonist/neurokinin 1 antagonist activity with a 20,60-dimethyl-L-tyrosine: in vitro, in vivo, and NMR-based structural studies. *J Med Chem* 2011;**54**:2029–38.
35. Zhou Q, Carlsson A, Hallberg M, Nyberg F. Substance P N-terminal fragment SP(1–7) attenuates chronic morphine tolerance and affects dynorphin B and nociceptin in rats. *Peptides* 2011;**32**:1661–5.

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Chapter 217

POMC-Derived Opioid Peptides

Patricia J. McLaughlin and Ian S. Zagon

ABSTRACT

Proopiomelanocortin (POMC) prohormone is processed to yield many fragments including adrenocorticotropin hormone (ACTH) and β-lipotropin hormone (β-LPH). These proteins are actively cleaved to yield multiple bioactive peptides, one of which is β-endorphin. Concentrated in the anterior pituitary and *pars intermedia*, β-endorphin is distributed widely throughout the body and has been detected in nervous tissue as well as adrenals, heart, liver, and placenta. Concomitant with its broad distribution, the physiological activity of β-endorphin directly or indirectly includes neurotransmitter functions, suppressive effects on respiration, analgesia, maintenance of cardiovascular homeostasis, and even regulation of T- and B-cell function in the immune system. β-endorphin's activity is mediated through classical μ and δ opioid receptors and perhaps a more selective epsilon receptor. Continued research is necessary to further define the signaling pathways associated with β-endorphin's roles in the pathophysiology of pain management, obesity, and even immunological responsiveness.

HISTORY

Endorphins were the first identified endogenous opioid peptides. The term 'endorphins' remains broader than the specific molecules that are truly endorphin derivatives. Endogenous neuropeptides were first identified and named by two independent laboratories in the mid-1970s. In Scotland, the laboratory of John Hughes and Hans Kosterlitz isolated a small peptide sequence from brain tissue isolated from pig and termed it "enkephalin."[6,7] During the same period, investigators in the United States isolated an active molecule that they considered to have morphine-like activity and termed it "endorphin."[14,17] As the field of neuropeptides blossomed, the term "endorphins" was used loosely in reference to all endogenous peptides with opioid-like activity, particularly morphine-like properties. Thus, the term "endorphins" has been assigned to numerous peptides with multiple functions ranging from pain cessation and analgesia to euphoria and neurotransmission.[15]

In common language, an "endorphin-high" is attributed to any euphoric feeling elicited from either physical or emotional challenge, pain, or stress. Frequently, the runner's high experienced near the end of a long, challenging race is attributed to endorphin release and a composite surge of pain-relief peptides that block sensory receptors. Whether this function is actually facilitated solely by β-endorphin molecules remains in debate.

STRUCTURE OF β-ENDORPHIN

Scientifically, the family of endorphins is derived from a single prohormone termed proopiomelanocortin, POMC. The POMC gene has 7665 bp consisting of three exons and two introns. Studies using the cell line AtT-20 from mouse anterior pituitary revealed that POMC processing yields distinct products that were not merely degradation entities.[11] The products included the N-terminal fragment, adrenocorticotropin hormone (ACTH, 16 kD) and β-lipotropin hormone (β-LPH). Neither of these larger proteins (i.e., ACTH, β-LPH) has inherent activity and requires additional processing. Further translation yields the corticotropin-like intermediate protein (CLIP), various forms of melanocyte stimulating hormone (α-MSH, β-MSH, and γ-MSH), and β-endorphin.

Sequences of the POMC gene from many species suggest that there is a single copy of the gene that has been substantially conserved throughout evolution. Data from the National Center for Biotechnology Information document that the POMC gene is located on chromosome 2p23.3 in humans, chromosome 6p14 in rats, and chromosome 12 in mice. The primary products are derived from exons 2 and 3, with all of the bioactive peptides, including β-endorphin being flanked by standard dibasic amino acid cleavage residues. The amino acid structure for β-endorphin is Tyr-Gly-Gly-Phe-Met, Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu-Phe-Lys-Asn-Ala-Ile-Ile-Lys-Asn-Ala-Tyr-Lys-Lys-Gly-Glu. Interestingly, the first 5 amino acids code for [Met^5]-enkephalin, one of the original opioid

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00217-7
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peptides discovered based on binding assays. However, POMC is not considered a primary source for enkephalins. Endorphin substances were originally isolated from camel pituitary and later characterized in rodents.[10] There are at least 20 different endorphins, many of which have activity as neurotransmitters, pain modulators, and anxiety suppressors. Further processing of the 3′-end segment of β-LPH gives rise to γ-LPH and β-endorphin subfragments. These in turn are further processed into β-endorphin 1–31, acetylated β-endorphin, and β-endorphin 1–27. The latter two polypeptides have little or no activity.[11] The primary products include α-endorphin containing 16 amino acids, β-endorphin with 31 amino acids, and γ-endorphin-containing 17 amino acids. Cleavage of the prohormone, as well as the active subunits ACTH and β-LPH, occurs in an orderly manner with proteases PC2 and to a smaller extent PC1, responsible for processing.

LOCATION AND MECHANISM OF ACTION

POMC is primarily expressed in the anterior and intermediate lobes of the pituitary, with each lobe being responsible for different peptide products. Corticotroph cells in the anterior pituitary secrete POMC peptides that control adrenal function, while melanotrophs of the *pars intermedia* secrete POMC-associated peptides (e.g., α-MSH) that influence hair and skin pigmentation and have behavioral and EEG effects on the brain. Although the major source of POMC is the pituitary, radioimmunoassay, western blotting, and ELISAs have found recordable levels in the hypothalamus, arcuate nucleus, medial basal hypothalamus, and median eminence.[4] Lesser amounts of endorphin have been detected in the amygdaloid nucleus, periaqueductal gray, locus coeruleus, and substantia nigra.

Several nonneural tissues have also been shown to express and/or process POMC including the adrenal, small intestines, reproductive tract, spleen, lung, liver, heart, and placenta. With this diffuse presence throughout the body, POMC has many diverse functions.[15] The presence of POMC-related peptides in these tissues, and possibly roles for β-endorphin, are discussed below.

Opioid activity associated with β-endorphin is dependent on its C-terminal residues, and loss of these amino acids substantially decreases the analgesic property of the peptide. Numerous studies have shown that β-endorphin shares many of the physiological actions of exogenous opiates such as morphine and has been documented in animal studies to have effects on analgesia, respiratory depression, vasopressin release, and cardiovascular homeostasis.[8] β-Endorphin can be easily measured in plasma and increases during pregnancy, with elevated levels reported particularly during labor and delivery. Moreover, β-endorphin has heightened presence throughout surgery, and in some pituitary-related diseases such as Cushing's disease and Addison's disease; conversely, pituitary resection results in diminished levels. Cerebrospinal fluid also contains detectable levels of endorphins, with elevated levels after surgically induced analgesia.

Once released into the blood stream, β-endorphin binds to an opioid receptor to elicit its activity. Studies over the last few decades have suggested that endorphins can bind to any or all of the classical opioid receptors (mu, delta, and kappa), and some studies have suggested that there is a specific or selective receptor for endorphin termed the epsilon (ε) receptor. This latter receptor is very much understudied, and its true existence and function are uncertain. The consensus of studies suggests that the highest affinity may be for mu (μ) opioid receptors (MOR1), moderate affinity for MOR2, and delta (δ) opioid receptor (DOR), and little or no affinity for the third subclass of classical opioid receptors known as kappa opioid receptors. Most reports infer that the euphoria associated with the 'runner's high' results from binding at both the traditional pain mediated mu receptors and the kappa opioid receptors.

Genetic POMC deficiencies are compatible with life depending on the extent of mutational rearrangement and/or deletion, and whether the ACTH deficiency can be medically handled by hydrocortisone treatment. In general, the adrenal insufficiency related to genetic POMC deletions is not much different from other clinically based (e.g., tumors) pituitary conditions.

FUNCTION

As with all of the opioid peptides, the function of β-endorphin changes depending on the receptor to which it is binding and the region of brain or location in the whole body. Although the literature supports the fact that β-endorphin binds to μ, δ, and κ classical opioid receptors, as well as to possibly that it has its own receptor termed epsilon (ε), the majority of actions of endorphin can be localized to μ and δ opioid receptors. As with other neuropeptides, such as methionine enkephalin, the ubiquity and generalization of function of endorphin are widespread. Endorphins function in analgesia and pain cessation, satiation and food metabolism, cardiovascular regulation, T- and B- cell proliferation and function in the immune system, as well as the euphoric responses attributed to higher order emotional and neurological systems.

β-ENDORPHIN AND MU RECEPTOR FUNCTIONS

β-Endorphin serves as an agonist for mu opioid receptors and *in vitro* and *in vivo* studies have supported its function at these receptors to be neuroendocrine or neuroimmune in nature. Some studies suggest that β-endorphin has few central nervous system mediated effects when administered systemically

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because of the inherent difficulty for β-endorphin to cross the blood–brain barrier. Consequently, the classic effects of mu receptors including confirmation of analgesia and respiratory depression are not attributed to the endogenous peptide β-endorphin.[18] *In vivo* studies of the function of β-endorphin at mu opioid receptors suggest that prolactin release after systemic injections mediate many of the neuroendocrine responses.[1] Nonhuman primate studies in which β-endorphin is injected intravenously reported that this peptide most probably acts at mu receptors on the hypothalamus. However, the *in vivo* studies are not completely confirmatory as it is well known that β-endorphin is rapidly metabolized in the plasma and that other bioactive peptides may yield high affinity to opioid receptors and confer the function.

β-ENDORPHIN AND OBESITY

The relationship of POMC and obesity has been well established because POMC is the precursor for ACTH which must be homeostatically secreted to maintain normal adrenal function.[12] Failure to secrete ACTH, related to pituitary dysfunction, results in weight loss. Conversely, POMC deficiency seems to result in both rodent models and humans with increased appetite and weight gain. Targeted genetic manipulation of POMC results in obese mice. These studies suggested that POMC neurons in the hypothalamus and their possible melanocortin-related receptors may have control over the pituitary function of weight control. Polymorphic variations among persons with weight issues fail to support a singular gene association between POMC and obesity.

Some interesting functions of β-endorphin were discovered with the development of a POMC-null mouse.[20] This mouse was hyperpigmented, overweight, and had defective adrenal development. As additional studies were conducted using the mouse model, as well as other transgenic mice, knowledge about the role of leptin activation mediated by POMC neurons and regulation of obesity was uncovered[3] These investigators recorded from POMC neurons and showed that leptin reduced neuropeptide Y/GABA circuitry and further that melanocortin peptides also inhibited this signaling pathway. However, leptin receptors alone are not the sole regulator of body weight homeostasis. Further studies on excitation of POMC neurons in the arcuate nucleus, another center for control of feeding, resolved that more than leptin receptors are involved in weight and eating regulation.

β-ENDORPHIN AS A NEUROMODULATOR OF THE CARDIOVASCULAR SYSTEM

Because cardiac muscle possesses a high density of opioid receptors, the involvement of endogenous opioids, particularly endorphin, in cardiac function has led to many reports implicating a role for endorphins in the management of hypertension, as well as a contraindication of morphine or as a mimic of the exogenous agonists.

In a similar manner to exogenous opioids such as morphine, intravenous administration of β-endorphin causes an immediate drop in blood pressure in rodents, cats, and dogs. Dose-dependent vasodepressor effects along with bradycardia are antagonized by naloxone, suggesting that the effects are opioid receptor mediated.[5] However, the effects of endorphins on the cardiovascular system are dependent on the route of administration and dose. The sympathetic nervous system seems to mediate the drop in pressure and vagal nerve tracts seem to be responsible for bradycardia.[8] Other cardiovascular responses to endorphin may involve a variety of other hormones and effector compounds including serotonin, catecholamines, vasopressin, angiotensin, and aldosterone. Serotonin has been linked with hypotension seen in animals injected with endorphins, as well as in reuptake transmitter models implicating an endogenous release of the compound on stimulation. Endorphins and exogenous opioids such as morphine act on presynaptic receptors in sympathetic nerves to inhibit the release of norepinephrine; however, epinephrine also causes the release of β-endorphin, while β-endorphin seems to invoke a general increase in plasma levels of several neurotransmitters including dopamine, norepinephrine, and epinephrine.[19]

β-ENDORPHIN AND THE IMMUNE SYSTEM

In addition to its role as a regulator of homeostatic functions including respiration, cardiovascular control, and its role as a neurotransmitter, endorphins have shown functional relevance in the immune system. Most of the lymphocyte subclasses (i.e., T and B cells) express one or more of the classical opioid receptors,[2,16] as well as the opioid growth factor receptor.[21,22] Endorphins have been reported to suppress phagocytosis activity of macrophages and to modulate cytokine production and proliferation of T- and B-lymphocytes through binding to δ-opioid receptors or the opioid growth factor receptor.[16,21,22] Some reports suggest that the class of endorphin molecules, and in particular enkephalins, inhibit CD4+ and CD8+ T-cell proliferation in populations of lymphocytes isolated from murine spleen. The responses of endorphins are mediated by delta opioid receptors although the evidence for this singular receptor is circumstantial. Specific data such as that which could be resolved by knockout delta opioid receptor mouse studies are lacking. Several studies examining the role of opioid receptor binding activity using radioactively labeled antagonists (e.g., naloxone), synthetic agonists (e.g., deltorphin), or partial amino acid sequences representative of proopiomelanocortin show the presence of selective and saturable binding for both kappa and delta opioid receptors on lymphoid cells, particularly T-lymphocytes. These results support some level of functional activity by endorphins in the immune system.

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MISCELLANEOUS ROLES OF β-ENDORPHIN

Sequence homology between the heavy chain of IgG and ACTH and endorphin has provided an incentive for a few researchers to ask what is the role of β-endorphin at nonopioid receptors. Studies have shown that a β-endorphin-like decapeptide, with sequence homology to human IgG, exhibits some biological activity in the immune system. The peptide activated human and rodent immunocompetent cells both *in vitro* and *in vivo* and to enhance proliferation of leukemia cell lines (Jurkat), blastomeres, and preimplantational mouse embryos.[9,13] This peptide termed immunorphine binds to naloxone-insensitive receptors located on T-lymphocytes, mouse macrophages, and selected rat brain homogenates. Radioactively labeled binding assays confirm nonopioid β-endorphin receptors in nonneural tissues including rodent heart and spleen, as well as brain and adrenal glands. A significant level of research is required to substantiate this role for β-endorphin.

SUMMARY

As with other endogenous neuropeptides, β-endorphin is widely distributed in the central nervous system, as well as other neural-related and nonneural tissues. Consequently, the physiological functions of β-endorphin are diverse and distributed throughout all the major organ systems. With additional research, new, nonfunctional roles for endorphins are continuing to be discovered, and new pathways and mechanisms are being validated. As the specificity of receptors increases and the classes of mu and delta opioid receptors expands, the relationship between receptors and endorphins becomes clearer and more refined. Nonetheless, endorphins remain a large body of peptide/hormones that impact most of the major biological systems in the human body. Research should be encouraged into defining the specific roles played by endorphins in disease states as well as maintaining homeostasis in the developing and adult organism.

REFERENCES

1. Butelman ER, Reed B, Chait BR, Mandau M, Yuferov V, Kreek MJ. Limited effects of β-endorphin compared to loperaminde or fentanyl in a neuroendocrine biomarker assay in non-human primates. *Psychoneuroendocrinology* 2007;**33**:292–304.
2. Carr DJ, Carpenter GW. Morphine-induced suppression of cytotoxic T lymphocyte activity in alloimmunized mice is not mediated through a naltrindole-sensitive delta opioid receptor. *Neuroimmunomodulation* 1995;**2**:44–53.
3. Cowley MA, Smart JL, Rubinstein M, Cerdan MG, Diano S, Horath TL, et al. Leptin activates anorexigenic POMC neurons through a neural network in the arcuate nucleus. *Nature* 2001;**411**:480–4.
4. Fredrickson RCA, Burgis V, Edwards JD. Hyperalgesia induced by naloxone follows diurnal rhythm in responsivity to painful stimuli. *Science* 1977;**198**:756–8.
5. Goldstein A, Lowery PJ. Effects of the opiate antagonist naloxone on body temperature in rats. *Life Sci* 1975;**17**:927–31.
6. Hughes J. Isolation of an endogenous compound from the brain with pharmacological properties similar to morphine. *Brain Res* 1975;**88**:295–308.
7. Hughes J, Smith TW, Kosterlitz HW, Fothergill LA, Morgan BA, Morris HR. Identification of two related pentapeptides from the brain with potent opiate agonist activity. *Nature* 1975;**258**:577–9.
8. Johnson MW, Mitch WE, Wilcox CS. The cardiovascular actions of morphine and the endogenous opioid peptides. *Prog Cardiovas Dis* 1985;**27**:435–50.
9. Kovalitskaya YA, Navolotskaya EV. Nonopioid effect of β-endorphin. *Biochemistry (Moscow)* 2011;**76**:379–93.
10. Loh HH, Tseng LF, Wei E, Li CH. β-Endorphin is a potent analgesic agent. *Proc Natl Acad Sci U S A* 1976;**73**:2895–8.
11. Mains RE, Eipper BA. Coordinate synthesis of corticotrophins and endorphins by mouse pituitary tumor cells. *J Biol Chem* 1978;**253**: 651–5.
12. Morley JE. The endocrinology of the opiates and opioid peptides. *Metabolism* 1981;**30**:195–209.
13. Navolotskaya EV, Kovalitskaya YA, Zolotarev YA, Kolobov AA, Kampe-Nemm EA, Malkova NV, et al. Characteristics of non-opioid β-endorphin receptor. *Biochemistry* 2004;**69**:394–400.
14. Pert CB, Pasternak GW, Snyder SH. Opiate agonists and antagonists discriminated by receptor binding in brain. *Science* 1973;**182**:1359–61.
15. Raffin-Sanson ML, de Keyzer Y, Bertagna X. Pro-opiomelanocortin, a polypeptide precursor with multiple functions: from physiology to pathological conditions. *Eur J Endocrinol* 2003;**149**:79–90.
16. Sharp BM. Multiple opioid receptors on immune cells modulate intracellular signaling. *Brain Behav Immun* 2006;**20**:9–14.
17. Simantov R, Snyder S. Morphine-like peptides in mammalian brain: isolation, structure elucidation, and interactions with the opiate receptor. *Proc Natl Acad Sci U S A* 1976;**73**:2515–9.
18. Tseng LF, Loh HH, Li CH. β-Endorphin is a potent analgesic by intravenous injection. *Nature* 1976;**263**:3239–40.
19. Van Loon GR, Appel NM. β-Endorphin induced increases in plasma dopamine, norepinephrine, and epinephrine. *Res Commun Chem Pathol Pharmacol* 1980;**27**:607–10.
20. Yaswen L, Diehl N, Brennan MB, Hochgeschwender U. Obesity in the mouse model of pro-opiomelanocortin deficiency responds to peripheral melanocortin. *Nat Med* 1999;**5**:1066–70.
21. Zagon IS, Donahue RN, Bonneau RH, McLaughlin PJ. T lymphocyte proliferation is suppressed by the opioid growth factor ([Met^5]-enkephalin)-opioid growth factor receptor axis: implication for the treatment of autoimmune diseases. *Immunobiology* 2011;**216**: 579–90.
22. Zagon IS, Donahue RN, Bonneau RH, McLaughlin PH. B lymphocyte proliferation is suppressed by the opioid growth factor-opioid growth factor receptor axis: Implication of the treatment of autoimmune diseases. *Immunobiology* 2011;**216**:173–83.

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Chapter 218

Prodynorphin-Derived Peptides

Santi Spampinato, Andrea Bedini and Monica Baiula

ABSTRACT

The dynorphin family consists of multiple peptides generated by posttranslational processing of prodynorphin. They are widely distributed in the central nervous system and occur in peripheral tissues, such as the heart, the gastrointestinal tract, and the adrenal gland. Dynorphin A(l–17) has been widely investigated because it can represent an endogenous ligand for the K-opioid receptor. It displays several biological functions, including the regulation of pain transmission, feeding, and pituitary hormone release, and it may influence myocardial function. C-terminal fragments of dynorphin A(l–17), lacking the N-terminal tyrosine, do not bind to opioid receptors and are excitotoxic by interacting with glutamate receptors. Increased prodynorphin expression, observed in several pathophysiological conditions such as neuropathic pain and heart ischemia, may tilt the balance toward neurotoxic dynorphin derivatives that may contribute to tissue damage. In recent years, implications of dynorphins in drug addiction, neurodegenerative disease, stress, and depression have been shown.

DISCOVERY

Dynorphins include multiple bioactive peptides generated by posttranslational processing of the large precursor prodynorphin;[8] they include α-neoendorphin (α-NE), big dynorphin (Dyn A 1–32), leumorphin (Dyn B 1–29), dynorphin A (Dyn A 1–17), dynorphin B (Dyn B 1–13), leucine–enkephalin–arginine (Leu–enkephalin–Arg), and potentially leucine–enkephalin (Leu–enkephalin) (Fig. 1).

In 1979, Avram Goldstein et al. reported the characterization and partial sequence of a highly potent opioid peptide obtained from pituitary extracts. This tridecapeptide contained the amino acid sequence for Leu–enkephalin at its N-terminus,[11] and in the guinea pig ileum longitudinal muscle preparation, it was approximately 700 times more potent than Leu–enkephalin. For this reason, it was named dynorphin (1–13), from the greek prefix *dyn* "strength or power." Later, the same laboratory reported the complete sequence of the endogenous heptadecapeptide named Dyn A(l–17). Soon thereafter, other laboratories described the sequence and distribution of the other dynorphins.[10]

STRUCTURE OF THE PRECURSOR mRNA/GENE

The cloning of the prodynorphin cDNA from a porcine hypothalamic library[16] confirmed that all dynorphins are contained and produced within the same protein-inactive precursor and are generated by cleavage at basic residues. The sequence of the prodynorphin gene has been reported for several species, including human. The coding gene contains four exons separated by three introns (Fig. 1). As reported in other opioid peptide genes, exons 1 and 4, which contain nontranslated sequences, are very large (1.4 and 2.2 kb, respectively). Exons 3 and 4 contain the translated regions; prodynorphin mRNA possesses a very long nontranslated 3′-terminal end of approximately 1550 nucleotides. A variety of mechanisms have been shown to lead to species-, tissue-, and treatment-specific differences in the structure of prodynorphin mRNA transcripts, and some of these different structural forms affect function. For example, in rat testis, alternative splicing of the prodynorphin mRNA yields a mature mRNA that lacks exon 2. This exon codes for part of the 5′ untranslated region; the lack of this exon has no apparent effect on translational efficiency.[21] At the peptide level, Dyn A(l–17) is completely conserved across all mammalian species in which its sequence has been determined; however, at the level of the mRNA primary structure, an assortment of mechanisms, including alternative splicing, differential transcription initiation, and site-specific cleavage, lead to the generation of species-, tissue-, and treatment-specific mRNAs for this peptide precursor.[21] The study of mRNA transcripts from the prodynorphin gene continues to provide examples of general and specific mechanisms of regulation of mRNA structure and also, in some instances, mRNA function. The functional significance of the longer form of the prodynorphin mRNA is as yet unknown, but may possibly alter translational efficiency or mRNA stability. Several transcription factor binding sites were identified in the promoter region of the prodynorphin gene and found to be responsible for the enhanced prodynorphin mRNA expression in response to AMP or protein kinase A in the rat.[8]

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00218-9
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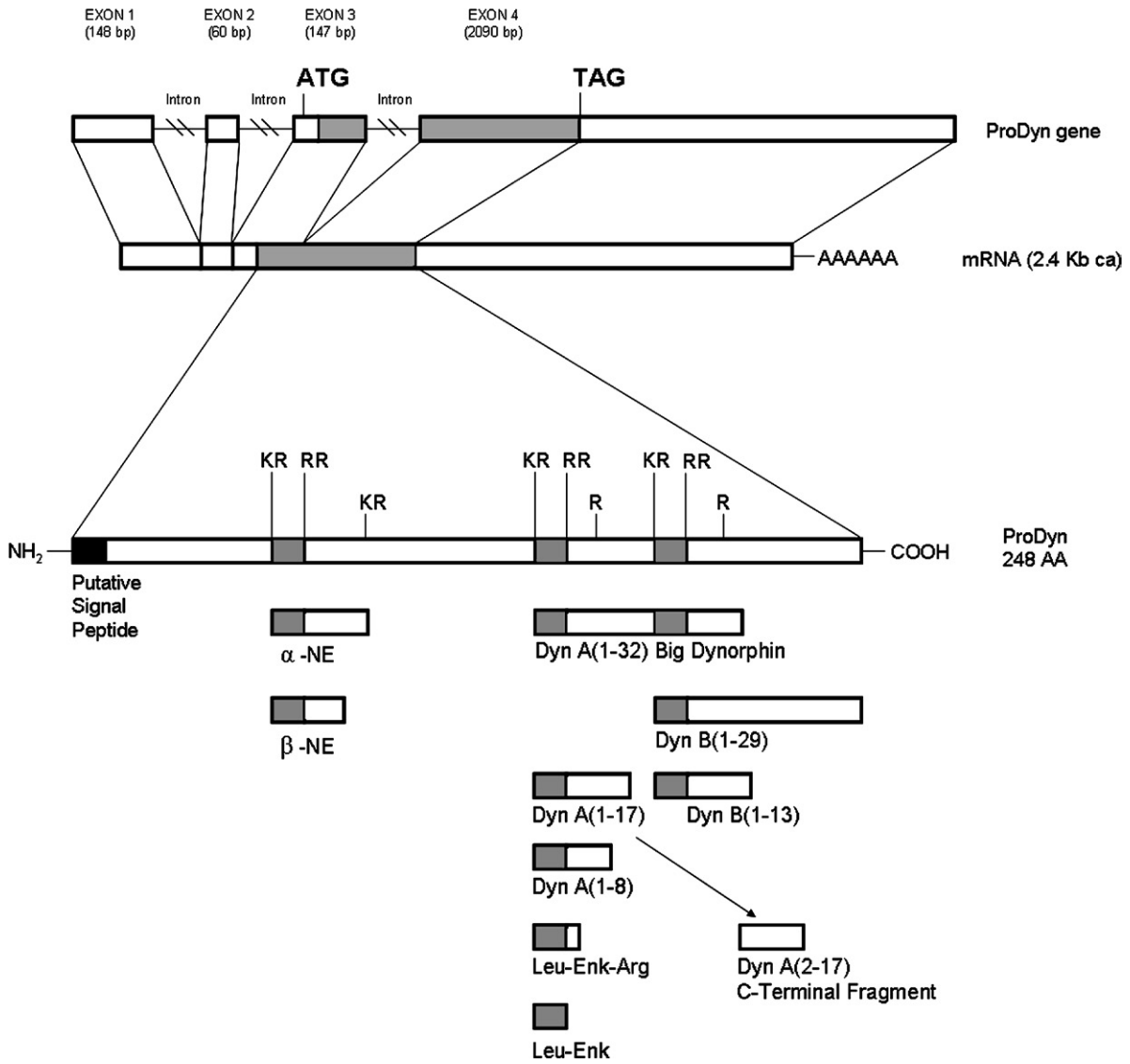

FIGURE 1 Prodynorphin (ProDyn) gene, messenger RNA, protein precursor structure, and processing pathways. Basic amino acid cleavage sites in the precursor are denoted by their single letter codes. K, lysine; R, arginine. Solid box corresponds to the sequence of Leu–enkephalin.

PROCESSING

The most common processing signal sequences of the prodynorphin precursor, which is highly basic, are the lysine–arginine (KR), the lysine–lysine (KK), and the arginine–argine (RR) residues; these are cleaved on the carboxyl side of the basic amino acids and followed by removal of the C-terminal basic residue. An endopeptidase-designated dynorphin-converting enzyme (DCE) cleaves neuropeptides such as dynorphins at monobasic sites. After endopeptidase activity, carboxypeptidases remove the basic amino acids from the C-terminus of peptides. Aminopeptidases capable of removing N-terminal Arg or Lys residues from the processed peptides have also been reported.[7] The relative levels of endopeptidase can have a large effect on the bioactivity of neuropeptides because differential proteolysis of a single precursor can result in the generation of several neuropeptides that activate a diverse set of receptors. (See the Peptide Biosynthesis/Processing section of the book for more details.) Thus, processing enzymes play a key role in the modulation of neuropeptide gene expression. Additional bioactive derivatives of prodynorphin probably exist in addition to those previously indicated. Many of the C-terminal fragments of dynorphin A [most likely Dyn A(2–17)], which are intrinsically neurotoxic, have been isolated from neural tissue *in vivo* or *in vitro*.[13] Moreover, neuropeptide processing may occur after secretion.

Initial studies carried out to isolate dynorphins focused on the characterization of small fragments corresponding to the end products of prodynorphin precursor processing.

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However, with antibodies directed against Dyn A or Dyn B, radioimmunoassay analysis of gel-fractioned tissue extracts revealed in certain tissues large-molecular-weight intermediates that were subsequently characterized.[6] The anterior pituitary gland contains several prodynorphin-processing intermediates: a 4-kDa peptide containing Dyn A and Dyn B [Dyn A (1–32)], a 6-kDa fragment comprising Dyn A(l–32) and a C-terminal fragment, an 8-kDa peptide having α-neoendorphin at the N-terminal position of the 6-kDa fragment; and, finally, a 16-kDa fragment comprising the N-terminal portion of the precursor plus α-neoendorphin and a 10-kDa fragment containing α-neoendorphin and Dyn A(l–32). Other intermediates, occurring in a small amount (~10–20%) have not been well characterized. In other brain areas containing dynorphins, these intermediates are present only in trace amounts, and the major components are the small dynorphins such as Dyn A(l–17), Dyn B, and α-neoendorphin.[6] Thus, this suggests that a tissue-specific processing requires the expression of particular processing enzymes.

RECEPTORS

Radioreceptor-binding assays in brain membrane fragments and bioassays in isolated intact tissues have ascertained that Dyn A(l–17) and the other dynorphins bind to κ-opioid receptors (KOR) with a somewhat higher affinity than μ- or δ-opioid receptors (MOR and DOR, respectively), and Dyn A(l–l7) is considered to be an endogenous ligand for the KOR.[10] Dyn A(l–8) and α-neoendorphin have lower affinities for the KOR, with significant affinity for the MOR and DOR sites. In agreement with the preferential binding to KOR, Dyn A(l–l7)-elicited effects on isolated tissue preparations can be blocked by selective KOR antagonists such as nor-binaltorphine.[5]

CONFORMATIONAL ANALYSIS OF DYN A(1–17)

Structural studies of dynorphin in the solid state and the membrane environment show that the N-terminus forms an a-helical structure and is inserted into the membrane with the helical axis almost perpendicular to the membrane surface. It has been suggested that the helical region of the extracellular loop II of the KOR may interact with the helical region of dynorphin with a high affinity in the membrane environment.[22]

DYNORPHIN EXPRESSION IN THE CENTRAL NERVOUS SYSTEM AND POSSIBLE BIOLOGICAL ACTIONS

Initial immunocytochemical studies of dynorphins were carried out in the rat hypothalamus, where Dyn (1–13) was found to be colocalized with vasopressin in magnocellular neurons of the supraoptic and paraventricular nuclei.[17] The immunocytochemical distribution of peptides derived from the prodynorphin precursor in the brain of the rhesus monkey (*Macaca mulatta*) indicates a widespread neuronal localization of immunoreactivity from the cerebral cortex to the caudal medulla. Immunoreactive perikarya are located in numerous brain loci, including the cingulate cortex, caudate nucleus, amygdala, hypothalamus, thalamus, substantia grisea centralis, parabrachial nucleus, nucleus tractus solitarius, and spinal cord dorsal gray laminae. In addition, fiber and terminal immunoreactivity are seen in varying densities in the striatum and pallidum, substantia innominata, hypothalamus, substantia nigra pars reticulata, parabrachial nucleus, spinal trigeminal nucleus, and other areas. The distribution of prodynorphin peptides in the brain of the monkey is similar to that described for the rat brain; however, significant differences also exist. *In situ* hybridization histochemistry has been adopted as a technique to investigate the localization of prodynorphin mRNA in the central nervous system; this approach has confirmed that prodynorphin-containing cells are relatively widespread throughout brain regions that contain dynorphin peptides.[17]

Dynorphins play a role in a wide variety of physiological parameters, including motor activity, cardiovascular regulation, respiration, temperature regulation, feeding behavior, and hormone release.[25] Although they do not elevate pain threshold when injected in the brain, they antagonize opioid analgesia in naive animals and potentiate it in tolerant animals. Dynorphins have beneficial effects on stroke that are like those of opioid antagonists rather than like those of agonists.[29]

Prodynorphin-positive neurons are widely distributed in brain and spinal cord areas involved in the transmission of nociceptive stimuli.[17] Dynorphins may be involved in a local circuit within the spinal cord, and in supraspinal functions. Interestingly, dynorphins were also detectable in cutaneous nerves with a distribution similar to that of calcitonin-gene-related peptide, a specific marker for sensory neurons. A moderate density of KOR binding sites has been seen in the central and peripheral neuronal nociceptive system and in various immune cells.[14] A lack of an antinociceptive action of dynorphin after its administration into the lateral brain ventricle[24] and some slight antinociceptive activity after intrathecal injection[24,29] have been reported. Other KOR agonists also possess some antinociceptive activity after their intrathecal administration. However, the effect is much weaker on a molar basis than that evoked by MOR or DOR agonists. On the other hand, in electrophysiological experiments in spinalized rats, KOR agonists reduce reflexes stimulated by thermal and mechanical nociceptive stimuli to the same extent and in a dose-dependent manner.[20,24] Dynorphin-mediated analgesia has been ascribed to its inhibitory action on neurons at KOR. Electrophysiological evidence

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supports a KOR-mediated inhibitory effect of dynorphins on synaptic transmission of nociceptive neurons in the spinal dorsal horn.[24] Dynorphins, and other neurotransmitters, can be released and can induce the hyperpolarization of neurons, potentially through a KOR-coupled enhancement of potassium conductance. In addition, dynorphin-mediated activation of KOR suppresses calcium currents and calcium-dependent secretion.[4] Further, dynorphin has been shown to inhibit substance P release in the spinal cord in a KOR-mediated manner.[20] The endogenous dynorphin–KOR system has been suggested to elicit antinociception during inflammation, pregnancy,[24] and acupuncture.[12] These studies support an antinociceptive function of dynorphin by negatively modulating transmission of nociceptive information.

PATHOPHYSIOLOGICAL IMPLICATIONS

Observation of the antinociceptive actions of dynorphin in the spinal cord is hindered by its neurotoxic effects. These can result from a nonopioidergic mechanism of action of the peptide.[13,20] The involvement of spinal dynorphins in animal models of inflammatory pain has been studied in great detail. Profound alterations in the spinal prodynorphin system has been observed when there is peripheral inflammation or chronic arthritis.[27,33] The levels of immunoreactive dynorphin are dramatically increased in the lumbar part of the spinal cord of rats with local inflammation of a hind limb. Further, an increase in prodynorphin mRNA levels induced by acute and chronic inflammation has also been observed in the spinal cord. Thus, many forms of peripheral inflammation induce a dramatic upregulation of prodynorphin biosynthesis in nociceptive neurons of the spinal dorsal horn, which parallels the behavioral hyperalgesia associated with the inflammation. There is much evidence that nerve injury is associated with elevated levels of spinal dynorphins. Spinal injury, which may cause neuropathic pain, also increases the dynorphin level in the spinal cord. Both prodynorphin mRNA and dynorphin levels were significantly increased, whereas those of proenkephalin mRNA and Met-enkephalin remained unchanged 3 days after injury. The increase in the spinal levels of prodynorphin mRNA was the highest in the areas dose to the side of transaction.[20] Thus, endogenous dynorphin levels increase under various conditions associated with neuropathic pain after damage to the spinal cord and injury of peripheral nerves.

The site and time of increase of the dynorphin content in the spinal cord after peripheral nerve injury seem to correlate with the appearance of neuropathic pain. Further, a single intrathecal injection of Dyn A(l–17) to mice induced mechanical allodynia and cold allodynia (acetone applied to the dorsal hind paw) lasting several days.[24] Interestingly, Dyn A(2–17), a nonopioid peptide, induced cold and tactile allodynia analogous to that induced by Dyn A(l–17), indicating the involvement of nonopioid receptors. Further, Dyn A(l–17) has nonopioid activity and may damage the spinal cord when given in high doses and may produce hind-limb paralysis when administered intrathecally to rats. This effect is mediated at least partly via nonopioid receptors because the des-Tyr fragment of dynorphin can induce a similar effect.[24] Dyn A(l–32), intrathecally administered in mice, is a more potent nociceptive peptide than Dyn A(l–17), and its effect was not mediated by opioid receptors; rather, it involved NMDA receptors.[13]

Considerable evidence reveals that dynorphin is actually pronociceptive in chronic pain states. The idea that dynorphins may contribute to the maintenance of neuropathic pain states has been tested in prodynorphin knockout mice.[26] They develop neuropathic pain after nerve injury but fail to sustain it for extended periods of time, and, by 10 days after injury, they are no more sensitive than they were before injury. By contrast, wildtype animals continue to display neuropathic pain. Thus, spinal dynorphins are critical for the maintenance of neuropathic pain, and their upregulation may represent a mechanism for the maintenance of the neuropathic pain state. Several investigators have shown that neurological damage observed after intrathecal dynorphin administration can be prevented by antagonists and modulators of the NMDA receptor, which is known to be an important mediator of neuropathic pain states. Moreover, the long-lasting hyperresponsiveness to both innocuous and noxious stimuli that is elicited by intrathecal dynorphin is not blocked by the opioid receptor antagonist naloxone, but it is prevented by pretreatment with the NMDA receptor antagonist MK-801.[20] This finding suggests that the NMDA receptor is probably a mediator of the nonopioid actions of dynorphin.

The interaction of endogenous dynorphins and NMDA receptors in promoting neuropathic pain may be a consequence of binding of dynorphins to NMDA or they may act as a positive allosteric modulator. Interestingly, Dyn A(2–17) binds to NMDA receptor R1/R2A subunits showing high-affinity binding. Substantial evidence suggests that dynorphin facilitates excitatory amino acid release, and because the binding of dynorphin to NMDA receptors is attenuated by NMDA agonists, dynorphin may not interact appreciably with NMDA receptors under neuronal hyper excitable conditions. It has been proposed that physiological concentrations of dynorphins are antinociceptive and neuroprotective through opiate receptor activation, whereas extremely elevated levels are pronociceptive and even excitotoxic in an NMDA receptor-dependent fashion.[13] Dynorphins can exert protective or proapoptotic effects depending on whether they activate opioid receptors or nonopioid mechanisms (i.e., glutamate receptors or protein–protein interactions) and that they seem to contribute to nervous system pathology through complex interactions involving multiple receptors and pathways.[13]

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Contribution of prodynorphin mutations to spinocerebellar ataxia type 23, an inherited neurodegenerative disorder, could open new vistas on the role of this endogenous opioid system[2] and any dysregulation of dynorphins in Alzheimer disease.[34]

In recent years, new insights, provided by investigations on prodynorphin- and opioid receptor-deficient mice, have proved any involvement of dynorphins in neurological and psychiatric diseases. There is increasing evidence for a potential involvement of dynorphin/KOR in schizophrenia,[25] in stress and depression.[18] Bruchas et al.[3] have reported that the anxiogenic properties of stress are encoded by endogenous dynorphins acting in the basolateral amygdala. Further, the anticonvulsant and antiepileptic effects of dynorphin in animal models have been investigated.[25]

DRUG ABUSE AND DYNORPHINS

Rewarding effects and compulsive drug-seeking behavior, characterizing drug addiction, are induced by various drugs of abuse and may require an increase of dopamine neurotransmission in the nucleus accumbens and in the striatum and a glutamate-dependent neuroplasticity. Dynorphins, locally released, may modulate dopamine neurotransmission and interfere with psychostimulants and other drugs that increase it.[19] The dynorphin/KOR system serves as an essential function in opposing alterations in behavior and in opposing alterations in behavior and brain neurochemistry that occur as a consequence of repeated drug use and that aberrant activity of this system may not only contribute to the dysregulation of behavior that characterizes addiction but to individual differences in vulnerability to the pharmacological actions of cocaine and alcohol.[28] Interestingly, in rats exposed to the anabolic androgenic steroid nandrolone, elevated dynorphin levels were observed in brain regions regulating emotions, dependence, and defensive reactions.[15] The authors have proposed that brain dynorphins may participate in behavioral modifications induced by anabolic steroids. Therefore, it has been suggested that the increase of endogenous dynorphins, caused by drugs of abuse, may counteract the effects elicited by these compounds on dopamine neurotransmission.[13] However, it cannot be excluded that considerable higher levels of dynorphins may also interfere with glutamate receptors and may promote, instead of counteract, neuroplastic changes that promote drug-seeking behavior. Exposure to high doses of psychostimulants may cause a neurotoxic loss of neurons and apoptotic cell death.[13] Therefore, as previously described for the spinal effects of dynorphins, it remains to be established whether these peptides may have a dichotomy of actions: at lower levels, they counteract drug addiction, whereas at supraphysiological levels, they may contribute to drug abuse toxicity. In this regard, it should be mentioned that dynorphin dysregulation might be associated with various neurological disorders such as Parkinson's and Huntington's disease; moreover, dynorphin alternations have been associated with the pathogenesis of refractory seizures.[13]

DYNORPHIN EXPRESSION IN PERIPHERAL TISSUES

In the periphery, numerous tissues express prodynorphin-derived peptides. Initially, Spampinato and Goldstein[31] reported the presence of immunoreactive dynorphin in the following rat tissues: stomach, intestine, adrenal gland, ovary, testis, skeletal muscle, and heart. Gel-permeation chromatography revealed a larger apparent molecule than that of Dyn A(l–17). Moreover, dynorphins have been detected in rat and human plasma.[31,32] However, the processing and the physiological role played by dynorphins in peripheral tissues are still largely unknown. The heart has drawn particular attention for its capacity to biosynthesize dynorphins in the rat atrial and ventricular tissue; dynorphins also occur in the human heart.[30] In single cardiac myocytes, Dyn A(1–17) elicited an increase of cytosolic calcium via KOR. These peptides could play an early but crucial role in ischemic tolerance; in the rabbit model, this was explained by the unique role kappa opioids provide in adrenergic regulation. Only KORs are found on presynaptic nerve terminals, and nerve terminal secretory vesicles contain dynorphin peptides. Both dynorphins and norepinephrine are released from nerve terminals during ischemia, but dynorphin activation of KOR then results in negative feedback on norepinephrine release.[23] As a governor of catecholamine stimulation during ischemia, dynorphins may reduce postischemic injury caused by deleterious, adrenergically enhanced cellular metabolism and contractile function. Nonselective blockade of opioid receptors increases the canine cardiac response to adrenergic stimulation, supporting the regulatory role of endogenous opioids. The influence of KOR agonists may thus be confined to early events in ischemic injury to temper sympathetic stimulation. However, in the isolated heart Dyn A(l–17) may exacerbate infarct size and increase hydroxyl radical production.[1] Thus, this peptide may exert opposing effects on the ischemic heart depending on concentration, exposure time, and experimental model. Finally, circulating dynorphins may contribute to pressor effects observed in hypertensive patients during episodes of stress-induced blood pressure increase.[9]

PERSPECTIVE

Dynorphins are multifunctional peptides acting through different receptors in either a protective or toxic way. These interactions depend on the manner in which dynorphins are

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processed and degraded. Dynorphins display multiple effects in the central nervous system and in peripheral tissues. Prodynorphin mutations could contribute to neurodegenerative diseases. In recent years, novel approaches based on the use of prodynorphin knockout mice and animal models of diseases have provided new vistas on the role played by these peptides as modulators of injury and disease outcome in the central nervous system and in the heart.

REFERENCES

1. Aitchison KA, Baxter GF, Awan MM, Smith RM, Yellon DM, Opie LH. Opposing effects on infarction of delta and kappa opioid receptor activation in the isolated rat heart: implications for ischemic preconditioning. *Basic Res Cardiol* 2000;**95**:1–10.
2. Balkalkin G, Watanabe H, Jezierska J, Depoorter C, Verschuuren-Bemelmans C, Bazov I, Artemenko KA, et al. Prodynorphin mutations cause the neurodegenerative disorder spinocerebellar ataxia type 23. *Am J Hum Genet* 2010;**87**:593–603.
3. Bruchas MR, Land BB, Chavkin C. The dynorphin/kappa opioid system as a modulator of stress-induced and pro-addictive behaviors. *Brain Res* 2010;**1314**:44–55.
4. Chavkin C. Dynorphins are endogenous opioid peptides released from granule cells to act neurohumorly and inhibit excitatory neurotransmission in the hippocampus. *Prog Brain Res* 2000;**125**:363–7.
5. Corbett AD, Paterson SJ, Kosterlitz HW. Selectivity of ligands for opioid receptors. In: Herz A, editor. *Opioids I*. Berlin: Springer-Verlag; 1993. p. 645–79.
6. Day R, Trujillo KA, Akil H. Prodynorphin biosynthesis and post-translational processing. In: Herz A, editor. *Opioids I*. Berlin: Springer-Verlag; 1993. p. 449–70.
7. Devi LA. Dynorphin processing endoprotease. In: Woessner F, Rawling N, Barrett AJ, editors. *Handbook of proteolytic enzymes*. San Diego: Academic Press; 1998. p. 1449–50.
8. Douglass J, McKinzie AA, Pollock KM. Identification of multiple DNA elements regulating basal and protein kinase A-induced transcriptional expression of the rat prodynorphin gene. *Mol Endocrinol* 1994;**8**:333–44.
9. Fontana F, Bernardi P, Spampinato S, Boschi S, De Iasio R, Grossi G. Pressor effects of endogenous opioid system during acute episodes of blood pressure increases in hypertensive patients. *Hypertension* 1997;**29**:105–10.
10. Goldstein A. In: Udenfriend S, Meienhofer J, editors. *The peptides: analysis, synthesis, biology*. New York: Academic Press; 1984. p. 95–123.
11. Goldstein A, Tachibana S, Lowney LI, Hunkapillar M, Hood L. Dynorphin (1-13), an extraordinary potent opioid peptide. *Proc Natl Acad Sci USA* 1979;**76**:6666–70.
12. Han JS. Acupuncture and endorphins. *Neurosci Lett* 2004;**361**:258–61.
13. Hauser KF, Aldrich JF, Anderson KJ, Bakalkin G, Chri Hall ED, et al. Pathobiology of dynorphins in trauma and disease. *Frontiers Biosci* 2005;**10**:216–35.
14. Herz A. Peripheral opioid analgesia—facts and mechanisms. *Prog Brain Res* 1996;**110**:95–104.
15. Johansson P, Hallberg M, Kindlundh A, Nyberg F. The effect on opioid peptides in the rat brain, after chronic treatment with the anabolic androgenic steroid, nandrolone decanoate. *Brain Res Bull* 2000;**51**:413–8.
16. Kakidani H, Furutani Y, Takahashi H, Noda M, Morimoto Y, Hirose T, et al. Cloning and sequence analysis of cDNA porcine beta-neoendorphin/dynorphin precursor. *Nature* 1982;**298**:245–9.
17. Khachaturian H, Schaefer MKH, Lewis ME. Anatomy and function of the endogenous opioid systems. In: Herz A, editor. *Opioids I*. Berlin: Springer-Verlag; 1993, p. 471–97.
18. Knoll AT, Carlezon Jr WA. Dynorphin, stress, and depression. *Brain Res* 2010;**1314**:56–73.
19. Kreek MJ. Cocaine, dopamine and the endogenous opioid system. *J Addict Dis* 1996;**15**:73–96.
20. Lai J, Ossipov MH, Vanderah TW, Malan Jr TP, Porreca F. Neuropathic pain: the paradox of dynorphin. *Mol Interventions* 2001;**1**: 160–7.
21. Mayer P, Hollt V. Allelic and somatic variations in the endogenous opioid system of humans. *Pharmacol Ther* 2001;**91**:67–177.
22. Naito A, Nishimura K. Conformational analysis of opioid peptides in the solid states and the membrane environments by NMR spectroscopy. *Curr Top Med Chem* 2004;**4**:135–45.
23. Pepe S, van den Brink OW, Lakatta EG, Xiao RP. Cross-talk of opioid peptide receptor and beta-adrenergic receptor signalling in the heart. *Cardiovasc Res* 2004;**63**:414–22.
24. Przewlocki R, Przewlocka B. Opioids in chronic pain. *Eur J Pharmacol* 2001;**429**:79–91.
25. Schwarzer C. 30 years of dynorphins—new insights on their functions in neuropsychiatric diseases. *Pharmacol Ther* 2009;**123**:353–70.
26. Sharifi N, Diehl N, Yaswen L, Brennan MB, Hochgeschwender U. Generation of dynorphin knockout mice. *Mol Brain Res* 2001;**86**: 70–5.
27. Sharma HS, Olsson Y, Nyberg F. Influence of dynorphin antibodies on the formation of edema and cell changes in spinal cord trauma. *Prog Brain Res* 1995;**104**:401–16.
28. Shippenberg TS, Zapata A, Chefer VI. Dynorphin and the pathophysiology of drug addiction. *Pharmacol Ther* 2007;**116**:306–21.
29. Smith AP, Lee NM. Pharmacology of dynorphin. *Ann Rev Pharmacol Toxicol* 1988;**28**:123–40.
30. Spampinato S, Canossa M, Ventura C, Bachetti T, Ventura C, Bastagli L, Bernardi P, Ferri S. Heterogeneity of immunoreactive dynorphin B-like material in human, rat, rabbit and guinea pig heart. *Life Sci* 1991;**48**:551–9.
31. Spampinato S, Goldstein A. Immunoreactive dynorphin in rat tissues and plasma. *Neuropeptides* 1983;**3**:193–212.
32. Spampinato S, Paradisi R, Canossa M, Campana G, Frank C, Flamigni F, Ferri S. Immunoreactive dynorphin A-like material in extracted human hypothalamic hypophysial plasma. *Life Sci* 1992;**52**:223–30.
33. Weihe E, Millan MJ, Hollt V, Nohr D, Herz A. Induction of the gene encoding prodynorphin by experimentally induced arthritis enhances staining for dynorphin in the spinal cord of rats. *Neuroscience* 1989;**31**:77–95.
34. Yakovleva T, Marinova Z, Kuzmin A, Seidah NG, Haroutunian V, Terenius L, et al. Dysregulation of dynorphins in Alzheimer disease. *Neurobiol Aging* 2007;**28**:1700–8.

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Chapter 219

Proenkephalin-Derived Peptides

Patricia J. McLaughlin

ABSTRACT

Proenkephalin-derived opioid peptides include the active pentapeptides [Met^5]-enkephalin and [Leu^5]-enkephalin. These peptides are processed from the preproenkephalin A gene and are distributed widely throughout the central nervous system and other nonneural systems of the body. Although these opioid peptides were initially reported to bind to classical μ and δ opioid receptors, and to function as neuromodulators, new receptors and new roles have been discovered that markedly expand their importance in maintaining mammalian homeostasis. One opioid peptide, [Met^5]-enkephalin, also termed opioid growth factor (OGF), is a potent regulator of cell proliferation, acting through a tonically active inhibitory axis, and is dependent on opioid growth factor receptor (OGFr), a novel nuclear-associated receptor. OGF uses the p16 and/or p21 cyclin-dependent inhibitory kinase pathway, and requires clathrin-mediated endocytosis for its inhibitory activity. Recently, enkephalins have been implicated in the pathogenesis of diseases including complications of diabetes, autoimmune diseases (e.g., multiple sclerosis, Crohn's), and cancer.

DISCOVERY OF THE ENKEPHALINS

The discovery of endogenous opioid peptides in 1975 by Hughes et al.[9] followed the identification of native opioid receptors in the brain and gastrointestinal tract that occurred in 1973 in three different laboratories.[19,22,25] With the new approach of using radioactive ligand binding, brain extracts containing opioid-like activity were isolated and two enkephalins, [Met^5]-enkephalin and [Leu^5]-enkephalin, were identified that mimicked morphine in a manner that was blocked by the opioid antagonist naloxone.[24] [Met^5]-enkephalin has the amino acid structure of Tyr-Gly-Gly-Phe-Met (i.e., YGGFM), whereas [Leu^5]-enkephalin differs in the fifth amino acid position by having a leucine molecule in place of methionine (i.e., YGGFL).

In addition to the pentapeptides, there are many larger peptide fragments that include all or some of the enkephalin sequence. Some of these larger peptides (e.g., octapeptide) have functions similar to that of the pentapeptides, whereas others do not resemble the neurotransmitter functions of methionine or leucine enkephalin. Numerous derivatives have been synthesized that deleted an amino acid, added a chemical modification, and/or reoriented the amino acids to alter their function.[24] However, to date, the pentapeptides remain the most active byproducts of preproenkephalin. The tyrosine and glycine residues have potential to function in many other nonenkephalinergic functions suggesting that the original enkephalins are members of, and play a role in, powerful dopaminergic, glycinergic, and enkephalinergic pathways.

STRUCTURE OF THE PRECURSOR GENE

The gene for the enkephalin pentapeptides is preproenkephalin A (PPE)[18] from which both [Met^5]-enkephalin and [Leu^5]-enkephalin are derived. Under normal biosynthesis, six copies of [Met^5]-enkephalin are produced and one copy of [Leu^5]-enkephalin. The opioid products from all three genes associated with endogenous opioids (proopiomelanocortin, prodynorphin, and proenkephalin) share an N-terminal core sequence but have different C-terminal amino acids and bind to different opioid receptors.[7] Comparison of the genes for proenkephalin, proopiomelanocortin (POMC), and prodynorphin indicates a highly conserved open reading frame with nearly 90% of the open reading frame in each gene being coded on a single, terminal exonr.[7]

DISTRIBUTION OF ENKEPHALIN mRNA AND PEPTIDE

Autoradiographic studies revealing the distribution of enkephalins were integral in the process of isolating and characterizing them. Initially, their virtual coexistence with opioid receptors was a key factor in their identification. The use of autoradiographic studies to determine the location of receptors provided a good estimate of enkephalin distribution.[22] Once the peptides were isolated and purified, antibodies were produced that facilitated the visualization of enkephalins.[24] Early immunohistochemical studies detected

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00219-0
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enkephalins in axon terminals and cell bodies, and these observations strongly supported the role of the enkephalins as neurotransmitters,[8] as did the presence of enkephalins in small interneurons of the spinal cord. Not until the advent of highly specific antisera for each peptide could a distinct location of [Met^5]-enkephalin be discerned from that for [Leu^5]-enkephalin, and the wide distribution of these peptides be fully appreciated.

Although each pentapeptide has distinct locations wherein there are enriched pools of peptide such as the [Met^5]-enkephalin-enriched hippocampus and thalamus, many neuronal populations share both peptides and support the concept of "redundant" expression, whereby either peptide can function if one or the other is defective. [Met^5]-enkephalin is located in the CNS structures including globus pallidus, hypothalamus, periaquaductal gray area, amygdala, and spinal cord. Because the two pentapeptides differ by only one amino acid that is coded by only one different base pair, it can be conjectured how the two peptides might be colocalized. PPE gene expression has been identified predominately in the posterior pituitary unlike POMC gene expression which is found in the anterior pituitary, and in the spinal cord, and sympathetic nervous system. However, gene expression of preproenkephalin has been detected by Northern blot analysis and/or *in situ* hybridization studies in many other noncentral nervous system structures such as the adrenal medulla, ocular tissue, and gastrointestinal system including pancreas and intestine, cardiovascular system, and the placenta.[15,35] In many cases, the gene expression of PPE may coincide with the presence of the smaller peptides in a variety of nonneural tissues, including neoplasia.

PROCESSING

The PPE gene undergoes transcription, RNA processing, and translation to the preprohormone and prohormone of the same name—preproenkephalin A and proenkephalin, respectively. This prohormone undergoes further proteolysis and peptide cleavage to give rise to a variety of active proteins including [Leu^5]-enkephalin, [Met^5]-enkephalin, Met-enkephalin-Arg-Phe (active heptapeptide), and an octapeptide termed proenkephalin. PPE codes for six copies of [Met^5]-enkephalin and one copy of [Leu^5]-enkephalin, and the two pentapeptides have received the most attention for their widespread distribution and multifunctionality. The primary site for processing of these peptides is within secretory granules of the Golgi apparatus, and typically the cleavage occurs between pairs of basic amino acids such as Arg–Arg, Arg–Lys, and Lys–Lys.

Despite differences in phosphorylation and/or post-translational processing, the enkephalins share the same four initial amino acids—tyrosine, glycine, glycine, and phenyalanine (YGGF)—with the N-terminus signifying distinctions in the resulting peptides. Processing of the prohormones is an ongoing event in order to sustain levels of rapid degradable peptides, which have been shown to biodegrade within minutes. In the case of enkephalins, hydrolysis and cleavage of the tyrosine–glycine bond is an initial catabolic step. Further degradation occurs when amino peptidase or nonspecific enkephalinases carve up the pentapeptides into 2, 3, or 4 amino acids, each of which retain some biological activity and serve as signaling entities or as precursors to inhibitory neurotransmitters.[14] Two aminopeptidases termed NAP and NAP-2 are responsible for the tissue degradation of these peptides. Additionally, aminopeptidases CD10 and CD13 are specific membrane peptidases reportedly in abundance in plasma and responsible for the very short half-lives of enkephalins ranging from 2 to 5 min in circulating blood.[29]

A discussion on the processing of opioid genes would be remiss if gene duplication from an evolutionary viewpoint was not mentioned.[7] The striking similarity in the genes for POMC, prodynorphin, and proenkephalin, and their products suggests that gene duplication leads to the formation of gene families in which there was some divergence in function, as well as duplication of functional end products, throughout phylogenetic development. There are many examples showing that peptides from one or more of the opioid genes (i.e., prodynorphin and proenkephalin genes) serve the same function (e.g., as analgesic molecules) in the same or different region of the central nervous system.

RECEPTORS

Classical Opioid Receptors

Opioid receptors were identified by three independent teams of investigators who, at about the same time, observed specific and saturable binding of radioactively labeled opiates to membrane receptors in the nervous tissue.[19,22,25] Further biochemical studies of the receptors identified the specific opioid binding sites as members of the rhodopsin-like 7 transmembrane G-coupled-signaling molecule.[15,24] Molecular cloning of the three classical receptors revealed homology among all receptors at both the gene and protein level, with 60% amino acid sequence identity.[11] Distinctions in the receptors seem to be a result of small changes in the N-terminal domains. Subsequent studies were able to readily generate knockout animals lacking in a specific mu, delta, or kappa opioid receptor,[11] and this enabled determination of selective, or at least somewhat specific, agonists for each receptor. Enkephalins seem to be more ubiquitous in their binding capability and thus are effective agonists with different affinities for several receptors, each having different functions. Methionine enkephalin binds predominantly to δ (delta) or μ (mu) opioid receptors, of which there are several subsets

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with various terminology including DOR1, DOR2 or δ_1, δ_2 and MOR1, MOR2 or μ_1, μ_2. The coupling of enkephalins to mu and delta opioid receptors confers the physiological cellular response and in this case results in hyperpolarization and subsequent inhibition of neurons, whereas agonist-activated kappa opioid receptors seem to possess both inhibitory and activated signaling pathways. The interaction between endogenous opioid agonists and their receptors activates transduction pathways that result in intracellular events such as calcium concentration changes, increased calcium influx into cells, increases in cGMP levels, and inhibition of phosphotidylinositol production.[24] This cascade of events is similar regardless of the peptide and receptor pairing. Classical opioid receptors for enkephalins are located throughout the body but are found in concentrated regions primarily in the olfactory system, neocortex, dorsal horn of the CNS, primary afferent terminals, and the limbic nuclei. Mu opioid receptors are historically associated with pain mediation and are distributed throughout the central and peripheral nervous system. Delta opioid receptors have also been identified in cardiovascular tissues, cancerous cells, gastrointestinal tract, and a variety of other nonneural related cells and tissues,[24] and thus their activation by enkephalins broadens the role of these peptides and their receptors in other physiological and pathological functions.

Along with the natural endogenous ligands, there are several well-known exogenous agonist ligands including DPDPE ([D-Pen^2, D-Pen^2]enkephalin), DADLE (D-ala^2, D-Leu^5]enkephalin), DSLET ([D-Ser^2, Leu^5]enkephalin), and DTLET ([D-threonine^2, Leu^5]enkephalin), deltorphin I (Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH_2), and deltorphin II (Tyr-D-Ala-Phe-Glu-Val-Val-Gly-NH_2). Antagonists for δ and μ receptors, and thus for blocking the action of enkephalins, include nonspecific antagonists such as naloxone, naltrexone, and BNTX (7-benzylidenealtrexone maleate), and more specific antagonists such as naltrindole, ICI-174,864, norbinaltrophimine (GNTI), and the synthetic peptides CTAP and CTOP. The range of antagonists and agonists that activate opioid receptors continues to grow, supporting their breadth of presence and function in mammalian homeostasis.

Nonclassical Opioid Receptor—Opioid Growth Factor Receptor (OGFr)

In addition to the classical δ and μ receptors serving as binding sites for enkephalins, the opioid growth factor receptor (OGFr), originally termed the zeta (ζ) opioid receptor, has been identified, isolated, characterized, cloned, and sequenced in mouse, rat, and human.[34] OGFr was initially discovered by pharmacological studies with radioactively labeled OGF in binding assays of developing rat brain;[34] specific and saturable binding, with a one-site model of kinetics was identified in the nuclear enriched fraction of postnatal rat brain and cerebellar homogenates. Subcellular fractionation studies showed that the OGFr is an integral membrane protein associated with the nucleus.[34] Antibodies generated to a binding fragment of OGFr facilitated the cloning and sequencing of this receptor in human and rodent.[33,34] Chromosomal identification of OGFr in humans links it to chromosome 20q13.3.[33]

Classical opioid receptors, predominately μ and δ, and the OGFr differ from each other in a variety of structural and molecular characteristics.[33,34] The commonality between them is their hallmark opioid pharmacology. That is, their ability to bind the same endogenous opioid peptide in a specific and saturable manner, and to do so, in a naloxone-reversible manner. Binding to OGFr is dependent on protein concentration, time, temperature, and pH, was sensitive to 100 nM Na^+, Ca^{2+}, and Mg^{2+}, but is not markedly reduced by the addition of guanyl nucleotides such as GppNHp. Optimal binding to the OGFr requires protease inhibitors and pretreatment of the cell homogenates; trypsin markedly reduces [^{3}H]-[Met^5]-enkephalin binding, suggesting that the binding site is proteinaceous in character. Displacement experiments indicate that [Met^5]-enkephalin, but not [Leu^5]-enkephalin, is the most potent displacer of [^{3}H]-[Met^5]-enkephalin, although [Met^5, Arg^6, Gly^7, Leu^8]-enkephalin and [D-Ala^5, D-Leu^6]-enkephalin also have K_is within approximately 3-fold that of [Met^5]-enkephalin in whole-cell homogenate preparations. Drug displacement studies reveal a requirement of an N-terminus tyrosine group. Moreover, binding of radiolabeled [Met^5]-enkephalin displayed stereospecificity, with (−)-naloxone being 18-fold more potent than (+)-naloxone. The genetic profile for OGFr and the subcellular location for this receptor provide distinctions between classical opioid receptors and OGFr. The molecular and protein structures of OGFr have little resemblance to that of any classical opioid receptor and have no significant homologies to known domains or functional motifs with the exception of a bipartite nuclear localization signal. Immunoelectron microscopy and immunocytochemistry investigations, including colocalization studies, have detected OGFr on the outer nuclear envelope where it interfaces with OGF.[32] It is currently postulated that the peptide–receptor complex associates with karyopherin, translocates through the nuclear pore and can be observed in concert with the inner nuclear matrix or at the periphery of nuclear heterochromatin. By immunoelectron microscopy and postembedding technique, double labeling experiments have revealed that complexes of OGF–OGFr colocalize on the outer nuclear envelope, in the paranuclear cytoplasm, perpendicular to the nuclear envelope in a putative nuclear pore complex, and in the nucleus adjacent to heterochromatin.[32] These results support the existence of an additional receptor for the endogenous peptide [Met^5]-enkephalin (i.e., OGF), and also indicate that

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signal transduction for cell proliferation (discussed below) seems to involve an OGF–OGFr complex that interfaces with chromatin in the nucleus.

OGFr Location and Function

Complexes of OGF–OGFr are colocalized on the outer nuclear envelope, in the paranuclear cytoplasm, and have been visualized perpendicular to the nuclear envelope in a putative nuclear pore complex.[32] OGFr is frequently identified associated with karyopherin β. These immunoultrastructural studies suggest that OGFr resides on the outer nuclear envelop where OGF interacts with the receptor rather than on the cytoplasmic membrane as documented for classical opioid receptors. The OGF-OGFr complex translocates between cytoplasm and nucleus through the nuclear localization pore,[32] using one of three nuclear localization signals found in OGFr to facilitate this process. Molecular biology studies taking advantage of siRNA technology to systematically inactivate each nuclear localization signal, and monitor OGFr using OGFr–eGFP transfected cell lines, confirmed this requirement for activity at the OGFr site.

The specific transport factors required for movement of OGF complexed with OGFr from cytoplasm to the nucleus have been identified by siRNA knockdown studies of karyopherin α1, α2, α3, α4, α6, karyopherin β2, and Ran. Regulation of cell proliferation by the OGF–OGFr axis seems to be dependent on nucleocytoplasmic transport by karyopherin β1 as well as the RanGTP/RanGDP gradient across the nuclear envelope. Other adaptor molecules related to karyopherin do not seem to be essential for the passage and function of this enkephalin-based axis.[6]

Molecular Regulation of OGFr

The bioactive OGF–OGFr axis tonically regulates the growth of neoplasia and normal proliferating cells. The repercussions of amplifying OGFr were investigated in several human cancers including ovarian, pancreatic adenocarcinoma and squamous cell carcinoma of the head and neck.[16] pcDNA3.1+ plasmids engineered with OGFr were transfected into cell lines and studied both *in vitro* and *in vivo*. Saturable binding of OGFr was increased 2- to 8-fold in head and neck cancer cell lines overexpressing OGFr relative to wildtype cancer cells. Proliferation rates were reduced up to 68% in these OGFr clonal lines relative to wildtype, with doubling times being nearly two-thirds longer. The addition of exogenous OGF further reduced replication in these neoplasias suggesting that the OGFr receptor is integral to cell division and that genetic modifications of receptor and/or regulation of endogenous opioids, specifically OGF (i.e., methionine enkephalin) may be effective as biotherapies.

BIOLOGICAL ACTIVITY OF ENKEPHALINS

Enkephalins as Neurotransmitters

The role of enkephalins has been widely studied and continues to develop as more specific antagonists and agonists are discovered. In general, enkephalins play a role in neurotransmission and pain modulation.[1] Their role as neurotransmitters was initially determined from localization studies in which immunohistochemical mapping revealed that enkephalins were in close proximity to autoradiographic mapping of opiate receptors.[21,24] The cellular localization of enkephalin suggested that its role was to act on opiate receptors positioned on terminals of sensory pain fibers and inhibit the release of neurotransmitters such as substance P,[17] vasopressin, or dopamine. Along with the inhibitory role of enkephalins to prevent release of transmitters is the modulatory role of enkephalins to alter calcium influx.[17] The direct hyperpolarization of neurons by enkephalins also supports their inclusion in the long list of neurotransmitters. Nonetheless, enkephalins have been implicated in a variety of functions in addition to "classic" neurotransmission.

Enkephalins as Neuromodulators

Total body mapping of enkephalin-processing neurons and their specific locations have been aided by immunohistochemistry.[24] Enkephalin-enriched neuronal regions process inhibitory sensory information; however, enkephalins are also modulators of synaptic activity outside of being neurotransmitters. In regions of the spinal cord (e.g., substantia gelatinosa), pain perception is integrated by enkephalin-enriched fiber tracts. The periaqueductal gray region is rich in enkephalins that resolve analgesia and inhibit the release of excitatory neurotransmitters.[24] High concentrations of enkephalins in the hypothalamus suggest a role for endocrine modulation. Other major enkephalin pathways are associated with motor activity, intestinal tract motility, and peristalsis, limbic system regulation of emotional behavioral, and the hypothalamic neuroendocrine axis.

Studies on enkephalins and endorphins as modulators of pain and addiction are currently an active area of research.[3] Although many of the reward pathways associated with addiction and dependence involve mu or kappa opioid receptors, the distinctions in addictive properties between exogenous agonists such as morphine and endogenous agonists such as enkephalins are still unclear. Nicotine addiction may involve endogenous enkephalins and endorphins acting on mu opioid receptors to inhibit nicotine-rewarding effects, and play a role in the development of physical dependence,[3] whereas prodynorphin peptides participate in nicotine aversive events. Animal studies using mu and kappa knockout mice have reported attenuated self-administration of alcohol in animals without these opioid receptors.[11]

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The functional role of enkephalins has expanded into the physiology of respiration. Krajnik[13] has mapped proenkephalin, and methionine enkephalin, as well as processing enzymes to alveolar macrophages, submucosal glands, and pulmonary neuroendocrine cells of bronchial epithelium. The precise functional roles of the opioid peptides are still under investigation.

Enkephalins as Growth Factors

During the early 1980s, the hypothesis was put forth that endogenous opioid peptides, particularly [Met^5]-enkephalin are involved in growth regulation of normal and abnormal cells and tissues. [Met^5]-enkephalin was renamed OGF to distinguish the function of the peptide as a neuromodulator[1] from that of an inhibitory growth factor.[28,34,35] In support of this hypothesis, many reports have put forth evidence to support the role of OGF in governing the growth of developing, neoplastic, renewing, and healing tissues, with documentation of growth regulation extending from prokaryotes through higher orders of eukaryotes.[2,16,26,29,34,35] OGF is a potent, reversible, species unspecific, tissue nonspecific negative growth regulator with action that is opioid receptor mediated.[34] The peptide is autocrine and paracrine produced, secreted, and effective at concentrations consistent with physiological behavior. OGF is rapid in biologic action and obedient to intrinsic rhythms of the cell (e.g., circadian rhythm).

Our knowledge and understanding of OGF as a growth factor emanates from investigations conducted in the 1980s on normal neural tissue, namely, the developing rat cerebellum and/or brain, and abnormal neural tissues, namely, neuroblastoma cells grown *in vitro* or transplanted into nude mice to produce tumors.[28–31] Nearly all of the characteristics of OGF as an inhibitory growth factor have been reproduced in all three models (i.e., normal brain, cancer cells *in vitro*, and tumors). To briefly summarize, OGF inhibits DNA synthesis in a receptor-mediated (i.e., OGFr) manner. Continuous blockade of the OGF–OGFr interaction results in accelerated proliferation. OGF has been detected immunologically in tissues where the growth effects have been documented, and the specificity of OGF has been studied extensively. In murine neuroblastoma cells (both *in vitro* and tumor transplantation),[34] and in subsequent studies with the developing rat cerebellum,[28,29] [Met^5]-enkephalin (OGF) was discovered to be the most potent opioid peptide associated with growth. In the case of tumor cells, peptide concentrations as low as 10^{-10} M inhibit the growth of log-phase S20Y neuroblastoma cells exposed to drug for 48 hr. Other PPE peptide derivatives such as the heptapeptide ([Met^5, Arg^6, Phe7]-enkephalin), octapeptide/proenkephalin ([Met^5, Arg^6, Gly^7, Leu^8]-enkephalin), and [Leu^5]-enkephalin also exhibited inhibitory properties but to a much lesser extent. The chemical structure of [Met^5]-enkephalin is also selective and specific. For example, no changes in cell growth occur when the amino acids Tyr^1 or Met^5 are deleted or when smaller fragments of the [Met^5]-enkephalin molecule such as Tyr–Gly or Tyr-Gly-Gly are used. Alterations in the basic structure of [Met^5]-enkephalin by N-terminal modifications (e.g., [Met^5]-enkephalinamide, [Met^5]-enkephalin sulfoxide) yield compounds that are ineffective in growth regulation. Other opioid peptides and analogs related to the prodynorphin and POMC genes (e.g., β-endorphin, α-neo-endorphin, dynorphin B/rimorphin), compounds selective for μ, δ, or κ opioid receptors (e.g., β-FNA, DADLE, EKC), have no significant influence on cultured cells even at concentrations as great as 10^{-6} M. In a wide variety of cells and tissues examined (e.g., colon cancer, heart, squamous cell carcinoma of the head and neck, pancreatic cancer, developing nervous system) in humans and animals, both *in vitro* and *in vivo*, [Met^5]-enkephalin has been identified as the primary opioid peptide involved with growth.

Opioid modulation of cell proliferation in zebra finches[12] confirms earlier studies that opioid antagonist treatment of cultured ventricular zone neurons enriched for enkephalin resulted in increased cell proliferation as measured by 5-bromo-2-deoxyuridine incorporation. *In vivo* studies with administration of methionine enkephalin to the finches directly resulted in decreased neurogenesis and gliogenesis in the ventricular brain regions.

Peptide Location

The subcellular distribution of methionine enkephalin in epithelium reveals that this neuropeptide, and its receptor OGFr, colocalize on the paranuclear cytoplasm and in the nuclei of keratinocytes in the *stratum basale*. Immunoelectron microscopic studies with 5- and 10-nm gold particles show that OGF is not always bound to the OGFr,[32] which in turn is associated with the outer nuclear envelope (see above for more discussion of OGFr transport).

Because of the ubiquitous nature of the presence of OGF in normal and neoplastic cells, human and vertebrate, as well as the quickness of effect observed after addition of OGF, the passage of OGF into cells was studied with OGF labeled with 5,6-tetramethylrhodamine (RhoOGF). In a cell line that is devoid of classical opioid receptors, COS-7 (African green monkey kidney cells), OGF internalization is temperature dependent. Within 15 min of adding RhoOGF to culture, it is detected in the cytoplasm of cells incubated at 37 °C, and within the nucleus after 30 min. No movement is detected at 4 °C. OGF enters cells by active transport with internalization being dependent on clathrin-mediated endocytosis.[5]

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Mechanisms of Action

OGF is a tonic, stereospecific, reversible, noncytotoxic peptide. Studies analyzing the effect of OGF on migration, chemotaxis, invasion, or adhesion of multiple types of cancer cells reveal that at concentrations ranging from 10^{-4} to 10^{-6} M, OGF has no effect on migration or invasion. Moreover, OGF does not change adhesion properties to collage I, collage IV, fibronectin, laminin, or vitronection. Other endogenous opioids and at least one other proenkephalin-derived peptide—leucine enkephalin—evaluated under the same conditions have no effect on migration, invasion, or adhesion.[31]

OGF is not cytotoxic and does not induce apoptosis as studied by Annexin V and TUNEL assays in a variety of neoplasias, nor does it seem to play a major role in differentiation.[30] Neither process outgrowth nor distributions of βIII-tubulin, involucrin, or actin were altered by OGF treatment of neural and nonneural human cancer cell lines.[30]

One target for the mechanism of OGF activity seems to be cell replication. In-depth cell cycle analysis shows that the G_0/S phases of the cell cycle are targeted by the inhibitory effects of OGF.[4] Specifically, OGF down regulation of the retinoblastoma protein by upregulation of the cyclin-dependent inhibitory kinases p16 or p21 leads to the inhibitory activity of the opioid peptide. Different cancers use separate CKI pathways. Squamous cell carcinoma of the head and neck uses the p16 CKI pathway, whereas pancreatic adenocarcinoma uses the p21 pathway. Normal cell lines including human umbilical vein endothelial cells, human epidermal keratinocytes, human dermal fibroblasts, and mesenchymal stem cells are depressed in replication by OGF modulation of the $p16^{INK4a}$ and $p21^{WAF1/CIP1}$ pathways; OGF did not affect p15, p18, p19, or p27 protein expression in either cell type. Exploration of the involved pathways show that OGF decreases the phosphorylation of retinoblastoma protein without decreasing total Rb protein, and reduces Cdk4 or Cdk2, kinase activity, but not total Cdk4 or Cdk2.[4] Upstream of these kinases, OGF increases the cyclin-dependent kinase inhibitors p16 or p21 depending on the presence of CKI in cell lines.[4] Inhibition of p16 (INK4a) activation by p16-specific siRNAs blocks the repression action of OGF in head and neck cancer cell lines, whereas this pathway is mutated/deleted in pancreatic adenocarcinoma. The p21 CKI pathway functions to activate OGF repression in pancreatic cancer cells.

PATHOPHYSIOLOGICAL IMPLICATIONS

The basic science and clinical implications of the enkephalins are wide ranging and have enormous potential. The native pentapeptides are not only produced by multiple genes, but they also have divergent functions and different sites of activity (i.e., receptors). Known to be conserved phylogenetically enkephalins play a role in two very necessary processes of animal survival—growth and neurotransmission. As such, enkephalins have a broad reaching impact on maintaining mammalian homeostasis and play a role in reversing disease states as well. OGFr mRNA has been detected to a certain level in all organs of adult rodents. It may be that homeostatic functions in these organs are minimal but that the genetic machinery is retained for cell restoration in the case of injury. The OGF–OGFr axis has been shown to be involved in regulation of neoplasia, wound healing, normal homeostatic functions, embryonic development, angiogenesis, with other areas of activity possibly not yet discovered. Mediation of enkephalin activity and harnessing the inhibitory action of enkephalins for therapeutic treatment and/or diagnostics has great clinical potential.

Role in Immunobiology and Autoimmune Diseases

OGF represses the incidence and magnitude of a mouse model of multiple sclerosis, myelin oligodendrocytic glycoprotein (MOG)-induced autoimmune encephalomyelitis (EAE). Daily injections of OGF (10 mg/kg, i.p.) initiated at the time of disease induction delay appearance of EAE behavior and attenuated behavioral signs for 60 days.[20] Neuropathology related to EAE including activated astrocytes, neuronal damage, and demyelination is significantly reduced in OGF treated mice at 10, 30, and 60 days after the induction of disease.[20]

Based on these findings, the role of methionine enkephalin on T and B lymphocyte proliferation was evaluated in culture. Lymphocytes were isolated from the spleens of normal adult male mice, dissociated and seeded into culture. Cells were stimulated with lipopolysaccharide to activate B cells or with phytohemagglutin to activate T cells. Both OGF peptide and receptor were detected on T and B lymphocytes by immunohistochemistry, and OGF treatment (10^{-6} M) markedly depressed the number of stimulated T and B cells.[27] The inhibitory action of OGF is mediated by the OGFr axis as siRNA knockdown of the mu, delta, and kappa opioid receptors does not alter the repressive effects of OGF on T and B cell number. The function of OGF on T and B cell activities *in vivo* in cells isolated from normal mice, as well as mice with autoimmune diseases such as multiple sclerosis and Crohn's disease, is currently being studied. Whether the role of OGF in autoimmunity is strictly related to proliferation, or also involves other modulatory activity as described more than two decades ago,[1] is unclear.

CLINICAL STUDIES WITH OGF

With the widespread, off-label use of low dosages of naltrexone (i.e., LDN), shown to upregulate enkephalins and

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opioid receptors, the importance of enkephalins for therapeutic treatment of both autoimmune diseases, as well as cancer, has taken a more prominent place in biology. In particular, exogenously administered OGF has been shown to lack toxicity when given intravenously to pancreatic patients. In this Phase I trial, the end-stage patients had failed all standard therapy, yet some remarkable observations were made regarding improved life style and survival time supporting further study with OGF. Phase II clinical trials with 250 μg/kg of OGF intravenously showed clinical benefit to 53% of the patients in comparison with patients receiving gemcitabine or 5-fluorouracil.[23] OGF-treated patients survived three times longer than untreated patients, and no adverse effects were noted. Clinical trials with OGF alone or in combination with other chemotherapy are in various stages for treatment of hepatocellular carcinoma and squamous cell carcinoma of the head and neck.

Clinical trials with low dosages of naltrexone that upregulate the production of enkephalins and receptors have also shown promise in the treatment of autoimmune diseases such as Crohn's, lupus, and multiple sclerosis. It is evident at these early stages of clinical research that both direct therapy with enkephalins (i.e., OGF), and indirect treatment using LDN, have shown positive outcomes for these low-cost opioid peptide-based biotherapeutics.

REFERENCES

1. Akil H, Watson SJ, Young E, Lewis ME, Katchaturian H, Walter JM. Endogenous opioids: biology and function. *Annu Rev Neurosci* 1984;**7**:223–55.
2. Barg J, Belcheva M, McHale R, Levy R, Vogel Z, Coscia CJ. β-Endorphin is a potent inhibitor of thymidine incorporation into DNA via μ- and κ-opioid receptors in fetal rat brain cell aggregates in culture. *J Neurochem* 1993;**60**:765–7.
3. Berrendero F, Robledo P, Trigo JM, Martin-Garcia E, Maldonado R. Neurobiological mechanisms involved in nicotine dependence and reward: participation of the endogenous opioid system. *Neurosci Biobehav Rev* 2010;**35**:220–31.
4. Cheng F, McLaughlin PJ, Verderame MF, Zagon IS. The OGF–OGFr axis utilizes the $p16^{INK4a}$ and $p21^{WAF1/CIP1}$ pathways to restrict normal cell proliferation. *Mol Biol Cell* 2009;**20**:319–27.
5. Cheng F, McLaughlin PJ, Banks WA, Zagon IS. Internalization of the opioid growth factor, [Met^5]-enkephalin, is dependent on clathrin-mediated endocytosis for downregulation of cell proliferation. *Am J Physiol* 2010;**299**:R774–85.
6. Cheng F, McLaughlin PJ, Zagon IS. Regulation of cell proliferation by the opioid growth factor is dependent on karyopherin β and Ran for nucleocytoplasmic trafficking. *Exp Biol Med* 2010;**235**:1093–101.
7. Danielson PB, Dores RM. Molecular evolution of the opioid/orphanin gene family. *Gen Comp Endocrinol* 1999;**113**:169–86.
8. Hokfelt T, Ljungdahl A, Terenius L, Elde RP, Nilsson G. Immunohistochemical analysis of peptide pathways possibly related to pain and analgesia: enkephalin and substance P. *Proc Natl Acad Sci U S A* 1977;**74**:3081–5.
9. Hughes J, Smith TW, Kosterlitz HW, Fothergill LH, Morgan BA, Morris HR. Identification of two pentapeptides from the brain with potent opiate agonist activity. *Nature* 1975;**258**:577–9.
10. Hui K-S. Brain-specific aminopeptidase: from enkephalinase to protector against neurodegeneration. *Neurochem Res* 2007;**32**: 2062–71.
11. Kieffer BL, Evans CJ. Opioid receptors: from binding sites to visible molecules in vivo. *Neuropharmacology* 2009;**56**:205–12.
12. Khurshid N, Hameed Ls, Mohanasundaram S, Iyengar S. Opioid modulation of cell proliferation in the ventricular zone of adult zebra finches (*Taenopygia guttata*). *FASEB J* 2010;**24**:3681–95.
13. Krajnik M, Scafer M, Sobanski P, Kowalewski J, Bloch-Boguslawska E, Zulics Z, et al. Enkephalin, its precursor, processing enzymes, and receptor as part of a local opioid network throughout the respiratory system of lung cancer patients. *Hum Pathol* 2010;**41**:632–42.
14. Lukiw WJ. Endogenous signaling complexity in neuropeptidase-leucine- and methionine-enkephalin. *Cell Mol Neurobiol* 2006;**26**: 1003–10.
15. Martin R. Opioid antagonists. *Pharmacol Rev* 1967;**19**:462–521.
16. McLaughlin PJ, Verderame MF, Hankins JL, Zagon IS. Overexpression of the opioid growth factor receptor downregulates cell proliferation of human squamous carcinoma cells of the head and neck. *Int J Mol Med* 2007;**17**:421–8.
17. Mudge AW, Leeman SE, Fischbach GD. Enkephalin inhibits release of substance P from sensory neurons in culture and decreases action potential duration. *Proc Natl Acad Sci U S A* 1979;**77**:526–30.
18. Noda M, Furutani H, Takahashi M, Toyosato M, Notake S, Hakanishi S, et al. Isolation and structural organization of the human pre-proenkephalin gene. *Nature* 1982;**297**:431–4.
19. Pert CB, Snyder SH. Opiate receptor demonstration in nervous tissue. *Science* 1973;**179**:1011–4.
20. Rahn KA, McLaughlin PJ, Zagon IS. Prevention and diminished expression of experimental autoimmune encephalomyelitis by low dose naltrexone (LDN) or opioid growth factor (OGF) for an extended period: therapeutic implications for multiple sclerosis. *Brain Res* 2011;**1381**:243–53.
21. Simantov R, Snyder SH. Morphine-like peptides in mammalian brain: isolation, structure elucidation, and interactions with the opiate receptor. *Proc Natl Acad Sci U S A* 1976;**73**:2515–9.
22. Simon EJ, Hiller JM, Edelman I. Stereospecific binding of the potent narcotic analgesic [3H]-etorphine to rat-brain homogenate. *Proc Natl Acad Sci U S A* 1973;**70**:1947–9.
23. Smith JP, Bingaman SI, Mauger DT, Harvey HH, Demers LM, Zagon IS. Opioid growth factor improves clinical benefit and survival in patients with advanced pancreatic cancer. *Open Access J Clin Trials* 2010;**2**:37–48.
24. Snyder SH. Opiate receptors and beyond: 30 years of neural signaling research. *Neuropharmacology* 2004;**47**:274–85.
25. Terenius L. Characteristics of the 'receptor' for narcotic analgesics in synaptic plasma membrane from rat brain. *Acta Pharmacol Toxicol* 1973;**33**:377–84.
26. Villiger PJ, Lotz M. Expression of prepro-enkephalin in human articular chondrocytes is linked to cell proliferation. *EMBO J* 1992;**11**: 135–43.
27. Zagon IS, Donahue RN, Bonneau RH, McLaughlin PJ. T lymphocyte proliferation is suppressed by the opioid growth factor ([Met^5]-enkephalin)-opioid growth factor receptor axis: implication for the treatment of autoimmune diseases. *Immunobiology* 2011;**216**: 579–90.

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28. Zagon IS, McLaughlin PJ. Identification of opioid peptides regulating proliferation of neurons and glia in the developing nervous system. *Brain Res* 1991;**542**:318–23.
29. Zagon IS, McLaughlin PJ. Opioid growth factor in the developing nervous system. In: Zagon IS, McLaughlin PJ, editors. *Receptors in the developing nervous system. Growth factors and hormones*, vol. 1 London: Chapman and Hall; 1993. p. 39–62.
30. Zagon IS, McLaughlin PJ. Opioids and differentiation in human cancer cells. *Neuropeptides* 2005;**39**:495–505.
31. Zagon IS, Rahn KA, McLaughlin PJ. Opioids and migration, chemotaxis, invasion, and adhesion of human cancer cells. *Neuropeptides* 2007;**41**:441–52.
32. Zagon IS, Ruth RB, McLaughlin PJ. Nucleocytoplasmic distribution of opioid growth factor and its receptor in tongue epithelium. *Anat Rec* 2005;**282A**:24–37.
33. Zagon IS, Verderame MF, Allen S, McLaughlin PJ. Cloning, sequencing, chromosomal location, and function of cDNAs encoding an opioid growth factor receptor (OGFr) in humans. *Brain Res* 2000;**856**:75–83.
34. Zagon IS, Verderame MF, McLaughlin PJ. The biology of the opioid growth factor receptor (OGFr). *Brain Res Rev* 2002;**38**:351–76.
35. Zagon IS, Wu Y, McLaughlin PJ. Opioid growth factor and organ development in rat and human embryos. *Brain Res* 1999;**839**: 313–22.

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Chapter 220

Activity-Dependent Neuroprotective Protein (ADNP) and Davunetide (NAP)

I. Gozes and P.W. Baas

ABSTRACT

Activity-dependent neuroprotective protein (ADNP) is essential for mouse brain formation and embryogenesis. At the neuronal level, ADNP is important for neurite outgrowth. ADNP haploinsuffiency in mice results in a neurodegenerative phenotype associated with age-dependent cognitive and social deficits, neurofibrillary tangle formation, and neuronal death. ADNP is a member of a protein family including ADNP2, which is important for cellular survival. The ratio of expression of ADNP and ADNP2 is dysregulated in schizophrenia. ADNP-binding partners include heterochromatin protein alpha1 and members of the chromatin remodeling complex, SWI/SNF—a complex that is important for neurodifferentiation. NAP, a peptide derived from ADNP, partially protects against ADNP deficiency. NAP (subsequently named davunetide) exhibits potent neurotrophic/neuroprotective action in multiple models of neurodegenerative disorders, protecting the microtubule cytoskeleton against breakdown. Davunetide is currently in phase II/III clinical studies in Progressive Supranuclear Palsy, a neurodegenerative disease that is characterized by neurofibrillary tangle formation.

DISCOVERY AND STRUCTURE OF ADNP AND DAVUNETIDE (NAP)

Activity-dependent neuroprotective protein (ADNP) was discovered in the laboratory of Illana Gozes (one of the authors of the current Book Chapter), as part of an ongoing collaboration with the laboratory of Douglas Brenneman.[1] The team originally isolated neuroprotective proteins from conditioned medium of astrocytes exposed to the major neuropeptide vasoactive intestinal peptide (VIP). The working hypothesis was that neuronal VIP activates the brain support cells (in this case astrocytes) to provide neuronal upkeep, enhancing the crosstalk between neurons and glia in the brain.[3] Thus, activity-dependent neurotrophic factor (ADNF) was discovered. In dissecting the ADNF structure, a small peptide, ADNF-9 (SAL) was identified, which provided the smallest epitope within ADNF that exhibits potent neuroprotective characteristics. ADNF antibodies recognizing ADNF-9 were further used to identify similar epitopes in other proteins, allowing the identification and isolation of ADNP by expression cloning from mouse P19 teratocarcinoma cells induced to differentiate into neurons and glial cells.[1] The ADNF-9-like epitope (S**A**LLR**SIP**A) on ADNP was identified as NAP (and later given the generic name, davunetide)—N**A**PV**SIP**Q. The shared amino acids between ADNF-9 and NAP are shown in bold type.

The cloning of the human ADNP (hADNP) gene[33] from a fetal brain cDNA library was followed by bioinformatic analysis and showed 90% identity with the mouse sequence at the mRNA level, including several single nucleotide polymorphic sites. The deduced protein structure contained nine zinc fingers, a proline-rich region, a nuclear bipartite localization signal, and a homeobox domain profile, suggesting a transcription factor function. Further comparative analysis identified an ADNP paralog (33% identity and 46% similarity), indicating that these genes belong to a novel protein family with a nine-zinc finger motif followed by a homeobox domain. However, the ADNP2 protein does not contain the NAP motif. Importantly, within its homeobox profile, ADNP contains a nuclear export signal and an import signal, signifying possible extracellular functions. Further, a potential interaction with cytoplasmic microtubules has been observed,[10] and this interaction provides part of the basis for our proposed mode of action of NAP to positively impact neurons and glial cells of diseased or injured animals and humans.

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00220-7
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THE ADNP GENE

The hADNP gene structure spans approximately 40 kb and includes five exons and four introns with alternative splicing of an untranslated second exon. The hADNP gene was mapped to chromosome 20q12–13.2, a region associated with aggressive tumor growth, frequently amplified in many neoplasias, including breast, bladder, ovarian, pancreatic, and colon cancers.[33] In depth analysis of the ADNP gene chromosome locus shows that it is flanked by type-II-diabetes-linked genes (http://ensembl.rzpd.de/Homo_sapiens/contigview?c_20:48584983;w_50000.5). For example, the ADNP gene is separated by 3438 bp from DPM1 (D20S196; http://www.ncbi.nlm.nih.gov/genome/sts/sts.cgi?uid_57739) that has been linked to type-II diabetes (p _ 0.00010). In this respect, type-II diabetes has been associated with poor cognitive performance and dementia, particularly in elderly patients.[15,32]

Structural comparison of the hADNP gene versus human ADNP2 gene (chromosome 18q23) (NCBI, UCSC, and Gozes published data[33]) revealed five exons for the ADNP gene (with exon 2 that can be alternatively spliced) and four exons for ADNP2. EMBOSS Pairwise Alignment Algorithms in the EMBL-EBI was used showing that exons 1 and 2 in ADNP correlate with exon 1 in ADNP2, exon 3 of ADNP with exon 2 of ADNP2, exon 4 of ADNP with exon 3 of ADNP2, and exon 5 of ADNP with exon 4 of ADNP2. In both cases, the major protein coding domain is on the last exon.[16]

ADNP EXPRESSION

Mouse ADNP RNA is expressed at the time of neural tube closure, detected at E7.5 and increased on E9.5. Expression is augmented in the brain (E12.5), sustained throughout embryogenesis, and regulated by VIP.[25] ADNP2 expression mimicked in part the expression pattern of ADNP. In non-differentiated P19 cells, there is a very close linear correlation between ADNP and ADNP2 mRNA levels ($r = 0.999$, $p = 0.001$). In the neurodifferentiated state, the expression level of both ADNP and ADNP2,[19,16] is increased and a significant correlation is maintained ($r = 0.98$, $p = 0.001$).[8] ADNP RNA expression is enriched in the adult mouse hippocampus and cerebellum[1] in comparison with other tested tissues. In the adult human, enrichment is also seen in the cerebellum, the cortex, skeletal muscle, kidney, and placenta.[33]

Alcohol exposure during embryonic development results in ADNP dysregulation as follows.[26] When timed, pregnant C57Bl6/J mice were treated with alcohol on day 8 of pregnancy, embryonic ADNP mRNA was significantly increased in the ethanol-exposed embryos compared with control embryos, 1 and 10 days after ethanol exposure. The authors explained this increase as a potential compensatory mechanism, given the neuroprotective activity of ADNP. In a study of chronic rather than acute ethanol exposure,[24] female rats received liquid diet (ethanol or control) for a minimum of 40 days before mating with male rats. After mating, the dams were placed in separate cages and kept on ethanol or control liquid diet during the gestation. Primary cortical astrocytes prepared from gestation day 21 fetuses showed a reduction in ADNP mRNA, which was associated with decreased neurotrophic/neuroprotective activity.[24]

Another study suggested that the anesthetic gas xenon that provides tissue protection also regulates ADNP expression in neonatal rat brain. It was further suggested that the differential expression of ADNP in the rat neonatal brain may account for the preconditioning and neuroprotective effects exerted by xenon.[35]

Closed head injury is associated with delayed increases in ADNP mRNA levels one month after the injury. Similarly, closed head injury is associated with a delayed increase in the expression of the immune cell surface glycoprotein Mac-1 (CD11B antigen). By immunocytochemistry, the increase in ADNP was localized to microglia and astrocyte-like cells.[14] The increase in ADNP in injured brains is suggested to be a part of an endogenous compensatory mechanism.

By using kainic acid (KA)-induced epileptic seizures, the regulation of ADNP expression in the hippocampus was suggested to be associated with the nitric oxide (NO)-cGMP pathway. Specifically, inhibitors of NO synthase (NOS) or soluble guanylyl cyclase (sGC) activity reduce ADNP expression in the absence of seizures when measured 30 h after dosing. KA-induced seizures increase neuronal NOS in neurons and inducible NOS in glia cells and suppress ADNP-immunoreactivity in the hippocampus. Under those conditions, repeated treatments with the NOS inhibitor N(G)-nitro-L: -arginine methyl ester (L: -NAME) before KA cause increases in ADNP mRNA expression in granule cells as measured 3 h after the KA treatment.[5] Interestingly, ADNP expression was suggested to exert autocrine regulation.[18]

As brain protection may be dysregulated in schizophrenia, the Gozes laboratory evaluated a possible involvement of ADNP and ADNP2 in the pathophysiology of schizophrenia in humans, measuring relative brain mRNA transcripts of both proteins compared with control subjects. Quantitative real-time polymerase chain reaction in postmortem hippocampal specimens from normal control subjects exhibits a significant ADNP to ADNP2 transcript level correlation ($r = 0.931$, $p < 0.001$). By contrast, in the hippocampus of matched schizophrenia patients, this correlation ($r = 0.637$, $p = 0.014$) is significantly decreased ($p = 0.03$), mirroring disease-associated increased ADNP2 transcripts. Thus, imbalance in ADNP/ADNP2 expression in the brain may influence disease progression in schizophrenia.[8]

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ADNP INTERACTING PROTEINS

Bioinformatics search identified a conserved potential heterochromatin protein1α (HP1 α) binding motif PxVxL on human, rat and mouse ADNP.[18] Nuclear proteins extracted from P19 cells using Pierce NE-PER nuclear extraction buffer (Pierce, Rockford, IL) supplemented with protease inhibitors cocktail (Sigma St. Louis, MO) were therefore used for ADNP/HP1 α immunoprecipitation using anti-ADNP (Bethyl, Montgomery, TX), HP1 α (Upstate, Lake Placid, NY) or nonspecific rabbit IgG antibodies. Using coimmunoprecipitation on differentiated and nondifferentiated P19 nuclear protein extracts, the Gozes laboratory showed that a low amount of HP1 α-like immunoreactivity is recovered after immunoprecipitation with ADNP antibodies and similarly ADNP-like immunoreactivity is recovered after immunoprecipitation with HP1 α antibodies, suggesting that HP1 α may be a binding partner of ADNP.[18] These original findings were corroborated and extended to show that HP1 recruits ADNP to H3 lysine 9 trimethylation (H3K9me3) marked pericentromeric heterochromatin for silencing of major satellite repeats.[23] In short, H3K9me3 is a histone posttranslational modification (PTM), a hallmark of pericentromeric heterochromatin. Mammalian cells contain three HP1 proteins, HP1 α, HP1 β, and HP1 γ all binding to H3K9me3 and mediating this histone PTM. The interaction of ADNP with H3K9me3 is mediated by all three HP1 isoforms *in vitro*, whereas, in cells, ADNP localization to areas of pericentromeric heterochromatin is only dependent on HP1 α and HP1 β. ADNP silencing is associated with major satellite repeats, thus regulating transcription.[23]

To further discover ADNP interacting proteins, the HEK293 human embryonic kidney cell line that allows efficient transfection with recombinant DNA was used. By using coimmunoprecipitation assays, the essential SWI/SNF (mating type switching/sucrose nonfermenting) chromatin remodeling complex is associated with ADNP. In short, in-gel protein digests and mass spectrometry identified BRG1, BAF250a, and BAF170 of the SWI/SNF chromatin remodeling complex, as proteins that coimmunoprecipitate with ADNP. This complex has been shown before to interact with HP1 α, connecting the HP1 results with the SWI/SNF interaction.[17]

ADNP BIOLOGICAL ACTIONS

The SWI/SNF complex was previously shown to be associated with neurodifferentiation. Using ADNP short hairpin RNA downregulation, the Gozes laboratory has shown microtubule reorganization and changes in cell morphology including reduction in cell process formation and cell number, using the pluripotent teratocarcinoma cell line P19 as a neuroglia differentiating model. An approximately 80% decrease in ADNP led to a substantial reduction in embryoid body formation and a reduction (~50%) in neurite numbers. These results directly associate ADNP with neuronal cell differentiation and maturation.[19] As indicated above, these morphological changes are closely associated with the SWI/SNF complex multifunctionality. Taking ADNP from the cellular scenario to the whole animal, complete knockout of ADNP results in embryonic lethality at the time of neural tube formation. Detailed analysis reveals cranial neural tube closure failure and death on E8.5–9.0 of the ADNP-knockout embryos. Oct4 expression, associated with germ-line maintenance, is markedly increased in the knockout embryos, whereas Pax6, crucial for cerebral cortex formation, is not detected in the brain primordial tissue of the ADNP-knockout embryos.[25] To further elucidate ADNP associated pathways, Affymetrix 22,690-oligonucleotide-based microarrays were used on ADNP-knockout and control mouse embryos (E9) separated completely from extra embryonic tissue.[18] More than 400 genes show significant differences in expression profiles between ADNP-deficient embryos and ADNP-expressing embryos. In detail: a group of dramatically upregulated gene transcripts in the ADNP-deficient embryos are clustered into a family encoding for proteins enriched in the visceral endoderm such as apolipoproteins, cathepsins and methallotioninins. A down-regulated gene cluster, associated with ADNP deficiency in the developing embryo, consisted of organogenesis markers including neurogenesis (Ngfr, neurogenin1, neurod1) and development of the heart (Myl2). Together, the results demonstrate the pivotal developmental role of ADNP.

Original studies have also associated ADNP with cancer growth and inhibition of ADNP expression in the cancer cells is related to reduction in cancer growth.[33]

In mouse astrocytes, partial loss in ADNP expression (ADNP haploinsufficiency) is associated with reduced capacity of the astrocytes to provide the required neuronal support.[32] Similar results were obtained with astrocytes from prenatal ethanol-exposed fetuses.[24] In the haplo insuffient mouse (ADNP+/−), the animals show cognitive deficits and age-dependent increase in tau pathology, hyperphosphorylation, and tangle-like formation emanating from astrocytes. This is coupled to age-related increase in brain cell death.[32] These findings of tau pathology are usually associated with decreased microtubule function, which are believed to be the basis of the ADNP-derived peptide—NAP (davunetide) protective activity, which protects partly against ADNP deficiencies (see below).

THE ADNP-DERIVED NEUROPROTECTIVE PEPTIDE NAP: BIOLOGICAL ACTIONS AND MICROTUBULE CONNECTION

As indicated above, NAP was discovered as a collaborative work of the laboratories of Illana Gozes and Douglas Brenneman.[1] Originally, NAP was shown to provide a

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broad range of neuroprotection.[1] It was then shown to interact with microtubules,[7] with increased interaction with the neuron-specific beta III tubulin isotype.[6] NAP activity at substoichiometric levels suggested that it could not conceivably substitute for any known microtubule-related protein or act in a fashion similar to any known microtubule-active drug.[1,6,7] NAP also shows neurotrophic activities enhancing neurite outgrowth *in vitro*[12] and protecting synaptic structures *in vivo*.[15] It further provides protection to astrocytes both *in vitro*[7] and *in vivo*.[15]

Here, we speculate a novel mode of action, wherein NAP subtly alters the microtubule's lattice so that it interacts with microtubule-related proteins differently than it otherwise would. For example, when tau is depleted from neurons, microtubules become abnormally sensitive to the microtubule-severing protein katanin, but this abnormal sensitivity to katanin is ameliorated by treatment with NAP.[31] Theoretically, this could extend to an array of other proteins relevant to the integrity of the microtubule such as proteins that promote depolymerization (such as stathmin), stabilization (such as tau or MAP6), and degradation (such as proteases). In addition, such changes in the microtubule lattice could alter the manner by which the microtubule interacts with molecular motor proteins relevant to axonal transport.

As NAP is associated with highly potent *in vitro* activity, it is plausible that NAP modulates kinase activities leading to a cascade of events that modulate the microtubule lattice. Evidence for such a potential cascade comes from the following observations. NAP potentiates axonal outgrowth in cerebellar granule neurons by activating the sequential tyrosine phosphorylation of Fyn kinase and the scaffold protein Crk-associated substrate (Cas). Pharmacological inhibition of Fyn kinase or expression of a Fyn kinase siRNA abolishes NAP-mediated axonal outgrowth. Concentrations of ethanol that are considered to be readily attained after social drinking (25 mM ethanol) block NAP-mediated axonal outgrowth by inhibiting NAP activation of Fyn kinase and Cas.[4] Interestingly, other studies have shown interactions of Fyn kinase with tau (information freely available on the web: jcs.biologists.org/content/111/21/3167/full.pdf). These findings tie in with other *in vitro* studies associating NAP activity with the Akt and ERK (externally regulated kinase) pathways as Fyn kinase is associated with these pathways, protecting against oxidative stress. Thus, cortical neurons co-cultured with astrocytes obtained from prenatal ethanol exposed fetuses[24] have reduced dendrite number and shorter dendritic branching than neuron-control glia co-cultures. In this system, NAP significantly increases dendritic and axonal growth or branching as well as the density of the synaptic structures as stained by SNAP-25 and synaptophysin. NAP treatment also stimulates the activity of ERK and Akt in a time-dependent manner. Further, NAP increases cAMP response element-binding protein (CREB) phosphorylation, which is known to be a downstream target of the MAPK/ERK and PI-3K/Akt pathways stimulated by growth factors in neurons. CREB is an important transcription factor, and accumulating results suggest that patterns of transcription regulation represent the molecular signatures of long-term synaptic changes and memory formation, implicating NAP activity in transcriptional regulation processes associated with memory.[24]

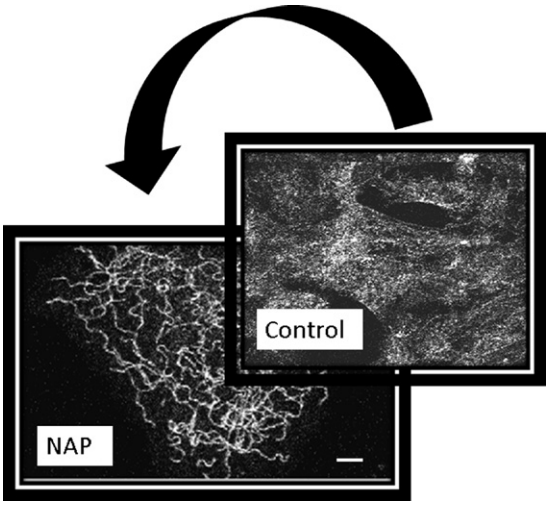

FIGURE 1 NAP and microtubule reorganization.[7] Confocal microscopy. NAP (10^{-15} M) was added to two-week-old rat cortical astrocyte cultures and incubated for 4 h. Control peptide (C2 = VLGGGSALL, 10^{-15} M) was incubated for the same time periods. Tubulin was detected with mouse monoclonal antibodies (as cited above) and secondary rhodamin-labeled goat antimouse IgG (Bars represent 20 μm), an extreme difference is shown.

Fig. 1, based on the original findings from the Gozes laboratory,[7] shows changes in the microtubule lattice in rat astrocytes after 4 h of 10^{-10} M NAP treatment. Interacting proteins, outcomes, and protection against toxicity are the subjects of future research.

CYTOSKELETON PROTECTION, *IN VITRO*

As indicated above, studies at the Gozes laboratory evaluating the mechanism of action of NAP suggested an interaction with tubulin.[7] Several follow-up studies at the same laboratory explored the ability of NAP (davunetide) to protect against microtubule disruption using a variety of microtubule poisons. These experiments in cell cultures (neuronal and glial) treated with NAP (davunetide) are as follows: [i] NAP (davunetide) treatment protected against zinc-induced toxic aggregation of tubulin.[6,7] [ii] After microtubule depolymerization in the presence of nocodazole, NAP (davunetide) treatment enhances rapid microtubule reassembly and stimulates neurite outgrowth.[11] [iii] NAP (davunetide) provides protection against microtubule

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breakdown associated with oxygen–glucose deprivation.[35] [iv] NAP (davunetide) protects against amyloid-beta-related increases in tau hyperphosphorylation.[29] Studies from the Baas laboratory show that neurons contain sufficient levels of the microtubule-severing protein katanin to completely breakdown the axonal microtubule array if not attenuated by microtubule-associated proteins, chief of which is tau. In neurons depleted of tau, NAP (davunetide) diminishes microtubule loss resulting from katanin activity.[31]

Importantly, in various systems, NAP (davunetide) provides neurotrophic functions promoting neurite outgrowth as originally discovered *in vitro* as a collaboration with Smith-Swintosky, Gozes, Brenneman, D'Andrea, and Plata-Salaman later confirmed and reviewed.[12] Further, these authors suggested that NAP increases synapse formation by increasing synaptophysin expression, as confirmed *in vitro*,[24] leading to *in vivo* studies.

CYTOSKELETON PROTECTION, *IN VIVO*

Several collaborative studies between the Gozes laboratory and the Grigoriadis, the Rosenmann as well as the Aisen-Matusuoka laboratories extend the NAP (davunetide) protection against tau hyperphosphorylation *in vitro*[29] into animal cognitive protection in three *in vivo* transgenic lines including: [i] a model of ADNP haploinsufficiency;[32] [ii] a model of tauopathy (expressing the human double mutant tau protein [P301S;K257T] fused to the tau promotor);[28] and [iii] a triple transgenic Alzheimer's disease model that recapitulates both tau and amyloid-beta pathology.[20] [i] NAP (davunetide) protects against tau hyperphosphorylation and increases performance in the Morris water maze in the model of ADNP haploinsufficiency.[32] [ii] NAP (davunetide) protects against tau hyperphosphorylation and tangle-like formation coupled to increased performance in the water maze, in the mouse model of tauopathy.[28] [iii] NAP protects against tau hyperphosphorylation and increased performance in two cognitive tasks, habituation–dishabituation and the water maze, in the triple transgenic Alzheimer's disease model.[20]

Collaborative studies between the Gozes and the Andrieux laboratories extended NAP (davunetide) to models of deficiencies of microtubule-associated proteins other than tau. Knockout mice for MAP6 (also called STOP—stable tubule only protein) are a model for schizophrenia. Mice exhibiting STOP haploinsufficiency show schizophrenia-like symptoms (hyperactivity) that are ameliorated by chronic treatment with the antipsychotic drug, clozapine. NAP (davunetide) treatment significantly decreases hyperactivity in these STOP-deficient mice and protects visual memory in the object recognition test.[21] Further, microtubule protection is translated to a broad range of neuroprotective activities *in vivo*.

BROAD NEUROPROTECTIVE SCOPE

Together, the above-mentioned reports suggest a broad neuroprotective scope for NAP (davunetide). More recent (2009–2011) publications further extend the scope of NAP (davunetide) neuroprotection as follows: [i] In a rat model of kainic acid toxicity mimicking epilepsy, the Gozes laboratory in collaboration with the Sapolsky laboratory at Stanford showed that NAP (davunetide) treatment protects against kainic-acid-induced hippocampal (CA3) neuronal death.[34] These results extend the neuroprotective scope of NAP (davunetide) and showed parallel regulation of key genes associated with proconvulsant properties and with long-lasting changes that underlie the epileptic state. [ii] In mice overexpressing alpha-synuclein—a model for idiopathic Parkinson disease—intranasal NAP (davunetide) improves motor function and reduced alpha-synuclein inclusions; these *in vivo* studies were performed in collaboration with MF Chesselet, UCLA, and Allon Therapeutics.[9] [iii] In a mouse model of brain lesion mimicking cerebral palsy, NAP (davunetide) potently protects against ibotenate-induced excitotoxic damage in the cortical plate and the white matter of P5 mice as well as against brain lesions of P0 mice.[30] Here, the mechanism of protection involves the PI-3K/Akt pathway in the cortical plate or both PI-3K/Akt and MAPK/MEK1 kinases in the white matter (a collaboration of Pierre Gressen's laboratory and the Gozes laboratory). [iv] NAP (davunetide) neuroprotective effects also occurs in laser-induced retinal lesions in rats. Intravitreal treatment has an early short-term effect, whereas the effect of systemic administration is delayed and prolonged.[2] [v] NAP (davunetide) protects against cognitive decline in a diabetes rat model, coupled with structural protection of the brain, protecting glial cells against apoptosis and against synaptic loss (synaptophysin expression).[15] [vi] Lastly, corroborating work in the literature shows that intranasal NAP (davunetide) administration in conditions of hypobaric hypoxia results in increased expression of Nrf2, the master regulator of antioxidant defense system coupled to improved performance in memory in rats.[27]

GENERAL CONSIDERATION FOR MICROTUBULE PROTECTION

Microtubules are critically important cytoskeletal elements in neurons that act as both architectural struts and railways for organelle transport. A growing body of evidence suggests that microtubules may be vulnerable to loss or distortion in a number of different disease and injury scenarios as also described in our respective publications.[20,28,29,31] This may be the primary reason for axonal degeneration. Traditional microtubule-stabilizing drugs such as taxol provide fortification of the microtubules and in theory could

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be helpful for staving off axonal degeneration resulting from loss of microtubules. Indeed, such drugs show promising indications of enabling functional recovery in animal models of diseased and injured neurons as discussed in our previous publications.[20,21] Although this is good proof-of-principle, prolonged use of taxol is notorious for causing neuropathy, as a portion of the microtubule array must remain dynamic for neurons to remain healthy.[20] The ideal drug would fortify microtubules against loss and also correct microtubule distortions that may have already occurred, while still allowing the microtubules to participate in their normal dynamics. Although such a drug has been elusive, previous studies indicate that NAP (davunetide) may hold promise in this regard.

CLINICAL PROGRESS

Based on these preclinical results, NAP (davunetide) has progressed through a drug development program.[13] Preclinical and clinical assessments indicated that davunetide is safe and well tolerated with brain bioavailability after intranasal or intravenous systemic administration.[22] The protection against microtubule disruption and tauopathy suggests a broad range of potential clinical applications as exemplified in the animal models described above.

NAP (davunetide) is currently in Phase II/III clinical-trials for Progressive Supernuclear Palsy (patients exhibiting microtubule dysfunction in the form of tau pathology or tauopathy), (Allon Therapeutics Inc.; ClinicalTrials.gov Identifier: NCT01110720). NAP (davunetide) has shown positive indications of increasing cognitive scores in patients with mild cognitive impairment (another tauopathy): Schmechel D, Gerard G, Vatakis NG, Harper L, Ross JS, Bari M, et al. A phase 2, double-blind, placebo-controlled study to evaluate the safety, tolerability, and effect on cognitive function of AL-108 after 12 weeks of intranasal administration in subjects with mild cognitive impairment Alzheimer's and Dementia. 2008;4:T483. Activity of davunetide in these trials is supportive of the hypothesis linking microtubule protection to reduced neurodegeneration.

WORKING HYPOTHESIS AND FUTURE PERSPECTIVES

ADNP

At the level of ADNP, an open question is its regulation in tauopathies and the mechanism by which ADNP deficiencies lead to tauopathy. Examination of the genes that ADNP regulates during development, apolipoprotein E is upregulated at least 15-folds on ADNP knockdown.[18] Further, ADNP specifically interacts with the apolipoprotein E gene promoter in neuroglia-differentiated P19 cells.[18] Apolipoprotein E4 gene is a major risk factor gene for Alzheimer's disease with suggested mechanisms of action that influence tauopathy as described also by Daniel Michaelson group, working with apolipoprotein E-deficient mice (original works reviewed in Ref. 1). Thus, findings reviewed here encourage further interest in the evaluation of the involvement of ADNP expression and function in Alzheimer's disease and other tauopathies.

NAP (davunetide)

It is entirely possible that NAP (davunetide) functions in a variety of different ways, some of which fortify microtubules and some of which provide other neuroprotective benefits. However, there seems to be a growing body of evidence supporting the hypothesis that the primary mode of action of NAP is to interact with microtubules and through this interaction, stave off and even reverse neuronal degeneration. Whether this primarily results from a direct interaction between NAP and the microtubule remains to be seen, as the possibility exists that NAP may impact various pathways that impact microtubules. Understanding of the mechanism of action of NAP is not only important for its optimal use in the clinic, but also for devising future strategies that could potentially be even more powerful than NAP at fortifying the microtubule array against loss and/or distortion. Our underlying hypothesis is that NAP interacts with microtubules, resulting in a change in its lattice structure. This could, in turn, cascade into a variety of changes throughout the neuron, given how essential microtubules are to various signaling pathways. Additional influences and cascades of events may include activation of kinase-associated pathways. Future studies elucidating the mechanism of its action will provide for better understanding of how davunetide can be used to treat a variety of diseases and injuries of the nervous system, and also provide insights into how potentially even more potent drugs can be developed to preserve microtubules against loss as well as correct distortions of microtubules caused by such pathologies.

CONFLICT OF INTEREST

Professor Gozes serves as the Scientific Cofounder and a Director at Allon Therapeutics Inc., the company that develops davunetide (NAP) (www.allontherapeutics.com).

ACKNOWLEDGMENTS

The authors thank Drs. Michael Gold, Bruce Morimoto and Alistair Stewart (Allon Therapeutics) as well as Saar Oz (the Gozes laboratory at Tel Aviv University) for excellent input and editorial suggestions.

Support was provided by the AMN Foundation, Canadian Friends of Tel Aviv University—Montreal Circle of Friend, Joe and Grace Alter, Barbara

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and Don Seal, the Oberfield family, the Adams family, Australian Friends of Tel Aviv University, Eva Selwyn and Allon Therapeutics Inc. Professor Illana Gozes is the incumbent of the Lily and Avraham Gildor Chair for the Investigation of Growth Factors, and the Director of the Adams Super Center for Brain Studies, the Levie-Edersheim-Gitter Institute for Functional Brain Imaging and the Dr. Diana and Zelman Elton (Elbaum) Laboratory for Molecular Neuroendocrinology at Tel Aviv University.

REFERENCES

1. Bassan M, Zamostiano R, Davidson A, Pinhasov A, Giladi E, Perl O, et al. Complete sequence of a novel protein containing a femtomolar-activity-dependent neuroprotective peptide. *J Neurochem* 1999;**72**:1283–93.
2. Belokopytov M, Shulman S, Dubinsky G, Gozes I, Belkin M, Rosner M. Ameliorative effect of NAP on laser-induced retinal damage. *Acta Ophthalmol* 2011;**89**:e126–31.
3. Brenneman DE, Gozes I. A femtomolar-acting neuroprotective peptide. *J Clin Invest* 1996;**97**:2299–307.
4. Chen S, Charness ME. Ethanol inhibits neuronal differentiation by disrupting activity-dependent neuroprotective protein signaling. *Proc Natl Acad Sci U S A* 2008;**105**:19962–7.
5. Cosgrave AS, McKay JS, Morris R, Quinn JP, Thippeswamy T. Nitric oxide regulates activity-dependent neuroprotective protein (ADNP) in the dentate gyrus of the rodent model of kainic acid-induced seizure. *J Mol Neurosci* 2009;**39**:9–21.
6. Divinski I, Holtser-Cochav M, Vulih-Schultzman I, Steingart RA, Gozes I. Peptide neuroprotection through specific interaction with brain tubulin. *J Neurochem* 2006;**98**:973–84.
7. Divinski I, Mittelman L, Gozes I. A femtomolar acting octapeptide interacts with tubulin and protects astrocytes against zinc intoxication. *J Biol Chem* 2004;**279**:28531–8.
8. Dresner E, Agam G, Gozes I. Activity-dependent neuroprotective protein (ADNP) expression level is correlated with the expression of the sister protein ADNP2: deregulation in schizophrenia. *Eur Neuropsychopharmacol* 2011;**21**:355–61.
9. Fleming SM, Mulligan CK, Richter F, Mortazavi F, Lemesre V, Frias C, et al. A pilot trial of the microtubule-interacting peptide (NAP) in mice overexpressing alpha-synuclein shows improvement in motor function and reduction of alpha-synuclein inclusions. *Mol Cell Neurosci* 2011;**46**:597–606.
10. Furman S, Steingart RA, Mandel S, Hauser JM, Brenneman DE, Gozes I. Subcellular localization and secretion of activity-dependent neuroprotective protein in astrocytes. *Neuron Glia Biol* 2004;**1**: 193–9.
11. Gozes I, Divinski I. NAP, a neuroprotective drug candidate in clinical trials, stimulates microtubule assembly in the living cell. *Curr Alzheimer Res* 2007;**4**:507–9.
12. Gozes I, Spivak-Pohis I. Neurotrophic effects of the peptide NAP: a novel neuroprotective drug candidate. *Curr Alzheimer Res* 2006;**3**:197–9.
13. Gozes I, Stewart A, Morimoto B, Fox A, Sutherland K, Schmechel D. Addressing Alzheimer's disease tangles: from NAP to AL-108. *Curr Alzheimer Res* 2009;**6**:455–60.
14. Gozes I, Zaltzman R, Hauser J, Brenneman DE, Shohami E, Hill JM. The expression of activity-dependent neuroprotective protein (ADNP) is regulated by brain damage and treatment of mice with the ADNP derived peptide, NAP, reduces the severity of traumatic head injury. *Curr Alzheimer Res* 2005;**2**:149–53.
15. Idan-Feldman A, Schirer Y, Polyzoidou E, Touloumi O, Lagoudaki R, Grigoriadis NC, et al. Davunetide (NAP) as a preventative treatment for central nervous system complications in a diabetes rat model. *Neurobiol Dis* 2011;**44**:327–39.
16. Kushnir M, Dresner E, Mandel S, Gozes I. Silencing of the ADNP-family member, ADNP2, results in changes in cellular viability under oxidative stress. *J Neurochem* 2008;**105**:537–45.
17. Mandel S, Gozes I. Activity-dependent neuroprotective protein constitutes a novel element in the SWI/SNF chromatin remodeling complex. *J Biol Chem* 2007;**282**:34448–56.
18. Mandel S, Rechavi G, Gozes I. Activity-dependent neuroprotective protein (ADNP) differentially interacts with chromatin to regulate genes essential for embryogenesis. *Dev Biol* 2007;**303**:814–24.
19. Mandel S, Spivak-Pohis I, Gozes I. ADNP differential nucleus/cytoplasm localization in neurons suggests multiple roles in neuronal differentiation and maintenance. *J Mol Neurosci* 2008;**35**:127–41.
20. Matsuoka Y, Jouroukhin Y, Gray AJ, Ma L, Hirata-Fukae C, Li HF, et al. A neuronal microtubule-interacting agent, NAPVSIPQ, reduces tau pathology and enhances cognitive function in a mouse model of Alzheimer's disease. *J Pharmacol Exp Ther* 2008;**325**:146–53.
21. Merenlender-Wagner A, Pikman R, Giladi E, Andrieux A, Gozes I. NAP (davunetide) enhances cognitive behavior in the STOP heterozygous mouse—a microtubule-deficient model of schizophrenia. *Peptides* 2010;**31**:1368–73.
22. Morimoto BH, de Lannoy I, Fox AW, Gozes I, Stewart AJ. Davunetide: pharmacokinetics and distribution to brain after intravenous or intranasal administration to rat. *Chim Oggi/Chemistry Today* 2009;**27**:16–20.
23. Mosch K, Franz H, Soeroes S, Singh PB, Fischle W. HP1 recruits activity-dependent neuroprotective protein to H3K9me3 marked pericentromeric heterochromatin for silencing of major satellite repeats. *PLoS One* 2011;**6**:e15894.
24. Pascual M, Guerri C. The peptide NAP promotes neuronal growth and differentiation through extracellular signal-regulated protein kinase and Akt pathways, and protects neurons co-cultured with astrocytes damaged by ethanol. *J Neurochem* 2007;**103**:557–68.
25. Pinhasov A, Mandel S, Torchinsky A, Giladi E, Pittel Z, Goldsweig AM, et al. Activity-dependent neuroprotective protein: a novel gene essential for brain formation. *Brain Res Dev Brain Res* 2003;**144**:83–90.
26. Poggi SH, Goodwin K, Hill JM, Brenneman DE, Tendi E, Schinelli S, et al. The role of activity-dependent neuroprotective protein in a mouse model of fetal alcohol syndrome. *Am J Obstet Gynecol* 2003;**189**:790–3.
27. Sharma NK, Sethy NK, Meena RN, Ilavazhagan G, Das M, Bhargava K. Activity-dependent neuroprotective protein (ADNP)-derived peptide (NAP) ameliorates hypobaric hypoxia induced oxidative stress in rat brain. *Peptides* 2011;**32**:1217–24.
28. Shiryaev N, Jouroukhin Y, Giladi E, Polyzoidou E, Grigoriadis NC, Rosenmann H, et al. NAP protects memory, increases soluble tau and reduces tau hyperphosphorylation in a tauopathy model. *Neurobiol Dis* 2009;**34**:381–8.
29. Shiryaev N, Pickman R, Giladi E, Gozes I. Protection against tauopathy by the drug candidates NAP (Davunetide) and D-SAL: biochemical, cellular and behavioral aspects. *Curr Pharm Design* 2011;**17**:2603–12, Review.
30. Sokolowska P, Passemard S, Mok A, Schwendimann L, Gozes I, Gressens P. Neuroprotective effects of NAP against excitotoxic brain damage in the newborn mice: implications for cerebral palsy. *Neuroscience* 2011;**173**:156–68.

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31. Sudo H, Baas PW. Strategies for diminishing katanin-based loss of microtubules in tauopathic neurodegenerative diseases. *Hum Mol Genet* 2011;**20**:763–78.
32. Vulih-Shultzman I, Pinhasov A, Mandel S, Grigoriadis N, Touloumi O, Pittel Z, et al. Activity-dependent neuroprotective protein snippet NAP reduces tau hyperphosphorylation and enhances learning in a novel transgenic mouse model. *J Pharmacol Exp Ther* 2007;**323**:438–49.
33. Zamostiano R, Pinhasov A, Gelber E, Steingart RA, Seroussi E, Giladi E, et al. Cloning and characterization of the human activity-dependent neuroprotective protein. *J Biol Chem* 2001;**276**:708–14.
34. Zemlyak I, Manley N, Vulih-Shultzman I, Cutler AB, Graber K, Sapolsky RM, et al. The microtubule interacting drug candidate NAP protects against kainic acid toxicity in a rat model of epilepsy. *J Neurochem* 2009;**111**:1252–63.
35. Zemlyak I, Sapolsky R, Gozes I. NAP protects against cytochrome c release: inhibition of the initiation of apoptosis. *Eur J Pharmacol* 2009;**618**:9–14.

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Chapter 221

Erythropoietin

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ABSTRACT

Erythropoietin (Epo), the major hormone that regulates erythropoiesis, is produced mainly in the kidney of adults in response to hypoxia. Recombinant human Epo (rHuEpo) is considered a major treatment for various types of anemias. The past decade has revealed extrahematopoietic sites of Epo production along with abundance of Epo receptors (Epo-Rs) in various tissues and cell lines, suggesting that this hormone may have pleiotropic activities. The unexpected discovery that Epo and its receptor are expressed also in cells of the central nervous system (CNS) has opened new avenues for research regarding the biological effects of Epo in the brain. This chapter reviews the classical well-known features of Epo and the more novel findings with emphasis on its effects in the CNS, and their possible underlying mechanisms.

DISCOVERY AND STRUCTURE OF EPO

The first milestones in the discovery of Epo were the pioneering experiments by Carnot and Deflandre in 1906. They demonstrated that plasma from a donor rabbit that is anemic resulting from hemorrhage, elicited reticulocytosis in the recipient rabbit. The phenomenon was attributed to a "hemopoietin" factor. Its production in the kidney was revealed from the observation that bilaterally nephroctomized rabbits or rats did not respond to hemorrhage with the expected increase in erythropoietic activity. The paucity of Epo necessitated several thousands of liters of human urine to get a pure Epo preparation that was obtained in the late 1970s.[9] Partial amino acid sequences of Epo were thus deduced.

In the mid-1980s, the human Epo gene was cloned, and recombinant DNA technology allowed for the large-scale production of erythropoiesis-stimulating agents (ESAs) that mimic the cytokine's physiological function. The first commercially available rHuEpo, also known as epoetin, was a full-length unmodified Epo polypeptide that has an identical amino acid sequence to that of endogenous Epo and is widely used to correct low hemoglobin levels in anemia.[5]

The crystal structure of human Epo complexed to its receptor was identified in 1998 [Protein Data Bank (PDB) ID code 1EER].[30] Epo consists of a single polypeptide chain backbone that contains four alfa helices (helices A–D) connected by loops. Helices A (residues 8–26) and D (residues 138–161), are held together by a disulfide bridge between Cys 7 and Cys 161. Helices B (residues 55–83) and C (residues 90–112) are linked by a short loop. A second disulfide bond, cysteine 29 to cysteine 33, links the end of the A helix with part of the loop between helices A and B.[30] These disulfide bonds stabilize and preserve the molecular structure of Epo.[15]

THE EPO GENE

The human Epo gene is located on the long arm of chromosome 7 and occupies a 5.4-kb region of genomic DNA. It encodes a 193-amino-acid polypeptide chain. The mature circulating protein consists of 165 amino acids resulting from cleavage of the 27 N terminal amino acid leader sequence and loss of the C terminal arginine during post-translational modification.[15]

The protein sequence of Epo from various mammals displays 80–90% identity to the human Epo.[32] Although the Epo protein in remote species, such as fish, displays only 32% identity to human Epo, the overall structure of the gene, and the hydrophilicity plots of the molecule, display striking similarities to those of human Epo.[6]

Hypoxia and anemia are the main inducers of Epo gene expression. The Epo gene is subjected to induction by hypoxia through a 50-bp hypoxia-inducible enhancer. The hypoxia responsive elements (HRE) are differentially located in the Epo genes in the kidney and the liver. Tissue-specific transcription factors bind to regulatory sequences and control gene transcription.[5]

DISTRIBUTION OF EPO AND EPO mRNA

In the fetus and in neonatal life, the Epo gene is expressed mainly in the liver, whereas after birth, it is expressed in the kidney which then becomes the predominant site of Epo

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00221-9
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production. This differential site of expression between prenatal and postnatal life is regulated at the gene transcription level. In the fetal liver, hepatocytes surrounding central veins contain Epo mRNA. Hepatic Ito cells also seem to be Epo producers. Until recently, Epo-producing kidney cells were not clearly identified. However, the peritubular interstitial cells of the kidney are believed to be the main producers of Epo in postnatal life.[5,15]

Aside from the kidney and the liver, Epo mRNA was detected in nonerythroid compartments, suggesting that this hormone is by far more pleiotropic than initially anticipated. Epo and Epo-R are expressed in the brain and in the cardiovascular, digestive, endocrine, female and male reproductive, and respiratory systems. In the CNS, neurons express Epo-R, and astrocytes produce Epo. Thus, the CNS can be regarded as a paracrine Epo/Epo-R system. In peripheral nerves, Epo-R is expressed only in myelin sheaths (Schwann cells). In the cardiovascular system, Epo and Epo-R are expressed in endothelial cells and in smooth muscle cells. Epo-R is also expressed in cardiac myocytes. With respect to the digestive tract, cells in the gastric mucosa and enterocytes in the fetal and neonatal small intestine of the rat express Epo-R. In the female reproductive tract of humans, Epo is expressed in the cervix, endometrium, ovary, and oviduct. Although Epo production in the kidney, liver, and brain is hypoxia inducible, in the oviduct and endometrium, it is modulated by estrogen and the supply of oxygen. With respect to the male reproductive system, the testes produce both Epo and Epo-R. Sertoli and peritubular myoid cells in particular express Epo mRNA, whereas Leydig cells express only Epo-R. Epo production has also been demonstrated in the epididymis.[15]

Generally, serum concentrations of Epo range between 4 and 27 mU/mL[5] and the terminal half-life ($t_{1/2}$) of Epo is 5 hours.[8] High concentrations of Epo are present also in human milk and milk from other species, such as the rat, suggesting that it plays a role in the erythropoiesis, neurodevelopment, and maturation of the gut of neonates.[15] In most of these organs, the Epo-R is expressed in cells that are in close proximity to the cells expressing the Epo protein, thus pointing to a local paracrine feedback system.[17]

PROCESSING OF EPO

Mature Epo is a glycoprotein of molecular mass 30.4 kDa. It consists of a 165 amino acid (aa) polypeptide core, which is generated by the cleavage of a 27 aa signal peptide and the loss of the C terminal arginine during posttranslational modification from the 193 aa precursor.[10]

The polypeptide moiety of Epo is sufficient for binding to its receptor. The carbohydrate portion, which consists of three N-linked and one O-linked acidic oligosaccharide side chains, comprises about 40% of the Epo molecule. The carbohydrate moiety of Epo is required for secretion and *in vivo* stability of the mature hormone and can protect the molecule from damage caused by oxygen radicals. Minor quantitative differences in the composition of the N and O glycans exist between urinary and serum Epo. The functional significance of these differences is not clear.[10]

A variety of erythropoiesis-stimulating agents (ESAs) including epoetin alfa, epoetin beta, epoetin delta, epoetin omega, and darbepoetin alfa (DPO) are used in the clinic. Epoetin alfa, beta, delta, and omega are four isoforms of rHuEpo. DPO is produced by glycoengineering, containing two additional N-linked carbohydrate chains to improve the stability and pharmacokinetic properties.[4] Although the affinity of DPO for the Epo-R is lower than that of the natural ligand, it has an advantage over other commercially available rHuEpo preparations, because of its threefold higher plasma half-life, and its increased biological potency *in vivo*.[10] These recombinant products are identical to the natural human Epo with respect to their primary and secondary structures; however, they differ slightly in their carbohydrate structure, which facilitates the detection of rHuEpo doping in athletes.[10]

THE EPO RECEPTOR

Epo-R is a type I transmembrane protein with a mass ranging between 59 and 70 kDa, which belongs to the cytokine receptor superfamily. In the bone marrow, Epo-R is expressed in erythroid progenitors and in megakaryocytes, myeloid cells, and endothelial progenitors. Recently, Epo-R was identified in nonerythroid cells in the heart, lung, brain, and the immune system as well as in several cancer types. Although the activity of the Epo-R on the tumor cells is still controversial,[1] its presence in malignant cells raises the concern that activation of Epo-R by treating cancer patients with Epo may lead to their undesirable proliferation.[19]

One of the hallmarks of the Epo-R is its low cell surface expression and its high levels in the endoplasmic reticulum (ER).[34] The numbers of receptor molecules per cell range from 34 to about 3,000.[15] The Epo-R consists of an approximately 230 aa extracellular ligand-binding domain, a single transmembrane segment, and an approximately 230 aa cytosolic domain. Its extracellular domain includes four conserved cysteines that form two disulfide bonds and the WSXWS motif near the membrane-spanning segment. The proximal region of the cytosolic domain contains three conserved segments: box 1, box 2, and the region between boxes 1 and 2.[26]

A single Epo molecule binds to 2 adjacent Epo-Rs on the target cell membrane and begins an intracellular signaling

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cascade. Like other cytokine receptors, including those of growth hormone, prolactin, thrombopoietin, oncostatin M, and several interleukins, Epo-R has no endogenous tyrosine kinase activity. Dimerization of Epo-R activates the receptor-associated tyrosine kinase (Janus Kinase 2, Jak2) by transphosphorylation.[15] The box regions of the cytosolic domain of the receptor are implicated in the association of Epo-R with Jak2.[26] Jak2 then phosphorylates tyrosine residues on the receptor's cytoplasmic domain.[15] This process creates docking sites for SH-2 containing adaptor molecules, which subsequently recruit the transcription factor signal transducer and activator of transcription (STAT), mitogen-activated protein kinase (MAPK), phospholipase-Cγ, phosphatidylinositol 3-kinase (PI3-K), and the protein tyrosine phosphatases SHP1 and SHP2. Numerous other adaptors and intermediates are involved in Epo-R signaling, generating multiple interactions that produce a complex interplay of signaling cascades[26] (Fig. 1).

Beta common receptor (βcR), a shared receptor subunit of interleukin-3 (IL-3), IL-5, and granulocyte-macrophage colony stimulation factor (GM-CSF) receptors, mediate several nonhematopoietic effects of Epo in various types of cells. It is involved in Epo-induced nitric oxide (NO) production and phosphorylation of AKT, Src, and Jak2[29] (Fig. 1).

EPO BIOLOGICAL ACTIONS

Erythropoiesis

The key biological role of Epo in erythropoiesis has been well known for the past 40 years. However, the under-

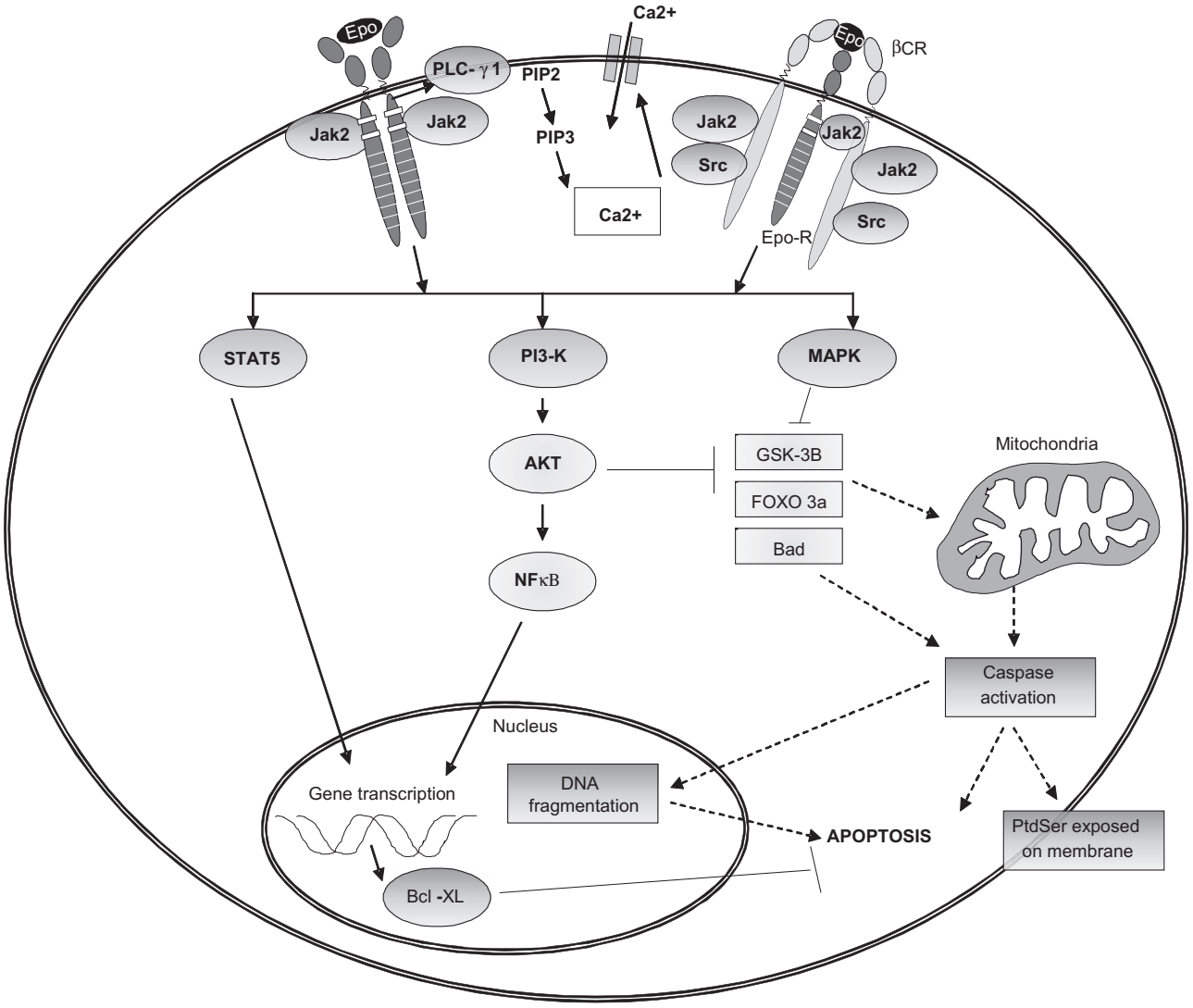

FIGURE 1 Epo-mediated intracellular signal transduction pathways. It is proposed that signaling pathways in the CNS are activated via Epo binding to its cognate Epo-R or to an Epo-R-βcR complex.

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lying mechanisms have been elucidated only in the last decade. Elaborate reviews have been published regarding the role of Epo in erythropoiesis, the signal transduction pathways controlling the process and the interaction with additional cytokines that lead to normal red cell production.[5]

Under hypoxia conditions, Epo production is induced in adult kidney through the activation of HIF and the downregulation of GATA transcription factors.[5] Epo then circulates to the bone marrow, where it binds to Epo-Rs on the surface of erythroid progenitor cells. Its primary target cells in the bone marrow are colony-forming unit erythroid (CFU-E) cells.[15]

Ligand binding to the Epo-R initiates a series of second-messenger complex cascades that ultimately transmit signals to the nucleus. These signals orchestrate the complex regulation of erythroid-specific gene expression to prevent erythroid apoptosis and to support terminal proliferation and differentiation.[15,26] Molecules that are involved in these processes include Jak2, STAT, MAPK, phospholipase-Cγ, PI3-K, AKT1, Ras, and Ca^{2+}.

Epo is the critical growth factor that acts on bone marrow erythroid progenitor cells to prevent them from undergoing apoptosis.[15] Two main signaling pathways activated by Epo-R are responsible for preventing apoptosis: (**1**) the Jak2/STAT5 signal transduction pathway, which activates numerous target genes, including the apoptosis-inhibitor Bcl-XL; a member of the Bcl-2 proteins and required for the survival of erythroid cells at differentiation and maturation stages and (**2**) the PI3-K pathway which activates the protein kinase B (PKB)/AKT1 and subsequently a member of the Forkhead family (FH) of transcription factors FKHRL1, which apparently regulates the generation of Epo-dependent anti-apoptotic signals.[26]

Beyond Erythropoiesis: Epo and Epo-R in the CNS

The finding of additional Epo-producing organs such as the liver, uterus, heart, immune system cells, and brain, has led to the discovery of additional biological functions of Epo, broadly beyond erythropoiesis, as summarized in Table 1.

The unexpected discovery that Epo and its receptor are expressed in the brain has elicited great interest in the scientific and medical communities, because of its potential activity as a neuroprotective agent and its possible clinical relevance in various brain pathologies. In the brain, as in the kidney and liver, hypoxia is a major stimulus of Epo transcription mediated by the HIF-1 pathway. Other factors such as insulin,[16] reactive oxygen species (ROS), injury, metabolic stress,[25] and certain cytokines such as insulin-like growth factor-1 (IGF-1), tumor necrosis factor α (TNFα), IL-1β, and IL-6, may also regulate Epo and Epo-R expression in the brain.[16]

Neurons and astrocytes are the main sites of Epo production in the CNS.[23] Epo and Epo-R mRNA have been identified in various cell types such as mesenchephalic (midbrain), hippocampal and cortical neurons, astrocytes, oligodendrocytes, and brain capillary endothelial cells. Their expression is increased after exposure to hypoxia, leading to increased sensitivity to Epo that may play a role in the activation of the endogenous neuroprotective pathways.[1] Moreover, Epo-R is expressed on the myelin sheath of peripheral nerves.[15] Brain-derived Epo and Epo-R molecules are smaller than their peripheral counterparts, resulting from the differences in sialic acid content.[3] The observation that Epo is produced by astrocytes and subsequently binds to the Epo-R on neighboring neurons,[15] suggests that Epo mediates paracrine and autocrine effects in neurons.

Neurotrophic and neuroprotective effects of Epo were shown *in vivo* in response to neuronal injury caused by hypoxia, ischemia, or brain hemorrhage, as well as by a variety of stressors such as glutamate, NO[23] and glucose deprivation.[1] These occurred when Epo was administered directly into sections of the brain, or systemically by crossing the blood–brain barrier.[23] *In vitro* studies in neural cell lines and primary cultures have shown that Epo stimulates neuronal function and viability by activating calcium channels and release of choline acetyltransferase and of neurotransmitters such as dopamine and acetylcholine.[18] In a mouse model of parkinsonism induced by drug treatment, exogenous Epo injected above the substantia nigra improved locomotor activity and survival of dopaminergic neurons. Adenoviral vector encoding Epo injected into rabbit brain augmented endothelium-dependent relaxation to acetylcholine.[23] The mechanisms by which Epo acts as a neuroprotectant can be summarized as follows:

(1) Decrease in glutamate toxicity

Glutamate toxicity is regarded as the main cause of ischemia-induced neuron death. This effect is accomplished by inducing a rapid, transient increase in intracellular Ca^{2+} concentration in neuronal cells, by raising the Ca^{2+} influx from outside of the cells. Epo blocks this calcium-induced exocytotic glutamate release and could interfere *in vitro* at any step within the well-described cascade of neuronal cell death, induced by exposure to glutamate or glutamate-receptor agonists.[18]

(2) Effects on apopotosis

Epo acts by upregulation of antiapoptotic genes or by the suppression of caspases. This is similar to the case in

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TABLE 1 Summary of the Biological Actions of Epo

Organ/cell type	Epo action
Erythroid progenitors*	Inhibition of apoptosis Support of final stages of erythropoiesis in fetal liver, bone marrow, and spleen
CNS†	
Neurons	***In Vitro:*** Neurotrophic effects Increase in choline acetyltransferase activity in primary cultured neurons Regeneration of cholinergic neurons Enhanced survival and dopaminergic differentiation of neural precursors Neurogenesis after hypoxia Regulation of calcium flux in neural cell lines Elevation of intracellular concentration of monoamines Stimulation of neurotransmitter release Survival promotion by the activation of antiapoptotic genes (Bcl-2, Bcl-XL, XIAP, c-IAP2) and the suppression of caspases Decrease of DNA fragmentation and membrane PtdSer exposure Decrease of glutamate toxicity Decrease of NO-mediated injury ***In Vivo:*** Neuronal survival and recovery Improvement in neuronal functional activity Increased locomotor activity and survival of dopaminergic neurons Neuroprotection
Glial cells	Maturation and differentiation of oligodendrocytes and proliferation of astrocytes Survival Reduction of astrocyte activation Reduction of the release of proinflammatory and neurotoxic factors
CNS vasculature	Decrease in BBB permeability and integrity maintenance Angiogenesis Augmentation of endothelium-dependent relaxation to acetylcholine
Spinal cord	Decreased motor neuron apoptosis and inflammation Improvement in functional recovery Improvement in blood flow to ischemic cells

TABLE 1 Summary of the Biological Actions of Epo—*Cont'd*

Organ/cell type	Epo action
Endothelial cells†	Mitotic and chemotactic effect Survival Angiogenesis and embryonic vasculogenesis Increased NO production under hypoxia conditions
Uterus†	Inhibition of angiogenesis and estrus cycle-dependent endometrial growth
Immune system**	Normalization of CD4:CD8 T cell ratio Increased antibody production in response to antigen Polymorphonuclear cells (PMN) survival Decreased superoxide production in PMN Enhanced LPS-mediated splenocytes proliferation Enhanced survival and function of dendritic cells Stimulation of bone-marrow-derived macrophages and splenic macrophages
Fetal Liver‡	Epo produced by liver is essential for fetal erythropoiesis
Heart†,‡,§	Protection of cardiomyocytes from apoptosis Improved myocardial energy preservation and function Decreased expression of inflammatory cytokines TNFα and IL-6
Vascular smooth muscle	Elevation of cytosolic Ca^{2+} concentration Increased response to norepinephrine, angiotensin II, endothelin-1 Mitogenic effects
Cardiomyocytes	Enhanced proliferation (neonatal) Stimulation of Na^+, K^+ activity
Megakarocytes§	Promotion of megakaryocytes colony formation Maturation
Glucose metabolism and body weight¶	***In Vivo:*** Reduction in blood glucose levels and hemoglobin A1c Attenuation of body weight gain

*Reviewed in Ref.[26].
†Reviewed in Refs.[1,3,15–18,23].
**Reviewed in Refs.[12–14].
‡Reviewed in Ref.[5]
§Reviewed in Ref.[28]
¶Ref.[11]

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erythroid precursor cells where, as mentioned above, Epo activates the expression of antiapoptotic genes such as Bcl-2 and Bcl-XL.[18] In addition, Epo increases the expression of the apoptosis-inhibitor genes *XIAP* and *c-IAP2* in cerebrocortical cells.[1]

(3) Effects on inflammation

Epo acts by reducing inflammatory processes that play a key role in many forms of brain injury. Studies on mice showed that Epo causes reduction of astrocyte activation and the recruitment of leukocytes and microglia into an infarction produced by middle cerebral artery occlusion. In this model, neuronal injury-related inflammation is attenuated by an indirect mechanism involving Epo-mediated reduction of neuronal apoptosis and increased resistance to secondary inflammatory injury.[17] In a mouse model of autoimmune encephalomyelitis, Epo treatment at the onset of paresis significantly improves neurological functional recovery associated with a significant reduction in inflammatory infiltrates and demyelination.[1] Noteworthy, ischemia-induced production of the proinflammatory cytokines TNFα, IL-6, and monocyte chemoattractant protein 1 concentration are reduced after rHuEpo administration.[17,18] In addition, Epo prevents phagocytosis of cells that are tagged by membrane phosphatidylserine exposure induced by anoxia and NO, and can thus precipitate a latent cellular inflammation. These processes are involved in various neurodegenerative diseases such as Alzheimer's disease and other dementias.[16]

(4) Effects on NO production

Epo effects on nitric oxide synthase and NO production may depend on cell type or conditions. In endothelial cell cultures, although short-term exposure to Epo decreases or leaves unchanged endothelial nitric oxide synthase (eNOS) and endothelin-1 expression, the combination of Epo and hypoxia increases Epo-R and eNOS expression as well as NO and cGMP production, showing a direct effect of Epo on eNOS and NO production. The participation of Epo in the regulation of NO production and blood flow may contribute to its neuroprotective role in brain ischemia. However, Epo inhibits the expression of inducible nitric oxide synthase (iNOS) in the hippocampus after bilateral carotid occlusion and in primary oligodendrocyte cultures exposed to inflammatory stimuli.[23]

(5) Effects on angiogenesis

Epo leads to increased angiogenesis, thus enabling additional transport of erythrocytes and thereby increasing the amount of oxygen delivered to the hypoxic brain.[18] Nevertheless, in an *in vitro* model of the bovine blood–brain barrier, Epo protected the blood–brain barrier against vascular endothelial growth factor (VEGF)-induced permeability. In addition to its direct protective effect on neurons, protection of the blood–brain barrier may contribute indirectly to Epo neuroprotection.[23]

The signal transduction pathways by which Epo protects neuronal cells from apoptosis have not yet been fully elucidated; however, it has been shown that the signal transduction pathways leading to CNS cell protection involve Jak2, MAPK, and PI3-K. Phosphorylation of AKT1 through the PI3-K pathways is central to the ability of Epo to prevent several pathways of cell death. It includes those pathways that involve glycogen synthase kinase 3β and forkhead transcription factor FOXO3a.[3] In addition, Bcl-2-associated death promoter (Bad), an AKT downstream substrate, is phosphorylated by activated AKT and thus becomes inactivated. Through AKT activation, Epo also maintains mitochondrial membrane potential preventing the release of cytochrome c and caspase 1, 3, and 8 -like activities. Inhibition of AKT partially reduces Epo neuroprotection, suggesting the participation of additional signaling pathways in Epo neuroprotection. Epo activation of Jak2 also results in the phosphorylation of the inhibitor of NF-κB (IκB), subsequent nuclear translocation of the transcription factor NF-κB, and NF-κB-dependent transcription of neuroprotective genes.[23]

A few years ago, an alternative Epo-R signaling pathway has been postulated. This was based on the generation of Epo derivatives (e.g. carbamylated Epo; CEpo) that bind to the Epo-R and display a tissue-protective effect, but not an erythropoietic one.[3] The premise is that CEpo-mediated tissue protection in the CNS and in myocardial tissue is carried out through a heteroreceptor complex that comprises an Epo-R monomer and a dimer of the common β receptor (βcR) subunit (CD131), which is the signal-transducing element, common to the granulocyte-macrophage colony stimulating factor (GM-CSF), IL-3, and IL-5 receptors. βcR provides increased ligand-binding affinity to the receptor complex.[3]

Epo also plays a role in neural progenitor cell survival and neuronal cell generation in the developing brain. The lack of Epo-R signaling affects murine brain development as early as E10.5, resulting in a reduction of neural progenitor cells and increased apoptosis, and in a marked reduction in the survival of neuronal cell cultures exposed to hypoxia.[1]

Figure 1 illustrates the possible signals mediated by Epo.

PATHOPHYSIOLOGICAL IMPLICATIONS

Abnormal Serum Levels of Epo

Low serum levels of Epo are typical in patients with end-stage renal failure, which is corrected by rHuEpo injections.[5] This treatment results in increased hemoglobin (Hb), hematocrit (Hct) and red blood cell counts, as well as

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reduction in the blood transfusion requirements (BTR), and on the whole an improved quality of life.

Other clinical situations requiring treatment by Epo include anemia associated with chronic disease, as well as perisurgical use in an attempt to reduce BTR in elective surgery.[31] Cancer-related anemia (CRA) has become a major indication for rHuEpo administration.[19] In these patients, the level of serum Epo—albeit higher than normal, is still inadequate, or rather low—relatively to the value predicted because of their anemia.[21] These patients, suffering from symptomatic anemia with relatively low serum Epo levels (in contrast to the true Epo deficiency found in end-stage renal disease) also respond to treatment with rHuEpo.[19]

High serum levels of Epo are commonly detected in patients with chronic hypoxia often associated with chronic lung diseases or other hypoxic situations (heart disease). High serum levels of Epo have also been documented in cases such as renal cell cancer, hepatocellular carcinoma, polycystic kidney disease, and uterine leiomyoma.[20] The high serum Epo levels are associated with polycythemia (erythrocytosis), resulting in the related clinical implications. In the classical hypoxic states such as chronic lung disease, high serum Epo levels are associated with secondary polycythemia (erythrocytosis), resulting in the related clinical implications including blood viscosity leading to thromboembloic complications. Recently, similar complications have been reported with CRA treatment when Hb level was allowed to increase too high.[27]

Exogenous use of rHuEpo injections (doping) has become popular among athletes, especially bike riders,[10] resulting in higher Hb and Hct levels that improve their physical performance. Unfortunately, young athletes are not always aware of the iatrogenic risks of such a treatment.

Epo and Epo-R Variants in Clinical States

Once the Epo and Epo-R genes and proteins have been cloned and characterized, attempts were made to identify related abnormalities that could explain certain clinical disorders. One possibility to be considered is an abnormal protein structure of Epo, but this so far has not been reported. A more complex picture emerges regarding the involvement of Epo-R in disease states. Primary familial and congenital polycythemia (PFCP) is a rare cause of inherited erythrocytosis resulting from mutations in the Epo-R genes. PFCP was first described in a family of Finnish descent, in which erythrocytosis was inherited in an autosomal dominant fashion. Subsequent testing of these kindred showed a heterozygous mutation in Epo-R. To date, 16 additional mutations in Epo-R have been described in PFCP. Some of these mutations result in the truncation of the cytoplasmic domain of the receptor impairing downregulation of signaling via the Epo-R and increasing the sensitivity of erythroid progenitors to Epo.[24] In experimental states, it has been shown that mutations in the exoplasmic domain of the Epo-R lead to constitutive activation of the receptor and tumorigenicity.[35] These were not described as yet, in clinical states in human.

Clinical Uses of rHuEpo—Perspectives

Cloning of the Epo gene has led to the introduction of ESAs into clinical practice in various anemias, including cases that are associated with kidney disease and cancer.[4] Treatment with rHuEpo has increased Hb and Hct, reduced blood transfusion requirements and improved the quality of life in anemic, cancer, and hemodialysis patients. Apart from alleviating anemia, Epo might have additional effects such as antineoplastic,[22] neuroprotective,[23] and immunomodulatory.[14]

Despite that, clinical trials since 2003 have linked rHuEpo therapy to increased risk of tumor progression, thrombovascular events, and mortality and thus prompted implementation of restrictions to their use to minimize potential for harm.[19] The increased risk of tumor progression in cancer patients treated by rHuEpo for their primary or treatment-associated anemia has been attributed to the presence of Epo-R on malignant cells. It should however be noted that several meta-analyses regarding the effect of rHuEpo therapy on cancer cells have not been conclusive.[2,33] A recent report summarizing a long-term surveillance of >7 years, in children with lymphoma and solid tumors treated with rHuEpo, confirmed the role of this hormone treatment in improving their anemia and failed to detect a significant shorter survival.[7]

Further research is needed to devise methods that will enable to identify the cases in which adverse effects of Epo might be predicted, and distinguish them from those where rHuEpo treatment would be beneficial. Until this important issue is clarified, one should adhere to clinical guidelines for the use of Epo, such as the recently published joint ASCO/ASH guidelines.[27]

REFERENCES

1. Arcasoy MO. The non-haematopoietic biological effects of erythropoietin. *Br J Haematol* 2008;**141**:14–31.
2. Bohlius J, Schmidlin K, Brillant C, Schwarzer G, Trelle S, Seidenfeld J, et al. Erythropoietin or Darbepoetin for patients with cancer—meta-analysis based on individual patient data. *Cochrane Database Syst Rev* 2009:CD007303.
3. Brines M, Cerami A. Emerging biological roles for erythropoietin in the nervous system. *Nat Rev Neurosci* 2005;**6**:484–94.
4. Chang ZY, Chiang CH, Lu DW, Yeh MK. Erythropoiesis-stimulating protein delivery in providing erythropoiesis and neuroprotection. *Expert Opin Drug Deliv* 2008;**5**:1313–21.
5. Chateauvieux S, Grigorakaki C, Morceau F, Dicato M, Diederich M. Erythropoietin, erythropoiesis and beyond. *Biochem Pharmacol* 2011;**226**:3330–9.

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6. Chou CF, Tohari S, Brenner S, Venkatesh B. Erythropoietin gene from a teleost fish, *Fugu rubripes*. *Blood* 2004;**104**:1498–503.
7. Durmaz O, Demirkaya M, Sevinir B. Recombinant human erythropoietin beta: the effect of weekly dosing on anemia, quality of life, and long-term outcomes in pediatric cancer patients. *Pediatr Hematol Oncol* 2011;**28**(6):461–8.
8. Eckardt KU, Mollmann M, Neumann R, Brunkhorst R, Burger HU, Lonnemann G, et al. Erythropoietin in polycystic kidneys. *J Clin Invest* 1989;**84**:1160–6.
9. Fisher JW. In: Jelkman W, editor. *From the elusive "hemopoietine" to the anti-anemic drug erythropoietin. Erytheopoietin: molecular biology and clinical use*. Johnson City, TN: FP Graham Publishing Co; 2003. p. 3–2.
10. Jelkmann W. Molecular biology of erythropoietin. *Intern Med* 2004;**43**:649–59.
11. Katz O, Stuible M, Golishevski N, Lifshitz L, Tremblay ML, Gassmann M, et al. Erythropoietin treatment leads to reduced blood glucose levels and body mass: insights from murine models. *J Endocrinol* 2010;**205**:87–95.
12. Kristal B, Shurtz-Swirski R, Shasha SM, Manaster J, Shapiro G, Furmanov M, et al. Interaction between erythropoietin and peripheral polymorphonuclear leukocytes in hemodialysis patients. *Nephron* 1999;**81**:406–13.
13. Lifshitz L, Prutchi-Sagiv S, Avneon M, Gassmann M, Mittelman M, Neumann D. Non-erythroid activities of erythropoietin: functional effects on murine dendritic cells. *Mol Immunol* 2009;**46**:713–21.
14. Lifshitz L, Tabak G, Gassmann M, Mittelman M, Neumann D. Macrophages as novel target cells for erythropoietin. *Haematologica* 2010;**95**:1823–31.
15. Lombardero M, Kovacs K, Scheithauer BW. Erythropoietin: a hormone with multiple functions. *Pathobiology* 2010;**78**:41–53.
16. Maiese K, Li F, Chong ZZ. Erythropoietin in the brain: can the promise to protect be fulfilled? *Trends Pharmacol Sci* 2004;**25**:577–83.
17. Maiese K, Li F, Chong ZZ. New avenues of exploration for erythropoietin. *JAMA* 2005;**293**:90–5.
18. Marti HH. Erythropoietin and the hypoxic brain. *J Exp Biol* 2004; **207**:3233–42.
19. McKinney M, Arcasoy MO. Erythropoietin for oncology supportive care. *Exp Cell Res* 2011;**317**:1246–54.
20. McMullin MF. Idiopathic erythrocytosis: a disappearing entity. *Hematology Am Soc Hematol Educ Program* 2009:629–35.
21. Miller CB, Jones RJ, Piantadosi S, Abeloff MD, Spivak JL. Decreased erythropoietin response in patients with the anemia of cancer. *N Engl J Med* 1990;**322**:1689–92.
22. Mittelman M, Zeidman A, Kanter P, Katz O, Oster H, Rund D, et al. Erythropoietin has an anti-myeloma effect—a hypothesis based on a clinical observation supported by animal studies. *Eur J Haematol* 2004;**72**:155–65.
23. Noguchi CT, Asavaritikrai P, Teng R, Jia Y. Role of erythropoietin in the brain. *Crit Rev Oncol Hematol* 2007;**64**:159–71.
24. O'Rourke K, Fairbairn DJ, Jackson KA, Morris KL, Tey SK, Kennedy GA. A novel mutation of the erythropoietin receptor gene associated with primary familial and congenital polycythaemia. *Int J Hematol* 2011;**93**:542–4.
25. Ostrowski D, Ehrenreich H, Heinrich R. Erythropoietin promotes survival and regeneration of insect neurons in vivo and in vitro. *Neuroscience* 2011;**188**:95–108.
26. Richmond TD, Chohan M, Barber DL. Turning cells red: signal transduction mediated by erythropoietin. *Trends Cell Biol* 2005;**15**: 146–55.
27. Rizzo JD, Brouwers M, Hurley P, Seidenfeld J, Arcasoy MO, Spivak JL, et al. American Society of Hematology/American Society of Clinical Oncology clinical practice guideline update on the use of epoetin and darbepoetin in adult patients with cancer. *Blood* 2010;**116**:4045–59.
28. Smith KJ, Bleyer AJ, Little WC, Sane DC. The cardiovascular effects of erythropoietin. *Cardiovasc Res* 2003;**59**:538–48.
29. Su KH, Shyue SK, Kou YR, Ching LC, Chiang AN, Yu YB, et al. beta common receptor integrates the erythropoietin signaling in activation of endothelial nitric oxide synthase. *J Cell Physiol* 2011. [Epub ahead of print].
30. Syed RS, Reid SW, Li C, Cheetham JC, Aoki KH, Liu B, et al. Efficiency of signalling through cytokine receptors depends critically on receptor orientation. *Nature* 1998;**395**:511–6.
31. Tamir L, Fradin Z, Fridlander M, Ashkenazi U, Zeidman A, Cohen AM, et al. Recombinant human erythropoietin reduces allogeneic blood transfusion requirements in patients undergoing major orthopedic surgery. *Haematologia (Budap)* 2000;**30**:193–201.
32. Wen D, Boissel JP, Tracy TE, Gruninger RH, Mulcahy LS, Czelusniak J, et al. Erythropoietin structure–function relationships: high degree of sequence homology among mammals. *Blood* 1993; **82**:1507–16.
33. Wilson J, Yao GL, Raftery J, Bohlius J, Brunskill S, Sandercock J, et al. A systematic review and economic evaluation of epoetin alpha, epoetin beta and darbepoetin alpha in anaemia associated with cancer, especially that attributable to cancer treatment. *Health Technol Assess* 2007;**11**:1–202:iii-iv.
34. Yoshimura A, D'Andrea AD, Lodish HF. Friend spleen focus-forming virus glycoprotein gp55 interacts with the erythropoietin receptor in the endoplasmic reticulum and affects receptor metabolism. *Proc Natl Acad Sci U S A* 1990;**87**:4139–43.
35. Yoshimura A, Longmore G, Lodish HF. Point mutation in the exoplasmic domain of the erythropoietin receptor resulting in hormone-independent activation and tumorigenicity. *Nature* 1990; **348**:647–9.

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Chapter 222

Insulin-Like Growth Factor-1

Itay Bentov and Haim Werner

ABSTRACT

The insulin-like growth factor-1 (IGF-1) is a pleiotropic growth factor with multiple roles in various aspects of normal and pathological growth and differentiation. The biological actions of IGF-1 are mediated by the IGF-1 receptor (IGF1R), a cell-surface heterotetramer structurally and functionally related to the insulin receptor. The effects of IGF-1 are modulated by a family of binding proteins (IGFBPs) that carry the ligand in the circulation and extracellular fluids. IGF-1 is widely expressed in the central nervous system (CNS) where it promotes proliferation, survival, and differentiation of neuronal and nonneuronal cells. Further, IGF-1 is a potent neurotrophic factor, rescuing neurons from apoptosis and enhancing neuronal growth and myelination. In addition, the IGF-1-signaling pathway has a key role in cancer development and attempts to target this pathway for therapeutic purposes are currently in progress. Understanding the role and regulation of the IGF-1 system will be of significant basic and clinical relevance.

DISCOVERY

The discovery of insulin-like growth factor-1 (IGF-1) is dated to the late 1950s, when the anabolic effects of growth hormone (GH, or somatotropin) in hypophysectomized rats were studied. The model measured incorporation of radioactive sulfate (in the form of chondroitin sulfate and keratin sulfate) into the hyaluronan macromolecule, the protein core of cartilage. Serum from these animals (with low GH levels) failed to stimulate incorporation of sulfate into cartilage. Moreover, the addition of GH to the incubation medium had no effect on sulfate incorporation. By contrast, serum of GH-treated hypophysectomized rats significantly enhanced sulfate uptake. Therefore, it was concluded that incorporation of sulfate was not a direct effect of GH, but rather that GH stimulated the action or production of a secondary, serum-borne factor, which was termed "Sulfation Factor."[27] The list of effects of the sulfation factor on cartilage development expanded in the following decade to include stimulation of thymidine incorporation into DNA, proline into collagen, and uridine into RNA. Furthermore, the global nature of the sulfation factor was established by studies showing that its growth-promoting activities were not limited to cartilage. Subsequently, the term "somatomedin" was introduced, reflecting the fact that the serum factor mediated the effects of somatotropin at somatic target tissues.

During the 1970s, the list of actions of somatomedin further expanded to incorporate many activities that were not restricted to growth, including an insulin-like activity in the diaphragm and the stimulation of glucose uptake in adipose tissue. Further, two closely related peptides with growth-promoting activities were discovered in the serum fraction. Although these peptides displayed a striking homology between their N-terminal domains and the insulin B chain, their insulin-like activities were not suppressed by insulin antibodies.[24] Thus, the factors that were originally termed "sulfation factor," "somatomedins," and "NSILA" (nonsuppressible insulin-like activity) were finally named insulin-like growth factor-1 and insulin-like growth factor-2 (IGF-1 and IGF-2). Although bearing a strong structural and functional resemblance, both peptides represent distinct entities that are the products of two different genes, the IGF-1 gene located on chromosome 12 and the IGF-2 gene on chromosome 11. This chapter will focus on IGF-1.

STRUCTURE OF PRECURSOR mRNA/GENE

The IGF-1 gene, located on chromosome band 12q22-q24, is about 100-kb long and contains six exons. Because of alternative transcription initiation, alternative splicing, and different polyadenylation sequences, numerous IGF-1 mRNAs are produced. These transcripts slightly differ in their coding sequences but mostly diverge in their 5′ and 3′ untranslated regions (UTRs). Exons 1 and 2 are alternative first exons, encoding each one a different 5′-UTR and an alternative N-terminal sequence of the IGF-1 precursor. Extrahepatic production of IGF-1 mRNA is almost exclusively from exon 1, whereas hepatic production, although predominantly from exon 1, can occur also from exon 2.

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00222-0
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The differences between transcripts stem not only from the use of either exon 1 or 2. Both exons contain a cluster of start sites (four start sites in exon 1 and two sites in exon 2), which indicates the existence of a complex control mechanism. Further, the usage of different transcription start sites in the IGF-1 gene is modulated by the nutritional status of the animal as well as by GH levels. Exon 3 encodes part of the signal peptide whereas exon 4 encodes a common N-terminal domain that is similar in humans, rats, and mice. Exons 5 and 6 encode each a different C-terminal product, containing each of them a stop codon. Alternative splicing results in two isoforms of the E peptide moiety of the prohormone—Ea and Eb. In humans, exon 4 is spliced directly either to exon 5 to encode the Eb isoform, or to exon 6 to produce the Ea isoform. The effect of genetic polymorphism within the IGF-1 gene on circulating IGF-1 levels and cancer risk is variable. Genetic variation in the 3′ region of the IGF-1 gene influence circulating levels of IGF-1. Specific IGF-1 single nucleotide polymorphisms (SNPs) account for up to 5% change in circulating IGF-1 levels, but no associations were observed between this polymorphism and breast cancer risk.[22] Common haplotypes in the block covering the 3′ region of the IGF-1 gene (using haplotype tagging assessment of SNPs) showed global association with prostate cancer risk.[16] Interestingly, in a study that examined prostate cancer risk, a single SNP was found to be associated with increased risk without changing IGF-1 circulating levels.[28]

In rodents, IGF-1 mRNA contains either exons 4, 5, and 6, or exon 4 spliced directly to exon 6. Exon 6 contains multiple polyadenylation sites, resulting in molecules containing alternative 3′-UTR domains of several hundred nucleotides in length. IGF-1 mRNA in humans and rats exists in three sizes: 0.8–1.2, 2.2, and 7.5 kb. The large isoform contains a long (~6.5 kb) AU-rich sequence, which results from the use of a downstream polyadenylation site and which renders the 7.5-kb IGF-1 mRNA molecule remarkably unstable.

DISTRIBUTION OF THE mRNA

In rodents, very low IGF-1 levels and high IGF-2 levels are observed during the prenatal period, while postnatal stages are characterized by an increase in circulating IGF-1 values and disappearance of IGF-2. These early observations might have led to an erroneous broad interpretation of the roles of IGF-2 and IGF-1 as fetal and pubertal growth factors, respectively. In humans, however, this expression pattern does not exist, and both ligands are produced from prenatal to postnatal periods. In fact, circulating IGF-2 levels in adults are higher than IGF-1 levels.

Circulating (endocrine) IGF-1 levels are mostly dependent on liver production, which is tightly controlled by GH. In addition to its hepatic production, many extrahepatic tissues also produce IGF-1, which usually exhibits paracrine and autocrine modes of action in these organs. Numerous factors have been shown to control circulating IGF-1 levels. These factors include both direct effectors (e.g., GH, protein calorie intake, thyroxine, catabolic stressors) and indirect factors (e.g., age, body fat, exercise, various hormones). The relative importance of liver-produced (endocrine) IGF-1 *versus* locally produced (paracrine/autocrine) IGF-1, was studied in several mouse models. When the liver IGF-1 gene is knocked-out using the cre–lox recombinase system, circulating IGF-1 levels are reduced by approximately 75%, and GH levels are elevated compared with normal littermates. However, there is almost no phenotypic difference, nor is there a disparity in tissue IGF-1 mRNA production that might have acted as a local compensatory mechanism.[29,35] However, in another mouse model, IGF-1 cDNA was inserted by knock-in into a mutated, inactive IGF-1 locus and was conditionally expressed from cognate promoters exclusively in the liver. In this model, liver-produced IGF-1 contributes about 30% of the adult body size and sustains postnatal development, including reproductive functions.[30] It is generally agreed that liver-derived and locally produced IGF-1 have overlapping growth-promoting effects in the maintenance of normal longitudinal bone growth. However, the paracrine/autocrine IGF-1 cannot replace liver-derived IGF-1 for the regulation of a large number of other parameters including GH secretion, cortical bone mass, progression of some tumors, and other functions.

PROCESSING

The IGFs are approximately 7-kDa peptides, which share a remarkable structural homology with proinsulin. The amino acid sequence of IGF-1 has been elucidated for >10 mammalian species and about the same number of non-mammalian vertebrate species. In most species, including humans, IGF-1 is a 70-amino acid, linear, basic peptide. It consists of B and A domains that are homologous to the B and A domains of insulin. The N-terminal B domain contains 29 residues while the A domain contains 21 residues. By a few substitutions in the A and B domains, it is possible to create an IGF-1 analog that binds to the insulin receptor with an affinity that is similar to that of insulin.[10] In addition, between the A and B domains lies a 12-residue C domain. Unlike insulin, the C domain is not cleaved out, and this may explain the immunologic difference between IGFs and insulin that led to the historical finding of the nonsuppressible insulin-like activity of IGF-1. The IGF's 8-residue D domain, located in the C-terminal end, is not found in insulin. The E domain, located in the C-terminus, is cleaved during secretion in the Golgi apparatus. The sizes of the E domain differ; thus, the IGF-1A protein contains a 35-residue E domain, whereas IGF-1B contains a 77-residue E domain. Because part of the E domain is

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encoded by exon 4 in both proteins, the initial 16 residues are identical.

Although the mammalian IGF-1 is highly conserved, a more significant variability is observed among IGF-1 molecules derived from nonmammalian vertebrates. Conserved amino acids include six cysteines that form three disulfide bonds (two between the A and B domains and one inside the A domain), suggesting that the overall spatial 3-D topography of the molecule is conserved. In addition, three tyrosine residues are conserved (analogous to positions B24, C31, and A60 in the human IGF-1 protein), and it was suggested that these residues are critical in high-affinity binding to the receptor.

RECEPTORS

IGF-1 and IGF-2 activate a common, ubiquitously expressed receptor, the IGF-1 receptor (IGF1R) or Type 1 IGF receptor, which signals mitogenic, antiapoptotic, and transforming activities. The IGF-2/mannose 6-phosphate receptor (IGF-2/M6PR) does not participate in IGF signaling, and its main role is to target IGF-2 for lysosomal degradation. The IGF1R gene is located on the long arm of chromosome 15 (15q25-q26) and it encodes a 1367-amino acid precursor protein that is proteolytically processed to yield mature α and β subunits. The functional cell-surface receptor is composed of two extracellular α-subunits, involved in ligand binding, and two transmembrane β-subunits containing a cytoplasmic tyrosine kinase domain. The IGF1R is coupled to several intracellular second messenger pathways, including the *ras-raf*-MAPK and PI3′K signaling cascades.[18]

IGF1R action is vital for cell survival, as illustrated by the lethal phenotype of mice in which the IGF1R gene was disrupted by homologous recombination. During normal ontogenesis, the IGF1R is expressed at every developmental period, including the oocyte stage. Late embryonic and adult stages, in which the percentage of dividing cells declines, are associated with an overall reduction in IGF1R mRNA levels. The seminal role of the IGF1R in normal and pathological growth has been demonstrated in animal models and in patients with chromosomal abnormalities that disrupt the IGF1R locus and in patients with specific IGF1R gene mutations. In mice, disruption of the IGF1R gene by homologous recombination results in animals weighing 45% of control littermates at the time of birth.[2] These animals exhibit generalized developmental abnormalities, including hypoplasia, abnormal skin formation, delayed bone development, and abnormal central nervous system (CNS) morphology, and they die in the immediate postnatal period from respiratory failure.

Chromosomal aberrations involving the 15q26 locus (e.g., ring chromosome 15) were shown to be associated with hemizygosity for the IGF1R locus and severe growth failure.[23] However, a patient with three copies of the gene resulting from partial duplication of the long arm of chromosome 15, presented with height and weight above the 97th percentile and showed accelerated cellular growth.[21] Combined, these studies underscore the link between IGF1R number and cellular proliferation. Two children with mutations in the IGF1R gene were described in two pediatric cohorts; the first cohort consisted of children with unexplained intrauterine growth retardation and subsequent short stature, and the second group included children with short stature and high circulating IGF-1 levels.[1] A girl from the first group was identified as a compound heterozygote for point mutations in exon 2 of the IGF1R gene. Fibroblasts cultured from the patient had decreased IGF1R binding and phosphorylation. In the second cohort, a boy was identified with a nonsense mutation, leading to reduced IGF1R expression. Additional mutations within the IGF1R gene that are associated with growth retardation and short stature were recently described. Reports included mutations that result in a kinase-deficient IGF1R, nucleotide duplication, which introduces a premature termination codon, and even a mutation causing retention of the nascent IGF1R in the endoplasmic reticulum.

Finally, examination of various tumors (including breast, ovary, prostate, colon, hematopoietic, rhabdomyosarcoma, and kidney) showed an abundant expression of IGF1R mRNA and protein, suggesting that the upregulation of the IGF1R gene constitutes a common paradigm in different types of cancer.

ACTIVE AND/OR SOLUTION CONFORMATION

Most of the IGF-1 peptide in the circulation is found in a ternary complex that includes, in addition to the IGF-1 molecule, a liver-derived glycoprotein (the acid-labile subunit, ALS) and an high-affinity-binding protein, the IGF-binding protein 3 (IGFBP3). The portion of free (active) IGF-1 is usually very low. Six IGFBPs (IGFBP1–6) and many IGFBP-related proteins have been characterized to date. IGFBP3, the predominant IGFBP in serum, is the largest and, therefore, the only one that cannot traverse the capillary membrane. The ternary complex between IGF-1, IGFBP3, and ALS modulates IGF-1 action by protecting the growth factor from proteolysis and prolonging its half live in the circulation. In addition, some IGFBPs exert their biological effects in an IGF-independent manner. In general, the IGFBPs inhibit the metabolic and proliferative actions of IGF, although some IGFBPs display IGF-potentiating effects as well. IGFBP3 in particular is recognized as an inhibitor of proliferation, driving the cell to apoptosis. Several potential mechanisms of action were postulated to explain IGFBP3 inhibition of IGF action, including sequestration of IGF-1 from the receptor and binding competition with the IGF1R.

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BIOLOGICAL ACTIONS

The structural and functional similarities between insulin and IGF-1 suggest that both molecules are derived from a common ancestral precursor that probably participated in food intake and regulation of cellular growth.[25] A divergence of functions most likely occurred before the appearance of the first vertebrates, with insulin mostly active in the regulation of metabolism and IGF-1 in growth processes. In view of their common evolutionary origins and semiconserved architecture, however, there is a certain degree of crosstalk between insulin, IGF-1, and their receptors. Accordingly, insulin exhibits many IGF-1-like activities, including growth stimulation, and IGF-1, on the other hand, exerts certain metabolic effects. In addition, the fact that IGF-1, but not insulin, is carried in the circulation and extracellular fluid by IGFBPs, may further contribute to the divergent actions of both ligands.

At the cellular level, IGF-1 stimulates a mitogenic response and inhibits cell death in a wide variety of cell types, including primary cultures and cancer cell lines. Quiescent cells in G_0 can be induced to enter G_1 by competence factors (e.g., PDGF, bFGF). Once the cell enters into G_1, subphysiological doses of IGF-1 will allow it to evade arrest in G_1 and to progress through the cell cycle. Thus, IGF-1 functions as a progression factor. IGF-1 can also induce differentiation and this activity can be blocked by specific antisense oligonucleotides. IGF-1 exhibits a variety of cellular functions, including regulation of hormone synthesis and secretion, chemoattractant migration, immune cell recognition, and neuromodulation. Metabolic effects of IGF-1 include elevation of glucose uptake and hypoglycemia, without lowering free fatty acid levels. In addition, IGF-1 was suggested to improve renal function by increasing renal blood flow and glomerular filtration rate.

The essential role played by the IGF system in growth and development was demonstrated by the severe growth deficits observed in mice in which various components of the IGF system were disrupted by homologous recombination. Thus, heterozygous mice for a disrupted IGF-1 allele had a body weight at the time of birth that was about 10–20% less than of wild-type animals, whereas null homozygotes weighed about 40% less than did wild type. Further, null homozygotes had very high perinatal mortality rates and many phenotypic aberrations. Interestingly, screening of IGF levels in species with distinct phenotypic features revealed that in different breeds of dogs circulating IGF-1 levels are matched with breed size. As a general rule, smaller breeds have lower IGF-1 levels than larger breeds. Further, many epidemiological observations in human populations highlight the pivotal role of the IGF axis in normal growth. For example, circulating IGF-1 levels in African pygmies are markedly lower than in the general population. Moreover, GH and IGF-2 levels are normal in pygmies, whereas IGF-1 levels in adolescent males and females are significantly lower. As a result of this reduction, the growth spurt associated with puberty is absent.

PATHOPHYSIOLOGICAL IMPLICATIONS

Growth Disorders

The importance of IGF-1 in normal growth is classically illustrated by the Laron Syndrome (LS).[26] LS patients are characterized by mutations in the GH receptor, leading to resistance or insensitivity to GH action and diminished IGF-1 production (despite normal or even high GH levels). LS patients' height is four to ten standard deviations below median normal height, and acceleration of linear growth was demonstrated by continuous, long-term treatment of affected patients with recombinant IGF-1. More recently, a homozygous partial deletion of the IGF-1 gene has been reported in a 15-year-old boy with severe prenatal and postnatal growth deficiency, sensorineural deafness, and mental retardation.[34] An additional component of the IGF system whose mutation was shown to have an impact on growth processes is the ALS subunit of the ternary complex. Mutation of the ALS gene in a 17-year-old boy resulted in undetectable levels of circulating IGF-1 (due to instability of the ternary complex), delayed onset of puberty, and slow pubertal progress.[8] This mutation, however, had only a minimal effect on linear growth. Finally, the effects of mutations in the IGF1R gene were described above.

Cancer

The proliferative and antiapoptotic effects of IGF-1 led to several observational studies that were intended to elucidate the possible connection between circulating IGF-1 levels and an increased risk of neoplasia. Large epidemiological studies have suggested that high circulating IGF-1 levels are associated with an increased risk for breast, prostate, lung, and colorectal cancer. Specifically, in a prospective nested control study (the Nurse's Health Study) the relative risk (RR) of breast cancer in premenopausal women was 4.6 in the upper tertile of IGF-1 values, in comparison to women in the lower tertile.[13] The RR increased to 7.3 when the concentrations of IGFBP3 were included in the analysis. Likewise, the RR of prostate cancer in men (evaluated in the Physician's Health Study) in the upper quartile of IGF-1 values was 2.4 (4.3 when normalized for IGFBP3).[4,5] In addition to hormone-dependent prostate and breast carcinomas, the importance of IGF-1 as a risk factor was evaluated in various nonhormone-dependent types of cancers. Analyses of colon cancer risk in the Nurse's Health Study and the Physician's Health Study showed an increased risk in individuals with the highest IGF-1 values (although a lower RR was observed).[11,12]

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Several mechanisms have been postulated to explain the possible role of the IGF system in the initiation and/or progression of neoplasia.[14] Although IGF-1 was shown to increase chromosomal fragility under experimental conditions, it is usually considered to be nongenotoxic. The proliferative actions of IGF-1, however, have been well characterized. Once a malignant transformation has occurred, cell survival of transformed cells depends on IGF-1 action.[3] The disruption of internal checks and control mechanisms associated with the neoplastic phenotype is further emphasized by the finding that IGF-1 action can override the cellular signals of apoptosis. Several monoclonal antibodies and small-molecule inhibitors that target the IGF1R and its autophosphorylation have been tested in preclinical studies and early-phase clinical studies. Early clinical trials indicate that these drugs are reasonably safe, and there is pharmacodynamic evidence that IGF1R inhibition is possible in patients.[19]

Longevity

Inhibition of the IGF-1 system results in stress resistant, long-lived worms,[17] flies,[32] and mice.[15] Interestingly, in a cohort of Jewish Ashkenazi centenarians, their offspring, and offspring-matched controls, the biochemical, phenotypic, and genetic variations demonstrated a gender-specific increase in serum IGF-1. This change was associated with a smaller stature in female offspring of centenarians. Sequence analysis of the IGF1R gene of female centenarians showed overrepresentation of heterozygous mutations that were associated with high serum IGF-1 levels and reduced activity of the IGF1R as measured in transformed lymphocytes. Thus, altered IGF signaling pathways are suggested to play a role in modulation of human lifespan.[31]

Neuroprotection

IGF-1 is widely expressed in the CNS where it promotes proliferation, survival, and differentiation of neuronal and nonneuronal cells. IGF-1 is a potent trophic factor for motor and sensory neurons as well as for glial cells. Further, circulating IGF-1 is an important determinant of brain function and its effects range from classical neurotrophic actions, such as housekeeping and antiapoptotic/prosurvival effects, to modulation of blood–brain barrier permeability, neuronal excitability, myelination, and new neuron formation.[9] Stimulation of sensory neurons by capsaicin increases tissue levels of IGF-1 and IGF-1 mRNA in various organs.[20] Upregulation of IGF-1 in the CNS was observed 1–7 days after various insults such as hypoxic-ischemic brain injury, brain contusion, and penetrating brain trauma, suggesting a role for IGF-1 in repair processes.[33] In a model of cerebral infarct, IGF-1 treatment reduced ischemia and improved neurological function. Interestingly, administration of IGF-1 in combination with erythropoietin (a neuroprotective agent in itself) resulted in a synergistic neuroprotective effect, whereas blockade of IGF-1 entry into the brain prevented the exercise-induced increase in neurogenesis.[7] The effect of IGF-1 is not limited to brain injury. Central administration of IGF-1 has been shown to result in an antidepressant effect. IGF-regulated mechanism that protects from amyloid beta toxicity (the main constituent of amyloid plaques found in the brains of patients with Alzheimer's disease) is conserved from worms to mammals and suggests that modulation of this signaling pathway can aid in the development of Alzheimer's disease therapy.[6] In conclusion, the potential therapeutic applications of IGF-1 in the treatment of common debilitating neurological conditions is showing great promise and is currently under extensive investigation.

CONCLUSIONS AND FUTURE DIRECTIONS

The IGF-1 axis has an important role in multiple growth and developmental processes. Derangement in IGF-1 action is linked to several pathologies, ranging from growth impairment, malignancies, and CNS anomalies, including Alzheimer's disease. In addition, the IGF-1 axis plays a role in the modulation of lifespan. Of importance, the IGF-1 axis emerged as a promising molecular target for therapeutic purposes. A better understanding of the biochemical and molecular mechanisms responsible for the regulation of the IGF-1 system will have important basic and translational implications, including the possibility of delivering more efficient and rationale therapies in pathologies in which the IGF-1 axis is involved.

REFERENCES

1. Abuzzahab MJ, Schneider A, Goddard A, Grigorescu F, Lautier C, Keller E, et al. Intrauterine Growth Retardation (IUGR) Study Group. IGF-I receptor mutations resulting in intrauterine and postnatal growth retardation. *N Engl J Med* 2003;**349**:2211–22.
2. Baker J, Liu JP, Robertson EJ, Efstratiadis A. Role of insulin-like growth factors in embryonic and postnatal growth. *Cell* 1993;**75**:73–82.
3. Baserga R, Peruzzi F, Reiss K. The IGF-1 receptor in cancer biology. *Int J Cancer* 2003;**107**:873–7.
4. Chan JM, Stampfer MJ, Giovannucci E, Gann PH, Ma J, Wilkinson P, et al. Plasma insulin-like growth factor-I and prostate cancer risk: a prospective study. *Science* 1998;**279**:563–6.
5. Chan JM, Stampfer MJ, Ma J, Gann P, Gaziano JM, Pollak M. Insulin-like growth factor-I (IGF-I) and IGF binding protein-3 as predictors of advanced-stage prostate cancer. *J Natl Cancer Inst* 2002;**94**:1099–106.
6. Cohen E, Paulsson JF, Blinder P, Burstyn-Cohen T, Du D, Estepa G, et al. Reduced IGF-1 signaling delays age-associated proteotoxicity in mice. *Cell* 2009;**139**:1157–69.
7. Digicaylioglu M, Garden G, Timberlake S, Fletcher S, Lipton SA. Acute neuroprotective synergy of erythropoietin and insulin-like growth factor I. *Proc Natl Acad Sci U S A* 2004;**101**:9855–60.

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8. Domene HM, Bengolea SV, Martinez AS, Ropelato G, Pennisi P, Scaglia P, et al. Deficiency of the circulating insulin-like growth factor system associated with inactivation of the acid-labile subunit gene. *New Engl J Med* 2004;**350**:570–7.
9. Dore S, Kar S, Quirion R. Insulin-like growth factor I protects and rescues hippocampal neurons against beta-amyloid- and human amylin-induced toxicity. *Proc Natl Acad Sci U S A* 1997;**94**:4772–7.
10. Gauguin L, Klaproth B, Sajid W, Andersen AS, McNeil KA, Forbes BE, et al. Structural basis for the lower affinity of the insulin-like growth factors for the insulin receptor. *J Biol Chem* 2008;**283**:2604–13.
11. Giovannucci E, Pollak MN, Platz EA, Willett WC, Stampfer MJ, Majeed N, et al. A prospective study of plasma insulin-like growth factor-1 and binding protein-3 and risk of colorectal neoplasia in women. *Cancer Epidemiol Biomarkers Prev* 2000;**9**:345–9.
12. Godowski PJ, Leung DW, Meacham LR, Galgani JP, Hellmiss R, Keret R, et al. Characterization of the human growth hormone receptor gene and demonstration of a partial gene deletion in two patients with Laron-type dwarfism. *Proc Natl Acad Sci U S A* 1989;**86**:8083–7.
13. Hankinson SE, Willett WC, Colditz GA, Hunter DJ, Michaud DS, Deroo B, et al. Circulating concentrations of insulin-like growth factor-I and risk of breast cancer. *The Lancet* 1998;**351**:1393–6.
14. Holly JM, Gunnell DJ, Davey Smith G. Growth hormone, IGF-I and cancer. Less intervention to avoid cancer? More intervention to prevent cancer? *J Endocrinol* 1999;**162**:321–30.
15. Holzenberger M, Dupont J, Ducos B, Leneuve P, Géloën A, Even PC, et al. IGF-1 receptor regulates lifespan and resistance to oxidative stress in mice. *Nature* 2003;**421**:182–7.
16. Johansson M, McKay JD, Stattin P, Canzian F, Boillot C, Wiklund F, et al. Comprehensive evaluation of genetic variation in the IGF1 gene and risk of prostate cancer. *Int J Cancer* 2007;**120**:539–42.
17. Kenyon C, Chang J, Gensch E, Rudner A, Tabtiang R. A *C. elegans* mutant that lives twice as long as wild type. *Nature* 1993;**366**:461–4.
18. LeRoith D, Werner H, Beitner-Johnson D, Roberts Jr CT. Molecular and cellular aspects of the insulin-like growth factor I receptor. *Endocr Rev* 1995;**16**:143–63.
19. López-Calderero I, Sánchez Chávez E, García-Carbonero R. The insulin-like growth factor pathway as a target for cancer therapy. *Clin Transl Oncol* 2010;**12**:326–38.
20. Okajima K, Harada N. Promotion of insulin-like growth factor-I production by sensory neuron stimulation; molecular mechanism(s) and therapeutic implications. *Curr Med Chem* 2008;**15**:3095–112.
21. Okubo Y, Siddle K, Firth H, O'Rahilly S, Wilson LC, Willatt L, et al. Cell proliferation activities on skin fibroblasts from a short child with absence of one copy of the type 1 insulin-like growth factor receptor (IGF1R) gene and a tall child with three copies of the IGF1R gene. *J Clin Endocrinol Metab* 2003;**88**:5981–8.
22. Patel AV, Cheng I, Canzian F, Le Marchand L, Thun MJ, Berg CD, et al. Breast and prostate cancer cohort Consortium. IGF-1, IGFBP-1, and IGFBP-3 polymorphisms predict circulating IGF levels but not breast cancer risk: findings from the breast and prostate cancer cohort Consortium (BPC3). *PLoS One* 2008;**3**(7):e2578.
23. Peoples R, Milatovich A, Francke U. Hemizygosity at the insulin-like growth factor I receptor (IGF1R) locus and growth failure in the ring chromosome 15 syndrome. *Cytogenet Cell Genet* 1995;**70**:228–34.
24. Rinderknecht E, Humbel RE. Polypeptides with nonsuppressible insulin-like and cell-growth promoting activities in human serum: isolation, chemical characterization, and some biological properties of forms I and II. *Proc Natl Acad Sci U S A* 1976;**73**:2365–9.
25. Rinderknecht E, Humbel RE. The amino acid sequence of human insulin-like growth factor I and its structural homology with proinsulin. *J Biol Chem* 1978;**253**:2769–76.
26. Rosenfeld RG. Insulin-like growth factors and the basis of growth. *N Engl J Med* 2003;**349**:2184–6.
27. Salmon Jr WD, Daughaday WH. A hormonally controlled serum factor which stimulates sulfate incorporation by cartilage in vitro. *J Lab Clin Med* 1957;**49**:825–36.
28. Schumacher FR, Cheng I, Freedman ML, Mucci L, Allen NE, Pollak MN, et al. A comprehensive analysis of common IGF1, IGFBP1 and IGFBP3 genetic variation with prospective IGF-I and IGFBP-3 blood levels and prostate cancer risk among Caucasians. *Hum Mol Genet* 2010;**19**:3089–101.
29. Sjögren K, Liu JL, Blad K, Skrtic S, Vidal O, Wallenius V, et al. Liver-derived insulin-like growth factor I (IGF-I) is the principal source of IGF-I in blood but is not required for postnatal body growth in mice. *Proc Natl Acad Sci U S A* 1999;**96**:7088–92.
30. Stratikopoulos E, Szabolcs M, Dragatsis I, Klinakis A, Efstratiadis A. The hormonal action of IGF1 in postnatal mouse growth. *Proc Natl Acad Sci U S A* 2008;**105**:19,378–83.
31. Suh Y, Atzmon G, Cho MO, Hwang D, Liu B, Leahy DJ, et al. Functionally significant insulin-like growth factor I receptor mutations in centenarians. *Proc Natl Acad Sci U S A* 2008;**105**:3438–42.
32. Tatar M, Kopelman A, Epstein D, Tu MP, Yin CM, Garofalo RS. A mutant Drosophila insulin receptor homolog that extends lifespan and impairs neuroendocrine function. *Science* 2001;**292**:107–10.
33. Walter HJ, Berry M, Hill DJ, Logan A. Spatial and temporal changes in the insulin-like growth factor (IGF) axis indicate autocrine/paracrine actions of IGF-I within wounds of the rat brain. *Endocrinology* 1997;**138**:3024–34.
34. Woods KA, Camacho-Hubner C, Savage MO, Clark AJ. Intrauterine growth retardation and postnatal growth failure associated with deletion of the insulin-like growth factor I gene. *New Engl J Med* 1996;**335**:1363–7.
35. Yakar S, Liu JL, Stannard B, Butler A, Accili D, Sauer B, et al. Normal growth and development in the absence of hepatic insulin-like growth factor I. *Proc Natl Acad Sci U S A* 1999;**96**:7324–9.

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Chapter 223

Neuregulins

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ABSTRACT

Neuregulins are pleiotrophic growth factors that influence cell survival, proliferation, differentiation, and organogenesis throughout the body. Their effects are mediated via interactions with the ErbB family of transmembrane receptor protein tyrosine kinases and subsequent activation of downstream intracellular signaling events. This chapter focuses on the expression and emerging neurotrophic, neuroprotective, and neuromodulatory actions of neuregulins in the central nervous system. Potential pathogenic and/or reparative roles for neuregulin/ErbB signaling in several neurological disorders are also discussed.

DISCOVERY AND STRUCTURE

Neuregulins (NRGs) are a family of structurally related signaling proteins that bind to receptor tyrosine kinases of the ErbB family and mediate a myriad of cellular functions including survival, proliferation, and differentiation in both neuronal and non-neural systems. Discovered independently over a decade ago by several different groups, these peptide growth factors were originally described as neu differentiation factor (NDF), heregulins, glial growth factors (GGFs), acetylcholine receptor-inducing activity (ARIA), and sensory and motor neuron-derived factor (SMDF) (see Refs. 12, 13 for reviews). It is now known that all these NRG proteins are encoded by the same gene, named NRG-1. Four genes (termed NRG-1, NRG-2, NRG-3, NRG-4) have been identified that encode NRGs in vertebrates, and the best characterized of these is the NRG-1 gene. The entire human NRG-1 gene has been sequenced.[23]

More than 30 isoforms are generated by multiple promotor usage and alternative splicing of the NRG-1 gene, including the above-mentioned NDFs, heregulins, GGFs, ARIA, and SMDF (see Refs. 3, 12, 13, 18 and original references therein). Multiple splice variants are also produced from each of the NRG-2, NRG-3, and NRG-4 genes. The most important portion of the NRG molecule is the EGF-like domain shared by all the isoforms because this alone is sufficient for receptor binding and the stimulation of intracellular signaling pathways; hence, the EGF domain is both necessary and sufficient for biological activity. This extracellular domain contains about 50 amino acids and is characterized by three pairs of cysteines that are important for its tertiary structure and biological function, and the alternate splice variant at this domain gives rise to α and β isoforms. Six types of NRG-1 (types I, II, III, IV, V, VI) are also divided based on the differences in their extracellular amino-terminal domains: types I, II, IV, and V have an immunoglobulin-like (IgG-like) domain followed, for example, by a glycosylation-rich region (type I) or a GGF-specific (kringle) domain (type II). Type-III contains the typical extracellular EGF domain, but is unique in containing a cysteine-rich domain (CRD) that loops back intracellularly along with its N-terminal sequence. In addition, it seems that all the types of NRG-1 have both transmembrane forms and secreted forms (except Type III NRG-1) depending on whether the isoform is initially synthesized as a transmembrane or nonmembrane protein.[3,13,18]

DISTRIBUTION AND PROCESSING

Neuregulin-1 transcripts are broadly but discretely expressed throughout the nervous system during development as well as in adulthood (see Refs. 2, 3, 12, 13, 18, 19 and original references therein), with the highest levels of expression in brainstem motor and sensory nuclei, spinal cord motor neurons, and sensory ganglia. Neuregulin-1 mRNAs are also found in the cerebral cortex, diencephalon, hippocampus, basal forebrain, substantia nigra, and cerebellum, as well as in the proliferative forebrain subventricular zone. Neuregulin-2 and -3 mRNAs are also expressed in the brain, but exhibit distinct expression patterns from NRG-1, with NRG-2 primarily restricted to the olfactory bulb, cerebellum, and hippocampal dentate gyrus in the adult, whereas NRG-3 expression seems widespread. Within the rodent central nervous system (CNS), intriguing differences between NRG-1 and NRG-2 have been identified.[17] For example, with respect to mRNA expression, the pattern of NRG-2 is essentially distinct from and sometimes complementary to sites mapped for NRG-1 expression. Thus, the products of these two different genes could have

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00223-2
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distinct functions in different brain circuits. The temporal patterns of expression in the brain also differ between the two genes. NRG-1 mRNA expression is relatively high perinatally, followed by downregulation during maturation into adulthood. By contrast, NRG-2 mRNA expression in the brain is the opposite, low prenatally and high in adulthood.[17] It has thus been hypothesized that NRG-1 might function preferentially in early prenatal and perinatal developmental events, whereas NRG-2 may be the preferred NRG ligand in the mature brain.[24] Finally, the subcellular distribution of the two NRGs differ in that NRG-2 is targeted to dendrites whereas NRG-1 is mainly localized to axonal and somatic compartments,[17] suggesting different functional roles for the ligands in the establishment, maintenance, and plasticity of synaptic connections. Taken together, the specific and distinct molecular and biochemical interactions of the two NRG ligands with their receptors, as well as their differential spatial, temporal and subcellular expression patterns in the brain, raise the possibility of differing functions of NRG-1 vs NRG-2 in the CNS. Neuregulin-4 transcripts have not been detected in the CNS. For the most part, immunohistochemical demonstrations of NRG-1 and NRG-2 proteins correspond fairly well with their mRNA localization in both rodent and human brain.

The proteolytic processing of NRG proproteins has been reviewed.[13,18] Briefly, most (but not all) NRGs in the nervous system are initially synthesized as single transmembrane proproteins with an extracellular N-terminal ectodomain (containing the biologically active EGF domain), a juxtamembrane stalk region, the transmembrane domain, and the intracellular cytoplasmic tail. A bioactive ectodomain fragment is generated and released via metalloprotease-mediated cleavage of the proprotein in the stalk region. Other NRGs are produced directly as soluble, secreted proteins (e.g. GGF2). The type-III NRG proproteins have two transmemembrane domains including an N-terminal region that loops back through the membrane resulting in an intracellular N-terminus, in addition to the intracellular C-terminus. Proteolytic cleavage at the stalk region results in an anchored N-terminal fragment (with the bioactive EGF-like region); further juxtamembrane proteolytic processing may release the fragment.[13] Another mode of NRG processing is back-signaling in which, upon NRG typeIII/ErbB binding, the intracellular C-terminal domain is proteolytically released into the cytoplasm where it subsequently travels to the nucleus and modulates gene expression.[18] The ultimate result of these various avenues of NRG isoform processing is that NRGs garner the capacity to signal via autocrine, paracrine, juxtacrine, and possibly intracrine mechanisms.

RECEPTORS

Neuregulins interact directly or indirectly with members of a subfamily of receptor tyrosine kinase proteins called ErbB receptors. The four members of the ErbB family include the epidermal growth factor (EGF) receptor (also known as ErbB1; the human homolog is termed HER1, human EGF receptor 1), ErbB2/Neu/HER2, ErbB3/HER3, and ErbB4/HER4. All family members have in common an extracellular ligand-binding N-terminal domain, a single transmembrane portion, a cytoplasmic tyrosine kinase domain, and a cytoplasmic C-terminal domain containing autophophorylation sites. Upon ligand binding, the receptors form homo- or heterodimers, which consequently stimulate the intrinsic tyrosine kinase activity of the receptors and triggers autophosphorylation of specific tyrosine residues within the cytoplasmic domain.[6,8,18] These phosphorylated residues serve as docking sites for signaling molecules involved in the regulation of intracellular signaling cascades, mainly through stimulation of the Ras/Raf/ERK mitogen-activated protein kinase (MAPK) pathway, the phosphatidylinositol-3 kinase (PI3 K) pathway, the PLCγ/PKC pathway, and the JAK/STAT pathway.[8] An alternative signaling mechanism for ErbB4 upon ligand binding includes presenilin-dependent intramembrane proteolysis by gamma-secretase to release an intracellular ErbB4 C-terminal fragment that translocates to the nucleus to regulate gene expression.[6,18] The ErbB receptors form a complex hierarchical network based on their relative affinity for the ligands, intrinsic tyrosine kinase activities, ability to recruit specific signaling molecules, and cellular distribution.[26] This network is critical to the ErbB signaling pathway because ErbB2, which exhibits the highest degree of kinase activity, has no known direct ligands and ErbB3 is kinase-defective; therefore, neither of them can function by homodimerization. However, by heterodimerizing with other ErbB receptors, they can form potent signaling assemblies (i.e. ErbB2/ErbB3, ErbB3/ErbB4, etc.) and ErbB2 is the most potent partner to any ErbB family member. ErbB4 itself contains moderate kinase activity; therefore, its presence alone is sufficient for biological activity, and it can function via both homodimerization and heterodimerization.

Neuregulins can directly bind to only the ErbB3 and ErbB4 receptors, which can then heterodimerize with ErbB2 (an orphan receptor) and ErbB1 to activate and/or potentiate subsequent signaling activity. All four NRG gene products can bind ErbB4, whereas only NRG-1 and NRG-2 proteins can bind ErbB3.[6,8] ErbB4 is also noteworthy for the fact that multiple isoforms of the receptor may exist based on sequence alterations in the extracellular juxtamembrane (JM) region and in the cytoplasmic (CYT) C-terminal domain. These ErbB4 splice variants differentially activate downstream signaling cascades, resulting in unique functions for each isoform.[18,28] Interestingly, some members of the EGF ligand family, including betacellulin, epiregulin, and heparin-binding EGF-like growth factor (HB-EGF), can bind and activate the ErbB4

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receptor.[6] Thus, not only are there complex interactions among each of the ErbB receptor family members after NRG binding, but the possibility of receptor crossactivation with select EGF family ligands must be taken into consideration.

ErbB receptor mRNAs are differentially expressed throughout the nervous system during development, both spatially and temporally, and are continuously expressed throughout adulthood. ErbB2, ErbB3, and ErbB4 transcripts and protein are broadly expressed throughout all areas of adult rat brain and spinal cord, including cortex, amygdala, hippocampus, medial habenula, reticular thalamic nucleus, substantia nigra pars compacta, ventral tegmental area (VTA), cerebellum, pons, and medulla (see Refs. 2, 3, 6, 12, 18, 19 and original references therein). These three receptors are differentially expressed in neurons and glia. In general, ErbB4 is more prevalent in neurons and ErbB3 in glia in white matter tracts, whereas ErbB2 is present in both neural cell types at lower density. Notably, ErbB4 expression defines a large subset of GABAergic interneurons in forebrain regions[2,19] (see Fig. 1) and dopaminergic neurons in the ventral midbrain,[33–35] suggesting some specific neurotransmitter-related functions for NRG/ErbB4 signaling in the brain. Moreover, the widespread expression of ErbB receptors in the adult brain points to numerous roles for NRG in the mature nervous system.

BIOLOGICAL ACTIONS

First recognized as glial cell mitogens, the NRGs are now believed to play essential roles in cell survival, migration, and differentiation in both neural and non-neural cells. Outside the nervous system, NRGs and their receptors are essential, for example, for proper development and/or function of the heart, mammary gland, skin, lungs, stomach, pancreas, skeletal muscle, and gonads. In terms of dysfunction, NRG/ErbB signaling is associated with many different cancers including those of the breast, lung, stomach, pancreas, kidney, bladder, ovary, prostate, colon, and brain.[6,8,13,22] Targeting of ErbB/HER receptors in human cancers (e.g. anti-HER monoclonal antibodies) has proven to be a useful therapy in some cases.[22] With regard to cardiac disease, initial results from a recent clinical trial indicate that treatment with recombinant human NRG-1 was safe and tolerable and improved cardiac function in patients with chronic heart failure.[15] The remainder of this review will focus on the broad array of effects of NRGs and their receptors in the nervous system, and particularly in the CNS.

The most well-characterized functions of NRGs and ErbB receptors associated with the nervous system include roles in glial and neuronal cell proliferation, migration, maturation, differentiation, and survival, as well as neuromuscular acetylcholine receptor induction, (e.g., see

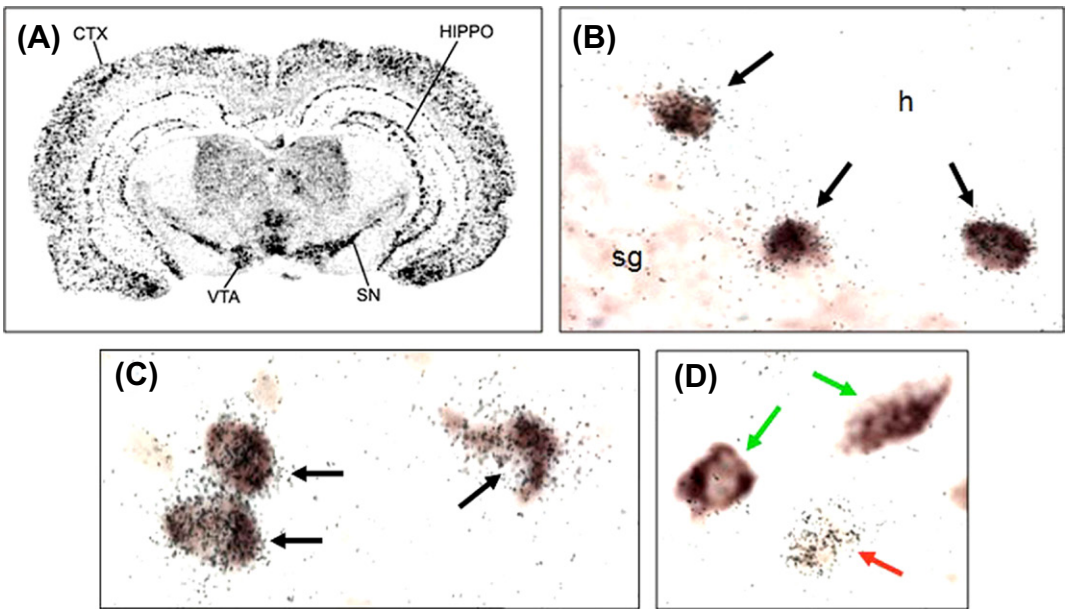

FIGURE 1 (A) Distribution of ErbB4 mRNA-expressing neurons in rat cortex and midbrain as revealed by in situ hybridization. The vast majority of the scattered ErbB4 mRNA-labeled cells in the cerebral cortex (CTX) and hippocampal formation (HIPPO) represent GABAergic interneurons. In the substantia nigra (SN) and ventral tegmental area (VTA) of the midbrain, ErbB4 is expressed by virtually all of the dopaminergic mesotelencephalic neurons. (B,C) Double-labeling in situ hybridization demonstration of the extensive coexistence of ^{35}S-labeled ErbB4 mRNA and digoxygenin-labeled glutamic acid decarboxylase (GAD, the biosynthetic enzyme for GABA) mRNA in neurons of the rat hippocampal dentate gyrus (B) and primary motor cortex (C). Black arrows denote double-labeled cells. (D) Demonstration of the *non-coexistence* of ^{35}S-labeled ErbB4 mRNA (red arrow) and digoxygenin-labeled GAD mRNA (green arrows) in the rat substantia nigra. h, hilus; sg, stratum granulosum. See color plate 60.

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Refs. 3, 13, 18 for reviews). Analysis of mutant knockout mice deficient in either NRG-1, ErbB2, ErbB3, or ErbB4 reveals severe reduction in several neural crest-derived cell populations including Schwann cells, neural crest–derived cranial sensory neurons, and sympathetic neurons and demonstrates critical roles for the receptors and ligand in aspects of hindbrain development.[1] Findings from additional genetic mouse mutation studies are described below when discussing the relationship of NRG/ErbB4 signaling and schizophrenia.

Neuregulins can regulate multiple neurotransmitter receptors in the brain, including GABAergic, glutamatergic, and acetylcholine receptors. Findings in cerebellar and hippocampal slices and cultures show that NRGs increase the expression of an NMDA receptor subunit (NR2C) and modulate expression of $GABA_A$ receptor α and β2 subunits. In hippocampal cultures, NRGs upregulate several subunits of nicotinic acetylcholine receptors.[12] Trophic functions are suggested by findings showing that NRGs enhance neurite outgrowth from cultured cerebellar granule cells, hippocampal neurons, dopaminergic neurons, and PC12 cells.[6,12,34] The potential role of NRGs and their receptors in activity-dependent neuronal mechanisms is underscored by the demonstration that several forms of activity (seizures, forced locomotion, LTP) induce an upregulation of NRG and ErbB4 expression in hippocampal, neocortical, and/or amygdaloid neurons.[11]

Neuregulins also exert a modulatory role in synaptogenesis and adult brain synaptic plasticity. ErbB4 is enriched in brain postsynaptic densities (mainly somatodendritic and axon initial segment compartments) and interacts functionally with PSD-95 in cortical and hippocampal synapses.[12,19,25] Neuregulins stimulate synaptogenesis, enhance existing synapses, and facilitate excitory synaptic transmission in GABAergic interneurons, likely via stabilization of PSD-95.[2,19,25] Most evidence indicates that NRG/ErbB4 signaling inhibits LTP in the hippocampus and reduces glutamatergic neurotransmission in the cortex.[2] Thus, NRGs regulate both inhibitory and excitatory cortical circuitries that ultimately exert a strong influence upon pyramidal cell output. Dysfunction of NRG/ErbB4 signaling and resulting implications for schizophrenia and other neurological disorders are discussed below.

Taken together, the above findings illustrate that NRG/ErbB signaling influences multiple activities of neurons and provide a strong basis to postulate neurotrophic and/or neuromodulatory functions for NRGs and their receptors in brain neuronal systems.

Neuregulins are Neurotrophic for Nigrostriatal Dopaminergic Neurons

Accumulating results suggest that ventral mesencephalic dopaminergic neurons are responsive to the neurotrophic and neuroprotective effects of NRGs. ErbB4 mRNA is expressed within a substantial population of dopaminergic neurons within the substantia nigra and VTA of both rodents and primates, including human.[33–35] Because ErbB2 and ErbB3 are sparsely expressed in the ventral mesencephalon, it is likely that any functional effects of NRGs on midbrain dopaminergic neurons (see below) are mediated via ErbB4 receptor activation. Interestingly, there is a significant reduction of both mRNA and protein for the ErbB4 receptor in the substantia nigra of aged rats.[9] These findings raise the possibility that diminished NRG/ErbB4 signaling could contribute to the increased susceptibility of aged midbrain dopaminergic neurons to degeneration and may have implications for the etiology of Parkinson's disease (PD).

As determined by *in vivo* microdialysis, a single supranigral injection of the NRGs heregulin-β1 or GGF2 induced a substantial increase in dopamine overflow in the ipsilateral striatum.[32] This was among the first *in vivo* demonstrations of NRG actions in the brain and provided initial evidence that NRGs can augment dopaminergic nigrostriatal function. A subsequent microdialysis study showed that NRG-1β stimulated dopamine release in the hippocampus via the activation of dopamine D4 receptors,[2] indicating modulatory actions of NRGs upon dopaminergic mesolimbic systems. In each case, a direct effect of NRGs on dopaminergic release is suggested by the substantial colocalization of ErbB4 and tyrosine hydroxylase in neurons of the substantia nigra and VTA, which give rise to the dopaminergic mesotelencephalic system.

Consistent with these findings, *in vitro* results indicate substantial neurotrophic and neuroprotective roles for GGF2 in primary cell cultures derived from fetal rat mesencephalon.[34] The effects of GGF2 were tested on developing dopaminergic neurons in the midbrain cultures under both serum-free and 6-OHDA-challenged conditions. GGF2 significantly promotes dopaminergic neuron survival, increases dopamine uptake, and enhances dopaminergic fiber outgrowth under both conditions compared with controls.[34] The presence of glial cells is absent or extremely rare in these serum-free midbrain cultures, suggesting that the neurotrophic effects of GGF2 on the dopaminergic cells are direct, and not mediated via glia. Notably, in separate experiments in midbrain cultures, the NRG-2 protein NRG-2β also enhances dopamine neuron survival and process outgrowth, and protects the dopamine cells against 6-OHDA-induced degeneration (D.M. Yurek and K.B. Seroogy, unpublished). Finally, human post-mitotic dopaminergic neurons are protected against 6-OHDA-induced toxicity *in vitro* by the extracellular domain of NRG-1-β1 [NRG-1β1-ECD; Ref. 5], raising

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the possibility that adult human midbrain dopaminergic neurons could be responsive to NRGs *in vivo*.

Evidence suggests that administration of NRG *in vivo* is neuroprotective and perhaps neurorestorative for dopaminergic neurons in rodent models of PD. In the rat 6-OHDA model, supranigral infusion of GGF2 or NRG-2β via osmotic minipumps is protective and partially restorative in terms of increased survival of nigral dopaminergic neurons and preservation of associated motor behaviors. Similar results were also obtained with GGF2 in an aged rat model of PD, suggesting that treatment with this NRG can overcome the age-related loss of nigral ErbB4 expression [J.W. Dickerson and K.B. Seroogy, unpublished]. In a 6-OHDA mouse model of PD, intraperitoneal injection of NRG-1β1-ECD is also neuroprotective and neurorestorative for injured midbrain dopaminergic cells.[5] The intriguing, but perhaps unexpected, finding that systemic administration of NRG-1 successfully improves survival of damaged dopaminergic neurons inside the brain is partially explained by the ability of NRG-1 to cross the blood–brain barrier.[16,20] This peripheral route of administration may hold promise in terms of potential therapeutic delivery of NRGs for the treatment of PD and other CNS disorders.

Neuregulins and Neurological Disorders

Increasing evidence of roles for NRG/ErbB signaling in the etiology and treatment of several neurological disorders is rapidly accruing. The amount of NRG is decreased in active multiple sclerosis (MS) lesions suggesting that insufficient levels of NRG may account, in part, for the demyelination seen in the disease.[27] In the experimental autoimmune encephalitis (EAE) mouse model of MS, administration of GGF2 results in significant beneficial effects including a reduction in inflammation and demyelination and enhanced remyelination.[4] Supported by the well known trophic effects of NRGs on oligodendrocytes, and the suppression of activated microglial responses by GGF2,[10] these findings raise the possibility of NRGs as a treatment for demyelinating disorders such as MS.

In animal models of stroke, both NRG and ErbB receptors are upregulated in the peri-infarct region in both neurons and macrophages/microglia. Pretreatment and acute post-treatment with NRG-1 in these transient focal ischemic stroke paradigms reduce infarct volume, block secondary neuronal degeneration and pro-inflammatory responses, and improve functional outcome indicating a neuroprotective role for NRGs in ischemic brain injury.[31] In a model of permanent focal ischemic stroke, functional recovery is promoted by GGF2 with treatment starting as long as one week post-occlusion, indicating neurorestorative effects.[14]

The expression of NRG-1 within neuritic plaques and of both NRG-1 and ErbB4 within reactive astrocytes and microglia surrounding the neuritic plaques has been identified in human Alzheimer's disease (AD) brain.[7] Upregulation of ErbB4 immunoreactivity also occurs in neurons of the hippocampus, amygdala, superior frontal gyrus, and basal forebrain of postmortem AD brains and in the hippocampus and cerebral cortex of the amyloid precursor protein/presenilin 1 (APP/PS1) transgenic mouse model of AD.[30] These findings raise the possibility of aberrant NRG/ErbB4 mechanisms in the pathogenesis of this debilitating neurodegenerative disorder.

The identification of the NRG-1 and ErbB4 genes as probable susceptibility genes for schizophrenia[21,23] has spawned a wealth of studies aimed at deciphering the role of NRG/ErbB4 signaling in the psychiatric disorder (see Refs. 2, 18, 19 for recent reviews). In particular, numerous mouse genetic manipulation studies indicate that NRG-1 and ErbB4 mutant mice (e.g. heterozygous knockouts, conditional knockouts) exhibit behavioral, electrophysiological and morphological phenotypes relevant to schizophrenia (see Ref. 19 for an excellent summary table). These consistently include defective long-term potentiation, impaired working memory, impaired prepulse inhibition, locomotion hyperactivity, impaired synapse formation, reduced number of cortical interneurons (GABAergic, parvalbumin-positive) impaired GABAergic transmission, hyperactive cortical pyramidal cells, disrupted dendritic morphology and spine density, and dysfunctional NMDA receptors. Key findings point to cortical developmental deficiencies, disrupted inhibitory circuits, and associated excitatory pyramidal cell dysfunction. Thus, emerging evidence strongly suggests that defects in cortical NRG/ErbB signaling contribute to the molecular, cellular, and physiological basis of schizophrenia. The NRG-3 gene has also been identified as a risk factor for schizophrenia;[29] possible roles of this gene and its cognate proteins in the pathogenesis of schizophrenia remain to be determined.

ACKNOWLEDGMENTS

This work was supported by the National Institutes of Health (NS39128), the Department of Defense (DAMD17-02-1-0174), the Selma Schottenstein Harris Laboratory for Research in Parkinson's, the Sunflower Revolution/University of Cincinnati Neuroscience Institute, the Gardner Family Center for Parkinson's Disease and Movement Disorders, and the Parkinson's Disease Support Network of Ohio, Kentucky and Indiana. J.W. Dickerson was supported by a Scottish Rite Foundation Fellowship and S.N. Cassella was supported by NIH Training Grant T32 NS007453. We thank Kerstin Lundgren and Ann Hemmerle for their invaluable assistance.

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REFERENCES

1. Birchmeier C. ErbB receptors and the development of the nervous system. *Exp Cell Res* 2009;**315**:611–8.
2. Buonanno A. The neuregulin signaling pathway and schizophrenia: from genes to synapses and neural circuits. *Brain Res Bull* 2010;**83**: 122–31.
3. Buonanno A, Fischbach GD. Neuregulin and ErbB receptor signaling pathways in the nervous system. *Curr Opin Neurobiol* 2001;**11**:287–96.
4. Cannella B, Hoban CJ, Gao Y, Garcia-Arenas R, Lawson D, Marchionni M, et al. The neuregulin, glial growth factor 2, diminishes autoimmune demyelination and enhances remyelination in a chronic relapsing model of multiple sclerosis. *Proc Natl Acad Sci U S A* 1998;**95**:10100–5.
5. Carlsson T, Schindler FR, Höllerhage M, Depboylu C, Arias-Carrión O, Schnurrbusch S, et al. Systemic administration of neuregulin-1β_1 protects dopaminergic neurons in a mouse model of Parkinson's disease. *J Neurochem* 2011;**117**:1066–74.
6. Carpenter G. ErbB-4: mechanism of action and biology. *Exp Cell Res* 2003;**284**:66–77.
7. Chaudhury AR, Gerecke KM, Wyss JM, Morgan DG, Gordon MN, Carroll SL. Neuregulin-1 and erbB4 immunoreactivity is associated with neuritic plaques in Alzheimer disease brain and in a transgenic model of Alzheimer disease. *J Neuropathol Exp Neurol* 2003;**62**:42–54.
8. Citri A, Skaria KB, Yarden Y. The deaf and the dumb: the biology of ErbB-2 and ErbB-3. *Exp Cell Res* 2003;**284**:54–65.
9. Dickerson JW, Hemmerle AM, Numan S, Lundgren KH, Seroogy KB. Decreased expression of ErbB4 and tyrosine hydroxylase mRNA and protein in the ventral midbrain of aged rats. *Neuroscience* 2009;**163**: 482–9.
10. Dimayuga FO, Ding Q, Keller JN, Marchionni MA, Seroogy KB, Bruce-Keller AJ. The neuregulin GGF2 attenuates free radical release from activated microglial cells. *J Neuroimmunol* 2003;**136**:67–74.
11. Eilam R, Pinkas-Kramarski R, Ratzkin BJ, Segal M, Yarden Y. Activity-dependent regulation of Neu differentiation factor/neuregulin expression in rat brain. *Proc Natl Acad Sci U S A* 1998;**95**: 1888–93.
12. Esper RM, Pankonin MS, Loeb JA. Neuregulins: versatile growth and differentiation factors in nervous system development and human disease. *Brain Res Rev* 2006;**51**:161–75.
13. Falls DL. Neuregulins: functions, forms, and signaling strategies. *Exp Cell Res* 2003;**284**:14–30.
14. Iaci JF, Ganguly A, Finklestein SP, Parry TJ, Ren J, Saha S, et al. Glial growth factor 2 promotes functional recovery with treatment initiated up to 7 days after permanent focal ischemic stroke. *Neuropharmacology* 2010;**59**:640–9.
15. Jabbour A, Hayward CS, Keough AM, Koltyar E, McCrohon JA, England JF, et al. Parenteral administration of recombinant human neuregulin-1 to patients with stable chronic heart failure produces favorable acute and chronic haemodynamic responses. *Eur J Heart Fail* 2011;**13**:83–92.
16. Kastin AJ, Akerstrom V, Pan W. Neuregulin-1-beta1 enters brain and spinal cord by receptor-mediated transport. *J Neurochem* 2004;**88**:965–70.
17. Longart M, Liu Y, Karavanova I, Buonanno A. Neuregulin-2 is developmentally regulated and targeted to dendrites of central neurons. *J Comp Neurol* 2004;**472**:156–72.
18. Mei L, Xiong W- C. Neuregulin 1 in neural development, synaptic plasticity and schizophrenia. *Nature Rev Neurosci* 2008;**9**:437–52.
19. Rico B, Marin O. Neuregulin signaling, cortical circuitry development and schizophrenia. *Curr Opin Genet Dev* 2011;**21**:262–70.
20. Rösler TW, Depboylu C, Arias-Carrión O, Wozny W, Carlsson C, Höllerhage M, et al. Biodistribution and brain permeability of the extracellular domain of neuregulin-1-β1. *Neuropharmacol* 2011;**61**:1413–8.
21. Silberberg G, Darvasi A, Pinkas-Kramarski R, Navon R. The involvement of ErbB4 with schizophrenia: association and expression studies. *Am J Med Genet B Neuropsychiatr Genet* 2006;**141B**:142–8.
22. Sithanandam G, Anderson LM. The ERBB3 receptor in cancer and cancer gene therapy. *Cancer Gene Therapy* 2008;**15**:413–48.
23. Stefansson H, Sigurdsson E, Steinthorsdottir V, Bjornsdottir S, Sigmundsson T, Ghosh S, et al. Neuregulin 1 and susceptibility to schizophrenia. *Am J Hum Genet* 2002;**71**:877–92.
24. Talmage DA, Role LW. Multiple personalities of neuregulin gene family members. *J Comp Neurol* 2004;**472**:134–9.
25. Ting AK, Chen Y, Wen L, Yin D-M, Shen C, Tao Y, et al. Neuregulin 1 promotes excitatory synapse development and function in GABAergic interneurons. *J Neurosci* 2011;**31**:15–25.
26. Tzahar E, Waterman H, Chen X, Levkowitz G, Karunagaran D, Lavi S, et al. A hierarchical network of interreceptor interactions determines signal transduction by Neu differentiation factor/neuregulin and epidermal growth factor. *Mol Cell Biol* 1996;**16**:5276–87.
27. Viehover A, Miller RH, Park SK, Fischbach G, Vartanian T. Neuregulin: an oligodendrocyte growth factor absent in active multiple sclerosis lesions. *Dev Neurosci* 2001;**23**:377–86.
28. Veikkolainen V, Vaparanta K, Halkilahti K, Iljin K, Sundvall M, Elenius K. Function of ERBB4 is determined by alternative splicing. *Cell Cycle* 2011;**10**:2647–57.
29. Wang YC, Chen JY, Chen ML, Chen CH, Lai IC, Chen TT, et al. Neuregulin 3 genetic variations and susceptibility to schizophrenia in a Chinese population. *Biol Psychiatry* 2008;**64**:1093–6.
30. Woo RS, Lee JH, Yu HN, Song DY, Baik TK. Expression of ErbB4 in the neurons of Alzheimer's disease brain and APP/PS1 mice, a model of Alzheimer's disease. *Anat Cell Biol* 2011;**44**:116–27.
31. Xu Z, Croslan DR, Harris AE, Ford GD, Ford BD. Extended therapeutic window and functional recovery after intraarterial administration of neuregulin-1 after focal ischemic stroke. *J Cereb Blood Flow Metab* 2006;**26**:527–35.
32. Yurek DM, Zhang L, Fletcher-Turner A, Seroogy KB. Supranigral injection of neuregulin1-β induces striatal dopamine overflow. *Brain Res* 2004;**1028**:116–9.
33. Yurek DM, Seroogy KB. Neurotrophic factor protection of dopaminergic neurons. In: Mochetti I, editor. *Neurobiology of the neurotrophins*. New York: Sulburger & Graham Publishing; 2001. p. 355–97.
34. Zhang L, Fletcher-Turner A, Lundgren KH, Marchionni M, Yurek DM, Seroogy KB. Neurotrophic and neuroprotective effects of the neuregulin glial growth factor-2 on dopaminergic neurons in rat primary midbrain cultures. *J Neurochem* 2004;**91**:1358–68.
35. Zheng Y, Watakabe A, Takada M, Kakita A, Namba H, Takahashi H, et al. Expression of ErbB4 in substantia nigra dopamine neurons of monkeys and humans. *Prog Neuropsychopharmacol Biol Psychiatry* 2009;**33**:701–6.

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Chapter 224

Neurotrophins

Ljubica Ivanisevic and H. Uri Saragovi

ABSTRACT

The neurotrophins belong to a family of homodimeric polypeptide growth factors that regulate neuronal development, survival or death, neuritogenesis, synaptic strength, and plasticity, and nervous system function. Moreover, other cell types also respond to neurotrophins. Nerve Growth Factor (NGF) is the prototype Neurotrophin. It is synthesized as a precursor proNGF that is further processed to a mature polypeptide, and each form has distinct activities. On binding to its receptors—the common p75 receptor (TNF-superfamily member) and a selective Trk tyrosine kinase receptor—the neurotrophins activate signaling. Each receptor can signal independently, and when coexpressed, the receptors regulate each other in terms of ligand affinity, net signal effects, and resulting biological endpoints. Neurotrophins and their receptors are implicated in human neurodegenerative diseases, pain, inflammation, and certain types of cancer, making these proteins targets for treatment.

DISCOVERY OF NERVE GROWTH FACTOR

Nerve Growth Factor (NGF) was discovered in the 1950s by Rita Levy Montalcini and Viktor Hamburger while studying the developing nervous system and the peripheral structures it innervates. A nerve growth-promoting factor essential for the survival and maintenance of sensory and sympathetic neurons was identified, on the basis of a functional assay measuring the effects of a secreted soluble factor that enabled specific sensory neuron fiber outgrowth and hypertrophy from dorsal root ganglia, but not motor neurons. *In vitro* experiments performed by Cohen et al. led to the identification of a nerve growth-promoting protein that was later cloned and identified as NGF.

From this work, the Neurotrophic Factor Hypothesis was developed, stating that developing neurons that innervate or are attempting to target a tissue are in competition for limited supplies of trophic factors made by the target tissue, and neurons that make appropriate connections obtain growth factors that help them survive while neurons that do not obtain growth factors die.[3]

After the discovery of NGF and its physiological roles in the central and peripheral nervous system, other neuron survival promoting neurotrophic factors were cloned and identified including brain derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4/5 (NT-4). Each of these neurotrophic factors affects a different neuronal population, with limited overlap, because different mature neurons express different types of neurotrophin receptors.[17]

STRUCTURE OF THE mRNA/GENE

The human NGF gene is located on the short arm of chromosome 1 (1p22) with the human beta NGF gene being highly homologous to mouse NGF gene. The NGF gene is >43 kbp and contains 4 exons: exons IA and B, exon II, exons IIIA and B, and exon IV. Part of exons IA, II, IIIB, and IV code for NGF precursor. Exon IV codes for all mature NGF (118 amino acids) and additional 125 amino acids in the amino terminus.[28] Exon IIIA is noncoding (Fig. 1).

There are four different NGF transcripts, with transcripts A and B being the most abundant and differing at the alternative splicing at exon II. There is a characteristic expression pattern of these transcripts, with B being more abundant than A in all the tissues except for submaxillary gland and placenta.[29] Therefore, the NGF gene is regulated by transcription initiation and RNA processing. In addition, basic residues in the NGF precursors are cleavage sites that once processed result in the mature NGF proteins.

Although there is little information about the physiological roles of each NGF transcript, much is known about alternative spliced proteins for BDNF. For example, different transcripts can be regulated epigenetically, and they play different roles in some types of memory.[18]

DISTRIBUTION OF THE mRNA

Distribution of NGF mRNA was determined by in situ hybridization and northern blot analysis in different tissues. NGF mRNA is expressed by different cells of the peripheral and central nervous system. In the periphery, NGF mRNA is expressed in the tissues that are targets of sensory and sympathetic ganglion neurons. The highest levels of NGF mRNA are present in the heart and the spleen.[29]

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00224-4
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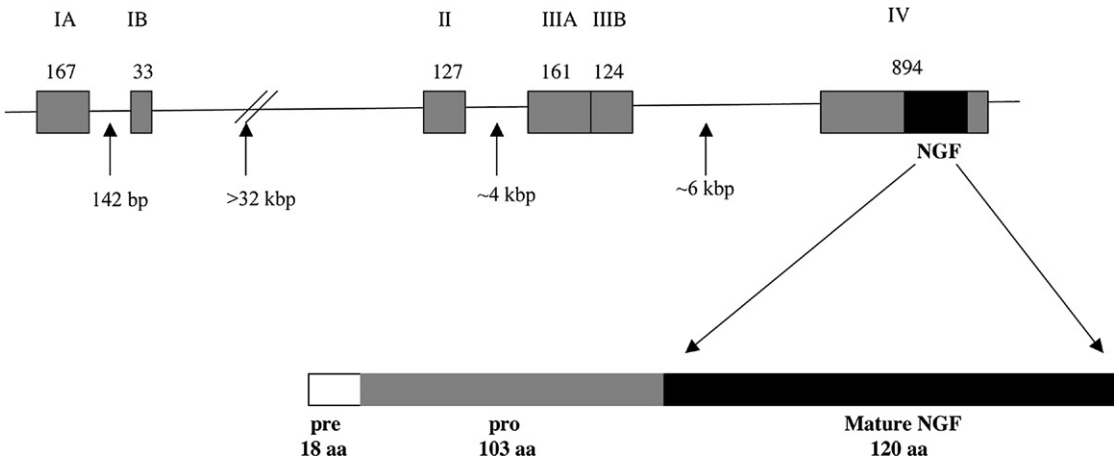

FIGURE 1 Structure of the NGF gene. Introns are shown as a line, and exons as a box. The size of exons and introns in base pairs (bp) is shown. There are 3 ATG start codons in different exons—exon IA, II and IV. The black box represents exon IV coding for mature NGF.The structure of preproNGF precursor is shown underneath. 142 bp >32 kbp ~4 kbp ~6 kbp. Modified from Selby et al., *Mol Cell Biol* 1987;**7**:3057–64.

NGF mRNA is also present in epithelial cells, smooth muscle cells, fibroblasts, and male germ cells. In the brain, the highest levels of NGF mRNA are expressed at postnatal week 3. NGF mRNA expressing cells are found in all areas of the hippocampus, almost exclusively in neurons.

Precursor Protein: Processing and Activity

NGF is synthesized as precursor, 31–35 kDa preproNGF that is processed until it is secreted as mature homodimeric NGF of 28 kDa. The pre-mRNA codes for a signal sequence that directs the synthesis of the protein at endoplasmatic reticulum (ER) ribosomes. Subsequently, the protein is sequestered in the ER, with cleavage of the signal peptide resulting in proNGF, which then forms spontaneous noncovalently linked proNGF homodimers.

Further processing of NGF occurs in the ER with N-linked glycosylation in the prodomain that affords protein stability to NGF. ProNGF homodimers then transit to the Golgi and accumulate in the membrane stacks of the trans-Golgi network.[35]

Sulfation of N-linked oligosaccharides and cleaving of the N-terminal domain of proNGF give rise to mature NGF. ProNGF is cleaved by furin and proconvertases, PACE4 and PC5/6B to yield C-terminal mature neurotrophin dimers. Intracellular cleavage of proNGF to produce mature NGF is at the Arg-Ser-Lys/Arg-Arg sequence.[28]

From the Golgi, NGF can undergo secretion through the constitutive or the secretory pathways, depending on the cell type. Cells have the ability to process and secrete biologically active NGF, and also proNGF can be secreted. Curiously, although mature NGF binds 2 receptors (TrkA and p75) and can afford neuronal survival, proNGF binds in a more restricted manner (p75 only) or to a different subset of receptors and signals toward death in neuronal cells, smooth muscle cells, and oligodendrocytes.

The p75-mediated apoptotic signals are carried through a functional (and physical) association of p75 receptor with sortilin. The prodomain of NGF interacts with the cysteine rich region of the sortilin extracellular domain, whereas the mature NGF domain interacts with p75.[12]

CONFORMATION

The mature NGF is cleaved from the preproNGF precursor (240–260 amino acids). It is a noncovalent homodimer with each monomer having a mass of 13 kDa or 118–129 amino acids. NGF is a member of cysteine knot family because of a double loop formed by two disulfide bonds, which is then penetrated by a third disulfide bond. The crystal structure of NGF reveals the monomer structure consisting of mostly β structure domains, with seven β strands A, A′, A″, A‴, B, C and D, which contribute to three antiparallel pairs of β strands. The core of the NGF monomer is formed by a pair of two-stranded, twisted β sheets with a cysteine knot motif and a reverse turn on one side and three β-hairpin loops on the other side. The large twist between each pair of β strands and the interface between the A′–A″ and B–C double stranded sheets form the hydrophobic core.

The cysteine knot motif and several side-chain to side-chain hydrogen bonds also contribute to hydrophobic core and give rise to the same overall three-dimensional structure of NGF and other members of the neurotrophin family. Two NGF homodimers interact in a parallel manner through hydrophobic interactions, and this explains the high association constant for the two subunits. A comparison of neurotrophin family proteins shows that most of the variable residues are located in the N-terminus, the three β-hairpin

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loops (residues 29–35, 43–48, and 92–98), and the reverse turn from residues 59–66.[20] The other characteristic feature of the NGF dimer is that it is a basic protein with isoelectric point >9 and clustered positive charged surface close to β-hairpin Asp-30 to Lys 34 that might be involved in binding to the acidic p75 receptor that has an isoelectric point of 4.4.

Variable residues are involved in the selective receptor binding of NGF to its selective TrkA receptor, whereas conserved residues between all the members of neurotrophin family might enable the binding to common neurotrophin receptor p75. The crystal structure of human TrkA-IgC2 domain in complex with human NGF has been elucidated. The ligand–receptor interface consists of two patches, one involving the central beta-sheet at the core of dimeric NGF and the loops at the TrkA-IgC2 domain C-terminal end, and second patch with contacts between N-terminal region of NGF and "ABED" sheet of TrkA-IgC2.[32]

The crystal structure of extracellular domain of p75 in complex with NGF was also determined. p75 Binds along the homodimeric interface of NGF, which disables the second p75 binding site through an allosteric conformational change. Therefore, p75 receptors may bind NGF in a 1:2 stoichiometry.[9] Although it has not been possible to cocrystalize p75-Trk or p75-NGF-Trk heteromeric complexes, there is compelling ligand binding, functional, and biochemical evidence that these complexes may exist.

RECEPTORS—BINDING SITES

NGF elicits its function by binding to two families of transmembrane receptors—the selective and specific TrkA tyrosine kinase receptor and the common p75 neurotrophin receptor. The first NGF binding protein that was cloned was p75, but later, it was discovered that it binds all members of neurotrophin family (BDNF, NT-3, NT-4). TrkA was initially discovered as an oncogenic fusion protein and was an "orphan receptor" until it was discovered that NGF induces its fast tyrosine phosphorylation.

Cells expressing TrkA alone display a small number of high affinity NGF binding sites (K_d 10^{-12} M), but the majority of sites have intermediate binding affinity (K_d 10^{-10} to 10^{-11} M), with a very slow association rate. The p75 receptor binds all mature neurotrophins with low affinity (K_d 10^{-9} M) but with different kinetics. Coexpression of p75 with TrkA receptors leads to the formation of a larger number of NGF high affinity binding sites (K_d 10^{-12}) and a faster association rate. The formation of high affinity NGF binding sites depends on the ratio of the numbers of p75 and TrkA receptors on the cell surface, and the juxtamembrane and transmembane domains of TrkA and p75 are required.

Other members of Trk receptor family include TrkB that binds preferentially BDNF and TrkC that binds preferentially NT-3. The picture is even more complex, because high affinity NT-3 binding to p75 has been reported, and p75 in complex with sortilin also creates highaffinity binding sites for proNGF proBDNF and proNT-3. Indeed, proNGF can activate p75 signaling more efficiently than does mature NGF. However, the biological consequences of proNGF activation of p75 differ from mature NGF: proNGF is generally thought to cause cell death.

TRKA AND P75 RECEPTORS—STRUCTURE

All Trk receptors are type I receptor tyrosine kinases. They have an extracellular ligand-binding domain, a single transmembrane domain and a cytoplasmic part with tyrosine kinase catalytic activity.

The extracellular domain is a ligand-binding domain, and it is glycosilated with 40 kDa of carbohydrate moiety. The extracellular domain has a characteristic subdomain organization. On the N-terminus, there is one cysteine rich domain (also known as D1) that is separated from the second cysteine rich domain (also known as D3) by three consecutive leucine rich motifs (also known as D2). The C-terminal region of the extracellular domain has two immunoglobulin (IgG) like domains, IgC1 and IgC2 (also termed D4 and D5 respectively) (Fig. 2). There is 75% sequence identity in the tyrosine kinase catalytic domains of Trk family members, and around 50% sequence homology in the extracellular domains.

Domain swapping studies between TrkA, TrkB, and TrkC identified their IgC2 domain as the ligand-binding domain and functional activation domain (also termed a "receptor hot spot"); and this was confirmed by crystallography.[30] The IgC1 domain has a role in regulating receptor dimerization and preventing constitutive receptor activation, likely acting as a hinge region. In addition, the D1 domain seems to be an allosteric receptor hot-spot which is unmasked when p75 receptors are coexpressed on the cell.[33]

Biochemical and mutational studies indicate that the Trk transmembrane domain linking the extracellular and intracellular domains plays important roles in ligand activation of the receptor, in the formation of Trk receptor dimers, and in association with p75 receptors.

The p75 receptor is a member of tumor necrosis factor (TNF) superfamily. Structurally, it has a ligand-binding extracellular domain, a single transmembrane domain and an intracellular domain. The extracellular domain has four cysteine repeats (CR1–CR4) that create binding sites for all neurotrophins and proneurotrophins. The cytoplasmic domain has no catalytic activity and has a strong sequence homology to the death domain of the proteins of the TNF superfamily. Thus, p75 has no intrinsic catalytic activity, but it can signal through several adaptor proteins.[4] In addition, the transmembrane domain of p75 has a reduced Cysteine

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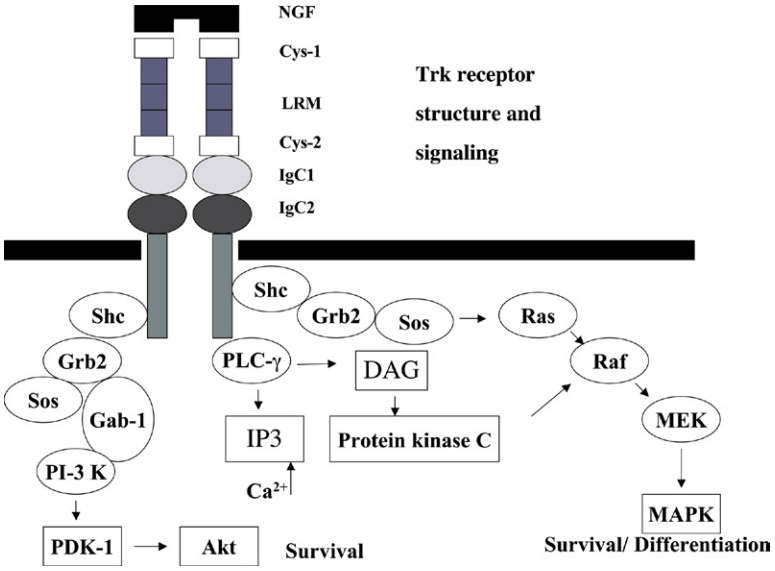

FIGURE 2

that under certain conditions is able to disulfide bond to create stable p75 homodimers that can signal without ligand.[31]

TRK RECEPTOR SIGNALING

The first step in Trk tyrosine kinase receptor activation is a ligand-induced receptor homodimerization followed by transphosphorylation of tyrosine residues in the activation loop. This leads to autophosphorylation of tyrosine residues that serve as recruitment sites for specific signaling proteins and adaptors. Binding of adaptor proteins containing phosphotyrosine binding (PTB) or Src-homology (SH-2) motifs to phosphtyrosines of Trk couples the receptor to intracellular signaling pathways, including Ras-MAPK protein kinase pathway, the phosphatidylinositol-3 kinase (PI-3 kinase)/Akt kinase pathway and phospholipase C-γ (PLC-γ) pathway.[13]

On TrkA activation, phosphorylation of tyrosine 490 leads to recruitment and phosphorylation of Shc that recruits the Grb–SOS complex via SH-2 domain, leading to the activation of MAPK kinase family extracellular signal-related kinases 1 and 2 (Erk1/2) via Ras/Raf/MEK-1 activation. On activation, Erk1/2 translocate to the nucleus where they activate transcription of genes regulated by NGF leading to cell survival.

Direct activation of PI-3 kinase by Ras is a major survival-promoting pathway in most neurons. Phosphatidylinositides generated by PI-3 kinase activate the protein kinase B (Akt) via phosphoinositide dependent kinase-1 (PDK-1). Phosphorylation of Akt by PDK-1 on threonine 308 followed by autophosphorylation on Ser 473 activates Akt and allows it to facilitate survival by phosphorylating different substrates, including transcription factors that modulate cell survival.[10]

Phosphorylation of TrkA at Y785 recruits and activates PLC-γ directly. Activated PLC-γ induces hydrolization of phosphatidyl inositide 4,5 biphosphate to generate inositol-3 phosphate (IP_3) and diacylglycerol (DAG). IP_3 induces Ca^{2+} release resulting in the activation of various protein kinases and phosphatases activated by cytoplasmic Ca^{2+}. DAG activates DAG-regulated protein kinase C isoforms (PKC). PKCs are a family of intracellular serine-threonine kinases that can activate Raf and MAPK cascade regulating cell survival or neuronal differentiation (Fig. 2).

P75 RECEPTOR SIGNALING

p75 Receptors can modulate Trk receptor signaling, but they also signal independently of Trks. Depending on the developmental stage, cell type, whether they are unbound or ligand bound, and the type of ligand, p75 can activate apoptosis or survival, regulate Schwann cell migration, myelination, axonal growth, and regeneration.

During development, p75 signals toward apoptosis to eliminate unnecessary neurons. The signaling pathways of p75 induced apoptosis involve activation of JNK via the recruitment of p75 interacting proteins NRIF (neurotrophin receptor interacting factor), NADE (neurotrophin associated cell death executor), NRAGE (neurotrophin receptor interacting MAGE homolog), and tumor necrosis factor associated factors (TRAFs).

Neurotrophin binding to p75 can activate the MAP kinase pathway leading to cell survival or apoptosis depending on the cell type or ligand type (Fig. 3). Different outcomes of p75 signaling may be caused by differential subcellular

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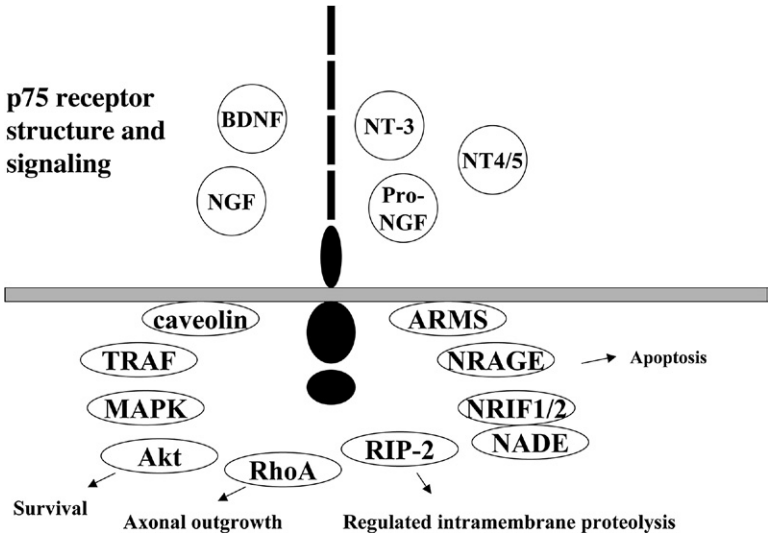

FIGURE 3

localization and transport of p75 complexes. Mature NGF induces very slow internalization of p75, whereas proNGF in the presence of sortilin enables much faster kinetics of internalization of p75-NGF complex.

Another signaling paradigm where p75 participates includes a receptor complex with Nogo receptor and Lingo-1 that mediates growth inhibition in response to myelin-based growth inhibitory proteins (Nogo), myelin-associated glycoprotein, and oligodendrocyte–myelin glycoprotein. p75 plays a role in RhoA activation that is required for growth inhibition. The role of regulated intramembrane proteolysis (RIP) of p75 by α and γ secretases is important in the p75-mediated activation in response to myelin-based growth inhibitory proteins.[6]

The field of p75 signaling is evolving, with new ligands and intracellular interactors being identified, and the identification of p75 homodimers that dissociate on ligand binding, thus the monomers perhaps function differently. However, p75 is a very complex receptor, with varying and sometimes opposite functions, dependent on the cellular context, and therefore, work continues to delineate the molecular pathways that enable p75 to regulate neuronal life, death, motility, and growth inhibition.

NEUROTROPHINS—RETROGRADE TRANSPORT

During development, retrogradely transported NGF promotes survival of appropriately connected neurons. NGF bound to TrkA at the axon terminals is internalized into signaling endosomes and retrogradely transported to the cell bodies. Activated phospho-TrkA in signaling endosomes is linked to downstream signaling pathways that promote neuronal survival and differentiation, whereas surface NGF bound TrkA receptors regulate axonal growth.[14]

After endocytosis, signaling receptors move into early endosomes. From early endosomes, Trk receptors can either return to the plasma membrane for reuse or go along the degradation pathway in a microtubule-dependent fashion, reaching late endosomes and the physical destruction of Trk receptor takes place in lysosomes.

The p75 receptor internalizes and recycles relatively slowly, although ligands do accelerate the rate of internalization. Perhaps one additional regulatory function for p75 is to retain the activated Trk at the cell surface, by reducing the internalization of ligand–Trk complexes, thereby altering the physiological outcomes.[27] These studies show the relevance of receptor location in stimulation, and afford a possible explanation of how different biological outcomes may be achieved by the activation of cell surface versus internalized Trks.

NEUROTROPHINS—PHYSIOLOGICAL ROLE AND ROLES IN PATHOLOGY

Neurotrophins induce cell survival and neurite outgrowth on different neuronal populations. In the PNS, NGF supports DRG neurons during development, the maintenance of mature cholinergic neurons, and regulates arborization and neurotransmitter release. One of the major roles of NGF is to mediate the selective survival of a proportion of developing sympathetic and sensory neurons as they innervate their particular target tissues. In the CNS, NGF also influences synaptic function and plasticity, and phenotypic (and probably also functional) maintenance in the mature cholinergic system.

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NGF is trophic to small-diameter sensory fibers and regulates nociception. Being a proinflammatory protein, NGF also plays a role in inflammatory pain. Thus, NGF antagonists could be useful in the treatment of certain forms of pain. The TrkA ectodomain fused to the Fc portion of an immunoglobulin reduces inflammatory pain, presumably by mopping NGF. Anti-NGF antibodies have been used with similar results.[26]

Neurotrophins and their receptors also play a role in the progression of certain types of cancer—neuroblastoma (TrkA, NGF), prostate cancer (TrkA, NGF), and medulloblastoma (TrkC) just to name a few.[25]

There is evidence for neurotrophic factors being effective at delaying neurodegenerative diseases in animal models and humans. NGF has been shown to have an effect in models of diabetic peripheral neuropathy and Alzheimer's disease. Phase II clinical trials using NGF for diabetic neuropathy had initial positive results, but in larger scale phase III trials, there was no beneficial effect.

Because a quantitative decline in TrkA expression in cortex and nucleus basalis correlates well with cognitive decline and stage of disease in Alzheimer's disease, in aged-associated cognitive impairment, and in Down's syndrome,[21] it is conceivable that neurotrophic factors may be useful as therapeutics.

However, problems associated with the use of neurotrophins as drugs are numerous: proteins are not orally bioavailable, they tend not to cross the blood–brain barrier in large amounts, they have a poor pharmacokinetic profile, and they can elicit immune reactions. Also, neurotrophins bind to at least two receptors, and the receptors are present sometimes on the same tissue and sometimes on different tissues, where they can carry out differing signals and biology.

Because neurotrophins have potential for the treatment of neurodegenerative diseases, several approaches have been used to circumvent some of the problems listed above.[24,25] Approaches include gene therapy, cell therapy, and microencapsulated slow-release implants. Gene therapy has entered a nonrandomized, nonplacebo clinical trial with somewhat promising results. However, all these approaches require surgery and are still in the early stages.

A more traditional pharmaceutical approach has been the development of small molecule nonpeptidic neurotrophin mimetics. Such mimetic compounds, defined on the basis of their activity, can have different mechanisms of action: activating receptors directly as ligands, indirectly by upregulating the production of endogenous neurotrophins, affecting receptor signaling pathways, or affecting coreceptors that regulate neurotrophin receptor activity.

The first rationally derived TrkA agonist is a peptidomimetic compound termed D3. D3 is a proteolytically stable monovalent small molecule, a selective TrkA partial agonist that binds the IgC2 (D5) domain of TrkA. *In vivo* D3 binds to TrkA receptors and affords a significant and long-term rescue of cholinergic neurons in the cortex and in the nucleus basalis that correlates well with an enhanced cholinergic phenotype and a significant improvement of memory/learning in cognitively impaired aged rats.[5]

Small molecule TrkA agonists are neuroprotective and can enhance cognition and short-term memory in a mouse overexpressing amyloid-β as a model of Alzheimer's Disease,[1] can delay neuronal death in a traumatic model of optic nerve injury,[15] and delay the death of retinal neurons in animal models of glaucoma.[2]

CLINICAL PROSPECTS

Neurotrophins belong to a family of polypeptide growth factors that control the apoptotic death or survival, growth, and differentiation of neurons. NTFs also regulate several other cell populations such as lymphoid, epithelial, oligoglia, and mast cells. Altered activity (upregulated or down regulated) of neurotrophins or their receptors plays a well-defined role in certain human pathologies. Consequently, the neurotrophins and their receptors are important therapeutic targets, and pharmacological modulation may have applications ranging from treatment of chronic or acute neurodegeneration and some forms of cancer and chronic pain with agonists, as well as some forms of cancer or acute pain with antagonists.

Several clinical trials have been attempted with neurotrophins, some of them seemingly premature and burdened with serious pharmacokinetic/pharmacodynamic issues, low stability, low efficacy, pleiotrophic effects because of the binding of multiple receptors, and high cost.

Although neurotrophins as therapeutics in the treatment of neurodegenerative diseases have been widely tested experimentally and in some human clinical trials, their use as drugs is limited by their poor pharmacokinetic and the high costs associated with their manufacturing. These obstacles have driven the scientific community toward the identification of small molecule neurotrophin mimetics, with more favorable drug-like properties.[23,25]

Therapeutic rationales targeting Trk receptors include the use of compounds that directly bind to and activate Trk (selective agonists of neuroprotective pathways); compounds that enhance or potentiate release of endogenous neurotrophins (secretagogs); and compounds that regulate signaling pathways (potentiators or biological response modifiers).

Therapeutic rationales targeting p75 receptors include the use of compounds that directly bind to and inactivate p75 (selective receptor antagonists or antagonism of neurodegenerative pathways); compounds that modulate or antagonize proneurotrophins; and compounds that prevent death signaling pathways (biological response modifiers).

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Neurotrophin mimetics are small molecules that resemble the structure and function of key domains or hot spots of the proteins. They have been designed as cyclic peptides[16,26] or organic peptidomimetics.[19,34] The term mimetic is also used for compounds that have little relationship to the neurotrophins, such as Gambogic amide, but which seem to directly regulate signal pathways through TrkA.[7,11] Similar strategies have been used to develop antagonists of p75 receptors.

Some Trk-agonists and p75 antagonists have been developed preclinically and have shown efficacy in animal models for the diseases, including glaucoma, motor neuron disease, traumatic nerve injury or stroke, and neuropathic pain.[2,8,22] At this time, one small molecule mimetic agonist of TrkA is under late clinical development for ophthalmic indications as topical eye-drops.

As we learn more about these important ligands and receptors, and as pharmacological tools evolve and are refined, we may still see the successful targeting of neurotrophin receptors or neurotrophin proteins in human disease.

REFERENCES

1. Aboulkassim T, Tong XK, Chung Tse Y, Wong TP, Woo SB, Neet KE, et al. Ligand-dependent TRKA activity in brain differentially affects spatial learning and long-term memory. *Mol Pharmacol* 2011;**80**: 498–508.
2. Bai Y, Dergham P, Nedev H, Xu J, Galan A, Rivera JC, et al. In chronic and in acute models of retinal neurodegeneration TrkA activity is neuroprotective while p75NTR activity is neurotoxic through a paracrine mechanism. *J Biol Chem* 2010;**285**:39392–400.
3. Barde YA. Trophic factors and neuronal survival. *Neuron* 1989;**2**: 1525–34.
4. Barker PA. p75NTR: a study in contrasts. *Cell Death Differ* 1998;**5**:346–56.
5. Bruno MA, Clarke PB, Seltzer A, Quirion R, Burgess K, Cuello AC, et al. Long-lasting rescue of age-associated deficits in cognition and the CNS cholinergic phenotype by a partial agonist peptidomimetic ligand of TrkA. *J Neurosci* 2004;**24**:8009–18.
6. Ceni C, Barker PA. Getting RIP'd stunts your growth. *Neuron* 2005;**46**:839–40.
7. Chan CB, Liu X, Jang SW, Hsu SI, Williams I, Kang S, et al. NGF inhibits human leukemia proliferation by downregulating cyclin A1 expression through promoting acinus/CtBP2 association. *Oncogene* 2009;**28**:3825–36.
8. Colangelo AM, Bianco MR, Vitagliano L, Cavaliere C, Cirillo G, De Gioia L, et al. A new nerve growth factor-mimetic peptide active on neuropathic pain in rats. *J Neurosci* 2008;**28**:2698–709.
9. He XL, Garcia KC. Structure of nerve growth factor complexed with the shared neurotrophin receptor p75. *Science* 2004;**304**:870–5.
10. Huang EJ, Reichardt LF. Trk receptors: roles in neuronal signal transduction. *Annu Rev Biochem* 2003;**72**:609–42.
11. Jang SW, Okada M, Sayeed I, Xiao G, Stein D, Jin P, et al. Gambogic amide, a selective agonist for TrkA receptor that possesses robust neurotrophic activity, prevents neuronal cell death. *Proc Natl Acad Sci U S A* 2007;**104**:16329–34.
12. Jansen P, Giehl K, Nyengaard JR, Teng K, Lioubinski O, Sjoegaard SS, et al. Roles for the pro-neurotrophin receptor sortilin in neuronal development, aging and brain injury. *Nat Neurosci* 2007;**10**: 1449–57.
13. Kaplan DR, Miller FD. Signal transduction by the neurotrophin receptors. *Curr Opin Cell Biol* 1997;**9**:213–21.
14. Kuruvilla R, Ye H, Ginty DD. Spatially and functionally distinct roles of the PI3-K effector pathway during NGF signaling in sympathic neurons. *Neuron* 2000;**27**:499–512.
15. Lebrun-Julien F, Morquette B, Douillette A, Saragovi HU, Di Polo A. Inhibition of p75(NTR) in glia potentiates TrkA-mediated survival of injured retinal ganglion cells. *Mol Cell Neurosci* 2009;**40**:410–20.
16. LeSauteur L, Wei L, Gibbs BF, Saragovi HU. Small peptide mimics of nerve growth factor bind TrkA receptors and affect biological responses. *J Biol Chem* 1995;**270**:6564–9.
17. Lewin GR, Barde Y-A. Physiology of the neurotrophins. *Annu Rev Neurosci* 1996;**19**:289–317.
18. Lubin FD, Roth TL, Sweatt JD. Epigenetic regulation of BDNF gene transcription in the consolidation of fear memory. *J Neurosci* 2008;**28**:10576–86.
19. Maliartchouk S, Feng Y, Ivanisevic L, Debeir T, Cuello AC, Burgess K, et al. A designed peptidomimetic agonistic ligand of TrkA nerve growth factor receptors. *Mol Pharmacol* 2000;**57**:385–91.
20. McDonald NQ, Chao MV. Structural determinants of neurotrophin action. *J Biol Chem* 1995;**270**:19669–72.
21. Mufson EJ, Ma SY, Cochran EJ, Bennett DA, Beckett LA, Jaffar S, et al. Loss of nucleus basalis neurons containing trkA immunoreactivity in individuals with mild cognitive impairment and early Alzheimer's disease. *J Comp Neurol* 2000;**427**:19–30.
22. Pehar M, Cassina P, Vargas MR, Xie Y, Beckman JS, Massa SM, et al. Modulation of p75-dependent motor neuron death by a small non-peptidyl mimetic of the neurotrophin loop 1 domain. *Eur J Neurosci* 2006;**24**:1575–80.
23. Price RD, Milne SA, Sharkey J, Matsuoka N. Advances in small molecules promoting neurotrophic function. *Pharmacol Ther* 2007;**115**:292–306.
24. Saragovi HU, Burgess K. Small molecule and protein-based neurotrophic ligands: agonists and antagonists as therapeutic agents. *Expert Opin Ther Pat* 1999;**9**:737–51.
25. Saragovi HU, Gehring K. Development of pharmacological agents for targeting neurotrophins and their receptors. *Trends Pharmacol Sci* 2000;**21**:93–8.
26. Saragovi HU, Zaccaro MC. Small molecule peptidomimetic ligands of neurotrophin receptors, identifying binding sites, activation sites and regulatory sites. *Curr Pharm Des* 2002;**8**:2201–16.
27. Saragovi HU, Zheng W, Maliartchouk S, DiGugliemo GM, Mawal YR, Kamen A, et al. A TrkA-selective, fast internalizing nerve growth factor-antibody complex induces trophic but not neuritogenic signals. *J Biol Chem* 1998;**273**:34933–40.
28. Seidah NG, Benjannet S, Pareek S, Savaria D, Hamelin J, Goulet B, et al. Cellular processing of the nerve growth factor precursor by the mammalian pro-protein convertases. *Biochem J* 1996;**314**(Pt 3): 951–60.
29. Selby MJ, Edwards R, Sharp F, Rutter WJ. Mouse nerve growth factor gene: structure and expression. *Mol Cell Biol* 1987;**7**:3057–64.
30. Ultsch MH, Wiesmann C, Simmons LC, Henrich J, Yang M, Reilly D, et al. Crystal structures of the neurotrophin-binding domain of TrkA, TrkB and TrkC. *J Mol Biol* 1999;**290**:149–59.

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31. Vilar M, Charalampopoulos I, Kenchappa RS, Reversi A, Klos-Applequist JM, Karaca E, et al. Ligand-independent signaling by disulfide-crosslinked dimers of the p75 neurotrophin receptor. *J Cell Sci* 2009;**122**:3351–7.
32. Wiesmann C, deVos AM. Nerve growth factor: structure and function. *Cell Mol Life Sci* 2001;**58**:748–59.
33. Zaccaro MC, Ivanisevic L, Perez P, Meakin SO, Saragovi HU. p75 Co-receptors regulate ligand-dependent and ligand-independent Trk receptor activation, in part by altering Trk docking subdomains. *J Biol Chem* 2001;**276**:31023–9.
34. Zaccaro MC, Lee HB, Pattarawarapan M, Xia Z, Caron A, L'Heureux PJ, et al. Selective small molecule peptidomimetic ligands of TrkC and TrkA receptors afford discrete or complete neurotrophic activities. *Chem Biol* 2005;**12**:1015–28.
35. Zhou A, Webb G, Zhu X, Steiner DF. Proteolytic processing in the secretory pathway. *J Biol Chem* 1999;**274**:20745–8.

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Chapter 225

TGF-β

Veronica Lifshitz and Dan Frenkel

ABSTRACT

Transforming growth factor β (TGF-β), a pleiotropic polypeptide, regulates multiple biological processes, including embryonic development, adult stem cell differentiation, immune regulation, wound healing, and inflammation. TGF-β family members are synthesized as prepropeptide precursors and are then processed and secreted as homodimers or heterodimers. Most ligands of this family signal through transmembrane serine/threonine kinase receptors and Smad proteins to regulate cellular functions. Alterations of specific components of the TGF-β-signaling pathway may contribute to a broad range of pathologies such as cancer, autoimmune diseases, tissue fibrosis, and cardiovascular pathology. This chapter focuses on the diverse properties of TGF-β in physiological and pathological conditions.

DISCOVERY

Todaro and De Larco,[11] in 1978, made the important discovery that murine sarcoma virus-transformed 3T3 cells produced a "factor" that induced nonneoplastic rat kidney fibroblast cells (NRK) to assume a neoplastic phenotype. They called this factor "sarcoma growth factor" (SGF) and assayed its transforming activity by the ability of cells to grow in soft agar.[11] It was suggested that SGF was a mixture of at least two constituents.[11] One fraction, which competed with epidermal growth factor (EGF) for receptor sites and induced formation of only a few small colonies of NRK cells in soft agar, was named "transforming growth factor-a" (TGF-α).[1] The second fraction, which induced the growth of many large colonies of NRK cells in the soft agar assay in the presence of small amounts of EGF, was named "transforming growth factor- β" (TGF-β).[1]

The TGF-β superfamily consists of >35 members in vertebrates, including TGF-β isoforms (TGF-β1, TGF-β2, and TGF-β3), bone morphogenetic proteins (BMPs), growth differentiation factors (GDFs), activins, inhibins, Nodals, and Müllerian inhibitory factor (MIF).[25] Members of the extended superfamily are found in a wide variety of species as diverse as insects, amphibia, birds, and human.[25]

STRUCTURE OF THE RNA/GENE

Currently, five distinct isoforms of TGF-β have been identified, and they share 64–82% identity; three of these (TGF-β1, TGF-β2, and TGF-β3) are expressed in mammalian tissues.[23] Among mammals, the sequence homology within a given isoform can reach as high as 100%. In humans, the three isoforms are located on three different chromosomes, 19q13, 1q41, and 14q24, respectively.[23] All genes have a length of >100 kb and contain seven exons.

PROCESSING OF THE PRECURSOR

The three mammalian TGF-β isoforms, TGF-β1, TGF-β2, and TGF-β3 are synthesized as latent precursor proteins (LTGF-β), 390–412 amino acids in length, with a longer N-terminal propeptide followed by a shorter C-terminal mature polypeptide.[28] The LTGF-βs are cleaved intracellularly by furin into a larger C-terminal proregion, also known as latency-associated peptide (LAP), and a shorter N-terminal active peptide, which forms the mature TGF-β-homodimers (25 kDa)[28] (see the furin chapter in Peptide Biosynthesis/Processing section of the book). LAP remains noncovalently associated with the mature TGF-β 25-kDa dimer forming a small latent complex (SLC; ~100 kDa). LAP serves as a chaperone during exocytosis of the complex and keeps TGF-β in an inactive form. LAP also mediates the deposition of TGF-β in the extracellular matrix (ECM), through its covalent association with large secreted proteins called latent TGF-β-binding proteins (LTBPs) and with ECM proteins. The binding of the SLC to LTBP produces the large latent complex (LLC; 235–260 kDa).[28] Often, the LLC is bound to collagen and other tissue matrix proteins through binding sites on the LTBP. Most cell types secrete TGF-β as part of LLC, although some cells (such as the bone cell line UMR-106) secrete SLC.[28]

The mature TGF-β protein can be released from the inactive complex by either proteolytic cleavage by various proteases (such as plasmin, thrombin, plasma transglutaminase, or endoglycosylases), or by physical interactions of the LAP with proteins, such as thrombospondin-1 and integrins

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00225-6
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αvβ6 and αvβ8.[28] After TGF-β is released from its latency-associated peptide, it is able to initiate its diverse cellular responses by binding to specific cell-surface receptors.[28]

RECEPTORS AND SIGNALING CASCADES

TGF-β Receptors

Each member of the TGF-β superfamily can bind to cell-surface serine/threonine kinase TGF-β receptors types I and II (TβRI and TβRII, respectively, see Fig. 1). Five type II and seven type I receptors also named ALKs (activin-like receptor kinases) have been identified in vertebrates.[31] There are additional receptors for TGF-β including the type III (TβRIII) and type V (TβRV). The type IV TGF-β receptor has been identified only in pituitary cells but has not been well characterized. TβRIII, also known as betaglycan, is a proteoglycan containing glycoprotein that functions as a coreceptor by presenting ligand to TβRII 14. TβRV, also known as IGFBP-3R or LRP-1, is a receptor for TGF-β, and IGFBP-3 and is coexpressed with TβRI, TβRII, and TβRIII in most cell types.[17]

Ligand binding to the constitutively active TβRII induces the assembly of type I and type II receptors into complexes, in which TβRII phosphorylates TβRI in the 'GS domain' (domain which is rich in glycine and serine residues).[21] Signaling from activated TβRI to the nucleus occurs predominantly by phosphorylation of cytoplasmic proteins known as Smad proteins (Fig. 1).

Smad Proteins

Smad proteins (Sma and Mad proteins from *Caenorhabditis elegans* and *Drosophila*, respectively) convey signals from TGF-β receptors to the nucleus (Fig. 1). Different members of the Smad family have different roles in signaling. The receptor-associated Smads (R-Smads: Smad1, 2, 3, 5, and 8) are recruited to the activated TβRI by auxiliary proteins such as Smad Anchor for Receptor Activation (SARA).[31] ALK1, ALK2, ALK3, and ALK6 phosphorylate Smad1, Smad5, and Smad8, whereas ALK4, ALK5, and ALK7 phosphorylate Smad2 and Smad3 (Fig. 1). Smad1, Smad5, and Smad8 are specific for signaling of BMPs, whereas Smad2 and Smad3 transduce signals for TGF-βs and activins[21] (Fig. 1). However, in endothelial cells that express both ALK1 and ALK5, TGF-β signaling is mediated by two different pathways, namely, Smad1/5/8 and Smad2/3[28] (Fig. 1). Activated

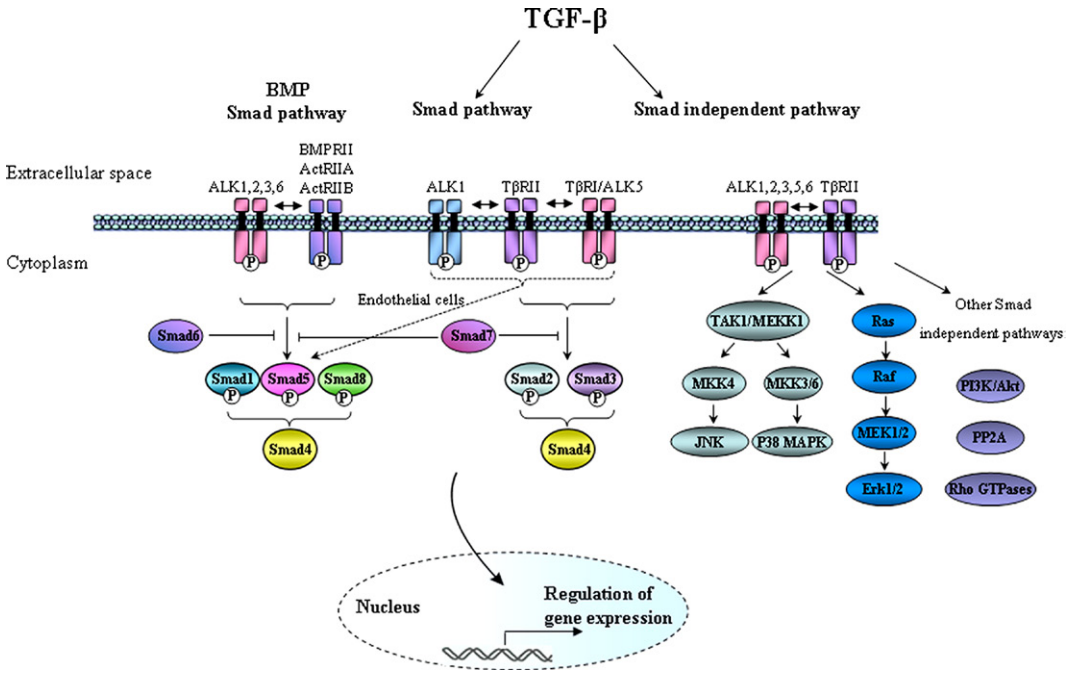

FIGURE 1 TGF-β signaling through Smad-dependent and Smad-independent pathways. TGF-β binds a complex of transmembrane receptor serine/threonine kinases (TβRII and TβRI) and induces phosphorylation and activation TβRI by TβRII. In most cells, TGF-β interacts with TβRII and ALK5, but in endothelial cells, it can also signal via ALK1. BMPs signal via BMPRII, ActRIIA, and ActRIIB, and type I receptors ALK1, 2, 3, and 6. Activated type I receptors induce the phosphorylation of specific receptor regulated Smads (R-Smads: Smad1, 2, 3, 5, and 8). ALK5 induces phosphorylation of Smad2 and Smad3, and ALK1, 2, 3, and 6 mediate phosphorylation of Smad1, Smad5, and Smad8. Activated R-Smads form heteromeric complexes with common mediator Smad4, which accumulate in the nucleus, where they can act as transcription factor complexes and regulate the expression of specific target genes. Inhibitory Smads, such as Smad6 and Smad7, can antagonize TGF-β signaling by inhibiting the activation of R-Smads. TGF-β activated receptor complex (TβRI and TβRII) can activates Smad-independent signaling pathways, such as extracellular-signal regulated kinase (Erk), p38 mitogen-activated protein kinase (p38 MAPK), c-Jun N-terminal kinase (JNK), phosphatidylinositol-3-kinase (PI3 K/Akt), Src, and pathways involving Rho GTPases. See color plate 61.

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R-Smads form heteromeric complexes with the common mediator, Smad4. The resulting Smad complex then moves into the nucleus, leading to the expression of different type of genes (Fig. 1). The inhibitory Smads, Smad6 and Smad7, compete with R-Smads for binding to activated TβRI and thus inhibit the phosphorylation of R-Smads and/or recruit E3-ubiquitin ligases to activated TβRI, resulting in receptor degradation. Additionally, they may recruit protein phosphatase-1 (PP1) to the receptor complex, resulting in the dephosphorylation, thus inactivation, of the receptors via the catalytic subunit of PP1, GADD45.[31]

Smad signaling is essential for most, but not all, TGF-β gene responses. TGF-β can also exert its biological effects through the activation of Smad-independent pathways, including extracellular-signal-regulated kinase (Erk), p38 mitogen-activated protein kinase (p38 MAPK), c-Jun N-terminal kinase (JNK), phosphatidylinositol-3-kinase (PI3 K/Akt), Src, and pathways involving Rho GTPases.[13,14]

DISTRIBUTION OF THE mRNA/PEPTIDE

TGF-β1-3 are widely distributed in both embryonic and adult tissues. Virtually every cell in the body, including epithelial, endothelial, hematopoietic, neuronal, and connective tissue cells, produces TGF-β and expresses receptors for it.[10] TGF-β1 mRNA is expressed in endothelial, hematopoietic, and connective tissue cells. TGF-β2 mRNA is mostly expressed in epithelial and neuronal cell, and TGF-β3 mRNA is primarily expressed in mesenchymal cells.[10] During development, TGF-β1 and TGF-β3 are expressed early in structures undergoing morphogenesis, whereas TGF-β2 is expressed later in mature and differentiating epithelium.[10]

BIOLOGICAL ACTIONS

TGF-β has been implicated as an important regulator of almost all major cell behaviors and activities, including cell growth, apoptosis, differentiation, adhesion, migration, extracellular matrix production, immune functions, hematopoesis, angiogenesis, wound repair, and mammalian embryogenesis and organogenesis.[25] In each cell type and each context, different sets of genes and signaling responses are activated or repressed in response to TGF-β signals, leading to distinct cellular responses in each case.[25]

The importance of TGF-β isoforms in mammalian biology is highlighted by the lack of viability in TGF-β knockout mice. TGF-β1 null mice die within 3 weeks after birth because of widespread inflammatory disease.[27] TGF-β2 null mice die in the perinatal period because of developmental defects in areas including the heart, lung, limbs, spinal column, eye, inner ear, and urogenital system. TGF-β3 null mice die soon after birth because of an impaired lung maturation and defective palatogenesis.[10]

TGF-β Effects on the Extracellular Matrix

TGF-β, through Smad2/3/4 signaling, regulates the synthesis and deposition of extracellular matrix proteins (ECM) such as collagen, fibronectin, osteonectin, and thrombospondin.[24] TGF-β also influences matrix degradation by regulating the expression of proteases such as MMP1, collagenase, transin, and plasminogen activator, while increasing the production of protease inhibitors such as plasminogen activator inhibitor-1 (PAI-1) and tissue inhibitors of metalloproteinases-3 (TIMP-3) as well.[24] Furthermore, TGF-β induces or represses numerous members of the integrin family, which are the key mediators of the cell-ECM interactions. Because of these differential effects, TGF-β has a role in cell attachment, migration, invasion, wound healing, and fibrosis.[24]

TGF-β Effects on Cell Proliferation

TGF-β inhibits the growth of most cell types, including epithelial cells, endothelial cells, and hematopoietic and glial cells, but stimulates the growth of certain mesenchymal cells (e.g. skin fibroblasts) and some other specific cell types.[17] Signaling by TGF-β family members can block a cells transition from the G1 to S phase, a process known as growth arrest, by down regulating the activity of cyclin-dependent kinases (cdks), which regulate progression through the cell cycle.[17] TGF-β signaling influences cdk activity by modulating the transcriptional activity of cdk inhibitors, p15 and p21.[23] In addition to the prominent TβRI/TβRII Smad2/3/4 signaling pathway, it has been suggested that the Ras/ERK signaling pathway, linked to the Smad-independent pathway, is involved in mediating TGF-β–induced growth inhibition in certain epithelial cell systems. The TβRV signaling cascade seems to cooperate with the TβRI/TβRII signaling cascade in mediating TGF-β–induced growth inhibition.[17]

TGF-β Effects on Apoptosis

TGF-β-mediated apoptosis plays an important part during tissue formation and remodeling and during the phase of ontogenetic neuron death.[26] TGF-β serves as a potent inducer of apoptosis in hepatoma cell lines by inducing the transcription of death-associated protein kinase (DAP kinase), Bim in human gastric epithelial cells and programmed cell death 4 (PDCD4) in human hepatocellular carcinoma cells.[26] TGF-β can also repress the expression of antiapoptotic genes, such as B-cell leukemia/lymphoma 2 (Bcl-2) in hematopoietic cells and breast cancer cells.[26] The apoptotic effect of TGF-β is implicated in the elimination of immature lymphocytes and prostatic epithelial cells.[26] However, it is also clear that the death-inducing capacity of TGF-β is context dependent, that is, it is restricted to certain cell types, to a certain state of differentiation, and

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most notably, to the presence or absence of other growth factors.[26]

TGF-β can also protect against apoptosis through (i) the induction of antiapoptotic gene expression (such as Bcl-2 in rat hippocampal neurons and pre-B cell lines), (ii) repression of proapoptotic gene expression (such as Bad in rat hippocampal cells, CD95 ligand in rat hepatic stellate cells), (iii) cooperation with other survival pathways (by activating Akt and inducing retention of apoptotic transcriptional factor FKHRL1).[26]

TGF-β Effects on Cell Differentiation

During development, TGF-β signaling frequently acts to promote the differentiation of pluripotent cells into specific cell types.[12] TGF-β family members have essential roles in self-renewal, the differentiation potential of embryonic and somatic stem cells, selection of the differentiation lineage and progression along that lineage.[12] TGF-β family proteins actively participate in redirecting the differentiation of ectodermal and epithelial cells toward mesodermal and mesenchymal differentiation (EMT).[12] In osteoblast, skeletal myoblast, and adipocyte cells, TGF-β can inhibit differentiation and stimulates the proliferation of these cells before they reach full maturation.[12]

TGF-β Effects on Angiogenesis

TGF-β signaling is critical for both the formation of the primary vascular plexus (vasculogenesis) and the subsequent extension and remodeling into a complex vascular network (angiogenesis).[4] Gene-targeting studies in mice have shown that inactivation of TGF-β1 causes midgestation lethality resulting from defects in the yolk sac vasculature and hematopoietic system. Moreover, the vessels of the TGF-β1 mutant embryos have decreased wall integrity caused by a defect in endothelial cells differentiation into capillary-like tubules.[4] A similar phenotype is exhibited by mice lacking the TβRII and in mice with null mutations of the genes encoding the TGF-β-signaling components ALK5, ALK1, endoglin, and Smad5.[4]

TGF-β Effects on Nervous System Physiology

Early in mammalian embryology, all three TGF-β isoforms and their TGF-β receptors are expressed in the central nervous system (CNS).[18] In the adult, TGF-β2 and TGF-β3 can be found in all areas of the CNS, whereas TGF-β1 is widely expressed in the choroid plexus and meninges, and in some specific regions of the brain such as the hippocampus and the cortex.[18] TGF-β1 expression is drastically upregulated in the CNS during injury and neurodegeneration, where it is secreted predominantly by activated microglial cells.

TGF-β2 and TGF-β3 are involved in the regulation of precursor cell divisions, migration of neural precursors, neuron and glial cell survival, differentiation, and synaptic plasticity.[6] TGF-β1 has been clearly implicated in several processes of brain development, such as expression of glial fibrillary acidic protein (GFAP) gene expression and astrocyte differentiation, neuronal migration, and cortex lamination, and neuronal survival. TGF-β1 protects neurons against damage induced by excitotoxins, hypoxia/ischemia, and deprivation of trophic factors. TGF-β1 prevents neuronal apoptosis through the inhibition of caspase-3 activation and an increased expression of the antiapoptotic proteins, Bcl-2 and Bcl-xl.[26] TGF-β1 can also activate the ERK pathway in neurons, thus promoting the phosphorylation and subsequent inhibition of the proapoptotic protein, Bad.[8] Furthermore, TGF-β1 enhances the expression of brain derived neurotrophic factor (BDNF)[8] and is required for a full neuroprotective activity of nerve growth factor (NGF), glial-derived neurotrophic factor (GDNF) and BDNF, thus contributing to neuronal survival.[8] There is increasing evidence that hippocampal learning correlates strongly with neurogenesis in the adult brain.[7] Glia secrete the TGF-β ligand myoglianin to instruct developmental neural remodeling in Drosophila.[3] This may suggest that TGF-β plays an important role in remodeling of neuronal network.

TGF-β Effects on the Immune System

TGF-βs have key roles in immune cell activation, inflammation and repair after injury.[30]The gut-associated lymphoid tissue is the largest immune organ in the body and is the primary route by which we are exposed to antigens.[32] Tolerance induction is the default immune pathway in the gut that is important for the maintenance of homeostasis following feeding.[32] TGF-β inhibits the proliferation of T cells and cytokine production via Foxp3-dependent and independent mechanisms. Furthermore, Th3-type T regulatory cells (Foxp3-positive T cells) express LAP on their surface and secrete TGF-β. Th3-type T regulatory cells were suggested to mediate oral tolerance.[32] Smad2 and Smad3 may play essential roles in Foxp3 induction and anti-inflammatory cytokine response, whereas Th17 differentiation and pro-inflammatory response (secretion of IL-17) is promoted via the Smad-independent pathway. Mutual suppression of signaling between TGF-β and inflammatory cytokines is necessary for the balance of immunity and tolerance.[35]

PATHOPHYSIOLOGICAL IMPLICATIONS OF TGF-β

Aberrant TGF-β signaling has been linked to various developmental disorders and diseases, such as cancer,[22] vascular disorders,[5,19] fibrosis,[15] reproductive disorders,[15] autoimmunity,[32] wound healing disorders,[15] and neurodegenerative diseases.[8,29]

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Some of these diseases are hereditary conditions such as HHT (hereditary hemorrhagic telangiectasia), familial primary pulmonary hypertension, and JP (juvenile polyposis).[5]

Developmental Disorders

Appropriate TGF-β superfamily signaling is critical during embryogenesis from the initial stages of blastula formation, during gastrulation, and throughout the multiple stages of organ development.[10] Defects in TGF-β signals may disrupt overall embryonic patterning, leading to premature termination of human embryogenesis. At later developmental stages, they often result in patterning defects and malformation of embryonic organs and have been linked to several human birth defects.[10] Thus, TGF-β2 knockout mice and ALK3 conditional knockout mice have specific defects in the development of valves and septa of the heart.[10] Embryos lacking ALK1, ALK5, TβRII, or endoglin die during midgestation because of impaired vascular development, exhibiting hyperdilated and leaky vessels.[10] A variety of craniofacial malformations have been associated with mutations in TGF-β superfamily members.[10] Mice lacking TGF-β3 have cleft palate and abnormal lung development.[12] Polymorphisms in the gene for TGF-β3 also have been linked to the development of cleft palate in humans.[10]

Mutations in genes encoding TGF-β superfamily proteins, their receptors, and binding proteins result in a variety of skeletal malformations including complete absence of skeletal structures, formation of additional skeletal elements, failure of segmentation of a structure, undergrowth, or abnormal morphology.[10]

Autoimmunity

Deficiency of molecules involved in TGF-β signaling, such as TGF-β1 and TβRII, causes spontaneous lymphocyte activation and differentiation, cell adhesion molecule expression, autoantibody production, multiorgan inflammation and cell apoptosis.[2] Spontaneous initiation of autoimmunity in the TGF-β1 knockout mouse suggests that dysregulation of TGF-β signaling may potentially underlie a number of autoimmune disorders,[32] including multiple sclerosis, experimental autoimmune encephalomyelitis, and inflammatory bowel disease.[2] More detailed examination of the role of TGF-β in inflammation and inflammatory diseases suggests that it has a critical role in the induction of Th17 cells, which play a major role in autoimmune diseases.[32]

Fibrosis in Different Organs

TGF-β is a critical regulator of extracellular matrix production and therefore, increased TGF-β signaling is a critical contributor to the progression of fibrotic diseases. Fibrotic diseases are characterized by excessive scarring resulting from excessive and abnormal deposition of extracellular matrix (ECM), consisting of fibronectin, laminin, vitronectin, and collagen. This occurs by long term activation of myofibroblasts.[15] If the build-up of ECM occurs in organs such as the lungs, liver, kidneys, and skin, it can interfere with organ function, and unabated, results in organ failure.[15] Examples of fibrotic diseases include diabetic nephropathy, liver cirrhosis, rheumatoid arthritis, and arteriosclerosis.[15] TGF-β regulates the expression of proteins involved in executing the fibrotic cascade through (i) regulation of many matrix proteins expression, (ii) regulation of the expression and activity of matrix-degrading proteases and their inhibitors, (iii) TGF-β promotes connective tissue growth factor (CTGF) expression in fibroblasts, which promotes collagen synthesis and myofibroblast differentiation, (iv) TGF-β-induces epithelial to mesenchymal transition (EMT) in renal tubular epithelial cells, which is associated with increased expression of fibroblast-associated proteins, such as alpha smooth muscle actin (SMA), (v) increasing the expression of the vasoconstrictive peptide endothelin-1, which contributes to persistent fibrosis by inducing fibroblasts to produce and secrete ECM.[15]

Cancer

The TGF-β-signaling pathway has complex and contradictory roles in the pathogenesis of human cancers.[15] In normal and premalignant cells, TGF-β enforces homeostasis and suppresses tumor progression directly through cell-autonomous tumor-suppressive effects (cytostasis, differentiation, apoptosis) or indirectly through effects on the stroma (suppression of inflammation and stroma-derived mitogens).[22] However, when cancer cells lose TGF-β tumor-suppressive responses, they can use TGF-β to their advantage to initiate immune evasion, growth factor production, motility, invasion, and metastasis of tumors that have formed.[22]

Malignant cells can circumvent the suppressive effects of TGF-β either through (i) inactivation of core components of the pathway, such as TGF-β receptors, Smad proteins, etc. or (ii) selectively amputate the tumor-suppressive arm of the pathway. In particular, biallelic inactivation of TβRII by mutations that truncate the receptor protein or inactivate its kinase domain occur in colon, gastric, biliary, pulmonary, ovarian, esophageal, and head and neck carcinomas.[22] In addition, frameshift and missense mutations in the TβRI coding region are present in subsets of ovarian, esophageal, and head and neck cancers.[22] Decreased expression of TβRI or TβRII occurs frequently in lung, gastric, prostate, and bladder cancers.[22] Smad2 mutations have been detected at low levels in colon, head and neck, and lung carcinomas. Smad3 mutations have been noted in gastric cancer and T cell lymphoblastic leukemia.[22] Smad4 mutations are present in more than half of pancreatic carcinomas, sporadic colorectal tumors without microsatellite, and in a high proportion of esophageal tumors.[22]

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Cardiovascular Disorders

TGF-β superfamily members critically regulate many different processes within the cardiovascular system (CVS), including cardiac development and angiogenesis.[15] Mutations in endoglin or the ALK1 receptor have been linked to an autosomal-dominant disorder called hereditary hemorrhagic telangiectasia (HHT). The most common clinical manifestations of HHT are nose bleeds and mucocutaneous, small, localized vascular malformations called telangiectasias, and arteriovenous malformations in the brain, lungs, liver, and gastrointestinal tract.[5] Another common genetic defect in TGF-β superfamily entails a mutation in BMP receptor type II gene, which leads to pulmonary arterial hypertension (PAH). PAH is a disease caused by constriction of small pulmonary arteries leading to an increase in blood pressure.[5]

In atherosclerosis, TGF-β regulates lesion phenotype by controlling T-cell responses and stimulating smooth muscle cells to produce collagen. Although TGF-β is believed to protect from atherosclerosis, several lines of evidence support increased TGF-β signaling as a major contributor to restenosis. Restenosis contributes to excessive intimal fibrocellular proliferation and inward remodeling. TGF-β contributes to restenosis by augmenting neointimal cell proliferation and collagen accumulation.[5]

Neurodegenerative Diseases

An impairment of TGF-β1 signaling critically contributes to the pathogenesis of diverse neurodegenerative disorders, including motor neuron diseases, Huntington's disease and Alzheimer's disease (AD).[8,29]

Role of TGF-β1 in Alzheimer's Disease (AD)

Alzheimer's disease is the most common type of dementia occurring in the elderly. The main cause of AD is generally attributed to the increased production and accumulation of amyloid-β (Aβ), as well as neurofibrillary tangle (NFT) consisting of intracellular hyperphosphorylated tau protein, and loss of synapses and neurons.[19]

Many reports have also shown a significant impairment of TGF-β1 signaling in AD brain.[29] Tesseur et al.[29] showed that the expression of TβRII by neurons is reduced very early in the course of AD. Furthermore, neuronal expression of the kinase deficient TβRII in AD transgenic mice promotes Aβ deposition and neurodegeneration, by suppression of neurotrophic factor expression.[29] In addition, genetic studies have revealed that single nucleotide polymorphisms (SNPs) of the TGF-β1 gene at codons +10 (T(C)) and +25 (G/C), which affect the levels of expression of TGF-β1, have been associated with an increased conversion of mild cognitive impairment (MCI) in AD.[8,19] Increased TGF-β1 levels have been found in cerebrospinal fluid and the brain parenchyma of AD patients, whereas a reduction of inactive (50 kDa) forms has been reported in AD plasma.[8] Furthermore, the levels of TGF-β mRNA are 3-fold higher in the frontal cortex of AD brains than control brains.[8] Additionally, in AD, an ectopic localization of phosphorylated Smad2/3 has been detected in the cytoplasm rather than the nucleus of hippocampal neurons[9] in addition to colocalization of phosphorylated Smad2/3 with amyloid plaques and NFT. *In vitro* studies have shown that TGF-β1 prevents Aβ-challenged cortical neurons from entering the S-phase of the cell cycle.[8] Cell-cycle inhibition is one of the mechanisms by which TGF-β1 exerts its neuroprotective effect against Aβ toxicity, another being the prevention of Aβ-induced tau hyperphosphorylation via the inhibition of the tau-phosphorylating enzyme, GSK-3β.[8] Furthermore, TGF-β1 has a constitutive role in the suppression of inflammation and seems to control the degree of microglial activation in the CNS.[8]

Cerebral Amyloid Angiopathy (CAA)

CAA is defined as the deposition of vascular amyloid in the walls of the meningeal and parenchymal arteries, arterioles, capillaries, and, albeit rarely, veins. The vascular amyloid deposits in CAA consist primarily of the β-amyloid peptide (Aβ) and a number of other molecules, such as extracellular matrix proteins, α1-antichymotrypsin, apolipoprotein E, acetylcholinesterase, and cystatin C. The prevalence of CAA, estimated from autopsy series, is approximately 10%–40% in the general elderly population and almost 80% of AD cases. The most widely recognized clinical manifestation of CAA is intracerebral hemorrhage (ICH) and cognitive impairment.[16] Cortical TGF-β1 messenger ribonucleic acid (mRNA) levels correlate positively with the degree of cerebrovascular amyloid deposition in AD cases, and TGF-β1 immunoreactivity in such cases is elevated along the cerebral blood vessels.[34] Heterozygous transgenic mice that overexpress TGF-β1 in astrocytes develop vascular Aβ deposits. Using magnetic resonance imaging (MRI) we found[20] that TGF-β1 mice show a significant cerebrovascular pathology that correlates with CAA. Further, these mice suffer from cognitive impairment that may reflect vascular dementia in patients.

Town et al. (2008) have found that blocking TGF-β/Smad2/3 signaling in peripheral macrophages reduced cerebrovascular Aβ deposits and Aβ abundance in Tg2576 mice, and these events result in promotion of Smad1/5/8 signaling with increased infiltration of Aβ-containing peripheral macrophages around cerebral vessels and β-amyloid plaques.[30]

We have suggested the importance of healthy interactions between endothelial cells (EC) and peripheral immunity to prevent toxic deposition of cerebrovascular amyloid.[20] This crosstalk may be disrupted after pathological expression of TGF-β1 by astrocytes along the cerebrovascular system.[20] We have shown[33] that TGF-β1 affects EC and peripheral immunity crosstalk, leading to a reduction in macrophage

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activity as measured by protein levels and migration ability. The investigation of TGF-β1 mediated cellular pathways in EC may provide useful therapeutic intervention targets for cerebrovascular amyloidosis diseases such as AD.

SUMMARY

The crucial role of TGF-β in different cycles of the cell ensures it as a major target for modulation. Better understanding of the molecular mechanisms of TGF-β signaling in pathological events may be an important basis for understanding and treating different pathological diseases.

ACKNOWLEDGMENTS

This work is supported by grants from the HFSP organization, ISF-Legacy Heritage Biomedical Science Partnership 862/09 (to D. Frenkel). The authors thank Inbal Sukenik-Buahnik for editing the article.

REFERENCES

1. Anzano MA, Roberts AB, Meyers CA, Komoriya A, Lamb LC, Smith JM,et al. Synergistic interaction of two classes of transforming growth factors from murine sarcoma cells. *Cancer Res* 1982;**42**:4776–8.
2. Aoki CA, Borchers AT, Li M, Flavell RA, Bowlus CL, Ansari AA, et al. Transforming growth factor beta (TGF-beta) and autoimmunity. *Autoimmun Rev* 2005;**4**:450–9.
3. Awasaki T, Huang Y, O'Connor MB, Lee T. Glia instruct developmental neuronal remodeling through TGF-beta signaling. *Nat Neurosci* 2011;**14**:821–3.
4. Bertolino P, Deckers M, Lebrin F, ten Dijke P. Transforming growth factor-beta signal transduction in angiogenesis and vascular disorders. *Chest* 2005;**128**:585S–90S.
5. Bobik A. Transforming growth factor-betas and vascular disorders. *Arterioscler Thromb Vasc Biol* 2006;**26**:1712–20.
6. Bottner M, Krieglstein K, Unsicker K. The transforming growth factor-betas: structure, signaling, and roles in nervous system development and functions. *J Neurochem* 2000;**75**:2227–40.
7. Buckwalter MS, Yamane M, Coleman BS, Ormerod BK, Chin JT, Palmer T, et al. Chronically increased transforming growth factor-beta1 strongly inhibits hippocampal neurogenesis in aged mice. *Am J Pathol* 2006;**169**:154–64.
8. Caraci F, Spampinato S, Sortino MA, Bosco P, Battaglia G, Bruno V, et al. Dysfunction of TGF-beta1 signaling in Alzheimer's disease: perspectives for neuroprotection. *Cell Tissue Res* 2011.
9. Chalmers KA, Love S. Neurofibrillary tangles may interfere with Smad 2/3 signaling in neurons. *J Neuropathol Exp Neurol* 2007;**66**:158–67.
10. Chang H, Brown CW, Matzuk MM. Genetic analysis of the mammalian transforming growth factor-beta superfamily. *Endocr Rev* 2002;**23**:787–823.
11. de Larco JE, Todaro GJ. Growth factors from murine sarcoma virus-transformed cells. *Proc Natl Acad Sci U S A* 1978;**75**:4001–5.
12. Derynck R, Akhurst RJ. Differentiation plasticity regulated by TGF-beta family proteins in development and disease. *Nat Cell Biol* 2007;**9**:1000–4.
13. Derynck R, Zhang YE. Smad-dependent and Smad-independent pathways in TGF-beta family signalling. *Nature* 2003;**425**:577–84.
14. Ehrlich M, Horbelt D, Marom B, Knaus P, Henis YI. Homomeric and heteromeric complexes among TGF-beta and BMP receptors and their roles in signaling. *Cell Signal* 2011;**23**:1424–32.
15. Gordon KJ, Blobe GC. Role of transforming growth factor-beta superfamily signaling pathways in human disease. *Biochim Biophys Acta* 2008;**1782**:197–228.
16. Greenberg SM. Cerebral amyloid angiopathy and vessel dysfunction. *Cerebrovasc Dis (Basel, Switzerland)* 2002;**13**(Suppl. 2):42–7.
17. Huang SS, Huang JS. TGF-beta control of cell proliferation. *J Cell Biochem* 2005;**96**:447–62.
18. Krieglstein K, Zheng F, Unsicker K, Alzheimer C. More than being protective: functional roles for TGF-beta/activin signaling pathways at central synapses. *Trends Neurosci* 2011;**34**:421–9.
19. Lifshitz V, Frenkel D. Animal models for cerebrovascular impairment and its relevance in vascular dementia. In: Landow ML, editor. *Cognitive impairment: causes, diagnosis and treatments*. Hauppauge, NY: Nova Science Publishers, Inc.; 2009.
20. Lifshitz V, Weiss R, Benromano T, Kfir E, Blumenfeld-Katzir T, Tempel-Brami C, et al. Immunotherapy of cerebrovascular amyloidosis in a transgenic mouse model. *Neurobiol Aging* 2011.
21. Massague J. TGF-beta signal transduction. *Annu Rev Biochem* 1998;**67**:753–91.
22. Massague J. TGFbeta in Cancer. *Cell* 2008;**134**:215–30.
23. Roberts AB. Molecular and cell biology of TGF-beta. *Miner Electrolyte Metab* 1998;**24**:111–9.
24. Ruiz-Ortega M, Rodriguez-Vita J, Sanchez-Lopez E, Carvajal G, Egido J. TGF-beta signaling in vascular fibrosis. *Cardiovasc Res* 2007;**74**:196–206.
25. Santibanez JF, Quintanilla M, Bernabeu C. TGF-beta/TGF-beta receptor system and its role in physiological and pathological conditions. *Clin Sci (Lond)* 2011;**121**:233–51.
26. Schuster N, Krieglstein K. Mechanisms of TGF-beta-mediated apoptosis. *Cell Tissue Res* 2002;**307**:1–4.
27. Shull MM, Ormsby I, Kier AB, Pawlowski S, Diebold RJ, Yin M, et al. Targeted disruption of the mouse transforming growth factor-beta 1 gene results in multifocal inflammatory disease. *Nature* 1992;**359**:693–9.
28. ten Dijke P, Arthur HM. Extracellular control of TGFbeta signalling in vascular development and disease. *Nat Rev Mol Cell Biol* 2007;**8**:857–69.
29. Tesseur I, Zou K, Esposito L, Bard F, Berber E, Can JV, et al. Deficiency in neuronal TGF-beta signaling promotes neurodegeneration and Alzheimer's pathology. *J Clin Invest* 2006;**116**:3060–9.
30. Town T, Laouar Y, Pittenger C, Mori T, Szekely CA, Tan J, et al. Blocking TGF-beta-Smad2/3 innate immune signaling mitigates Alzheimer-like pathology. *Nat Med* 2008;**14**:681–7.
31. Verrecchia F, Mauviel A. Transforming growth factor-beta and fibrosis. *World J Gastroenterol* 2007;**13**:3056–62.
32. Weiner HL, da Cunha AP, Quintana F, Wu H. Oral tolerance. *Immunol Rev* 2011;**241**:241–59.
33. Weiss R, Lifshitz V, Frenkel D. TGF-beta1 affects endothelial cell interaction with macrophages and T cells leading to the development of cerebrovascular amyloidosis. *Brain Behav Immun* 2011;**25**:1017–24.
34. Wyss-Coray T, Lin C, von Euw D, Masliah E, Mucke L, Lacombe P. Alzheimer's disease-like cerebrovascular pathology in transforming growth factor-beta 1 transgenic mice and functional metabolic correlates. *Ann N Y Acad Sci* 2000;**903**:317–23.
35. Yoshimura A, Muto G. TGF-beta function in immune suppression. *Curr Top Microbiol Immunol* 2011;**350**:127–47.

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Chapter 226

Augurin and *Ecrg4*-derived Neuropeptides

Conrad Johanson, Ana Gonzalez, Miles Miller, Sonia Podvin, Edward Stopa and Andrew Baird

ABSTRACT

The candidate tumor suppressor gene Ecgr4 encodes a preproneuropeptide ligand-like precursor protein that is proteolytically processed into smaller peptides. The Ecrg4 product augurin (14 kDa) is particularly abundant in the choroid plexus (CP) epithelium and detected in the cerebrospinal fluid (CSF). On secretion by CP, augurin anchors to the CSF-facing membrane, poised for rapid release after CNS injury. After traumatic brain injury (TBI) in rats by cortical stabbing, there is immediate CP release of Ecrg4-encoded peptides in early inflammation (*activation phase*). This may protect the subventricular zone (SVZ) from an immune-activated surge of growth factors and cytokines. Shortly thereafter, Ecrg4 gene expression in CP is transiently downregulated in response to TBI. A lower level of Ecrg4 in CP-CSF coincides with the proliferation phase of neural progenitor cells (NPCs) in the neurogenic niches of the SVZ. The working hypothesis is as follows: CP-derived Ecrg4 normally inhibits NPC mitosis, but when its synthesis/release into CSF post-TBI is diminished, the resultant NPC dysinhibition enhances subependymal neurogenesis.

INTRODUCTION

The discovery of the polypeptide hormone augurin in choroid plexus (CP), the major source of cerebrospinal fluid (CSF), opens up new neuroscience research vistas. A promising area of investigation is neuroendocrine modulation of the brain interior: its development, response to injury, and aging.[10,15,18] Peptides and proteins secreted by CP into the ventricles control subependymal cell growth and homeostasis, preserve brain fluid balance, and regulate neuronal functions. Augurin is one of the protein products of *ecrg4*, a gene that is down regulated in epithelial cancers. It seemed reasonable to explore how the altered expression of Ecrg4 in the CNS might impact the epithelial linings of the ventricular system. These include CP, ependyma and subependymal cells. A knowledge about how Ecrg4 controls these periventricular regions may also provide an insight into the generation of CNS primary tumors. For the purposes here, it also provides information on the dynamics of nascent neuron generation in ontogeny, neuronal replacement in senescence, and the compensatory response to brain trauma.

This chapter describes how the use of Markov modeling[13] identified the novel prohormone-like gene, describes the gene products of *ecrg4*, and specifies the elevated Ecrg4 expression sites around CNS ventricles. It also highlights how some experimental manipulations that alter levels of Ecrg4 gene expression modify the associated degree of mitosis in neurogenic niches (NNs). Because CP-derived ecrg4 gene products evidently modulate stem cell targets in NNs via CSF endocrine distribution mechanisms, another topic treated herein is basic CSF neuropeptide physiology. Documented CSF transport of neuroactive materials[31] helps to bolster a neuroendocrine-like role for Ecrg4-derived peptides in regulating neurogenesis at sites distant from CP. Before describing Ecrg4 biological activities, we first discuss the genomic features, molecular structure, and expression patterns of this newly introduced neurohormone-like peptide precursor.

DISCOVERY OF PREPONDERANT EXPRESSION OF AUGURIN IN CHOROID PLEXUS

The initial step in uncovering Ecrg4 as a CP-CSF expressed peptide was enabled by the prediction of its existence by Hidden Markov Modeling (HMM).[13] On the basis of amino acid sequence information, obtained from the alignment of peptides registered in multiple proteomics databases, the mature peptide product now known as augurin was predicted from HMM. An in-depth search into the human genome, using an HMM bioinformatics approach, revealed that the *Ecrg4* gene contains the features of a neuropeptide precursor within its primary sequence and at least one highly conserved secreted polypeptide. Mirabeau et al. coined the name augurin for one of these anticipated neurohormones[13] encoded by Ecrg4(31–148).

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00226-8
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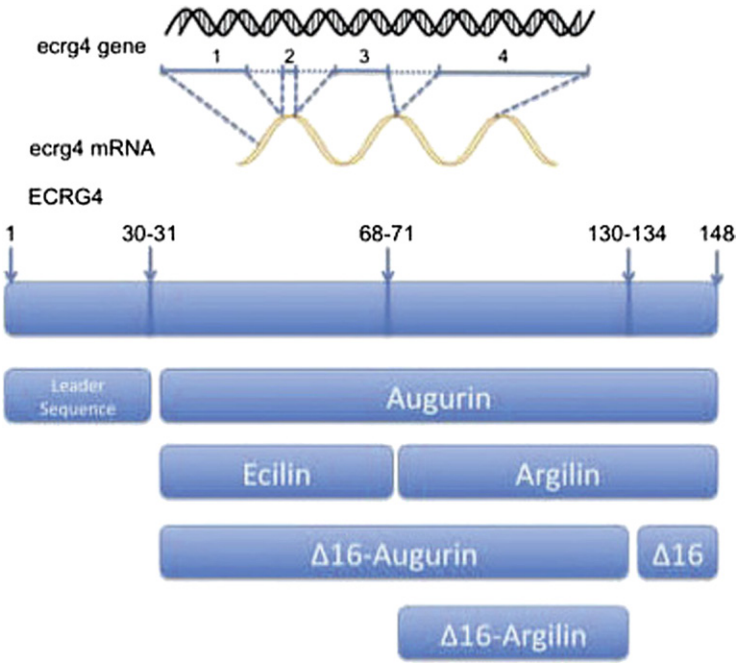

FIGURE 1 Candidate peptides generated from *Ecrg4* gene expression. Algorithms predict that the single augurin gene can produce 8 potential protein/peptide cleavage products. Intact augurin, including the 30-amino-acid leader sequence, consists of 148 amino acids. Ecilin and argilin consist of amino acid fragments 31–70 and 71–148, respectively. Δ16C-terminal cleaved homologs include the Δ16 peptide itself as well as CΔ16-augurin and CΔ16-argilin. Consensus processing sites include those for removing the signal peptide, and for cleavage by furin-like hormones and thrombin.

Accordingly, augurin (also known as Ecrg4(71–148)) is produced by proteolytic processing of the esophageal-cancer-related gene (ecrg4) holoprotein. Including the leader sequence (amino acids 1–30), the augurin precursor molecule consists of 148 amino acids. *Ecrg4* gene products are schematized in Fig. 1. This novel neuropeptide and its cleavage products include an amino terminal "**EC**" domain which we call ecilin (amino acids Ecrg4 (31–70)), a carboxyl terminal "**RG**" domain which we call argilin (Ecrg4(71–148)), and a variety of other potential products depending on whether the 16 C-terminal peptide domain (CΔ16) is also processed from the precursor protein. No gene product had a known biological function at the time of prediction.

Ecrg4 amino acid sequences for a variety of species are aligned in Table 1. Open reading frame (ORF) comparisons reveal a high degree of conservation across diverse species (Table 2). Homology is particularly high (99%) when the chimpanzee *Pan troglodytes* is compared with the ORF for humans. Mice and rats have 84% and 83% homology, consistent with the crossreactivity of the antibody with augurin (recombinant human) in all three species analyzed by us.

The amino acid sequence of augurin within the *Ecrg4* ORF has all the features of a neuropeptide-like precursor. The sequence fits a highly conserved secreted hormone. Therefore, we decided to focus the initial investigations on CP and secretory tissues known for neuroendocrine mediation and homeostasis. Significant expression of Ecrg4 occurs in the pancreas and adrenal gland, but the greatest expression by far occurs in the CP epithelium (Fig. 1A).[5] A distinguishing feature of the CP-CSF function is the secretion/transport of multiple peptides/proteins that influence the state of neurogenesis in periventricular regions.[8] This applies to normal brain development[26] and to periventricular homeostatic responses in adult CNS injury.

In contrast to many neural and nonneural tissues, the mRNA for augurin is strikingly abundant in CP (Fig. 2A). Ecrg4 transcript in CP is several orders of magnitude greater than in the heart, lungs, kidney, and cerebral cortex (Fig. 2B).[5] Immunostaining of rat tissues displays the augurin signal in the CP and adjacent ependymal wall (Fig. 3A). Moreover, immunohistochemistry of human CP reveals the 14 kDa augurin localized to epithelial cells, especially prominent at the CSF-facing apical membrane (Fig. 3B). Immunoblotting of rat CP confirms the presence of a manufactured 14 kDa molecule, that is, augurin (Fig. 3C).

Both human and rat CP release augurin at the epithelial apical surface. Cultured adult human CP epithelium transfected by adenovirus with Ecrg4 cDNA express augurin,[5] but the secreted peptide detected in the conditioned medium by an ELISA[5] is of lower molecular weight suggesting that

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TABLE 1 *ECGR4* **Sequences in Various Species Used for Investigative Work**

	1	10	20	30	40	50	60
HUMAN	MAASP	ARPAV LALTG	LALLL LLCWG	PGGIS GNKLK	LMLQK REAPV	PTKTK VAVDE	NTAKE
CHIMPANZEE	MAASP	ARPAV LALTG	LALLL LLCWG	PGGIS GNKLK	LMLQK REAPV	PTKTK VAVDE	NTAKE
RHESUS	MAASP	ARPAV LALTG	LALLL LLCWG	PGGIS GNKLK	LMLQK REAPV	PTKTK VAVDE	NTAKE
MOUSE	MSTSS	ARPAV LALAG	LALLL LLCLG	PDGIS GNKLK	KMLQK REGPV	PSKTN VAVAE	NTAKE
RAT	MGTSS	ARPAV LALAG	LALLL LLCLG	PGDVS GNKLK	KMLQK REGPV	PSKTN VAVSE	HTAKE
BAT	MASSL	VRPAI LATTA	LALFL LLCLG	PSATG GNTLK	KMLQK REASA	PKKAD LAVDE	SKAKS
DOG	MAASS	ARPAV LALTA	LALLL LLCLG	PGGIS GNKLK	LLLQK REAPA	PAKTN VAVGE	SKAKE
COW	MANSS	ARPAF LVMTA	LALLL LLCVG	PGGIS GNKLK	LLLRK REAPA	PTMTP VAVQE	SRAKE
CHICKEN	MPPPC	PRGAL PG--A	FLLFF LLFVV	P---- -----	-LLCA APAPI	ATKPE VSLKE	EAAKG
ZEBRAFISH	ML--S	EKFHL HLLTL	LTLLT ALALT	DVASE SKLEK	LLMKR VDRDV	KPAAA CAVSP	SKAKE
	61	70	80	90	100	110	120
HUMAN	FLGSL	KRQKR QLWDR	TRPEV QQWYQ	QFLYM GFDEA	KFEDD ITYWL	NRDRN GHEYY	GDYYQ
CHIMPANZEE	FLGSL	KRQKR QLWDR	TRPEV QQWYQ	QFLYM GFDEA	KFEDD ITYWL	NRDRN GHEYY	GDYYQ
RHESUS	FLGSL	KRQKR QLWDR	TRPEV QQWYQ	QFLYM GFDEA	KFEDD ITYWL	NRDRN GHEYY	GDYYQ
MOUSE	FLGGL	KRAKR QLWDR	TRPEV QQWYQ	QFLYM GFDEA	KFEDD VNYWL	NRNRN GHDYY	GDYYQ
RAT	FLGGL	KRAKR QLWDR	TRPEV QQWYQ	QFLYM GFDEA	KFEDD VNYWL	NRNRN GHDYY	GDYYQ
BAT	FLGSL	KRQRR QLWDR	TRPEV QQWYQ	QFLYM GFDEA	KFEDD VTYWL	NRNRN GHDYY	-DYHH
DOG	FLSGL	KRQKR QLWDR	TRPEV QQWYQ	QFLYM GFDEA	KFEDD ITYWL	NRDRN GHDY-	-DYYQ
COW	FLSSL	RRPKR QLWDR	SRPEV QQWYQ	HFLYL GFDEA	KFEDD ISYWL	NRNRN GHDYY	-DYYQ
CHICKEN	FLRSL	RRPKR QLWDR	SQPDV QQWYQ	QFLYL GFDEA	KFEDD MSYWT	SLGRG GSEYY	GGYYQ
ZEBRAFISH	FLTSL	KRPKR NLWDR	SRPDV QQWIQ	QFMYM GFDEA	RLETD LAYWM	DQHRS SDQGR	----Q
	121	130	140	148			
HUMAN	RHYDE	DSAIG PRSPY	GFRHG ASVNY	DDY			
CHIMPANZEE	RHYEE	DSAIG PRSPY	GFRHG ASVNY	DDY			
RHESUS	RHYDE	DSAIG ARSPY	SFRHG ASVNY	DDY			
MOUSE	RHYDE	DAAIG PHSRE	SFRHG ASVNY	DDY			
RAT	RHYDE	DAAIG PRSRE	GFRHG ASVNY	DDY			
BAT	RHYDE	DAAIG PRSPD	SFRHG ASVNY	DDY			
DOG	RHYDE	DAAIG PQSPY	SFRHG ASVNY	DDY			
COW	RHYDE	DSAIG PRSAH	SFRHG ASVNY	DDY			
CHICKEN	HHYDE	DSPIG PRNPH	TFRHG AGVNY	DDY			
ZEBRAFISH	HHYDE	NAALG PRGAA	SYRGA NVVNY	DDY			

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TABLE 2 High Evolutionary Conservation of the *ECGR4* Open Reading Frame

Species	Gene	Protein	Homology to human
Homo sapiens	C2orf40	NP_115787.1	------
Mus musculus	1500015010Rik	NP_077245.1	84%
Rattus norvegicus	RGD1305645	XP_343563.1	83%
Pan troglodytes	LOC459473	XP_001164223.1	99%
Bos taurus	ECRG4	NP_001033202.1	81%
Canis lupus familaris	LOC611190	XP_853925.1	89%
Gallus gallus	LOC771055	XP_001234361.1	58%
Dana rerio (A)	Zgc112443	NP_001017697	47%
Dana rerio (B)	BC095747.1	P0CAX4.1	42%

posttranslational processing occurs on the cell surface or after shedding. CP also releases Ecrg4-derived peptides *in vivo* into the ventricles as suggested in follow-up experiments that assayed augurin in rat CSF .[17] Taken together, the various methods and species analyses (mouse, rat, and human) constitute strong evidence for augurin prominence in the mammalian CP-CSF-ependymal nexus.

EXPLORING PHYSIOLOGIC ROLES FOR AUGURIN

Early evidence points to multiple homeostatic roles for Ecrg4. These include regulation of cell proliferation, senescence, inflammation, tumor progression. and stress responses involving the hypothalamus–pituitary–adrenal axis. Few systematic studies have been conducted on Ecrg4 to date.[5,17,28] The aforementioned initial observations, however, serve to guide experimentation for establishing its cell biology and physiology.

Another candidate role for augurin and Ecrg4, of particular interest to our research group, is related to the ability of the CSF circulation to carry ventricular neuropeptides to target cells within the brain. Peptides secreted by CP are extensively involved in neuronal homeostatic phenomena.[10] This is because of the brisk CSF bulk flow that rapidly distributes macromolecules to regions near the ventricular wall, for example, the SVZ and hippocampal dentate gyrus (DG). Therefore, CSF hydrodynamics efficiently conveys peptides and proteins from CP secretory sites to receptors in periventricular neuronal networks. Neural progenitor cells (NPCs) are housed in specialized subependymal niches, the SVZ and DG, where mitosis is ongoing. All in all, these observations prompt the working model that augurin participates in the control of subependymal neurogenesis.

CSF AS A CIRCULATORY LINK BETWEEN CP AND BRAIN

Many reports indicate that CSF functionally couples choroidal and cerebral metabolic activities.[9,11] CSF performs diverse endocrine functions.[15] Acting like a ventricular "neuroendocrine organ,"[6] the CP is strategically positioned to provide hormones and trophic factors to the CSF-brain axis.[1] As a distributing medium, the CSF circulation conveys peptides and proteins to multiple target cells in the hippocampus, SVZ, and hypothalamus. Targeted cells include ependyma in the ventricular wall and immature neurons in the hippocampal neurogenic nitches (NNs). Hypothalamic nuclei such as the arcuate nucleus also contain peptide receptor targets.[19] Periventricular target cells are accessible to CSF-borne peptides by way of bulk flow and cytoskeletal transport mechanisms in tanycyte fibers. Being a permeable interface, the ependyma in adult mammals permit great diffusional exchange of large molecules between ventricular CSF and subependymal ISF.[9]

Neuropeptides, by way of CSF signaling mechanisms, mediate diverse homeostatic responses in the CNS. Homeostasis entails the regulation of fluid balance and cell metabolism, as well as the production of new neurons after causing physical harm to the brain. Nascent neurons are needed in increased numbers after ischemic and traumatic injuries that destroy neuronal networks.

CSF circulation is an essential component of all CNS transport models. Accordingly, a major convective system for translocating peptides destined for the NNs is the CP-CSF nexus. The CP at the blood–CSF interface engages in transport (hormones from blood) and secretion (choroid epithelial synthesis) of peptides into the ventricles. In fetal brain, a plethora of growth factors and neurotrophins are

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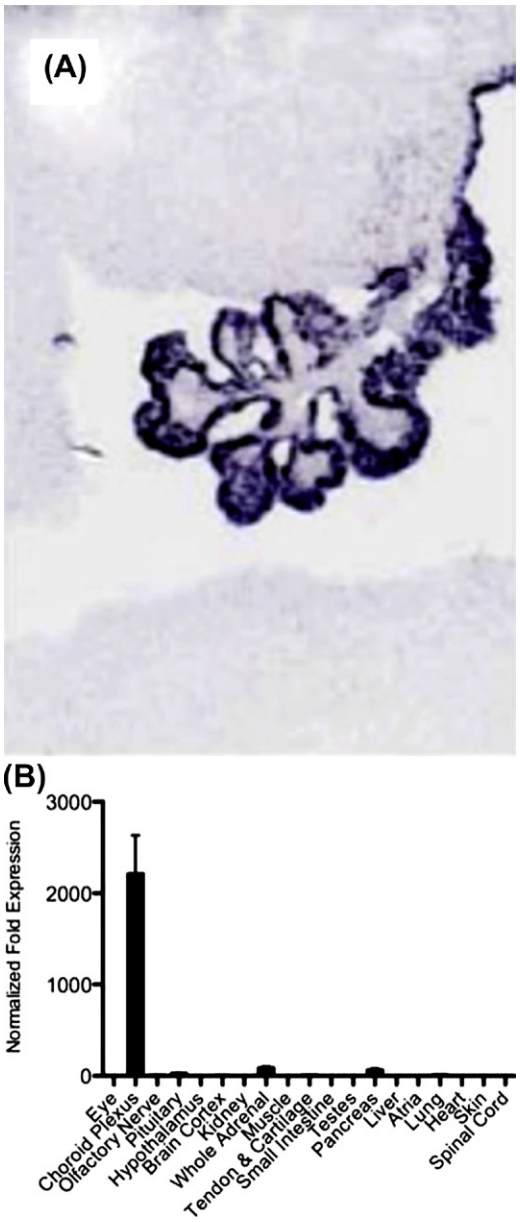

FIGURE 2 Major sites of *Ecrg4* expression in CNS. **(A)** *Ecrg4* gene expression in mouse CP and ependymal lining. *In situ* hybridization signal (mRNA) localizes primarily to the tuft (frond) of choroid plexus epithelial cells (center of figure) and, to a lesser extent, the single layer of ventricular ependymal cells lining the brain interior (top right). **(B)** Quantitative *Ecrg4* gene expression by RT-qPCR. After extraction of tissues from donor adult mice, highest *Ecrg4* expression is found in dissected CP; very low expression is observed in the cerebral cortex. Data, calculated by ΔCt, were normalized to values from testes.[5] A similar expression magnitude occurs in male rats (data not shown). Low gene expression is detectable in all tissues. Error bars are mean ± standard deviation, n = 5.

distributed along the CP–CSF ependyma. These regulatory molecules promote mitosis, differentiation, and migration in the subependymal NNs. Such events occur during ontogeny when the expanding brain is relatively avascular and therefore dependent on micronutrient (vitamin) and hormonal provisions from CSF.[26] In later adult life when the brain suffers harm and requires trophic support for restitution, the CP again takes on a critical role in copiously supplying peptides/hormones to NNs in hippocampus and SVZ. This suggests an emerging role for ecrg4 and augurin production in regulating the proliferation of neural progenitors.

PEPTIDE TRAFFIC THROUGH THE BLOOD–CSF BARRIER VS. BLOOD–BRAIN BARRIER

For solute distribution, the CP–CSF nexus works in parallel with the brain microvessel–neuron interface to furnish substances to the CNS. Thus, another major source of hormonal peptides and micronutrients for NPCs and neuronal networks is the cortical endothelium of the BBB. Accordingly, the cerebral endothelial cells transport and secrete peptides across the capillary abluminal membrane into interstitial fluid (ISF).[34] Concurrent fluid secretions from the CP epithelium via CSF, and the BBB endothelium via ISF, thereby support the NPCs in the SVZ and DG. Unique expression profiles of transporters and translocated peptides at the BCSFB vs. BBB enable variegated effects on NPCs by the respective secretions. Because CP displays the highest expression of Ecrg4 among >15 tissues analyzed (including cortex), it is interesting to speculate that augurin distribution and kinetics at the BCSFB–CSF interface and in adjacent ventricular ependymal regions are involved in CNS homeostasis.

CP translocates a host of polypeptides in the CSF secretory process. Peptides are derived from the circulating blood in the plexus by transport,[2] and from the choroidal epithelium by *de novo* synthesis.[3] Examples of such peptides conveyed from the BCSFB into CSF are insulin-like growth factor (I and II), basic fibroblast growth factor, extradermal growth factor, nerve growth factor, hepatocyte growth factor, as well as brain derived- and glial-derived neurotrophic factors. CSF-convected hormones, received from CP epithelial secretions, include leptin,[29] prolactin,[23] and insulin. Many principles and characteristics of CSF distribution of the aforementioned established hormones probably apply to augurin. By volume transmission or bulk flow, these peptides/proteins are swept down the neuraxis from the lateral ventricles to CSF cisterns at the base of the brain. In transit through the cerebroventricles of the telencephalon, diencephalon, and myencephalon, the CSF-borne growth factors and neurotrophins permeate the ventricular wall and gain access to NNs. NPC cell mitosis, growth, differentiation and migration are stimulated (or inhibited) by these polypeptides that move from ventricular CSF into the brain interior. Hemodynamic perturbation of CP[16] or hydrocephalic distortion of the ventricles[33] thwarts CSF delivery of peptides to neurogenic regions.[14] Interrupted provision of CSF peptides to neural stem cells

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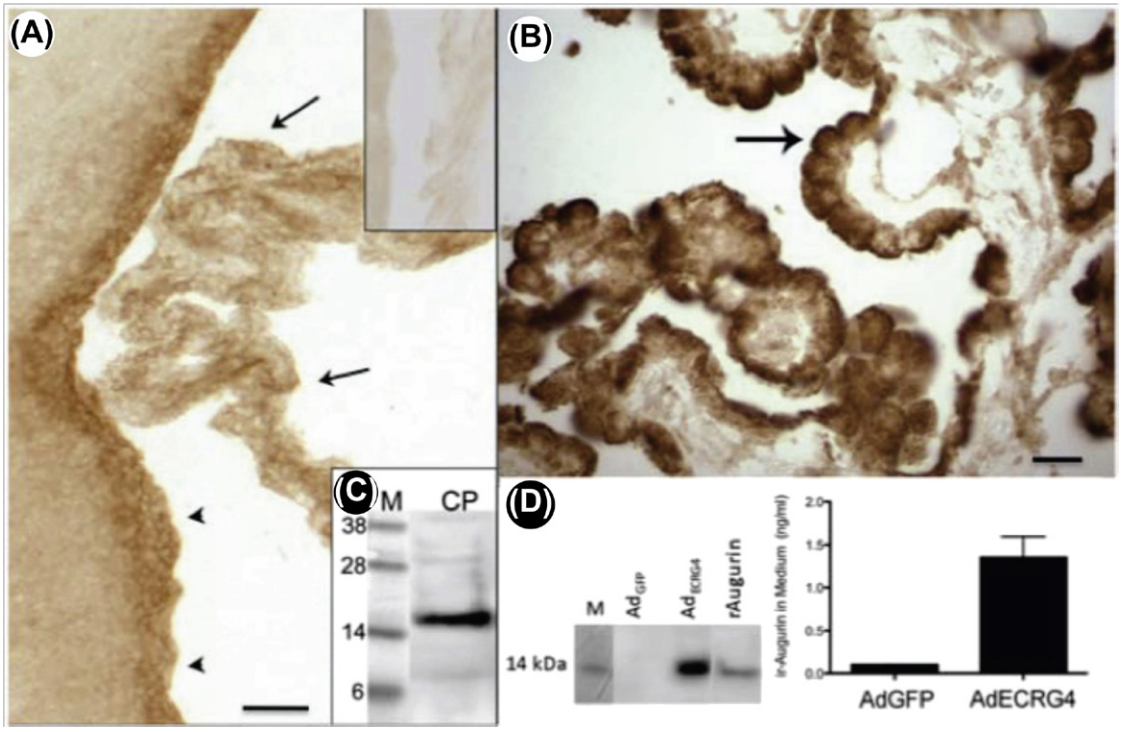

FIGURE 3 Choroid plexus expression of augurin, a 14-kDa processed protein of the *ECRG4* ORF (amino acids 31–148). **(A)** Immunoreactive augurin in adult rat CP (arrow) and ependyma (arrowhead) of third third ventricle, compared with preimmune IgY control (top inset). Scale bar = 20 μm. **(B)** Adult human tissue shows similar augurin staining localized primarily to CPe (black arrow). Bar = 20 μm. **(C)** Rat CP immunoblotting reveals a 14-kDa band, corresponding to the anticipated molecular weight of augurin. **(D)** Augurin secretion *in vitro*: Adult primary human CPe cells, transduced with adenovirus vector (Ad) containing green fluorescent protein gene (Ad_{GFP}), fluoresced (data not shown); and like untreated CP cells in culture, do not express augurin. Cells transduced with Ad containing *Ecrg4* cDNA (Ad_{Ecrg4}) express augurin protein, detectable in RIPA lysates by immunoblotting. Ad_{ECRG4}-transduced CPe secreted augurin into conditioned medium, measurable by ELISA but not by immunoblotting. M = molecular weight marker. rAugurin = recombinant augurin purified from *E coli* and used as standard. Images are from Ref. 5.

can compromise restorative adaptation to diseases and injuries.

CSF-PEPTIDE-MEDIATED HOMEOSTATIC RESPONSES IN CNS PATHOPHYSIOLOGY

Multiple homeostatic mechanisms in CP work to maintain brain fluid composition in a steady state.[10] Marked disturbances of the *status quo* physiology, occurring in stroke and trauma, trigger compensatory actions to restore equilibrium. Diverse insults to the CNS by hyperthermia,[20] TBI, hydrocephalus, hypertension, ischemia, and Alzheimer's disease[27] generate common pathophysiologic sequelae. These include disrupted barriers, ventriculomegaly, cerebral edema, and injured neurons.[21,22,35] Homeostatically upregulated or downregulated peptides have a prominent role in the biochemical and functional adjustments to stress-induced pathophysiology.

Are brain "injury signals" transmitted to the CP to elicit a compensatory response for repairing damaged neural tissue? Supporting evidence comes from the seminal finding of Walter et al.[32] who showed in a rat TBI model that insulin-like growth factor-II (IGF-II), upregulated in CP after a cortical stab wound, is carried by CSF flow to the injury site to promote healing. The CP is sensitive to chemical changes in nearby CSF.[7] Receptors for cytokines and neuropeptides abound at the choroid apical (CSF-facing) membrane.[10] We theorize that "injury signal" peptides (e.g. cytokines), released at the damage site in cortex, diffuse or flow into ventricular CSF. There, cytokine transport and binding to CP epithelial receptors initiate a compensatory response that includes altered peptide synthesis and secretion into the ventricles. CSF thus constitutes the medium for receiving "injury signals" (afferent arm) and then dispersing the CP-derived response peptide (efferent arm) back to the injury site to facilitate healing. Evidence presented herein indicates that CP–CSF augurin, like IGF-II, participates in the efferent arm of an injury response. The augurin injury response, that is, a postulated reduced secretion of augurin into CSF, by CP in which Ecrg4 is downregulated (Fig. 4), may therefore be permissive to stimulate neurogenesis for recovery from TBI.

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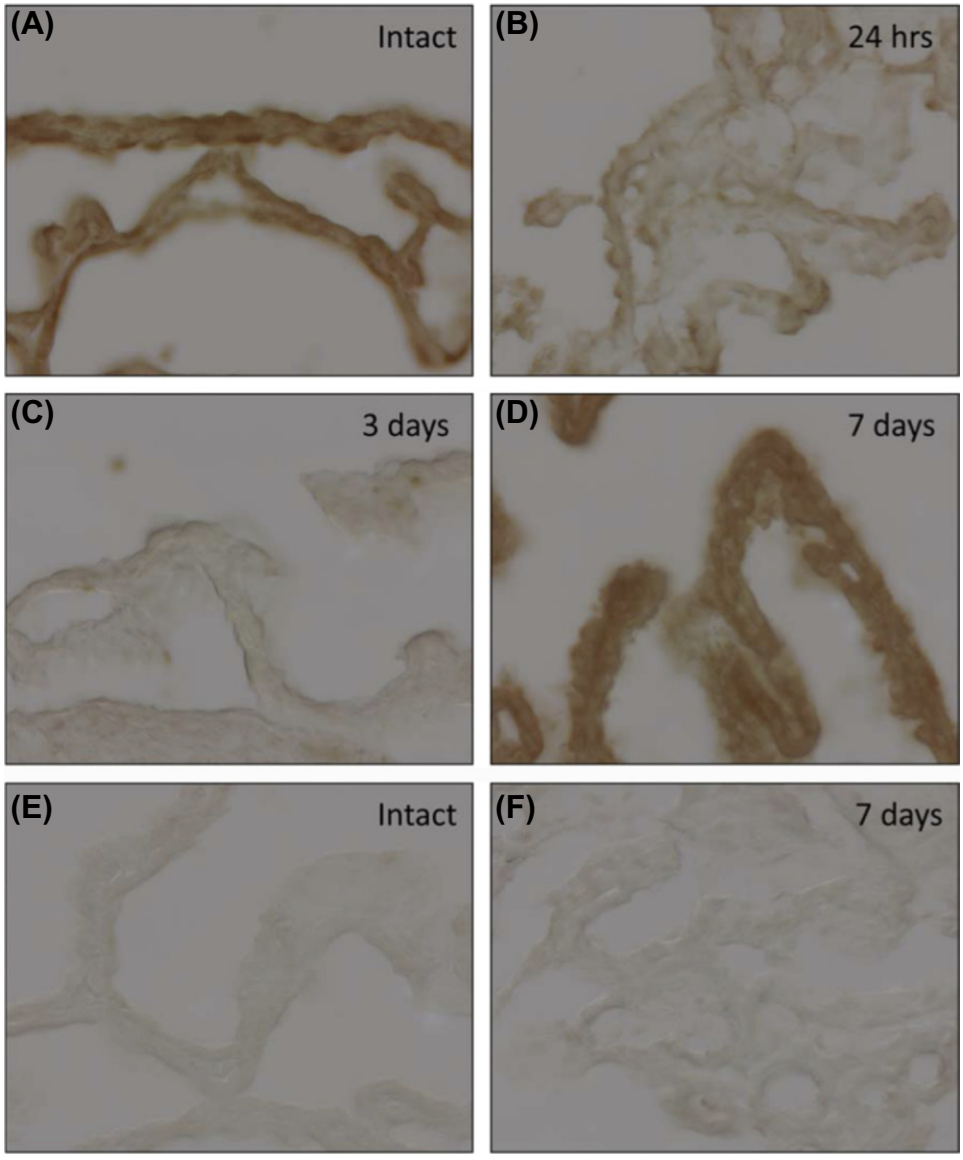

FIGURE 4 Decreased expression levels of *Ecrg4* in CP epithelium after TBI. Brain was stabbed by ophthalmic knife penetration of adult rat dorsal cortex to transect the corpus callosum 3 mm deep and lateral to the right lateral ventricle. Gene expression was evaluated by *in situ* hybridization (binding of an Ecrg4 mRNA antisense probe) in the contralateral CP in intact rat brains **(A)**; and in postinjured specimens at 24 hr **(B)**, 72 hr **(C)**, and 7 d **(D)**. The greatly reduced *Ecrg4* signal (brown reaction product) at 3 d post-TBI is restored to control levels at 7 d. A synthetic, negative control mRNA probe was generated from the pSPT18-Neo plasmid control backbone and used to assess background signal in control (E) and 7 day (F) sections. Images represent 3 rats for each group. Images are from Ref. 17.

ELEMENTS OF AN INJURY RESPONSE FEEDBACK LOOP

Ecrg4 is a candidate neuropeptide gene for responding homeostatically to TBI. Criteria for servomechanistic injury repair, presented below, are offered to encourage future analyses. In modeling CSF–brain homeostasis, the CP is a useful reference point for (i) receiving peptides that function as injury signals, (ii) binding molecular 'signals' released from the trauma site and transported to the blood-CSF interface, (iii) upregulating or downregulating, and secreting, a response peptide (e.g. augurin and/or a growth factor), and then (iv) decreasing or increasing the dispersal of the modulating agent (e.g. augurin or IGF-II) to other CNS regions for beneficial effects that are either a) neurogenic (affecting NPCs) or b) nonneurogenic (promoting wound healing). Criteria i and ii are part of the afferent arm of the response, although iii and iv are related to the efferent arm.

Criterion i awaits elucidation. Candidates include cytokines/peptides released at the injury site. Leukocytes in the inflammation zone, secondary to trauma, may also

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transfer "signals" to first responder cells, for example, CP. By convection or diffusion from brain to ventricular CSF, such 'injury molecules' or cells reach the CP. The second criterion involves receptors for 'injury signals.' Substantial evidence shows that the CSF-facing membrane of CP is studded with receptors for cytokines, growth factors, hormones, and fluid-regulating peptides. This apical membrane also houses Kolmer cells,[24] the antigen-presenting macrophages that are activated when CSF immune molecule concentration increases. Criterion iii deals with altered expression/secretion of peptides by CP post-TBI. It is backed by evidence for IGF-II[32] and augurin.[5,17] Finally, criterion iv involves a circulatory-distributive mechanism carrying CP-secreted peptides to remediate hippocampal and cortical injury sites. This is accomplished by CSF bulk flow from ventricular CP to SVZ/DG, and to cortex enveloping the brain (proximate to subarachnoid CSF). Moreover, ependymal and pia-glial surface permeability[25] allows CSF-borne peptides to readily penetrate the target regions in the injured neural parenchyma.

LEVEL OF AUGURIN PEPTIDE EXPRESSION AND DEGREE OF NPC MITOSIS IN TBI

TBI and other CNS injuries lead to compensatory enhancement of SVZ mitosis and neurogenesis. Neuropeptide mobilization, or its attenuation, has been implicated in the boosted neurogenic response.[8] Interestingly, NPC proliferation occurs in the SVZ/DG of the brain interior, even though the injury site is in overlying cortex distant from CP, CSF, and periventricular NNs. The rescue or improvement of cognition, after TBI damage, depends on greater conversion of NPCs to nascent neurons.

A wide array of neuropeptides modulates neurogenesis. Ecrg4 overexpression and hence augurin overproduction, can regulate cell division of NPCs. An established role of *Ecrg4* as a tumor suppressor gene intimates the ability of its product, augurin, to curtail mitosis even in noncancerous tissue. Therefore, we suggest that augurin normally supplied by CP-CSF to subependymal NPCs constituitively inhibits proliferation in the SVZ/DG. Relieving the inhibitory tone on mitosis, by decreased Ecrg4 expression and curtailed delivery of augurin to the SVZ after injury, would consequently dysinhibit the cells. Enhanced neurogenesis would be the outcome.

Any model implicating a homeostatic feedback role for the BCSFB in response to CNS disturbances must provide convincing evidence that the CP expression level of peptide is sensitive to pathophysiology at an injury site distant from the choroidal epithelium. Dysinhibition pathology could result from neural disease, stroke, or trauma. We selected TBI in part because of the previous observation that another peptide, IGF-II, undergoes enhanced secretion by CP into CSF after a stab wound.[32] Two days after stabbing the rat cortex, there is downregulated Ecrg4 gene expression and augurin protein in CP epithelium and concurrent increase of neurogenesis in the nearby SVZ.[5,17] This fits the notion that after TBI, a progressively decreasing titer of CP–CSF augurin, a probable suppressor of mitosis, is relieving constitutive inhibitory tone on NPC cell division in the SVZ.

What happens in the SVZ after cortical trauma? Observations in rats document an increase both in BrdU incorporation (reflecting greater mitosis) and in the immunoreactive signal for nestin (a biomarker for NPCs). This fits the proposed augmented neurogenesis in subependymal regions such as SVZ. Consequently, a faster rate of production of nascent neurons, and their subsequent incorporation into injured neuronal circuits, would be expected to counter the cognitive diminution suffered in TBI. The potential for Ecrg4-derived peptides such as augurin along with other neuropeptides to facilitate these putative homeostatic mechanisms, which promote neurogenesis, prompts consideration of additional endogenous peptide factors in restoration of the brain after TBI.[8]

TBI has been widely used for analyzing the response of subependymal NNs to CNS injury.[8] Consistently, SVZ neurogenesis is upregulated after physical brain trauma. Moreover, periventricular mitosis is also stimulated by cerebral ischemia.[4,12,30] Although the specific role of augurin in TBI recovery has been implicated, data from other Ecrg4 manipulation experiments (below) bolster the working model that neurogenesis (or SVZ mitosis) is inversely related to the CSF augurin level.

TITRATING SVZ PROLIFERATION BY ALTERING AUGURIN IN THE CP–CSF–EPENDYMAL NEXUS

We sought more evidence for the Ecrg4-neurogenesis model with experiments that ranged from creating injury pathology (plus augurin transfection) to instilling vector-carrying Ecrg4 into intact ventricular CSF or cultured CP epithelium (Table 3). All together, these transfection experiments furnish evidence that CSF augurin from CP/ependyma is a peptide factor that regulates NPC activity level in subependymal regions of the lateral ventricle (Table 3).

Human CP epithelial cells in culture express Ecrg4 and secrete peptides into the *in vitro* conditioned medium (fluid analogous to CSF). Augurin presence in the cultured epithelium was confirmed by ELISA of whole cell lysates, but the smaller peptide fragments were found in media.[5,17] In an *in vivo* experiment on rats, also involving transfection of CP/ependyma by viral vector (ADEcrg4 icv), to boost the level of augurin synthesized *in vivo*, it was the 14 kDa parent augurin peptide that was detected in endogenous CSF by immunoblotting.[17] These two lines of investigation

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thus show that mammalian CP epithelium and ependyma produce Ecrg4-derived peptides for release into ventricular CSF (Table 3). Documentation of these steps of synthesis and release is essential to show in our model that Ecrg4/augurin participates in the CP–CSF modulation of subependymal NPC proliferation.

The ability of Ecrg4/augurin-derived peptides to modify neurogenesis was tested by other approaches. If augurin exerts neurogenic effects by receptor-mediated action, then experimentally changing the concentration of augurin in CP, CSF, and subependymal regions, should reset the response level of the SVZ to TBI. Levels of augurin in the CNS can be "titrated" by experimental manipulation like transduction with Ecrg4 (Table 3). Knowing that icv viral vector transduction of *Ecrg4* alone increases augurin in CSF-SVZ, we sought to reverse the effects of TBI (which normally decreases the peptide level) by first instilling AD_{Ecrg4} into a lateral ventricle *and* then stabbing the cortex.[5] Using BrdU and nestin immunostaining intensity to reflect degree of neurogenesis (endpoint), we obtained evidence that stimulated neurogenesis in TBI is countered (abolished) by swamping CSF with excess exogenous augurin (Table 3). This reinforces our working model that the augurin level in CSF-ependyma-SVZ impacts the brain neurogenic adjustment to TBI.

Advantages accrue to the use of diverse species and stages of development to differentiate the effects of *ecrg4* gene expression. Zebrafish *Ecrg4* has substantially less homology to the human gene than do rodents tested in the TBI model (Tables 1 and 2). With an *Ecrg4* homology of 43–45%, the gene structure in zebrafish is more dissimilar to that of humans than that of rodents (83–84% homology). Accordingly, the polypeptide cleavage products (Fig. 1) may differ substantially across species (fish vs. mammal), leading to unique phenotypes (and responses to injury) when genes are upregulated by vectors or knocked down by morpholinos. Limitations exist in comparing data in Table 3, because of variables in the stage of development and methods for altering gene expression. Nonetheless, a pilot study with zebrafish[5] provides additional clues on how augurin expression in CSF brain impacts CNS fluid homeostasis and progenitor division in the NNs.[5] Knockdown of *ecrg4* in embryonic zebrafish (Table 3) results in disturbed fluid balance (hydrocephalus-like edema) and increased proliferation of GFAP-positive cells. Mitotic activity in NNs includes both neurogenesis and gliogenesis. Consequently, Ecrg4/augurin

TABLE 3 Manipulated Augurin Expression in the CP–CSF–ependyma-SVZ: Impact on Neurogenesis

Species	Model	Treatment or manipulation	Result	Significance	Reference
Human (adult)	Primary culture of CP epithelium	*In vitro* transduction by adenovirus with Ecrg4 transgene	Augurin peptide found in cell lysate and in conditioned medium	CP synthesizes and secretes augurin out of choroid epithelial cells	5,17
Rat (adult)	Viral vector delivery of transgene into the ventricle	ADEcrg4 transgene injected into CSF (exposure for 2 days)	Augurin became tethered to the CP apical surface; CSF augurin (14 kDa) found by immunoblot analysis	Augurin synthesized in CP is secreted for autocrine and endocrine-like actions	17
Rat (adult)	Traumatic brain injury (TBI)	Stab injury to parietofrontal cortex	In CP ↓ immunoreactive augurin and ↓ Ecgr4 gene mRNA	↓ Augurin in CP is a response to TBI and leads to ↑ neurogenesis in SVZ	5,17
Rat (adult)	TBI *plus* overexpression of Ecrg4 in ependyma	Stab wound *plus* ADEcrg4 delivery to ventricular CSF and ependyma	↓ BrdU incorp-oration by SVZ progenitors; ↓ immunoreactive nestin (NPCs)	Ecgr4 overexpression can override TBI effect on the neurogenesis	5
Zebrafish (embryo)	Gene knockdown with morpholino	↓ Expression of Ecrg4 in CSF-brain nexus	↑ Proliferation of GFAP+ cells in brain and induced hydrocephalus and edema	Rescue by coinjected antisense (Ecrg4) suggests augurin-specific effect	5

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may help to control the formation of both neurons and glia in development. Cross-species variation in gene products (augurin, ecilin, argilin, and other peptide fragments) also gives rise to the possibility that injury responses and homeostatic mechanisms vary considerably with differentials in expression and posttranslational processing. Nevertheless, despite phenotypic differences in mammals vs. nonmammalian systems, there is evidence for common effects across phyla and that Ecrg4 expression/augurin production can modulate NPCs in the NNs (Table 3).

AUGURIN RELEASE: RELATION TO AUTOCRINE VS. ENDOCRINE HOMEOSTATIC MECHANISMS

Homeostatic adjustment to CNS injury spans several phases (Fig. 5). Some peptides act in more than one phase of the injury response to foster repair. This seems to be the case for augurin released from CP in brains subjected to physical trauma. Autocrine (local to the same cell) and paracrine/endocrine (distant from the peptide-releasing cell) release/distribution processes are common in peptide physiology. An autocrine function of augurin may contribute to the relatively low rate of tumor formation in plexus epithelium despite great choroidal vascularity and blood flow.[10] The neuroendocrine-like function of augurin, distributed by CSF bulk flow after release from CP, may relate more to peptide conveyance to targeted regions in hypothalamus and SVZ.[8] Accordingly, Ecrg4 expression and augurin modulation of germinal cells in the SVZ have implications for the prevention of primary tumor formation and for adaptive responses of the brain to injury and disease.

CP and ependymal cells that abut CSF prominently regulate cell division in regions surrounding the ventricles. Peptide release mechanisms at the BCSFB and CSF–brain interface, in response to stressful states, are an integral part of the homeostatic control machinery. Various mechanisms and organelles in epithelial cells mediate the extrusion of proteins and polypeptides. Augurin immunostaining of CP epithelium occurs in cytoplasmic vesicles (punctate distribution) and at the apical surface where staining is uniformly dense.[17] Both

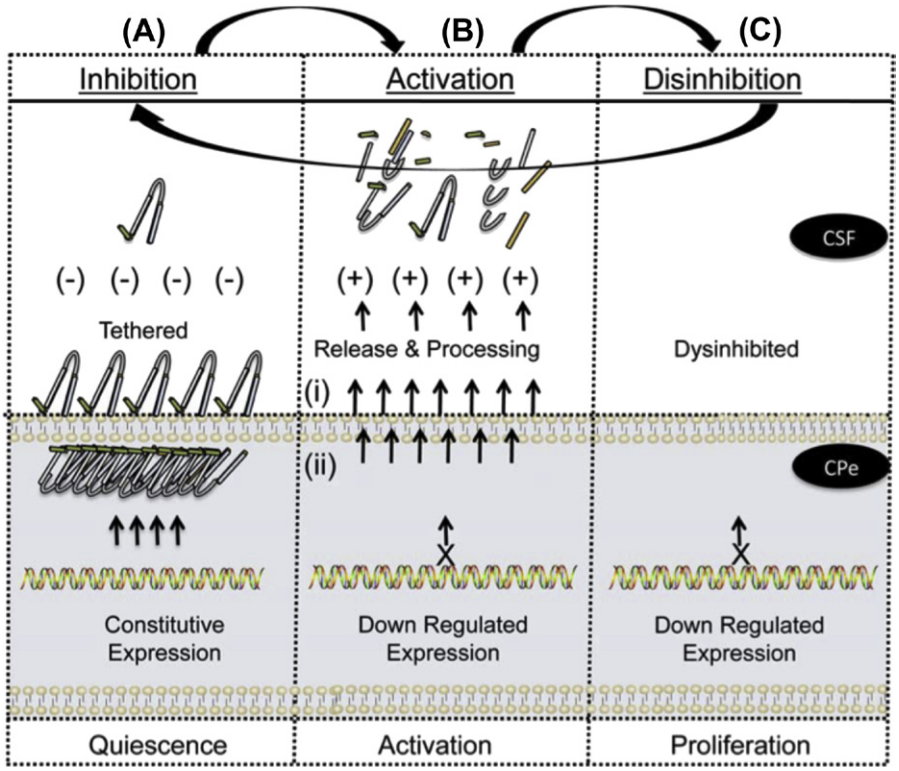

FIGURE 5 Attenuated augurin activity and central neurogenic dysinhibition: a multiphasic model for states of quiescence **(A)**, activation **(B)** And proliferation **(C)**. Choroid epithelial cells (CPe) normally express *Ecrg4*, containing the 14 kDa augurin protein in an inhibitory conformation. Unprocessed peptide is compartmentalized in (i) Cellular vesicles (punctate staining), (ii) the CSF-facing apical membrane (condensed polarized staining), and (iii) the ventricular apex of epithelium to which it tethers. On TBI, there is a sudden release of CP augurin and cleavage apically. Subsequent augurin extrusion (B) from intracellular stores is a "panic signal" to indicate central (brain) injury. The initial proinflammatory response shuts down *Ecrg4* for 1–3 hr post-TBI. Consequently, repair cells proliferate during the early injury stage because the normally constitutive inhibition of neurogenesis by augurin is absent. When *Ecrg4* expression returns to CP and ependyma, periventricular quiescence is restored and CNS homeostasis reestablished. Diagram is from Ref. 17.

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regions are potential release sites for the peptide being translocated from cell to CSF. By separating and analyzing the apical membrane, with the use of subcellular fractionation and confocal microscopy, we identified augurin at the CSF-facing brush border and a smaller fragment that is released from *in vitro* CP cells into the conditioned medium.[17] This 6–8 kDa peptide is a candidate for release into CSF soon after TBI insult and could be distinct from the constituitive release of 14 kDa augurin. Spatially, the proximity of the apical site to CSF favors this region as a rapid responder to any molecular signal brought from injured cortex to induce augurin release from CP. Cytoplasmic vesicle release of augurin, eventually reaching CSF, may represent another mechanism for exerting actions on target cells (NPCs or injured neurons) distant from CP. Peptidergic release mechanisms in CP, hypothetically activated following damage to cortex, need delineation to ascertain the applicability to the initial *immune* response and the later proliferative phase (Fig. 5).

CONCLUSIONS

Neuropeptide modulation of neurogenic niches, especially in regard to generating nascent neurons to maintain sound cognition, has vast translational research potential. Heretofore, several peptides and proteins supplied by the CSF circulation have been strongly implicated in nourishing and protecting stem cells in the NNs bordering the lateral ventricles. Augurin is yet another polypeptide with marked effects on germinal cells, by way of fortifying the interstitial microenviroment in SVZ/DG niches and controlling the mitosis of NPCs. Investigations of the bioactivity profiles of products derived from the Ecrg4-augurin precursor (e.g. ecilin, argilin, and CΔ16), and the cloning of their cognate receptors, will be critically important in advancing basic research and clinical progress with this newly-identified peptide. Although this chapter emphasizes the CP–ependyma–CSF source of augurin, other cells may furnish ecrg4-derived peptides to NNs. If so, they may also eventually prove useful in devising therapeutic regimens to enhance the viability of the NNs and associated cognitive processes.

REFERENCES

1. Chodobski A, Szmydynger-Chodobska J. Choroid plexus: target for polypeptides and site of their synthesis. *Microsc Res Tech* 2001;**52**:65–82.
2. Dietrich MO, Muller A, Bolos M, Carro E, Perry ML, Portela LV, et al. Western style diet impairs entrance of blood-borne insulin-like growth factor-1 into the brain. *Neuromolecular Med* 2007;**9**:324–30.
3. Emerich DF, Skinner SJ, Borlongan CV, Vasconcellos AV, Thanos CG. The choroid plexus in the rise, fall and repair of the brain. *Bioessays* 2005;**27**:262–74.
4. Font MA, Arboix A, Krupinski J. Angiogenesis, neurogenesis and neuroplasticity in ischemic stroke. *Curr Cardiol Rev* 2010;**6**:238–44.
5. Gonzalez AM, Podvin S, Lin SY, Miller MC, Botfield H, Leadbeater WE, et al. Ecrg4 expression and its product augurin in the choroid plexus: impact on fetal brain development, cerebrospinal fluid homeostasis and neuroprogenitor cell response to CNS injury. *Fluids Barriers CNS* 2011;**8**:6.
6. Gonzalez AM, Taylor WM, Johanson CE, King JC, Leadbeater WE, Stopa EG, et al. Co-localization and regulation of basic fibroblast growth factor and arginine vasopressin in neuroendocrine cells of the rat and human brain. *Cerebrospinal Fluid Res* 2010;**7**:13.
7. Johanson C, McMillan P, Tavares R, Spangenberger A, Duncan J, Silverberg G, et al. Homeostatic capabilities of the choroid plexus epithelium in Alzheimer's disease. *Cerebrospinal Fluid Res* 2004;**1**:3.
8. Johanson C, Stopa E, Baird A, Sharma H. Traumatic brain injury and recovery mechanisms: peptide modulation of periventricular neurogenic regions by the choroid plexus-CSF nexus. *J Neural Transm* 2011;**118**:115–33.
9. Johanson C, Stopa E, McMillan P, Roth D, Funk J, Krinke G. The distributional nexus of choroid plexus to cerebrospinal fluid, ependyma and brain: toxicologic/pathologic phenomena, periventricular destabilization, and lesion spread. *Toxicol Pathol* 2011;**39**: 186–212.
10. Johanson CE, Duncan 3rd JA, Klinge PM, Brinker T, Stopa EG, Silverberg GD. Multiplicity of cerebrospinal fluid functions: new challenges in health and disease. *Cerebrospinal Fluid Res* 2008;**5**:10.
11. Johanson CE, Palm DE, Primiano MJ, McMillan PN, Chan P, Knuckey NW, et al. Choroid plexus recovery after transient forebrain ischemia: role of growth factors and other repair mechanisms. *Cell Mol Neurobiol* 2000;**20**:197–216.
12. Kernie SG, Parent JM. Forebrain neurogenesis after focal ischemic and traumatic brain injury. *Neurobiol Dis* 2010;**37**:267–74.
13. Mirabeau O, Perlas E, Severini C, Audero E, Gascuel O, Possenti R, et al. Identification of novel peptide hormones in the human proteome by hidden Markov model screening. *Genome Res* 2007;**17**:320–7.
14. Miyan JA, Nabiyouni M, Zendah M. Development of the brain: a vital role for cerebrospinal fluid. *Can J Physiol Pharmacol* 2003;**81**:317–28.
15. Nilsson C, Lindvall-Axelsson M, Owman C. Neuroendocrine regulatory mechanisms in the choroid plexus-cerebrospinal fluid system. *Brain Res Brain Res Rev* 1992;**17**:109–38.
16. Palm D, Knuckey N, Guglielmo M, Watson P, Primiano M, Johanson C. Choroid plexus electrolytes and ultrastructure following transient forebrain ischemia. *Am J Physiol* 1995;**269**:R73–9.
17. Podvin S, Gonzalez AM, Miller MC, Dang X, Botfield H, Donahue JE, et al. Esophageal cancer related gene-4 is a choroid plexus-derived injury response gene: evidence for a biphasic response in early and late brain injury. *PLoS One* 2011;**6**:e24609.
18. Redzic ZB, Preston JE, Duncan JA, Chodobski A, Szmydynger-Chodobska J. The choroid plexus-cerebrospinal fluid system: from development to aging. *Curr Top Dev Biol* 2005;**71**:1–52.
19. Rodriguez EM, Blazquez JL, Guerra M. The design of barriers in the hypothalamus allows the median eminence and the arcuate nucleus to enjoy private milieus: the former opens to the portal blood and the latter to the cerebrospinal fluid. *Peptides* 2010;**31**:757–76.
20. Sharma HS, Duncan JA, Johanson CE. Whole-body hyperthermia in the rat disrupts the blood-cerebrospinal fluid barrier and induces brain edema. *Acta Neurochir Suppl* 2006;**96**:426–31.
21. Sharma HS, Johanson CE. Blood-cerebrospinal fluid barrier in hyperthermia. *Prog Brain Res* 2007;**162**:459–78.

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22. Sharma HS, Zimmermann-Meinzingen S, Johanson CE. Cerebrolysin reduces blood–cerebrospinal fluid barrier permeability change, brain pathology, and functional deficits following traumatic brain injury in the rat. *Ann N Y Acad Sci* 2010;**1199**:125–37.
23. Shingo T, Gregg C, Enwere E, Fujikawa H, Hassam R, Geary C, et al. Pregnancy-stimulated neurogenesis in the adult female forebrain mediated by prolactin. *Science* 2003;**299**:117–20.
24. Singhrao SK, Neal JW, Rushmere NK, Morgan BP, Gasque P. Differential expression of individual complement regulators in the brain and choroid plexus. *Lab Invest* 1999;**79**:1247–59.
25. Smith DE, Johanson CE, Keep RF. Peptide and peptide analog transport systems at the blood-CSF barrier. *Adv Drug Deliv Rev* 2004;**56**:1765–91.
26. Spector R, Johanson CE. The mammalian choroid plexus. *Sci Am* 1989;**261**:68–74.
27. Stopa EG, Berzin TM, Kim S, Song P, Kuo-LeBlanc V, Rodriguez-Wolf M, et al. Human choroid plexus growth factors: what are the implications for CSF dynamics in Alzheimer's disease? *Exp Neurol* 2001;**167**:40–7.
28. Tadross JA, Patterson M, Suzuki K, Beale KE, Boughton CK, Smith KL, et al. Augurin stimulates the hypothalamo–pituitary–adrenal axis via the release of corticotrophin-releasing factor in rats. *Br J Pharmacol* 2010;**159**:1663–71.
29. Thomas SA, Preston JE, Wilson MR, Farrell CL, Segal MB. Leptin transport at the blood–cerebrospinal fluid barrier using the perfused sheep choroid plexus model. *Brain Res* 2001;**895**:283–90.
30. Tsai YW, Yang YR, Wang PS, Wang RY. Intermittent hypoxia after transient focal ischemia induces hippocampal neurogenesis and c-fos expression and reverses spatial memory deficits in rats. *PLoS One* 2011;**6**:e24001.
31. Veening JG, Barendregt HP. The regulation of brain states by neuroactive substances distributed via the cerebrospinal fluid. *Cerebrospinal Fluid Res* 2010;**7**.
32. Walter HJ, Berry M, Hill DJ, Cwyfan-Hughes S, Holly JM, Logan A. Distinct sites of insulin-like growth factor (IGF)-II expression and localization in lesioned rat brain: possible roles of IGF binding proteins (IGFBPs) in the mediation of IGF-II activity. *Endocrinology* 1999;**140**:520–32.
33. Weaver C, McMillan P, Duncan JA, Stopa E, Johanson C. Hydrocephalus disorders: their biophysical and neuroendocrine impact on the choroid plexus epithelium. In: Hertz L, editor. *Non-neuronal cells of the nervous system: function and dysfunction*. Amsterdam: Elsevier Press; 2004. p. 269–93.
34. Zhao LR, Navalitloha Y, Singhal S, Mehta J, Piao CS, Guo WP, et al. Hematopoietic growth factors pass through the blood-brain barrier in intact rats. *Exp Neurol* 2007;**204**:569–73.
35. Zipser BD, Johanson CE, Gonzalez L, Berzin TM, Tavares R, Hulette CM, et al. Microvascular injury and blood-brain barrier leakage in Alzheimer's disease. *Neurobiol Aging* 2007;**28**:977–86.

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Chapter 227

Diseases Mediated by the BBB: Alzheimer's, Obesity, and Beyond

William A. Banks

ABSTRACT

The blood–brain barrier (BBB) interfaces between the central nervous system (CNS) and circulation and regulates the exchange of informational molecules, including peptides and regulatory proteins. When this regulation is faulty, diseases can occur. Examples of BBB dysfunctions and their associated diseases are impaired transport of amyloid beta protein out of the brain and Alzheimer's disease, impaired transport of leptin into the brain and obesity, recovery of methionine enkephalin efflux in animals dependent on alcohol and alcohol withdrawal seizures, impaired transport of glucose into the brain and developmental retardation, enhanced insulin transport into brain and the insulin resistance of sepsis.

INTRODUCTION

The blood–brain barrier (BBB) prevents the unrestricted leakage of substances between the blood and the central nervous system (CNS) fluids (the brain's interstitial fluid and the cerebrospinal fluid). The BBB also transports into the brain the glucose, amino acids, vitamins, minerals, and other nutrients required for brain function and transports out of the brain toxins, electrolytes, and xenobiotics. Some peptides and regulatory proteins cross the BBB, in the blood-to-brain direction, the brain-to-blood direction, or bidirectionally. Immune cells are increasingly recognized as being able to cross the BBB under physiological, not under just pathological, conditions. Taken together, this means that the BBB has protective, nutritive, homeostatic, and communication roles to play toward the brain. When the BBB fails to perform these roles properly, diseases can arise.

It is well appreciated that the BBB can, like other tissues of the body, be a target of diseases. But this chapter will emphasize a different category of disease: those in which the BBB plays a central role, even a causal role, in disease.

LEPTIN AND OBESITY

A hallmark of obesity in humans is a resistance to the anorectic actions of leptin. Leptin is a 16- kDa protein secreted from fat cells, which acts at the arcuate nucleus in the hypothalamus to inhibit feeding and to promote thermogenesis.[15] Leptin is one-fourth the size of albumin and is too large to cross the BBB without the aid of a saturable transporter. Such a transporter has been shown *in vivo* and *in vitro* in brain endothelial cells and *in vivo* at the choroid plexus. Leptin is transported across the BBB into all major brain regions, but transport is especially high at the arcuate nucleus.

Resistance to the CNS actions of leptin arises in obese humans and in rodents with diet-induced obesity.[15] In general, this resistance first occurs at the level of the BBB.[3] At this stage, rodents can still respond to leptin given directly into the brain but no longer respond to leptin given peripherally. Analysis of the cerebrospinal fluid (CSF) and serum levels of leptin show that in obese humans the major level of resistance is also at the BBB.[8]

Several laboratories have shown that leptin transport across the BBB is decreased in obese animals. Exogenous leptin is blocked from entering the CNS in part because of competitive inhibition by the high levels of leptin in the serum of obese animals. However, this self-inhibition explains only a minor part of the BBB defect seen in obesity. Other components, including those not immediately ascribable to circulating factors, are major contributors to an impaired transport of leptin across the BBB. These defects in BBB transport are acquired in tandem with increasing obesity and reversible with weight loss. Starvation also impairs leptin transport across the BBB.

The findings of inducible and reversible defects in leptin transport show that the transporter is not static, but responsive to metabolic needs. This, in turn, implies that the leptin transporter is itself regulated. To date, several agents have

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00227-X
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been noted to modulate leptin transport: insulin, cholecystokinin, glucose, adrenergic agents, and triglycerides.[1,4,12,17] These agents are themselves major players in energy use in general and involved in other aspects of leptin metabolism.

Leptin transport is enhanced by the administration of alpha$_1$-adrenergics.[1] Transport can be more than tripled with the intravenous or intraperitoneal administration of epinephrine, but central administration is without effect. Enhanced transport of the anorectic leptin into the brain could be one mechanism by which peripheral administration of epinephrine induces weight loss. Leptin enhances sympathetic tone to fat and other tissues and stimulates the release of epinephrine into blood by binding to leptin receptors located on the adrenal gland's epinephrine-secreting cells. Hence, mice that lack leptin have decreased blood epinephrine levels. This increased sympathetic tone, in turn, suppresses leptin secretion from fat cells by binding to β3 adrenergic receptors. This forms a negative feedback loop between leptin and epinephrine and explains the inverse relation between blood levels of epinephrine and leptin. Epinephrine-enhanced transport of leptin would reinforce this inverse relation between serum epinephrine and leptin levels in two ways. First, by elevating the brain levels of leptin, increased serum levels of epinephrine would be increasing the anorectic signal to the brain. This would eventually lead to a decrease in body fat, resulting in lower leptin levels. Second, leptin and epinephrine have opposite effects in the CNS than in the periphery. Specifically, leptin inhibits epinephrine release from neurons and CNS epinephrine stimulates feeding. Thus, peripheral epinephrine reinforces its anorectic effect by indirectly, through leptin, inhibiting its antianorectic CNS levels.

Leptin transport is inhibited by the peripheral administration of triglycerides.[4] Triglycerides are increased in obesity, and so, this is probably a major mechanism by which obesity mediates a decrease in leptin transport. In fact, the rate of leptin transport is inversely related to serum triglyceride levels in diet-induced obesity and in fasted animals. Unlike most other metabolic effects induced by triglycerides, the effects on leptin transport are mediated by the triglycerides themselves and not by the free fatty acids (FFA), which can be hydrolyzed from them. The effects of triglycerides on leptin transport can also be shown by lowering of the triglycerides pharmacologically and by direct administration of triglycerides *in vivo* and *in vitro*.

As with epinephrine, the interaction between the BBB transporter for leptin and triglycerides is important in a negative feedback loop between leptin action and triglycerides. Leptin decreases triglyceride levels by promoting triglyceride hydrolysis and FFA oxidation and inhibiting FFA synthesis. Patients with severe lipodystrophy or lipoatrophy (i.e., little or no fat and so little or no leptin) also have very severe hypertriglyceridemia, which is reversed by treatment with leptin. The ability of triglycerides to induce leptin resistance at the level of the BBB would block the leptin-induced shift toward use of triglycerides as an energy source and so help to conserve fat stores and keep triglyceride levels elevated.

Triglyceride inhibition of leptin transport may also explain the fundamental paradox of leptin resistance in obesity: Why should leptin resistance lead to the development of obesity, when otherwise it would be so effective in maintaining body weight? Triglycerides are also elevated in starvation. During most of evolution, animals have been confronted with the problem of obtaining enough calories in the diet rather than having to deal with too many. Thus, healthy baboons living in the wild in nonfamine conditions have body fat mass and serum leptin levels seen only with anorectic conditions in Western humans.[22] Only when food sources become abnormally abundant do leptin levels approach those seen in Westerners and, with it, evidence of obesity-related diseases. Through evolutionary pressures, hypertriglyceridemia probably has come to represent to the brain a signal of starvation, rather than of obesity. The ability of triglycerides to inhibit the transport of an anorectic signal to the brain would provide great survival value to the starving animal.

AMYLOID β PROTEIN EFFLUX AND ALZHEIMER'S DISEASE

Amyloid β peptide (Abeta) has emerged as playing a major role in the cause of Alzheimer's disease. Abeta is neurotoxic and both transgenic mice and those with spontaneous mutations (the SAMP8 mouse) that overexpress Abeta develop cognitive impairments.[18] Reversals of histological changes and cognitive impairment have been shown in transgenics after active or passive vaccination to Abeta. In the SAMP8, antisense directed against amyloid precursor protein or antibodies directed against Abeta reverse cognitive impairments, reverse oxidative stress, and improve cholinergic tone.

The level of a substance in most tissues is the result of an equilibrium between synthesis and degradation. For substances in the brain, including Abeta, two additional factors are also important: transport into the brain from blood and efflux from the brain into blood.[26] In the case of Abeta, both of these events are mediated by saturable transporters located at the BBB. The receptor for advanced glycation end products, or RAGE, is the main influx transporter and both LDL receptor–related protein-1 and p-glycoprotein to act as efflux transporters.[14]

Evidence from both humans and animal models suggests that efflux of Abeta is impaired in Alzheimer's disease. Levels of LDL receptor–related protein-1 are reduced in the brain vasculature of patients with Alzheimer's disease.[23] Efflux

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is impaired in both transgenics and SAMP8 mice. Efflux of $Abeta_{1–42}$, the more toxic form of Abeta, is impaired more than is the efflux of $Abeta_{1–40}$ in the aged SAMP8.[9] However, LDL receptor–related protein-1 has a preference for $Abeta_{1–40}$. Whether separate transporters exist for the 1–40 and 1–42 forms is not currently established. The rate of reabsorption of cerebrospinal fluid into the blood, known as bulk flow, is also impaired in normal aging, in Alzheimer's disease, and in the SAMP8 model. Thus, both the saturable and the nonsaturable clearances of Abeta from brain are impaired.

Whether decreased efflux is caused from alterations in Abeta primary structure/conformation vs. alterations in transporter synthesis and function remains unknown. Either is possible, as mutations of Abeta (such as at codon[22]) can result in proteins not transported by the BBB and mutations in LDL receptor–related protein-1 results in accumulation of Abeta by brain. Work with SAMP8 mice suggests another mechanism, that of impairment induced by Abeta itself. Treatment of SAMP8 with antisense directed to Abeta restores both the saturable efflux of Abeta and bulk flow.[7] In addition, inflammatory processes inhibit Abeta efflux at the BBB.[16] Oxidative stress could be a common mechanism by which Abeta and inflammatory processes inhibit Abeta efflux.[19]

Lack of clearance from brain could be a major contributor to accumulation of Abeta levels in brain. This lack of clearance of Abeta from brain, in turn, could explain how potential therapies work. Passage and active immunization against Abeta results in antibodies in the circulation which bind Abeta and so prevent it from crossing from blood-to-brain.[20] This would prevent the influx mechanism from contributing to the brain levels of Abeta. It could also prevent any Abeta that is effluxed from brain into blood from returning to brain. CSF shunting has also been proposed as a mechanism by which unidentified toxins contributing to Alzheimer's disease are rid from the brain. Shunting could restore a type of clearance of Abeta from the brain.

In summary, Abeta is transported by saturable mechanisms both into and out of the CNS. Impairment of the BBB's mechanisms for efflux of Abeta from the brain is postulated to be a major mechanism by which this protein accumulates in the brain of Alzheimer's disease patients. Antisense directed toward Abeta can restore the BBB's efflux mechanisms. Further, treatment with antibodies or with CSF shunting may act by restoring a sort of balance between the influx and efflux rates of Abeta.

METHIONINE ENKEPHALIN AND ALCOHOL WITHDRAWAL

An inverse relation exists between the levels of methionine enkephalin in brain and the amount of alcohol an animal will voluntarily drink.[5,10] This relation has been shown to occur across strains that are alcohol-naive and in animals whose brain levels of methionine enkephalin have been manipulated. Physical dependence to alcohol also lowers the brain levels of methionine enkephalin.

A major determinant of the brain levels of methionine enkephalin is peptide transport system-1 (PTS-1). This is an efflux (brain-to-blood) saturable transport system located at the BBB, which has methionine enkephalin as one of its major ligands.[6] Studies across strains and among individuals within a strain suggest that PTS-1 accounts for one-third to half of the variation in brain levels of methionine enkephalin. PTS-1 activity is greatly reduced in rodents made physically dependent on alcohol. Because these animals have reduced levels of methionine enkephalin, the decrease in PTS-1 activity can be seen as adaptive as it would conserve the brain levels of methionine enkephalin. In physically dependent animals, mRNA and protein levels of preproenkephalin no longer correlate with the brain levels of methionine enkephalin. Thus, posttranslational mechanisms become the sole determinants of brain levels of methionine enkephalin.

Mice made physically dependent to alcohol have a reduction in their brain levels of methionine enkephalin; paradoxically, these mice have a further reduction in brain methionine enkephalin levels when they stop drinking. This further reduction is thought to underlie the withdrawal seizures these animals undergo. PTS-1 is a probable cause for this paradoxical decrease in brain methionine enkephalin levels. Although brain levels of methionine enkephalin remain under posttranslational control, PTS-1 recovers its activity within hours of cessation of alcohol drinking. Thus, although the mechanisms for increasing the synthesis of methionine enkephalin (increases in transcription and translation) have not recovered, the mechanism for reducing brain levels (saturable efflux) has. In theory, blockade of this early recovery of PTS-1 could prevent alcohol withdrawal seizures.

GLUT-1 DEFICIENCY SYNDROME

De Vivo et al have described a familial syndrome in a number of patients caused by mutations in the GLUT-1 transporter.[13] Glucose levels in CSF are usually <2.5 mmol/l (~50% below normal), and most cases are caused by a mutation in the SLC2A1 gene, with a number of missense, nonsense, insertion, and deletion mutations described. These patients are characterized by infantile seizures, developmental delays, acquired microencephalopathy, spastic ataxia, and low levels of glucose in the CSF. Because the red blood cell also has GLUT-1, a test measuring the uptake of methyl-D-glucose by red blood cells is useful in the diagnosis of this syndrome. The *V*max for the red cell GLUT-1 is about 50% of normal with no change in K_m.

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INSULIN RESISTANCE IN SEPSIS

Insulin and insulin receptors are found throughout the CNS. Originally, the source of CNS insulin was debated, but subsequent work has suggested that little or no mRNA for insulin is found in the CNS.[2] Thus, under normal circumstances most, if not all, of CNS insulin originates from the pancreas. Seminal work by Margolis and Altszuler in the 1960s and by Woods, Porte, colleagues, and others showed correlations between blood and CSF levels of insulin and established that insulin in the CNS has important effects on feeding and metabolism.[24] Work from several laboratories showed that many of the effects of CNS insulin are opposite to those of peripheral insulin. For example, CNS insulin decreases the levels of insulin in blood, increases glucose levels in blood, and inhibits feeding. Thus, insulin can act as its own counter-regulatory hormone. Short-term, CNS insulin would then be believed to produce a kind of insulin resistance. Paradoxically, the long-term effect of CNS insulin seems to be to prevent insulin resistance.[11] This latter conclusion is based on the work in which transgenic animals with a selective knockout of CNS insulin receptors are prone to diet-induced obesity, increased serum insulin levels, resistance to the hypoglycemic effects of insulin, and increased body fat. The paradox that both short- and long-term insulin have opposite effects on insulin resistance is easily reconciled if the most important effect of short-term insulin in the CNS is suppression of appetite. By acting to prevent obesity, CNS insulin would safeguard against this major cause of insulin resistance.

CNS insulin probably plays important roles in several pathophysiological conditions, including insulin resistance and body weight control in inflammatory conditions. CNS insulin can improve cognitive function in Alzheimer's disease.[21] These conditions are associated with both insulin resistance and body weight loss. Treatment of mice with lipopolysaccharide (LPS) enhances insulin transport across the BBB.[25] LPS is derived from the cell walls of gram-negative bacteria and induces the release of proinflammatory cytokines. These cytokines probably are responsible for the effects of LPS on the BBB transport of insulin. The enhanced transport of insulin across the BBB would counter peripheral insulin's effects and so provides a mechanism for insulin resistance in sepsis.

SUMMARY

The BBB is pivotal in an endocrine-like communication between the CNS and peripheral tissues. The BBB does this by regulating the exchange of information molecules such as peptides and proteins between the CNS and peripheral tissues. When the BBB does not perform this function correctly, diseases can occur. Here, five examples of BBB dysfunction and associated diseases were discussed. In most of these cases, the exact magnitude of the contribution of BBB dysfunction to the overall disease process is not established. However, these examples serve to illustrate the concept that the BBB is intimately involved in the onset and promotion of disease processes.

REFERENCES

1. Banks WA. Enhanced leptin transport across the blood–brain barrier by alpha1-adrenergic agents. *Brain Res* 2001;**899**:209–17.
2. Banks WA. The source of cerebral insulin. *Eur J Pharmacol* 2004;**490**:5–12.
3. Banks WA. The blood–brain barrier as a cause of obesity. *Curr Pharm Des* 2008;**14**:1606–14.
4. Banks WA, Coon AB, Robinson SM, Moinuddin A, Shultz JM, Nakaoke R, et al. Triglycerides induce leptin resistance at the blood–brain barrier. *Diabetes* 2004;**53**:1253–60.
5. Banks WA, Jaeger LB, Urayama A, Kumar VB, Hileman SM, Gaskin FS, et al. Preproenkephalin targeted antisenses cross the blood–brain barrier to reduce brain methionine enkephalin levels and increase voluntary drinking. *Peptides* 2006;**27**:784–96.
6. Banks WA, Kastin AJ. The role of the blood–brain barrier transporter PTS-1 in regulating concentrations of methionine enkephalin in blood and brain. *Alcohol* 1997;**14**:237–45.
7. Banks WA, Kumar VB, Farr SA, Nakaoke R, Robinson SM, Morley JE. Impairments in brain-go-blood transport of amyloid-beta and reabsorption of cerebrospinal fluid in an animal model of Alzheimer's disease are reversed by antisense directed against amyloid-beta protein precursor. *Journal of Alzheimer's Disease* 2011;**23**:599–605.
8. Banks WA, LeBel C. Strategies for the delivery of leptin to the CNS. *J Drug Target* 2002;**10**:297–308.
9. Banks WA, Robinson SM, Verma S, Morley JE. Efflux of human and mouse amyloid beta proteins 1–40 and 1–42 from brain: impairment in a mouse model of Alzheimer's disease. *Neuroscience* 2003;**121**:487–92.
10. Blum K, Elston SFA, DeLallo L, Briggs AH, Wallace JE. Ethanol acceptance as a function of genotype amounts of brain [met]-enkephalin. *Proc Natl Acad Sci U S A* 1983;**80**:6510–2.
11. Bruning JC, Gautam D, Burks DJ, Gillette J, Schubert M, Orban PC, et al. Role of brain insulin receptor in control of body weight and reproduction. *Science (Washington DC)* 2000;**289**:2122–5.
12. Cano V, Merino B, Ezquerra L, Somoza B, Ruiz-Gayo M. A cholecystokinin-1 receptor agonist (CCK-8) mediates increased permeability of brain barriers to leptin. *Br J Pharmacol* 2008;**154**:1009–15.
13. De Vivo DC, Trifiletti RR, Jacobson RI, Ronen GM, Behmand RA, Harik SI. Defective glucose transport across the blood-brain barrier as a cause of persistent hypoglycorrhachia, seizures, and developmental delay. *N Engl J Med* 1991;**325**:703–9.
14. Deane R, Sagare A, Zlokovic B. The role of the cell surface LRP and soluble LRP in blood–brain barrier Abeta clearance in Alzheimer's disease. *Curr Pharm Des* 2008;**14**:1601–5.
15. Friedman JM, Halaas JL. Leptin and the regulation of body weight in mammals. *Nature* 1998;**395**:763–70.
16. Jaeger JB, Dohgu S, Lynch JL, Fleegal-DeMotta MA, Banks WA. Effects of lipopolysaccharide on the blood–brain barrier transport of amyloid beta protein: a mechanism for inflammation in the progression of Alzheimer's disease. *Brain Behav Immun* 2009;**23**:507–17.
17. Kastin AJ, Akerstrom V. Glucose and insulin increase the transport of leptin through the blood–brain barrier in normal mice but not in streptozotocin-diabetic mice. *Neuroendocrinology* 2001;**73**:237–42.

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18. Morley JE, Farr SA, Kumar VB, Banks WA. Alzheimer's disease through the eye of a mouse: acceptance lecture for the 2001 Gayle A. Olson and Richard D. Olson prize. *Peptides* 2002;**23**:589–99.
19. Owen JB, Sultana R, Aluise CD, Erickson MA, Price TO, Bu G, et al. Oxidative modification to LDL receptor-related protein 1 in hippocampus from subjects with Alzheimer's disease: implications for Abeta accumulation in AD brain. *Free Radic Biol Med* 2010;**49**:1798–803.
20. Pan W, Solomon B, Maness LM, Kastin AJ. Antibodies to beta-amyloid decrease the blood-to-brain transfer of beta-amyloid peptide. *Exp Biol Med* 2002;**227**:609–15.
21. Reger MA, Watson GS, Green PS, Baker LD, Cholerton B, Fishel MA, et al. Intranasal insulin administration dose-dependently modulates verbal memory and plasma amyloid-beta in memory-impaired adults. *J Alzheimer's Disease* 2008;**13**:323–31.
22. Rutenberg GW, Coelho Jr , Lewis DS, Carey KD, McGill Jr . Body composition in baboons: evaluating a morphometric method. *Am J Primatol* 1987;**12**:275–85.
23. Shibata M, Yamada S, Kumar SR, Calero M, Bading J, Frangione B, et al. Clearance of Alzheimer's amyloid-beta 1-40 peptide from brain by LDL receptor-related protein-1 at the blood–brain barrier. *J Clin Invest* 2000;**106**:1489–99.
24. Woods SC, Seeley RJ, Baskin DG, Schwartz MW. Insulin and the blood–brain barrier. *Curr Pharm Des* 2003;**9**:795–800.
25. Xaio H, Banks WA, Niehoff ML, Morley JE. Effect of LPS on the permeability of the blood-brain barrier to insulin. *Brain Res* 2001;**896**:36–42.
26. Zlokovic BV, Yamada S, Holtzman D, Ghiso J, Frangione B. Clearance of amyloid beta-peptide from brain: transport or metabolism? *Nat Med* 2000;**6**:718–9.

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Chapter 228

Hypothalamic Neuropeptides

David J. Begley

ABSTRACT

Until the mid-1980s, it was generally believed that peptides did not cross the blood–brain barrier (BBB), as it was held that their relatively polar nature and high molecular weight would simply lead to their physical exclusion from the central nervous system by the combination of the endothelium and their paracellular tight junctions. In the intervening years, this notion has been quite clearly shown to be mistaken, with many peptides now shown to exert central actions by virtue of either passive or carrier-mediated movement across the BBB. This realization that peptides are part of the complex regulation of physiology via feedback phenomena between blood and brain, and vice versa, is in full accordance with the modern concept of the BBB as a dynamic, reactive, and adaptive interface between the brain and the peripheral tissues. In this chapter, the interaction of many peptides with the BBB, better known for their endocrine function via the pituitary gland, is reviewed.

INTRODUCTION

A large spectrum of biologically active peptides are synthesized by and released by the hypothalamus. These peptides are neuropeptides synthesized by neurons with neurotransmitter/neurohormonal roles, which either regulate the function of the anterior pituitary or in the case of vasopressin and oxytocin are released by the posterior pituitary into the systemic circulation. Additionally, within both the CNS generally and also within the hypothalamus, many of these peptides also have more traditional neurotransmitter roles acting at synapses between neurons.[32]

All the neuropeptides synthesized by the neurons of the hypothalamus as regulatory hypophysiotropic hormones of the anterior pituitary or as systemic hormones are released, at synaptic endings terminating outside of the blood–brain barrier (BBB) within anatomical sites where tight junctions between endothelial cells do not form and minimal BBB function exists. For the hypothalamic neuropeptides, the release structures are the median eminence and the neurohypophysis (posterior pituitary). These release areas, including the pineal gland, transfer neurohormones to blood, and are often referred to as neurohemal organs, which form part of the larger complex of circumventricular organs (CVOs) that are specialized sites in the brain where no BBB exists.[29]

The circumventricular organs	Function
Median eminence	Neurohemal
Neurohypophysis	Neurohemal
Pineal gland	Neurohemal
Choroid plexus	Neurohemal/sensory/ secretory (CSF)
Area postrema	Sensory
Subcommisural organ	Sensory
Subfornical organ	Sensory
Organum vasculosum lamina terminalis	Sensory

The function of the CVOs is to act as windows in the BBB and, thus by virtue of their lack of BBB, permit localization and equilibration of a small volume of parenchymal interstitial fluid to occur with plasma for a wide range of solutes. Within the CVOs, either sensitive dendrites or nerve endings can respond to changes in extracellular solute content, or neurosecretory neuronal terminals can specifically secrete their product into systemic blood. The interstitial fluid within the CVOs is effectively isolated from the bulk of the extracellular fluid in the brain by a layer of ependymal cell with tight junctions between them.[29] Without CVOs, the brain would be sealed off by the BBB from direct influence by blood-borne solutes and additionally could not secrete neurohormones into the systemic circulation. The combination of BBB and the CVOs allows the brain to maintain its own specialized independent internal environment and still to communicate freely and specifically with the soma via blood-borne signals.

Many active neuropeptides found in the hypothalamus are known to interact with the BBB at large and are able to either penetrate it directly by passive diffusion or are transported across the endothelial cells by specific saturable mechanisms. These BBB transport processes may also form part of a mechanism whereby feedback mechanisms for systemically secreted CNS peptides can operate and also

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00228-1
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provide a means by which peripherally released peptides from other endocrine sites can alter and influence CNS function. The ability of some hypothalamic peptides to cross the BBB also opens a door for the clinical use of these peptides or their analogs as therapeutic agents.[10]

A large variety of peptides are recognized as endogenous to the hypothalamus although this brain structure is not the exclusive site of their synthesis and release. The remainder of this handbook entry contains brief reviews of the interactions of selected hypothalamic peptides with the BBB. Some other peptides, which could be considered to be hypothalamic neuropeptides and whose interactions with the BBB are covered elsewhere in this section will not be included. The concept of hypophysiotropic hormones has also been somewhat modified in recent years with the realization that a single regulatory peptide may influence the release more than one anterior pituitary hormone. For example, growth hormone (GH) can be released by GHRH (GH-releasing hormone), ghrelin, TRH (thyrotropin-releasing hormone), and PACAP (pituitary adenylcyclase-activating polypeptide) and its release is inhibited by somatostatin, while in mammals, TRH will also release prolactin in addition to thyrotropin (TSH) and GnRH (gonadotropin-releasing hormone) releases FSH (follicle stimulating hormone) as well as LH (luteinizing hormone). Clearly, the influences are complex and much depends on the microanatomically specific release of neuropeptide delivered to a specific target either within the CNS or to a specific group of secondary endocrine response cells. In addition, neuropeptide influence on the CNS via the systemic route will differ, whether it is by direct influence through non-BBB areas or via transport across the BBB.

TRH

TRH is a tripeptide synthesized in the paraventricular nucleus releasing predominantly TSH from the anterior pituitary. Intact TRH seems to cross the BBB passively with a permeability thrice that of mannitol.[35] TRH is also subject to hydrolysis, apparently on contact with the BBB as in the experimental system there is no plasma protein present containing circulating peptidases. Its extraction, when radioactively labeled at the proline residue, can be reduced by approximately 50% if the peptidase inhibitor bacitracin (2 mM) is added to the experimental pefusate. The BBB permeability to TRH cannot be reduced further by the addition of 1 mM TRH in addition to the enzyme inhibitor indicating a lack of a saturable component to the transport.[35] A further series of experiments with the perfused sheep choroid plexus also shows a similar passive movement of TRH with peptide hydrolysis on contact with the tissue.[34]

TRH has central actions after iv administration and has antidepressive properties and improves cognition. However its short plasma half-life greatly limits its potency. The addition of methyl groups to the molecule to give a 3-methyprolinamide (RX74355), or a 3,3-dimethylprolinamide (RX77368), extends the plasma half-life, increases lipid solubility, and as a direct result enhances the CNS penetration and activity.[11]

GnRH

GnRH (also called LHRH) is a decapepetide synthesized by neurons with cell bodies located in the ventral hypothalamus and in the mammal releases both FSH and LH from the anterior pituitary.[31,32] Intravenous administration of GnRH potentiates sexual behavior in both male and female rodents and enhances the central actions of dopamine and serotonin. These effects are evident in castrated and hypophysectomized animal and are highly suggestive of transport across the BBB.[8]

GnRH is accumulated by many regions of the rat brain after intravenous injection.[18] GnRH has saturable bidirectional transport from blood to brain. Inclusion of excess10 nM/mL of GnRH into the vascular perfusate significantly inhibits brain uptake of the peptide by the BBB. AVP and Tyr-MIF 1 are without influence on blood-to-brain GnRH transport. Transport out of cerebrospinal fluid (CSF) occurs to an extent greater than would be expected from CSF turnover after intraventricular injection of the peptide. The introduction of excess GnRH, D-Tryp^6-GnRH, and GnRH 7–10 inhibits efflux from CSF. GnRH 1–3 and tyrosine do not inhibit efflux from CSF. It is not clear whether transport in both directions is the result of a single transporter or if two separate transporters are involved.[8]

CORTICOTROPIN-RELEASING HORMONE (CRH)

CRH (41aa) is a classic hypophysiotropic hormone releasing ACTH from the pituitary. It is synthesized and released by the parvicellular neurons of the paraventricular nucleus.[12] CRH is a member of a peptide family that includes urocortin (40aa), urocortin II (38aa), and urocortin III (38aa). The peptides have a high degree of homology. CRH and urocortin I bind to the same receptor CRH-R1, whereas urocortins I, II, and III, bind to CRH-R2.[24] All members of the family are found in the brain and modulate endocrine, autonomic, and behavioral responses to stress.[24] The peptides all inhibit feeding behavior but to different extents.[22]

Their interactions with the BBB are also very different. Urocortin I only enters the brain after a specific saturable carrier for the peptide is activated by circulating leptin. Urocortin II enters the brain passively as a result of its inherent lipophilicity by a nonsaturable mechanism, whereas urocortin III is degraded by BBB enzymes and does not seem to enter the brain intact. CRH itself enters the CNS by a separate saturable transport system, which is not sensitive

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to urocortins I, II, III, or leptin.[22] CRH is the only member of this family of peptides to have a saturable efflux system out of the brain with a half time of 15 min compared with that of 50 min for albumin.[25] Transport is inhibited by verapamil, ouabain, colchicine, TNFα, and β-endorphin, which might suggest the involvement of an ABC transporter. It is also interestingly enhanced by corticosterone but seems to be cyclosporin insensitive.[25]

VASOPRESSIN (ANTIDIURETIC HORMONE)

Vasopressin is a nonapeptide synthesized in the magnocellular cells of the supraoptic and parventricular nuclei of the hypothamus. After posttranslational processing of the proprotein, the hormone is transported to the posterior pituitary in the axons forming the hypothalamic–hypophysial tract where it is released into systemic circulation after appropriate osmotic and baroreceptor stimulation of the CNS. Its major target is usually considered as the posterior convoluted tubule and the collecting duct of the kidney via V_2 receptors where it stimulates aquaporin insertion into the tubular cell membranes and thus enhances water resorption. The name vasopressin however arises from the peptide's effect of promoting arteriolar smooth muscle contraction in response to significant hemorrhage by acting on the smooth muscle V_1 receptors.

In addition, vasopressin is also recognized as having significant CNS activity associated with an elevated plasma concentration and V_1 receptors are found in the brain. Vasopressin has long been associated with the reinforcement of memory and attention and avoidance behavior[14,27] and an increase in circulating vasopressin has been shown to reduce the blood-to-brain transport of certain large neutral amino acids.[13,30] This effect seems to result from a direct change in the activity of the large neutral amino acid transporter mediated by vasopressin at the BBB. After intracarotid injection vasopressin accumulates in all regions of the brain with a BBB to an extent greater than the reference marker inulin. This accumulation is more marked in pineal gland, the choroid plexus, the neural lobe, and the anterior pituitary, all lacking an endothelial BBB and the extraction ratio of vasopressin to inulin remains similar suggesting no preferential extraction of vasopressin.[17]

The CNS effects of circulating vasopressin centering around the consolidation and reinforcement of memory processes, however, suggest a central mode of action, and many studies have shown a carrier-mediated transport of vasopressin from blood to brain and CSF.[2,27] The blood-to-brain transport of vasopressin is significantly greater than mannitol, is saturable, and is inhibited with a V_1 receptor antagonist. Capillary depletion shows that vasopressin is transported intact across the BBB.

Vasopressin injected into the brain ventricles of the rat and guinea pig shows a rate of clearance from CSF with a terminal half-life of 26 min.[21,27] This is in excess of the rate of turnover of CSF in these species produced by the secretion and turnover of fluid and suggests a specific clearance mechanism for this peptide.

OXYTOCIN

Oxytocin is also a nonapeptide structurally closely related to vasopressin. It is also synthesized in the same hypothalamic nuclei as vasopressin but in separate neurons and is secreted into the systemic circulation by the posterior pituitary. Oxytocin acts on smooth muscle bearing appropriate oxytocin receptors and promotes contraction. In this context, its actions on the uterus during parturition and the smooth muscle of the lactating mammary gland are well described. It is however stored in the posterior pituitary in significant quantities in the nonpregnant and nonlactating state and in the posterior pituitaries of both sexes. It is released into the circulation of both sexes during orgasm.

Oxytocin has similar reinforcing effects on memory processes as vasopressin,[14,27] will reduce food intake and also reduces opioid self-administration in tolerant rats.[16] It also has effects on BBB permeability to orotic acid[23] and possibly other solutes. It is also accumulated in brain after intarcarotid injection with a similar regional distribution to vasopressin,[18] oxytocin also seems to penetrate the BBB and appears in CSF after intravenous injection, but a specific influx transporter is yet to be identified.[27] Oxytocin is also cleared from CSF[21] with a half-life of 19 minutes[16,27] by a carrier-mediated mechanism.[16] The clearance mechanisms for vasopressin and oxytocin seem to be distinct,[2] PTS1 seems to transport vasopressin and PTS2 oxytocin.[6]

SOMATOSTATIN

Somatostatin is a 14aa peptide cyclized by virtue of a disulfide bridge between residues 3 and 14. As its name implies, it inhibits anterior pituitary release of growth hormone. It seems to penetrate the BBB to a moderate extent, apparently by a nonsaturable passive process.[1] The volume of distribution is greater than that of radioactively iodinated serum albumin. The BBB transport of several octapeptide analogs of somatostatin has also been investigated in view of their ability to bind to somatostatin receptors expressed on brain tumors. Of the three analogs RC 161, 160, and 121, RC 161 seems to enter the brain most readily.[3] Interestingly, the brain entry of RC 160 increases >200% when administered in a serum free solution suggesting that protein binding may be a significant factor limiting the brain uptake of some of these analogs. The BBB uptake of octreotide (SMS 201-995) after iv injection has a similar brain uptake to RC 160 and RC 121.[9] For these three analogs of somatostatin, entry to the brain is passive. An *in vitro* study of octreotide movement across

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a monolayer of bovine cerebral endothelial cells also suggests passive movement with a significant contribution from the paracellular pathway, which is presumably not available in the intact BBB.[20] Interestingly, when injected icv, the clearance of RC 160 and RC 121 is significantly faster than could be explained by turnover of CSF. The efflux of RC160ias saturable and thus carrier-mediated.[5] A confocal study with isolated porcine cerebral microvessels has suggested that octreotide efflux is verapmil, cyclosporin A, and PSC 833 sensitive and is probably mediated`by the ABC transporters P-glycoprotein and multidrug resistance-associated protein 2.[19]

PACAP

PACAP is a member of a family of peptides that includes vasoactive intestinal peptide (28aa) (VIP), secretin (22aa), glucagon (29aa), glucagon-like peptides 1 (36aa) and 2 (33aa) (GLP1–2), gastric inhibiting peptide (42aa) (GIP), and growth hormone releasing hormone (45aa) (GHRH). These various peptides, described in detail in other chapters of this book, show different transport characteristics at the BBB.

PACAP has two principal forms PACAP 27 and PACAP 38. PACAP 27 shows passive diffusive entry into the CNS, while the larger PACAP 38 enters 30% faster and has a self-saturable component to entry.[4,15] PACAP entry to the brain is not inhibited by an analog peptide-T which antagonizes binding to vascular receptors.[4] Once within the brain, PACAP 38 seems to be more resistant to degradation than PACAP 27, but in spite of its passive entry, a greater percentage of the injected dose enters the brain of PACAP 27, as the AUC and pharmokinetics favor its entry.[4] PACAP 38 is transported into the CNS by peptide transport system 6.[7]

PACAP 38 is a potent neurotrophic and neuroprotective hormone, and its rate of BBB entry is significantly faster in the hypothalamus and hippocampus, (4- to 8-fold) than in other brain regions, the pons-medulla showing no measurable PACAP 38 penetration. This suggests that it may have clinical applications in neurodegenerative diseases such as Alzheimers involving the hippocampus,[28] and memory loss and may underpin its protective effect on CA1 neurons after experimental ischemia.[33]

VIP, secretin, and GLP1 all appear to enter the CNS by passive diffusion.[15] Both glucagon and GHRH seem to cross the BBB but the mechanism has not been examined.[1]

REFERENCES

1. Banks WA, Kastin AJ. Peptides and the blood–brain barrier: lipophilicity as a predictor of permeability. *Brain Res Bull* 1985;**15**:287–92.
2. Banks WA, Kastin AJ, Horvath A, Michals EA. Carrier-mediated transport of vasopressin across the blood–brain barrier of the mouse. *J Neurosci Res* 1987;**18**:326–32.
3. Banks WA, Schally AV, Barrera CM, Fasold MB, Durham DA, Csernus VJ, et al. Permeability of the murine blood–brain barrier to some octapeptide analogs of somatosatin. *Proc Natl Acad Sci USA* 1990;**87**:6762–6.
4. Banks WA, Kastin AJ, Komaki G, Arimura A. Passage of pituitary adenylate cyclase activating peptide 1–27 and pituitary adenylate cyclase activating polypeptide 1-38 across the blood–brain barrier. *J Pharm Exp Ther* 1993;**267**:690–6.
5. Banks WA, Kastin AJ, Sam HM, Cao VT, King B, Maness LM, et al. Saturable efflux of the peptides RC-160 and Tyr-MIF-1 by different parts of the blood–brain barrier. *Brain Res Bull* 1994;**35**: 179–82.
6. Banks WA, Kastin AJ. Peptide transport system 1. In: Greenwood J, Segal MB, Begley DJ, editors. *New concepts of a blood–brain barrier*. NY: Plenum Press; 1995. p. 111–7.
7. Banks WA, Kastin AJ, Arimura A. Effects of spinal cord injury on the permeability of the blood brain and blood-spinal cord barriers to the neurotrophin PACAP. *Exp Neurol* 1998;**151**:116–23.
8. Barrera CM, Kastin AJ, Fasold MB, Banks WA. Bidirectional saturable transport of LHRH across the blood–brain barrier. *Am J Physiol* 1991;**261**:E312–8.
9. Begley DJ. Peptides and the blood–brain barrier. In: Bradbury MWB, editor. *Physiology and pharmacology of the blood–brain barrier. Handbook of experimental pharmacology 103*. Berlin: Springer Verlag; 1992. p. 151–203.
10. Begley DJ. Peptides and the blood–brain barrier: the status of our understanding. In: Strand FL, Beckwith W, Chronwall B, Sandman CA, editors. *Models of neuropetide action*. *Ann NY Acad Sci* 1994; **739**:89–100.
11. Begley DJ. The blood–brain barrier: principles for targeting peptides and drugs to the central nervous system. *J Pharm Pharmacol* 1996;**48**: 136–46.
12. Bruhn TO, Plotsky PM, Vale WW. Effect of paraventricular lesions on corticotrophin-releasing factor (CRF-like immunoreactivity) in the stalk-median eminence: studies on the adrenocorticotropin response to ether stress and exogenous CRF. *Endocrinology* 1984;**114**:57–62.
13. Brust P, Diemer NH. Blood–brain transfer of L-phenylalanine declines after peripheral but not central administration of vasopressin. *J Neurochem* 1990;**55**:2098–104.
14. De Weid D, Bohus B, Urban I, van WimersmaGreidanus TjB, Gispen WH. Pituitary peptides and memory. In: Walter R, Meinhofer J, editors. *Peptides: chemistry, structure and biology. Proceedings of the fourth American peptide symposium*. Ann Arbour Science Publ. Inc.; 1975. p. 635–43.
15. Doggrukol-Ak D, Tore F, Tuncel N. Passage of VIP/PACP/ secretin family across the blood–brain barrier: therapeutic effects. *Curr Pharm Des* 2004;**10**:1325–40.
16. Durham DA, Banks WA, Kastin AJ. Carrier-mediated transport of labelled oxytocin from blood to brain. *Neuroendocrinology* 1990;**53**:447–52.
17. Ermisch A, Barth T, Rühle H-J, Škopková J, Hrbas P, Landgraf R. On the blood–brain barrier to peptides: accumulation of labelled vasopressin, DesGlyNH2-vasopressin and oxytocin by brain regions. *Endocrinol Experiment* 1985;**19**:29–37.
18. Ermisch A, Ruhle HJ, Klasschenz E, Kretzschmar R. On the blood–brain barrier to peptide: [^{3}H]-gonadotropin releasing hormone accumulation by eighteen regions of the rat brain and by anterior pituitary. *Exp Clin Endocrinol* 1984;**84**:112–6.

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19. Fricker G, Nobmann S, Miller DS. Permeability of the porcine blood–brain barrier to somatostatin analogues. *Brit J Pharmacol* 2002;**135**:1308–14.
20. Jaehde U, Masereeuw R, De Boer AG, Fricker G, Nagelkerde JF, Vonderscher J, et al. Quantification and visualization of the transport of octreotide, a somatostatin analogue, across monolayers of cerebrovascular endothelial cells. *Pharm Res* 1994;**11**:442–8.
21. Jones PM, Robinson ICAF. Differential clearance of neurophysin and neurohypophysial peptides from the cerebrospinal fluid in conscious guinea pigs. *Neuroendocrinology* 1983;**34**:454–8.
22. Kastin AJ, Akerstrom V. Differential interactions of urocortin/corticotrophin-releasing hormone peptides with the blood–brain barrier. *Neuroendocrinology* 2002;**75**:367–74.
23. Landgraff R, Hess J, Ermisch A. The influence of vasopressin on the regional uptake of [^{3}H] orotic acid by the rat brain. *Acta Biol et Med Germ* 1978;**37**:655–8.
24. Lewis K, Li C, Perrin MH, Blount A, Kunitake K, Donaldson C, et al. Identification of urocortin III, an additional member of the corticotrophin-releasing factor (CRF) family with high affinity for the CRF2 receptor. *Proc Natl Acad Sci USA* 2001;**98**:7570–5.
25. Martins M, Banks WA, Kastin AJ. Acute modulation of active carrier-mediated brain-to-blood transport of corticotrophin releasing hormone. *Am J Physiol* 1997;**272**:E312–9.
26. Mc Ewen BB. Brain-fluid barriers: relevance for theoretical controversies regarding vasopressin and oxytocin memory research. *Adv Pharmacol* 2004;**50**:531–92.
27. Mens WBJ, Witter A, van Wimersma Greidanus TB. Penetration of neurohypophysial hormones from cerebrospinal fluid (CSF): half-times of disappearance of these neuropeptides from CSF. *Brain Res* 1983;**262**:143–9.
28. Nonaka N, Shioda S, Banks WA. Effects of lipopolysaccharide on the transport of pituitary adenylate cyclase activating polypeptide across the blood–brain barrier. *Exp Neurol* 2005;**191**:137–44.
29. Prescott L, Brightman MW. Circumventricular organs of the brain. In: Pardridge WM, editor. *Introduction to the blood–brain barrier*. UK: Cambridge University Press; 1998. p. 270–6.
30. Reichel A, Begley DJ, Ermisch A. Changes in amino acid levels in rat plasma, cisternal cerebrospinal fluid, and brain tissue induced by intravenously infused arginine vasopressin. *Peptides* 1995;**16**:965–71.
31. Schally AV, Arimura A, Kastin AJ. Hypothalamic regulatory hormones. *Science* 1973;**179**:341–50.
32. Strand F. *Neuropeptides. Regulators of physiological processes*. Cambridge, MA: MIT Press; 1999.
33. Uchida D, Arimura A, Somogyvari-Vigh A, Shioda S, Banks WA. Prevention of ischemia-induced death of hippocampal neurons by pituitary adenlate cyclase activating polypeptide. *Brain Res* 1996;**736**: 280–6.
34. Zloković BV, Segal MB, Begley DJ. Permeability of the isolated choroid plexus of the sheep to thyrotropin-releasing hormone. In: Yudilevich DL, Mann GE, editors. *Carrier mediated transport of solutes from blood to tissue*. London: Longman; 1985. p. 307–12.
35. Zloković BV, Lipovac MN, Begley DJ, Davson H, Rakić L. Slow penetration of thyrotropin releasing hormone across the blood–brain barrier of an in situ perfused guinea pig brain. *J Neurochem* 1988;**51**:252–7.

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Chapter 229

Ingestive Peptides

William A. Banks

ABSTRACT

The passage across the BBB of substances such as leptin, ghrelin, and insulin has emerged in the last decade as a major mechanism by which the central nervous system and gastrointestinal tract communicate. Besides regulation of feeding, many of these substances have effects on cognition and neurogenesis, raising the possibility of their use in the treatment of neurodegenerative diseases as well as obesity and anorexia syndromes. Saturable transport systems in the brain or blood directions exist for many of these substances and others cross by nonsaturable transmembrane diffusion. The transporters are themselves regulated, and their dysregulation results in disease. For example, inflammatory inhibition of insulin transport could explain the insulin resistance of sepsis and impaired transport of leptin is an early cause of the leptin resistance seen in obesity. In summary, many of the central nervous system effects of gastrointestinal substances depend on their transport across the blood–brain barrier.

INTRODUCTION

The discovery in the late 1970s that the central nervous system (CNS) and gastrointestinal (GI) tract contain many of the same peptides and regulatory proteins renewed speculations about how these regions communicate.[9] The blood–brain barrier (BBB) plays a major role in communication between the GI tract and the CNS.[12,14] Clear examples of this communication are provided by peptides and regulatory proteins that arise from the GI tract and reach their targets in the CNS by being transported by saturable systems across the BBB. It was assumed for many years that even small peptides would be too large to cross the BBB. However, when experiments were carefully conducted, it was discovered that many of the principles governing the passage of other substances also applied to peptides and even the larger regulatory proteins.[3,7] Specifically, accumulation of substances by the brain from the blood is a balance between influx and efflux rates. Influx rates and CNS accumulation are primarily determined by the presence or absence of saturable transporters, the physicochemical properties of the substance that determine its rate of nonsaturable transmembrane diffusion, pharmacokinetic parameters (clearance from blood and volume of distribution), capillary sequestration, and binding in plasma. Efflux rates and CNS retention are determined by many factors that include the presence or absence of saturable efflux transporters, cerebrospinal fluid (CSF) reabsorption, degradation within the CNS, and uptake and sequestration by CNS tissues.

The saturable transport systems are themselves regulated by physiological parameters and can be affected in disease states. Taken together, this means that the BBB is a pivotal point in a humorally based communication between the CNS and the GI tract. Understanding this role of the BBB is necessary to understand how the GI tract and CNS interact under physiological conditions and is needed to explain some aspects of disease. Below, we discuss selected examples of peptides and regulatory proteins and some of the major factors regulating their interactions with the BBB.

INSULIN

The question of whether insulin crosses the BBB was investigated in as early as 1954. Insulin has powerful effects on feeding and energy regulation mediated through its receptors in peripheral tissues.[21] However, insulin also has receptors in the brain. Within the CNS, the largest concentration of insulin, insulin receptors, and insulin-degrading enzymes is in the olfactory bulb. Insulin through its CNS receptors has effects on body weight, feeding, glucose, and energy regulation that are often opposite to those mediated through its peripheral receptors.[23] Insulin within the CNS thus may act as its own counterregulatory hormone. CNS insulin has other effects on brain function, including effects on cognition and baroreceptor activity.

Margolis and Altszuler in 1967 proposed that insulin crossed the BBB because levels of insulin in the CSF increased after peripheral administration of insulin. Because the relation between CSF and blood levels of insulin was not linear, they proposed that insulin crossed the BBB by a saturable transport system. However, several other laboratories could not find insulin in the CSF or found insulin in the CNS of streptozotocin-treated rodents. These studies appeared well into the 1980s and were interpreted as

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00229-3
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showing that insulin did not cross the BBB and that it was produced within the CNS. In a series of classic studies beginning in 1977, Woods, Porte, and collaborators clearly showed the presence of insulin in the CSF and its nonlinear relation with serum. As it became increasingly clear that little or no insulin is produced in the CSF, it also became clear that these studies showed that there was a saturable transporter for insulin. Studies from the laboratory of Pardridge reinforced this idea of insulin transport. His laboratory demonstrated binding sites on the endothelial cells that comprise the BBB. At least some of these binding sites would represent insulin receptors coupled to intracellular machinery, thus providing a mechanism to explain why insulin has effects on brain endothelial cell function. But some of these binding sites could also represent a transporter mechanism.

Further work by several laboratories, including our own, characterized many aspects of insulin transport across the BBB.[5] The use of radioactive insulin and of species-specific insulin radioimmunoassays not only definitively showed that insulin crosses the BBB but also measured its influx rate and demonstrated the saturable nature of its passage.

The insulin transporter is not static, but it is regulated in various conditions. For example, the correlation between CSF and serum insulin is lost during hibernation, suggesting that the transporter is turned off. Because the hibernating animal is using but not acquiring energy, this finding would suggest that CNS insulin is more important in the regulation of aspects of energy intake than in states of energy use. Insulin transport is also impaired in obesity, as is leptin transport. Unlike leptin, this obesity-related inhibition is not mediated by triglycerides. Insulin transport is also increased in streptozotocin-induced diabetes. This is not just an apparent increase caused simply by less endogenous insulin being available to compete for transport with the radioactive insulin used to measure influx rate. The increased transport of insulin is seen in diabetic mice even when assessed by the brain perfusion method in buffer rather than blood, which obviates the effects of serum insulin.

Recent *in vitro* work has shown that glucose itself is a regulator of insulin transport at the BBB.[15] Hyperglycemia induces a faster rate of transport across brain endothelial cells cocultured with pericytes and astrocytes. This induction, however, does not seem to be mediated directly at the level of the endothelial cell but is dependent on the crosstalk among the endothelial cells, astrocytes, and pericytes.

Insulin transport is also modulated in mice treated with lipopolysaccharide (LPS), a derivative of the cell wall from Gram negative bacteria.[24] LPS induces an immune reaction mediated by cytokine release. LPS has multiple effects on BBB function, including disruption of the BBB in extreme cases and effects through various mechanisms on some saturable transporters. LPS induces an increase in the saturable transport of insulin that is mediated through nitric oxide. Inhibition of nitric oxide synthase potentiates the effects of LPS on insulin transport, although inhibition of nitric oxide synthase in non-LPS treated mice has no effect on insulin transport. This suggests that LPS induces two pathways affecting insulin transport across the BBB; one enhances insulin transport and the other, mediated through nitric oxide, inhibits it. Because CNS insulin counters the effects of peripheral insulin, enhanced transport of insulin into the CNS by LPS could be a mechanism by which sepsis induces insulin resistance. A possible role for the BBB in mediating the insulin resistance of sepsis is discussed in the chapter "Diseases Mediated by the BBB: Alzheimer's, Obesity, and Beyond."

LEPTIN

Leptin is a 16-kDa protein secreted from fat cells, which acts at the arcuate nucleus in the hypothalamus to inhibit feeding and to promote thermogenesis.[11] An alternate route proposed for leptin uptake that does not exist in the adult is leakage out of the adjacent median eminence. The median eminence contains capillaries that do not form a BBB, and so leptin can leak into this tissue. However, the median eminence is isolated from the ventricular CSF and adjacent hypothalamic tissue by epithelial and tanycytic arms of the BBB.[18,20] These barriers are so robust that even a small molecule like glutamate cannot penetrate them. The tanycytic barrier develops in rodents after birth. It is for this reason that monosodium glutamate can destroy the hypothalamic feeding centers when given to the neonate but not when given to the adults.[18]

Resistance to the anorectic and thermogenic effects of leptin is a hallmark of human obesity and of diet-induced obesity in many strains of rodents.[11] This resistance can occur at three levels: impaired transport at the BBB, receptor/postreceptor impairments, and impairments in the anorectic downstream neuronal circuitries stimulated by leptin. Studies with diet-induced obesity show that resistance first occurs at the level of the BBB.[4] At this stage, rodents can still respond to leptin given directly into the brain but no longer respond to leptin given peripherally. Analysis of the CSF and serum levels obtained from humans with moderate obesity also showed that the major level of resistance is at the BBB. The BBB is a major level of resistance in sheep as well.[1] As discussed in the chapter "Diseases Mediated by the BBB: Alzheimer's, Obesity, and Beyond," this resistance at the BBB may be a major cause of leptin-related obesity.

The leptin transporter is also not static but modulated by various substances and by various conditions. Obesity, starvation, and loss of ovarian function decrease transport of leptin across the BBB.[13] Glucose, insulin, and $alpha_1$

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adrenergics increase leptin transport, whereas triglycerides inhibit it. There is also a diurnal rhythm to leptin's transport rate.

These aspects of leptin's interaction with the BBB can be used to develop potential therapeutics for the treatments of anorexia and obesity. An analog of leptin designed so as to block the leptin receptor and transporter can act as a potent antagonist to endogenous leptin activity.[19] Such an action recapitulates a leptin-resistance type picture with mice immediately gaining weight. An analog of leptin that can cross the BBB independently of the leptin transporter but still activate the leptin receptor would be active in the face of peripheral leptin resistance.[10] Such a drug would be an anorectic even in obese mice.

GHRELIN

Ghrelin is a 28 amino acid residue peptide hormone characterized by a novel posttranslational acylation that is required for its bioactivity.[22] Ghrelin is secreted by the fundus of the stomach into the circulation before the onset of meals. Current thinking suggests that ghrelin is a major orexigenic peptide stimulating the sensation of hunger from sites within the CNS. As such, it would be logical to assume that ghrelin crosses the BBB. Human octanoyl ghrelin is transported across the mouse BBB bidirectionally; that is, there is a saturable component to both its influx and its efflux.[8] Paradoxically, however, mouse ghrelin is treated differently than human ghrelin. Mouse octanoyl ghrelin, the biologically active form of ghrelin, and mouse des-octanoyl ghrelin are not transported by the influx system. Because human and mouse ghrelin differ by only two amino acids, this shows that these two amino acids are crucial for recognition by the influx transporter. Mouse octanoyl ghrelin, but not the des-octanoyl form, was transported by the efflux system.

Ghrelin transport across the BBB has a complicated interaction with events related to feeding and fasting. As with leptin, ghrelin transport is decreased in obese mice. Unlike leptin, epinephrine has no effect on ghrelin transport and triglycerides or fasting increases transport.[6]

These results show that, as expected, ghrelin crosses the BBB. However, the paradoxical interactions of mouse octanoyl ghrelin with the BBB show that there are aspects of transport yet to be clarified.

OTHER INGESTIVE PEPTIDES AND PROTEINS: SPECIAL INTERACTIONS WITH THE BBB

A number of other ingestive peptides and proteins have been examined for their ability to cross the BBB. Several of these have saturable transport systems and others, although able to penetrate the BBB, could not be shown to be inhibited by unlabeled peptide. These are categorized in Table 1 as having crossed the BBB by the nonsaturable mechanism of transmembrane diffusion. However, it is always possible that saturation may have been shown if a higher dose of unlabeled material had been used.

Many of these peptides and proteins have special aspects affecting their brain uptake and BBB penetration. For example, whereas orexin A crosses the BBB by a nonsaturable mechanism, orexin B is so rapidly degraded in blood that it is difficult to study. Although the nonsaturable transport of orexin A provides a mechanism explaining how it can stimulate appetite after peripheral administration, the rapid degradation of orexin B makes it unlikely that it reaches the brain. Similarly, glucagon-like peptide is very unstable in blood, but it can be shown to cross the BBB when studied by brain perfusion. A more stable analog, [Ser8]GLP, crosses by a nonsaturable system. Amylin is co-secreted with insulin from the pancreas and, like insulin, crosses the BBB. Insulin and amylin have distinct distribution patterns in the brain and, whereas insulin clearly is transported across the BBB by a saturable transporter, amylin transport has not been shown to be self-inhibitable. However, amylin transport is inhibited in mice pretreated with aluminum, a maneuver known to inhibit some saturable transporters. It may be that an amylin transporter exists but with a very high capacity.

Binding in serum, either by serum proteins or by some factor in serum promoting aggregation, influences the BBB permeation of some ingestive peptides and proteins. IGF-1, galanin-like peptide, and adrenomedullin cross the BBB, but a saturable component to that passage is not demonstrable after their intravenous injection. However, the saturable transporter can be demonstrated when studied by the brain perfusion method. This method precludes the influence of circulating substances and allows the study of interactions directly between the BBB and the substance of interest. In the cases of IGF-1, galanin-like peptide, and adrenomedullin, it is likely that binding to serum proteins competes with the BBB transporters in such a way as to make it difficult to show the saturable nature of blood-to-brain uptake. Such a mechanism has been shown to be operable for free fatty acids. Binding in serum also interferes with the uptake of Phe^{13},Tyr^{19}-melanin-concentrating hormone (MCH). No uptake can be observed when studied after iv injection, but a low, nonsaturable entry can be seen when studied by brain perfusion.

Urocortin does not cross the BBB under normal conditions. However, a saturable transporter for it can be induced either by treatment with leptin or with glucose. Galanin-like peptide transport is increased with glucose and inhibited by food deprivation. Secretin was the first hormone to be discovered. Bayliss and Starling first described its actions on pancreatic exocrine secretions in 1902. About 80 years later, secretin was discovered in the brain. Very low doses of secretin

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TABLE 1 Transport Systems for Ingestive Peptides and Proteins

Substance	Influx	Efflux	Action with CNS
Leptin	Sat	Nonsat	Anorectic
Insulin	Sat	Ret	Anorectic
Ghrelin*	Nonsat	Sat	Orexigenic
Des Ghrelin*	Nonsat	Nonsat	Inactive
Orexin A	Nonsat	Nonsat	Orexigenic
Orexin B	None	NS	Orexigenic
GLP	None	NS	–
Exendin	Sat	Nonsat	Anorectic
PP	Sat	Nonsat	Anorectic
Ser^8-GLP	Nonsat	NS	–
$ARP_{83–132}$	Nonsat	Nonsat	Orexigenic
Amylin	Nonsat?	NS	–
IGF-1	Sat	NS	–
Adrenomedullin	Sat	Nonsat	Anorectic
$Mahogany_{1377–1428}$	Nonsat	–	Anorectic
$PYY_{3–36}$	Nonsat	Nonsat	Anorectic
MSH	Nonsat	NS	Anorectic
MCH	None	Nonsat	Orexigenic
Galanin-like peptide	Sat	Nonsat	Anorectic
CART	Nonsat	Nonsat	Anorectic
NPY	Nonsat	Nonsat	Orexigenic
Urocortin	None/inducible sat	Nonsat	Anorectic
Secretin	Sat (CSF)	NS	–
–	Nonsat (Vascular)	–	–
Nesfatin	Nonsat	Nonsat	Anorectic
Adiponectin	None/Nonsat	–	–

Sat = saturable transport; Nonsat = passage by nonsaturable mechanisms (transmembrane diffusion for influx and bulk flow for efflux); Ret = retained by brain (little or no efflux) after intracerebroventricular injection; None = no blood-to-brain uptake after intravenous injection; NS = not studied.
GLP = glucagon-like peptide; PP = pancreatic polypeptide; ARP = agouti-related peptide; IGF-1 = insulin-like growth factor; MSH = alpha melanocyte-stimulating hormone; MCH = Phe^{13},Tyr^{19}-melanin-concentrating hormone; CART = cocaine- and amphetamine-related transcript; neuropeptide Y.
Special notes: murine ghrelin as studied in the mouse.

administered into the brain also induce pancreatic secretions. Secretin crosses the vascular BBB at a low rate by a nonsaturable mechanism but crosses the blood–CSF barrier at a much higher rate and by a saturable system. Adiponectin accumulates in the CNS less well than albumin and in some studies its penetration into the CNS cannot be demonstrated at all. However, it binds to brain endothelial cells, the cells that comprise the vascular BBB, to alter their secretion of interleukin-6; this cytokine, in turn, can have effects on feeding.

Many other peptides and regulatory proteins which are not considered as ingestive substances nevertheless have profound effects on appetite and metabolism. These include opiate peptides, corticotrophin-releasing hormone (CRH), and cytokines.[17] These are not considered here except to contrast their BBB interactions with the substances discussed above. For example, most cytokines found to have significant entry into the brain are transported across the BBB by a saturable system. CRH and endogenous opiate peptides are substrates for various efflux systems, most notably P-glycoprotein and peptide transport system-1. No saturable influx system has yet been described for an opiate peptide and only one saturable efflux system for a cytokine (IL-2) has been described.

ACTIONS OTHER THAN FEEDING

The gastrointestinal hormones are emerging as pleuripotent substances with actions on the CNS beyond those immediately related to feeding or appetite. These include effects on cognition, neurogenesis, reproduction, respiration/breathing, thermoregulation, and baroreceptor function. Conditions from Alzheimer's disease to preeclampsia have been postulated to arise in part from an impaired transport of insulin across the BBB.[2,16] Future studies will likely find other conditions that result from altered transport of hormones across the BBB.

SUMMARY

The BBB is a major regulatory interface coordinating a humoral-based communication between the GI tract and the CNS. Leptin, insulin, and ghrelin are important examples of substances involved in this communication. Their interactions with the BBB illustrate several principles, including the importance of saturable and nonsaturable mechanisms of passage, the regulation of BBB transporters, and the occurrence of disease with dysregulation of passage across the BBB.

REFERENCES

1. Adam CL, Findlay PA. Decreased blood–brain leptin transfer in an ovine model of obesity and weight loss: resolving the cause of leptin resistance. *Int J Obes* 2010;**34**:980–8.
2. Azar AS, Brooks VL. Impaired baroreflex gain during pregnancy in conscious rats: role of brain insulin. *Hypertension* 2011;**57**:283–8.

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3. Banks WA. Physiology and pathophysiology of the blood–brain barrier: implications for microbial pathogenesis, drug delivery and neurodegenerative disorders. *J Neurovirol* 1999;**5**:538–55.
4. Banks WA. Is obesity a disease of the blood-brain barrier? physiological, pathological, and evolutionary considerations. *Curr Pharm Des* 2003;**9**:801–9.
5. Banks WA. The source of cerebral insulin. *Eur J Pharmacol* 2004;**490**:5–12.
6. Banks WA, Burney BO, Robinson SM. Effects of triglycerides, obesity, and starvation on ghrelin transport across the blood–brain barrier. *Peptides* 2008;**29**:2061–5.
7. Banks WA, Kastin AJ. Editorial review: peptide transport systems for opiates across the blood–brain barrier. *Am J Physiol* 1990;**259**:E1–10.
8. Banks WA, Tschop M, Robinson SM, Heiman ML. Extent and direction of ghrelin transport across the blood–brain barrier is determined by its unique primary structure. *J Pharmacol Exp Ther* 2002;**302**:822–7.
9. Bloom SR. Gut and brain–endocrine connections. the Goulstonian lecture 1979. *J R Col Physicians Lond* 1980;**14**:51–7.
10. Elinav E, Niv-Spector L, Katz M, Price TO, Ali M, Yacobovitz M, et al. Pegylated leptin antagonist is a potent orexigenic agent: preparation and mechanism of activity. *Endocrinology* 2009;**150**:3083–91.
11. Friedman JM, Halaas JL. Leptin and the regulation of body weight in mammals. *Nature* 1998;**395**:763–70.
12. Kastin AJ, Pan W. Feeding peptides interact in several ways with the blood–brain barrier. *Curr Pharm Des* 2003;**9**:789–94.
13. Kastin AJ, Pan W. Blood–brain barrier and feeding: regulatory roles of saturable transport systems for ingestive peptides. *Curr Pharm Des* 2008;**14**:1615–9.
14. Morley JE. Minireview: the ascent of cholecystokinin (CCK)—from gut to brain. *Life Sci* 1982;**30**:479–93.
15. Nakaoke R, Verma S, Niwa M, Dohgu S, Banks WA. Glucose-regulated blood–brain barrier transport of insulin: pericyte–astrocyte–endothelia cell cross talk. *Int J Neuroprot Neuroregener* 2007;**3**:195–200.
16. Neumann KF, Rojo L, Navarrete LP, Farias G, Reyes P, Maccioni RB. Insulin resistance and Alzheimer's disease: molecular links and clinical implications. *Curr Alzheimer Res* 2008;**5**:438–47.
17. Pan W, Kastin AJ. Interactions of cytokines with blood–brain barrier: implications for feeding. *Curr Pharm Des* 2003;**9**:827–31.
18. Peruzzo B, Pastor FE, Blazquez JL, Schobitz K, Pelaez B, Amat P, et al. A second look at the barriers of the medial basal hypothalamus. *Exp Brain Res* 2000;**132**:10–26.
19. Price TO, Farr SA, Yi X, Vinogradov SV, Batrakova E, Banks WA, et al. Transport across the blood–brain barrier of pluronic leptin. *J Pham Exp Ther* 2010;**333**:253–63.
20. Rodriguez EM, Blazquez JL, Guerra M. The design of barriers in the hypothalamus allows the median eminence and the arcuate nucleus to enjoy private milieus: the former opens to the portal blood and the latter to the cerebrospinal fluid. *Peptides* 2010;**31**:757–76.
21. Schwartz MW, Figlewicz DP, Baskin DG, Woods SC, Porte Jr D. Insulin in the brain: a hormonal regulator of energy balance. *Endocrine Rev* 1992;**13**:387–414.
22. van der Lely AJ, Tschop M, Heiman ML, Ghigo E. Biological, physiological, pathophysiological, and pharmacological aspects of ghrelin. *Endocrine Rev* 2004;**25**:426–57.
23. Woods SC, Seeley RJ, Baskin DG, Schwartz MW. Insulin and the blood–brain barrier. *Curr Pharm Des* 2003;**9**:795–800.
24. Xaio H, Banks WA, Niehoff ML, Morley JE. Effect of LPS on the permeability of the blood–brain barrier to insulin. *Brain Res* 2001;**896**:36–42.

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Chapter 230

Neurotrophic Peptides

Weihong Pan and Abba J. Kastin

ABSTRACT

Neurotrophic peptides are not only essential for normal CNS function but they also reduce neurodegeneration after trauma, ischemia, and neuroinflammation. Many neurotrophic peptides permeate the blood–brain barrier (BBB) by specific transport systems and/or they can modulate BBB function. In conditions with partial disruption of the BBB, there are regulatory changes of specific transport systems for selective neurotrophic peptides. Naturally occurring neurotrophic peptides for which interactions with the BBB have been studied include epidermal growth factor (EGF), transforming growth factors (TGFs) α and β, insulin-like growth factors (IGF) I and II, acidic and basic fibroblast growth factor (FGF), colony-stimulating factors, pituitary adenylate cyclase activating polypeptide (PACAP), and several basic peptides. Here, we review their pharmacokinetics, cellular endocytosis and trafficking, and specific effects in CNS disease models. We also summarize the current understanding of peptide-based drug delivery systems.

INTRODUCTION

The functions of neurotrophic peptides are discussed elsewhere in this book. Three points should be stressed: (1) They can be produced both within the CNS and elsewhere, yet their induced expression after an insult to the CNS may not be sufficient to rescue neurodegeneration. (2) Like typical cytokines, they are multipotent and may not always be beneficial or neuroprotective. (3) Endogenous neurotrophic peptides can function as autocrine or paracrine signals and serve as neuromodulators, whereas exogenous neurotrophic peptides introduced by targeted delivery across the blood–brain barrier (BBB), or by implanted cells and tissue, could have a greater influence on CNS function.

Here, we discuss interactions of these peptides with the BBB. The reason that this is an important and critical topic is because the BBB serves as a diffusion barrier and regulatory interface at the same time, and is an essential target when addressing peptide delivery to treat CNS disorders. As a neurovascular interface, the BBB also provides a bidirectional route of communication. An efflux transport system explains part of the peripheral effects of brain-derived neuropeptides, determines whether a peptide is retained in the CNS after influx, and is also important for the clearance of some peptides that are potentially cytotoxic, such as β-amyloid. Deposition and specific interactions of peptides with the cerebral and spinal vasculature precede the influx transfer of selective peptides. Because of their importance in vascular pathology and therapeutic potential in CNS diseases, the blood-to-brain (and spinal cord) influx of neurotrophic peptides is a main focus of this chapter.

Rather than direct permeation across the BBB, some peptides alter endothelial signaling and induce subsequent functional changes. Exocytotic release of secondary signals from the basolateral side of the BBB endothelia can thus modulate cerebral function. Vasoactive peptides are specific examples, as there may be robust changes of cerebral blood flow and metabolism even in the absence of direct permeation of the peptides across the BBB.

Regional specificity of enzymes and specific receptors dictates the pharmacokinetics of peptides in different brain and spinal cord areas. The actions of peptides, of course, are coupled with the phenotypes of neurons and glia in the particular regions. This can make a peptide neurotrophic in one situation, but can stimulate other actions such as feeding suppression or antidepression in another. All these factors need to be taken into consideration when discussing their interactions with the heterogeneous BBB.

The studies of interactions of neurotrophic peptides with the BBB originated from our initial observation that peripheral peptides can exert unconfounded CNS effects — a well-known fact that was controversial four decades ago. The ability of a peptide to cross the BBB in intact form, like its longer biological actions than the half-life in blood, are among the concepts that we presented in the early stage of peptide-BBB research.[13] The BBB is undoubtedly a huge neurovascular unit for information exchange between the CNS and the periphery. As Abbott points out, "For the typical human brain, capillary length is around 650 km (distance from Paris to Dublin). This provides an enormous 12–20 m^2 of surface area for molecular exchange across the capillary wall. The mean distance between capillaries is approximately 40 μm, so that no neuron is more than about 8 μm from a capillary." Here, we summarize the interac-

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00230-X
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tions between neurotrophic peptides and the BBB in regard to permeability and endothelial cell signaling, mainly discussing normal physiology but also commenting on studies related to neurological diseases where exogenous neurotrophic support would be beneficial.

RESOLUTION OF MYTHS ABOUT PEPTIDES AND THEIR CNS EFFECTS: *IN VIVO* AND *IN VITRO* METHODOLOGIES

Although the endothelial cells forming the BBB make a thin monolayer, they are joined by tight junctions and lined by a continuous basement membrane. This is further reinforced by pericytes, astroglial endfeet, and extracellular matrix. Besides the anatomical structure, intracellular degradative enzymes and extracellular matrix proteases also reinforce a biochemical barrier. Merely a few decades ago, when we first proposed the CNS affects of peptides, it was considered inconceivable that peptides (and even some larger proteins) in the periphery might effect the CNS. It was a long journey of more than a decade to convince the neuropeptide community that peptides can indeed cross the BBB.

The first stage of showing neurotrophic peptides can cross the BBB in intact form involves pharmacokinetic assays. If the overall structure of the BBB is considered as a semipermeable membrane, the permeation of peptides and proteins is slow, because they are relatively large and lipid insoluble. Permeation is often incomplete, which means that proper mathematical modeling and correction for partial degradation are necessary.

With the exception of several vasoactive peptides, permeation of peptides and proteins across the BBB is not dependent on blood flow. Banks and Kastin established the method of multiple-time regression analysis for *in vivo* studies . The peptide to be studied is radioactively labeled to ensure sensitive and specific detection and delivered by intravenous bolus injection to anesthetized animals. In mouse studies, each time point is represented by individual animals. A period during which most of the peptide remains intact in circulation is reconstructed by the use of multiple mice sacrificed at different time points. The brain/serum ratio over time reflects BBB permeability to the particular peptide. To correct for the disappearance of peptide in the blood over time, and to provide a steady-state value, the theoretical "exposure time" is calculated.[9] Two parameters of permeability can thus be derived from multiple-time regression analysis: the unidirectional influx rate (how fast) and the volume of distribution (how much). The analysis can be extended to different regions of brain and spinal cord and can be applicable to peripheral organs. With this method, permeability of different peptides can be compared, and the effects of potential modulators tested.

There are certain questions beyond the resolution of the two-compartmental model of the BBB that are answered by other techniques. The capillary depletion studies assists in addressing the events in the endothelial cell compartment in addition to cerebral vascular space and brain parenchyma. Analysis of isolated cerebral microvessel endothelial cells *in vitro* further provides another powerful tool to identify the mechanisms of permeation. Internalization of a peptide can be determined after removal of specific surface binding by mild acid wash, trypsin digestion, or other similar procedures.

Cultured primary cerebral microvessel endothelial cells can form relatively tight junctions. This enables measurement of apical-to-basolateral flux (simulating blood-to-brain transfer) and basolateral-to-apical flux (mimicking brain-to-blood permeation) when the cells are grown on a supportive monolayer, such as the side-by-side diffusion chamber or the transwell insert. Because a relatively large quantity of viable cells is needed, many researchers choose bovine or porcine primary cells; however, studies are also performed on cells of rat or mouse origin. A tight monolayer is a prerequisite for *in vitro* permeability studies, so that the nonspecific paracellular diffusion is low. Nonetheless, endocytosis studies can also be performed on freshly isolated microvessels or immortalized endothelial cells. The list of available cell lines suitable for BBB studies are growing. Rat cell lines include RBE4 and TR-BBB cells; mouse cell lines are represented by TM-BBB4, b.End3, and b.End5 cells; whereas the best characterized human cell line is hCMEC/D3 cells.

The second stage to determine transcytosis and regulatory changes of peptides across the BBB involves morphological studies. Fluorescent conjugation and confocal microscopic imaging techniques have shown membrane interactions of peptides and their intracellular trafficking. Theoretically, total internal reflection fluorescence microscope (TIRFM) technology should enable the analysis of exocytic events. Electron microscopy (EM) and EM autoradiography are useful tools to determine the vectorial transport of peptides across the BBB. Studies of molecular trafficking in non-CNS cells have progressed much further than those in BBB cells, and it is yet to be determined whether cerebral endothelia and other cellular constituents process peptides differently during the course of transport. This will be further discussed below.

The third issue is the direct effects of peptides after permeating the BBB vs cellular signaling and secondary mediators generated at the BBB level.[26] Many neurotrophic peptides may provide secondary signals by their effects on BBB endothelia, thus affecting CNS function. The specific examples of vasoactive peptides and cell-penetrating peptides such as human immunodeficiency virus (HIV)-specific protein transactivator of transcription (Tat) are discussed elsewhere.

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BINDING PROTEINS THAT AFFECT THE STABILITY AND PERMEATION KINETICS: INTRAVENOUS DELIVERY VS IN SITU BRAIN PERFUSION

A complex of the peptide with its binding protein(s) usually makes the peptide more stable and less susceptible to degradative enzymes in the blood circulation and provides a reservoir for its permeation across the BBB. A specific example is epidermal growth factor (EGF). Binding to proteins and cell-surface heparin sulfate proteoglycans (HSPG) can also accelerate peripheral degradation and reduce BBB permeation, such as occurs with the receptor-associated protein (RAP). Further, aggregation and polymerization of a peptide may deter or even abolish the permeation across the BBB, such as occurs with the chemokine MIP-1[1] and the feeding-related peptide AgRP (83-132).[8]

To accurately assess the permeability of the BBB, the first two issues can be resolved by in situ brain perfusion in blood-free physiological buffer. The radioactively labeled tracers are delivered at a constant rate to the brain at a time when the BBB is still viable and functional (usually within 10 min), and multiple-time regression analysis can be performed. This method also detects rapid influx within the first few minutes that might have been missed by *in vivo* studies. The last issue of aggregation and polymerization can be addressed by chemical methods such as gel chromatography or capillary electrophoresis.

PRESENCE OF RECEPTORS AT THE APICAL OR BASOLATERAL SURFACE OF THE BBB: USEFUL OR NOT FOR PEPTIDE TRANSPORT?

Cell-surface biotinylation and ligand binding methods can determine the relative distribution of receptors on the apical and basolateral surfaces. There are also experimental approaches that differ from whole cell biology, involving preparation of apical or basolateral membranes of the endothelial cells or plasma membrane-derived inside-out vesicles. Thus far, EGF and bFGF are the two neurotrophic peptides for which the distribution of receptors in endothelial cell subdomains is well known. EGFR is a peptide with characteristic 6 half-cystines in the primary structure that is mainly located at the basolateral surface of the epithelial cell. It is known that EGFR mediates the endocytosis of EGF from the basolateral surface. However, most trafficking studies have been performed on nonpolarized cells in which the sorting motifs in the EGFR (located at the apical surface) determine its fate including lysosomal degradation,[15] and sorting nexin 1 also is involved in the trafficking.[16] Intact EGF can be exocytosed from the basolateral to apical side in intact form in MDCK cells of kidney origin, with exocytosis being the rate-limiting step of transport.[33] In animal studies of the BBB, the blood-to-brain transfer of EGF is a saturable process, indicating the presence of a specific transport system.[22] Nonetheless, this influx does not seem to be an EGF receptor-mediated process, because TGFα that binds to the same receptor does not cross the BBB efficiently. Rather, it is retained in the vascular space of the brain.[27] Thus, in this case, the cell surface receptor for EGF and TGFα does not mediate blood-to-brain transport.

Acidic FGF (aFGF, or FGF-1) has limited stability in the blood circulation and therefore cannot be studied by our multiple-time regression analysis. However, basic FGF (bFGF, or FGF-2) has been shown to cross the BBB by adsorptive endocytosis.[5] Perlecan, a heparin sulfate proteoglycan, is involved in its endocytosis from the apical surface of the endothelial cells. Thus, the receptor does not play a significant role in the transport of bFGF across the BBB.

Although a receptor is not always the transporter for a peptide across the BBB, there are many examples of receptor-mediated transport, such as for insulin, IGF-1, tumor necrosis factor (TNF)α, and transferrin. The crossinhibition of insulin and IGF-1 permeability indicates that the transport systems are at least partially shared.

ADSORPTIVE ENDOCYTOSIS AND CHEMICAL MODIFICATIONS THAT ENHANCE DELIVERY ACROSS THE BBB

Several neurotrophic peptides are basic and therefore bear positive charges in solution. For instance, the isoelectric point (pI) of mouse bFGF is 9.59, making it possible to enter cells by adsorptive endocytosis. Binding of bFGF to heparin facilitates HSPG-mediated interaction with endothelial cells.

For peptides crossing the BBB by simple diffusion, physiochemical properties such as lipophilicity, hydrogen bonding, conformation, size, and polymerization are important determinants of permeability. Thus, chemical modifications to enhance stability, lipophilicity, and selective interactions with cerebral endothelial cells will help. Among the posttranslational modifications, glycosylation increases the permeability of some proteins.[29] Pegylation enhances the stability of a peptide in blood but might reduce its permeation across the BBB.[30,31] Some of the modifications, especially immunoliposomes, have been used successfully to increase the bioavailability of a peptide drug to the CNS.

THE INTRACELLULAR TRAFFICKING OF PEPTIDES THAT LEADS TO EXOCYTOSIS

The intracellular trafficking of neurotrophic peptides in the endothelial cells composing the BBB has not been extensively studied. Our unpublished results with urocortin

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indicates that substantial amounts of fluorescently labeled urocortin are colocalized with endosomes within 2 min and translocate to the Golgi complex and proteasomes at a later time. Urocortin is a 4 kDa peptide that binds to CRH receptors and is primarily involved in feeding behavior and energy expenditure as well as being neurotrophic in certain circumstances. More than 10% of radioactively labeled urocortin can be exocytosed by cultured cerebral microvessel endothelial cells over a time course of 5 min to 1 h in intact form.[35] It is yet to be confirmed whether endocytosis is the rate-limiting step for transport, and much needs to be learned about the trafficking routes and any cytoskeletal motors, ions, or energy processes driving the exocytosis.

Exocytosis of endocytosed peptides and proteins has not received much attention, but valuable information can be learned from studies of those newly synthesized. Although not a complete analogy, the trafficking patterns of a newly synthesized peptide or protein illustrate the diversity of potential pathways encoded by its structure and its interacting partners. The classical secretory route involves the Golgi complex. Sorting may be related to the signal peptide that is cleaved from the mature peptide, associated with sorting nexins, and dependent on chaperon proteins. There are also alternative exocytosis pathways, such as multivesicular bodies and exosome-like vesicles, exovesicles related to membrane blebbing, transporters residing at the plasma membrane, and lysosomal secretion.[32]

SPECIFIC EXAMPLES OF THE PHARMACOKINETICS OF BBB PERMEATION

It seems that cerebral endothelial cells render a different fate to neurotrophic peptides than that shown in other cells such as fibroblasts, lymphocytes, and hepatocytes. Earlier work with radioactively labeled EGF in fibroblasts and other cell types clearly showed its intracellular degradation.[4] Later, polarized sorting was shown to be different when EGF was applied apically vs basolaterally. Kozu et al. provided strong evidence that EGF can be exocytosed in its intact form and that exocytosis is the rate-limiting step of transport.[14] *In vivo* studies in mice also showed that EGF is relatively stable in blood and crosses the BBB by a specific transport system. The amount of EGF in brain parenchyma at 10 min accounts for >50% of the total radioactivity, as contrasted with only 20% in blood vessels.[22] TGFα, although sharing the same receptor as EGF, is retained in the cerebral vasculature without meaningful penetration of the BBB after intravenous injection.[27] TGFβ1 is stable in circulating blood but does not show influx from the blood to the brain.[10]

IGF1 delivered into the blood is mainly protein bound; however, it has a saturable transport system at the BBB. The passage of IGF-1 across the BBB might partially explain the effect of peripheral growth hormone at the CNS, because growth hormone itself has limited permeation of the BBB by simple diffusion.[28] The rate of entry of ^{125}I-IGF1 from blood to CNS shows apparent regional differences, and excess unlabeled IGF1 paradoxically increases the influx of the radioactively labeled tracer in each region tested, probably explained by the displacement of ^{125}I-IGF1 from its binding proteins in the periphery.[23]

Receptor-mediated endocytosis of insulin in cerebral microvessel endothelial cells has been studied both *in vivo* and *in vitro* by several groups. There are regional differences of insulin transport and its regulation in diabetes and various models of obesity. Based on its capability to transport insulin, the insulin receptor has been a target for the design of vehicles (antibody-based cargos) to deliver other neurotrophic peptides and proteins from blood to brain.

Granulocyte macrophage-colony stimulating factor (GM-CSF) enters the brain and spinal cord by a saturable transport system in both mice and rats.[18] Its presence in blood and relative stability in both blood and CNS indicate that GM-CSF plays an important role in modulating the physiological functions of neurons and astrocytes.

As mentioned above, the uptake of bFGF occurs by adsorptive endocytosis and involves HSPG. PACAP is a peptide related to secretin, glucagon, GLP1, and GHRH and it binds to PACAP/VIP receptors as discussed elsewhere in this book. PACAP illustrates how the size of the primary structure does not determine the behavior of its permeation across the BBB. $PACAP_{1–27}$ has a slower influx that is not saturable, whereas the larger $PACAP_{1–38}$ has a faster and saturable influx.[2] The efflux of these two PACAP peptides from brain to blood is impaired in a mouse model of Alzheimer's disease (SAMP8).[3] The structurally related VIP is more vasoactive; it is able to enter the brain from the blood by simple diffusion and is retained in the CNS.[7]

THE SITUATION OF THE PARTIALLY DISRUPTED BBB AND SELECTIVE REGULATION OF THE TRANSPORT SYSTEM IN PATHOPHYSIOLOGY

Neurotrauma, ischemia, and inflammation are associated with increased permeability of the BBB, altered axonal transport, and disrupted neuronal and glial functions. When the BBB is partially disrupted, certain transport systems for cytokines are not abolished but upregulated. This has been specifically shown by TNFα transport after spinal cord injury,[20] head trauma,[25] experimental allergic encephalomyelitis (EAE),[21] and lipopolysaccharide (LPS) administration.[19] The enhanced transport is related to increased mRNA expression of the transporting receptors.[24] Similar regulation of TNFα transport is also present after focal cerebral ischemia.

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BBB permeability of $PACAP_{1-38}$ is decreased at selected times (24 h) in rats after 2 h of middle cerebral artery occlusion followed by reperfusion, and the change seems to be bilateral rather than unilateral.[34] One common feature found for both TNFα and $PACAP_{1-38}$ is that regulation is not only confined to the damaged area but also extends to remote regions, such as the hemisphere contralateral to ischemia. This suggests a general and possibly hormonal factor in the regulation of transport.

However, for neurotrophic proteins that enter the brain and spinal cord by adsorptive endocytosis or other non-receptor-mediated mechanisms, regulation does not always lead to increased uptake. Ebiratide—a synthetic analog of an MSH/ACTH peptide and potent neurotrophic factor—has a high baseline penetration across the BBB, and spinal cord injury does not further facilitate its permeation from blood to brain and spinal cord.[20] EGF uptake by the injured spinal cord also fails to show a significant increase over time in different CNS regions.

ENHANCED DELIVERY OF NEUROTROPHIC PEPTIDES: HOW IT AFFECTS NEUROREGENERATION

Unlike classical hormones and neurotrophins, the neurotrophic peptides have a broader distribution in both synthesis and targets of action. Because insults to the CNS often affect multiple populations of cells and extracellular matrix, the provision of trophic support by these peptides is extremely useful. To deliver neurotrophic peptides as CNS therapeutics, the peripheral route is more advantageous than invasive application directly to the CNS. However, there are only a small proportion of neurotrophic peptides crossing the BBB by specific transport systems; the rest depend on the time- and space-related partial disruption of the BBB. Even for those normally entering the CNS by saturable transport, there does not seem to be substantial enhancement of transport in the diseased state. With the additional concern of peripheral degradation, peptides are often linked to delivery vehicles for adequate transfer. These experimental approaches include immunoliposomes, phage display, viral vectors, stem cells, fusion proteins, and stable, more lipophilic synthetic analogs.

Apart from the delivery of blood-borne neurotrophic peptides such as by intravenous, subcutaneous, and intraperitoneal injections, and by intravenous infusions, an alternative pathway is nasal delivery. Although the detailed mechanism of nasal mucosa and olfactory nerve-mediated transfer is not clear, nasal delivered IGF-1 can reduce the infarct volume and neurological deficit in rats after focal stroke.[17] Transmission within the CSF has enabled the neuropeptides to rescue neuronal cell death after neurodegeneration and cerebral ischemia.

Despite progress in the attempts to use neurotrophic peptides as CNS therapeutics, there have not been many successful long-term effects. Part of the overall picture is that their actions are time and situation dependent, and that other factors, especially inhibitory molecules for neuroregeneration, are also present in the local environment.[12] Nonetheless, the effects of neurotrophic peptides probably extend beyond their roles as cell survival factors to contribute to neuroplasticity both structurally and physiologically.[6]

SUMMARY

Neurotrophic peptides not only affect the functions of endothelial cells and other components of the BBB, but they also may cross the BBB themselves. The permeation of neurotrophic peptides across the BBB is determined by physiochemical properties of the peptides (for those permeating by simple diffusion) or the presence of a carrier system (for those involving receptor-mediated transport, adsorptive endocytosis, or other specific interactions with the cell surface). There are multiple approaches to enhance the permeability of these peptides across the BBB, to conjugate them to targeted delivery systems, or to bypass the BBB. The long-term effect of exogenous neurotrophic peptides on CNS regeneration and neurobehavioral changes are finally receiving more extensive study, about 40 years after the first notion that peripheral peptides have direct CNS effects.[11] It is now established that BBB permeation of neurotrophic peptides can be involved in neuroendocrine changes and neuroplasticity.

REFERENCES

1. Banks WA, Kastin AJ. Reversible association of the cytokines MIP-1α and MIP-1β with the endothelia of the blood-brain barrier. *Neurosci Lett* 1996;**205**:202–6.
2. Banks WA, Kastin AJ, Komaki G, Arimura A. Passage of pituitary adenylate cyclase activating $polypeptide_{1-27}$ and pituitary adenylate cyclase activating $polypeptide_{1-38}$ across the blood–brain barrier. *J Pharmacol Exp Ther* 1993;**267**:690–6.
3. Banks WA, Robinson SM, Verma S, Morley JE. Efflux of human and mouse amyloid β proteins 1–40 and 1–42 from brain: impairment in a mouse model of Alzheimer's disease. *Neurosci* 2003;**121**:487–92.
4. Carpenter G, Cohen S. ^{125}I-labeled human epidermal growth factor. Binding, internalization, and degradation in human fibroblasts. *J Cell Biol* 1976;**71**:159–71.
5. Deguchi Y, Okutsu H, Okura T, Yamada S, Kimura R, Yuge T, et al. Internalization of basic fibroblast growth factor at the mouse blood–brain barrier involves perlecan, a heparan sulfate proteoglycan. *J Neurochem* 2002;**83**:381–9.
6. Ding Y, Kastin AJ, Pan W. Neural plasticity after spinal cord injury. *Curr Pharm Des* 2005;**11**(11):1441–50.
7. Dogrukol-Ak D, Banks WA, Tuncel N, Tuncel M. Passage of vasoactive intestinal peptide across the blood–brain barrier. *Peptides* 2003;**24**:437–44.

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8. Kastin AJ, Akerstrom V, Hackler L. Agouti-related protein (83-132) aggregates and crosses the blood–brain barrier slowly. *Metabolism* 2000;**49**:1444–8.
9. Kastin AJ, Akerstrom V, Pan W. Validity of multiple-time regression analysis in measurement of tritiated and iodinated leptin crossing the blood–brain barrier: meaningful controls. *Peptides* 2001;**22**:2127–36.
10. Kastin AJ, Akerstrom V, Pan W. Circulating TGF-β1 does not cross the intact blood–brain barrier. *J Mol Neurosci* 2003;**21**:43–8.
11. Kastin AJ, Olson RD, Schally AV, Coy DH. CNS effects of peripherally administered brain peptides. *Life Sci* 1979;**25**:401–14.
12. Kastin AJ, Pan W. Targeting neurite growth inhibitors to induce CNS regeneration. *Curr Pharm Des* 2005;**11**(10):1247–53.
13. Kastin AJ, Pan W. Concepts for biologically active peptides. *Curr Pharm Des* 2010 Aug;**16**:3390–400.
14. Kozu A, Kato Y, Shitara Y, Sugiyama Y. Kinetic analysis of transcytosis of epidermal growth factor in Madin–Darby canine kidney epithelial cells. *Pharm Res* 1997;**14**:1228–35.
15. Kurten RC. Sorting motifs in receptor trafficking. *Advanced Drug Delivery Reviews* 2003 Nov;**55**(11):1405–19.
16. Kurten RC, Cadena DL, Gill GN. Enhanced degradation of EGF receptors by a sorting nexin, SNX1. *Science* 1996 May; **272**(5264):1008–10.
17. Liu XF, Fawcett JR, Thorne RG, Frey WH. Non-invasive intranasal insulin-like growth factor-I reduces infarct volume and improves neurologic function in rats following middle cerebral artery occlusion. *Neurosci Lett* 2001 Aug;**308**(2):91–4.
18. McLay RN, Kimura M, Banks WA, Kastin AJ. Granulocyte-macrophage colony-stimulating factor crosses the blood–brain and blood–spinal cord barriers. *Brain* 1997;**120**:2083–91.
19. Osburg B, Peiser C, Dömling D, Schomburg L, Ko YT, Viogt K, et al. Effect of endotoxin on expression of TNF receptors and transport of TNF-α at the blood–brain barrier of the rat. *Am J Physiol* 2002;**283**:E899–908.
20. Pan W, Banks WA, Kastin AJ. Blood-brain barrier permeability to ebiratide and TNF in acute spinal cord injury. *Exp Neurol* 1997;**146**:367–73.
21. Pan W, Banks WA, Kennedy MK, Gutierrez EG, Kastin AJ. Differential permeability of the BBB in acute EAE: enhanced transport of TNF-α. *Am J Physiol* 1996;**271**:E636–42.
22. Pan W, Kastin AJ. Entry of EGF into brain is rapid and saturable. *Peptides* 1999;**20**:1091–8.
23. Pan W, Kastin AJ. Interactions of IGF-1 with the blood–brain barrier in vivo and in situ. *Neuroendocrinology* 2000;**72**:171–8.
24. Pan W, Kastin AJ. TNFα transport across the blood–brain barrier is abolished in receptor knockout mice. *Exp Neurol* 2002;**174**:193–200.
25. Pan W, Kastin AJ, McLay RN, Rigai T, Pick CG. Increased hippocampal uptake of TNFα and behavioral changes in mice. *Exp Brain Res* 2003;**149**:195–9.
26. Pan W, Stone KP, Hsuchou H, Manda VK, Zhang Y, Kastin AJ. Cytokine signaling modulates blood–brain barrier function. *Curr Pharm Des* 2011 Nov;**17**(33):3729–40.
27. Pan W, Vallance K, Kastin AJ. TGFα and the blood–brain barrier: accumulation in cerebral vasculature. *Exp Neurol* 1999;**160**:454–9.
28. Pan W, Yu Y, Cain CM, Nyberg F, Couraud P-O, Kastin AJ. Permeation of growth hormone across the blood–brain barrier. *Endocrinol* 2005;**146**:4898–904.
29. Poduslo JF, Curran GL. Glycation increases the permeability of proteins across the blood–nerve and blood–brain barriers. *Mol Brain Res* 1993;**23**:157–62.
30. Reddy KR. Controlled-release, pegylation, liposomal formulations: new mechanisms in the delivery of injectable drugs. *Ann Pharmacother* 2000 Jul;**34**(7–8):915–23.
31. Sakane T, Wu D, Pardridge WM. Neuropeptide pegylation: reduction in peripheral peptide metabolism causes parallel reduction in apparent brain neuropeptide uptake. *FASEB J* 1997 Feb;**11**(3):3628.
32. Schafer T, Zentgraf H, Zehe C, Brugger B, Bernhagen J, Nickel W. Unconventional secretion of fibroblast growth factor 2 is mediated by direct translocation across the plasma membrane of mammalian cells. *J Biol Chem* 2004 Feb;**279**(8):6244–51.
33. Shitara Y, Kato Y, Sugiyama Y. Effect of brefeldin A and lysosomotropic reagents on intracellular trafficking of epidermal growth factor and transferrin in Madin-Darby canine kidney epithelial cells. *J Control Release* 1998 Oct;**55**(1):35–43.
34. Somogyvari-Vigh A, Pan W, Reglodi D, Kastin AJ, Arimura A. Effect of middle cerebral artery occlusion on the passage of pituitary adenylate cyclase activating polypeptide across the blood–brain barrier in the rat. *Regul Peptides* 2000;**28**:89–95.
35. Tu H, Kastin AJ, Bjorbaek C, Pan W. Urocortin trafficking in cerebral microvessel endothelial cells. *J Mol Neurosci* 2007;**31**:171–82.

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Chapter 231

Oligopeptide and Peptide-Like Drug Transport

Richard F. Keep and David E. Smith

ABSTRACT

A knowledge of peptide transport at the blood–brain and blood–CSF barriers is important in understanding both endogenous neuropeptide activity and delivery of oligopeptide or peptide-like drugs to the brain. Although some oligopeptides use specific transport systems to gain entry into brain, the low lipophilicity of many small peptides and the presence of peptidases at the blood–brain barriers limit their entry into the central nervous system. In addition, the presence of several transporters that clear peptides from the brain or the CSF to the blood, such as PEPT2, P-glycoprotein, and PTS-1, has a major impact on brain peptide concentrations.

INTRODUCTION

The ability of some peptides to affect cell function means that their concentrations in the extracellular space are critical. This is particularly true in the brain where many peptides are neurotransmitters or neuromodulators. Perturbations in peptide concentration may come from brain parenchymal cell release, protein/peptide degradation within the brain, and blood-borne peptides. The aim of this chapter is to summarize our knowledge of the mechanisms that may influence the movement of oligopeptides (focusing on peptides with fewer than 10 amino acids) between the blood and brain and, thus, regulate peptide activity within the brain. A knowledge of such mechanisms is important in understanding the effects of endogenous neuropeptides, but it is also important for designing ways of delivering peptide or peptidomimetic therapeutic agents to the brain.

BLOOD–BRAIN BARRIERS: GENERAL PROPERTIES

Passive Permeability

The movement of peptides between the blood and brain is regulated by two barrier systems, the blood–brain barrier (BBB) and the blood–cerebrospinal fluid barrier (BCSFB). The BBB is formed by the cerebral endothelial cells and the tight junctions that link them. The BCSFB is formed by choroid plexus (CP) epithelial cells, situated in the two lateral, third and fourth cerebral ventricles, and the cells of the arachnoid membrane that overlays the surface of the brain. These cells are also linked by tight junctions that limit paracellular permeability of polar compounds (such as many peptides). By contrast, even some large compounds, such as horseradish peroxidase, can permeate between the brain and the CSF across the ependyma. Thus, homeostasis of the brain microenvironment relies on the coordinated action of both the BBB and the BCSFB.

Although tight junctions limit paracellular diffusion at the BBB and the BCSFB, there are major quantitative differences between these two barrier systems. Thus, the transendothelial electrical resistance of the BBB is about 3000–8000 $\Omega\,cm^2$, whereas the transepithelial resistance of the CP is about 150–175 $\Omega\,cm^2$. This is reflected in the passive diffusion of polar compounds across the barriers. The permeability of mannitol (MW 182) and inulin (MW 5000) is about 10- and 100-fold greater at the BCSFB compared with that of the BBB.[28]

CSF Sink Action

In addition to the low permeability of the BBB and BCSFB to polar compounds, the continual replenishment of CSF will also help limit the penetration of polar compounds from the blood to the brain or CSF. The CSF is primarily produced by the CPs and drains back into the blood at the arachnoid villi and other drainage sites. This turnover of CSF will continually clear compounds that have entered the brain from the blood, and this has been termed the CSF sink action. Thus, even during chronic administration, inulin concentrations in CSF only reach 1–3% of levels found in plasma, whereas sucrose (MW 342) may only reach 4–9% of plasma levels.[28]

Transport Properties

Although the blood–brain barriers were first described in terms of their ability to exclude dyes from the CNS, brain

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00231-1
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homeostasis requires more than a simple physical barrier. Thus, for example, the brain requires acquisition of nutrients from, and removal of waste products to, blood. Both these functions are potentially hindered by the occlusion of the BBB and BCSFB paracellular pathways by tight junction proteins. There are, therefore, a series of transport proteins at the BBB and BCSFB to fulfill these functions. Thus, Glut1 is present at the BBB to facilitate the entry of glucose from blood to brain, while there is an array of organic anion transporters at the BBB and BCSFB to remove metabolic waste products.[7,15] Although some barrier transporters are facilitative and enhance the rate of transport without imparting directionality (e.g., Glut1), others are coupled directly (e.g., P-glycoprotein) or indirectly (e.g., the peptide transporter, PEPT2) to ATP utilization. Many transporters are polarized in distribution (i.e., present on the blood-facing or brain-facing membrane of the barrier tissue) and involved specifically in blood to brain (CSF) or brain (CSF) to blood transport.[7,15]

For larger molecules, such as polypeptides and proteins, blood to brain uptake can involve either fluid-phase, absorptive, or receptor-mediated endocytosis.[3,5] It should be noted, however, that the level of endocytosis in cerebral endothelial cells is low compared with that in systemic capillaries and that endocytosis does not necessarily indicate transcytosis across the barrier tissue into the brain parenchyma or CSF. Thus, endocytosed compounds may accumulate in the barrier tissue, undergo degradation, or be recycled back to the blood.

Enzymatic Barriers

Apart from the low paracellular permeability of the BBB and BCSFB and the presence of efflux transporters at those barrier tissues that clear compounds from the brain, the entry of organic compounds into the brain from the blood can also be prevented by enzymatic degradation at the level of the BBB or BCSFB. For example, the BBB contains a variety of neurotransmitter-metabolizing enzymes such as monoamine oxidase, cholinesterases, GABA transaminase, aminopeptidases, and endopeptidases that will help prevent the entry of neurotransmitters from the blood to the brain.[3]

Nasal Delivery

There has been considerable interest in delivering peptide therapeutics to brain across the nasal epithelium to circumvent the blood–brain and blood–CSF barriers. Oligopeptides (and larger peptides) can be delivered by this route in animals and humans.[2,8] The delivery of peptides by intranasal delivery is very brain region specific (i.e., high to the olfactory bulb and along the trigeminal nerve[31]).

OLIGOPEPTIDE TRANSPORT AT THE BBB

Blood to Brain Transport at the BBB

Because of potential pharmacological and physiological importance, many studies have examined whether a variety of peptides can cross the BBB.[3,5,19,32,35] The BBB permeabilities of the dipeptides Gly-Phe, Gly-Leu, and carnosine are all very low.[35] It seems that these compounds enter the brain by passive diffusion as their permeabilities are not different from mannitol, a compound of similar molecular weight. Cyclo (His-Pro) has a greater BBB permeability, although it is still low.[1] Its uptake into brain is nonsaturable, and the greater brain uptake results from its greater lipophilicity and its resistance to enzymatic degradation.[1] Although it has a low permeability, cyclo (His-Pro) has central nervous system effects when administered systemically, showing the important point that peptides may have physiological effects within the brain at very low concentrations. The tripeptide, glutathione, has a much greater brain uptake than the dipeptides studied. This reflects the presence of a specific sodium-dependent glutathione transporter at the BBB.[35]

Several larger oligopeptides have higher BBB permeabilities than would be predicted from their molecular weight and lipophilicity,[35] such as Leu-enkephalin and delta sleep inducing peptide. This observation, as with glutathione, reflects the presence of carrier-mediated transport at the luminal (blood-facing) membrane of the BBB.[35] It should be noted, however, that for Leu-enkephalin, peptidase activity may prevent the appearance of intact peptide in the brain parenchyma.[35]

It is now evident that the BBB can selectively transport a variety of oligo- and polypeptides.[3,5,19,35] In terms of small oligopeptides, the BBB expresses many different solute carriers that transport organic compounds.[7] These transporters often have a wide range of substrates and overlapping substrate specificities. The affinity of these different transporters for different types of peptides has not been examined in detail. However, the organic anion-transporting polypeptide-2 (Oatp2) has affinity for the opioid peptide [D-pen^{2,5}]enkephalin (DPDPE). Oatp2 is believed to act as a bidirectional transporter on both the luminal and abluminal (brain-facing) membranes of the BBB, and to participate in DPDPE uptake into the brain.[7] Human OATP transports DPDPE and deltorphin II.[7] For larger peptides and polypeptides, which are outside the scope of this chapter, transcytosis is an important mechanism for blood to brain transport.[3,5,19,35]

Brain to Blood Transport at the BBB

In the past decade, considerable attention has been focused on efflux transporters at the BBB, particularly in relation to P-glycoprotein (Pgp) and its role in preventing the access

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of a wide range of drugs into the brain. The brain:blood concentration ratio of the opioid peptide DPDPE after systemic administration is 3- to 4-fold greater in mice deficient in Pgp.[6] This effect of Pgp on peptide distribution, however, probably is limited to those peptides that are relatively lipophilic. An analog of Leu-enkephalin, H-Tyr–D-Ala–Gly–Phe–D-Leu–OH (DADLE), does not seem to be significantly effluxed from the brain by Pgp, but the cyclic prodrugs of DADLE are.[18]

Other efflux transporters at the BBB can also affect the distribution of peptides. Thus, peptide transport system (PTS)-1 clears Tyr-MIF-1, Met-enkephalin, and oxytocin from the brain.[5] Somatostatin and its analogs also have a saturable efflux system that is separate from PTS-1.[5] PTS-6 also clears peptides from the brain at the BBB, but the size restriction of that transporter is unclear as it has so far been shown to transport only large peptides, pituitary adenylate cyclase-activating peptide and derivatives.[4] Such transporters may play an important role in clearing endogenous neuropeptides from the brain, but they may also play an important role in limiting the entry of neuroactive peptides (endogenous or exogenous) from the blood to the brain.

PEPTIDASES AT THE BBB

The cerebrovasculature expresses a wide range of enzymes that degrade peptides including carboxy-, amino-, and endopeptidases.[3,33] Some of these enzymes may be enriched both with respect to brain and with respect to systemic capillaries. The metabolic substrates for such peptidases include a wide range of compounds including enkephalins, neurotensin, bradykinin, angiotensin I and II, substance P, endothelin, dynorphin, luteinizing hormone releasing hormone, growth hormone releasing factor, and kallidin.[33] Along with the low passive diffusion across the BBB, the presence of these peptidases on the cerebrovasculature may aid in preventing the entry of potentially neuroactive peptides into the brain from the blood. It should be noted, though, that some peptides are vasoactive and neuroactive (e.g., angiotensin) and some peptidases may be present to limit vascular effects.

OLIGOPEPTIDE TRANSPORTERS AT THE CHOROID PLEXUS

Molecular Basis for Oligopeptide Transporter Heterogeneity

In mammals, the proton-coupled oligopeptide transporter (POT) family consists of four members (i.e., PEPT1, PEPT2, PHT1, and PHT2) and is responsible for the transport of small peptides/mimetics across biological membranes via an inwardly directed proton gradient and negative membrane potential.[12,21] The POT proteins vary in size from 572 to 729 amino acids and are predicted to contain 12 transmembrane domains, with the N- and C-termini facing the cytosol. The encoded proteins have many potential protein kinase recognition domains (i.e., 0–3 for PKA, 1–11 for PKC) and glycosylation sites (i.e., 2–7); however, their relevance has not been proven experimentally. Although PEPT1 and PEPT2 have high homology between species (about 80% in rat, rabbit, human, and mouse), the homology between these transporters for a given species is low (about 50%). Rat PHT1 and PHT2 have an amino acid identity of about 50%, but they show little homology to either PEPT1 or PEPT2 (<20%).

Tissue Distribution

PEPT1 is found primarily in the intestine and, to a lesser extent, in kidney.[12,21] It is a high-capacity, low-affinity transporter with *K*m values in the millimolar range. PEPT2 is the predominant POT in the kidney and, in contrast to PEPT1, is a low-capacity, high-affinity transporter with *K*m values in the micromolar range.[12,21] Together, PEPT1 and PEPT2 work in concert at the apical surface of epithelial cells to efficiently absorb and conserve protein digestive products arising in the intestine and kidney. However, PEPT2 has a wider tissue distribution that PEPT1 and, as demonstrated by immunolocalization in rats, is found extensively throughout the brain.[24] Although PEPT2 has its strongest expression in cerebral cortex, strong expression is also observed in the olfactory bulb, basal ganglia, cerebellum, and hindbrain. PEPT2 is also expressed abundantly in epithelial cells of the CP as well as in ependymal cells. This transporter is expressed exclusively on the apical membrane (i.e., CSF facing) of CP epithelia in both adult and neonatal animals. PEPT2 is differentially expressed in cerebral cortex as a function of age, with much greater levels in fetal and neonatal tissue than in adult. Moreover, PEPT2 is expressed in neurons (adult and neonate) as well as in astrocytes (neonate but not adult). The age-related decline of PEPT2 in cerebral cortex may reflect a loss of astrocytic PEPT2. There is no molecular or functional evidence for PEPT2 in endothelial cells of the BBB. Likewise, PEPT1 seems to be absent from brain. The apical expression of PEPT2 in CP suggests (along with functional studies; see below) that this transporter is involved in the export of neuropeptides, peptide fragments, and peptide-like drugs from CSF. Its expression in neurons and astrocytes further suggests that PEPT2 may play a role in regulating the concentration of neuropeptides in extracellular fluid.

Crystal Structure of a Prokaryotic Homolog of the POT Family

A significant discovery in advancing modern drug design and the targeted delivery of drugs within the body was made by determining the crystal structure of a prokaryotic peptide

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transporter, $PeptT_{So}$, from *Shewanella oneidensis*.[16] This structure contains 14 transmembrane helices, was found in a ligand-bound occluded state, and shares a high degree of sequence similarity and functional characteristics with the human PepT1 and PepT2 proteins. Fundamental structural biology questions remain such as which residues are involved in protonation and peptide binding, how specificity is achieved within the binding pocket, and what does the structure look like for peptide transporters in different conformational states along the transport cycle. Once these, and other questions of broader biochemistry, are solved, it may be possible to engineer the targeted delivery of future drug molecules to the central nervous system where PEPT2 has a significant role in peptide/mimetic disposition and dynamics.

Basolateral Transporter

The cloning, membrane localization, and functional activity of PEPT2 in CP epithelium have provided the fundamental basis for how peptides/mimetics are transported from CSF into the cell. However, the precise nature of the basolateral peptide transporter in CP is yet to be resolved (as is also the case in the kidney and intestine). Still, a low-affinity transporter was observed for glycylsarcosine (GlySar) uptake at the basolateral membrane of rat CP epithelial cells.[26] It has been suggested[30] that a single facilitative peptide transporter is expressed at the basolateral membrane of Caco-2 cells, and that PEPT1 and the basolateral peptide transporter cooperate in the efficient transepithelial transport of small peptides and peptide-like drugs. Thus, PEPT1 (at the apical membrane of intestine) mediates the active uptake of these substrates from the lumen against a concentration gradient, followed by the facilitated cellular efflux into blood down a concentration gradient. It is reasonable to conjecture that the same mechanism could apply for the transepithelial transport of peptides/mimetics across the CP. Notwithstanding this uncertainty, the CP basolateral transporter does not seem to be PEPT1.[24,26]

Intracellular Transporters

Information on the tissue distribution, cellular localization, and transport properties of the peptide/histidine transporters, PHT1 and PHT2, is limited. PHT1 transcripts are abundantly expressed in rat eye and brain, including the CP.[34] Moreover, PHT1 expressed in *Xenopus laevis* oocytes transports L-histidine with high affinity (K_m of 17 μM) and the dipeptide carnosine. In contrast, PHT2 transcripts are abundant in the rat lung, spleen, and thymus but detected faintly in brain.[22] Although the uptake of L-histidine and histidyl-leucine was observed when PHT2 protein was reconstituted into liposomes, the transport kinetics was not determined. PHT1 and PHT2 are unlikely to be involved in peptide/mimetic transport at the blood-CSF barrier (apical or basolateral membrane) because functional studies have failed to show inhibition of oligopeptide uptake by excess L-histidine.[12] Despite the uncertain function of PHT1 and PHT2 in the brain, they may have a role in the intracellular trafficking of small peptides, as suggested by lysosomal expression studies in rat PHT2-transfected BHK and HEK-293T cells.[22]

Larger Peptide Transporters

Three members of the organic-anion-transporting polypeptide (Oatp) gene family of membrane transporters have been shown to transport the δ-opioid peptides DPDPE (5 amino acids) and deltorphin II (7 amino acids).[7] Although rat Oatp1 and Oatp2 are localized at the apical and basolateral domains, respectively, of CP epithelium, Oatp2 is also expressed strongly at the BBB. Moreover, the human OATP (called OATP-A) exhibits especially strong expression in brain microvessels and capillary endothelial cells. These findings suggest that certain members of the Oatp/OATP gene family may play an important role in carrier-mediated translocation of opioid peptides across the mammalian BBB and BCSFB.

PEPTIDASES AT THE CHOROID PLEXUS

A wide range of peptidases have been identified at the CP.[3,28] Some are located on the blood vessels of the CP (e.g., angiotensin-converting enzyme) and may be involved in regulating vasoactive hormones. Others, however, are present on the epithelium plasma membrane and have a polarized distribution. Thus, both endopeptidase 24.11 and aminopeptidase N are found on the CP apical membrane. Both these peptidases cleave enkephalins (and other substrates). It is probable that such peptidases serve to keep the concentrations of such neuroactive compounds low in CSF. They may act in concert with PEPT2 present on the apical membrane, with PEPT2 clearing peptide fragments produced by peptidase action.

In addition to cleaving peptides present in the CSF, CP peptidases may be involved in preventing the entry of peptides from the blood to the CSF. Peptidases on the CP vasculature or the epithelial basolateral membrane could prevent access of neuroactive peptides to the CSF. In addition, intracellular CP peptidases may degrade peptides taken up by endocystosis and other processes. Thus, cathepsin D and tripeptidyl peptidase I are both present in the CP lysosomal compartment.[28]

PEPTIDE AND PEPTIDE-LIKE DRUG TRANSPORT AT THE BCSFB

Although our understanding of blood–CNS barriers has increased greatly over the past few years, a significant gap still remains in our appreciation of drug efflux transporters

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and their role in affecting the CNS exposure and pharmacological response to nutrients, drugs, and potential neuropharmaceuticals. Because of its unique localization within the brain, PEPT2 may represent an important determinant of CNS exposure and response to peptide (or mimetic) drugs, as well as endogenous peptides and their "oligo" fragments (some of which may be pharmacologically active or toxic).

In Vitro Disposition at the BCSFB

Studies in adult rat CP whole tissue, neonatal rat CP epithelial cells in primary culture, and genetically modified mice showed that PEPT2, located at the apical (CSF facing) as opposed to basolateral (blood-facing) membrane, is the primary member of the POT family responsible for the removal of neuropeptides, peptide fragments, and peptide-like drugs from the CSF.[12,28] In particular, *Pept2*-deficient mice have marked reductions (≥90%) in their ability to transport GlySar, carnosine, and 5-aminolevulinic acid in CP whole tissue. Moreover, the CP uptake of cefadroxil (a β-lactam antibiotic), as determined in wild-type and PEPT2 null mice, is primarily mediated by PEPT2 (80–85%) and to a minor extent by organic anion transporters (10–15%) and nonspecific mechanisms (5%). This finding suggests that aminocephalosporins, which are recognized by PEPT2, may have reasonable access to the CSF but are rapidly removed by drug efflux transporters making them ineffective for the treatment of bacterial infections in the CNS. However, one must be careful in extrapolating the results with cefadroxil to other aminocephalosporin drugs. As reported by Kuroda et al.,[13] small differences in chemical structure can substantially influence the mechanism of drug efflux from CSF. For example, cefaclor (3-chloro in dihydrothiazine ring) is eliminated from rat CSF by a benzylpenicillin-sensitive mechanism distinct from OAT3, whereas cephalexin (3-methyl in dihydrothiazine ring) is removed by a GlySar-sensitive transporter, presumably PEPT2.

In Vivo Disposition at the BCSFB

The development of *Pept2* knockout mice[25] has afforded a unique opportunity to explore the *in vivo* disposition of peptides and peptide-like drugs. Initial "proof of concept" studies with GlySar (a hydrolysis/peptidase resistant compound) showed that PEPT2 ablation has a significant effect on dipeptide disposition. In particular, *Pept2* knockout mice have a 2-fold greater concentration of GlySar in CSF, despite having much lower blood concentrations (as a result of a greater clearance by the kidney).[17] Thus, the CSF-to-blood concentration ratio of GlySar is 4-fold greater in *Pept2* null animals as compared with that of wild-type controls ($p<0.001$; Fig. 1). In the absence of PEPT2, the CSF-to-blood ratio is about 0.9, indicating that GlySar can achieve concentrations in the CSF similar to

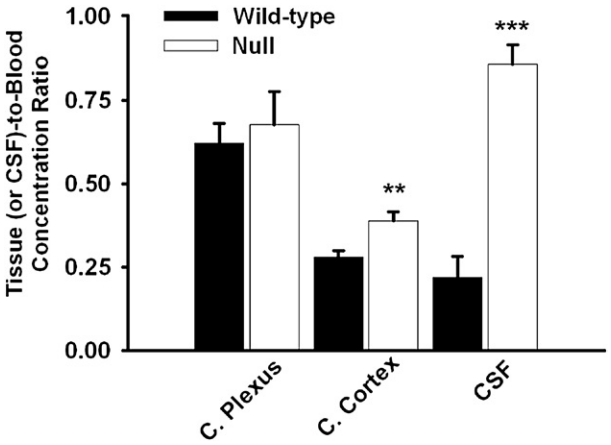

FIGURE 1 Tissue (or CSF)-to-blood concentration ratios of GlySar in the choroid plexus (c. plexus), cerebral cortex (c. cortex), and CSF of wild-type and PEPT2 null mice, 60 min after an intravenous bolus dose of dipeptide (0.05 μmol/g of body weight). Data are expressed as mean ± SE ($n=6$–7). $^{**}p<0.01$, and $^{***}p<0.001$ compared with wild-type mice.

that in blood. However, when PEPT2 is present, GlySar's persistence in the CSF is substantially reduced (i.e., CSF-to-blood concentration ratio of about 0.2). Although a modest 40% increase is observed in the cerebral cortex-to-blood concentration the ratio of GlySar in *Pept2*-deficient mice (i.e., 0.28 in wild-type vs. 0.39 in *Pept2* null animals, $p<0.01$; Fig. 1), there is no difference between genotypes in the CP-to-blood ratio (i.e., 0.62 in wild-type vs. 0.68 in *Pept2* null animals, $p>0.50$; Fig. 1). However, a substantial difference is observed in the CP-to-CSF concentration ratio of GlySar, where wild-type animals have a 5-fold greater tissue accumulation relative to medium at the transporter interface (i.e., 4.2 in wild-type vs. 0.8 in *Pept2* null animals, $p<0.01$).

Similar results were observed subsequently with the antibiotic drug cefadroxil in which the CSF-to-blood concentration ratio is 6-fold greater in *Pept2* knockout mice compared with that of wild-type animals.[23] Likewise, *Pept2* knockout mice have an 8-fold higher CSF-to-plasma concentration ratio of the dietary supplement carnosine after intravenous dosing (0.70 for null vs. 0.08 for wild-type mice).[11] By contrast, endogenous levels of carnosine are not different in the plasma (2.8 vs. 2.5 μM in null and wild-type mice, respectively) or CSF (8.9 vs. 8.6 μM in null and wild-type mice, respectively) for both genotypes. Taken as a whole, these findings clearly show that, under physiologic *in vivo* conditions, PEPT2 acts to efflux substrates from CSF into CP. It is also apparent that the body can adapt so that plasma and CSF levels of endogenous substrate (i.e., carnosine) are maintained during PEPT2 ablation. The nature of this adaptation remains unclear at present but deserves further attention. A proposed model for the transepithelial transport of di/tripeptides (and peptide-like drugs) in CP is shown in Fig. 2.

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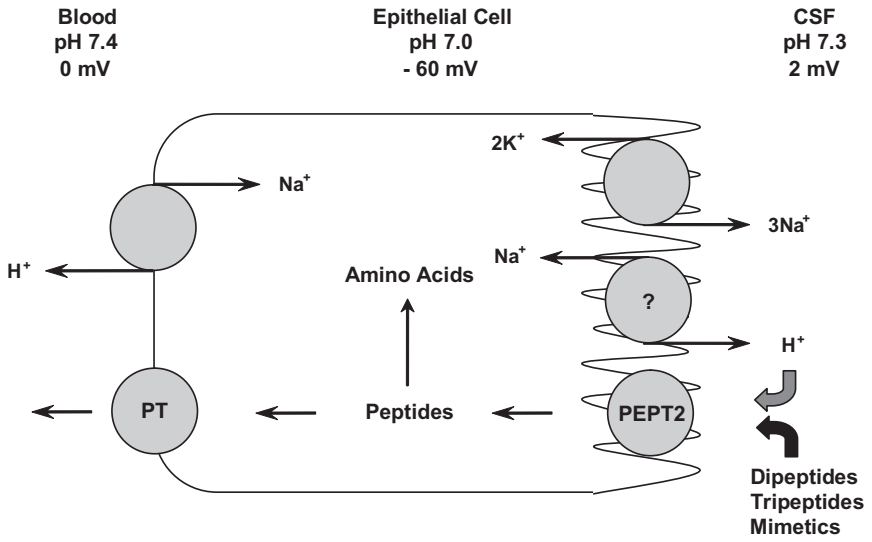

FIGURE 2 Proposed model for the transepithelial transport of di/tripeptides (and peptidomimetic drugs) in the choroid plexus. It is uncertain whether or not an acid microclimate (an area of low pH adjacent to the apical membrane) exists at choroidal brush border membranes, such as that observed in the intestine and the kidney. However, in the absence of a pH microclimate, accumulation could be driven by the negative membrane potential that exists in choroid plexus epithelium. Note that in the epithelial cells of the intestine and kidney, polarity of Na^+/K^+ ATPase and the Na^+/H^+ exchanger is reversed; still, PEPT2 is localized to the apical membrane. PT is the basolateral peptide transporter and PEPT2 is the apical proton-coupled peptide transporter.

Quantitative autoradiography of [^{14}C]GlySar in wild-type and *Pept2* knockout mice permits assessment of the parenchymal distribution of dipeptide in brain with high spatial resolution.[27] Autoradiographic analysis (performed 60 min after intracerebroventricular injection) shows that GlySar concentrations in the lateral, third, and fourth ventricle CPs are higher in wild-type than in *Pept2* null mice. Although GlySar uptake is higher in the ependymal–subependymal layer and septal region of wild-type mice, the half-distance of penetration into parenchyma is significantly less in *Pept2*-competent animals. The latter finding probably reflects the clearance of GlySar from interstitial fluid by neurons expressing PEPT2, which limits further penetration. A schematic illustrating as to how PEPT2 modifies the regional distribution of GlySar (and presumably other peptides and peptide-like drugs) in brain is shown in Fig. 3.

In Vivo Dynamics at the BCSFB

Regional modulation of peptide/mimetic disposition in brain by PEPT2 has relevance in pharmacology and toxicology. There is definitive evidence that PEPT2 can influence the antinociceptive response to a PEPT2 substrate, L-kyotorphin (L-KTP).[10] Specifically, the CSF efflux of L-KTP is slower in *Pept2* knockout mice than in wild-type animals, and this difference is reflected in greater L-KTP induced analgesia during PEPT2 ablation. In dose–response studies, the ED_{50} of L-KTP in *Pept2*-deficient mice is one-fifth the value of that observed in *Pept2*-competent mice (4 vs. 21 nmol, respectively). By contrast, the ED_{50} values of its diastereomer (D-KTP), a non-PEPT2 substrate, is not different between the two genotypes (9–10 nmol). The findings suggest that PEPT2 might function in the control of analgesia through the regulation of endogenous neuropeptides (and potential drug candidates) in the CSF or other compartments in brain. Another study showed a neuroprotective role for PEPT2 in modulating the toxicity of a heme precursor, 5-aminolevulinic acid (5-ALA).[9] This showed that survivability is reduced after high-dose acute administration and that neuromuscular function worsens after chronic dosing of 5-ALA in *Pept2* knockout mice. Pharmacokinetic studies indicate that CSF concentrations of 5-ALA are 8–30 times higher with PEPT2 ablation, suggesting that the greater toxicity of 5-ALA in *Pept2* knockout mice is because of changes in CSF (and presumably brain extracellular fluid) concentrations.

REGULATION OF PEPTIDE TRANSPORT

There is growing interest in the regulation of BBB and BCSFB transport and how that may affect brain drug delivery. Thus, BBB Pgp expression is affected by xenobiotics, stress, and disease states[15] and this will alter the brain distribution of peptide substrates of that transporter. Similarly, there is evidence that stress and disease can change BBB organic anion transport.[20]

The regulation of PEPT2 at the blood–CSF barrier has received limited attention. In this regard, mutations in the scaffolding protein PDZK1 can reduce the functional activity of PEPT2 in HEK293 cells,[29] although the mechanism of this interaction and relevance to brain function are unclear. Likewise, the serum- and glucocorticoid-inducible kinase SGK1 can enhance PEPT2 function and surface abundance

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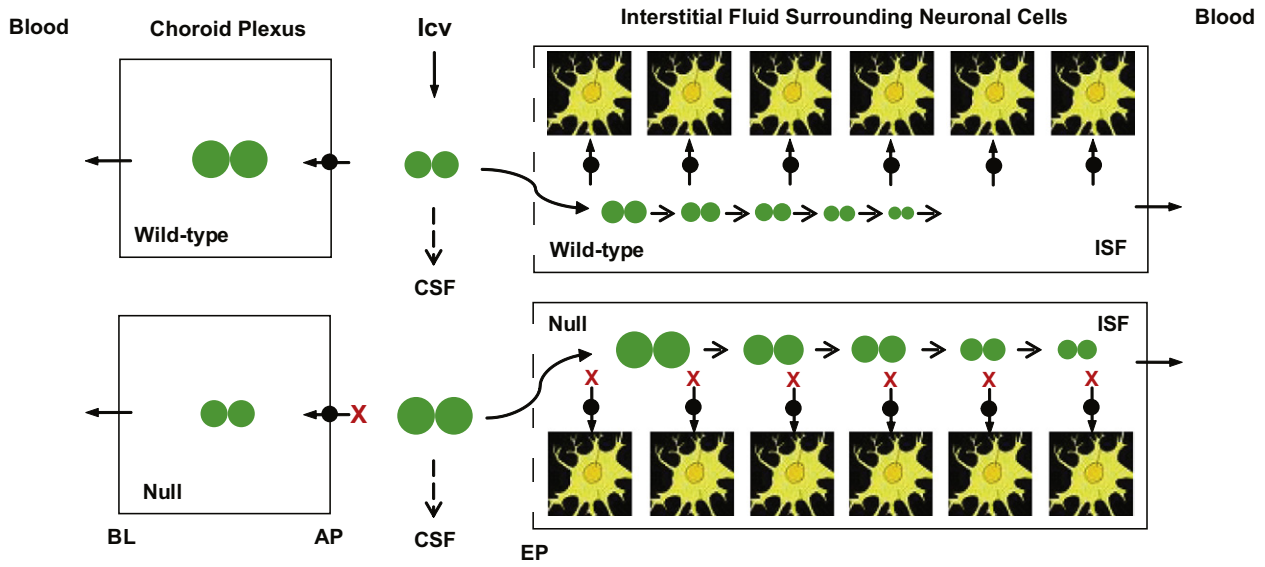

FIGURE 3 Schematic of how the proton-coupled oligopeptide transporter SLC15A2 (PEPT2 shown as ◄●–) affects the distribution of GlySar (displayed as dipeptide ●●) in different regions of the brain after an icv injection (adapted from Ref. 27). Interstitial fluid (ISF) concentrations of GlySar are reduced in wild-type mice because of PEPT2-mediated uptake in choroid plexus (CP), along with reduced coronal penetration of GlySar as it moves along the ISF–parenchymal axis and is taken up and trapped by PEPT2-containing neurons (shown as ✸). In null mice, substantially lower concentrations of GlySar are observed in CP epithelial cells, and higher concentrations of GlySar are observed in cerebrospinal fluid (CSF) and ISF surrounding the neurons. The solid arrows (——►) represent GlySar transport through membranes and the dashed arrows (- - ->) represent GlySar diffusion/bulk flow. Icv, intracerebroventricular; BL, basolateral membrane; AP, apical membrane; EP, ependyma (shown as dashed lines for both wild-type and null mice). The solid X symbols (X) represent lack of PEPT2 activity in the null mice.

in *Xenopus oocytes*,[14] but the nature of this interaction in neuronal function and disease is unknown. Finally, as discussed before,[24] PEPT2 is developmentally regulated in rat cerebral cortex and, as shown by immunolocalization studies, is expressed in rat neurons (neonate and adult) and astrocytes (neonate only).

SUMMARY

Although some peptides use specific transport systems at the blood–brain barriers to gain entry into brain, the low lipophilicity of many small peptides and the presence of peptidases at the BBB and BCSFB limit the entry of oligopeptides into the central nervous system. The presence of efflux transporters may further limit such entry. In particular, the activity of transporters such as Pgp and PTS-1 at the BBB, and PEPT2 at the BCSFB serves to clear neuropeptides, peptide fragments, and potential peptidomimetic drugs from the brain. As a result, a better understanding of efflux transport will be critical to the successful design, delivery, and targeting of neuropharmaceuticals. Moreover, such knowledge will facilitate insight into the regulation of endogenous neuropeptide activity.

ACKNOWLEDGMENTS

This work was supported in part by Grants R01 GM035498 (D.E.S.) and R01 NS034709 (R.F.K.) from the National Institutes of Health.

REFERENCES

1. Banks WA, Kastin AJ, Akerstrom V, Jaspan JB. Radioactively iodinated cyclo(His-Pro) crosses the blood–brain barrier and reverses ethanol-induced narcosis. *Am J Physiol* 1993;**264**:E723–9.
2. Born J, Lange T, Kern W, McGregor GP, Bickel U, Fehm HL. Sniffing neuropeptides: a transnasal approach to the human brain. *Nature Neuroscience* 2002;**5**:514–6.
3. Brasnjevic I, Steinbusch HWM, Schmitz C, Martinez-Martinez P. European nanobiopharmaceutics research I. Delivery of peptide and protein drugs over the blood–brain barrier. *Prog Neurobiol* 2009;**87**:212–51.
4. Dogrukol-Ak D, Kumar VB, Ryerse JS, Farr SA, Verma S, Nonaka N, et al. Isolation of peptide transport system-6 from brain endothelial cells: therapeutic effects with antisense inhibition in Alzheimer and stroke models. *J Cereb Blood Flow Metabol* 2009;**29**:411–22.
5. Egleton RD, Davis TP. Bioavailability and transport of peptides and peptide drugs into the brain. *Peptides* 1997;**18**:1431–9.
6. Golden PL, Pollack GM. Blood–brain barrier efflux transport. *J Pharm Sci* 2003;**92**:1739–53.
7. Graff CL, Pollack GM. Drug transport at the blood–brain barrier and the choroid plexus. *Curr Drug Metab* 2004;**5**:95–108.
8. Hallschmid M, Benedict C, Schultes B, Perras B, Fehm H-L, Kern W, et al. Towards the therapeutic use of intranasal neuropeptide administration in metabolic and cognitive disorders. *Regul Pept* 2008;**149**:79–83.
9. Hu Y, Shen H, Keep RF, Smith DE. Peptide transporter 2 (PEPT2) expression in brain protects against 5-aminolevulinic acid neurotoxicity. *J Neurochem* 2007;**103**:2058–65.
10. Jiang H, Hu Y, Keep RF, Smith DE. Enhanced antinociceptive response to intracerebroventricular kyotorphin in Pept2 null mice. *J Neurochem* 2009;**109**:1536–43.

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11. Kamal MA, Jiang H, Hu Y, Keep RF, Smith DE. Influence of genetic knockout of Pept2 on the in vivo disposition of endogenous and exogenous carnosine in wild-type and Pept2 null mice. *Am J Physiol Regul Integr Compar Physiol* 2009;**296**:R986–91.
12. Kamal MA, Keep RF, Smith DE. Role and relevance of PEPT2 in drug disposition, dynamics, and toxicity. *Drug Metabol Pharmacok* 2008;**23**:236–42.
13. Kuroda M, Kusuhara H, Endou H, Sugiyama Y. Rapid elimination of cefaclor from the cerebrospinal fluid is mediated by a benzylpenicillin-sensitive mechanism distinct from organic anion transporter 3. *J Pharmacol Exp Ther* 2005;**314**:855–61.
14. Lang F, Strutz-Seebohm N, Seebohm G, Lang UE. Significance of SGK1 in the regulation of neuronal function. *J Physiol London* 2010;**588**:3349–54.
15. Miller DS. Regulation of P-glycoprotein and other ABC drug transporters at the blood–brain barrier. *Trends Pharmacol Sci* 2010;**31**:246–54.
16. Newstead S, Drew D, Cameron AD, Postis VLG, Xia XB, Fowler PW, et al. Crystal structure of a prokaryotic homologue of the mammalian oligopeptide-proton symporters, PepT1 and PepT2. *EMBO J* 2011;**30**:417–26.
17. Ocheltree SM, Shen H, Hu Y, Keep RF, Smith DE. Role and relevance of peptide transporter 2 (PEPT2) in the kidney and choroid plexus: in vivo studies with glycylsarcosine in wild-type and PEPT2 knockout mice. *J Pharmacol Exp Ther* 2005;**315**:240–7.
18. Ouyang H, Andersen TE, Chen W, Nofsinger R, Steffansen B, Borchardt RT. A comparison of the effects of p-glycoprotein inhibitors on the blood–brain barrier permeation of cyclic prodrugs of an opioid peptide (DADLE). *J Pharm Sci* 2009;**98**:2227–36.
19. Pan W, Kastin AJ. Why study transport of peptides and proteins at the neurovascular interface. *Brain Res Brain Res Rev* 2004;**46**:32–43.
20. Ronaldson PT, Finch JD, Demarco KM, Quigley CE, Davis TP. Inflammatory pain signals an increase in functional expression of organic anion transporting polypeptide 1a4 at the blood-brain barrier. *J Pharmacol Exp Ther* 2011;**336**:827–39.
21. Rubio-Aliaga I, Daniel H. Peptide transporters and their roles in physiological processes and drug disposition. *Xenobiotica* 2008;**38**:1022–42.
22. Sakata K, Yamashita T, Maeda M, Moriyama Y, Shimada S, Tohyama M. Cloning of a lymphatic peptide/histidine transporter. *Biochem J* 2001;**356**:53–60.
23. Shen H, Ocheltree SM, Hu Y, Keep RF, Smith DE. Impact of genetic knockout of PEPT2 on cefadroxil pharmacokinetics, renal tubular reabsorption, and brain penetration in mice. *Drug Metab Dispos* 2007;**35**:1209–16.
24. Shen H, Smith DE, Keep RF, Brosius III FC. Immunolocalization of the proton-coupled oligopeptide transporter PEPT2 in developing rat brain. *Mol Pharm* 2004;**1**:248–56.
25. Shen H, Smith DE, Keep RF, Xiang J, Brosius III FC. Targeted disruption of the PEPT2 gene markedly reduces dipeptide uptake in choroid plexus. *J Biol Chem* 2003;**278**:4786–91.
26. Shu C, Shen H, Teuscher NS, Lorenzi PJ, Keep RF, Smith DE. Role of PEPT2 in peptide/mimetic trafficking at the blood-cerebrospinal fluid barrier: studies in rat choroid plexus epithelial cells in primary culture. *J Pharmacol Exp Ther* 2002;**301**:820–9.
27. Smith DE, Hu Y, Shen H, Nagaraja TN, Fenstermacher JD, Keep RF. Distribution of glycylsarcosine and cefadroxil among cerebrospinal fluid, choroid plexus, and brain parenchyma after intracerebroventricular injection is markedly different between wild-type and Pept2 null mice. *J Cereb Blood Flow Metab* 2011;**31**:250–61.
28. Smith DE, Johanson CE, Keep RF. Peptide and peptide analog transport systems at the blood–CSF barrier. *Adv Drug Deliv Rev* 2004;**56**:1765–91.
29. Sugiura T, Kato Y, Kubo Y, Tsuji A. Mutation in an adaptor protein PDZK1 affects transport activity of organic cation transporter OCTNs and oligopeptide transporter PEPT2. *Drug Metab Pharmacok* 2006;**21**:375–83.
30. Terada T, Sawada K, Saito H, Hashimoto Y, Inui K. Functional characteristics of basolateral peptide transporter in the human intestinal cell line Caco-2. *Am J Physiol* 1999;**276**:G1435–41.
31. Thorne RG, Hanson LR, Ross TM, Tung D, Frey II WH. Delivery of interferon-beta to the monkey nervous system following intranasal administration. *Neuroscience* 2008;**152**:785–97.
32. Van Dorpe S, Adriaens A, Polis I, Peremans K, Van Bocxlaer J, De Spiegeleer B. Analytical characterization and comparison of the blood–brain barrier permeability of eight opioid peptides. *Peptides* 2010;**31**:1390–9.
33. Witt KA, Gillespie TJ, Huber JD, Egleton RD, Davis TP. Peptide drugmodificationstoenhancebioavailabilityandblood–brainbarrierpermeability. *Peptides* 2001;**22**:2329–43.
34. Yamashita T, Shimada S, Guo W, Sato K, Kohmura E, Hayakawa T, et al. Cloning and functional expression of a brain peptide/histidine transporter. *J Biol Chem* 1997;**272**:10,205–11.
35. Zlokovic BV. Cerebrovascular permeability to peptides: manipulations of transport systems at the blood–brain barrier. *Pharm Res* 1995;**12**:1395–406.

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Chapter 232

Opioid Peptides

Richard D. Egleton, Ken A. Witt and Thomas P. Davis

ABSTRACT

Opioid peptide research over the last 30 years has revealed considerable information regarding the function of the opioid receptors and their role in regulating pain and behavioral processes. Despite this, there remains a lack of viable peptide-based therapeutics capable of central nervous system action. This is largely because of the limited ability of opioid peptides to transverse the blood–brain barrier (BBB) intact. The BBB acts as a metabolic and transport barrier preventing peptide delivery to the brain. This chapter update addresses opioid peptide development for the enhancement of brain delivery.

INTRODUCTION

The *endogenous opioid system* modulates the perception of pain and many other behavioral responses (e.g. memory, pleasure, hunger) through a complex series of neuronal networks.[2] This system is regulated by three principle opioid receptor types (μ, δ, κ) expressed throughout the central nervous system (CNS). The endogenous substrates for these receptors (i.e. enkephalins, endorphins, endomorphins, and dynorphins) each have differential binding to the respective opioid receptor subtypes. Over the past two decades, numerous opioid peptide analogs have been synthesized. Although such peptides have led to a significant increase in the understanding of opioid receptors, none have shown to be a practical clinical alternative to morphine or other non-peptide-based therapeutics. Moreover, given the recent insights of opioid receptor action in various behavior processes,[2] there is a pressing need for viable receptor subtype-specific opioids for both treatment and study. Nevertheless, the development of CNS acting opioid peptides faces a significant hurdle, that being the BBB. The BBB plays a critical role in maintaining brain homeostasis, which it does through the interaction of three barrier systems: a physical barrier, a transport barrier (i.e. efflux mechanisms), and a metabolic barrier. These barrier systems directly impede peptide uptake into the brain. In this chapter update, we focus on studies evaluating opioid peptide drug delivery to the brain, within the context of the BBB. We further address how diseases may impact peptide drug uptake in the context of BBB alterations.

STABILITY

The metabolic stability of any drug is a primary factor in the determination of brain bioavailability. As opioid peptides are rapidly degraded by endogenous peptidases, one approach to enhancing BBB penetration is to increase the metabolic stability. This is highlighted by the study of Brownson et al. who showed that in the presence of enzyme inhibitors, transport of Met-enkephalin across an *in vitro* BBB was increased.[3] By contrast, there was no effect on the transport of metabolically stable opioid peptides,[3] indicating that metabolic stability is a vital first step toward improving peptide-based drug delivery to the brain. Furthermore, the Brownson study underscores the enzymatic activity of the BBB endothelium itself. Although not as metabolically active as the liver, the BBB endothelium does express enzymes capable of rapidly degrading peptide-based drugs, including a significant number of cytochrome P450 enzymes (Table 1).

Several strategies have been used to increase the metabolic stability of opioid peptides, such as insertion/addition of unnatural amino acids, cyclization of linear peptides, and glycosylation (see Ref. 31 for review of methods). One of the common features of opioid peptides is that they share a similar amino terminal sequence of Tyr-Gly-Gly-Phe-X. This amino acid sequence is believed to be essential for opioid receptor binding and activation. This makes the opioid peptides particularly sensitive to peptidase cleavage of the N-terminal TYR residue, as this cleavage will render the peptide inactive. A successful strategy for protecting the amino nitrogen amino acid is to replace the Gly at position 2 with a D-amino acid. Several amino acids have been used at this position, such as Ala (e.g. DAMGO, DADLE) and Ser (e.g. DSLET). Such amino acid substitutions when coupled with C-terminal amidation have shown to substantially enhance enzymatic stability.[24,28] Altering the structural design of a peptide via constrainment or bridging may also improve stability. A prime example is shown with the Met-enkephalin analog D-penicillamine (2,5)-enkephalin (DPDPE), which is constrained by a disulfide bridge. Not only does this design enhance stability

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00232-3
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TABLE 1 Common Enzymes at the BBB

Peptidases (alternate names)
Angiotensin-Converting Enzyme (Peptidyl-dipeptidase hydrolase) (Peptidyl dipeptidase A) (Kinase II)
Aminopeptidase M (N)
Enkephalinase (Endopeptidase 24.11) (Neutral endopeptidase)
Endopeptidase (Endopeptidase 24.15)
Carboxypeptidase N (Kinase I)
Cytochrome P450s
CYP1A1, CYP1B1, CY2A6, CYB2B6, CYP2C, CYP2C9, CYP2E1, CYP2J2, CYP3A4, CYP4A11, CYP11B

with a serum and brain half-life of approximately 500 min but it also enhances selectivity and affinity for the δ-opioid receptor.[3] Another consistently applied method to increase stability is glycosylation. The addition of a carbohydrate moiety to a peptide (i.e. glycopeptide) may significantly impact the pharmacokinetic properties, not only improving metabolic stability but also increasing plasma half-life and membrane permeability.[31] Glycosylation of the opioid analog TY027 (Tyr-D-Ala-Gly-Phe-Met-Pro-Leu-Trp) by replacing the terminal Trp with a Ser-glycoside results in an increased metabolic half-life from 4.8 h to over 24 h (rat plasma evaluation).[35] Other sugar substitutions, such as fructose, also improve the metabolic stability, thus increasing the half-life of peptide drugs.[13] The principle caveat with opioid peptide glycosylation, or any other addition/modification, is that the placement of the moiety must occur at a location within the peptide so as not to interfere with the receptor binding.

Endomorphins (EMs) are endogenous opioid peptides that differ from the typical opioid structure. Instead of having the opioid receptor motif of Tyr-Gly-Gly-Phe-X, endomorphin-1 (EM-1) has a Tyr-Pro-Trp-Phe-NH_2 sequence, whereas endomorphin-2 (EM-2) has a Tyr-Pro-Phe-Phe-NH_2 sequence. They are also a bit unusual that instead of being full agonists at the opioid receptors (like the other endogenous opioids), they are partial agonists. The Pro in position 2 induces what has been described as a "kink" in the peptide structure that places the aromatic group at positions 1 and 3 into the correct alignment for binding to the μ-receptor. Initial studies replacing the Pro with a D-Pro also increased enzymatic stability but greatly reduced receptor binding affinity. An alternate strategy is to incorporate unnatural amino acids with six-membered heterocyclic rings, such as piperidine-2-, 3-, and 4-carboxylic acids, which mimic Pro. Perlikowska[23] showed that this substitution significantly increases metabolic stability in brain and subsequently increases intracerebroventricular analgesic activity.[22] Other research groups have found that conjugation of EM-1 with lipoamino acids such as 2-aminododecanoic acid increases the metabolic stability.[15] However, such conjugations tend to make the EM-1 too lipophilic and decrease delivery to the brain.[15] This highlights that increasing the metabolic stability of peptides is only one part of the equation for opioid peptide delivery to the brain.

TRANSPORT

Although numerous opioid peptide modifications have been explored to enhance stability and receptor selectivity, the question remains as to how these modifications affect the ability of the peptide to traverse the BBB. The nature of the BBB, with the presence of "tight junctions" with high transendothelial cell resistance, limits the paracellular entry of drugs into the brain. Thus, the transport of opioid peptides between endothelial cells is not viable. Beyond invasive methods, a number of structural/pharmacological modifications can be used to circumvent the BBB. Moreover, there are a number of transporters that can be targeted to enhance brain delivery (Fig. 1).

The number of hydroxyl groups within a peptide, and in turn hydrogen-bonding potential, dictates its partition coefficient. In this regard, the type of amino acids (polar vs. nonpolar) and their respective positions to one another dictates the overall lipophilicity of a peptide. Generally, moderately increasing the lipophilicity of a peptide drug promotes its ability to diffuse across the membrane. Historically, several different methods have been used to promote this, including methylation, halogenation, and reduction of hydrogen-bonding potential.[31] Methylation has shown to reduce hydrogen binding potential. Methylation [2,6-dimethyl-tyr] of the opioid peptide DPDPE has shown to result in enhanced bioavailability to the brain, resulting in a 10-fold increase in analgesia.[11] Tri-methylation of DPDPE was subsequently evaluated, with shifts in lipophilicity, BBB permeability, and an analgesic profile shown to be dependent on stereoconformation methyl groups.[34] Halogenation impact on lipophilicity and brain uptake is dependent on the conjugated halogen (i.e. Cl, Br, F). Addition of chlorine to DPDPE is particularly advantageous, increasing lipophilicity and BBB permeability with significant enhancement of analgesia.[29] A combination of a C-terminal chloro addition and an N-terminal guanidine addition (cationization), together with a D-Ala substitution in position 2 of EM-1, results in a guanidine- [D-Ala^2, *p*-Cl-Phe^4] EM-1 with substantial

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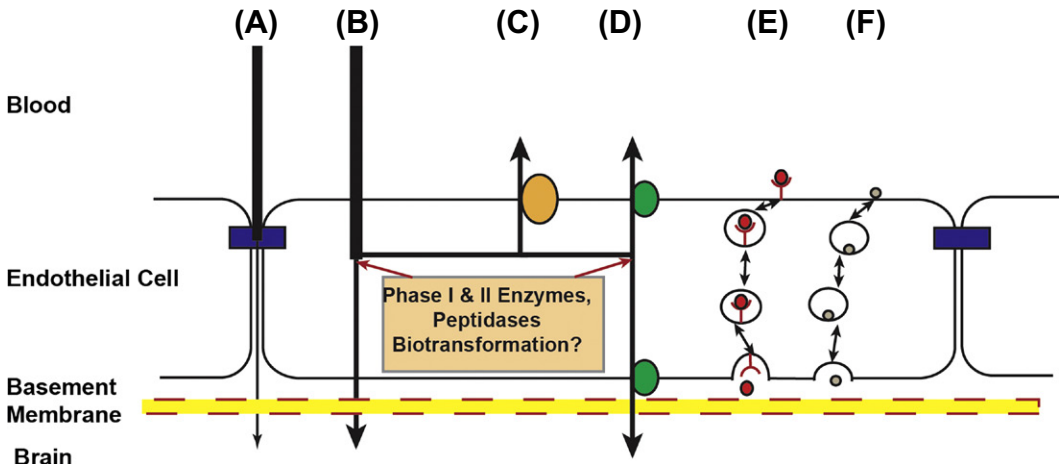

FIGURE 1 Potential transporter targets to improve opioid peptide delivery at the BBB. The transport systems at the BBB that have been targeted for increased opioid uptake include diffusion via increasing lipophilicity (B), masking efflux transport properties (C), targeting solute carriers (D), prodrugs/targeted immunoliposomes for receptor-mediated endocytosis (E), and changing charge/amphipacity for adsorptive-mediated endocytosis (F). We also have to take into account that the transporters can interact with each other and also with the metabolic barrier of the BBB. Additionally, the presence of phase-I and phase-II metabolic enzymes, and various peptidases, can turn a peptide into a substrate for the efflux transporters.

stability and 3-fold greater potency than the parent compound.[17] Such hybrid formulations using multiple methods to increase BBB permeability have great potential for future pharmacological use, yet the complexity of synthesis must be weighed against outcome benefits. For enkephalins, other lipophilic derivatives show promise not only in increasing metabolic stability, but also improving brain delivery. One evaluation showed that adding a synthetic lipid moiety such as 2-amino-D, L-octanoic acid hydrochloride (C8 lipoamino acid) or 2-amino-D, L-dodecanoic acid hydrochloride (C12 lipoamino acid) to the N-terminus of Leu-enkephalinamide increases lipophilicity and *in vitro* permeability.[4] Furthermore, in the subsequent *in vivo* evaluation, there was effective brain uptake of the C8-Leu-enkephalinamide via the nasal route.[4] The nasal route has significant advantages for delivery of highly lipophilic peptides as it avoids first pass metabolism through the liver, yields a high concentration of drug in the vicinity of the receptor, and reduces the potential of the compound from entering other organ beds. Although many strategies exist to enhance lipid solubility of opioid peptides, there are several limitations. Beyond maintenance of receptor activity and potential for enhanced peripheral distribution of the drug, there is also the potential for intracellular sequestration and enhanced affinity of efflux transporters at the BBB.

The BBB has a high level of efflux transporters [e.g. P-glycoprotein (P-gp), multidrug resistant protein (MRP), breast cancer resistant protein (BRCP)] that can prevent substances from entering the brain (Fig. 1). The substrate specificity for these transporters is rather broad and can be a considerable issue for opioid peptide delivery to the brain. Opioid peptides that are substrates for efflux mechanisms include a number of cyclized DADLE (Tyr-D-Ala-Gly-Phe-D-Leu) prodrugs. Cyclization of opioid peptides has been used to promote enzymatic stability and also maintain the peptide drug structure in a rigid conformation that can promote receptor binding. A strategy used by one group was to cyclize prodrugs of DADLE to enhance BBB permeability. However, in this case, the DADLE prodrug design enhanced its affinity for P-gp, one of the highest expressed ATP dependent efflux pumps expressed at the BBB.[20] Inhibitors of P-gp, such as cyclosporine, significantly increase the uptake of these DADLE prodrugs into the brain, showing this is indeed the key mechanism in inhibiting their uptake.[20] DPDPE, another cyclized peptide encephalin analog, is also a substrate for P-gp.[5] Administration of DPDPE to P-gp knockout mice, or in the presence of a P-gp inhibitor, increases brain accumulation of DPDPE and the analgesic response.[5] Several strategies have been used to mask opioids from efflux transporters. Pegylation (PEG) of DPDPE has been shown *in vitro* to mask the peptide structure from P-gp.[33] This modification improves brain distribution in mice and increases analgesia. Interestingly, the PEG seems to also act as a prodrug, improving the peripheral pharmacokinetics and promoting BBB entry. Once inside the brain, the PEG group is believed to undergo hydrolysis allowing the unconjugated DPDPE to act at the δ opioid receptor.[33] Other methods that have been used include the use of pluronic copolymer P85, which seems to inhibit DPDPE P-gp interaction as well. Regardless of the manner used, the critical aspect is to reduce efflux activity against the respective peptide, while maintaining BBB permeability and receptor affinity.

On the flipside, the BBB has several influx transporter mechanisms that can be targeted to improve brain delivery

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of peptides. Many highly active influx mechanisms consist of transporters that work either via facilitative diffusion or via receptor-mediated processes (Fig. 1). The incorporation of linkers and/or modification of an opioid peptide to enhance the affinity for such nutrient transports have shown some success. The potent opioid peptide analog biphalin [(Tyr-D-Ala-Gly-Phe-NH)2], targets the large neutral amino acid (LNAA) carrier for uptake across the BBB, a transporter also used for L-DOPA.[1] However, some enkephalins use the saturable transporter, peptide transport system-1 (PTS-1) for efflux.[18] Another proposed strategy aimed to increase brain uptake of opioid peptides is via targeting the glucose-1 transporter (Glut-1). In one study, a group glycosylated the cyclic Met-enkephalin analog [D-Cys^{2,5},Ser^{6},Gly^{7}]-enkephalin to increase brain uptake by Glut-1.[9] Although this strategy did improve brain uptake and analgesia, the increased brain uptake of the glycosylated analog was shown not to be mediated via the glut-1 transporter, but via a yet unidentified saturable mechanism.[9] Nevertheless, other groups subsequently used this method on a series of different peptides and found similar improvements in brain penetration in rodents.[13,35] Interestingly, the glycosylated peptides that show the best analgesia in rats and mice do not lead to any significant improvements in CNS-mediated analgesia in primates.[8] This further highlights another fundamental caveat often overlooked that different transport systems exist across species, and thus evaluations of peptide modifications often needs to be evaluated in multiple models.

Targeting receptor-mediated transcytosis (RMT) is another heavily explored strategy to improve BBB uptake. In this regard, vector-based methods have shown some success. The principle of the vector approach is to couple a peptide to a substance (e.g. antibody/biologics) with affinity for a highly expressed BBB transporter. In this manner, the peptide is not only targeted to the BBB but also actually taken up across the membrane. One such study used a vector approach that bound the opioid peptide DALDA to an antibody targeting the transferrin-receptor.[14] The BBB expresses high levels of the transferrin-receptor, so this complex acts as a specific target for the brain endothelium. Compared with nonvector peptide, the brain uptake of vectorized-DALDA was increased 18-fold to reach 0.12% of the injected dose per gram tissue.[14] Although an effective method for delivering the peptide, this approach using an antibody or Fc-conjugate is rather expensive and may not be an economically viable for continuous drug delivery to the brain. Nevertheless, this method has shown utility for targeting liposomes to the brain and has been used for both delivery of chemotherapeutics and genetic material.[21]

Another delivery avenue is to take advantage of adsorptive-mediated endocytotic/transcytotic (AMT) processes. Although RMT is a highly specific means of transport, AMT is by definition a nonspecific process. In this regard, the AMT pathways of BBB may provide a more effective means for continuous transport of opioid peptides through the endothelial cytoplasm. AMT-based approaches include structural modification, cationization, glycosylation, and binding to various carriers/vectors (see Ref. 12 for full review). Several dermorphin analogs have been shown to undergo AMT at the BBB. Interestingly, for this class of opioid analogs, there seems to be some differential rates of AMT. Two dermorphin analogs, N-alpha-amidino-Tyr-D-Arg-Phe-betaAla-OH (ADAB) and N-alpha-amidino-Tyr-D-Arg-Phe-MebetaAla-OH (ADAMB) use AMT to penetrate the BBB. Their respective membrane uptake is inhibited by a range of endocytosis inhibitors and polycations, though not 2,4-dinitrophenol, or by decreasing the temperature to 4 °C. By contrast, the related dermorphin Tyr-D-Arg-Phe-beta-Ala-OH (TAPA) is inhibited by 2,4-dinitrophenol and has a greater rate of uptake than either ADAMB or ADAB.[6,7] Further, the half-saturation constant of TAAP is approximately three times that of ADAMB and ADAB, indicating that the transport affinity is much higher.[6,7] Another approach is to enhance the cationized state of the peptide, which contributes to an enhanced interaction with anionic sites of the BBB endothelium (i.e. glycocalyx) and thereby increase the potential for AMT uptake. Some examples involve the binding of cationic compounds such as spermine or putrescine, or via making the peptide structure more basic, such as for the dynorphin peptide analog E-2078.[12] AMT is also a potential mechanism for the increased uptake for glycosylated peptides. Some studies have shown that the least energy confirmation for a group of enkephalin glycopeptides is an amphipathic-like structure. It is possible that this increases the interaction with the membrane of the cells to increase uptake. Many glycosylated opioid peptides, including Met-enkephalins and β-endorphins, have shown enhanced enzymatic stability and increased BBB transport.[31] Although the AMT approach shows viability, there is the potential for enhanced peripheral distribution and increased serum protein binding.

Lastly, opioid peptides may be encapsulated via liposomes or nanoparticles for the enhancement of brain delivery. Liposomes consist of phospholipid bilayer that can act as a carrier for either hydrophilic or hydrophobic drugs. Such liposomes increase serum half-life, decrease clearance, and reduce toxicity a high drug concentrations.[31] A variation on the liposome approach is the use of self-assembling amphiphilic block copolymers (SAABCs). One example of an SAABC is P85, which is arranged in a triblock construct, consists of a central poly (propylene-oxide) block with attached poly (ethylene-oxide) blocks on each end. The hydrophilic blocks form hydrogen bonds with aqueous surroundings and form a tight "shell" around a hydrophobic core, allowing maintenance of a micelle structure on dilution. Hydrophobic drugs have an affinity for the core region, entrapping them and protected them

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against enzymatic degradation and hydrolysis. Such formulations increase membrane transport and stability, while reducing immunogenicity, proteolysis, cellular efflux, and systemic clearance. The use of P85 with the opioid peptide analogs DPDPE and biphalin enhances analgesia, as well as inhibits P-gp and enhances BBB uptake *in vitro*[32] Nanoparticles have also been used to deliver opioids experimentally for a number of years. These are particularly useful for peptides such as EM-1 that has a short half-life. The benefit of nanoparticles is that if prepared with coatings, such as poly sorbate 80, they can readily cross the BBB and gradually release the peptide leading to a prolonged analgesia.[16]

BBB AND DISEASE, WILL IT CHANGE OPIOID PEPTIDE DELIVERY?

For many years, the BBB was considered to be a relatively static barrier. However, we now know that it is a highly flexible and dynamic barrier that can be modulated via physiological, pharmacological, and pathological signals. To date, several disease states have shown to influence the function of the BBB, including diabetes, Alzheimer's disease, acquired immune deficiency syndrome (AIDS), and Parkinson's disease.[10] Moreover, patients often require pain treatment over the course of such chronic diseases. The question is "How might drug transport be altered by disease and can opioids themselves alter BBB function?" Disease alters the expression and activity of ATP-binding cassette (ABC) transporters (e.g. P-gp, BCRP). TAT and other proteins, such as gp-120, which are regulated by the human immunodeficiency virus (HIV) are known regulators of BBB proteins, including P-gp.[31] Inflammatory pain has specifically shown to increase the expression of efflux transporters, potentially impacting the efficacy of opioids in producing centrally mediated analgesia.[25] P-gp has also been shown to be overexpressed at the BBB during morphine treatment, suggesting that the opioid drugs themselves have a direct or, more probably, an indirect action on efflux mechanism.[27] Changes in cerebral neurotransmitters during exposure to opioids and the release of cytokines during pain may well affect transporter synthesis. There is also evidence that opioids drugs can influence the BBB tight junctional proteins and increase paracellular permeability.[19] In an *in vitro* examination, a group investigated the effect of treating human brain microvessel endothelial cells (HBMEC) cells with morphine.[30] Morphine treatment led to increased paracellular permeability and decreased the expression of the tight junctional protein zona-occludens-1 (ZO-1). This increase in paracellular permeability by morphine was blocked by the use of the opioid receptor antagonist naltrexone. However, naltrexone only partially restored the expression of ZO-1. Conversely, in an *in vivo* study, in which rats were chronically treated with morphine for 12 days, paracellular permeability was significantly decreased, yet when withdrawn from morphine, significant increases in BBB permeability were observed in association with brain edema.[26] It is plausible that opioids may be producing the differential effects on paracellular permeability and P-gp via opioid receptors present on the endothelial cells of the BBB. Assessing the contribution of respective opioid receptors with regards to alterations in permeability and protein expression at the BBB may provide a significant insight into disease and treatment outcomes.

CONCLUSIONS

Opioid peptides have the potential to not only be valuable research tools but also be viable CNS acting therapeutics. Although the BBB remains a significant impediment, several delivery strategies show considerable promise in overcoming such obstacles. Therefore, rational drug design and customized delivery strategies must be tempered with a greater understanding of BBB dynamics in both health and disease.

REFERENCES

1. Abbruscato TJ, Thomas SA, Hruby VJ, Davis TP. Brain and spinal cord distribution of biphalin: correlation with opioid receptor density and mechanism of CNS entry. *J Neurochem* 1997;**69**:1236–45.
2. Bodnar RJ. Endogenous opiates and behavior. *Peptides* 2010;**2011**:32.
3. Brownson EA, Abbruscato TJ, Gillespie TJ, Hruby VJ, Davis TP. Effect of peptidases at the blood–brain barrier on the permeability of enkephalin. *J Pharmacol Exper Ther* 1994;**270**:675–80.
4. Cros CD, Toth I, Blanchfield JT. Lipophilic derivatives of leu-enkephalinamide: in vitro permeability, stability and in vivo nasal delivery. *Bioorg Med Chem*;**19**:1528–34.
5. Dagenais C, Graff CL, Pollack GM. Variable modulation of opioid brain uptake by P-glycoprotein in mice. *Biochem Pharmacol* 2004;**67**:269–76.
6. Deguchi Y, Miyakawa Y, Sakurada S, Naito Y, Morimoto K, Ohtsuki S, et al. Blood–brain barrier transport of a novel micro 1-specific opioid peptide, H-Tyr-D-Arg-Phe-beta-Ala-OH (TAPA). *J Neurochem* 2003;**84**:1154–61.
7. Deguchi Y, Naito Y, Ohtsuki S, Miyakawa Y, Morimoto K, Hosoya K, et al. Blood-brain barrier permeability of novel [D-arg2]dermorphin (1-4) analogs: transport property is related to the slow onset of antinociceptive activity in the central nervous system. *J Pharmacol Exp Ther* 2004;**310**:177–84.
8. Do Carmo GP, Polt R, Bilsky EJ, Rice KC, Negus SS. Behavioral pharmacology of the mu/delta opioid glycopeptide MMP2200 in rhesus monkeys. *J Pharmacol Exp Ther* 2008;**326**:939–48.
9. Egleton RD, Mitchell SA, Huber JD, Janders J, Stropova D, Polt R, et al. Improved bioavailability to the brain of glycosylated Met-enkephalin analogs. *Brain Res* 2000;**881**:37–46.
10. Grammas P, Martinez J, Miller B. Cerebral microvascular endothelium and the pathogenesis of neurodegenerative diseases. *Expert Rev Mol Med* 2011;**13**:e19.
11. Hansen Jr DW, Stapelfeld A, Savage MA, Reichman M, Hammond DL, Haaseth RC, et al. Systemic analgesic activity and delta-opioid selectivity in [2,6-dimethyl-Tyr1, D-Pen2, D-Pen5]enkephalin. *J Med Chem* 1992;**35**:684–7.

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12. Herve F, Ghinea N, Scherrmann JM. CNS delivery via adsorptive transcytosis. *AAPS J* 2008;**10**:455–72.
13. Jakas A, Vinkovic M, Smrecki V, Sporec M, Horvat S. Fructose-induced N-terminal glycation of enkephalins and related peptides. *J Pept Sci* 2008;**14**:936–45.
14. Kang YS, Voigt K, Bickel U. Stability of the disulfide bond in an avidin–biotin linked chimeric peptide during in vivo transcytosis through brain endothelial cells. *J Drug Target* 2000;**8**:425–34.
15. Koda Y, Liang MT, Blanchfield JT, Toth I. In vitro stability and permeability studies of liposomal delivery systems for a novel lipophilic endomorphin 1 analogue. *Int J Pharm* 2008;**356**:37–43.
16. Liu H, Ni J, Wang R. In vitro release performance and analgesic activity of endomorphin-1 loaded nanoparticles. *Pharmazie* 2006;**61**:450–2.
17. Liu HM, Liu XF, Yao JL, Wang CL, Yu Y, Wang R. Utilization of combined chemical modifications to enhance the blood-brain barrier permeability and pharmacological activity of endomorphin-1. *J Pharmacol Exper Ther* 2006;**319**:308–16.
18. Maresh GA, Kastin AJ, Brown TT, Zadina JE, Banks WA. Peptide transport system-1 (PTS-1) for Tyr-MIF-1 and Met-enkephalin differs from the receptors for either. *Brain Res* 1999;**839**:336–40.
19. McCaffrey G, Willis CL, Staatz WD, Nametz N, Quigley CA, Hom S, et al. Occludin oligomeric assemblies at tight junctions of the blood-brain barrier are altered by hypoxia and reoxygenation stress. *J Neurochem* 2009;**110**:58–71.
20. Ouyang H, Andersen TE, Chen W, Nofsinger R, Steffansen B, Borchardt RT. A comparison of the effects of p-glycoprotein inhibitors on the blood-brain barrier permeation of cyclic prodrugs of an opioid peptide (DADLE). *J Pharm Sci* 2009;**98**:2227–36.
21. Pardridge WM. Preparation of Trojan horse liposomes (THLs) for gene transfer across the blood-brain barrier. *Cold Spring Harb Protoc* 2010:pdb prot5407.
22. Perlikowska R, do-Rego JC, Cravezic A, Fichna J, Wyrebska A, Toth G, et al. Synthesis and biological evaluation of cyclic endomorphin-2 analogs. *Peptides*;**31**:339–45.
23. Perlikowska R, Gach K, Fichna J, Toth G, Walkowiak B, do-Rego JC, et al. Biological activity of endomorphin and [Dmt1]endomorphin analogs with six-membered proline surrogates in position 2. *Bioorg Med Chem* 2009;**17**:3789–94.
24. Roemer D, Pless J. Structure activity relationship of orally active enkephalin analogues as analgesics. *Life Sci* 1979;**24**:621–4.
25. Seelbach MJ, Brooks TA, Egleton RD, Davis TP. Peripheral inflammatory hyperalgesia modulates morphine delivery to the brain: a role for P-glycoprotein. *J Neurochem* 2007;**102**:1677–90.
26. Sharma HS, Sjoquist PO, Ali SF. Alterations in blood–brain barrier function and brain pathology by morphine in the rat. Neuroprotective effects of antioxidant H-290/51. *Acta Neurochir Suppl* 2010;**106**:61–6.
27. Tournier N, Decleves X, Saubamea B, Scherrmann JM, Cisternino S. Opioid transport by ATP-Binding Cassette (ABC) transporters at the blood–brain barrier: implications for neuropsychopharmacology. *Curr Pharm Des* 2011.
28. Uchiyama T, Kotani A, Kishida T, Tatsumi H, Okamoto A, Fujita T, et al. Effects of various protease inhibitors on the stability and permeability of [D-Ala2, D-Leu5]enkephalin in the rat intestine: comparison with leucine enkephalin. *J Pharm Sci* 1998;**87**:448–52.
29. Weber SJ, Greene DL, Sharma SD, Yamamura HI, Kramer TH, Burks TF, et al. Distribution and analgesia of [3H][D-Pen2, D-Pen5] enkephalin and two halogenated analogs after intravenous administration. *J Pharmacol Exper Ther* 1991;**259**:1109–17.
30. Wen H, Lu Y, Yao H, Buch S. Morphine induces expression of platelet-derived growth factor in human brain microvascular endothelial cells: implication for vascular permeability. *PLoS One*;**6**:e21707.
31. Witt KA, Davis TP. CNS drug delivery: opioid peptides and the blood–brain barrier. *AAPS J* 2006;**8**:E76–88.
32. Witt KA, Huber JD, Egleton RD, Davis TP. Pluronic p85 block copolymer enhances opioid peptide analgesia. *J Pharmacol Exp Ther* 2002;**303**:760–7.
33. Witt KA, Huber JD, Egleton RD, Roberts MJ, Bentley MD, Guo L, et al. Pharmacodynamic and pharmacokinetic characterization of poly(ethylene glycol) conjugation to met-enkephalin analog [D-Pen2, D-Pen5]-enkephalin (DPDPE). *J Pharmacol Exp Ther* 2001;**298**:848–56.
34. Witt KA, Slate CA, Egleton RD, Huber JD, Yamamura HI, Hruby VJ, et al. Assessment of stereoselectivity of trimethylphenylalanine analogues of delta-opioid [D-Pen(2), D-Pen(5)]-enkephalin. *J Neurochem* 2000;**75**:424–35.
35. Yamamoto T, Nair P, Jacobsen NE, Vagner J, Kulkarni V, Davis P, et al. Improving metabolic stability by glycosylation: bifunctional peptide derivatives that are opioid receptor agonists and neurokinin 1 receptor antagonists. *J Med Chem* 2009;**52**:5164–75.

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Chapter 233

Peptide Delivery

Paul Kiptoo, Marlyn D. Laksitorini and Teruna J. Siahaan

ABSTRACT

The development of peptides as drugs for treating brain diseases has been hampered by the difficulty to deliver them through the blood–brain barrier (BBB). Therefore, there is an urgent need to develop methods to enhance peptide delivery through the BBB. Most peptides have unfavorable physicochemical properties for unrestricted permeation through the transcellular pathway of the BBB. Although some peptides can permeate through the paracellular pathway, this route of transport is limited by the presence of tight junctions. Paracellular permeation of peptides through the BBB can be enhanced by modulating the protein–protein interaction in the intercellular tight junctions by the use of Zot, occludin, and cadherin peptides. To improve permeation through the transcellular pathway, peptides can be modified to cyclic peptide prodrugs, which have better BBB permeation than that of the parent peptide. BBB permeation of peptides can be increased by improving peptide enzymatic stability and targeting saturable and receptor-mediated transport.

INTRODUCTION

Many peptide drugs have been developed as therapeutics to treat various brain diseases (i.e., Alzheimer's, Parkinson's, multiple sclerosis, brain tumors); unfortunately, the development of these molecules has been hampered by the limited ability to deliver them to the brain. The challenge of delivering peptides to the brain results from their difficulty in crossing the blood–brain barrier (BBB). Although some peptides may penetrate the BBB through the paracellular pathway,[19,35] paracellular penetration is constrained by the presence of tight junctions. The size of the peptide is one of the obstacles for diffusion through the paracellular pathway; normally, peptides have a hydrodynamic radius larger than 11 Å, and they cannot freely diffuse through tight junctions.[19] The porosity of the BBB paracellular pathway can be increased on disruption of the interaction of intercellular junction proteins.[35]

Most peptides have physicochemical properties (i.e., hydrophilicity, hydrogen-bonding potential, size) that make them less favorable for transcellular penetration through the BBB. The transcellular route of transport includes passive diffusion, passive diffusion modified by efflux pumps, receptor-mediated saturable transport, and non-receptor-mediated saturable transport. In regular passive diffusion, the peptide has to partition into the membranes on the blood side and subsequently enter into the cells; this is followed by partitioning into the membranes on the side of the brain before entering the brain environment. To make peptides more readily permeate via the transcellular pathway, the physicochemical properties of the peptide can be temporarily altered to favor membrane partition. An increase in lipophilicity is one major way to do this. Physicochemical properties of the peptide can be altered transiently by forming cyclic peptide prodrugs.[24] The formation of cyclic prodrugs has been shown to improve BBB penetration by opioid peptides.[2]

Another problem in peptide delivery to the brain is the presence of metabolic enzymes encountered in the blood and/or during crossing the BBB that prevents the parent peptide molecules from reaching the target site. Thus, improving peptide enzymatic stability can prolong its delivery to the brain. The presence of efflux pumps (i.e., P-glycoprotein (Pgp) and multidrug resistance proteins (MRPs)) on the BBB may also prevent the entry of peptides into the brain.[9] This chapter focuses on improving delivery of peptides to the brain through the paracellular route by modulation of the intercellular junctions and through the transcellular route by changing the physicochemical properties and enzymatic stability of the peptides. A few examples of the way that targeting saturable and receptor-mediated transport systems could be used to enhance permeation of a limited number of peptides are also discussed.

MODELS TO STUDY BRAIN DELIVERY OF PEPTIDES

Many *in vitro* and *in vivo* BBB models have been established for evaluating brain delivery of drug molecules including peptides. There is no single *in vitro* cell culture model that

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00233-5
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can mimic the total properties of the *in vivo* BBB; nonetheless, many different *in vitro* BBB models have been very useful for preliminary evaluation or prediction of drug transport through the BBB. A good *in vitro* model should be easy to culture in a reproducible manner. These *in vitro* models normally have cellular architecture similar to that of the *in vivo* BBB, including tight junctions and transporters.[11] Rapid screenings of transport properties of drug molecules through the BBB have been conducted successfully with *in vitro* models, and the results from *in vitro* studies can be correlated to *in vivo* results.

To mimic the *in vivo* conditions, the model cell monolayer should have tight intercellular junctions with high transendothelial electrical resistance (TEER) to restrict the paracellular permeation of drug molecules. Unfortunately, it is difficult to mimic the *in vivo* cerebrovascular capillaries with a high TEER value (i.e., $>1000\,\Omega\,cm^2$). Although the bovine brain microvessel endothelial cell (BBMEC) monolayer is considered a good model for the BBB, it has a low TEER value (i.e., $60–200\,\Omega\,cm^2$); this model is suitable for studying transcellular transport of drugs. Its low TEER value reflects a leaky tight junction of the BBMEC monolayer; therefore, it is not a good model for studying the permeation of those peptides that penetrate the BBB via the paracellular pathway. By contrast, monolayer of porcine brain capillary endothelial cells is closer to the BBB *in vivo* situation with a high TEER value ($600–800\,\Omega\,cm^2$).[6] Because isolation of the BBMEC can be laborious, cell culture models such as bEND3 and bEND5 from immortalized brain endothelial cell lines were developed.[31] Similar to the BBMEC, these models are appropriate for studying drug uptake and efflux but not for studying paracellular transport because they have a low TEER ($60\,\Omega\,cm^2$). Madin–Darby canine kidney (MDCK) cell monolayers have also been used as an *in vitro* model to predict the passive transport of drug molecules through the BBB. Because MDCK has a tighter intercellular junction than BBMEC, it may be a better model for predicting the paracellular permeation of peptides through the BBB. Because the MDCK model does not express the proper efflux pumps on its surface, there are efforts to increase the expression of efflux pumps on the surface of MDCK cells.[8]

METHODS TO IMPROVE PARACELLULAR PEPTIDE DELIVERY THROUGH THE BBB

One way to improve brain delivery of peptides is by increasing their permeation via the paracellular pathway of the BBB. This can be done by modulation of the protein–protein interactions in the intercellular junctions of the BBB. Although there are many aspects of the structure and function of intercellular junctions that are not fully understood, it is clear that protein–protein interactions in the intercellular junctions are important in maintaining the integrity of the BBB.[35] The intercellular junction structure is divided into three regions—tight junctions, adherens junctions, and desmosomes. The tight junctions are located at the very top of the BBB close to the apical (AP) side or blood side and are mediated by transmembrane proteins such as occludins, claudins, and junctional adhesion molecules (JAM). Underneath the tight junctions is adherens junctions, which are mediated by calcium-dependent VE-cadherins and calcium-independent nectins.[35] Below the adherens junctions are the desmosmes, which contain desmocolline and desmoglein. The intercellular junctions are dynamic in nature and can assemble or disassemble during the physiological process. *In vitro*, the tight intercellular junction can be modulated by adjustment of the calcium concentration and use of peptides that inhibit protein–protein interactions. Next, the improvement of drug delivery (i.e., small molecules and peptides) through the BBB by disrupting protein–protein interactions in the intercellular junctions will be described.

Modulation of Cadherin–Cadherin Interactions

E-cadherin and VE-cadherin are found in the adherens junctions of the epithelial intestinal mucosa and vascular endothelial cells of the BBB, respectively. One hypothesis is that paracelluar permeation of large hydrophilic drugs (i.e., peptides) can be increased on modulation of cell-cell adhesion in the intercellular junctions by inhibiting protein–protein interactions.[18,19,23] It is proposed that the inhibition of protein–protein interactions increases the overall paracellular porosities of the intercellular junctions of the BBB. Thus, peptide molecules derived from the region of homophilic contact of the protein–protein interactions can be used to inhibit these interactions and loosen the intercellular junctions. An HAV hexapeptide (Ac-SHAVSS-NH_2) derived from the EC1 domain sequence of E-cadherin enhances permeation of ^{14}C-mannitol and ^{3}H-daunomycin through the BBB *in vivo*.[13] This result suggests that modulation of cadherin–cadherin interactions in the adherens junction can increase the porosity of the paracellular of the BBB.

Although VE-cadherin (cadherin-5) has been found in BBB microvessel endothelial cells,[29] anti-E-cadherin (DECMA-1) monoclonal antibody (mAb) can decorate the intercellular junctions of BBMEC monolayers[23] and block the single cell aggregation of BBMEC, suggesting the potential involvement of E-cadherin in the intercellular junctions.[18] Alternatively, the E-cadherin mAb has cross-reactivity with VE-cadherin. The alignment of VE-cadherin and E-cadherin shows that the VE-cadherin has the A**VIV**DK sequence instead of the S**HAV**SS found

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in E-cadherin; however, sequence homologies are found between VE-cadherin and E-cadherin at the flanking residues to the AVIVDK and SHAVSS sequences, respectively (see the alignment of E-cadherin and VE-cadherin in Table 1).

Initially, the activity of HAV peptides derived from the EC1 domain of E-cadherin was evaluated for the inhibition of E-cadherin-mediated cell–cell adhesion in a single cell aggregation assay of BBMEC.[18] In this situation, BBMEC are trypsinized in the presence of calcium to acquire single cells.[23] The presence of calcium in the medium prevents the digestion of cadherins on the cell surface. The single cells are incubated with 1.8 mM of calcium in the presence of different concentrations of HAV peptides and anti-E-cadherin mAb as well as in the absence of peptide. In the absence of peptides or mAb, the aggregation of BBMEC single cells is observed. In the presence of HAV peptides and anti-E-cadherin mAb, less aggregation of BBME single cells is observed; the IC_{50} of the cell aggregation inhibitor is determined by counting the number of cell particles as a function of peptide concentration. The HAV-24 peptide (Table 1) is the most potent HAV peptide for inhibiting BBMEC aggregation, with an IC_{50} of 0.008 μM.[18] When HAV-24 is reduced to HAV6 (Table 1), the potency of HAV6 peptide in inhibition of BBMEC cell aggregation is dramatically reduced to 20 μM. These *in vitro* results suggest several possibilities. First, both E-cadherin and VE-cadherin have a role in the formation of intercellular junctions of the BBB. Second, if E-cadherin is present in the adherens junctions of the BBB, the HAV peptide and anti-E-cadherin mAb modulate the E-cadherin-mediated membrane-membrane contact on the BBB.[18,23] Third, if VE-cadherin but not E-cadherin is present in the adherens junctions of the BBB, modulation of the BBB by HAV peptide and anti-E-cadherin mAb interactions may result from binding or cross-reactivity of these molecules to VE-cadherin. Further studies are necessary to elucidate the roles of E- and VE-cadherins in the adherens junctions of the BBB and to determine the mechanism of action of HAV peptides in modulating the BBB.

HAV and ADT peptides are two different peptides derived from the groove and bulge regions of EC1 domain of E-cadherin (cadherin-1); these peptides modulate the intercellular junctions of cell monolayers (i.e., BBMEC, Caco-2, and MDCK).[14,18,20,23] In epithelial cells, the homotypic adhesion of Caco-2 single cells to modified Caco-2 cell monolayers is largely dependent on extracellular Ca^{2+} concentrations, and the calcium-mediated Caco-2 cell–cell adhesion can be inhibited by anti-E-cadherin mAb and E-cadherin peptides (HAV and ADT peptides), indicating that the cell–cell adhesion is mediated by E-cadherins.[14] In MDCK cell monolayers, HAV and ADT peptides lower the TEER values as well as improve ^{14}C-mannitol transport, indicating paracellular porosity can be increased on modulation of the cell–cell adhesion.[20] As mentioned previously, HAV hexapeptide enhances the permeation of ^{14}C-mannitol and [^{3}H]-daunomycin through the BBB of the *in situ* rat brain perfusion model.[13] In addition, HAV6 peptide administered in combination with verapamil, an efflux pump inhibitor, has a synergistic effect in enhancing the absorption of daunomycin through the BBB. Flanking residues on

TABLE 1 Peptides that Modulate the Intercellular Junctions of the Biological Barriers

Peptide origin	Peptide name	Sequence
E-cadherin	HAV-24	Ac-DRERIATYTLFSHAVSSNGNAVED-NH_2
VE-cadherin	VE-Cad	L**DRE**N**I**SE**Y**HLTA<u>VIV</u>DKDT**G**ENL**E**T**P** (Bold letters are residues homologous with E-cadherin)
E-cadherin	HAV-6	Ac-SHAVSS-NH_2
E-cadherin	ADT6	Ac-ADTPPV-NH_2
ZOT	Delta-G	12 kDa fragment of ZOT protein
ZOT	AT-1002	FCIGRL
Occludin	OP90–135	DRGYGTSLLGGSVGYPYGGSGFGSYGSGYGYGYGYGYGYGGYTDPR-NH_2
Occludin	OP90–103	DRGYGTSLLGGSVG
Occludin	LIP-OP90–103	H_2N-CH($C_{12}H_{25}$)-CO-DRGYGTSLLGGSVG
Chick occludin 81–125	OCC1	DYGYGLGGAYGTGLGGFYGSNYYGSGLSYSYGYGGYYGGVNQRT
Chick occludin 184–227	OCC2	GVNPQAQMSSGYYYSPLLAMC(Acm)SQAYGSTYLNQYIYHYC(Acm)TVDPQE, note: Acm, acetamido methyl
Rat occludin 209–230	OCC2R	GSQIYTICSQFYTPGGTGLYVD

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the His–Ala–Val (HAV) sequence have an influence on the activity of HAV hexapeptides in inhibiting cadherin–cadherin interactions in cell aggregation and TEER evaluation assays. Mutation of some of the residues in Ac-SHAVSS-NH_2 peptide to alanine (i.e., Ac-SHAVAS-NH_2 and Ac-SHAVSA-NH_2) enhances the peptide activity in modulating the intercellular junctions of MDCK monolayers as measured by the lowering of TEER values and the enhancement of ^{14}C-mannitol.[13] The increase in ^{14}C-mannitol transport suggests that the peptides increase the paracellular porosity of the intercellular junctions on disruption of cadherin–cadherin interaction.

The potential mechanism of action of HAV peptides was evaluated in Caco-2 cell monolayers. Prolonged treatment with Ac-SHAVSS-NH_2 peptide (100 μM) for 24 h reduces the amount of E-cadherin protein in Caco-2 cells about 20%; however, there is no change in the E-cadherin gene expression.[1] Addition of HAV6 peptide to Caco-2 cell monolayers upregulates several different stress genes, including prostate differentiation factor, and several protective genes (i.e., ATP-binding cassette subfamily ABCA member 5, endothelin, and AMP-activated protein kinase alpha 2 catalytic subunit).[1] These results suggest that the mechanism of action of HAV6 peptide may involve not only disrupting the E-cadherin interactions in the intercellular junctions but also indirectly interfering with various intracellular pathways.[1]

Modulation of TJ Proteins by Occludin Peptides and ZOT Peptide

Occludin Peptides

Occludin and claudin are found in the tight junctions of biological barriers, and modulation of occludin interactions enhances the porosity of the tight junctions to allow molecules to penetrate biological barriers (i.e., BBB and intestinal mucosa barrier)[26] Occludin has four transmembrane helical domains and two extracellular loops with each loop containing 44 amino acid residues. Although none of the occludin peptides has been used to improve peptide delivery to the brain, it is useful to discuss the potential of occludin peptides in modulating the tight junctions of cell monolayers to increase paracellular permeation of hydrophilic molecules *in vitro*. Synthetic peptides called OCC1 and OCC2 from loop-1 and loop-2 of chick occluding, respectively, were evaluated in disrupting the tight junction integrity of *Xenopus* kidney epithelial A6 cell monolayers.[34] OCC2 but not OCC1 can modulate the tight junctions of the cell monolayers as reflected by the lowering of the TEER values of the monolayers. OCC2 increases paracellular penetration of large hydrophilic molecules (i.e., inulin, dextran-3000, and dextran-40,000) through the A6 cell monolayers.[34] The active OCC2 peptide has the thiol groups on both cysteine residues capped with acetamido-methyl groups; this protecting group prevents the formation of inactive OCC2 oligomers via a disulfide bond(s). A smaller fragment of the second loop of rat occludin (22 amino acid OCC2R, Table 1) could also disrupt the blood–testis barrier.[3,33] Tight junction modulation by OCC2 occurs via lowering of the total cellular levels of occludin, thus depleting the occludin molecules at the cellular boundaries of tight junctions. The lowering of occludin cellular level is not because of inhibition of occludin protein synthesis but to the faster turnover of occludin. OCC2 modulation of tight junctions does not change the distribution of E-cadherin and ZO-1 and ZO-2 proteins at the intercellular junctions.

Although the first loop of chick occludin (OCC1) does not show activity in the modulation of the tight junction of kidney epithelial A6 cell monolayers,[34] a 45-mer peptide called OP90–135 (Table 1) derived from the first extracellular loop region of human occludin modulates the intercellular junctions of the Caco-2 cell.[28] OP90–135 peptide lowers the TEER value of Caco-2 cell monolayers and improves ^{14}C-mannitol transport when added from the basolateral (BL) side but not from the apical (AP) side. The lack of modulatory activity from the AP side results from peptide metabolism at the AP side brush border.[28] It is also possible that, because of the large size of the peptide, OP90–135 cannot efficiently penetrate the tight junctions to modulate occludin–occludin interactions. OP90–135 was systematically shortened to OP90–103, and this peptide has better activity than OP90–135 in modulating the intercellular junctions. The modulatory activity of OP peptides is inhibited by dimer and oligomer formation. To overcome the oligomerization problem, the peptide was conjugated to the hydrocarbon (C-14) chain at the N-terminus to make "lipoamino" peptide (LIP-OP90–103). LIP-OP90–103 peptide has an 11-fold better activity than the parent peptide because LIP-OP90–103 does not form oligomers. By contrast, conjugation of the hydrocarbon (C-14) chain to the C-terminus of OP peptide does not improve the activity of the conjugate. In the future, it will be interesting to see whether OP peptides can be used to modulate the tight junctions of the BBB in *in vitro* and *in vivo* models.

ZOT Peptides

Zonula occludens toxin (Zot) and zonulin are proteins that can modulate the tight junctions of the intestinal mucosa and BBB to improve paracellular transport of large hydrophobic and hydrophilic drugs.[26,27] Zot is associated with Vibhrio cholerae and is involved in the opening of tight junctions in the pathology of cholera, causing passage

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of water and electrolytes during cholera-associated diarrhea.[17] Zot enhances transport of small-to-large hydrophilic molecules (i.e., mannitol, polyethylene glycol 4000, and inulin) or small hydrophobic molecules (e.g., paclitaxel, acyclovir, cyclosporine A, and doxorubicin) across Caco-2 cell and BBMEC monolayers.[27] Oral delivery of insulin is improved by Zot administration, and the blood glucose is effectively reduced compared with the use of insulin alone. In cell culture, Zot peptide enhances paracellular permeation of ^{14}C-inulin more than that of ^{14}C-PEG4000 and the peptide is stable for 10 days.[4] This higher inulin permeation presumably results from the globular shape of inulin compared with the rod shape of PEG. However, the peptide is unable to enhance the permeation of FITC-dextran-10,000 through Caco-2 cell monolayers, suggesting that there is a cut-off limit on the paracellular opening created by the peptide.

In vivo, a 12-kDa fragment of Zot protein called ΔG administered intraduodenally via a cannula significantly increases (up to 57-fold) the C_{max} and AUC of drugs such as ritonavir and cyclosporin in the presence of protease inhibitors (30 mg/kg bestatin + 30 mg/kg captopril + 67 mg/kg leupeptin).[27] The need to include protease inhibitors results from the rapid *in vivo* enzymatic degradation of ΔG protein, but. protease inhibitors alone increase the transport of cyclosporine and ritonavir. These results suggest that a mixture of ΔG and protease inhibitors provides an alternative to enhance drug delivery through the biological barriers (i.e., intestinal mucosa and BBB).

The proposed mechanism of action of Zot and zonulin is that these molecules activate intracellular signaling related to protein kinase C (PKC) and phospholipase C and induce actin polymerization.[17] Zot peptides can modulate PKC to cause rearrangement of the cytoskeleton that alters the tight junction protein–protein interactions and loosens the tight junctions in a reversible fashion.[4] In the brain, the plasma membranes contain Zot receptors that bind to Zot and zonulin proteins, and these receptors have structural similarity with MRP-8 (14-kDa), which is a member of S-100 family of calcium-binding proteins.[17]

AT-1002 is a hexapeptide (FCIGRL) derived from the Zot sequence that can modulate the tight junctions *in vitro* and *in vivo* to enhance paracellular permeation of marker molecules.[10] Although AT-1002 has not been investigated for enhancing drug delivery to the brain, it improves oral absorption of low molecular weight heparin[21] and pulmonary delivery of salmon calcitonin.[10] The mechanism of action of AT-1002 in modulating the tight junctions was evaluated in Caco-2 and IEC6 cell monolayers. The tight junction modulation results from the rearrangement of the cellular cytoskeleton caused by the disruption of stress fibers, lowering of the actin filament, and the disappearance of ZO-1 protein from the "kiss" region of the tight junctions.[10] The alteration of the cytoskeleton can be correlated with the phosphorylation of ZO-1 protein at the tyrosine residue due to the activation of Src kinase.

FORMATION OF CYCLIC PEPTIDES TO IMPROVE TRANSCELLULAR DELIVERY THROUGH THE BBB

Often, the physicochemical properties of peptides contribute to their difficulty in penetrating the BBB via the transcellular pathway. This could result from the size and high hydrogen-bonding potential to water that limits their partitioning into cell membranes. One way to improve brain delivery of peptides is to modify their physicochemical properties. The idea is that passive transcellular transport of peptide involves peptide partition into cell membranes of vascular endothelial cell microvessels of the BBB. Peptide partitioning into cell membranes can be optimized by reducing the hydrogen-bonding potential to water, increasing the octanol/water partition coefficient, increasing molecular structure stability by forming a secondary structure, and/or reducing the hydrodynamic radius of the peptide. Lipid solubility is particularly important and serves to emphasize the role of membrane permeation for peptides without transporters. The physicochemical properties of peptide also could be modified by the formation of cyclic peptides or cyclic peptide prodrugs. The hypothesis is that the formation of a cyclic structure induces intramolecular hydrogen bonding to suppress the hydrogen-bonding potential to water. Lowering the hydrogen-bonding potential enhances the octanol/water partition coefficient (e.g., hydrophobicity) of the peptide to favor partition into cellular membranes of the BBB. The formation of a cyclic structure increases the propensity of the peptide to form a stable secondary structure such as beta- and gamma-turns and reduces the hydrodynamic radius of the peptide, which also favors penetration through the paracellular pathway of the BBB. Although formation of cyclic peptides may enhance the biological activity of some peptides, others can be active only in the linear form. Thus, formation of irreversible cyclic peptides may not be desirable for this class of linear peptides. To overcome this hurdle, a cyclic peptide prodrug method was developed in which the cyclic peptide prodrug is a transient molecule that can be converted into an active linear peptide at the site of action after it crosses the BBB.

A cyclic peptide prodrug can be constructed by linking the N- and C-termini via an enzyme-sensitive promoiety; however, the cyclic structure can also be generated by linking the backbone terminus (i.e., N- or C-termini) to the side chain or by linking side chain to side chain via a promoiety linker. The promoiety can readily be cleaved by an enzyme (i.e., esterase) to produce the parent peptide. Several

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promoieties have been developed for the formation of cyclic peptide prodrugs, including acyloxy-alkoxy,[24] trimethyl-lock, coumarin,[30] and acyloxy-alkoxy coumarin linkers[2] (Fig. 1). The formation of cyclic peptide prodrugs has been applied to opioid peptides, delta sleep-inducing peptides (DSIP), and RGD peptides and peptidomimetics. In cell culture models of biological barriers (i.e., Caco-2 cell monolayers), cyclic peptide prodrugs penetrate the cell monolayer more readily (i.e., ~70 times) than the parent linear peptides. This suggests that the cyclic peptide prodrug has more desirable physicochemical properties for membrane partition than does the parent linear peptide. The cyclic prodrugs are readily converted by esterases to the parent linear peptides in the blood and tissues.[16] Structural studies indicate that cyclic peptide prodrugs normally have a stable secondary structure such as a beta-turn with an intramolecular hydrogen bond. They are also more hydrophobic and structurally more compact than the parent linear peptide. These structural properties contribute to their favorable transport properties through the transcellular pathway of the biological barriers.

The delivery of DADLE and its cyclic prodrugs (AOA-DADLE, OMCA-DADLE, and CA-DADLE) through the BBB has been determined by rat brain perfusion.[2] There is minimal brain disposition of DADLE and its cyclic prodrugs in the absence of a P-glycoprotein (Pgp) inhibitor, GF-120918. In the presence of GF-120918, the brain delivery of AOA-DADLE, OMCA-DADLE, and CA-DADLE is enhanced about 50, 170, and 460 times, respectively, compared with that of the parent DADLE. These results indicate that the cyclic prodrugs are recognized by Pgp to prevent their permeation through the BBB. The cyclic prodrugs have high metabolic stability, but they also have low bioconversion in the brain. The recognition of the DADLE cyclic prodrugs by Pgp results from the promoiety rather than the DADLE peptide. Different Pgp inhibitors (i.e., GF-120918, cyclosporine A (CsA), and PCS833) have different effects on enhancing permeation of cyclic prodrugs into the brain. GF-120918 has the highest impact on the permeation of cyclic prodrugs compared with CsA and PCS833; CSA and PCS833 have similar Pgp inhibitory activity.[22]

Several esterases are involved in converting cyclic prodrugs to the parent peptide, including carboxylesterase, cholinesterase, and acetylcholinesterase (AChE).[15] The cyclic prodrug should be stable while crossing the BBB and be converted by the esterase to parent peptide in the brain. AOA-DADLE, OMCA-DADLE, and CA-DADLE are stable crossing the BBB; unfortunately, they have low bioconversion in the brain, which is not a favorable property.[2] The optimal balance of stability in the blood stream and readability for enzymatic conversion has to be designed into the physicochemical properties of successful cyclic prodrugs. In other words, the

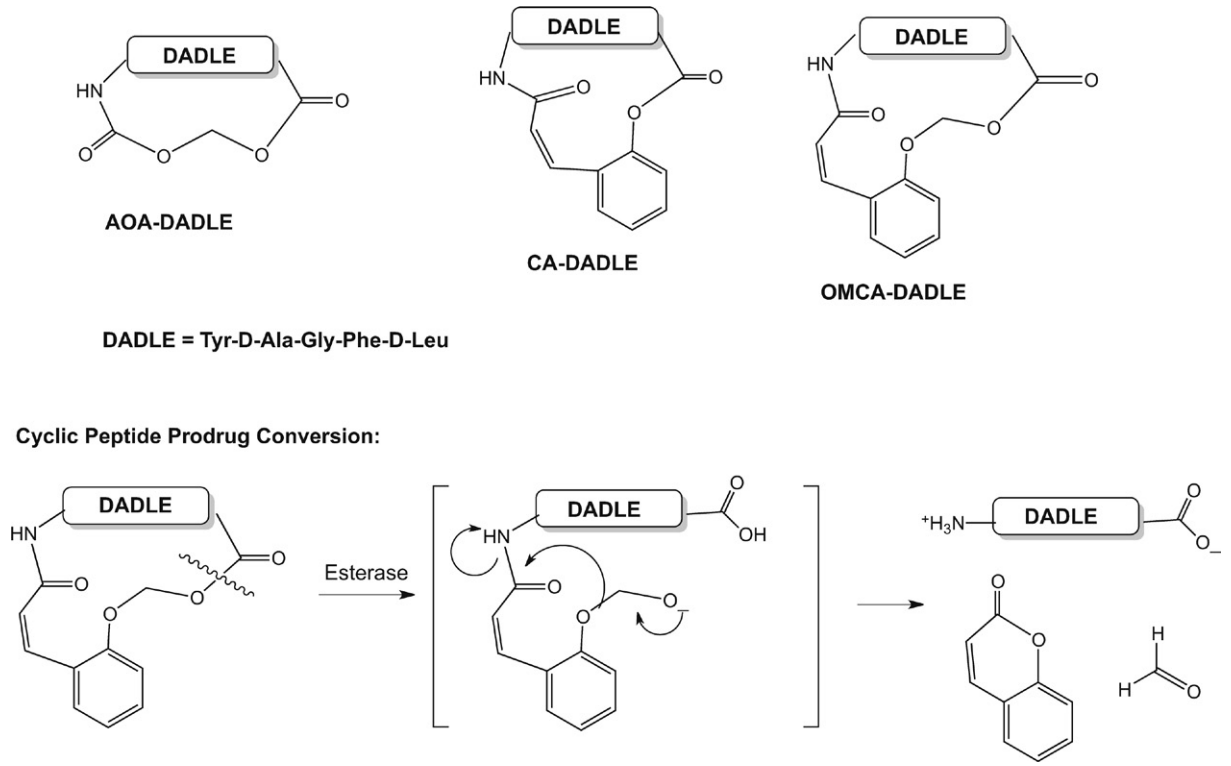

FIGURE 1 Structures of cyclic prodrug of DADLE with different promoeities, including AOA-DADLE, CA-DADLE, and OMCA-DADLE. The cyclic peptide prodrug can be converted to DADLE by esterase.

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cyclic prodrug should not be easily converted to the parent drug in the blood because that will produce hydrophilic linear peptides that pass less rapidly through the BBB. Conversely, the cyclic prodrug should be easily converted to the parent drug in the brain to produce its biological activity. It is known that esterase activity (i.e., carboxylesterase) is tissue dependent. In the case of DADLE cyclic prodrugs, the rate of hydrolysis in the brain is more pronounced than in plasma, and this rate depends on the chirality of the C-terminus amino acid. As predicted, the promoiety hydrolysis is more rapid when L-amino acid is the C-terminus rather than D-amino acid.

IMPROVING ENZYMATIC STABILITY

In addition to their physical barriers, peptides also suffer from enzymatic degradation in the blood, BBB, and brain. For example, the degradation of opioid peptides normally occurs in the extracellular space of the BBB and at the cytosol. Peptides can be degraded by endo- and exo-peptidases, and the rate of peptidase degradation is sequence dependent. Peptidase degradation can be prevented by acetylation and amidation of the N- and C-termini, respectively. Modifications of peptides by D-amino acid and cyclic peptide formation have been shown to slow peptide degradation.

Endomorphin-1 (Tyr–Pro–Trp–Phe–NH_2) is one of the more stable opioid peptides, and its degradation occurs at the peptide bond.[12] The C-terminal amidation prevents the degradation of endomorphin-1 by carboxypeptidases. In the brain preparation, endomorphin-1 is degraded by dipeptidyl peptidase IV (DPP IV) to produce a Tyr–Pro fragment. Endomorphin-1 is also degraded by aminopeptidase M (APM) from the N-terminus. Replacement of the L-Pro with D-Pro increases the enzymatic stability of endomorphin-1, but this modification lowers the activity. N-methylation of the peptide bonds of endomorphin-2 (Try–Pro–Phe–Phe–NH_2) could improve the enzymatic stability, but diminishes the biological activity. Replacement of the Pro with (NMe)Gly to give Tyr–(NMe)Gly–Phe–Phe–NH_2 improves the enzymatic stability and maintaining the activity. If pyperidine-2-carboxylic acid (Nip) replaces Pro-2 in endomorphin-2 to produce Tyr–Nip–Phe–Phe–NH_2, this modification enhances the enzymatic stability 10-fold in brain homogenates. Modification of the amino group of the Tyr residue to a guanidinium group in endomorphin-2 could also contribute to improved stability in brain homogenates.

Another strategy involves masking the enzyme cleavage site to improve metabolic stability. Endogenously produced Met-enkephalin (Tyr–Gly–Gly–Phe–Met) has a very short half-life ($t_{1/2} < 1$ min) because of rapid enzymatic degradation. A Met-enkephalin analog, DPDPE, formed through cyclization via two D-penicillamine moieties at positions 2 and 5, results in the formation of a conformationally strained peptide with increased metabolic stability ($t_{1/2} > 500$ min), leading to increased high BBB permeability and bioavailability.[32] However, subsequent studies showed that DPDPE had a significantly high clearance via the hepatic route because DPDPE is a substrate for many efflux pumps involved in billiary secretion, including Pgp and organic anion transporters (OATP).

TARGETING SATURABLE TRANSPORT SYSTEMS IN THE BBB TO IMPROVE DELIVERY INTO THE CNS

As indicated above, the presence of brain-to-blood saturable transport systems (efflux pumps) at the BBB is one of the major causes of low BBB permeability to peptides. In addition to the use of an enzyme inhibitor, one of the strategies is to counter the effect of efflux pumps such as Pgp is pegylation of the peptide. Polyethylene glycol (PEG) conjugation helps to reduce peptide affinity to Pgp. Masking of DPDPE with a PEG group reduces plasma clearance and increases brain uptake, thus leading to improved analgesia activity. Another peptide in which targeting saturable transport systems might be beneficial is the pituitary adenylate cyclase-activating polypeptide (PACAP). PACAP, a member of the secretin/glucagon/vasoactive intestinal peptides, is a pleuripotent peptide with neuroprotective, endocrine, and vasodilatory effects. *In vitro*, PACAP is neuroprotective in femtomole concentration amounts, but its brain uptake is limited by an efflux component of peptide transport system-6 (PTS-6). PTS-6 is identified as a β-F1 ATPase, and it is colocalized on the BBB endothelial cells with PACAP27, a C-terminal-truncated 27-aminoacid form of PACAP.[5] Inhibition of PTS-6 efflux with antisense to β-F1 ATPase increased PACAP concentration in the brain by increasing the uptake of peripherally administered PACAP and decreasing the loss of endogenously produced PACAP. *In vivo* findings show that treatment with antisense and PACAP27 improve cognition in a mouse model of Alzheimer's disease and reduced infarct size after cerebral ischemia.[5]

USE OF RECEPTOR-MEDIATED DRUG TRANSPORT TO IMPROVE PEPTIDE DELIVERY INTO THE BRAIN

Receptor-mediated drug transport involves the conjugation of a peptide and/or drug to a vector moiety, which has affinity for receptors of the target tissue to allow receptor-mediated transcytosis. Vectors that have been used include cationized albumin, liposomes, nanoparticles, and antibodies to receptors such as transferrin. Transferrin receptor is constitutively expressed at the BBB at higher levels than other capillary beds, and is involved in the transport of iron into the brain. The use of transferrin receptor has been investigated for the transport of various peptides across the BBB. For example, a murine monoclonal antibody to the rat transferring receptor OX26 has been successfully used to

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increase brain uptake of proteins and peptides in a receptor-mediated manner. In the transport of brain-derived neurotrophic factor (BDNF), the administration of BDNF–OX26 conjugate directly after an ischemic incident prevented significant neuronal damage compared with that of BDNF or OX26 alone; this suggests that the protective effect of the BDNF–OX26 results from its increased ability to cross the BBB.[7] Receptor-mediated transport has also been investigated in the delivery of angiopep-2 peptide, whose sequence is derived from ligands that bind to low-density lipoprotein receptor-related protein-1 (LRP-1). LRP-1 is highly expressed in the brain endothelial cells, neurons, and astrocytes as well as in brain tumor cells and can be used to deliver drugs to the brain. *In vivo* injection of ANG1005, a conjugate of containing Angiopep-2 and the antineoplastic drug paclitaxel, inhibited growth of orthotopic human glioblastoma brain tumors in mice more potently than paclitaxel alone and significantly increased animal survival rate.[25] It is thought that the increased uptake of ANG1005 into the brain is due to ANG1005 bypassing Pgp efflux pumps and crossing the BBB via receptor-mediated transcytosis.[25] Although receptor-mediated transport represents a potent strategy, it has some disadvantages. First, the vector used for receptor-mediated transport could lead to potential steric inhibition of the peptide or drug at the target receptor. Second, not many peptides can be delivered via this mechanism. Third, when targeting by a vector such as the transferrin receptor, it is important to consider how it impacts the transport of the native substrate. Studies with OX26 showed that pretreatment of animals with OX26 reduced Fe-transferrin delivery across the BBB by 30–40%, suggesting that receptor-mediated transport should be used in a limited fashion or with potent peptides that require only limited delivery to the brain.

CONCLUSIONS

Peptide delivery into the brain is still very challenging. Some progress has been made in improving the peptide delivery through the paracellular pathways of the BBB by modulation of the tight intercellular junctions. However, there are serious safety issues that arise from nonselectively increasing the paracellular porosity of the BBB, and these need to be addressed. In the future, further investigation of mechanisms of action and tissue selectivity of peptides that modulate the intercellular junctions will be necessary. Further, the increase in pore size opening created by junction modulators has not been characterized, and the favorable shapes of molecules that can penetrate the paracellular pathway of the BBB are still unknown. Progress also has been made in improving transcellular permeation of peptides through the BBB by the formation of cyclic peptide prodrugs, increased lipophilicity, and enzymatic resistance. However, recognition of the promoiety by Pgp hampers the transcellular passive diffusion of cyclic peptide prodrugs, and the slow brain conversion of cyclic peptide prodrugs also needs further exploration. Therefore, there is a need to design cyclic prodrugs and other forms that are not recognized by Pgp and are readily converted to the parent peptide or active forms in the brain. Saturable and receptor-mediated transport systems have been used to improve the permeation of a limited number of peptides through the BBB.

ACKNOWLEDGMENTS

T.J. Siahaan is supported by NIH Grants R01 NS-075374 and R56 AI-063002.

REFERENCES

1. Calcagno AM, Fostel JM, Reyner EL, Sinaga E, Alston JT, Mattes WB, et al. Effects of an E-cadherin-derived peptide on the gene expression of Caco-2 cells. *Pharm Res* 2004;**21**:2085–94.
2. Chen W, Yang JZ, Andersen R, Nielsen LH, Borchardt RT. Evaluation of the permeation characteristics of a model opioid peptide, H-Tyr–D-Ala–Gly–Phe–D-Leu–OH (DADLE), and its cyclic prodrugs across the blood–brain barrier using an in situ perfused rat brain model. *J Pharmacol Exp Ther* 2002;**303**:849–57.
3. Chung NP, Mruk D, Mo MY, Lee WM, Cheng CY. A 22-amino acid synthetic peptide corresponding to the second extracellular loop of rat occludin perturbs the blood–testis barrier and disrupts spermatogenesis reversibly in vivo. *Biol Reprod* 2001;**65**:1340–51.
4. Cox DS, Raje S, Gao H, Salama NN, Eddington ND. Enhanced permeability of molecular weight markers and poorly bioavailable compounds across Caco-2 cell monolayers using the absorption enhancer, zonula occludens toxin. *Pharm Res* 2002;**19**:1680–8.
5. Dogrukol-Ak D, Kumar VB, Ryerse JS, Farr SA, Verma S, Nonaka N, et al. Isolation of peptide transport system-6 from brain endothelial cells: therapeutic effects with antisense inhibition in Alzheimer and stroke models. *J Cereb Blood Flow Metab* 2009;**29**:411–22.
6. Franke H, Galla H, Beuckmann CT. Primary cultures of brain microvessel endothelial cells: a valid and flexible model to study drug transport through the blood–brain barrier in vitro. *Brain Res Brain Res Protoc* 2000;**5**:248–56.
7. Friden PM, Walus LR, Musso GF, Taylor MA, Malfroy B, Starzyk RM. Anti-transferrin receptor antibody and antibody–drug conjugates cross the blood–brain barrier. *Proc Natl Acad Sci U S A* 1991;**88**:4771–5.
8. Goh LB, Spears KJ, Yao D, Ayrton A, Morgan P, Roland Wolf C, et al. Endogenous drug transporters in in vitro and in vivo models for the prediction of drug disposition in man. *Biochem Pharmacol* 2002;**64**:1569–78.
9. Golden PL, Pollack GM. Blood–brain barrier efflux transport. *J Pharm Sci* 2003;**92**:1739–53.
10. Gopalakrishnan S, Pandey N, Tamiz AP, Vere J, Carrasco R, Somerville R, et al. Mechanism of action of ZOT-derived peptide AT-1002, a tight junction regulator and absorption enhancer. *Int J Pharm* 2009;**365**:121–30.
11. Gumbleton M, Audus KL. Progress and limitations in the use of in vitro cell cultures to serve as a permeability screen for the blood–brain barrier. *J Pharm Sci* 2001;**90**:1681–98.
12. Janecka A, Staniszewska R, Gach K, Fichna J. Enzymatic degradation of endomorphins. *Peptides* 2008;**29**:2066–73.

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13. Kiptoo P, Sinaga E, Calcagno AM, Zhao H, Kobayashi N, Tambunan US, et al. Enhancement of drug absorption through the blood–brain barrier and inhibition of intercellular tight junction resealing by E-cadherin peptides. *Mol Pharm* 2011;**8**:239–49.
14. Kobayashi N, Ikesue A, Majumdar S, Siahaan TJ. Inhibition of e-cadherin-mediated homotypic adhesion of Caco-2 cells: a novel evaluation assay for peptide activities in modulating cell–cell adhesion. *J Pharmacol Exp Ther* 2006;**317**:309–16.
15. Liederer BM, Borchardt RT. Enzymes involved in the bioconversion of ester-based prodrugs. *J Pharm Sci* 2006;**95**:1177–95.
16. Liederer BM, Borchardt RT. Stability of oxymethyl-modified coumarinic acid cyclic prodrugs of diastereomeric opioid peptides in biological media from various animal species including human. *J Pharm Sci* 2005;**94**:2198–206.
17. Lu R, Wang W, Uzzau S, Vigorito R, Zielke HR, Fasano A. Affinity purification and partial characterization of the zonulin/zonula occludens toxin (Zot) receptor from human brain. *J Neurochem* 2000;**74**:320–6.
18. Lutz KL, Siahaan TJ. Modulation of the cellular junction protein E-cadherin in bovine brain microvessel endothelial cells by cadherin peptides. *Drug Delivery* 1997;**4**:187–93.
19. Lutz KL, Siahaan TJ. Molecular structure of the apical junction complex and its contribution to the paracellular barrier. *J Pharm Sci* 1997;**86**:977–84.
20. Makagiansar IT, Avery M, Hu Y, Audus KL, Siahaan TJ. Improving the selectivity of HAV-peptides in modulating E-cadherin-E-cadherin interactions in the intercellular junction of MDCK cell monolayers. *Pharm Res* 2001;**18**:446–53.
21. Motlekar NA, Fasano A, Wachtel MS, Youan BB. Zonula occludens toxin synthetic peptide derivative AT1002 enhances in vitro and in vivo intestinal absorption of low molecular weight heparin. *J Drug Target* 2006;**14**:321–9.
22. Ouyang H, Andersen TE, Chen W, Nofsinger R, Steffansen B, Borchardt RT. A comparison of the effects of p-glycoprotein inhibitors on the blood–brain barrier permeation of cyclic prodrugs of an opioid peptide (DADLE). *J Pharm Sci* 2009;**98**: 2227–36.
23. Pal D, Audus KL, Siahaan TJ. Modulation of cellular adhesion in bovine brain microvessel endothelial cells by a decapeptide. *Brain Res* 1997;**747**:103–13.
24. Pauletti GM, Gangwar S, Okumu FW, Siahaan TJ, Stella VJ, Borchardt RT. Esterase-sensitive cyclic prodrugs of peptides: evaluation of an acyloxyalkoxy promoiety in a model hexapeptide. *Pharm Res* 1996;**13**:1615–23.
25. Regina A, Demeule M, Che C, Lavallee I, Poirier J, Gabathuler R, et al. Antitumour activity of ANG1005, a conjugate between paclitaxel and the new brain delivery vector Angiopep-2. *Br J Pharmacol* 2008;**155**:185–97.
26. Salama NN, Eddington ND, Fasano A. Tight junction modulation and its relationship to drug delivery. *Adv Drug Deliv Rev* 2006;**58**:15–28.
27. Salama NN, Fasano A, Thakar M, Eddington ND. The impact of DeltaG on the oral bioavailability of low bioavailable therapeutic agents. *J Pharmacol Exp Ther* 2005;**312**:199–205.
28. Tavelin S, Hashimoto K, Malkinson J, Lazorova L, Toth I, Artursson P. A new principle for tight junction modulation based on occludin peptides. *Mol Pharmacol* 2003;**64**:1530–40.
29. Vorbrodt AW, Dobrogowska DH. Molecular anatomy of interendothelial junctions in human blood-brain barrier microvessels. *Folia Histochem Cytobiol* 2004;**42**:67–75.
30. Wang B, Gangwar S, Pauletti G, Siahaan T, Borchardt RT. Synthesis of an esterase-sensitive cyclic prodrug of a model hexapeptide having enhanced membrane permeability and enzymatic stability using a 3-(2′-hydroxy-4′6′-dimethylphenyl)-3,3-dimethyl propionic Acid promoiety. *Methods Mol Med* 1999;**23**:53–69.
31. Williams RL, Risau W, Zerwes HG, Drexler H, Aguzzi A, Wagner EF. Endothelioma cells expressing the polyoma middle T oncogene induce hemangiomas by host cell recruitment. *Cell* 1989;**57**:1053–63.
32. Williams SA, Abbruscato TJ, Hruby VJ, Davis TP. Passage of a delta-opioid receptor selective enkephalin, [D-penicillamine2,5] enkephalin, across the blood–brain and the blood–cerebrospinal fluid barriers. *J Neurochem* 1996;**66**:1289–99.
33. Wong CH, Mruk DD, Lee WM, Cheng CY. Targeted and reversible disruption of the blood-testis barrier by an FSH mutant-occludin peptide conjugate. *FASEB J* 2007;**21**:438–48.
34. Wong V, Gumbiner BM. A synthetic peptide corresponding to the extracellular domain of occludin perturbs the tight junction permeability barrier. *J Cell Biol* 1997;**136**:399–409.
35. Zheng K, Trivedi M, Siahaan TJ. Structure and function of the intercellular junctions: barrier of paracellular drug delivery. *Curr Pharm Des* 2006;**12**:2813–24.

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Chapter 234

Acetylation

Qun Li, Iain J. Clarke and A. Ian Smith

ABSTRACT

Biologically active peptides and polypeptides are generated after proteolytic cleavage and other posttranslational modifications of precursor proteins. One of the most widely studied precursors is the prohormone precursor proopiomelanocortin (POMC), which gives rise to a number of peptide products with a range of biological activities. Interestingly, two of its posttranslational products (α-MSH and β-endorphin) belong to a very rare group of secreted, biologically active peptides that are acetylated. Acetylation of α-MSH enhances activity through increased biological stability, whereas acetylation abolishes activity of β-endorphin. These two peptides seem to have opposing roles in the regulation of energy balance, such that α-MSH inhibits feeding and β-endorphin stimulates feeding. Similarly, α-MSH stimulates reproduction, whereas β-endorphin is inhibitory. Thus, acetylation allows coordinate function of POMC-derived peptides, enhancing α-MSH function and reducing β-endorphin activity. The regulation of the relevant acetyl transferase enzyme(s), which remains to be identified, may provide a crucial regulatory point for the opposing physiological actions of these two peptides.

ACETYLATION OF BIOACTIVE PEPTIDES

Purification and molecular characterization of biologically active peptides have revealed that many are posttranslationally modified; this has a major effect on biological activity. Some commonly occurring posttranslational modifications include glycosylation of asparagine residues at the classic Asn-X-Ser/Thr sites, O-glycosylation of tyrosine residues, and the phosphorylation of serine residues. Less common peptide modifications include hydroxylation of lysine residues, serine octanoylation, sulfation, and finally, acetylation of N-terminal α-amino groups (serine and tyrosine) and, for α-MSH, the O-acetylation of the serine hydroxyl group. Since the discovery of an acetyl group at the amino-terminus of tobacco mosaic virus coat protein,[13] many acetylated proteins have been identified and characterized in animals and plants. For example, histone acetylation has been extensively studied; the lysine residues of histones undergo regulated acetylation and deacetylation, regulating the binding of these histones to DNA.[21,28] Acetylation is now considered a common modification of proteins in many organisms, and it has been suggested that more than half of the intracellular soluble proteins are N-acetylated. However, the acetylation of secreted bioactive peptides is considered a rare event. Indeed, only a handful of secreted proteins/peptides seem acetylated, and this group includes growth hormone where the acetylated form represents <5% total stored pituitary growth hormone and chaperonin 10;[4,29] in both of these, acetylation seems to have little impact on biological activity. Two other secreted hormones, α-MSH and β-endorphin, can be extensively acetylated and, for α-MSH, this can occur at two sites on the N-terminal serine residue (N,O) of the α-MSH molecule to produce diacetyl α-MSH.[15,31] Unlike chaperonin 10 and growth hormone, acetylation of α-MSH and β-endorphin has profound effects on biological activity.[19,32,33]

ACETYL TRANSFERASES

The acetylation of proteins and peptides is believed to be mediated by an acetyl transferase, which catalyzes the transfer of an acetyl group from a donor molecule, acetyl-CoA, to the target protein or peptide.[16] With respect to the mechanism of action, the acetyl transferases fall into two major classes. One class includes the enzyme that catalyzes acetylation of the newly synthesized peptide chain as it undergoes ribosomal synthesis. The enzymes in this class are likely ribosome and/or membrane bound. The second class of enzymes includes those that are responsible for processing bioactive peptides and mature proteins. For the second class of enzymes, it seems probable that different peptides and proteins are acetylated by substrate-specific enzymes. Glycine, alanine, serine, methionine, and aspartic acid are the preferred N-terminal amino acid of the α-N-acetylated proteins,[26] suggesting that the amino terminal residue may be a critical recognition site for these α-N-acetyl transferase(s).

DISTRIBUTION AND LOCALIZATION OF PEPTIDE ACETYL TRANSFERASE ACTIVITY

α-N-acetylation seems to occur in a tissue-specific manner given that different biological activities can be generated from one precursor molecule depending on the precise

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00234-7
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pattern of processing, which in turn is dependent on the suite of enzymes coexpressed with the precursor in the cell/tissue of interest. One well-studied example of differential precursor processing is that of the prohormone precursor proopiomelanocortin (POMC). POMC is expressed in a variety of different tissues including, brain, pituitary, heart, and the immune system.[31] The intermediate lobe of the pituitary gland is a highly specialized tissue, consisting of a dense population of melanotropes, which are the major source of α-MSH and β-endorphin that is found in the blood stream.[15,31] POMC cells are also found in the arcuate nucleus of the hypothalamus, and these particular cells play a major role in the regulation of energy homeostasis.[11] In both these regions, α-N-acetylation of the POMC peptides occurs, and this is believed to be localized to the secretory vesicles.[16] Examples of this mechanism of vesicle-associated α-N-acetylation seem early in evolution and as such seem highly conserved.[10] Pulse-chase type experiments suggest that α-MSH and β-endorphin are acetylated in parallel.[15] Although this may be affected by the same α-N-acetyl transferase, the identity of the enzyme is not known.[16,33] Studies conducted >30 years ago by Glembotski[16] partially characterized an α-N-acetyl transferase activity in the neurointermediate lobe of both bovine and rat pituitaries. Subsequent studies by the same group showed a potential secretory vesicle location for this enzyme activity in the rat neurointermediate pituitary.[15] These studies also suggested that the enzyme is not membrane associated, has a pH optimum of 7.0, and the K_m value for the donor acetyl CoA is 8 μM. K_m values for the peptide substrates range from 11 to 286 μM. Other studies showed that β-endorphin 1–27 competitively inhibits the acetylation of α-MSH, suggesting that the same enzyme is responsible for acetylating endorphins and melanocortins.

The mapping of acetyl transferase activity indicated its presence in the pituitary and the brain, though activity and substrate levels are not always concomitant.[7] Finally, studies of intermediate lobe acetyl transferase activity suggest that both the POMC gene and the enzyme activity are coregulated.[25] Definitive studies to examine the expression, tissue location, subcellular distribution, and regulation of activity await the unequivocal identification and molecular characterization of the relevant acetyl transferase(s) involved in secreted peptide acetylation.

Both α-MSH and β-endorphin are generated after extensive posttranslational processing that involves the sequential action of several enzymes such as the prohormone convertases 1 and 2 (PC1 and PC2), carboxypeptidase E (CPE) and peptidyl α-amidating monooxygenase (PAM). This is detailed elsewhere in this volume. Although it remains possible that the amounts of the end products are determined by the activity of these processing enzymes, it is the acetylation of the peptides that finally determines activity or inactivity.

PATHOPHYSIOLOGY OF ACETYLATED AND NONACETYLATED α-MSH AND β-ENDORPHIN

Melanocortins

α-MSH is a well-characterized peptide that is secreted into the blood stream and stimulates skin pigmentation.[34] More recently, however, α-MSH has also been shown to play a pivotal role in regulating inflammation/immunomodulation,[6] reproductive function,[3] and energy homeostasis.[11] It should be noted that the POMC precursor also encodes β-MSH and γ-MSH. All three forms of melanocortins bind to the four (MC1R, MC3R, MC4R, and MC5R) of the five melanocortin receptor subtypes (MC1-5R). Although they have similar affinities for MCR3, their affinities to MC1R, MC4R, and MC5R differ.[14] In the periphery, MCR-1 is the effector for melanocortin action in skin, MCR-2 is the adrenocorticotropin receptor found in the adrenal glands, and MCR-5 is found in muscle, where it may mediate thermoregulation. MCR-3 and MCR-4 are found in the brain and have a major role in the regulation of food intake and energy homeostasis.[8,20] An elegant study by Bjorbaek and colleagues[18] showed that acetylation of α-MSH is required for full potency in terms of ability to suppress food intake (Fig. 1).

Despite the differences in potency between des-acetyl-α-MSH and its acetylated congeners, several studies have failed to find evidence for substantial differences in binding affinities for any of the MSH receptors.[30] The one factor that seems to address the apparent differences in potency between acetylated and des-acetylated MSH peptides is that in a biological environment, the acetylated form of α-MSH is much more resistant to proteolytic degradation than des-acetyl-α-MSH. Degradation of the des-acetylated form of α-MSH is initiated by amino-peptidases, and this occurs more rapidly than the metabolism of the acetylated

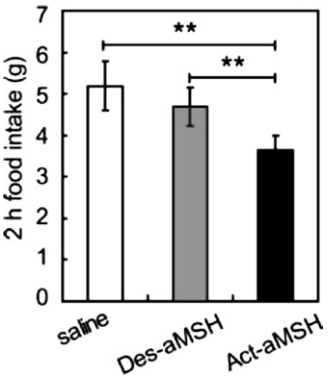

FIGURE 1 Mean (± SEM) food intake 2 h after an injection of des-acetyl-α-MSH (des-aMSH; gray column) or acetylated α-MSH (Acet-aMSH; black column) or vehicle (open column) into the third ventricle of rats ($n=6$) $^{**}P<0.005$. The figure is reproduced from Ref. (18) (permission requested). Note the stronger effect of the acetylated peptide.

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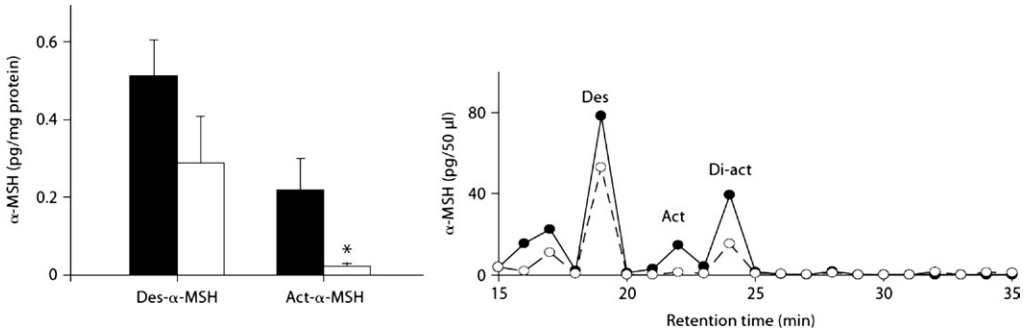

FIGURE 2 Mean (± SEM) levels of des-acetyl-α-MSH and acetylated α-MSH in the hypothalamus of sheep of normal (black columns) or lean body weight. *$P<0.05$ vs normal. The figure is reproduced from Ref. (2) with permission. High performance liquid chromatography was performed (right-hand panel) to separate the di-acetylated (Di-act), mono acetylated (Act) and des-acetylated forms of α-MSH (Des), before immunoassay.

forms of α-MSH. There is now some evidence to suggest that differences in specific G protein receptor-mediated activation of cellular signaling pathways may influence the relative functional activity of the different molecular forms of MSH.[33] Leptin stimulates the acetylation of α-MSH in the hypothalamus of rats [18] and in lean sheep, with low levels of leptin the acetylated form of α-MSH is reduced (Fig. 2).[2]

Endorphins

Although the 31-amino acid peptide β-endorphin was first characterized in 1976[22] after the isolation and amino acid sequencing of an opiate-like activity from camel pituitary, it was a series of studies in 1977, using both classical protein purification and cell free translation techniques[24,27] that showed that β-endorphin, β-LPH, and ACTH (which contains the α-MSH amino acid sequence) are synthesized in the ribosome as part of the same precursor molecule. The first study showing that β-endorphin was acetylated in the intermediate pituitary also showed that the acetylated form of β-endorphin lacked any opiate activity.[32]

The functions of β-endorphin have been extensively studied in the period after the identification of the endogenous opioids.[9] β-endorphin has analgesic properties with 20- to 30-fold greater potency than morphine;[23] this is a central function of the peptide, and the significance of peripherally circulating β-endorphin remains to be fully elucidated. It seems that the levels of β-endorphin in blood reflect cosecretion with α-MSH,[12] perhaps arising from the intermediate lobe, in the sheep at least. Acting centrally, β-endorphin stimulates food intake and inhibits the reproductive function.[5,17] It also modulates the function of most other neuroendocrine systems.[9,33]

The relative potency of acetylated and des-acetylated forms of the endorphins is primarily a function of receptor affinity. Modifications to both the N-terminus of β-endorphin (acetylation) and C-terminus (truncation) have quite profound effects on receptor binding.[1] Acetylation causes a dramatic, up to 1000-fold reduction in opiate binding efficiency, essentially rendering the peptide opiate inactive. C-terminal shortening, however, only marginally (~10-fold) reduces opiate receptor affinity.[1] The acetylated forms of β-endorphin simply do not bind significantly to either the k- or μ-type opiate receptors.[1]

CONCLUSIONS

Acetylation of α-MSH and β-endorphin has significant biological consequences in terms of melanogenesis; regulation of food intake; and reproduction, neural regeneration, and analgesia; these are detailed extensively.[33] Various endogenous and exogenous stimuli can impose physiological or pathological changes via the acetylation process, as indicated above for α-MSH. Thus, for α-MSH and β-endorphin, which have opposing activities, acetylation may represent a crucial switch whereby the physiological activities of these two peptides can be posttranslationally regulated.

REFERENCES

1. Akil H, Young E, Watson SJ, Coy DH. Opiate binding properties of naturally occurring N- and C-terminus modified beta-endorphins. *Peptides* 1981;**2**:289–92.
2. Backholer K, Bowden M, Gamber K, Bjorbaek C, Iqbal J, Clarke IJ. Melanocortins mimic the effects of leptin to restore reproductive function in lean hypogonadotropic ewes. *Neuroendocrinology* 2010;**91**:27–40.
3. Backholer K, Smith J, Clarke IJ. Melanocortins may stimulate reproduction by activating orexin neurons in the dorsomedial hypothalamus and kisspeptin neurons in the preoptic area of the ewe. *Endocrinology* 2009;**150**:5488–97.
4. Baumann G, Abramson EC. Urinary growth hormone in man: evidence for multiple molecular forms. *J Clin Endocrinol Metab* 1983;**56**:305–11.
5. Bonavera JJ, Kalra SP, Kalra PS. Evidence that luteinizing hormone suppression in response to inhibitory neuropeptides, beta-endorphin, interleukin-1 beta, and neuropeptide-K, may involve excitatory amino acids. *Endocrinology* 1993;**133**:178–82.

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6. Catania A, Airaghi L, Colombo G, Lipton JM. Alpha-melanocyte-stimulating hormone in normal human physiology and disease states. *Trends Endocrinol Metab* 2000;**11**:304–8.
7. Chappell MC, Loh YP, O'Donohue TL. Evidence for an opiomelanotropin acetyltransferase in the rat pituitary neurointermediate lobe. *Peptides* 1982;**3**:405–10.
8. Chen AS, Marsh DJ, Trumbauer ME, Frazier EG, Guan XM, Yu H, et al. Inactivation of the mouse melanocortin-3 receptor results in increased fat mass and reduced lean body mass. *Nat Genet* 2000;**26**:97–102.
9. Dalayeun JF, Nores JM, Bergal S. Physiology of beta-endorphins. A close-up view and a review of the literature. *Biomed Pharmacother* 1993;**47**:311–20.
10. Dores RM, Steveson TC, Lopez K. Differential mechanisms for the N-acetylation of alpha-melanocyte-stimulating hormone and beta-endorphin in the intermediate pituitary of the frog, *Xenopus laevis*. *Neuroendocrinology* 1991;**53**:54–62.
11. Ellacott KL, Cone RD. The central melanocortin system and the integration of short- and long-term regulators of energy homeostasis. *Recent Prog Horm Res* 2004;**59**:395–408.
12. Engler D, Pham T, Fullerton MJ, Clarke IJ, Funder JW. Evidence for an ultradian secretion of adrenocorticotropin, beta-endorphin and alpha-melanocyte-stimulating hormone by the ovine anterior and intermediate pituitary. *Neuroendocrinology* 1989;**49**:349–60.
13. Filner B, Marcus A. TMV coat protein synthesis in vivo: analysis of the N-terminal acetylation. *Virology* 1974;**61**:537–46.
14. Gantz I, Fong TM. The melanocortin system. *Am J Physiol Endocrinol Metab* 2003;**284**:E468–74.
15. Glembotski CC. Acetylation of alpha-melanotropin and beta-endorphin in the rat intermediate pituitary. Subcellular localization. *J Biol Chem* 1982;**257**:10493–500.
16. Glembotski CC. Characterization of the peptide acetyltransferase activity in bovine and rat intermediate pituitaries responsible for the acetylation of beta-endorphin and alpha-melanotropin. *J Biol Chem* 1982;**257**:10501–9.
17. Grandison L, Guidotti A. Stimulation of food intake by muscimol and beta endorphin. *Neuropharmacology* 1977;**16**:533–6.
18. Guo L, Munzberg H, Stuart RC, Nillni EA, Bjorbaek C. N-acetylation of hypothalamic alpha-melanocyte-stimulating hormone and regulation by leptin. *Proc Natl Acad Sci USA* 2004;**101**:11797–802.
19. Guttmann S, Boissonnas RA. Influence of the structure of the n-terminal extremity of alpha-MSH on the melanophore stimulating activity of this hormone. *Experientia* 1961;**17**:265–7.
20. Huszar D, Lynch CA, Fairchild-Huntress V, Dunmore JH, Fang Q, Berkemeier LR, et al. Targeted disruption of the melanocortin-4 receptor results in obesity in mice. *Cell* 1997;**88**:131–41.
21. Khochbin S, Verdel A, Lemercier C, Seigneurin-Berny D. Functional significance of histone deacetylase diversity. *Curr Opin Genet Dev* 2001;**11**:162–6.
22. Li CH, Chung D. Isolation and structure of an untriakontapeptide with opiate activity from camel pituitary glands. *Proc Natl Acad Sci U S A* 1976;**73**:1145–8.
23. Loh HH, Tseng LF, Wei E, Li CH. Beta-endorphin is a potent analgesic agent. *Proc Natl Acad Sci USA* 1976;**73**:2895–8.
24. Mains RE, Eipper BA, Ling N. Common precursor to corticotropins and endorphins. *Proc Natl Acad Sci USA* 1977;**74**:3014–8.
25. Millington WR, O'Donohue TL, Chappell MC, Roberts JL, Mueller GP. Coordinate regulation of peptide acetyltransferase activity and proopiomelanocortin gene expression in the intermediate lobe of the rat pituitary. *Endocrinology* 1986;**118**:2024–33.
26. Polevoda B, Sherman F. N-terminal acetyltransferases and sequence requirements for N-terminal acetylation of eukaryotic proteins. *J Mol Biol* 2003;**325**:595–622.
27. Roberts JL, Herbert E. Characterization of a common precursor to corticotropin and beta-lipotropin: identification of beta-lipotropin peptides and their arrangement relative to corticotropin in the precursor synthesized in a cell-free system. *Proc Natl Acad Sci USA* 1977;**74**:5300–4.
28. Ruiz-Carrillo A, Wangh LJ, Allfrey VG. Processing of newly synthesized histone molecules. *Science* 1975;**190**:117–28.
29. Ryan MT, Naylor DJ, Hoogenraad NJ, Hoj PB. Affinity purification, overexpression, and characterization of chaperonin 10 homologues synthesized with and without N-terminal acetylation. *J Biol Chem* 1995;**270**:22037–43.
30. Schioth HB, Muceniece R, Larsson M, Wikberg JE. The melanocortin 1, 3, 4 or 5 receptors do not have a binding epitope for ACTH beyond the sequence of alpha-MSH. *J Endocrinol* 1997;**155**:73–8.
31. Smith AI, Funder JW. Proopiomelanocortin processing in the pituitary, central nervous system, and peripheral tissues. *Endocr Rev* 1988;**9**:159–79.
32. Smyth DG, Massey DE, Zakarian S, Finnie MD. Endorphins are stored in biologically active and inactive forms: isolation of alpha-N-acetyl peptides. *Nature* 1979;**279**:252–4.
33. Wilkinson CW. Roles of acetylation and other post-translational modifications in melanocortin function and interactions with endorphins. *Peptides* 2006;**27**:453–71.
34. Wintzen M, Gilchrest BA. Proopiomelanocortin, its derived peptides, and the skin. *J Invest Dermatol* 1996;**106**:3–10.

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Chapter 235

Carboxypeptidases E and D

Lloyd D. Fricker

ABSTRACT

Most bioactive peptides are produced from proteolytic processing of precursors. In many cases, the processing sites contain basic amino acids (Lys, Arg), which are cleaved by proprotein convertases to generate processing intermediates containing C-terminal basic amino acids. These intermediates are rarely biologically active, and removal of the C-terminal basic residues is necessary to produce the mature form. Although the proprotein convertase gene family contains nine members in mammals, the metallocarboxypeptidase gene family contains 25 mammalian members. However, only two of these are believed to function in the generation of bioactive peptides within the secretory pathway. Carboxypeptidase E is the primary peptide-producing enzyme; it is active within secretory granules. Carboxypeptidase D also contributes to peptide processing but plays a minor role in the production of most neuropeptides; this enzyme is primarily located in the trans-Golgi network, cycles to the cell surface, and returns to the trans-Golgi.

NAME AND HISTORY

Carboxypeptidase E (CPE) was originally discovered in enkephalin-containing chromaffin vesicles of the adrenal medulla and was renamed enkephalin convertase for publication.[10] The original name of CPE was used once it became clear that the enzyme was involved in the production of many neuroendocrine peptides and was not specific for enkephalin. CPE is also known as carboxypeptidase H, among its other names. For many years, CPE was believed to be the only carboxypeptidase involved in neuroendocrine peptide processing. However, mice lacking CPE activity resulting from a naturally occurring point mutation produce low levels of many neuroendocrine peptides.[2,6,14,18,35] Although the low levels of peptides in these mice supported the idea that CPE is the carboxypeptidase involved with the biosynthesis of most neuroendocrine peptides, the finding that low levels of mature peptides are still produced in the absence of CPE raised the possibility of an additional peptide-processing carboxypeptidase. This led to the discovery of carboxypeptidase D (CPD).[23]

CPD is occasionally referred to as metallocarboxypeptidase D to distinguish it from serine carboxypeptidase D, an unrelated enzyme. CPD was independently discovered in 1995 by three separate groups working in distinct areas. One group identified and characterized bovine CPD based on its enzymatic properties.[23] A duck protein named gp180 was identified by its ability to bind avian hepatitis B virus particles, and when the cDNA was cloned and sequenced, gp180 was found to be a homolog of CPD.[13] A third group was working on the *Drosophila silver* gene mutation, which was originally discovered in 1918 and caused reduced viability, altered pigmentation, and a pointed wing shape. The *silver* gene was found to represent the *Drosophila* homolog of CPD.[19] Soon after the initial discovery of CPD, homologs were found in humans,[26] rats,[34] and other organisms.

FUNCTION IN PEPTIDE BIOSYNTHESIS

CPE functions in the cellular secretory pathway where it is the primary carboxypeptidase involved in the biosynthesis of neuroendocrine peptides such as insulin and enkephalin, as well as many others. CPE functions after endopeptidases, such as proprotein convertase 1 (also known as proprotein convertase 3) and proprotein convertase 2, cleave the peptide precursor into intermediates containing C-terminal basic residues (these endopeptidases are covered in another chapter in this section of the Handbook). Additional roles for CPE have been described, such as a role in prohormone sorting, but this function is controversial and not supported by numerous studies. CPE is found in all vertebrate animals and many other species, including *Caenorhabditis elegans*. This is consistent with its broad role in the biosynthesis of neuroendocrine peptides. However, *Drosophila* do not have an ortholog of CPE, and instead rely on CPD, which is alternatively spliced in this species to produce a number of short forms, some of which resemble CPE in terms of enzyme properties. Most bioactive peptides described in other chapters of this volume require CPE for their production, including vasopressin, oxytocin, thyrotropin-releasing hormone, gonadotropin-releasing hormone, somatostatin, neuropeptide Y, melanocyte-stimulating hormone, cocaine and

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00235-9
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amphetamine transcript-derived peptides, cholecystokinin, several tachykinins, enkephalins, dynorphins, nociceptins, and insulin. Other peptides also require CPE for their production, such as peptides derived from VGF, chromogranins A and B, secretogranin 2, 7B2, and proSAAS.[35]

Carboxypeptidase D has a broad tissue distribution and is believed to function primarily in the Golgi in the processing of proteins that transit the secretory pathway. Many secreted or cell-surface proteins require proteolytic processing at specific sites containing multiple basic residues; examples include the insulin receptor, and many of the molecules that serve as extracellular messengers (growth factors and neuroendocrine peptides). Endopeptidases such as furin initially cleave the precursors to the C-terminus of the basic amino acids, and the subsequent removal of these basic residues from the C-terminus of the intermediate is accomplished by CPD. Although the primary role of CPD is the processing of proteins, rather than neuropeptides, CPD is able to process peptides and proteins. The limiting factor is whether the endopeptidase processing occurs early enough in the secretory pathway for the intermediates to be exposed to CPD. For example, if furin or another proprotein convertase cleaves a neuropeptide precursor in the Golgi, then the intermediates will be exposed to CPD, and this enzyme will trim off the basic residues. However, CPD is not present in secretory granules, so if the prohormone processing occurs later in the pathway within the maturing granules, then CPD will not have access to the intermediates and cannot act on them. Because most neuropeptides are processed within the secretory granules, CPE was therefore predicted to function as the major carboxypeptidase in their biosynthesis, with CPD playing only a minor role. This prediction was borne out by the analysis of peptides in brain, pituitary, and other tissues of animals lacking CPE activity (described in another chapter in this section of the volume).

GENERAL PROPERTIES

CPE and CPD are both members of the same subfamily of metallocarboxypeptidases, named the N/E subfamily (after the first two enzymes discovered in this family, carboxypeptidase N and CPE). In addition to CPE, CPD, and carboxypeptidase N (a plasma enzyme), there are two other active enzymes in this subfamily: carboxypeptidase M, which is an extracellular membrane-bound enzyme, and carboxypeptidase Z, an extracellular matrix-bound enzyme. Three other members of this gene subfamily have no detectable carboxypeptidase activity (named CPX1, CPX2, and AEBP1/ACLP); these proteins are predicted to fold into the basic metallocarboxypeptidase structure but lack critical active site residues. CPE and CPD share approximately 50% amino acid sequence similarity and have many properties in common as well as some fundamental differences.

CPE is a single-chain protein of 52–56 kDa that contains an active site zinc ion. Although the crystal structure has not been determined, the structure of CPE has been modeled based on the crystal structure of one of the domains of CPD.[1] The two structures are similar, except for minor differences in the lengths and positions of the loops that shape the entrance to the active site.[1] The overall structure of CPE includes an approximately 300 residue domain that has homology to all 25 members of the M14 metallocarboxypeptidase gene family (i.e. eight members of the N/E subfamily and all members of the other three subfamilies). Following the general metallocarboxypeptidase domain, CPE contains an approximately 80 residue transthyretin-like domain (Fig. 1). Both the

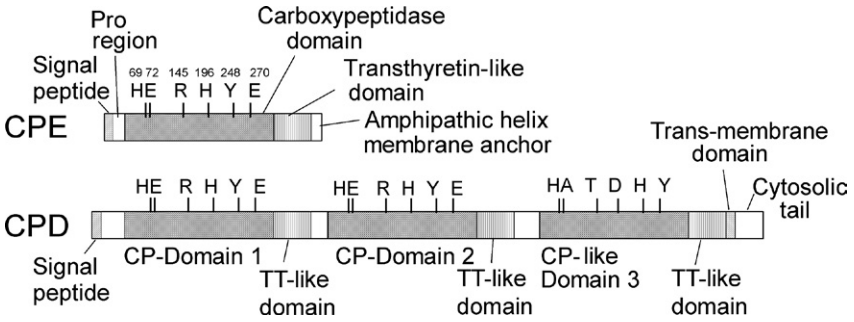

FIGURE 1 Schematic diagram of CPE and CPD, showing the various domains. CPE has an N-terminal signal peptide, a short proregion (which does not regulate enzyme activity, in contrast with most pro-domains), a carboxypeptidase domain with sequence and structural homology to carboxypeptidases A and B, a transthyretin-like domain that has structural homology but not sequence homology to the protein transthyretin, and a C-terminal domain that seems to form an amphipathic helix and bind the full length form of CPE to membranes. All forms detected in tissues lack the N-terminal signal peptide. Most forms lack the proregion, and soluble forms lack the C-terminal region. CPE is also modified by N-linked glycosylation at two distinct sites (not shown). Amino acids critical for Zn^{2+} binding (H69, E72, H196), substrate binding (R145, Y248), and catalytic activity (E270) are indicated; the numbering system is that of the active form of bovine carboxypeptidase A, by convention in the field. CPD has a signal peptide, two active carboxypeptidase (CP) domains and one inactive carboxypeptidase-like (CP-like) domain; each of these is followed by a transthyretin (TT)-like domain. CPD is membrane-bound through a transmembrane-spanning domain located near the C-terminus, followed by an approximately 50–60 residue C-terminal cytosolic tail. The CP domains face the interior of the Golgi, away from the cytosol. A number of N-linked glycosylation sites are present in the luminal part of the protein, and palmitate is added to the cytosolic tail at sites located near the transmembrane domain (not shown).

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general carboxypeptidase domain and the transthyretin-like domain within CPE are present in CPD and all other members of the N/E subfamily (carboxypeptidases M, N, Z, and three related proteins that have no detectable carboxypeptidase activity). Although the 80 residue transthyretin-like domain is not conserved in the other three subfamilies of metallocarboxypeptidases, most other family members have a comparable region of 80–100 amino acids that forms a beta-sheet-rich structure, even though there is no amino acid sequence homology among these domains between the different subfamilies.

In addition to the common metallocarboxypeptidase domain and transthyretin domain found in all members of the N/E subfamily, CPE contains a unique C-terminal domain of approximately 30 amino acids that is not found in other metallocarboxypeptidases.[8] The C-terminal domain of CPE directs association of CPE with membranes, and variable proteolysis of the C-terminal region gives rise to soluble forms of CPE. The membrane-association of CPE seems to be as a peripheral membrane protein not as a transmembrane protein.[30] It has been proposed that CPE is anchored to membranes via a transmembrane domain.[4] However, there is no suitable sequence on CPE that corresponds to a transmembrane-spanning domain.[8] Furthermore, numerous studies have shown that CPE is released from membranes with an intact C-terminus by moderate to high pH treatments,[5,7,16] this is not compatible with the transmembrane mechanism of attachment. Therefore, the peripheral association of CPE through an amphipathic C-terminal helix is more likely to be correct, based on the numerous published studies, although the possibility that CPE forms a novel type of interaction with membranes cannot be ruled out.

In addition to the C-terminal membrane-binding region, a form of CPE detected in cells and tissue contains a 14 residue extension on the N-terminus. This form, named "proCPE" shows full enzymatic activity.[16] The function of the 14 residue propeptide is not known; it does not influence enzyme activity or sorting to the secretory pathway. CPE is also known to undergo additional posttranslational modifications; it is glycosylated on two Asn-Xaa-Ser/Thr sites found in the protein. Glycosylation does not seem to affect enzyme activity, and the function of this modification remains unknown.

CPD is unique from CPE, and all other members of the metallocarboxypeptidase gene family, in several ways. First, CPD is the only member of the M14 metallocarboxypeptidase family that is a type 1 transmembrane protein. Second, carboxypeptidase D contains three distinct carboxypeptidase-like domains, the first two of which are enzymatically active, whereas the third is not (Fig. 1). This feature of two active carboxypeptidase domains followed by a third inactive domain and then a transmembrane domain and cytosolic tail is found in CPD proteins from *Drosophila* to humans. Because of the three carboxypeptidase domains, CPD is a large protein with a molecular weight of approximately 180 kDa in most species.[23,24] The first two metallocarboxypeptidase domains have 40–50% amino acid identity with other members of the CPD subfamily. The third domain shows slightly less homology to the other members of the subfamily, and lacks one or more residues believed to be critical for catalytic activity.[13,26,34] The C-terminal cytosolic tail of CPD directs the retention of this protein in the trans-Golgi network. This 50–60 residue domain is highly conserved among vertebrates, with only two conservative differences between ducks and humans. Although the sequence of the C-terminal tail of vertebrate CPD shows little amino acid sequence similarity with *Drosophila* CPD, the general domains are conserved between *Drosophila* and vertebrates.[29,31,33] These include acidic clusters, di-Leu motifs, and other motifs thought to be important for the trafficking of CPD from the trans-Golgi to the cell surface and back to the trans-Golgi.

The crystal structures of the first and second carboxypeptidase domains of duck CPD have been determined.[11,27] There is considerable structural homology between each carboxypeptidase domain of CPD and all other members of the larger metallocarboxypeptidase family. The first and second carboxypeptidase domains of CPD each contain a zinc atom, which is required for catalysis. The zinc-binding residues, the active site residues, and most of the substrate-binding residues are conserved among CPD and other metallocarboxypeptidases. Like CPE, after each of the carboxypeptidase domains in CPD is an 80-residue domain that folds into a β-sandwich that resembles the topology of transthyretin and other proteins.[11,27] Even though the third carboxypeptidase-like domain of CPD is inactive, modeling shows a generally similar structure as the catalytically active carboxypeptidase domains of CPD, including the presence of a transthyretin-like domain.[1] The function of this transthyretin-like domain in CPD (or in CPE and related subfamily members) is not currently known, although it is essential for activity; deletions of any portion of this domain lead to inactive enzyme. Thus, it has been proposed to function in protein folding.

SUBSTRATE SPECIFICITY AND REGULATION OF ACTIVITY

CPE removes C-terminal basic residues (i.e. Arg, Lys) from a variety of substrates, with only weak activity toward C-terminal His and no detectable activity toward nonbasic residues.[10,22] Most residues are well tolerated in the penultimate (P1) position, with Met the most favorable and Pro the least favorable. The Pro–Arg bond is slowly cleaved, with a rate that is orders of magnitude slower than substrates with other P1 residues.[3,22] CPE is much more active toward tripeptides than dipeptides, and longer peptides are also efficiently cleaved.

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Like CPE, CPD also removes C-terminal Lys or Arg residues from a variety of substrates, with no detectable activity toward nonbasic residues.[23] Both the first and second domains of CPD prefer an Ala in the penultimate position.[15] As with CPE, CPD also shows higher activity with tripeptides than with dipeptides. CPD has been assayed with the same substrates previously developed for CPE.[23] The first and second domains of CPD differ in their pH optima; the first domain of CPD is maximally active at neutral pH whereas the second domain is preferentially active at the acidic pH 5–6 range. The first and second domains of CPD also show different preferences for C-terminal Lys and Arg residues. The first domain cleaves C-terminal Arg residues more efficiently than Lys residues, whereas the second domain cleaves Lys residues as efficiently as Arg residues.

A number of different substrates have been used to detect CPE and CPD. Some of the assays make use of a solubility change that the peptide undergoes when the basic residue is removed from the C-terminus of the substrate (Dns-Phe-Ala-Arg; [^{3}H] Bz-Phe-Ala-Arg; or [^{125}I] Ac-Tyr-Ala-Arg). Once the basic residue is removed, the products readily partition into organic solvents such as chloroform from an acidic aqueous phase. Assays based on the extraction of the product into organic solvent permit the rapid analysis of a large number of samples. This assay is much faster than other assays developed for CPE that use HPLC or thin-layer chromatography to separate the product from the substrate.

The assays for CPE and CPD are not specific for these enzymes and will also detect other carboxypeptidases that cleave Arg and endopeptidases that cleave the substrate. The assay can be performed at pH 5.5 to minimize the contribution from cellular peptidases that are optimal at neutral pH values, but at pH 5.5, the assay will detect lysosomal enzymes that cleave the substrate. To increase the specificity of the assay for CPE and CPD, the assay can be performed in the presence and absence of selective inhibitors such as guanidinoethylmercaptosuccinic acid; this compound selectively inhibits metallocarboxypeptidases and not the lysosomal carboxypeptidases or various endopeptidases found in a cell. Unfortunately, the various assays do not allow for the discrimination of CPE versus CPD. However, it is possible to separate these two enzymes by partial purification on a substrate affinity column.[23] CPE elutes from *p*-aminobenzoyl-Arg-Sepharose when the pH is raised to 8, whereas CPD remains bound at this pH and is eluted with a competitive ligand such as Arg.[23]

CPE activity is optimal at pH 5.0–5.5, and activity falls off dramatically with increasing pH due to a change in K_m. It is likely that the sensitivity of CPE to pH over the range 5.5–7 serves as a mechanism for CPE to become activated when it moves from the neutral pH of the Golgi to the mature secretory vesicles (internal pH of 5–5.5). By contrast, CPD shows a broad pH optimum resulting from the two distinct carboxypeptidase domains that are active; the first domain is active at neutral pH, whereas the second is maximally active at acidic pH. Thus, CPD is able to function in the Golgi (where the pH is slightly acidic), on the cell surface (where the pH is neutral), and in the endocytic pathway (where the pH is acidic).

Both CPE and CPD are stimulated by millimolar concentrations of Co^{2+} and inhibited by 1,10-phenanthroline and by transition-state inhibitors and thiol-directed reagents such as *p*-chloromercuriphenylsulfonate and $HgCl_2$. None of these compounds or ions is thought to be involved in the physiological regulation of CPE or CPD. One ion that may play some role in the regulation of CPE is Ca^{2+}. CPE has a binding site for Ca^{2+}, and this ion affects the stability of CPE. However, the effect is small and Ca^{2+} does not seem to substantially affect enzyme activity. Increasing concentrations of Ca^{2+}, together with decreasing pH, cause the aggregation of CPE, which may contribute to the sorting of CPE into the regulated secretory pathway.

DISTRIBUTION/LOCALIZATION

The distribution of both CPE and CPD is consistent with broad roles for these enzymes in the production of numerous biologically active peptides. CPE is most abundant in brain, pituitary, and pancreatic islets, and it is also present in many other neuroendocrine tissues. At a subcellular level, CPE is enriched in peptide-containing secretory granules and is cosecreted along with the bioactive peptides.

The distribution of CPD is broader than that of CPE, consistent with a role in the processing of many proteins that transit either the regulated or constitutive secretory pathways.[29,31,33] CPD is found in virtually all tissues examined.[24,34] Within cells, CPD is enriched in the trans-Golgi network and is also found in immature secretory vesicles but not in mature secretory granules. Although CPD has been reported to exist within purified secretory granules, it is present in only a small subset of these granules and this presumably corresponds to the immature granules.[17,29,33] CPD cycles to the cell surface via a constitutive secretory pathway and returns to the trans-Golgi network via an endocytic pathway.[29,31,33] Cell surface CPD is enzymatically active and cleaves peptides containing C-terminal Arg residues, thereby releasing free Arg. It has been proposed that this Arg can then be a substrate for nitric oxide synthesis; treatments that lower the levels of cell surface CPD or which inhibit CPD enzyme activity have been shown to reduce the production of nitric oxide in cell culture systems.[12]

PATHOPHYSIOLOGICAL IMPLICATIONS

A spontaneously arising mouse mutation, named the *fat* mutation, represents a point mutation within the coding region of the *Cpe* gene.[14] This mutation causes a Ser to

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be replaced with a Pro, and this change eliminates CPE enzyme activity. The resulting mutant form of the protein is degraded in the early secretory pathway, before packaging into secretory vesicles.[32] Mice homozygous for the *fat* mutation have very low levels of the mature forms of most neuropeptides and greatly elevated levels of C-terminally extended peptide processing intermediates.[2,6,14,18,35] This result is consistent with the role of CPE activity in the production of many neuroendocrine peptides, and is described in more detail in another chapter in this section of the handbook. Mice with the *fat* mutation show juvenile-onset obesity, greatly reduced fertility, and other behavioral and physiological abnormalities.[9,25] Mice with a gene disruption in *Cpe* have been created and characterized; the phenotype of mice with a disruption of the *Cpe* gene is essentially the same as the phenotype of the *fat* mice.

A number of variants of human *CPE* have been found. A study of 269 Japanese subjects found three nucleotide variants, but none of these altered the amino acid sequence.[28] A study of 945 Ashkenazi subjects found five different single nucleotide polymorphisms.[3] Two of these variants altered the amino acid sequence, one of which was a highly-conserved residue (Arg283). The variant found in the Ashkenazi population encoded a Trp in this position (R283W). The enzymatic properties of the R283W form of CPE were examined; the *V*max of the R283W variant was reduced about 10-fold compared with that of wild-type CPE.[3] The R283W variant form of CPE was less stable to heat denaturation than that of wild-type CPE.[3] Although this variant may contribute to some cases of obesity and/or diabetes, the prevalence of the variant in the human population is too low to explain the vast majority of human obesity or diabetes.

The CPD gene is not associated with any known genetic mutations in humans or mice. A number of mutations have been found in the CPD ortholog in *Drosophila*.[21] Severe mutations of *Drosophila* CPD are lethal, with no survivors past the third instar stage;[20] these severe mutations do not produce any CPD protein. Mutations within the second carboxypeptidase domain of *Drosophila* CPD are viable; these mutants produce short forms of CPD that correspond to the first carboxypeptidase domain and this is sufficient to rescue the lethality. However, *Drosophila* mutants that lack the long CPD forms have altered wing shape and changes in behavior, suggesting that the full-length membrane-bound forms of CPD perform different functions than do the shorter forms of CPD produced in *Drosophila*.[21]

LATER FINDINGS

Despite having been discovered approximately 30 years ago, CPE is still yielding new insights. CPE is inhibited by catecholamines. Because the levels of catecholamines that are inhibitory to CPE are within the physiological range of their concentrations within adrenal medullary chromaffin granules, this raises the possibility that the production of bioactive peptides is controlled by endogenous levels of catecholamines. CPE is implicated in cancer, although the forms of CPE that seem to correlate with cancer are missing in their N-terminal region and therefore are not routed to the secretory pathway. Because CPE requires disulfide bond formation, and is inhibited when treated with agents that disrupt disulfide bonds, it is not probable that CPE produced in the cytosol will be enzymatically active. Still, there may be alternative roles for CPE protein that do not require enzyme activity. Another emerging area is the characterization of the behavior of mice lacking CPE activity because of the *fat* mutation. Originally reported to be overweight and sterile, they have additional behavioral problems, as expected for the broad role of neuropeptides in many neurological processes. Further studies to identify the peptides responsible for each of the aberrant behaviors in the mice lacking CPE activity may identify novel roles for existing peptides or novel neuropeptides.

CPD is also still revealing many new insights. One unsolved mystery is the reason for the unusual domain structure, with two carboxypeptidase domains and one carboxypeptidase-like domain present in CPD from human to *Drosophila*. In *C. elegans*, only two of these domains are present; the first is predicted to be enzymatically active, the second is predicted to be inactive based on the absence of critical active site residues. Therefore, the presence of an inactive carboxypeptidase-like domain seems to be an essential part of CPD function. Transgenic *Drosophila* lines have been created, which express forms of CPD that lack the inactive carboxypeptidase-like domain, or which have this domain replaced with one of the active domains. All of these lines show greatly reduced viability, compared with transgenic *Drosophila* in which constructs contained the inactive carboxypeptidase-like domain. These results support the hypothesis that this inactive carboxypeptidase-like domain has a function, and further studies are necessary to identify this function.

REFERENCES

1. Aloy P, Companys V, Vendrell J, Aviles FX, Fricker LD, Coll M, et al. The crystal structure of the inhibitor-complexed carboxypeptidase D domain II as a basis for the modelling of regulatory carboxypeptidases. *J Biol Chem* 2001;**276**:16177–84.
2. Che F-Y, Yan L, Li H, Mzhavia N, Devi L, Fricker LD. Identification of peptides from brain and pituitary of Cpe^{fat}/Cpe^{fat} mice. *Proc Natl Acad Sci U S A* 2001;**98**:9971–6.
3. Chen H, Jawahar S, Qian Y, Duong Q, Chan G, Parker A, et al. A missense polymorphism in the human carboxypeptidase E gene alters its enzymatic activity: possible implications in type 2 diabetes mellitus. *Hum Mutat* 2001;**18**:120–31.
4. Dhanvantari S, Arnaoutova I, Snell SR, Steinbach PJ, Hammond K, Caputo GA, et al. A prohormone sorting receptor, is anchored to secretory granules via a C-terminal transmembrane insertion. *Biochemistry* 2002;**41**:52–60.

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5. Fricker LD. Activation and membrane binding of carboxypeptidase E. *J Cell Biochem* 1988;**38**:279–89.
6. Fricker LD, Berman YL, Leiter EH, Devi LA. Carboxypeptidase E activity is deficient in mice with the fat mutation: effect on peptide processing. *J Biol Chem* 1996;**271**:30619–24.
7. Fricker LD, Das B, Angeletti RH. Identification of the pH-dependent membrane anchor of carboxypeptidase E (EC 3.4.17.10). *J Biol Chem* 1990;**265**:2476–82.
8. Fricker LD, Evans CJ, Esch FS, Herbert E. Cloning and sequence analysis of cDNA for bovine carboxypeptidase E. *Nature (Lond.)* 1986;**323**:461–4.
9. Fricker LD, Leiter EH. Peptides, enzymes, and obesity: new insights from a "dead" enzyme. *Trends Biochem Sci* 1999;**24**:390–3.
10. Fricker LD, Snyder SH. Enkephalin convertase: purification and characterization of a specific enkephalin-synthesizing carboxypeptidase localized to adrenal chromaffin granules. *Proc Natl Acad Sci U S A* 1982;**79**:3886–90.
11. Gomis-Ruth FX, Companys V, Qian Y, Fricker LD, Vendrell J, Aviles FX, et al. Crystal structure of avian carboxypeptidase D domain II: a prototype for the regulatory metallocarboxypeptidase subfamily. *EMBO J* 1999;**18**:5817–26.
12. Hadkar V, Skidgel RA. Carboxypeptidase D is up-regulated in raw 264.7 macrophages and stimulates nitric oxide synthesis by cells in arginine-free medium. *Mol Pharmacol* 2001;**59**:1324–32.
13. Kuroki K, Eng F, Ishikawa T, Turck C, Harada F, Ganem D. gp180, a host cell glycoprotein that binds duck hepatitis B virus particles, is encoded by a member of the carboxypeptidase gene family. *J Biol Chem* 1995;**270**:15022–8.
14. Naggert JK, Fricker LD, Varlamov O, Nishina PM, Rouille Y, Steiner DF, et al. Hyperproinsulinemia in obese *fat/fat* mice associated with a point mutation in the carboxypeptidase E gene and reduced carboxypeptidase E activity in the pancreatic islets. *Nat Genet* 1995;**10**:135–42.
15. Novikova EG, Eng FJ, Yan L, Qian Y, Fricker LD. Characterization of the enzymatic properties of the first and second domains of metallocarboxypeptidase D. *J Biol Chem* 1999;**274**:28887–92.
16. Parkinson D. Two soluble forms of bovine carboxypeptidase H have different NH_2-terminal sequences. *J Biol Chem* 1990;**265**:17101–5.
17. Rindler MJ, Xu CF, Gumper I, Smith NN, Neubert TA. Proteomic analysis of pancreatic zymogen granules: identification of new granule proteins. *J Proteome Res* 2007;**6**:2978–92.
18. Rovere C, Viale A, Nahon J, Kitabgi P. Impaired processing of brain proneurotensin and promelanin-concentrating hormone in obese *fat/fat* mice. *Endocrinology* 1996;**137**:2954–8.
19. Settle SHJ, Green MM, Burtis KC. The silver gene of *Drosophila* melanogaster encodes multiple carboxypeptidases similar to mammalian prohormone-processing enzymes. *Proc Natl Acad Sci U S A* 1995;**92**:9470–4.
20. Sidyelyeva G, Baker NE, Fricker LD. Characterization of the molecular basis of the *Drosophila* mutations in carboxypeptidase D. Effect on enzyme activity and expression. *J Biol Chem* 2006;**281**:13844–52.
21. Sidyelyeva G, Wegener C, Schoenfeld BP, Bell AJ, Baker NE, McBride SM, et al. Individual carboxypeptidase D domains have both redundant and unique functions in *Drosophila* development and behavior. *Cell Mol Life Sci* 2010;**67**:2991–3004.
22. Smyth DG, Maruthainar K, Darby NJ, Fricker LD. C-terminal processing of neuropeptides: involvement of carboxypeptidase H. *J Neurochem* 1989;**53**:489–93.
23. Song L, Fricker LD. Purification and characterization of carboxypeptidase D, a novel carboxypeptidase E-like enzyme, from bovine pituitary. *J Biol Chem* 1995;**270**:25007–13.
24. Song L, Fricker LD. Tissue distribution and characterization of soluble and membrane-bound forms of metallocarboxypeptidase D. *J Biol Chem* 1996;**271**:28884–9.
25. Srinivasan S, Bunch DO, Feng Y, Rodriguiz RM, Li M, Ravenell RL, et al. Deficits in reproduction and pro-gonadotropin-releasing hormone processing in male Cpefat mice. *Endocrinology* 2004;**145**:2023–34.
26. Tan F, Rehli M, Krause SW, Skidgel RA. Sequence of human carboxypeptidase D reveals it to be a member of the regulatory carboxypeptidase family with three tandem active site domains. *Biochem J* 1997;**327**:81–7.
27. Tanco S, Arolas JL, Guevara T, Lorenzo J, Aviles FX, Gomis-Ruth FX. Structure-function analysis of the short splicing variant carboxypeptidase encoded by *Drosophila melanogaster* silver. *J Mol Biol* 2010;**401**:465–77.
28. Utsunomiya N, Ohagi S, Sanke T, Tatsuta H, Hanabusa T, Nanjo K. Organization of the human carboxypeptidase E gene and molecular scanning for mutations in Japanese subjects with NIDDM or obesity. *Diabetologia* 1998;**41**:701–5.
29. Varlamov O, Eng FJ, Novikova EG, Fricker LD. Localization of metallocarboxypeptidase D in AtT-20 cells: potential role in prohormone processing. *J Biol Chem* 1999;**274**:14759–67.
30. Varlamov O, Fricker LD. The C-terminal region of carboxypeptidase E involved in membrane binding is distinct from the region involved with intracellular routing. *J Biol Chem* 1996;**271**:6077–83.
31. Varlamov O, Fricker LD. Intracellular trafficking of metallocarboxypeptidase D in AtT-20 cells: localization to the trans-Golgi network and recycling from the cell surface. *J Cell Sci* 1998;**111**:877–85.
32. Varlamov O, Leiter EH, Fricker LD. Induced and spontaneous mutations at Ser202 of carboxypeptidase E: effect on enzyme expression, activity, and intracellular routing. *J Biol Chem* 1996;**271**:13981–6.
33. Varlamov O, Wu F, Shields D, Fricker LD. Biosynthesis and packaging of carboxypeptidase D into nascent secretory vesicles in pituitary cell lines. *J Biol Chem* 1999;**274**:14040–5.
34. Xin X, Varlamov O, Day R, Dong W, Bridgett MM, Leiter EH, et al. Cloning and sequence analysis of cDNA encoding rat carboxypeptidase D. *DNA Cell Biol* 1997;**16**:897–909.
35. Zhang X, Che FY, Berezniuk I, Sonmez K, Toll L, Fricker LD. Peptidomics of Cpe(fat/fat) mouse brain regions: implications for neuropeptide processing. *J Neurochem* 2008;**107**:1596–613.

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Chapter 236

Disulfide Bond Formation

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ABSTRACT

'Spontaneous' disulfide bond formation is critically dependent on the local environment, and multiple host-cell gene products provide the environmental and compartment-specific control in which these reactions take place. In the secretory pathway of cells, rather than being spontaneous, disulfide bond formation in biologically active peptides is primarily catalyzed, by protein disulfide isomerase (PDI)-like oxidoreductases. *In vitro* studies provide a solid understanding of the spectrum of enzymatic capabilities of PDI and related family members. However, these *in vitro* studies cannot replicate the oxidoreductase working environment within the secretory pathway. In the yeast endoplasmic reticulum (ER) – which may be a more oxidizing environment than in the ER of mammalian cells – there is strong evidence that PDI facilitates disulfide bond formation in exportable protein substrates, with some evidence supporting a role for PDI in disulfide reduction or isomerization. In mammalian cells, the literature has tended to group PDI-like family members as if they were a single protein, but more recent evidence points to the idea that the distinct PDI-like family members are subspecialized for somewhat different (albeit overlapping) *in vivo* functions. This probably reflects differences in their relative redox setpoints, alterations/modifications of their catalytic CXXC sequences and neighboring residues, differences in their hydrophobic pockets for substrate binding and chaperone function, and differences in their ER resident partners that provide substrate specificity and function. A case in point is for proinsulin folding in pancreatic beta cells: *in vitro* results support a role for PDI in proinsulin disulfide bond formation while thus far limited results in beta cells provide no direct support for this view. As PDI-like family members may play roles in the pathogenesis or amelioration of disease, further work is needed to elucidate their respective roles in the spectrum of oxidoreductase activities in the context of their native working environment.

INTRODUCTION

Disulfide bonds are made in nearly one-third (7000) of the proteins in the eukaryotic proteome,[11] many of which are destined for contact with the relatively nonreducing extracellular environment as secretory or cell surface proteins. Disulfide bond formation involves a reaction between the sulfhydryl (SH) side chains of two cysteine residues: an S^- anion from one sulfhydryl group acts as a nucleophile, attacking the side chain of a second cysteine to create a disulfide bond, and in the process releases electrons (reducing equivalents) for transfer. Proper disulfide bonds provide stability to a protein, decreasing further entropic choices that facilitate folding progression toward the native state by limiting unfolded or improperly folded conformations. An increase in stability of the native structure resulting from the formation of a particular disulfide bond has been said to be directly proportional to the number of residues between the linked cysteines, i.e. the larger the number of residues in the 'disulfide loop,' the greater the stability provided to the native structure.[22]

The chemistry of protein disulfide bond formation is directly influenced three key factors: 1) the spatial accessibility/physical proximity of the partner cysteine residues forming the disulfide bond; 2) the difference between the pK_a of the involved thiol groups and the pH of the local environment (with lower pH limiting reactivity and higher pH favoring increased reactivity); and 3) the redox environment (with lesser reactivity under more reducing conditions and greater reactivity under more oxidizing conditions).[22] As the latter two factors are environmentally controlled, specific cellular compartments have evolved within cells to facilitate disulfide bond formation. In bacteria, disulfide bonds in bioactive peptides and polypeptides of the secretory pathway are formed in the periplasm; in eukaryotes, such (poly)peptides tend to acquire their disulfide bonds in the endoplasmic reticulum (ER) although disulfide bond formation can occasionally occur at other sites (including extracellularly). The ER is a vastly more common site than the cytosol (which is very rarely the site of disulfide bond formation) because the ER intralumenal environment is more oxidizing than that of the cytosol.[1] The redox environment is at least partly reflected by the ratio of oxidized glutathione (GSSG) to reduced glutathione (GSH), which in the case of the ER (and periplasm), is shifted in favor of GSSG.[1]

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00236-0
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THE CONCEPT OF SECRETORY PATHWAY CATALYSIS OF DISULFIDE BOND FORMATION

Although disulfide bonds can form spontaneously (albeit very slowly) via molecular oxygen or directly via GSSG as an electron acceptor, the main mechanism of disulfide bond formation in the secretory pathway seems to involve enzymatic catalysis, via oxidoreductase proteins bearing a catalytically active thioredoxin domain motif: CXXC (where 'X' residues tend to be hydrophobic amino acids) that is responsible for disulfide bond transfer from oxidoreductase to substrate.[11] (A thioredoxin domain, stemming from thioredoxin proteins, a class of redox proteins found in all organisms, consists of a βαβαβαββα fold motif.) In secretory proteins that contain more than one disulfide bond in their final structure, the formation of these bonds is typically not a linear process, insofar as their order of formation may vary *en route* to the native state. In general, newly synthesized secretory proteins have preferred folding intermediates (such as has been become well known from *in vitro* folding studies of ribonuclease and bovine pancreatic trypsin inhibitor[22]). Oxidoreductases may help to promote a particular folding pathway both by selective disulfide catalysis and by acting as molecular chaperones that limit off-pathway folding choices. Once a transient intramolecular disulfide bond has formed within the CXXC motif of the oxidoreductase, the catalytic event proceeds via a transient mixed-disulfide bond between the amino-terminal cysteine of the CXXC and the substrate protein, which is then resolved by completion of an intramolecular disulfide bond formed within the substrate protein (Fig. 1) in a process facilitated by both the carboxyl-terminal cysteine of the CXXC motif and neighboring residues that influence the CXXC redox potential. Catalyzed disulfide bond formation occurs very rapidly, with dynamic sampling and release of substrate proteins by oxidoreductase enzymes.[2,10] The reaction catalyzing disulfide transfer to the substrate protein does not result in a net loss or gain of disulfide bonds, as a reduced CXXC motif is regenerated in the disulfide transfer process, i.e. the transfer reaction involves oxidation of the substrate protein concomitant with reduction of the thioredoxin motif in the oxidoreductase. Thus, within the ER, there is a net flow of electrons from substrate proteins to oxidoreductases that catalyze disulfide bond formation. In the ER of eukaroyotes, protein disulfide isomerase (PDI) is the most prominent and most evolutionarily conserved of the oxidoreductases – but there are at least 21 PDI-like family members[11] – several of which shall be touched upon later (Table 1).

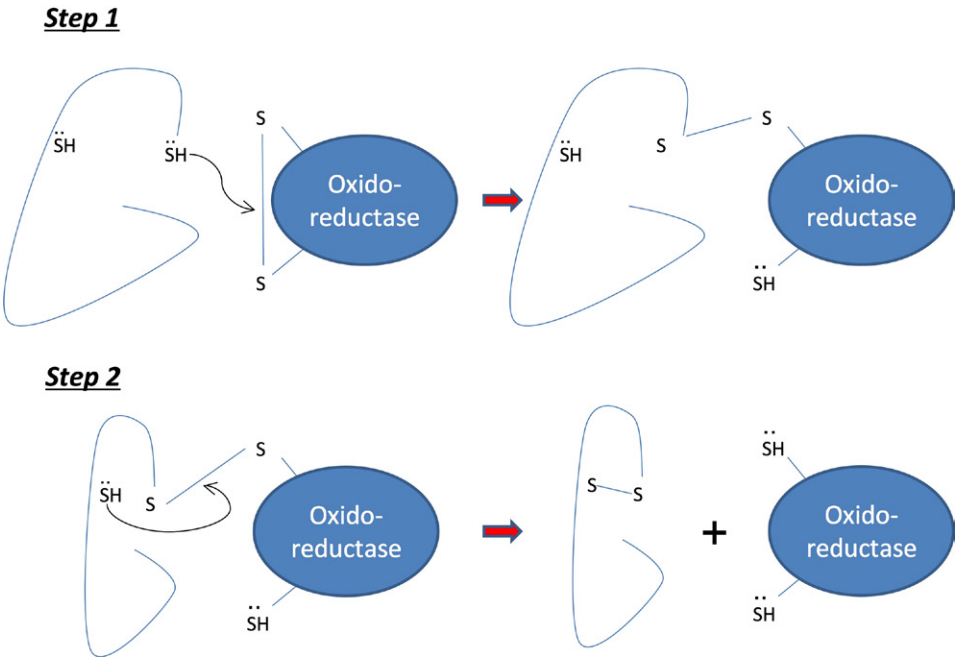

FIGURE 1 Oxidoreductase-catalyzed disulfide bond formation. In this diagram, the ER oxidoreductase (oval) oxidizes a secretory protein substrate (shown schematically, as a line, at *left*). In the first step, a sulfhydryl group in the substrate attacks a disulfide bond in the thioredoxin motif (CXXC) within the oxidoreductase, creating a transient mixed-disulfide bond between the two proteins. In the second step, folding changes in the substrate bring another cysteine sulfhydryl moiety into proximity with the mixed disulfide. The nucleophilic attack results in the formation of an intramolecular disulfide bond, creating a disulfide loop structure within the substrate, and leaving the CXXC motif of the oxidoreductase as reduced thiols (electrons represented by dots). This will require reoxidation in order that another round of catalyzed disulfide bond formation may proceed.

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The secretory pathway operates as a protein manufacturing and export company. Once properly folded, the most recent cohort of oxidized secretory proteins is exported, only to be immediately followed by a new cohort of incoming (newly synthesized) secretory proteins – and these new polypeptides begin their 'life' with a full complement of reduced cysteine thiols. In order to bring about another round of substrate protein oxidation, the catalytic oxidoreductases must again form a transient intramolecular disulfide bond within their CXXC motif(s); thus, they must become reoxidized. In bacteria, secretory protein oxidation by the disulfide bond formation family ("Dsb") of proteins begins with DsbA catalyzing initial disulfide bond formation in substrates. DsbA is reoxidized by electron transfer to the periplasmic membrane-bound DsbB (from which electrons are subsequently 'unloaded' to ubiquinone that is finally reoxidized by the respiratory chain involving molecular oxygen as the terminal electron acceptor[22]). In the secretory pathway of eukaryotes, the various ER oxidoreductases also must become reoxidized, and this is an extremely active area of ongoing investigation. What is clear is that PDI (encoded by the P4HB gene) can be specifically reoxidized by Endoplasmic Reticulum Oxidoreductin-1 (ERO1), an ER membrane–associated protein that serves as the functional analog of bacterial DsbB. In yeast, ERO1 was first identified because its genetic deletion resulted in a phenotype in which exportable proteins lacked disulfide bonds.[7] Indeed, ERO1 was found to form a transient mixed disulfide with PDI and yet ERO1 seemed unable to interact directly

TABLE 1 Mammalian PDI-like Family Members

	Gene name	Protein	Thioredoxin domains	Catalytic motif sequence
1	**P4HB**	**PDI**	**4 (2)**	**CGHC, CGHC**
2	**PDIA3**	**ERp57**	**4 (2)**	**CGHC, CGHC**
3	**PDIA4**	**ERp72**	**5 (3)**	**CGHC, CGHC, CGHC**
4	**PDIA6**	**P5**	**3 (2)**	**CGHC, CGHC**
5	**DNAJC10**	**ERdj5**	**6 (4)**	**CSHC, CPPC, CHPC, CGPC**
6	PDIA2	PDIp	4 (2)	CGHC, CTGC
7	PDIA5	PDIr	4 (3)	CSMC, CGHC, CPHC
8	TXNDC12	ERp18	1 (1)	CGAC
9	ERp27	ERp27	2 (0)	None
10	ERp29	ERp29	2 (0)	None
11	ERp44	ERp44	3 (1)	CRFS
12	ERp90	ERp90	5 (0)	None
13	TXNDC5	ERp46	3 (3)	CGHC, CGHC, CGHC
14	PDILT	PDILT	4 (2)	SKQS*, SKKC
15	Agr-2	Agr-2	1 (1)	CPHS
16	Agr-3	Agr-3	1 (1)	CQYS
17	TMX1	TMX1	1 (1)	CPAC
18	TMX2	TMX2	1 (1)	SDNC
19	TMX3	TMX3	3 (1)	CGHC
20	TMX4	TMX4	1 (1)	CPSC
21	TMX5	TMX5	1 (1)	CRFS

Gene names (first column) and protein alias (second column) are shown. The total number of thioredoxin domains in each gene product is indicated, with the number of catalytically active domains listed in parentheses. The specific CXXC sequences are listed in the last column; note that for PDILT (line 14) the SKQS motif bears strong resemblance to the A domain of PDI and is listed under the catalytic motif sequence, although it does exhibit catalytic activity. Family members highlighted with gray background signify transmembrane proteins and the first five members in large font denote those whose functions are specifically mentioned within the text. The bolded oxidoreductases are mentioned specifically in the text and the asterisk denotes a motif that cannot form a disulfide bond.

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with exportable protein substrates.[8] Moreover, overexpression of ERO1 itself cannot rescue perturbation of disulfide bond formation resulting from PDI knockout, strongly suggesting that, at least in *Saccharomyces cerevisiae*, oxidized PDI is required for ongoing disulfide bond formation in secretory proteins and ERO1 is required for the catalytic reoxidation of PDI.[8] From these and additional studies performed in higher eukaryotic cells and animals,[5] ERO1 proteins are now considered to be one of the major catalysts of disulfide bond formation in the ER lumen[27] – although still under active investigation are possible important roles for other oxidizers of lumenal ER oxidoreductases and substrate proteins – including oxidized glutathione (discussed above) plus additional enzymes (discussed below).[1]

TRANSFER OF REDUCING EQUIVALENTS FROM THE ER LUMEN TO THE CYTOSOL

ERO1, containing a variant of the thioredoxin motif known as a cysteine triad, provides a mechanism for delivery of reducing equivalents back to the cytosol, leading ultimately to molecular O_2.[27] In the case of the mammalian gene product, whereas the last two cysteines of the amino-terminal cysteine triad (residues C94 and C99) of ERO1 catalytically transfer a disulfide bond to the active site of PDI, the last two cysteines of the carboxyl-terminal triad (residues C394 and C397) of ERO1 are used to transfer reducing equivalents to FAD^+.[3] Reducing equivalents are internally shuttled within ERO1 from C94/C99 to C394/397, to make ready C94/C99 for another round of disulfide bond formation. The reduction of FAD^+ to $FADH_2$ (which, in the cytosol, can transfer these reducing equivalents to molecular oxygen as the terminal electron acceptor) is accompanied by formation of hydrogen peroxide (H_2O_2) in the ER, as a byproduct.[27] The stoichiometry is such that for every ERO1-catalyzed disulfide bond, one molecule of H_2O_2 is generated.

As H_2O_2 is one form of reactive oxygen species (ROS), the catalytic activity of ERO1 has been considered a contributor to cellular oxidative stress that is seen as an undesirable outcome,[27] although this has been debated.[1] New studies indicate that Peroxiredoxin 4 (PRDX4) links the reduction of cytoplasmic hydrogen peroxide to additional disulfide bond formation in PDI-like oxidoreductases of the ER lumen.[4,35] In the process of reducing H_2O_2, PRDX4 becomes chemically oxidized at the carboxyl-terminal cysteine of its CXXC motif, followed by formation of a mixed-disulfide bond that is transferred to PDI or other PDI-like oxidoreductase. Notably, mice bearing a double knockout of ERO1α and ERO1β (the two mammalian ERO1 genes) are still able to generate disulfide bonds in secretory proteins with only a minor delay, but a triple knockout of ERO1α, ERO1β, and PRDX4 displays a more severe oxidative folding defect.[35] In addition to PRDX4, PDI peroxidases GPX7 and GPX8 also reduce cytoplasmic H_2O_2 and may promote disulfide bond formation in the ER lumen, although these molecules are relatively understudied.[4]

Vitamin K–dependent OxidoReductase (VKOR) is another protein that can receive reducing equivalents from PDI-like family members. VKOR is an ER transmembrane protein that catalyzes reduction of Vitamin K epoxide to hydroquinone (a cytoplasmic co-factor needed for gamma-glutamyl carboxylation of newly synthesized blood clotting factors in the ER [of hepatocytes][30]), allowing the CXXC motif in VKOR itself to become oxidized. VKOR can then in turn receive reducing equivalents from PDI-like oxidoreductases, as shown by 'disulfide trap mutants' of oxidoreductases bearing the active site cysteine mutation CXXA that allows for creation/capturing of a stable mixed-disulfide complex with VKOR.[26]

Quiescin–Sulfhydryl Oxidase (QSOX), another potent source of direct disulfide bond oxidation in substrate proteins (in a manner entirely independent of PDI-like oxidoreductases), uses molecular oxygen as its terminal electron acceptor to very potently generate disulfide bonds directly in substrate proteins (preferentially over those in PDI-like family members), and produces H_2O_2 as a byproduct.[12] In yeast, the overexpression of QSOX, a monomeric flavoprotein, can rescue mutant phenotypes of *ero1* null mutants. Yet the physiological role of QSOX *in vivo* remains unknown because of its low expression level and its apparent localization in the Golgi complex rather than the ER where most secretory protein disulfide bonds are believed to form.

PROPER DISULFIDE BOND FORMATION IN THE SECRETORY PATHWAY MAY ALSO INCLUDE DISULFIDE REDUCTION AND ISOMERIZATION

Even after disulfide bond formation in the secretory pathway, there are times when existing disulfide bonds must be broken within the ER (or periplasmic space of bacteria). For example, initial disulfide bond formation may involve cysteine mispairing, leading an exportable protein into a nonnative 'off-pathway' conformation. Such mispaired disulfide bonds need either to be broken (reduced) or isomerized to native pairings. In higher eukaryotes, the large number of distinct PDI-like oxidoreductases makes it all the more likely that subspecialization exists among family members to function more as oxidases or more as reductases for specific substrates – even as all members must cycle their catalytic CXXC motifs between oxidized and reduced states. Although this is largely unproven in higher eukaryotes, it is plausible that evolution may have divided catalysts of disulfide bond formation versus disulfide reduction or isomerization into distinct gene products, because such specialization might allow for greater functionality. Even in bacteria, isomerization activity is carried out by

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DsbC and DsbD, which tend not to assist in initial disulfide bond formation within substrates.[23]

Nevertheless, PDI – which is certainly associated with disulfide bond formation in yeast and ER reoxidation after reductive stress in higher eukaryotic cells[1] – can also be clearly shown to exhibit disulfide isomerase activity. Indeed, disulfide isomerization catalyzed by PDI was one of the observed activities reported by Anfinsen in his Nobel prize-winning study of the *in vitro* refolding of denatured ribonuclease. By contrast, there are other agents, such as QSOX (and others, described below), that do not seem to be capable of reduction or isomerization of disulfide bonds in secretory proteins – providing support for the notion of specialization of enzymes in the secretory pathway primarily dedicated to substrate oxidation rather than to isomerization or reduction.

THE FAMILY OF PDI-LIKE ER OXIDOREDUCTASES

As PDI is the prototypic ER oxidoreductase, it is worth briefly reviewing its structure–function relationships. A 55-kDa protein containing two catalytic thioredoxin (a and a') domains, PDI also contains two noncatalytic (b and b') domains arranged as a–b–b'–a' (Fig. 2). The noncatalytic b and b' domain (and its following linker region) tend to contain hydrophobic pockets that contribute importantly to low-affinity binding of a wide variety of substrates.[10] The low affinity allows PDI to "sample" many potential substrates and thereby to potentially direct rapid catalysis – within the ER, the apparent K_m of the enzyme for a given substrate is at least as much about protein sampling as it is about catalytic activity. The a and a' domains may also contribute to substrate recognition.[10] PDI and many other members of the PDI-like family of ER oxidoreductases also contain the 4 amino acid carboxyl-terminal KDEL-like sequence that functions as a Golgi retrieval signal to enrich these proteins within the ER. With the combination of its four domains, PDI can also function as a molecular chaperone – preventing aggregation of proteins – in addition to its function as an enzyme. In yeast, catalytically-dead PDI rescues the lethal PDI null phenotype, suggesting that PDI's essential role in the ER goes beyond its role in disulfide bond formation.[18] Thus, it is thought that through both its catalytic and noncatalytic activities, PDI can minimize kinetic traps – helping to eliminate 'dead-end' folding intermediates.

Other PDI-like family members differ in length and activity, but share the common thioredoxin-like-fold,[1] being grouped on the basis of their content of a, a', b, or b' homology domains. The noncatalytic b and b' domains are often not well defined; moreover, some PDI-like family members are entirely noncatalytic as they contain no "a-domain" bearing a CXXC motif (a Ser residue is often substituted in place of Cys) – nevertheless, these family members assist in protein folding of substrates.[25] For a list of PDI-like ER oxidoreductases, please see Table 1. This broad family of PDI-like ER oxidoreductases displays a diversity of functions that continues to be discovered. As a complete review of all family members and their putative functions is beyond the scope of this Handbook, we will confine ourselves to a few limited examples. ERp57, like PDI, has structural units that include two redox-active a and a' domains.[11] However, for ERp57, the noncatalytic b domain contains no substrate-binding hydrophobic pocket. Instead, the positively charged noncatalytic domain of ERp57 seems to be specialized for interaction with the negatively charged tip of the P-domain of lectin-like chaperones calreticulin and calnexin – it is for this reason that ERp57 substrates almost exclusively comprise glycoproteins.[16] ERdj5 has been shown to function mainly as a reductase, breaking disulfide bonds in misfolded proteins.[29] ERp72 can act on the same protein substrate as PDI, yet ERp72 does not have an identified hydrophobic domain, and its phenotypic effects tend to be opposite to those of PDI. In one such example, PDI has been found to act as an unfoldase that enhances the retrotranslocation of cholera toxin from the ER lumen to the cytosol, whereas

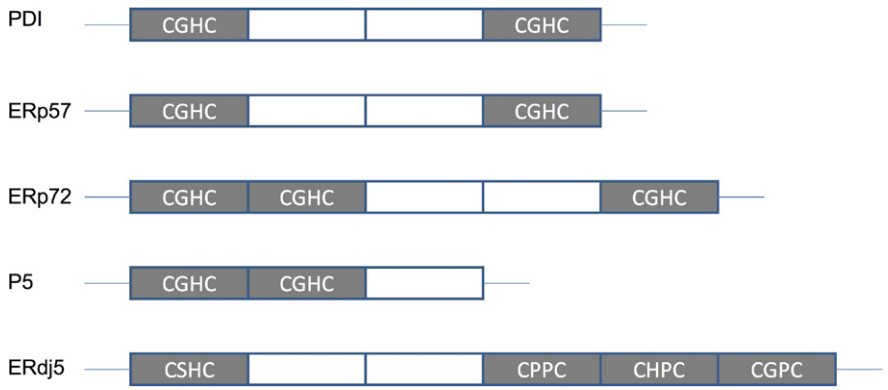

FIGURE 2 Domain structure of selected ER oxidoreductases. Schematic of PDI-like family members, comprising "A"-type thioredoxin domains that are catalytically active (shown in gray), and "B"-type noncatalytic thioredoxin-like domains.

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ERp72 limits cholera toxin retrotranslocation.[6] ERp72 and another PDI-like oxidoreductase, P5, have been found to complex with the molecular chaperone BiP (GRP78) and may also be involved in recognition of misfolded protein cargo. PDILT (restricted to testis) and PDIp (restricted to pancreas) interact with endogenous substrates specific to those tissues.[11] Overall, existing results tend to support the view that different ER oxidoreductases are selective about their choice of substrates – the central issues (besides cell type–specific gene expression) include their relative abundance in the ER, the molecular organization, and structure of their catalytic and noncatalytic domains, their redox potential (relative ease of reduction vs. oxidation of the catalytic CXXC), and the availability of ER resident partner proteins for the oxidoreductase that may bring it into close proximity with selective substrate proteins.

SELECTIVE ACTIVITIES OF OXIDOREDUCTASES WITHIN THE ER ENVIRONMENT

Although PDI-catalyzed substrate (re)folding has been extensively characterized *in vitro*, there remains little data on the role(s) of PDI-like oxidoreductases *in vivo*. It has been reported that siRNA-mediated knockdown of PDI in the HepG2 hepatocyte cell line results in a modest retardation in oxidative folding of various substrates. The effect is enhanced when combined with simultaneous knockdown of other oxidoreductases such as ERp57, resulting in delayed substrate exit from the ER – with the authors suggesting functional redundancy among members of the PDI-like family of ER oxidoreductases.[24] Conversely, it has previously been shown that siRNA-mediated depletion of ERp57 in mouse L cells causes a delay in the oxidative folding of MHC class I heavy chain and a delay in the export of class I molecules from the ER[33] – and ERp57 knockout mice die in utero, believed primarily to represent a B cell defect in the formation of MHC class I peptide-loading complexes.[9] Thus, it would seem that other ER oxidoreductases cannot adequately replace this essential physiologic function of ERp57. Likewise, neither ERp72 nor ERp57 can support normal retrotranslocation of cholera toxin A1 polypeptide from the ER to the cytosol – a function that seems to be facilitated selectively by PDI.[6] Indeed, PDI overexpression stimulates ER-Associated Degradation (ERAD, a process involving retrotranslocation for proteolysis via the ubiquitin–proteasome pathway) of mutant thyroglobulin, while ERp72 overexpression decreases ERAD rate for the same mutant.[6] Taken together, these examples suggest that ER oxidoreductases exhibit distinct behaviors within the ER. Nevertheless, multiple ER oxidoreductases are induced by ER stress (also known as the Unfolded Protein Response, or UPR), and deficiency of a variety of different ER oxidoreductases (including ERdj5) can sensitize cells to ER stress-mediated apoptosis[28] – at least in part resulting from deficiency of disulfide reductase activity that is needed for the ERAD process.[5,29]

OXIDATION OF SPECIFIC BIOACTIVE PEPTIDE SUBSTRATES WITHIN THE ER: THE CASE OF PROINSULIN

PDI has been studied with regard to disulfide bond formation within proinsulin/insulin; indeed, this is perhaps the best-studied example of a protein substrate for which PDI interaction has been analyzed both *in vivo* and *in vitro*. Proinsulin is the secretory polypeptide precursor to biologically active insulin. Insulin comprises two peptide chains (B and A), linked by two disulfide bonds [C(B7)–C(A7), and C(B19)–C(A20)]. Proinsulin is a single-chain molecule in which the two insulin chains are contiguous because of the presence of a 35-amino acid connecting peptide (C-peptide) plus flanking cleavage sites that are endoproteolytically excised in the final processing events leading to the synthesis of active insulin. Thus, after cleavage of the signal peptide, the proinsulin primary sequence, beginning from the amino terminus of the molecule, sequentially encodes B-chain, C-peptide, and A-chain.

Human proinsulin, produced as an 86-residue peptide (along with a 24-residue signal peptide that is cleaved), contains three intramolecular disulfide bonds: the two noted above interlink the B- and A-chains while a third is contained entirely within the A-chain [C(A6)–C(A11)]. Each of the three bonds is ultimately essential to the biological activity of insulin. In pancreatic beta cells, on folding to the native state (that includes of course the proper disulfide pairings), proinsulin exits the ER and is delivered to the Golgi complex from which newly made secretory granules are first formed – the compartment in which processing from proinsulin to insulin takes place. Ultimately, the insulin granules migrate to the periphery of beta cells to ready themselves for exocytosis in response to secretory stimuli such as elevated blood glucose.

The proinsulin gene product is just one member of the large insulin/IGF superfamily, each sharing the 3-disulfide structure. Multiple members of this family, including both IGF-I and IGF-II, have a branched protein folding pathway that can lead to distinct disulfide-linked final structures – including structures with fairly similar thermodynamic stability – although only one of these disulfide isomers (the native form) has optimal bioactivity.[13]

Quality control in the ER, mediated via recognition of nonnative structures by ER chaperones (and oxidoreductases), is designed to limit ER export of secretory protein substrates that are biologically inactive due to conformational immaturity (i.e.

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"unfolded") or from having formed inappropriate 'dead-end' folding products (i.e. "misfolded"). In this regard, the three disulfide bonds of proinsulin are not equal in importance. Specifically, without the C(B19)–C(A20) interchain disulfide bond, proinsulin is incompetent for export from the ER, indicating recognition and retention of the "open structure" by ER quality control mechanisms. Likewise, absence of the C(B7)–C(A7) interchain disulfide bond both impairs thermodynamic stability and dramatically decreases secretability. However, selective absence of the intrachain C(A6)–C(A11) disulfide bond creates only a modest structural perturbation of proinsulin (or insulin) and does not impair secretion.[21]

B/A-chain alignment is critical to achieving the C(B19)–C(A20) and C(B7)–C(A7) pairings. Early work suggested that biosynthesis of a single-chain insulin with direct contiguity of B- and A-chains (i.e. lacking C-peptide) results in efficient secretion of a structure isomorphous with native 2-chain insulin; however, it had not been appreciated that certain nonnative disulfide isomers (identifiable by abnormal mobility on nonreducing Tris–tricine–urea–SDS-PAGE) could also be secreted – as is known for other members of the IGF superfamily. In reality, such a protein is quantitatively misfolded, proving that, in addition to the primary sequence of B- and A-chains, a linking peptide between these chains is actually required for proper insulin disulfide bond formation in the ER,[21] which may represent an evolutionary pressure to retain the C-peptide that is otherwise of limited biological function.

Not only does improper disulfide bond formation critically perturb insulin function, it also seems to be linked to a chain of events that leads to full-blown diabetes with loss of pancreatic beta cell survival. This is quite a curious situation, as complete deletion of up to three of the four wild-type proinsulin-encoding alleles from the mouse genome (or loss of expression of one of the two wild-type alleles from the human genome) does not cause diabetes or beta cell death.[20] However, the presence of mutant proinsulins bearing cysteine substitutions inexorably leads to this autosomal-dominant phenotype. An interesting structural feature of wild-type proinsulin is that the C(B7)–C(A7) disulfide bond is nearly exposed at the surface of the molecule[14] potentially rendering it susceptible to persistent thiol attack. In fact, recent evidence suggests that the presence of "*Akita* proinsulin," a mouse mutant bearing the C(A7)Y substitution, blocks the secretion of coexpressed wild-type proinsulin and results in the production of aberrant disulfide-linked protein complexes in the ER that contain both wild-type and mutant gene products.[20] Moreover, studies of certain additional proinsulin coding sequence mutations (including those altering residues other than the cysteines themselves), leading to the syndrome of "Mutant INS-gene–induced Diabetes of Youth" (MIDY), also result in improper disulfide bond formation that triggers the same diabetes phenotype as that seen in the *Akita* mouse.[19] These findings validate a sensitive role of proper disulfide bond formation not only for the substrate itself, but also for innocent bystander proteins that may come under attack by coexpressed proteins that bear exposed, reactive cysteine thiols.[20] It is believed that proinsulin dimerizes in the ER[15]; what remains to be established is whether misfolded proinsulin triggering dominant-negative blockade of innocent bystander substrates is restricted exclusively to bystander proinsulin or may also have an impact – either through inappropriate mixed-disulfide bonds, or via a generalized ER stress response – on any other cysteine-bearing secretory proteins that happen to be coexpressed in the same ER.

PDI can catalyze the formation of proinsulin disulfide bonds *in vitro*,[31] yet the physiological role of PDI in the ER of pancreatic beta cells remains unknown. There are data that do not support the notion of PDI as a facilitator of the oxidative maturation of proinsulin in pancreatic beta cells. Specifically, overexpression of PDI in beta cell lines results in a decrease in insulin content and secretion, while proinsulin accumulates intracellularly (presumably in the ER).[32] Although it is possible that PDI overexpression can result in nonspecific effects, the results imply an apparent PDI-mediated inhibition of proinsulin export from the ER, which in turn suggests a probable defect in the proinsulin folding process. ERO1β has been reported to function as the primary oxidoreductin in pancreatic beta cells,[34] and, as described in detail above, ERO1 accepts reducing equivalents from PDI (and some other PDI-like family members[1]) to allow the ER oxidoreductases to form a catalytic disulfide bond that can be transferred to exportable protein substrates. Impaired expression of the ERO1β gene produces a phenotype – both in mouse islets and in cultured pancreatic beta cells –similar to that seen upon PDI *overexpression*, i.e. there is a delay in the ER exit of proinsulin with lower beta cell insulin content and lower insulin secretion.[17,34] Unlike in yeast, knockdown or deletion of PDI gene expression is not lethal to higher eukaryotic cells – undoubtedly because of the presence of many other PDI-like family members that can partially or fully replace its function.[24] Thus, based on the foregoing discussion, it is now essential that PDI knockdown or gene deletion in pancreatic beta cells be directly examined for its effects on proinsulin disulfide bond formation. In this way, we should finally be able to decide whether, in a cellular biological/physiological context, PDI is directly responsible for receiving reducing equivalents from proinsulin.

CONCLUSIONS

Proper disulfide bond formation is essential for the folding stability and export of numerous secretory proteins, including bioactive peptides. Several related genes have evolved to assist in the process of disulfide bond formation. In the

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yeast *S. cerevisiae*, five PDI-like family members have been identified whereas in mammalian systems at least 21 different PDI-like family members have been discovered. What remains unclear, however, is the spectrum of secretory protein substrates that each PDI-like protein participates in assisting, and whether, for a given family member, the assistance actually operates primarily in disulfide bond formation or disulfide isomerization or even reduction of its preferred substrate(s). As this field of investigation grows, the possibility emerges that some of the PDI-like ER oxidoreductases may specialize in unfolding protein substrates to assist in ERAD – while other PDI-like family members may operate in an entirely noncatalytic yet important way for secretory protein folding and export. As a case in point, for a protein as well studied as proinsulin/insulin, the oxidoreductase(s) that primarily assist(s) in nascent disulfide bond formation in the ER, has(ve) not yet been identified. In addition to probable substrate preferences of distinct PDI-like family members, a new appreciation is developing for ER redox proteins that contribute primarily to controlling the proper compartmental milieu while other gene products operate directly on the secretory protein substrates. Multiple pathways operate in parallel in the generation of secretory protein disulfide bonds, including proteins that are not classic members of the PDI-like family of oxidoreductases. An understanding of the specific functions of PDI-like family members in the *in vivo* folding of proinsulin and other bioactive secretory peptides is expected to emerge over the coming decade.

REFERENCES

1. Appenzeller-Herzog C. Glutathione- and non-glutathione-based oxidant control in the endoplasmic reticulum. *J Cell Sci* 2011;**124**: 847–55.
2. Appenzeller-Herzog C, Riemer J, Zito E, Chin KT, Ron D, Spiess M, et al. Disulphide production by Ero1alpha-PDI relay is rapid and effectively regulated. *EMBO J* 2010;**29**:3318–29.
3. Bertoli G, Simmen T, Anelli T, Molteni SN, Fesce R, Sitia R. Two conserved cysteine triads in human Ero1alpha cooperate for efficient disulfide bond formation in the endoplasmic reticulum. *J Biol Chem* 2004;**279**:30047–52.
4. Bulleid NJ, Ellgaard L. Multiple ways to make disulfides. *Trends Biochem Sci* 2011.
5. Dong M, Bridges JP, Apsley K, Xu Y, Weaver TE. ERdj4 and ERdj5 are required for endoplasmic reticulum-associated protein degradation of misfolded surfactant protein C. *Mol Biol Cell* 2008;**19**:2620–30.
6. Forster ML, Sivick K, Park YN, Arvan P, Lencer WI, Tsai B. Protein disulfide isomerase-like proteins play opposing roles during retrotranslocation. *J Cell Biol* 2006;**173**:853–9.
7. Frand AR, Kaiser CA. The ERO1 gene of yeast is required for oxidation of protein dithiols in the endoplasmic reticulum. *Mol Cell* 1998;**1**:161–70.
8. Frand AR, Kaiser CA. Ero1p oxidizes protein disulfide isomerase in a pathway for disulfide bond formation in the endoplasmic reticulum. *Mol Cell* 1999;**4**:469–77.
9. Garbi N, Tanaka S, Momburg F, Hammerling GJ. Impaired assembly of the major histocompatibility complex class I peptide-loading complex in mice deficient in the oxidoreductase ERp57. *Nature Immunol* 2006;**7**:93–102.
10. Hatahet F, Ruddock LW. Substrate recognition by the protein disulfide isomerases. *FEBS J* 2007;**274**:5223–34.
11. Hatahet F, Ruddock LW. Protein disulfide isomerase: a critical evaluation of its function in disulfide bond formation. *Antioxid Redox Signaling* 2009;**11**:2807–50.
12. Heckler EJ, Rancy PC, Kodali VK, Thorpe C. Generating disulfides with the Quiescin-sulfhydryl oxidases. *Biochim Biophys Acta* 2008;**1783**:567–77.
13. Hua QX, Gozani SN, Chance RE, Hoffmann JA, Frank BH, Weiss MA. Structure of a protein in a kinetic trap. *Nature Struct Biol* 1995;**2**:129–38.
14. Hua QX, Nakagawa SH, Jia W, Hu SQ, Chu YC, Katsoyannis PG, et al. Hierarchical protein folding: asymmetric unfolding of an insulin analogue lacking the A7–B7 interchain disulfide bridge. *Biochemistry* 2001;**40**:12299–311.
15. Huang XF, Arvan P. Intracellular transport of proinsulin in pancreatic beta-cells. Structural maturation probed by disulfide accessibility. *J Biol Chem* 1995;**270**:20417–23.
16. Jessop CE, Tavender TJ, Watkins RH, Chambers JE, Bulleid NJ. Substrate specificity of the oxidoreductase ERp57 is determined primarily by its interaction with calnexin and calreticulin. *J Biol Chem* 2009;**284**:2194–202.
17. Khoo C, Yang J, Rajpal G, Wang Y, Liu J, Arvan P, et al. Endoplasmic reticulum oxidoreductin-1-like beta (ERO1lbeta) regulates susceptibility to endoplasmic reticulum stress and is induced by insulin flux in beta-cells. *Endocrinology* 2011;**152**:2599–608.
18. LaMantia M, Lennarz WJ. The essential function of yeast protein disulfide isomerase does not reside in its isomerase activity. *Cell* 1993;**74**:899–908.
19. Liu M, Haataja L, Wright J, Wickramasinghe NP, Hua QX, Phillips NF, et al. Mutant INS-gene induced diabetes of youth: proinsulin cysteine residues impose dominant-negative inhibition on wild-type proinsulin transport. *PloS One* 2010;**5**:e13333.
20. Liu M, Hodish I, Haataja L, Lara-Lemus R, Rajpal G, Wright J, et al. Proinsulin misfolding and diabetes: mutant INS gene-induced diabetes of youth. *Trends Endocrinol Metab: TEM* 2010;**21**:652–9.
21. Liu M, Ramos-Castaneda J, Arvan P. Role of the connecting peptide in insulin biosynthesis. *J Biol Chem* 2003;**278**:14798–805.
22. Mamathambika BS, Bardwell JC. Disulfide-linked protein folding pathways. *Annu Rev Cell Develop Biol* 2008;**24**:211–35.
23. Rietsch A, Belin D, Martin N, Beckwith J. An in vivo pathway for disulfide bond isomerization in *Escherichia coli*. *Proc Natl Acad Sci U S A* 1996;**93**:13048–53.
24. Rutkevich LA, Cohen-Doyle MF, Brockmeier U, Williams DB. Functional relationship between protein disulfide isomerase family members during the oxidative folding of human secretory proteins. *Mol Biol Cell* 2010;**21**:3093–105.
25. Sargsyan E, Baryshev M, Szekely L, Sharipo A, Mkrtchian S. Identification of ERp29, an endoplasmic reticulum lumenal protein, as a new member of the thyroglobulin folding complex. *J Biol Chem* 2002;**277**:17009–15.
26. Schulman S, Wang B, Li W, Rapoport TA. Vitamin K epoxide reductase prefers ER membrane-anchored thioredoxin-like redox partners. *Proc Natl Acad Sci U S A* 2010;**107**:15027–32.

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27. Sevier CS, Kaiser CA. Ero1 and redox homeostasis in the endoplasmic reticulum. *Biochim Biophys* 2008;**1783**:549–56.
28. Thomas CG, Spyrou G. ERdj5 sensitizes neuroblastoma cells to endoplasmic reticulum stress-induced apoptosis. *J Biol Chem* 2009;**284**:6282–90.
29. Ushioda R, Hoseki J, Araki K, Jansen G, Thomas DY, Nagata K. ERdj5 is required as a disulfide reductase for degradation of misfolded proteins in the ER. *Science* 2008;**321**:569–72.
30. Wajih N, Hutson SM, Wallin R. Disulfide-dependent protein folding is linked to operation of the vitamin K cycle in the endoplasmic reticulum. A protein disulfide isomerase-VKORC1 redox enzyme complex appears to be responsible for vitamin K1 2,3-epoxide reduction. *J Biol Chem* 2007;**282**:2626–35.
31. Winter J, Klappa P, Freedman RB, Lilie H, Rudolph R. Catalytic activity and chaperone function of human protein-disulfide isomerase are required for the efficient refolding of proinsulin. *J Biol Chem* 2002;**277**:310–7.
32. Zhang L, Lai E, Teodoro T, Volchuk A. GRP78, but not protein-disulfide isomerase, partially reverses hyperglycemia-induced inhibition of insulin synthesis and secretion in pancreatic {beta}-cells. *J Biol Chem* 2009;**284**:5289–98.
33. Zhang Y, Baig E, Williams DB. Functions of ERp57 in the folding and assembly of major histocompatibility complex class I molecules. *J Biol Chem* 2006;**281**:14622–31.
34. Zito E, Chin KT, Blais J, Harding HP, Ron D. ERO1-beta, a pancreas-specific disulfide oxidase, promotes insulin biogenesis and glucose homeostasis. *J Cell Biol* 2010;**188**:821–32.
35. Zito E, Melo EP, Yang Y, Wahlander A, Neubert TA, Ron D. Oxidative protein folding by an endoplasmic reticulum-localized peroxiredoxin. *Mol Cell* 2010;**40**:787–97.

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Chapter 237

Ghrelin *O*-Acyltransferase (GOAT)

Masayasu Kojima and Hideko Ohgusu

ABSTRACT

Ghrelin is a gastric peptide hormone in which serine[3] (threonine[3] in frogs) is modified, primarily by an *n*-octanoic acid; this modification is essential for ghrelin's activity. The enzyme that transfers *n*-octanoic acid to the third serine residue of the ghrelin peptide has been identified and named (GOAT) ghrelin *O*-acyltransferase. GOAT is the only known enzyme that catalyzes acyl modification of ghrelin; it specifically modifies the third amino acid serine (or threonine) and does not modify other serine residues in ghrelin peptides. GOAT prefers *n*-hexanoyl-CoA over *n*-octanoyl-CoA as the acyl donor, although in the stomach, the *n*-octanoyl form is the main acyl-modified ghrelin.

INTRODUCTION

Ghrelin is a peptide hormone that is secreted from the stomach in response to hunger and starvation and serves as a peripheral orexigenic signal transmitting information to the brain and encouraging consumption of food.[1,10] Both intravenous and subcutaneous injections of ghrelin stimulate the hypothalamic arcuate nucleus, an important region in the control of appetite, via the vagus nerve.

Ghrelin has been identified in almost all vertebrate species thus far examined, including both mammalian and nonmammalian species, for example, frogs, birds, and fish.[9] In its active form, ghrelin bears an acyl modification at the third amino acid (serine in most species; threonine in the frog). This acyl modification is necessary for ghrelin to bind to the ghrelin receptor, GHS-R, and for it to exert biological activity.[8] The structure of ghrelin, particularly that of the acyl-modified regions, is highly conserved throughout all vertebrate species. The fatty acyl group that is used for the modification of ghrelin is primarily *n*-octanoic acid. The enzyme that transfers *n*-octanoic acid to the third serine residue of the ghrelin peptide was identified in 2008, and named GOAT.

NAME AND HISTORY OF GOAT

Ghrelin is the first and only known case of a peptide hormone modified by a fatty acid, and this acyl modification is essential for ghrelin's activity.[8] The enzyme that catalyzes this acyl transfer to ghrelin was identified in February 2008, when two independent groups reported the identification of GOAT (Fig. 1).[3,14]

The group led by Brown and Goldstein from The University of Texas Southwestern Medical Center searched the genome database and identified several orphan acyltransferases, meaning that the functions of the enzymes had not been clarified.[14] The authors expressed both ghrelin and putative enzyme cDNAs in a cell culture system, and examined the production of acyl-modified ghrelin. They finally identified (MBOAT4) membrane-bound *O*-acyltransferase as the GOAT that made the acyl modification of the ghrelin peptide.

Another group from Eli Lilly also identified several candidate acyltransferases in the genome database, and suppressed their expression by siRNA in TT cells, a thyroid medulla carcinoma cell line in which acyl-modified ghrelin is endogenously expressed.[3] They also identified MBOAT4 as the GOAT responsible for ghrelin's acyl modification. The group created MBOAT4 knockout mice and confirmed that ghrelin was not acyl-modified in the knockout mice.

Thus, MBOAT4 is the GOAT that catalyzes transfer *O*-acyl acid to the hydroxyl group of serine[3] (Ser[3]).

FUNCTIONS OF GOAT IN GHRELIN PROCESSING

The main molecular form of ghrelin in the stomach is a 28 amino acid peptide, in which Ser[3] is *n*-octanoylated. GOAT is the only known enzyme that catalyzes acyl modification of ghrelin. GOAT specifically modifies the third amino acid (serine) and does not modify other serine residues in human ghrelin. When the serine residues at the second, sixth and 18th amino acid positions in ghrelin are replaced by alanine, Ser[3] is still correctly modified by *n*-octanoic acid.[14] However, when the Ser[3] is replaced by alanine, GOAT cannot modify the peptide. The third amino acid of frog ghrelin is threonine, and is modified by *n*-octanoic acid just as Ser[3] is in other ghrelins.[7] When the third amino acid serine of rat

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00237-2
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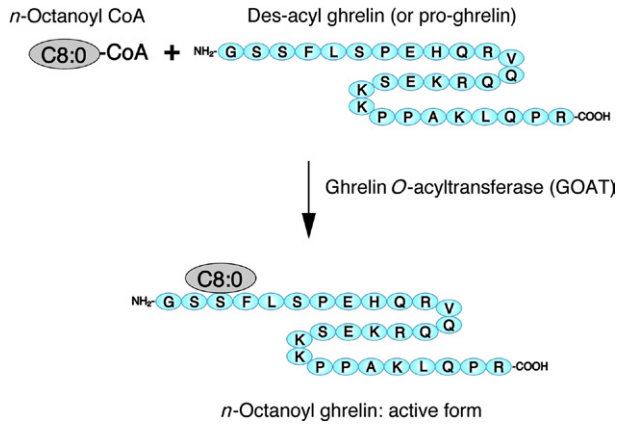

FIGURE 1 Des-acyl ghrelin (or proghrelin) is modified with an acyl acid (mainly *n*-octanoic acid) by GOAT to produce active ghrelin.

ghrelin is replaced with threonine, GOAT can modify the replaced threonine using *n*-octanoic acid.[14] Thus, the presence of serine or threonine as the third amino acid is important for *n*-octanoyl modification of ghrelin, and GOAT can modify not only serine but also threonine residues.

PROPERTIES

General Properties of GOAT

GOAT is a membrane-bound enzyme with multiple transmembrane domains. The number of membrane-spanning regions remains controversial and has been reported as eight (Yang et al.)[14] or five (Gutierrez et al.).[3] GOAT is a member of an acyltransferase family that includes at least 16 acyltransferase enzymes. Among these proteins, only GOAT has the ability to acyl-modify ghrelin. Just as ghrelin is present in all vertebrate species, GOAT is also found in mammals, birds, and fish.

The acyl group that is used for ghrelin modification is not a free acid but is instead a CoA-conjugated form.[12] The ghrelin modification reaction does not occur with free fatty acids or a mixture of free fatty acid and CoA.

Structure

The GOAT amino acid sequence is highly conserved among vertebrate species (Fig. 2). Amino acids 300–340 contain the acyltransferase catalytic region;[4] however, the role of other regions has not yet been revealed. The putative catalytic asparagine and histidine residues are conserved not only in mammalian GOAT but also in chicken, zebrafish, and *Xenopus tropicalis* GOATs.

Molecular Weight

The amino acid length of six mammalian GOATs (human, mouse, rat, chimpanzee, cow, and horse) is 435; the predicted molecular weights are 49,585 (human), 49,749 (mouse) and 49,877 Da (rat). The amino acid lengths of nonmammalian GOATs are 424 (chicken), 415 (zebrafish), and 429 (*X. tropicalis*); the predicted molecular weights are 47,345 (chicken), 47,495 (zebrafish), and 49,971 (*X. tropicalis*).

Effects of Detergents on GOAT Activity

Treatment of the membrane fraction with CHAPS or Tween 80 preserves GOAT activity.[12] By contrast, treatment with other six detergents (Triton X100, Lubrol, NP40, Brij96v, Triton X45 and NP9) attenuate or abolish GOAT activity. These results indicate that CHAPS and Tween 80 stabilize the conformation of GOAT and are useful for the solubilization of this protein.

STIMULATION OF GOAT

GOAT activity is enhanced by incubation with long-chain fatty acyl CoA in the GOAT reaction.[15] The GOAT reaction is stimulated equally by myristoyl-CoA (C14:0), palmitoyl-CoA (C16:0), and oleoyl-CoA (18:1). These long-chain fatty acids stimulate GOAT activity by inhibiting deacylation of *n*-octanoyl ghrelin.

Inhibitors

The pentapeptide derived from the ghrelin N-terminal peptide (GSSFL-NH_2, GSAFL-NH_2, GSSFL-COOH) inhibits *n*-octanoyl modification of ghrelin.[15] Moreover, when this peptide bears an *n*-octanoyl modification (GSS(C8:0) FL-NH_2), it also inhibits GOAT. In particular, when Ser^3 is replaced with (S)-2,3-diaminopropionic acid (Dap) and modified by *n*-octanoic acid, the pentapeptide potently inhibits the acyl modification activity of GOAT. [Dap]octanoyl-ghrelin (1-5)-NH_2 also exhibits potent GOAT inhibiting activity.

A synthetic bisubstrate inhibitor for GOAT, GO-CoA-Tat, has been developed (Fig. 3).[2] GO-CoA-Tat consists of three components: octanoyl-modified N-terminal 10 amino acid ghrelin peptide (GO), coenzyme A (CoA), and a Tat peptide, which is an 11 amino acid oligomer HIV Tat-derived peptide sequence intended to enhance cell penetration. Intraperitoneal injection of GO-CoA-Tat inhibits the production of acyl-modified ghrelin and suppresses body weight gain. Moreover, GO-CoA-Tat increases insulin concentration and improves glucose tolerance by lowering glucose concentration. Thus, GO-CoA-Tat may be a clinical target for the treatment of metabolic diseases, such as diabetes mellitus and obesity.

SUBSTRATE SPECIFICITY OF GOAT

Peptide Substrates

Not only the 28 amino acid des-acyl ghrelin but also the 94 amino acid proghrelin (human) can be acyl-modified by GOAT.[14] In fact, proghrelin is octanoylated before it is

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Human                1 --MEWLWLFFLHPISFYQGAAFPFALLFNYLCIMDSFSTRARYLFLLTGGGALAVAAMGS    58
Porcine              1 --MDWLQLFFLHPVLFYQGAAFPFALLFNYLCITDSFSTRARYLFLLAGGGALAVAAMGA    58
Mouse                1 --MDWLQLFFLHPLSFYQGAAFPFALLFNYLCILDTFSTRARYLFLLAGGGVLAFAAMGP    58
Chicken              1 --MRWADLLILLPAAWYQLAAFLFAALFHYLCALGHLSLTSRYIFLLAGGCLLAGTAMGS    58
Zebrafish            1 MIDLLWISSDGHPQLFYQFINIPFAFLFHCLSSQGHLSIINRYVYLAMGGFMLAIATMGP    60
Xenopus tropicalis   1 --MDYLQLVF-HPVALYQLAAFPWAFLYGYLSSFGHLPITPRFIYLLFGGIVLSCVSMGP    57

Human               59 YAVLVFTPAVCAVALLCSLAPQQVHRWTFCFQMSWQTLC-HLGLHYTEYYLHEPPSVRFC   117
Porcine             59 FAVLVFVPALGAVVLIHSLGPRHVHRPTFLFQMSWQTLC-HLGLHYTEYYLQESPSTRFC   117
Mouse               59 YSLLIFIPALCAVALVSFLSPQEVHRLTFFFQMGWQTLC-HLGLHYTEYYLGEPPPVRFY   117
Chicken             59 YATLLLIPAVSSVLMFLLVSPAYVHTWVFSLQMCWQTLC-HLGL---GSLVLESQDTRPA   114
Zebrafish           61 YSSLLFLSAIKLLLLIHYIHPMHLHRWILGLQMCWQT-CWHLYVQYQIYWLQEAPDSRLL   119
Xenopus tropicalis  58 YAVVIFIPALGSIVLFHIISWQSVHWWALALQMVWQTAC-HLWLLYKEYYLQEEITLRLS   116

Human              118 ITLSSLMLLTQRVTSLSLDICEGKVKA-ASGGFRSRSSLSEHVCKALPYFSYLLFFPALL   176
Porcine            118 ITLSSFMLLTQRVTSLSLDIFEGKVEV-ASEAVGSESSLPKRLWKALPYCSYLLFFPALL   176
Mouse              118 ITLSSLMLLTQRVTSLSLDICEGKVEA-PRRGIRSKSSFSEHLWDALPHFSYLLFFPALL   176
Chicken            115 VTLSAIMLLTQKVTSLALDIHEGTVLPQPGQGL---------LQRALPLCSYLLFFPALL   165
Zebrafish          120 LAISALMLMTQRISSLSLDFQEGTI---------------SNQSILIPFLTYSLYFPALL   164
Xenopus tropicalis 117 IMISALMLLTQKITTLALDIHERKVRIIPVDGGMKNWFFSGSAHNILIFLSYLLFFPALL   176

Human              177 GGSLCSFQRFQARVQGSSALHPRHSFWALSWRGLQILGLECLNVAVSRVVDAGAGLTDCQ   236
Porcine            177 GGPLCSFRRFQSHVQGPRSWDPRHSLWALTCQGLQIVGLECLKVAMRWAVSVGAGLTDCR   236
Mouse              177 GGSLCSFRRFQACVQRSSSLYPSISFRALTWRGLQILGLECLKVALRSAVSAGAGLDDCQ   236
Chicken            166 GGPLCSFSKFQAQVMSLGA--VPCPLRAVCWRYLGVLVLQVLRAWLEGWLPCMQGWAS--   221
Zebrafish          165 GGPLCSFNAFVQSVERQHT-SMTSYLGNLTSKISQVIVLVWIKQLFSELLKSATFNIDSV   223
Xenopus tropicalis 177 GGPLCSFVEFHHHVTVAPR-CNYLCFKQVAKGFFFALILQMLRSLVSVNLSFQLSLMTCR   235

Human              237 QFECIYVVWTTAGLFKLTYYSHWILDDSLLHAAGFGPELGQSPGEEGY--VPDADIWTLE   294
Porcine            237 QLQCVYVMWSMAGLFKLTYYSHWILDESLLQAAGFGSGFGGSTGEESF--VPDADIWTLE   294
Mouse              237 RLECIYLMWSTAWLFKLTYYSHWILDDSLLHAAGFGAEAGQGPGEEGY--VPDVDIWTLE   294
Chicken            222 ----MGHMWAQALLFRLAYYSQWVLDEALLEAAGFGAAV-----EHRD--LSGHDLWTLE   270
Zebrafish          224 CLDVLWI-WIFSLTLRLNYYAHWKMSECVNNAAGLGVYFHKHSGQTSWDELSDGSVLVTE   282
Xenopus tropicalis 236 HLNCVCIMWTTALLFKLTYYFHWLLDESLFCAAGFLTAYHEDGFQVTF--C-DTDIWTLE   292
                                  *                                   *
Human              295 RTHRISVFSRKWNQSTARWLRRLVFQHSRAWPLLQTFAFSAWWHGLHPGQVFGFVCWAVM   354
Porcine            295 TTHKLSLFTRKWNHSTAQWLRRLVFQQGRTWPLLQTFVFSAWWHGLHPGQVFGFLCWAVM   354
Mouse              295 TTHRISLFARQWNRSTALWLRRLVFRKSRRWPLLQTFAFSAWWHGLHPGQVFGFLCWSVM   354
Chicken            271 TTHRLSVFSRTWNRSTSLWLRRLVFQRCPVQPLLATFAFSAWWHGLRPGQIFGFLCWAIM   330
Zebrafish          283 ASSRPSVFARKWNQTTVDWLRKIVFNRTSRSPLFMTFGFSALWHGLHPGQILGFLIWAVT   342
Xenopus tropicalis 293 TTHKISVFTRTWNKSTASWLRRIIFEKCKIGSLLMTFAFSAWWHGLHPGHIFGFLCWALL   352

Human              355 VEADYLIHSFANEFIRSWPMRLFYRTLTWAHTQLIIAYIMLAVEVRSLSSLWLLCNSYNS   414
Porcine            355 VKADYLIHTFAKVSIRSQLMWLLYRTLTWAHTQLIIAYIILAVEARSLSSLWLLCNSYNS   414
Mouse              355 VKADYLIHTFANVCIRSWPLRLLYRALTWAHTQLIIAYIMLAVEGRSLSSLCQLCCSYNS   414
Chicken            331 VEADYRIHPFLSSRATSCVAKLLYRGTTWVFTQLIIAYIQMAVETESFSMLCLLWTSYNS   390
Zebrafish          343 VQADYKLHRFSHPKLNSLWRKRLYVCVNWAFTQLTVACVVVCVELQSLASVKLLWSSCIA   402
Xenopus tropicalis 353 VKADYRIHKYFNPCQQSWCTRVLYRIFTWLHTQLIVAFLIVAIEMRSIKIIWSLCLSYNC   412

Human              415 VFPMVYCILLLLLAKRKHKCN-------------                             435
Porcine            415 VFPMVYCILFFLLAKRKHKCN-------------                             435
Mouse              415 LFPVMYGLLLFLLAERKDKRN-------------                             435
Chicken            391 ILPLTYGLVLLLLLLFAQKPKQNRACPGLTRLAG                             424
Zebrafish          403 VFPLLSALILIIL---------------------                             415
Xenopus tropicalis 413 YFPILYCLSLISSIKRK-----------------                             429
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FIGURE 2 Sequence comparison of six vertebrate GOATs. Identical amino acids in six species are shaded dark and amino acids conserved among four or five species are shaded pale. The asterisks (*) indicate putative catalytic asparagine and histidine residues.

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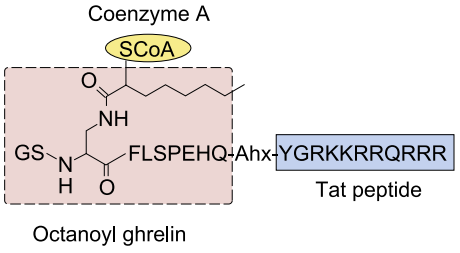

FIGURE 3 Structure of GOAT inihibitor, GO-CoA-Tat. Ahx, aminohexanoate, is the linker between ghrelin peptide and Tat peptide. Tat peptide is used for enhancing cell penetration.

cleaved to form mature 28 amino acid ghrelin.[17] These results indicate that although des-acyl ghrelin can serve as a substrate for GOAT, the acyl modification of ghrelin does not require previous protease processing.

With synthetic ghrelin N-terminal peptides, the minimum length of ghrelin peptide that can be acyl-modified by GOAT has been determined.[12] The synthetic N-terminal peptides used for these studies were shorter than des-acyl ghrelin and their C-termini had α-amide structures.

Immunoreactive octanoylated ghrelin concentration in the reactions revealed that the longer 8 amino acid peptide is acylated to 100-fold more efficiently than the shorter 4 amino acid peptide, and 4-fold higher than the 5 amino acid peptide. Because the amount of GOAT-treated 4 amino acid peptide produced (GSSF-NH_2) was very low, there was a possibility that the *n*-octanoyl modification of the 4 amino acid peptide was not a GOAT-specific reaction, but was instead a nonenzymatic reaction. However, in the absence of GOAT enzyme solution, no *n*-octanoyl modified peptide is produced.

These results indicate that these five synthetic peptides serve as substrates for GOAT and can be modified by *n*-octanoic acid. Thus, a peptide as short as four amino acids constitutes the core motif for substrate recognition by GOAT.

As described above, GOAT specifically modifies the third residue, serine or threonine. Interestingly, when the first amino acid (glycine) or the fourth amino acid (phenylalanine) is replaced by alanine, Ser^3 is not acyl-modified; however, replacements of Ser^2, Leu^5, Ser^6, or Pro^7 by Ala do not have an effect on the acyl modification.[14] In addition, N-terminal addition of two amino acids (Ser-Ala) significantly suppresses the acyl modification of Ser^3 of ghrelin. These results indicate that three amino acids in the N-terminus (Gly^1, Ser^3, and Phe^4) are important for the acyl modification reaction.

Acyl Donors

GOAT can use a wide range of substrates for acyl modification of ghrelin, ranging in size from acetic acid (C2:0) to tetradecanoic acid (C16:0).[3] However, the main molecular form of gastric ghrelin is modified by *n*-octanoic acid, and other acyl-modified forms of ghrelin are found only at low levels.

GOAT can modify des-acyl ghrelin peptide not only with *n*-octanoyl CoA, but also with other medium-chain acyl groups, such as *n*-hexanoyl-CoA and *n*-decanoyl-CoA. By contrast, very little acyl-modified products result from reaction with long-chain fatty acids. Kinetic studies using des-acyl ghrelin and three medium-chain acyl-CoAs as the acyl donor (*n*-hexanoyl-CoA, *n*-octanoyl-CoA and *n*-decanoyl-CoA), indicate that the order of the activities (as evaluated by *Vmax*/*Km*) is *n*-hexanoyl-CoA > *n*-octanoyl-CoA > > *n*-decanoyl-CoA.[12] The *Km* values for *n*-hexanoyl-CoA and *n*-octanoyl-CoA are 294 and 13.6 μM, respectively. Thus, GOAT prefers *n*-hexanoyl-CoA to *n*-octanoyl-CoA as the acyl donor. However, the concentration of *n*-hexanoyl ghrelin in the mouse stomach is very low relative to *n*-octanoyl ghrelin. Nishi et al. reported that ingestion of glyceryl trihexanoate drastically stimulates the production of *n*-hexanoyl ghrelin in the stomach.[11] This observation supports the idea that GOAT prefers *n*-hexanoyl-CoA as an acyl donor. These results indicate that the content of *n*-hexanoyl-CoA in the stomach may be lower than that of *n*-octanoyl-CoA, and these concentration differences may determine the relative production rates and concentrations of various acyl-modified ghrelins.

Multiple Acyl-modified Forms of Ghrelin

Several forms of acyl-modified ghrelin peptides have been isolated. In human, these can be classified into four groups by the type of acylation observed at Ser^3: nonacylated, octanoylated (C8:0), decanoylated (C10:0), and decenoylated (C10:1).[5] Additionally, there are several different acyl-modified forms of ghrelin in cat stomach: C8:0, C10:0, C10:1, C10:2, C13:0, C13:1.[6]

Because of its broad spectrum of acyl group substrates, GOAT is probably responsible for the transfer of all these modifications onto des-acyl ghrelin peptides.

REGULATION OF ACTIVITY

Ions

The following metals have no effect on GOAT activity: Mg^{2+}, Ca^{2+}, Na^+, and K^+. However, Fe^{2+} and Cu^{2+} potently inhibit GOAT activity.[12] GOAT activity is completely blocked >5 mM Fe^{3+} or 0.5 mM Cu^{2+}. EDTA and EGTA have no effect on GOAT activity, indicating that the enzyme has no absolute requirement for cations.

pH

The optimal pH for maximal specific activity of GOAT is 7.0–7.5 (Fig. 4).[12] The specific activity decreased rapidly <pH 6.5 and >pH 8.5.

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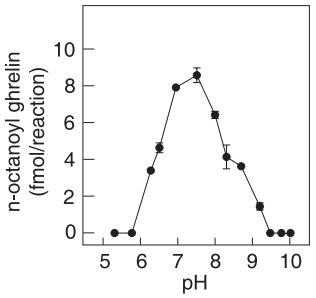

FIGURE 4 pH dependence of GOAT activity. *n*-Octanoyl ghrelin production activity was measured using standard assay conditions. *n*-Octanoyl ghrelin concentrations were measured by active ghrelin ELISA Kit. Results are expressed as the means ± S.D. ($n = 3$). The following buffers were used: 50 mM MES (pH 5–7), Tris-HCl (pH 7.5–8.5), and $NaHCO_3$ (pH 9–10).

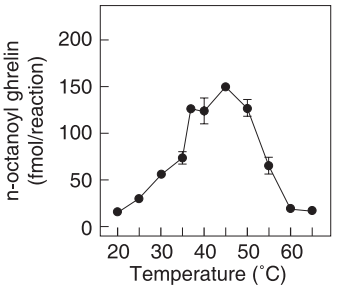

FIGURE 5 Optimal temperature of GOAT activity.

Because the optimal pH of the processing protease PC1/3 is rather acidic, and the pH value in the secretary granules is also acidic (pH 5–6), the acyl modification of ghrelin should occur before protease processing. In other words, it is probable that ghrelin will be modified before it matures, as preproghrelin and proghrelin. Indeed, proghrelin has been shown to be modified by *n*-octanoic acid.[14] Thus, acyl modification precedes protease cleavage.

Optimal Temperature

The optimal GOAT reaction temperature is 37–50 °C (Fig. 5).[12] GOAT still retains some enzyme activity at 55 °C, but the activity is abolished at temperatures >60 °C.

Distribution and Localization

The tissue distribution of GOAT is similar to that of ghrelin.[3,14] GOAT mRNA is most highly expressed in the stomach, followed by the small intestine and colon. GOAT mRNA is also expressed in testis, pancreas, total brain and especially in the hypothalamus. Ghrelin and GOAT are colocalized in the stomach.[13] However, there is a subset of ghrelin-immunoreactive cells in the stomach that do not contain GOAT mRNA.

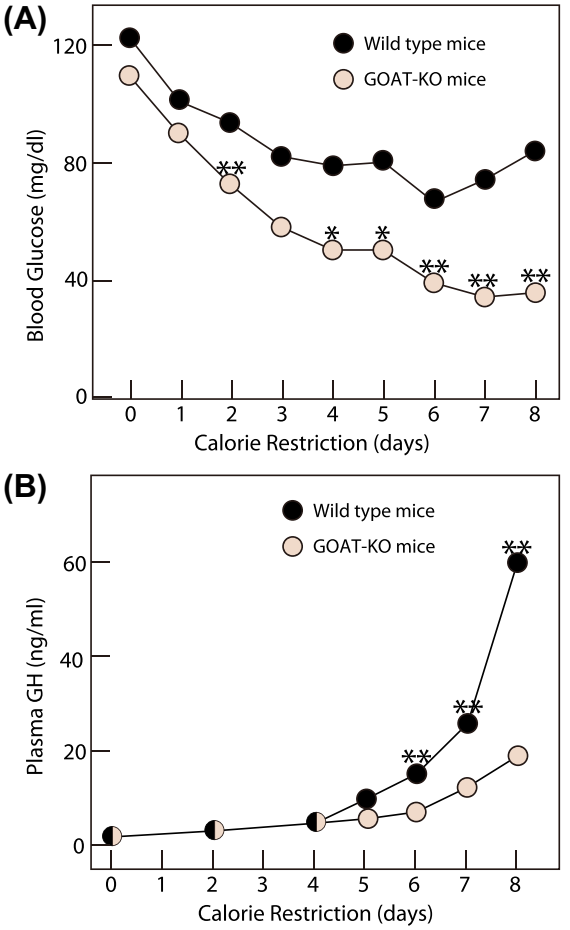

FIGURE 6 Blood glucose and plasma GH levels in 60% calorie-restricted mice. (A) Blood glucose levels in WT mice are stable after 4 days, whereas in GOAT-KO mice, they continue to decrease. (B) Plasma GH concentrations in GOAT-KO mice increase much more slowly than those of WT mice after 4 days. (These figures are modified from Reference 17.)

PATHOPHYSIOLOGICAL IMPLICATIONS

The growth and body weight of GOAT-KO mice are similar to those of WT mice when the GOAT-KO and WT mice are fed normal or high-fat diets. Under 60% caloric restriction, body weight and body fat are suppressed in both WT and GOAT-KO mice for the first four days, and their fasting blood glucose levels are decreased.[16] After four days of restriction, the blood glucose level in WT mice is maintained steadily at 58–76 mg/dl; however, the glucose level of GOAT-KO mice decreased gradually and reached 12–36 mg/dl after seven days (Fig. 6A). Under these conditions, WT mice showed normal activity, whereas GOAT-KO mice were close to death. In addition, the plasma GH level of the calorically restricted WT mice gradually increased, whereas the increase in plasma GH levels in the calorically restricted GOAT-KO mice was lower than that of WT mice (Fig. 6B). Ghrelin or GH injections into the calorically restricted GOAT-KO mice returned plasma glucose concentration to the normal level and prevented death.

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These results indicate that under highly calorically restricted conditions, ghrelin maintains normal plasma GH concentrations and prevents death. It is not clear whether ghrelin-KO and ghrelin receptorKO mice exhibit the same phenotypes as GOAT-KO mice.

REFERENCES

1. Andrews ZB. The extra-hypothalamic actions of ghrelin on neuronal function. *Trends Neurosci* 2011;**34**:31–40.
2. Barnett BP, Hwang Y, Taylor MS, Kirchner H, Pfluger PT, Bernard V, et al. Glucose and weight control in mice with a designed ghrelin *O*-acyltransferase inhibitor. *Science* 2010;**330**:1689–92.
3. Gutierrez JA, Solenberg PJ, Perkins DR, Willency JA, Knierman MD, Jin Z, et al. Ghrelin octanoylation mediated by an orphan lipid transferase. *Proc Natl Acad Sci U S A* 2008;**105**:6320–5.
4. Hofmann K. A superfamily of membrane-bound *O*-acyltransferases with implications for wnt signaling. *Trends Biochem Sci* 2000;**25**: 111–2.
5. Hosoda H, Kojima M, Mizushima T, Shimizu S, Kangawa K. Structural divergence of human ghrelin. Identification of multiple ghrelin-derived molecules produced by post-translational processing. *J Biol Chem* 2003;**278**:64–70.
6. Ida T, Miyazato M, Naganobu K, Nakahara K, Sato M, Lin XZ, et al. Purification and characterization of feline ghrelin and its possible role. *Domest Anim Endocrinol* 2007;**32**:93–105.
7. Kaiya H, Kojima M, Hosoda H, Koda A, Yamamoto K, Kitajima Y, et al. Bullfrog ghrelin is modified by *n*-octanoic acid at its third threonine residue. *J Biol Chem* 2001;**276**:40441–8.
8. Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H, Kangawa K. Ghrelin is a growth-hormone-releasing acylated peptide from stomach. *Nature* 1999;**402**:656–60.
9. Kojima M, Ida T, Sato T. Structure of mammalian and nonmammalian ghrelins. *Vitam Horm* 2008;**77**:31–46.
10. Kojima M, Kangawa K. Ghrelin: structure and function. *Physiol Rev* 2005;**85**:495–522.
11. Nishi Y, Hiejima H, Hosoda H, Kaiya H, Mori K, Fukue Y, et al. Ingested medium-chain fatty acids are directly utilized for the acyl modification of ghrelin. *Endocrinology* 2005;**146**:2255–64.
12. Ohgusu H, Shirouzu K, Nakamura Y, Nakashima Y, Ida T, Sato T, et al. Ghrelin *O*-acyltransferase (GOAT) has a preference for n-hexanoyl-CoA over *n*-octanoyl CoA as an acyl donor. *Biochem Biophys Res Commun* 2009;**386**:153–8.
13. Sakata I, Yang J, Lee CE, Osborne-Lawrence S, Rovinsky SA, Elmquist JK, et al. Colocalization of ghrelin *O*-acyltransferase and ghrelin in gastric mucosal cells. *Am J Physiol Endocrinol Metab* 2009;**297**:E134–41.
14. Yang J, Brown MS, Liang G, Grishin NV, Goldstein JL. Identification of the acyltransferase that octanoylates ghrelin, an appetite-stimulating peptide hormone. *Cell* 2008;**132**:387–96.
15. Yang J, Zhao TJ, Goldstein JL, Brown MS. Inhibition of ghrelin *O*-acyltransferase (GOAT) by octanoylated pentapeptides. *Proc Natl Acad Sci U S A* 2008;**105**:10750–5.
16. Zhao TJ, Liang G, Li RL, Xie X, Sleeman MW, Murphy AJ, et al. Ghrelin *O*-acyltransferase (GOAT) is essential for growth hormone-mediated survival of calorie-restricted mice. *Proc Natl Acad Sci U S A* 2010;**107**:7467–72.
17. Zhu X, Cao Y, Voogd K, Steiner DF. On the processing of proghrelin to ghrelin. *J Biol Chem* 2006;**281**:38867–70.

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Chapter 238

Glutaminyl Cyclases

Holger Cynis, Stephan Schilling and Hans-Ulrich Demuth

ABSTRACT

Posttranslational modifications such as glycosylation, amidation, or phosphorylation play an important role for the maturation of peptides and proteins. Frequently, the modifications alter their bioactivity and stability *in vivo*. Besides C-terminal modification by peptidylglycine alpha-amidating monooxygenase (PAM), the N-terminal modification by a 5-oxo-prolyl (pyroglutamyl, pGlu)-residue has been described for many peptide hormones and proteins, for example, thyrotropin-releasing hormone (TRH), gonadotropin-releasing hormone (GnRH), neurotensin, and fibronectin. The pGlu-residue originates from intramolecular cyclization of N-terminal glutamine and glutamate residues. This reaction is facilitated by glutaminyl cyclases (QC, isoQC, EC 2.3.2.5) and is found in bacteria, plants, and animals. Similar to C-terminal amidation, N-terminal pGlu-formation is important for the stability and/or bioactivity of the respective substrates. In addition to their important role in hormone function, several pGlu-peptides and proteins have been implicated in human disorders, such as Alzheimer's disease or monocyte-driven inflammatory disorders making glutaminyl cyclases attractive targets for drug discovery.

N-TERMINAL pGLU-MODIFICATION OF PEPTIDES AND PROTEINS

pGlu-Peptides of Plants

In plants, a few pGlu-modified proteins have been described. Most of these proteins are functionally related to plant defense reactions, for example, chitinases and glucanases. In addition, selected pathogenesis-related proteins (PR) are modified by an N-terminal pGlu-residue (Table 1). The role of the N-terminal pGlu-residue is unknown in either case, but because of the co-upregulation of proteases on infection, it might exert a stabilizing function to ensure stability of the respective pGlu-peptide in a highly proteolytic environment.

pGlu-Peptides of Invertebrates

In accordance with the distribution of glutaminyl cyclases among invertebrates and vertebrates, a broad spectrum of pGlu-peptides with differing functions have been described. The lowest metazoans, which contain pGlu-peptides, are cnidarians such as the sea anemone *Anthopleura elegantissima*. This organism expresses a number of pGlu-modified neuropeptides, and some of them were found to induce muscular constriction and were, therefore, considered as neurotransmitter or neuromodulators[30] (Table 2). Also, in the maritime snail *Aplysia californica*, pGlu-modified neuropeptides could be isolated. However, their specific functions remain unclear[9] (Table 2).

Among arthropods, a significant number of pGlu-peptides with different functions have been described so far. In this regard, Attacin-C is part of the immune response of *Drosophila melanogaster* showing antimicrobial activity against Gram-negative bacteria.[19] Further, the adipokinetic hormone of different arthropod species, for example, of *Locusta migratoria*, is a neuropeptide with importance for energy homeostasis. It is released when the insect flies and ensures the mobilization of fatty acids and carbohydrates from the fat body. In addition, pGlu-peptides are important components of the venom of arachnids. pGlu-discrepin, an irreversible potassium channel blocker inhibits potassium currents of cerebellar granule cells of rats and has been isolated from the venom of the scorpion *Tityus discrepans* (Table 2). Likewise, the Brazilian armed spider *Phoneutria nigriventer* also injects potent venom. By mass spectrometric analysis, this cocktail contains several pGlu-modified peptides structurally related to tachykinin peptides. Although detailed functional analysis of these peptides are lacking, it seems that these peptides are able to induce a constriction of the small intestine of guinea pigs, a function, which was described for tachykinin peptides[16] (Table 2). The role of the N-terminal pGlu-residue for the above-mentioned invertebrate pGlu-peptides is still unknown. However, it is tempting to speculate, that at least the pGlu-peptides in venom might have a stabilizing function to ensure a long bioactivity after injection.

In analogy to pGlu-peptides in venom of arachnids, the poison gland of the colubrid snakes *Boiga irregularis* and *Boiga dendrophila* generates a mixture of pGlu-peptides.[15] Therefore, the appearance of pGlu-peptides in animal venom is an evolutionary conserved hallmark among different phyla.

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00238-4
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TABLE 1 Selected pGlu-Peptides in Plants

Species	pGlu-protein	Function
Nicotiana tabacum	Glucan-endo-(1,3)-glucosidase pE-S-I-G-V…316	Hydrolysis of (1,3)-beta-D-glucosidic linkages
Solanum lycopersicum	Pathogenesis-related leaf protein 6 pE-N-S-P-Q-D…135	Plant defense
Arabidopsis thaliana	Pathogenesis-related protein 1 pE-D-S-P-Q-D…135	Plant defense

pGlu-Peptides of Vertebrates

The neuropeptides TRH and GnRH probably represent the best studied pGlu-peptides from vertebrates (Table 3). Both are formed in the hypothalamus and regulate the hypothalamus–pituitary–thyroid (TRH) and hypothalamus–pituitary–gonadal axes (GnRH), which are responsible for energy homeostasis and reproduction, respectively. TRH stimulates the release of prolactin and thyroid-stimulating hormone (TSH) from the anterior pituitary and is also known as a neurotransmitter and neuromodulator. The pGlu-residue of TRH is essential for its bioactivity.[1]

GnRH stimulates the release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the anterior pituitary. The N- and C-terminal amino acids are essential for receptor binding and the activation of the GnRH-receptor is facilitated via the N-terminal region. The most important residues for activation are His^2 and Trp^3, but $pGlu^1$ might also be involved.[31] In addition, the presence of the N-terminal pGlu-residue increases the circulation half-life of TRH and GnRH suggesting a stabilizing function presumably by protection against aminopeptidase cleavage.[24] Additional human pGlu-neuropeptides are neurotensin and orexin A.

Besides pGlu-hormones such as gastrin-17 leading to the gastric release of hydrochloric acid (Table 3) and fertilization promoting peptide (FPP), a TRH analog with importance for sperm fertilization (Table 3), pGlu-peptides and proteins play a pivotal role in the human immune system. This is mainly attributed to pGlu-chemokines such as "monocyte chemoattractant proteins" (MCPs) leading to the attraction of immune cells to inflamed tissues.

Chemokines are grouped on the basis of conservation of cysteine residues in the N-terminal region. Accordingly, the peptides constitute the four classes "C-," "CC-," "CXC-," and "CX_3C-." Among those, CCL2 (MCP-1) represents the best studied CC-chemokine showing a high potential to attract monocytes. On stimulation, CCL2 is released by a variety of cell types, such as endothelial cells, fibroblasts, smooth muscle cells, or neuronal cells. After secretion, CCL2 is bound by glycosaminoglycanes of the extracellular matrix building up a gradient pointing toward the inflamed tissue. In addition, CCL2 is connected to vascular endothelial cells leading to the recruitment of monocytes to the subendothelial space. The N-terminal pGlu-residues of human MCPs are essential for their bioactivity and stability. Mature pGlu-CCL2 has a higher potency to activate its main receptor CCR2 and, therefore, possesses a higher potential to attract monocytes compared with immature Gln-CCL2.[4] In addition, the N-terminal pGlu-residue protects CCL2 and its family members CCL7, CCL8, and CCL13 against proteolytic degradation by aminopeptidases such as dipeptidylpeptidase 4 (DP4) and aminopeptidase P (ApP). Interestingly, artificial elongation of the N-terminal

TABLE 2 Selected pGlu-Peptides of Invertebrates

Phylum	Species	pGlu-peptide	Function
Cnidaria	*Anthopleura elegantissima*	Antho-RFamide pE-G-R-F-NH_2	Neuropeptide
Mollusca	*Aplysia californica*	R3-14 peptide pE-E-V-F-D-D…26	Neuropeptide
Arthropoda	*Drosophila melanogaster*	Attacin-C pE-R-P-Y-T-Q…218	Immune defense
	Locusta migratoria	Adipokinetic hormone pE-L-N-F-T-P…41	Energy homeostasis
	Tityus discrepans	Discrepin pE-I-D-T-N-V…38	Poison component Potassium channel blocker
	Phoneutria nigriventer	PnTkP-XIII pE-K-K-D-K-K…13	Poison component Unknown mechanism

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TABLE 3 Selected pGlu-peptides of Vertebrates Grouped by Function

Group	pGlu-peptide	Main function
Neuropeptides	TRH pE-H-P-NH_2	Release of prolactin and TSH in pituitary Neurotransmitter/-modulator
	GnRH pE-H-W-S-Y-G-L-R-P-G-NH_2	Release of gonadotropins LH und FSH in pituitary
Hormones	FPP pE-E-P-NH_2	Sperm cell maturation
	Gastrin-17 pE-G-P-W-L-E…17	Stimulation of gastric HCl-release
Chemokines	CCL2 (MCP-1) pE-P-D-A-I-N…76	Chemoattractant for monocytes and lymphocytes
	CCL7 (MCP-3) pE-P-V-G-I-N…76	Chemoattractant for monocytes and eosinophils
	CCL8 (MCP2) pE-P-D-S-V-S…76	Chemoattractant for monocytes, lymphocytes, basophils, and neutrophils
	CCL13 (MCP-4) pE-P-D-A-L-N…75	Chemoattractant for monocytes, lymphocytes, basophils, and eosinophils
Others	Fibronectin pE-A-Q-Q-M-V…2355	Cell adhesion
	α-Amylase pE-Y-S-S-N-T…496	Salivary protein Cleavage of α1,4 O-glycosidic bonds
Amyloids	β-amyloid peptide (Aβ) pE-F-R-H-D-S…40/42	Amyloid peptide deposited in Alzheimer's disease
	ABri pE-A-S-N-C-F-…N-I-I-E-E-N_{34}	Amyloid peptide deposited in familial British dementia (FBD)
	ADan pE-A-S-N-C-F-…S-Q-E-K-H-Y_{34}	Amyloid peptide deposited in familial Danish dementia (FDD)

sequence or truncation by aminopeptidases leads to a loss of bioactivity of all MCPs, highlighting the importance of the pGlu-moiety.[4,18] Thus, the pGlu-residue mediates stabilizing and activating functions in human pGlu-MCPs.

pGlu-Amyloid Peptides as Exceptional Cases with Relevance to Pathophysiology

In contrast to the vast majority of peptides, in which the pGlu-moiety is generated from a glutaminyl precursor, pGlu is formed from N-terminal glutamic acid in few peptides. The cyclization reaction is also accelerated by glutaminyl cyclases[6] but apparently as a slow and physiologically irrelevant side reaction. Evidence for a QC-catalyzed cyclization of glutamyl residues came from analysis of human amyloid peptides. First hints toward pGlu-formation from Glu were obtained by studies on hormonal peptides. Neuronal extracts of *A. californica* catalyzed the conversion of a glutamyl- into a pGlu-residue.[9] Furthermore, analysis of the cleavage products of proopiomelanocortin (POMC), namely, β-lipotropin and the joining peptide revealed a partial pGlu-formation from N-terminal glutamate. However, this pGlu-formation seems to lack functional consequences underlining the role of an irrelevant side reaction to most of the proteins.[2]

In amyloid peptides, however, the pGlu-formation potently influences the biophysical properties, which likely contributes to the pathophysiology of disease. The neurodegenerative disorders Alzheimer's disease (AD), familial British dementia (FBD), and familial Danish dementia (FDD) are characterized by progressive accumulation of such pGlu-peptides[10,20] (Table 3). The pGlu-modification of Aβ renders the peptides more toxic and leads to an increase of the aggregation propensity. Therefore, pGlu-Aβ might contribute to the formation of initial aggregates, mediating disease progression.[29] In FBD, pGlu-ABri is deposited in the brain parenchyma and peripheral deposits, whereas the unmodified glutamate precursor is found in the circulation.[11]

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Apparently, the pGlu-modification contributes to the deposition, in turn highlighting a gain of toxic potential for certain pGlu-modified amyloid peptides.

GLUTAMINYL CYCLASES

Glutaminyl Cyclase (QC, QPCT, EC 2.3.2.5)

Glutaminyl cyclases are aminoacyltransferases catalyzing the intramolecular cyclization of N-terminal glutaminyl- and glutamyl residues under the concomitant release of ammonia or water, respectively[27] (Fig. 1). This reaction has been described in prokaryotes and eukaryotes. The first QC was isolated in 1963 from the latex of the tropical plant *Carica papaya*. Twenty-four years later the first mammalian enzyme was identified in bovine pituitary extracts. In addition, a prokaryotic QC from *Streptococcus bovis* was described in 1993. Bacterial/plant and animal QCs catalyze the same reaction. The proteins, however, do not share structural homology. Apparently, animal QCs evolved from ancestral zinc-dependent aminopeptidases, and both proteins share the typical α/β-hydrolase fold. By contrast, the homologous plant and bacterial QCs form a separate protein family, the polypeptides adopt an all-β conformation.[8]

Homologous plant QCs were identified in *Arabidopsis thaliana* and *Solanum lycopersicum*.[28] Mammalian QCs were described and characterized from bovine, rat, mouse, and human sources.[17,21,22,33] In addition, QCs from arthropods (*D. melanogaster*) and serpents (*Bioga spec.*) were reported.[15,25] Because of the broad distribution of pGlu-peptides and QCs among phyla, it is tempting to speculate, that QCs are conserved in most of the metazoan species.

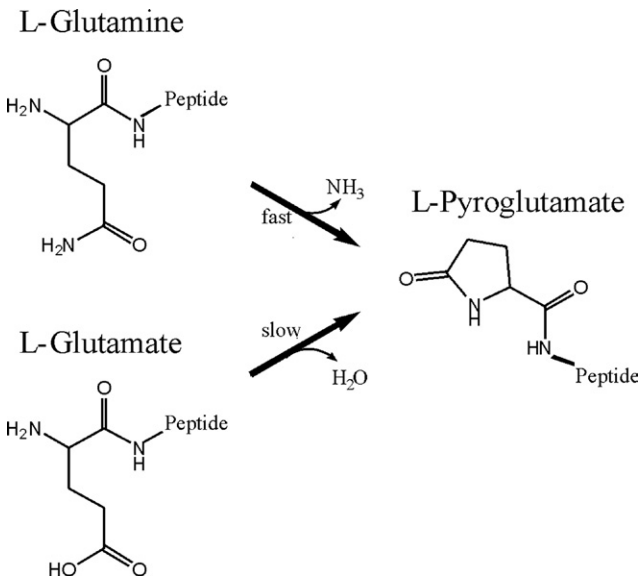

FIGURE 1 Cyclization of N-terminal glutaminyl- and glutamyl residues into pyroglutamate.

Mammalian QCs are monomeric glycoproteins of approximately 40 kDa. QCs are primarily expressed in neuronal tissue, such as cerebral cortex, striatum, and hippocampus.[5] In addition, QC expression and activity were identified in a broad range of cell lines in culture.[7]

QC is directed to the endoplasmatic reticulum (ER) by an N-terminal signal peptide. The enzyme passes the Golgi complex, is packed into secretory granules, and transported to the plasma membrane, where it is secreted from the cell. In hypothalamus, QC is colocalized with its substrates TRH and GnRH in dense core secretory granules. Apparently, those are transported together along the tractus hypothalamo hypophysialis.[3] Similarly, studies point toward cotransport and cosecretion of QC and Aβ-peptides from the entorhinal cortex to the hippocampus. The mechanism might have implications for formation of pGlu-Aβ containing deposits in Alzheimer's disease.[12] Support for the cosecretion and conversion of QC substrates within secretory granules is also obtained from QC knockout mice. Homozygous mice are fertile and do not show any overt abnormalities. The animals show a mild form of hypothyreodism, which is probably caused by reduced TRH production. Other hormonal cascades are apparently not affected, suggesting a therapeutic window for QC inhibition and clinical applications.[24]

Animal QCs are single-zinc metalloenzymes, which are inactivated by metal-chelating compounds such as 1, 10-phenantroline or dipicolinic acid. Animal QCs and the bacterial aminopeptidases (MEROPS ID: clan MH, family M28) share a conserved zinc-binding motif (H-D-E-D-H) (Fig. 2). However, in contrast to QCs, two zinc ions are coordinated in the active site of the aminopeptidases. The presence of one zinc ion in the active site of QC was confirmed by total X-ray fluorescence spectroscopy (TXRF), atomic absorption spectroscopy and X-ray crystallography.[14,27] The metal ion is coordinated by D^{159}, E^{202}, and H^{330} in human QC. The zinc ion represents the primary interaction site of competitive QC inhibitors. The most potent compounds contain imidazole or benzimidazole moieties as metal-binding groups. The interaction between the imidazole and the active site zinc ion was confirmed by cocrystallization of QC with imidazole derivatives such as 1-vinylimidazole.[14]

A characteristic feature of QCs from plants and animals is relaxed substrate specificity. The substrate spectrum ranges from peptide surrogates, such as glutamine-β-naphthylamine to protein substrates, for example, CCL2(1–76).[4,26] Moreover, QC also converts β-homoglutamine or glutamic acid. In contrast to glutamine, however, the conversion of glutamate into pyroglutamate proceeds with significantly reduced velocity. Apparently, N-terminal glutamic acid is only converted with uncharged γ-carboxyl and α-amino groups. The reaction shows a pH optimum at pH 5.2–6.5, which contrasts with the mildly basic pH optimum of QC-catalyzed cyclization of glutamine.[23]

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Iso-Glutaminyl Cyclase (isoQC, QPCTL, EC 2.3.2.5)

The presence of a QC isoenzyme was postulated for bovine spleen first; however, an enzyme could not be isolated. A genomic homology search led to the identification and isolation of different QC isoforms from *D. melanogaster*, highlighting the possibility of the presence of mammalian QC isoforms.[25] Consequently, an isoenzyme of human and mouse QC (isoQC) was isolated and characterized.[5] Human QC and isoQC represent homologous proteins sharing a sequence identity of 45%. The catalytically important zinc-binding motif (D–E–H) is conserved in all mammalian isoQCs (Fig. 2). Inhibition studies using heterocyclic metal-binding compounds supported a zinc-dependent catalysis of isoQCs, which was later substantiated by TXRF analysis and X-ray crystallography.[34] Likewise, the X-ray structure of QC and isoQC shows conspicuous similarity.[13]

Interestingly, the N-terminal parts of QC and isoQC display rather low homology mediating differential subcellular localizations of the sister enzymes. Initial studies suggest retention of isoQC in the Golgi complex, which is mediated by a signal anchor. By contrast, the N-terminus of QC contains a secretion signal, which is cleaved in the mature, secreted protein.[5] (Fig. 2).

Similar to QC, isoQC shows broad substrate specificity *in vitro*, although the absolute catalytic proficiency is lower. Studies *in vivo* suggest, however, a differential substrate

```
hQC             --MAGGRHRRVVGTLHLLLLVAALP--------WASRGVSPSASAWPEEKNYHQPAILNS
h-isoQC         MEPLLPPKRRLLPRVRLLPLLLALAVGSAFYTIWSGWHRRTEELPLGRELRVPLIGSLPE
SgAP            ----------------------------------------------------APDIPL
VpAP            ----MPPITQQATVTAWLPQVDASQITGT----------------------------IS

hQC             SALRQIAEGTSISEMWQNDLQPLLIERYPGSPGSYAARQHIMQR---IQRLQADWVLEID
h-isoQC         ARLRRVVGQLDPQRLWSTYLRPLLVVRTPGSPGNLQVRKFLEAT---LRSLTAGWHVELD
SGAP            ANVKAHLTQL------STIAANNGGNRAHGRPGYKASVDYVKAK---LDA--AGYTTTLQ
VpAP            -------------------SLESFTNRFYTTTSGAQASDWIASEWQALSA--SLPNASVK
                                          *    ..     . :      :    :     :.

hQC             TFLSQTPYGYRSFSNIISTLNPT-AKRHLVLACHYDSKYFSHWNNR-VFVGATDSAVPCA
h-isoQC         PFTASTPLGPVDFGNVVATLDPR-AARHLTLACHYDSKLFPP-GST-PFVGATDSAVPCA
SGAP            QFTSGGATGYNLIAN---WPGGD-PNKVLMAGAHLDSVS--------SGAGINDNGSGSA
VpAP            QVSHSGYNQ---KSVVMTITGSEAPDEWIVIGGHLDSTIGSHTNEQSVAPGADDDASGIA
                 .          .     .   . . :  . * **            *  *..   *

hQC             MMLELARALDKKLLSLKTVSDSKPDLSLQLIFFDGEEAFLHWSPQDSLYGSRHLAAKMAS
h-isoQC         LLLELAQALD---LELSRAKKQAAPVTLQLLFLDGEEALKEWGPKDSLYGSRHLAQLMES
SGAP            AVLETALAVSR--------AGYQPDKHLRFAWWGAEEL--------GLIGSKFYVNNLPS
VpAP            AVTEVIRVLSE--------NNFQPKRSIAFMAYAAEEV--------GLRGSQDLANQYKS
                 : *   .:.            .   : :    .**         .* **:  .    *

hQC             TPHPPGARGTSQLHGMDLLVLLDLI---GAPNPTFPNFFPNSARWFERLQAIEHELHELG
h-isoQC         IPHSP---GPTRIQAIELFMLLDLL---GAPNPTFYSHFPRTVRWFHRLRSIEKRLHRLN
SGAP            ADRS----------KLAGYLNFDMI---GSPNPGYFVYDDDPV--------IEKTFKNYF
VpAP            EGKN-----------VVSALQLDMTNYKGSAQDVVFITDYTDS--------NFTQYLTQ-
                  :            :   : :*:    *:.:

hQC             LLKDHSLEGRYFQNY----SYGGVIQDDHIPFLRRGVPVLHLIP----------------
h-isoQC         LLQSHPQEVMYFQPG----EPSGSVEDDHIPFLRRGVPVLHLIS----------------
SGAP            AGLNVPTEI----------ETEGDGRSDHAPFKNVGVPVGGLFTGAGYTKSAAQAQKWGG
VpAP            ------LMDEYLPSLTYGFDTCGYACSDHASWHNAGYPAAMPFE----------------
                                   .  *   .** .: . * *.   :

hQC             ---SPF----PEVWHTMDDNEENLDESTIDNLNKILQVFVLEYLHL-------------
h-isoQC         ---TPF----PAVWHTPADTEVNLHPPTVHNLCRILAVFLAEYLGL-------------
SGAP            TAGQAF----DRCYHSSCDSLSNINDTALDR-NSDAAAHAIWTLSS--GTGEPPT----
VpAP            ---SKFNDYNPRI-HTTQDTLANSDPTGSHA-KKFTQLGLAYAIEMGSATGDTPTPGNQ
                     *        *:  *.  * . .  .            :
```

FIGURE 2 Alignment of human QC (hQC) and human isoQC (h-isoQC) with two prototypes of zinc-dependent aminopeptidases from *Streptomyces griseus* (SGAP) and *Vibrio proteolytica* (VpAP). Note the conserved zinc-binding motif (H–D–E–D–H) in the human and bacterial sequences. In contrast to bacterial enzymes, hQC and h-isoQC complexes only one zinc ion in the active site. Complexation is fulfilled by an aspartyl-, a glutamyl, and a histidinyl residue.

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conversion by QC and isoQC. For instance, by using QC and isoQC knockout mice, it could be shown that pGlu-CCL2 is generated by isoQC. Apparently, QC does not contribute to CCL2 maturation. Although further studies are required, the subcellular localization and substrate processing appear to significantly influence whether a substrate is converted by QC or isoQC.[4] A comparison of isoQC expression in different tissues reveals an equal distribution among CNS and peripheral tissues contrasting the pronounced expression of QC in neuronal tissue. Many pGlu-hormones are formed in the late secretory pathway, requiring prohormone convertase cleavage before N-terminal cyclization. Because secretory granules apparently do not contain isoQC, pGlu-hormone maturation might represent the major function of QC. By contrast, isoQC topology shows similarities to glycosyltransferases, with a short cytosolic N-terminus, a transmembrane helix and a large luminal catalytic domain suggesting a major function of isoQC in ubiquitous, "house-keeping" protein maturation.

Although the formation of pGlu is obviously catalyzed in plants, bacteria, and animals, N-terminal glutaminyl residues tend to form pGlu spontaneously. *In vitro* studies reveal a rate of 10^{-6} s^{-1} or even higher, which suggests half-lives of hours to days.[32] The nonenzymatic cyclization of glutamine might be accelerated by anions such as phosphate or borate. The spontaneous cyclization of glutamine probably causes a residual pGlu formation even in the excess of specific inhibitors in cell culture experiments.[4] In comparison with glutamine, the spontaneous cyclization of N-terminal glutamic acid proceeds at rates, which are three orders of magnitude lower. QC accelerates this reaction by a factor of 2.2×10^5 revealing a lower but reliable proficiency for glutamate substrates *in vitro*.[32]

The spontaneous cyclization of glutaminyl precursors, resulting in a formation of pGlu-peptides and proteins independent of QC or isoQC activity, probably contributes to the very mild phenotypes of the knockout mouse models. These studies support, in turn, current endeavors to develop inhibitors of the enzymes as therapeutics in neurodegenerative disorders.

REFERENCES

1. Abraham GN, Podell DN. Pyroglutamic acid. Non-metabolic formation, function in proteins and peptides, and characteristics of the enzymes effecting its removal. *Mol Cell Biochem* 1981;**38**(Spec No (Pt 1)):181–90.
2. Bateman A, Solomon S, Bennett HP. Post-translational modification of bovine pro-opiomelanocortin. Tyrosine sulfation and pyroglutamate formation, a mass spectrometric study. *J Biol Chem* 1990;**265**(36):22130–6.
3. Bockers TM, Kreutz MR, Pohl T. Glutaminyl-cyclase expression in the bovine/porcine hypothalamus and pituitary. *J Neuroendocrinol* 1995;**7**(6):445–53.
4. Cynis H, Hoffmann T, Friedrich D, Kehlen A, Gans K, Kleinschmidt M, et al. The isoenzyme of glutaminyl cyclase is an important regulator of monocyte infiltration under inflammatory conditions. *EMBO Mol Med* 2011;**3**(9):545–58.
5. Cynis H, Rahfeld JU, Stephan A, Kehlen A, Koch B, Wermann M, et al. Isolation of an isoenzyme of human glutaminyl cyclase: retention in the golgi complex suggests involvement in the protein maturation machinery. *J Mol Biol* 2008;**379**:966–80.
6. Cynis H, Scheel E, Saido TC, Schilling S, Demuth HU. Amyloidogenic processing of amyloid precursor protein: evidence of a pivotal role of glutaminyl cyclase in generation of pyroglutamate-modified amyloid-beta. *Biochemistry* 2008;**47**(28):7405–13.
7. Cynis H, Schilling S, Bodnar M, Hoffmann T, Heiser U, Saido TC, et al. Inhibition of glutaminyl cyclase alters pyroglutamate formation in mammalian cells. *Biochim Biophys Acta* 2006;**1764**:1618–25.
8. Dahl SW, Slaughter C, Lauritzen C, Bateman Jr RC, Connerton I, Pedersen J. Carica papaya glutamine cyclotransferase belongs to a novel plant enzyme subfamily: cloning and characterization of the recombinant enzyme. *Protein Expr Purif* 2000;**20**(1):27–36.
9. Garden RW, Moroz TP, Gleeson JM, Floyd PD, Li L, Rubakhin SS, et al. Formation of N-pyroglutamyl peptides from N-Glu and N-Gln precursors in Aplysia neurons. *J Neurochem* 1999;**72**(2):676–81.
10. Ghiso J, Vidal R, Rostagno A, Mead S, Revesz T, Plant G, et al. A newly formed fragment due to a stop codon mutation causes familial British dementia. *Ann N Y Acad Sci* 2000;**903**:129–37.
11. Ghiso JA, Holton J, Miravalle L, Calero M, Lashley T, Vidal R, et al. Systemic amyloid deposits in familial British dementia. *J Biol Chem* 2001;**276**(47):43909–14.
12. Hartlage-Rubsamen M, Morawski M, Waniek A, Jager C, Zeitschel U, Koch B, et al. Glutaminyl cyclase contributes to the formation of focal and diffuse pyroglutamate (pGlu)-Abeta deposits in hippocampus via distinct cellular mechanisms. *Acta Neuropathol* 2011;**121**(6):705–19.
13. Huang KF, Liaw SS, Huang WL, Chia CY, Lo YC, Chen YL, et al. Structures of human golgi-resident glutaminyl cyclase and its complexes with inhibitors reveal a large loop movement upon inhibitor binding. *J Biol Chem* 2011;**286**(14):12439–49.
14. Huang KF, Liu YL, Cheng WJ, Ko TP, Wang AH. Crystal structures of human glutaminyl cyclase, an enzyme responsible for protein N-terminal pyroglutamate formation. *Proc Natl Acad Sci U S A* 2005;**102**(37):13117–22.
15. Pawlak J, Manjunatha KR. Snake venom glutaminyl cyclase. *Toxicon* 2006;**48**(3):278–86.
16. Pimenta AM, Rates B, Bloch Jr C, Gomes PC, Santoro MM, de Lima ME, et al. Electrospray ionization quadrupole time-of-flight and matrix-assisted laser desorption/ionization tandem time-of-flight mass spectrometric analyses to solve micro-heterogeneity in post-translationally modified peptides from *Phoneutria nigriventer* (Aranea, Ctenidae) venom. *Rapid Commun Mass Spectrom* 2005;**19**(1):31–7.
17. Pohl T, Zimmer M, Mugele K, Spiess J. Primary structure and functional expression of a glutaminyl cyclase. *Proc Natl Acad Sci U S A* 1991;**88**(22):10059–63.
18. Proost P, Struyf S, Couvreur M, Lenaerts JP, Conings R, Menten P, et al. Posttranslational modifications affect the activity of the human monocyte chemotactic proteins MCP-1 and MCP-2: identification of MCP-2(6-76) as a natural chemokine inhibitor. *J Immunol* 1998;**160**(8):4034–41.

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19. Rabel D, Charlet M, Ehret-Sabatier L, Cavicchioli L, Cudic M, Otvos Jr L, et al. Primary structure and in vitro antibacterial properties of the *Drosophila melanogaster* attacin C Pro-domain. *J Biol Chem* 2004;**279**(15):14853–9.
20. Saido TC, Iwatsubo T, Mann DM, Shimada H, Ihara Y, Kawashima S. Dominant and differential deposition of distinct beta-amyloid peptide species, a beta N3(pE), in senile plaques. *Neuron* 1995;**14**(2):457–66.
21. Schilling S, Appl T, Hoffmann T, Cynis H, Schulz K, Jagla W, et al. Inhibition of glutaminyl cyclase prevents pGlu-Abeta formation after intracortical/hippocampal microinjection in vivo/in situ. *J Neurochem* 2008;**106**(3):1225–36.
22. Schilling S, Cynis H, von Bohlen A, Hoffmann T, Wermann M, Heiser U, et al. Isolation, catalytic properties, and competitive inhibitors of the zinc-dependent murine glutaminyl cyclase. *Biochemistry* 2005;**44**(40):13415–24.
23. Schilling S, Hoffmann T, Manhart S, Hoffmann M, Demuth HU. Glutaminyl cyclases unfold glutamyl cyclase activity under mild acid conditions. *FEBS Lett* 2004;**563**(1–3):191–6.
24. Schilling S, Kohlmann S, Baeuscher C, Sedlmeier R, Koch B, Eichentopf R, et al. Glutaminyl cyclase (QC) knock out mice show mild hypothyreodism but absence of hypogonadism: implications for enzyme function and drug development. *J Biol Chem* 2011;**286**(16): 14199–208.
25. Schilling S, Lindner C, Koch B, Wermann M, Rahfeld JU, Bohlen AV, et al. Isolation and characterization of glutaminyl cyclases from drosophila: evidence for enzyme forms with different subcellular localization. *Biochemistry* 2007;**46**(38):10921–30.
26. Schilling S, Manhart S, Hoffmann T, Ludwig HH, Wasternack C, Demuth HU. Substrate specificity of glutaminyl cyclases from plants and animals. *Biol Chem* 2003;**384**(12):1583–92.
27. Schilling S, Niestroj AJ, Rahfeld JU, Hoffmann T, Wermann M, Zunkel K, et al. Identification of human glutaminyl cyclase as a metalloenzyme. Potent inhibition by imidazole derivatives and heterocyclic chelators. *J Biol Chem* 2003;**278**(50):49773–9.
28. Schilling S, Stenzel I, von Bohlen A, Wermann M, Schulz K, Demuth HU, et al. Isolation and characterization of the glutaminyl cyclases from *Solanum tuberosum* and *Arabidopsis thaliana*: implications for physiological functions. *Biol Chem* 2007;**388**(2):145–53.
29. Schilling S, Zeitschel U, Hoffmann T, Heiser U, Francke M, Kehlen A, et al. Glutaminyl cyclase inhibition attenuates pyroglutamate Abeta and Alzheimer's disease-like pathology. *Nat Med* 2008;**14**(10):1106–11.
30. Schmutzler C, Darmer D, Diekhoff D, Grimmelikhuijzen CJ. Identification of a novel type of processing sites in the precursor for the sea anemone neuropeptide Antho-RFamide (<Glu-Gly-Arg-Phe-NH_2) from *Anthopleura elegantissima*. *J Biol Chem* 1992;**267**(31): 22534–41.
31. Sealfon SC, Weinstein H, Millar RP. Molecular mechanisms of ligand interaction with the gonadotropin-releasing hormone receptor. *Endocr Rev* 1997;**18**(2):180–205.
32. Seifert F, Schulz K, Koch B, Manhart S, Demuth HU, Schilling S. Glutaminyl cyclases display significant catalytic proficiency for glutamyl substrates. *Biochemistry* 2009;**48**(50):11831–3.
33. Song I, Chuang CZ, Bateman Jr RC. Molecular cloning, sequence analysis and expression of human pituitary glutaminyl cyclase. *J Mol Endocrinol* 1994;**13**(1):77–86.
34. Stephan A, Wermann M, von Bohlen A, Koch B, Cynis H, Demuth HU, et al. Mammalian glutaminyl cyclases and their isoenzymes have identical enzymatic characteristics. *FEBS J* 2009;**276**(22):6522–36.

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Chapter 239

In Silico Search for Biologically Active Peptides

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ABSTRACT

Reverse pharmacology and orphan receptor strategy have successfully unraveled many endogenous compounds including peptides for orphan GPCRs, combined with a variety of screening assays. However, there are still >100 orphan GPCRs, and their ligands and functions remain to be identified for fundamental and clinical research. Many screening methods to deorphanize orphan GPCRs have so far been developed and successfully used, while few practical methods to predict novel ligand candidates have been reported. A potentially useful approach to predict bioactive peptide candidates may be the use of bioinformatics, based on information of public database of protein amino acid sequences derived from human genome sequence project. In this chapter, we describe an outline for systematic in silico prediction of bioactive peptide candidates with a C-terminal amide.

BACKGROUND OF GPCR DEORPHANIZATION

According to the reported data of the human genome sequence, approximately 800 genes are indicated to encode G protein-coupled receptors (GPCRs).[9,22] Excluding presumed odorant receptors and pheromone receptors (~400 GPCRs), there exist around 400 typical GPCRs in the human genome. Among them, about 100 GPCRs, whose endogenous ligands are still unknown, are called "orphan receptors."[1,4,6,8,23] A wide variety of chemicals are identified as ligands for GPCRs, including amines, amino acids, peptides, proteins, nucleosides, nucleotides, Ca^{2+} ions, and lipids. Among GPCRs for known ligands, the ratio of peptide receptors to nonpeptide receptors is approximately 90:100.[1] If the same ratio is assumed among orphan receptors, potentially about 50 peptide receptors may remain to be deorphanized, although it might be a little overestimated.

Formerly, the conventional approach of GPCR investigation was the first to discover natural ligands, then to search and identify their receptors, next to analyze the characteristics of the various compounds that inhibit binding of the ligand to the receptor, and finally to develop novel therapeutics relating to the receptor. However, in recent years, other strategies have been widely accepted in orphan receptor research. One such approach is to search the ligands in natural tissue extracts by monitoring responses of cells expressing orphan receptors (orphan receptor strategy),[11,15–17] and another one is to identify the ligands by combining orphan receptors and chemically known compounds whose receptors are unknown (reverse pharmacology strategy).[2,3,21] Both approaches have been successfully applied so far. In these 15 years, ligands for >60 GPCRs have been newly identified. Hunting of endogenous ligands for the remainder of the orphan GPCRs now becomes the target of intense worldwide severe competition between pharmaceutical industries, universities, and academic institutes, to develop novel therapeutic agents.[4,8,23] About 45% of the top selling drugs target GPCRs.[24]

In the usual methods to search for peptide ligands, extracts from animal tissues are used as sources of peptides. However, in the cases of peptides whose content in tissues is extraordinarily low, or which are expressed only at extremely limited sites, this method with tissue extracts may fail to identify the endogenous ligands. Peptides existing abundantly in tissues with potent biological activities may have already been identified easily, so that peptides with only trace or unique activities remain unknown.

PREDICTION OF BIOACTIVE PEPTIDES: BIOSYNTHETIC PATHWAY OF BIOACTIVE PEPTIDES AND THE EMPIRICAL CLEAVAGE RULES

N-terminal and C-terminal Structures of Bioactive Peptides

Bioactive peptides, including peptide hormones, neuropeptides, and neurotransmitters, are initially synthesized

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00239-6
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as precursor proteins, usually called "preprohormones." In this chapter, we use the term "preprohormone" for precursors of all kinds of bioactive peptides that may have various sizes and functional properties. Each preprohormone is cleaved into the signal peptide and the remaining part, "prohormone," containing bioactive peptide sequences. The presence of the signal peptide suggests that bioactive peptides are secreted to play physiological roles. The prohormones are further processed by proprotein convertases and carboxylpeptidases to yield mature bioactive peptides (Fig. 1). Although each prohormone possesses a distinct primary sequence, proteolytic processing occurs at dibasic amino acid residue sites that commonly flank the amino (N)- and carboxyl (C)-termini of bioactive peptides within their precursors. Then, the dibasic residues at the C-terminal are removed by carboxylpeptidases to produce mature bioactive peptides. Lys-Arg (KR) or Arg-Arg (RR) are the dibasic pairs most often found at the cleavage sites of known bioactive peptides.[7,18] However, processing also occurs at the dibasic residue sites Lys-Lys and sometimes Arg-Lys (Table 1). Processing occurs at monobasic Arg sites in certain cases, such as procholecystokinin, provasopressin, and prosomatostatin. For bioactive peptides with a C-terminal α-amide, including thyrotropin-releasing hormone, substance P, gastrin, oxytocin, and vasopressin, a specific sequence, Gly-Lys-Arg (GKR) or Gly-Arg-Arg (GRR) is located at the C-terminal sites of the bioactive peptides (Fig. 1). The glycine (Gly) residue in these sequences is catalyzed by amidation enzymes (peptidylglycine α-amidating monooxygenase and peptidylamido-glycolate lyase)[5,14] and serves as the amide donor for C-terminal amidation, that is essential for exerting most biological activity.

Biochemical Characteristics of Bioactive Peptides and Their Precursors

To predict bioactive peptides by in silico methods, we introduced several criteria for the characteristics of preprohormones deduced from full-length cDNA and genomic DNA sequences. (1) Each preprohormone should contain a signal peptide at the N-terminal site. (2) Preprohormones should not have membrane-spanning motifs in their sequences. (3) Peptide candidates produced from preprohormones should belong to the following six groups.

We classified bioactive peptides into six groups based on their specific sequences of the N-terminal and C-terminal sites franking mature bioactive peptides (Table 2). In type A, each peptide contains a most frequently cleaved signal, the dibasic pair (KR or RR) at both N- and C-terminal sites. Type B peptides contain an N-terminal dibasic pair and an amide signal, GKR or GRR, at the C-terminal. As shown in the following section, in the final version of the criteria in our in silico search of bioactive peptides, the dibasic pairs of KR and RR are accepted as processing sites and those of KK

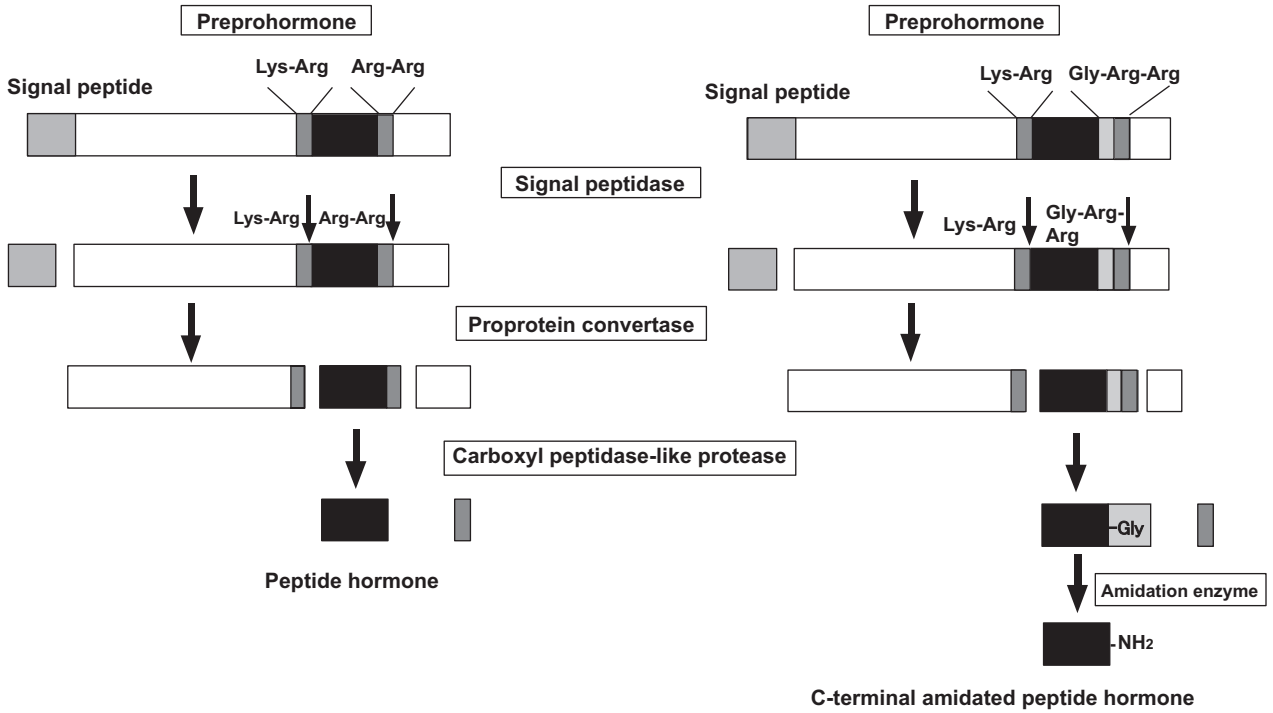

FIGURE 1 A schematic representation of preprohormone precursors and their posttranslational processing. Arrows indicate where prohormone convertases cleave the C-terminal side of a dibasic pair site (Lys-Arg, Arg-Arg, or Gly-Arg-Arg). C-terminal amides of mature peptide hormones are produced from Gly by amidation enzymes (peptidylglycine a-amidating monooxygenase and peptidylamido-glycolate lyase).

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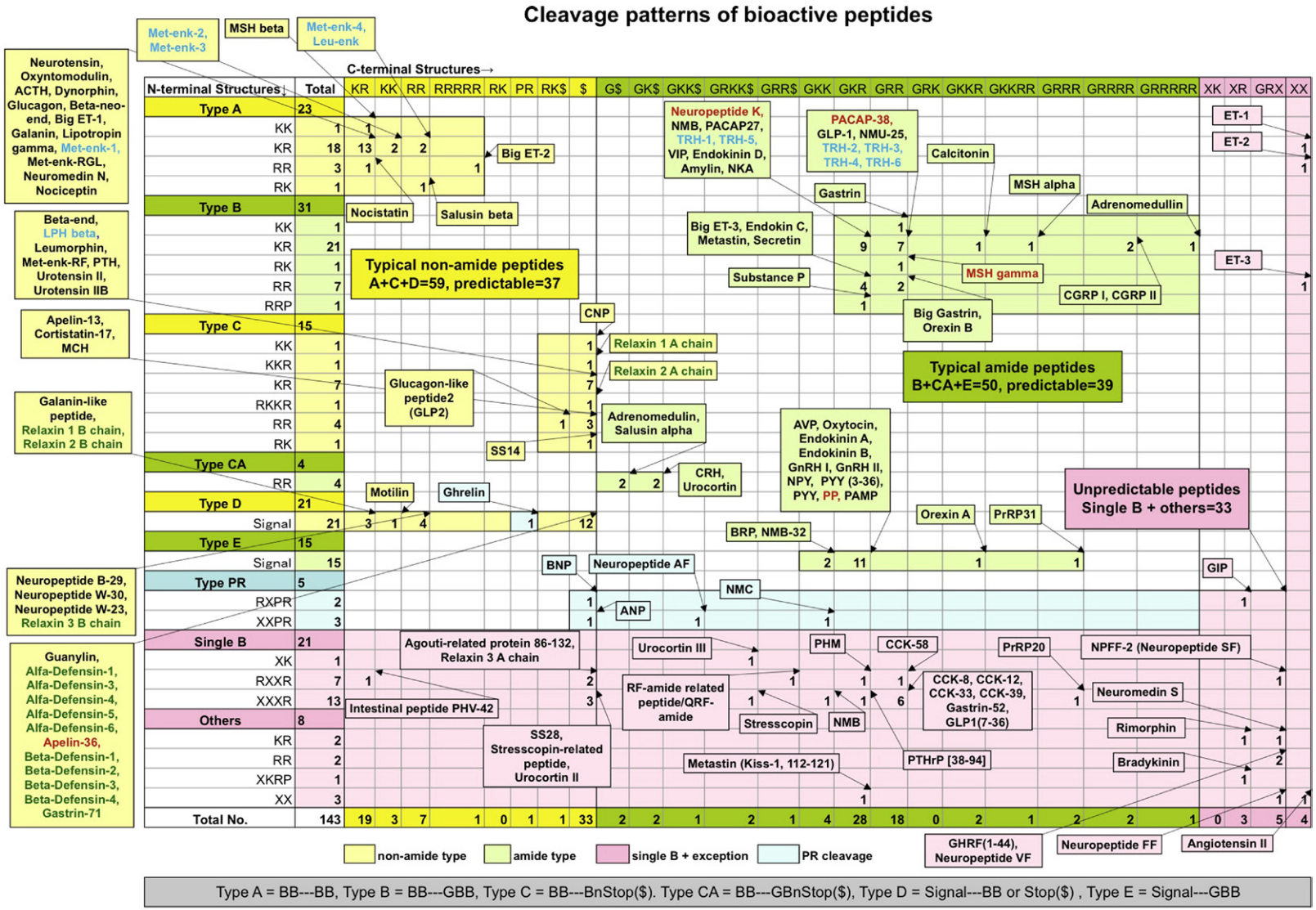
TABLE 1 Processing Patterns of Known Bioactive Peptides



Peptides written in red, blue, or green are difficult to predict, because those written in red contain KR and RR in the molecule, those written in blue are ≤5 or ≥61 amino acids in length, and those written in green contain more than 6 cysteine residues.

See color plate 62.

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TABLE 2 Classification of Bioactive Peptides with a Dibasic Pair at the N- or C-terminal

Cleavage type	Pattern*	Step 1	Step 2	Step 3	Known bioactive peptides†
Type A	([KR]R-**peptide**-[KR]R)	15,848	3,815	327	3
Type B	([KR]R-**peptide**-G[KR]R)-**amide**	1,789	1,789	170	16
Type C	([KR]R-**peptide**-[KR]n-STOP)	7,425	916	130	5
Type CA	([KR]R-**peptide**-G[KR]n-STOP)-**amide**	638	638	78	3
Type D	(signal peptide-**peptide**-[KR]R)	5,896	726	108	0
Type E	(signal peptide-**peptide**-G[KR]R)-**amide**	709	709	104	13
	No. of bioactive peptide candidates	32,302	8,593	917	40
	(C-terminal amide type peptides)	3,136	3,136	352	32
	(Non-C-terminal amide type peptides)	29,168	5,457	565	8

Step 1: 6 ≤ **peptides** ≤60, **precursors** ≤ 300, Cys residues (0, 2, or 4).
Step 2: calculated from precursors with a C-terminal amide (including sequence overlapping).
Step 3: Lys-Arg and Arg-Arg in mature peptide sequences, no overlapping sequences.
**G = Gly, K = Lys, R = Arg, [KR] = K or R,* n = *0-n.*
†Predicted numbers of known bioactive peptides.

and RK are omitted, because our analyses on the sequences of about 140 known bioactive peptides precursors showed that most of the peptides are processed at the dibasic pairs KR or RR, and not KK or RK at N- and C-terminal cleavage sites (Table 1). Similarly, only GKR and GRR are accepted as C-terminal cleavage sites of amide type peptides. On the other hand, the four types of dibasic pairs are found almost equally in the population of peptide candidates simply extracted in silico from the DNA databases. Type C peptides and type CA peptides contain an N-terminal dibasic pair and a stop signal at C-terminal. Type D peptides possess a signal peptide at N-terminal and a dibasic pair at C-terminal. Similarly, type E peptides possess a signal peptide and a C-terminal amide signal. Further, analysis of known bioactive peptides indicates their biochemical properties; (1) preprohormones consist of 50–300 amino acid residues, (2) most of the mature bioactive peptides are 6–60 amino acids in length (Fig. 2) and contain 0, 2, or 4 Cys residue(s).

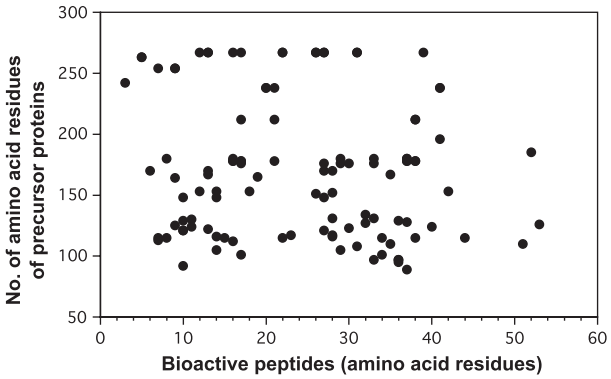

FIGURE 2 Relationship of the number of amino acid residues between mature peptide hormone and their preprohormone precursors. The number of amino acid residues of 94 peptide hormones and their precursors are indicated. Most of peptide hormones and their precursors contain <60 and 300 amino acid residues, respectively.

PREDICTION OF BIOACTIVE PEPTIDE CANDIDATES

Extraction of Protein Precursors

We used sequence information from three databases, Ensembl, IPI (International Protein Index), and H-InvDB (H-Invitational Database) (Table 3). In February 2006, we obtained about 25 million protein sequences with overlapping. Removal of overlapped sequences gave us about 200,000 protein sequences. Next, to extract the probable secreted precursors, we further applied the two criteria into our original soft program; (1) preprohormones contain a signal peptide, judged by SignalP and (2) contain no membrane-spanning region, judged by their membrane retention signal analyses. We finally obtained 33,119 proteins as starting precursor proteins for in silico prediction of bioactive peptide candidates (Table 3).

Extraction of Bioactive Peptide Candidates by Pattern Matching Methods

We started in silico analysis using 33,119 protein sequences to obtain bioactive peptide candidates matching to six groups of bioactive peptides (Table 2). We first tried a pattern matching analysis with a simple criterion that bioactive

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TABLE 3 Number of Prepropeptide Precursors Found in Three Databases (February, 2006)

Database	Precursors downloaded	Precursors without overlapping	Precursors with a signal peptide and without membrane spanning sequence
Ensembl*			
IPI (International Protein Index)†	24,535,323	197,080	33,119
H-InvDB (H-Invitational Database)**			

*http://hinvdb.ddbj.nig.ac.jp/ahg-db/ChgLang.do?lang=en&path=/index.jsp
†http://www.ebi.ac.uk/IPI/IPIhelp.html
**http://www.ensembl.org/index.html

peptides contain dibasic residues (KR, RR, RK, KK) at their N- and C-terminal sites. Surprisingly, enormous numbers of about 300,000 peptide candidates were obtained, suggesting that we need the more specific criteria based on biochemical features empirically obtained from biosynthetic pathways (Table 1). Next, we introduced several criteria into the computer program; (1) 6 ≤ amino acid residues of bioactive peptides ≤60 amino acids in length, (2) 50 ≤ amino acid residues of precursor proteins ≤300 amino acids in length, (3) number of Cys residues in a peptide sequence is 0, 2, or 4. These criteria gave us 32,302 peptide candidates with overlapping (C-terminal amide-type peptides 3,136; non-C-terminal amide-type 29,168) (step 1 in Table 2). We judged that these candidate numbers are still too many for making peptide libraries by chemical synthesis. Thus, we tried to limit the candidate proteins to those containing peptides with C-terminal amide sequences, for example, GKR, GRR, and G-stop (step 2 in Table 2). Further, we added two criteria; (4) bioactive peptide candidates not containing dibasic pair sequences (KR, RR, KK, and RK) within their mature peptide sequences, and (5) N- and C-terminal cleavage sites are KR or RR (as mentioned above, step 3 in Table 1). Finally, 352 C-terminal amide-type and 565 non-C-terminal amide-type peptide candidates were obtained (step 3 in Table 2). Among these predicted candidates, 32 peptides of C-terminal amide type and 8 peptides of the nonamide type matched known bioactive peptides, indicating that the prediction method described above may be practically useful. The number of finally predicted candidates may be acceptable for preparing a synthetic peptide library for screening. A part of these predicted peptides is now commercially available from PharmaDesign, Inc. (PharmaGPEP series, http://www.pharmadesign.co.jp/eng/index.html).

PERSPECTIVES

Screening methods used to search for biologically active ligand among the predicted peptide candidates were not described in this article because of the limited space. In signaling of many orphan GPCRs, it is difficult to specify the responsible type(s) of G protein α subunits and to choose adequate assay procedures. Therefore, multiple assay procedures are often combined for screening. Identification of a true ligand for an orphan receptor and elucidation of its physiological and pathophysiological roles require that researchers have much patience.

Discovery of novel peptide ligands is decreasing in recent years. It has also been pointed out by some investigators that an orphan GPCR and a known receptor can form a heterodimer. If so, in such situations, the orphan receptor itself might have no specific endogenous ligand and only serve as a regulatory protein through the dimer formation.[12,13] However, as described above, through the systematic in silico prediction method based on the information of the opened protein database, the number of amide type peptides predicted as GPCR ligand candidates may not be too many. A combination of well-established expression systems of orphan GPCRs and adequately tuned assay procedures may hopefully lead to the discovery of novel ligands. In contrast, as to nonamide type peptide candidates predicted from the systematic in silico search, the number of the predicted peptides are still too many for practical screening, and future development of more effective and more reliable prediction methods is awaited to find a more limited number of peptide candidates of good quality. For the detection of endogenous bioactive peptides whose tissue content is very low, development of multiple assay systems has been improved in recent years, and for identification of the detected peptides, technical sensitivity has been increased dramatically by the introduction of mass spectrometry. However, to overcome the difficulties for peptides whose tissue content is very low, or which is expressed at very limited sites, it may be necessary to improve in silico prediction methods by bioinformatics.[10,13,19,20]

Because identification of novel ligands of orphan GPCRs is inevitable to clarify a novel mechanism in biological regulation, continuous efforts are required to discover novel ligands. For this purpose, improved methods to predict peptide ligand candidates, together with improved screening technique, may be a powerful tool for researchers.

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REFERENCES

1. Alexander SPH, Mathie A, Peters JA. 7TM receptors. In: Guide to Receptors and Channels (GRAC), 4th ed. *Br J Pharmacol* 2009; 158:(Suppl. 1):S5–101.
2. Ames RS, Sarau HM, Chambers JK, Willette RN, Aiyar NV, Romanic AM, et al. Human urotensin-II is a potent vasoconstrictor and agonist for the orphan receptor GPR14. *Nature* 1999;**401**: 282–6.
3. Boels K, Schaller HC. Identification and characterisation of GPR100 as a novel human G protein-coupled bradykinin receptor. *Br J Pharmacol* 2003;**140**:932–8.
4. Civelli O. GPCR deorphanizations: the novel, the known and the unexpected transmitters. *Trends Pharmacol Sci* 2005;**26**:15–9.
5. Eipper BA, Milgram SL, Husten EJ, Yun HY, Mains RE. Peptidylglycine alpha-amidating monooxygenase: a multifunctional protein with catalytic, processing, and routing domains. *Protein Sci* 1993;**2**:489–97.
6. Foord SM. Receptor classification: post genome. *Curr Opin Pharmacol* 2002;**2**:561–6.
7. Hook V, Funkelstein L, Lu D, Bark S, Wegrzyn J, Hwang SR. Proteases for processing proneuropeptides into peptide neurotransmitters and hormones. *Annu Rev Pharmacol Toxicol* 2008;**48**:393–423.
8. Howard AD, McAllister G, Feighner SD, Liu Q, Nargund RP, Van der Ploeg LH, et al. Orphan G protein-coupled receptors and natural ligand discovery. *Trends Pharmacol Sci* 2001;**22**:132–40.
9. International Human Genome Sequencing Consortium. Initial sequencing and analysis of the human genome. *Nature* 2001;**409**:860–921.
10. Kliger Y. Computational approaches to therapeutic peptide discovery. *Biopolymers* 2010;**94**:701–10.
11. Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H, Kangawa K. Ghrelin is a growth-hormone-releasing acylated peptide from stomach. *Nature* 1999;**402**:656–60.
12. Levoye A, Dam J, Ayoub MA, Guillaume JL, Jockers R. Do orphan G protein-coupled receptors have ligand-independent functions? New insights from receptor heterodimers. *EMBO Rep* 2006;**7**:1094–8.
13. Levoye A, Clement N, Tenconi E, Jockers R. Past and future strategies for GPCR deorphanization. In: Gilchrist A, editor. *GPCR molecular pharmacology and drug targeting, shifting paradigms and new directions*. New Jersey: John Wiley & Sons Inc; 2010. p.165–90.
14. Nakayama K. Furin: a mammalian subtilisin/Kex2p-like endoprotease involved in processing of a wide variety of precursor proteins. *Biochem J* 1997;**327**:625–35.
15. Reinscheid RK, Nothacker HP, Bourson A, Ardati A, Henningsen RA, Bunzow JR, et al. Orphanin FQ: a neuropeptide that activates an opioidlike G protein-coupled receptor. *Science* 1995;**270**:792–4.
16. Saito Y, Nothacker HP, Wang Z, Lin SH, Leslie F, Civelli O. Molecular characterization of the melanin-concentrating-hormone receptor. *Nature* 1999;**400**:265–9.
17. Sakurai T, Amemiya A, Ishii M, Matsuzaki I, Chemelli RM, Tanaka H, et al. Orexins and orexin receptors: a family of hypothalamic neuropeptides and G protein-coupled receptors that regulate feeding behavior. *Cell* 1998;**92**:573–85.
18. Seidah NG, Mayer G, Zaid A, Rousselet E, Nassoury N, Poirier S, et al. The activation and physiological functions of the proprotein convertases. *Int J Biochem Cell Biol* 2008;**40**:1111–25.
19. Shemesh R, Toporik A, Levine Z, Hecht I, Rotman G, Wool A, et al. Discovery and validation of novel peptide agonists for G protein-coupled receptors. *J Biol Chem* 2008;**283**:34643–9.
20. Shichiri M, Ishimaru S, Ota T, Nishikawa T, Isogai T, Hirata Y. Salusins: newly identified bioactive peptides with hemodynamic and mitogenic activities. *Nat Med* 2003;**9**:1166–72.
21. Szekeres PG, Muir AI, Spinage LD, Miller JE, Butler SI, Smith A, et al. Neuromedin U is a potent agonist at the orphan G protein-coupled receptor FM3. *J Biol Chem* 2000;**275**:20247–50.
22. Venter JC, Adams MD, Myers EW, Li PW, Mural RJ, Sutton GG, et al. The sequence of the human genome. *Science* 2001;**291**:1304–51.
23. Wise A, Jupe SC, Rees S. The identification of ligands at orphan G protein coupled receptors. *Annu Rev Pharmacol Toxicol* 2004;**44**: 43–66.
24. Xiao SH, Reagan JD, Lee PH, Fu A, Schwandner R, Zhao X, et al. High throughput screening for orphan and liganded GPCRs. *Comb Chem High Throughput Screen* 2008;**11**:195–215.

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Chapter 240

New Mass Spectrometric Approaches for Uncovering Neuropeptides

Xiaowen Hou and Jonathan V. Sweedler

ABSTRACT

Neuropeptides are a diverse group of cell-to-cell signaling molecules; because of their chemical complexity and wide range of physiological concentrations, their characterization requires analytical techniques with low detection limits and high information content. Recent developments in mass spectrometry (MS)-based technologies, integrated with appropriate sample preparation and separation protocols, have enabled the characterization of hundreds of neuropeptides in a broad range of model organisms, from invertebrates to mammals. Sample sizes also vary widely, from single cells to whole animals. The wealth of knowledge about neuropeptides acquired using MS includes identity and quantity, localization and distribution, and temporal and spatial variation. In addition to providing lists of brain peptides, MS analysis now helps elucidate their function. With ongoing improvements in the figures of merit for MS-based profiling and sampling techniques, more rapid, sensitive, accurate, and higher-throughput approaches are emerging that enhance our understanding of neuropeptides.

INTRODUCTION

Neuropeptides, a broad class of structurally diverse cell-to-cell signaling molecules, are involved in a myriad of biological processes. Synthesized as protein precursors, they undergo complex enzymatic processing, are packaged into vesicles, and can be transported long distances to their cellular release sites. Many prediction programs are available that help identify cell-to-cell signaling peptides by predicting prohormone cleavage sites.[32] However, because the final bioactive peptide products are determined by the particular enzymes present and the order in which they act, and because they vary in a brain- and context-specific manner, it is difficult to confirm final products without measuring them. As one example, the cleavage of prohormones at basic amino acid (lysine and arginine) sites is common, but only a fraction of such sites are actually cleaved.[32] Although prediction methods are useful, oftentimes empirically measuring the peptide presence using mass spectrometry (MS)-based methods is required.

MS, assisted by separation techniques such as liquid chromatography (LC), offers the capability to fractionate and characterize complex samples to determine their chemical constituents. Since one of its first successes in characterizing thyrotropin-releasing hormone,[4] MS characteristics have advanced dramatically, leading to recent discoveries of large numbers of brain peptides in many animal models.[12,24] In addition to being used to unambiguously identify and characterize neuropeptides and their posttranslational modifications in complex biological systems—without previous knowledge of the peptide sequences—MS can also provide information about neuropeptide level changes under various physiological conditions. Further, recent advances in localization and imaging approaches have been combined with several MS platforms to reveal the distribution of neuropeptides in tissue slices and even whole animals.

No single method works equally well for all samples. Instruments vary in their analytical capabilities; thus, selection of the appropriate MS platform depends on many factors. These may include the characteristics of the peptides of interest (e.g. peptide charge states, mass range of detection), the experimental goals (e.g. peptide identification, quantification, or distribution), and the number of samples to be examined. Several MS technologies are available: electrospray ionization (ESI), quadrupole-time of flight (Q-TOF), ion trap (IT), matrix-assisted laser desorption/ionization (MALDI)-TOF, Fourier transform ion cyclotron resonance (FT-ICR), and others.[2,24]

When measuring peptides in a brain sample, one typically characterizes hundreds of peptides. Which ones are neuropeptides and what are the other peptides present? Although prohormones often encode multiple peptides, only a portion of these peptides are involved in cell-to-cell

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00240-2
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signaling. It has been suggested that the other peptides provide charge neutrality during acidification of the dense core vesicle, aid in correct folding and disulfide bond formation of their corresponding prohormones, and even participate in receptor localization. Another important point is that only a fraction of the peptides in a vesicle are expected to be *bone fide* neuropeptides. Contributing to the complexity of peptide measurements, one typically obtains a snapshot of prohormone processing (peptide formation), storage, and degradation. Thus, one can expect that the final products, as well as the processing intermediates and degradants, will be present in the sample. It should also be kept in mind that the tissue isolation process may lead to further degradation or other changes in the peptide forms.

Consequently, a peptidome measurement can result in long lists of peptides, only a fraction of which are involved in cell-to-cell signaling (and hence, only a fraction of which are neuropeptides). One can design sampling protocols to obtain information on function, transport, release, distribution, and changes in abundance based on treatments. In this chapter, we provide an overview of various MS-based approaches for uncovering neuropeptides by following their dynamics from transport to release, and from localization to heterogeneity, as well as their level changes based on physiological perturbations. The sample types range from single cells to whole animals. These measurements allow one to focus on specific sets of peptides based on their form, release, and level change or distribution, information that advances neuropeptide research.

MS-BASED NEUROPEPTIDE DISCOVERY

Peptide Transport Studies

Among the numerous peptides identified by MS, only a subset are expected to be transported long distances such as from a soma down nerves, and it is this subset of transported brain peptides that is more likely to function in cell-to-cell signaling. The ability to isolate subcellular structures associated with the transport process (vesicles) or release (synapses) results in fractions containing specific sets of peptides, thereby facilitating the identification of the localized neuropeptides. These transport studies can address questions such as: "Which neuropeptides are transported?" "Which endogenous neuropeptides are cleaved and generated in vesicles via *in vivo* events?"

Many approaches are available to examine these issues. One option is to ligate living nerve tissue between the central nervous system and periphery so that the ends of the nerve can be sampled after a suitable delay. Increased peptide levels can be observed at the nerve end toward the peptide source and reduced levels from the end located toward the destination of the peptides. A simple technique is to place the entire nerve onto a sample plate for the direct measurement of peptides by MALDI MS.[23] Alternatively, the nerve sample can be homogenized in peptide extraction solutions, such as acidified acetone, acidified methanol, or HCl.[35] The resulting solution contains a variety of peptides that were being transported when the nerve was isolated. These samples oftentimes are subjected to several clean-up steps (e.g. desalting) or separation by LC or other techniques, before identification by MS. Just as with the approach described above with nerves, one can isolate individual neurons for direct analysis via MALDI MS; this allows one to discriminate peptide patterns among soma, dendrites, and axons.[19,29] and thus determine which peptides are transported.

Another intriguing approach is to look for peptide packaging by isolating vesicles, such as large dense core vesicles, or to isolate specific locations such as synaptosomes. Typically after tissue or brain isolation, secretory vesicles are separated and collected through differential and sucrose density gradient centrifugations,[13] while synaptic components are enriched by subcellular fractionation.[27] These processes reduce the chemical complexity of the samples by concentrating the more abundant molecules in these fractions. The fractions are then homogenized in extraction solutions to recover peptides, followed by separation and MS identification. A variety of MS platforms have been used to detect and sequence the peptides from these subcellular compartments, or to create sets of peptides determined to likely play a role in cell-to-cell communication.[13,16,27] As another example, vesicles and synaptosomes from a region of interest have been isolated before and after a treatment (e.g. morphine administration); the changes in peptide levels determined by MS reflect the effects of the treatment.[27] Techniques used to accomplish these measurements are detailed later (see Peptide Quantification).

Peptide Release Studies

Cell-to-cell signaling peptides must be released from the cell under a stimulation condition in order to affect other cells. Which peptides are released, what are their forms on release, and under what conditions are they released? For example, upon various stimulations, the posterior lobe of the pituitary releases hormones such as oxytocin and vasopressin, while the anterior pituitary secretes other growth and reproductive hormones. The integration of MS measurements with *in vitro* and *in vivo* sampling approaches allows the characterization of peptide release; several examples of measuring peptide release are discussed below.

In vitro sampling of release has been reported using tissues from individual neurons and neuronal clusters in invertebrates[15] and from mammalian brain slices.[14] The stimulation and releasate collection are performed *in vitro* or ex vivo and the releasate profiles typically characterized by off-line MALDI-TOF MS. Once released, the peptides

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are diluted by orders of magnitude as they diffuse into the cellular media. Many techniques have been developed to improve neuropeptide recovery and to provide spatial information on the location of release. By using a solid-phase extraction (SPE) material embedded within pipettes or micrometer-sized beads to collect releasates, as shown in Fig. 1A, the peptides are collected and concentrated before their measurement. After desalting with water rinses, peptide analytes are eluted from the SPE material or beads for subsequent MS analysis. Because of its high sensitivity and tolerance for impurities, MALDI MS is often used for these measurements. In MALDI MS, the eluents are cocrystallized with a matrix (e.g. alpha-cyano-4-hydroxycinnamic acid), ionized with a laser, and the resultant ions analyzed by the appropriate mass analyzer. MALDI MS requires minimal sample preparation due to its high tolerance for salts and other background molecules. This approach provides high-quality mass profiles, allowing one to measure multiple released peptides and their posttranslational modifications (Fig. 1B). Because of these characteristics, MALDI MS has been widely implemented in peptide release studies.[14,15,18] Furthermore, by collection of releasates from various locations using these beads (Fig. 1C), or over a time course (Fig. 1D), spatial and temporal information is added to the chemical release profiles. To improve the detectability of this approach for specific peptide categories, several SPE collection columns have been developed, such as a micron-scale porous polymer monolithic column, which provides both high binding capacity and spatial resolution.[18]

Microdialysis is a well-used brain sampling technique that has been combined with MS and other separation techniques to examine the extracellular fluid of animals *in vivo*, including brain tissues and the cerebrospinal fluid.[26,28] A microdialysis probe can be used both to collect endogenous compounds (e.g. peptides) and administer exogenous substances, such as drugs, directly and locally into the tissue regions of interest. The system includes a pump to perfuse the probe with a physiological salt solution (perhaps with a drug) while the device collects compounds that diffuse from the extracellular space across the membrane; the heart of the approach is the semipermeable membrane that allows molecules smaller than a selected size to be sampled (Fig. 2A).[6] To provide optimum sampling efficiency, the probe needs to be carefully designed (e.g. the membrane material and size), and the experimental parameters well controlled (e.g. the composition of the perfusion fluid, the perfusion flow rate, temperature).[26] As shown in Fig. 2B, the probe can be placed in a freely moving animal to monitor intracerebral neuropeptide release.[17] SPE materials have also been incorporated into probes to further concentrate and/or desalt the releasates before MS analysis. MS enables mass

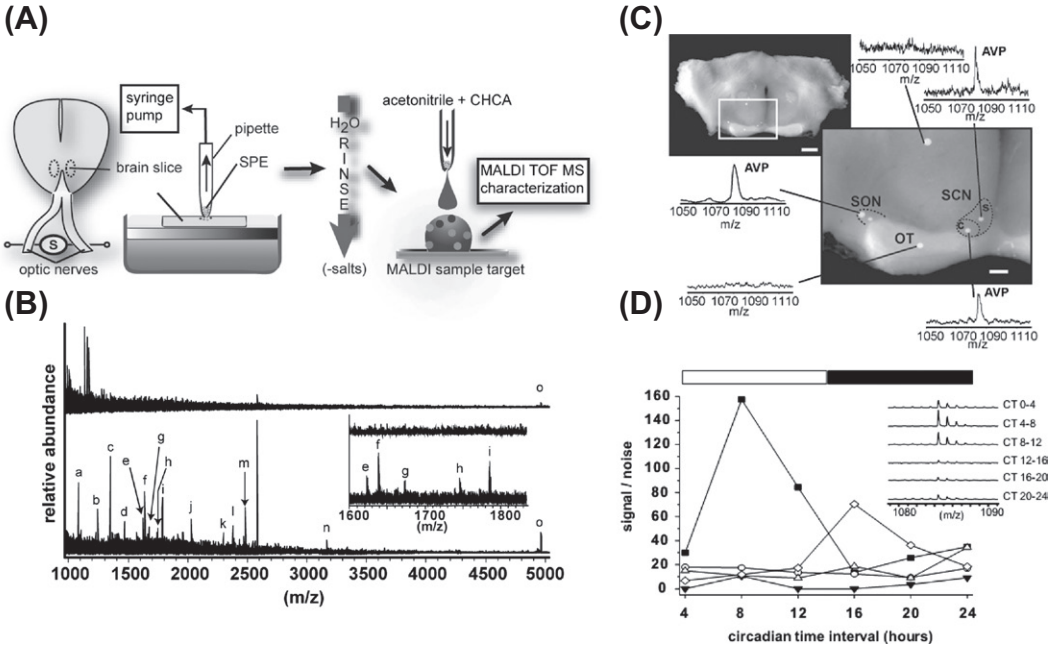

FIGURE 1 MS-based approaches for collecting peptide release from brain slices. (A) Solid-phase extraction pipette sampling allows the spatially resolved collection of released peptides and their subsequent characterization via MALDI-TOF MS. CHCA is alpha-cyano-4-hydroxycinnamic acid. (B) MALDI-TOF MS characterization of releasates before (upper panel) and after (lower panel) simulation. Peptide peaks are labeled as (a) to (o) and identified in the original work.[14] (C) Spatially resolved release of arginine vasopressin (AVP) peptide from a brain slice was performed by placing individual beads onto the slice, and then removing them for subsequent MS characterization; the beads were placed on four regions—suprachiasmatic nucleus (SCN), supraoptic nucleus (SON), anterior hypothalamic nucleus (AHP), and optic tract (OT). (D) Temporal release of five endogenous peptides over a time course of 24 h. The inset shows the release of AVP at 4-hour intervals. CT is circadian time. Figure adapted from Ref. 14 with copyright © 2008 by PNAS.

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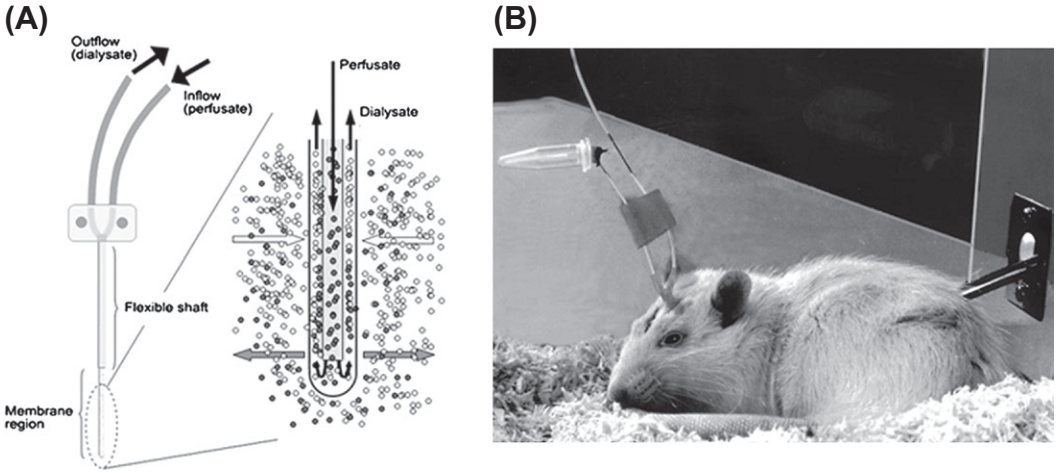

FIGURE 2 Microdialysis used for the *in vivo* collection of releasates from a behaving animal. (A) Microdialysis probe (on the left) and a schematic representation (on the right). The magnified membrane region demonstrates the net diffusion of analytes (open circles) into the probe, and the net diffusion of the perfusate containing stimuli or other substances (closed circles) to the extracellular space (Figure adapted and modified from Ref. 6 with permission). (B) An animal in the cage during microdialysis with a U-shaped probe. Figure adapted from Ref. 17 with permission from Elsevier © 2001.

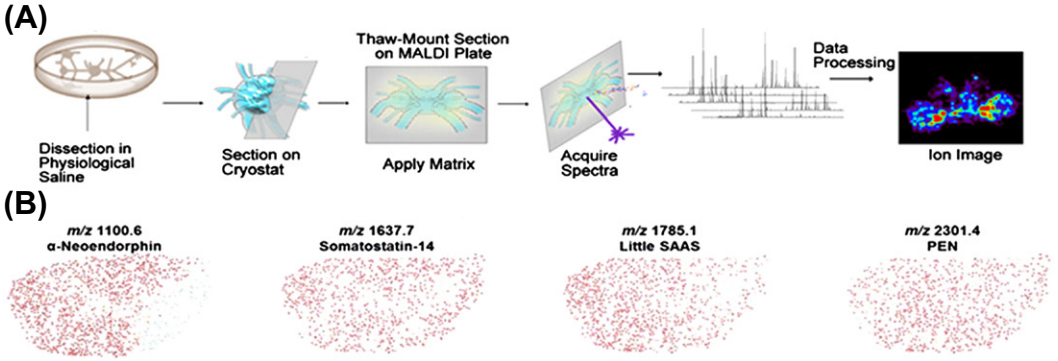

FIGURE 3 Schematic of MS imaging protocols with representative ion images. (A) Typical procedures for MSI. Figure adapted and modified from Ref. 9 with permission. (B) Molecular distribution maps of four endogenous peptides detected in spinal cord using the stretched sampling approach and MS imaging. Figure adapted and modified from Ref. 34. See color plate 63.

spectral comparisons between samples collected before, during and after the application of the selected stimuli, or over a specific time course, or at different tissue locations, all of which provide insights into the temporal and spatial chemistry occurring *in vivo*. Although accurate mass measurements are useful, more confident identifications require tandem MS capabilities. Because the number of peptides that can be fragmented by MS over a specific time period is limited, hyphenating MS to small-volume separations reduces sample complexity, yielding larger numbers of peptides that are identified via tandem MS. The combination of chemical, temporal, and spatial information on peptides that can be revealed by MS-based approaches, demonstrate the power of this technology as a discovery tool. For example, the peptide little SAAS is released from the suprachiasmatic nucleus at one time of day and directly affects circadian phase at that time of the day.[14]

Peptide Localization and Distribution Studies

Peptides often have sparse and highly localized distributions that change during development, treatment, and in some cases, even during specific behaviors. Methods that provide localization details are valuable to understanding peptide function and, in fact, optical imaging via microscopy has established that peptides have complex distributions within the brain. The caveat with microscope-based imaging is that selective and specific probes are often required to image specific peptides. By contrast, mass spectrometry imaging (MSI), which combines mass spectrometric approaches with imaging technologies, does not require preselection or labeling of analytes. It has been used to examine a range of biological tissues and provides both chemical and spatial information for known and unknown molecules, including neuropeptides.[5,9] Another advantage of MSI compared

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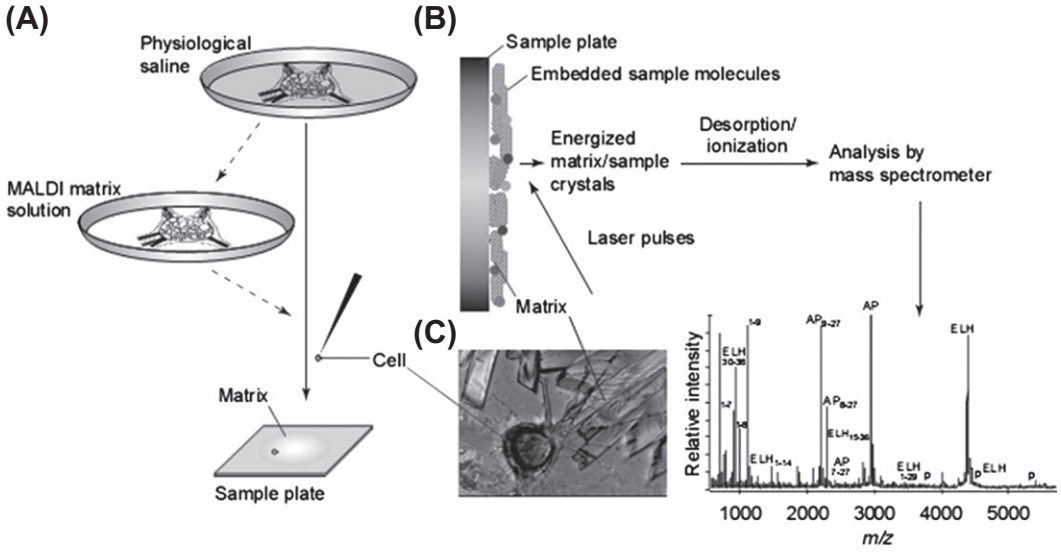

FIGURE 4 Overview of single-cell MALDI MS. (A) Cell isolation and sample preparation. (B) The MALDI ionization process. A typical mass spectrum of a single bag cell neuron from *Aplysia californica* is shown. (C) A cultured neuron with matrix crystals. Figure adapted from Ref. 22 with permission from Elsevier © 2000.

with traditional microscopy is that the distribution of a wide range of compounds can be obtained in a single experiment. The direct analysis of tissue samples and the minimal sample preparation requirements are also beneficial. Figure 3A illustrates a typical MSI procedure, from sample preparation to data analysis. Experimental animals are selected and the regions of interest (e.g. brain, spinal cord, nerves) are isolated and dissected out under physiological conditions. A slice of tissue is sectioned via a cryostat and then placed onto a MALDI target plate, incorporating additional sample preparation steps as needed, for example, sample stretching to improve spatial resolution.[34] After matrix application, mass spectra are acquired in an ordered array of positions across the sample. Selected ion images of neuropeptides are reconstructed by plotting the MS signal intensities with the *x*, *y* coordinates of each spot (Fig. 3B). Peak assignments are made either by mass-matching or via subsequent tandem MS. The localization of tens to hundreds of peptides can be imaged at spatial resolutions from the low micron to hundreds of microns size range, depending on the instrumental and protocol figures of merit.

For obtaining quality images of peptides with MSI, one has to rely heavily on choosing the appropriate sample preparation protocols, selected according to sample type, mass range of interest, and desired spatial resolution and sensitivity. Several measurement approaches can be considered, such as stigmatic imaging,[1] oversampling,[20] and stretched sampling.[34] Because each MS platform provides unique capabilities, choosing the right system is also important. MALDI MS is well suited for imaging peptides with a wide range of masses at spatial scales from single cells[29] to whole animals,[21] while secondary ion mass spectrometry, or SIMS imaging is often used to study peptides with lower molecular weight masses (usually <700 Da) at higher spatial resolution.[34] Other ionization techniques, such as ESI and desorption electrospray ionization, or DESI, are alternative approaches.[33]

Single-Cell Neuropeptide Studies

Neurons are heterogeneous cells with distinct chemical compositions and functions, even among adjacent cells. With optimized sample preparation protocols and appropriate MS platforms, individual larger neurons can be rogated;[19,22,30,31] however, such measurements are not common. Because the neuropeptide complement varies in a cell-specific fashion, the analysis of single cells tends to reduce sample complexity and prevent the dilution of a specific neuropeptide with contents from nearby cells. Therefore, neuropeptides present at high local levels, but low global abundances are more easily detected.

Many single-cell analyses use MALDI MS; as mentioned previously, it is tolerant of salts. It is also tolerant of lipids, compatible with solid samples, has excellent detection limits, and requires minimal sample preparation. An overview of single-cell MALDI MS is illustrated in Fig. 4. After animal dissection and single-cell isolation under physiological conditions, single cells are positioned onto the target plate, and an appropriate MALDI matrix is applied. This approach has successfully been used to assay large invertebrate neurons to mammalian cells, with cell sizes ranging from several to hundreds of microns.[30] These approaches are becoming higher-throughput, capable of identifying rare novel neuropeptides, and placing the resulting information

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into the context of the specific functions of individual neurons, and of following the cell-specific changes of peptide levels resulting from disease and exposure to drugs.[31]

Peptide Quantification

Details about which peptides are present within a brain region or tissue, including how these peptides are affected by the administration of drugs, gene knockout, or behavioral changes,[12] advances our understanding of peptide function. Quantitative peptidomic approaches that use MS-based methodologies allow us to compare changes in the relative amounts of many peptides simultaneously. There are several common MS-based approaches for quantifying peptides that use isotopic labeling[3,7,8,11,25] and label-free methods;[10] the general procedures are illustrated in Fig. 5. For isotopic labeling, the extracts from control and experimental samples are labeled with either light or heavy labeling reagents, which create labeled peptides with the same structure but a mass difference. Suitable isotopic labeling reagents should provide high reactivity and efficient labeling, stably labeled peptides during sample preparation and MS measurement, the coelution of differentially labeled peptides from commonly used LC columns, and a mass difference large enough for peak recognition and differentiation; these characteristics help ensure more reproducible and accurate results. Two types of "universal" reagents are widely employed for peptide labeling: one targets the N-terminus and the ε-amino group of lysine residues (e.g. succinic anhydride and trimethylammoniumbutyryl), and the other targets the C-terminus (e.g. methanol).[12] After labeling, samples are combined and simultaneously measured, with the MS instrument allowing the "identical" peptides to be differentiated; the difference in peptide abundance is quantified by determining the ratio of differentially labeled peaks in the same mass spectra (Fig. 5C).

Label-free measurements require fewer sample preparation steps after peptide extraction, and the samples are individually analyzed using LC–MS to obtain separate mass spectra for comparison (Fig. 5B). Because this approach handles samples separately throughout the process, it is essential to maintain experimental consistency during peptide extraction and sample preparation, and to carefully select the proper MS platform and parameters to obtain reproducible and comparable results. Although label-free quantification tends

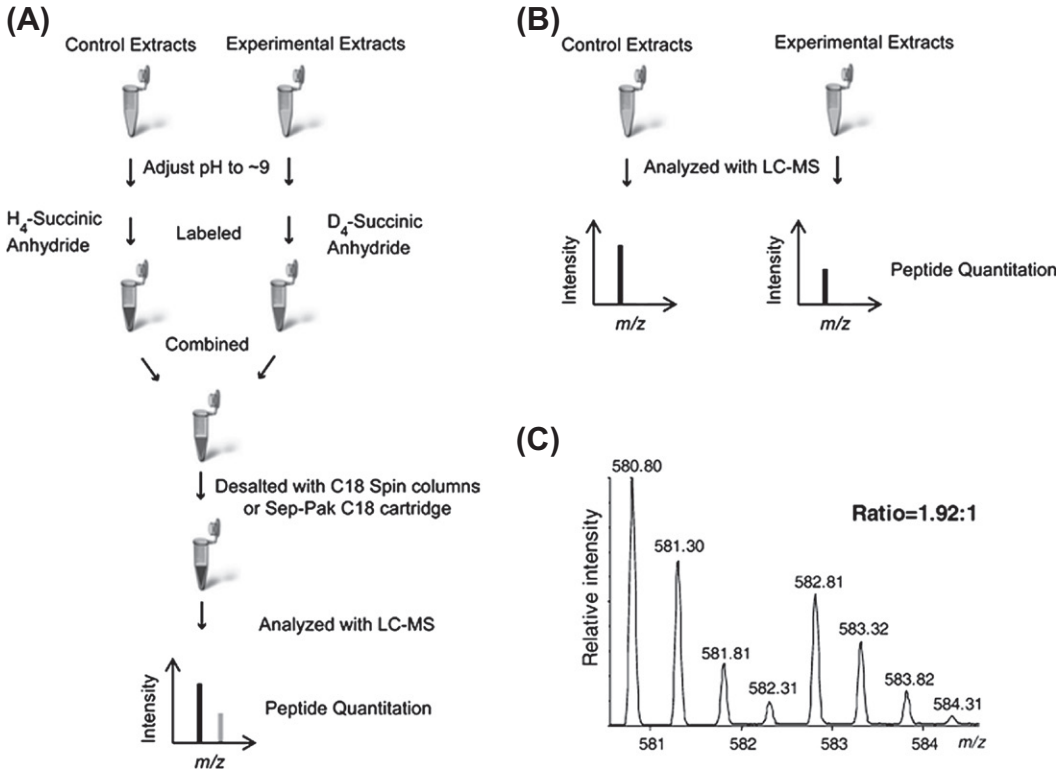

FIGURE 5 Neuropeptide quantification workflow with representative mass spectra shown. (A) Labeling approach from sample preparation and succinic anhydride labeling to LC–MS data analysis. (B) Label-free approach from sample preparation to LC–MS data analysis. (C) MS spectra of bradykinin standards differentially labeled with succinic anhydride (molecular weight = 1059.55 with masses 1159.62 (labeled with hydrogen)/1163.66 (labeled with deuterium) after labeling; charge state +2). The measured ratio is within 4% of the expected ratio, 2:1. Figure adapted and modified from Ref. 35 with permission.

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to work well for quantifying peptides in the smallest-volume samples and peptides in low abundance with no theoretical limitation to the number of samples being compared, labeling approaches combine the samples at an earlier stage and may thus provide a more accurate quantification. The current availability of labeling reagents for quantitative peptidomics allows a comparison of up to four samples,[3,25] but the development of optimized reagents may lead to the ability to label more samples in parallel. Both labeling and label-free approaches have been successfully applied to investigate the correlation of peptide level changes in examining foraging behavior in honey bees[3] and the effects of drug exposure[7,11] and food deprivation and exercise[8] in mice. We expect such peptide studies to continue.

CONCLUSIONS

Significant advances in the integration of MS measurement methods with various sampling techniques have combined to collect dynamic, temporal and spatial information for neuropeptidomics research. As a result, the number of peptides discovered from a range of model organisms has dramatically increased over the past decade, shedding light on potential neuropeptides in the context of their biological environments. The emergence of more advanced MS platforms capable of unambiguous peptide identification, lower detection limits, higher information content and higher-throughput, in concert with the continuous development of more localized and activity-dependent sampling techniques, will further advance our understanding of these important cell-to-cell signaling molecules.

ACKNOWLEDGMENTS

The project described was supported by the National Institute on Drug Abuse under Award No. P30DA018310. The content is solely the responsibility of the authors and does not necessarily represent the official views of NIDA or the National Institutes of Health.

REFERENCES

1. Altelaar AF, Luxembourg SL, McDonnell LA, Piersma SR, Heeren RM. Imaging mass spectrometry at cellular length scales. *Nat Protoc* 2007;**2**:1185–96.
2. Boonen K, Landuyt B, Baggerman G, Husson SJ, Huybrechts J, Schoofs L. Peptidomics: the integrated approach of MS, hyphenated techniques and bioinformatics for neuropeptide analysis. *J Sep Sci* 2008;**31**:427–45.
3. Brockmann A, Annangudi SP, Richmond TA, Ament SA, Xie F, Southey BR, et al. Quantitative peptidomics reveal brain peptide signatures of behavior. *Proc Natl Acad Sci U S A* 2009;**106**:2383–8.
4. Burgus R, Dunn TF, Desiderio D, Guillemin R. [Molecular structure of the hypothalamic hypophysiotropic TRF factor of ovine origin: mass spectrometry demonstration of the PCA-His-Pro-NH2 sequence]. *C R Acad Sci Hebd Seances Acad Sci D* 1969;**269**:1870–3.
5. Caprioli RM, Farmer TB, Gile J. Molecular imaging of biological samples: localization of peptides and proteins using MALDI-TOF MS. *Anal Chem* 1997;**69**:4751–60.
6. Chaurasia CS, Muller M, Bashaw ED, Benfeldt E, Bolinder J, Bullock R, et al. AAPS-FDA workshop white paper: microdialysis principles, application and regulatory perspectives. *Pharm Res* 2007;**24**:1014–25.
7. Che FY, Vathy I, Fricker LD. Quantitative peptidomics in mice: effect of cocaine treatment. *J Mol Neurosci* 2006;**28**:265–75.
8. Che FY, Yuan Q, Kalinina E, Fricker LD. Peptidomics of Cpe fat/fat mouse hypothalamus: effect of food deprivation and exercise on peptide levels. *J Biol Chem* 2005;**280**:4451–61.
9. Chen R, Li L. Mass spectral imaging and profiling of neuropeptides at the organ and cellular domains. *Anal Bioanal Chem* 2010;**397**:3185–93.
10. Collins 3rd JJ, Hou X, Romanova EV, Lambrus BG, Miller CM, Saberi A, et al. Genome-wide analyses reveal a role for peptide hormones in planarian germline development. *PLoS Biol* 2010;**8**:e1000509.
11. Decaillot FM, Che FY, Fricker LD, Devi LA. Peptidomics of Cpefat/fat mouse hypothalamus and striatum: effect of chronic morphine administration. *J Mol Neurosci* 2006;**28**:277–84.
12. Fricker LD, Lim J, Pan H, Che FY. Peptidomics: identification and quantification of endogenous peptides in neuroendocrine tissues. *Mass Spectrom Rev* 2006;**25**:327–44.
13. Gupta N, Bark SJ, Lu WD, Taupenot L, O'Connor DT, Pevzner P, et al. Mass spectrometry-based neuropeptidomics of secretory vesicles from human adrenal medullary pheochromocytoma reveals novel peptide products of prohormone processing. *J Proteome Res* 2010;**9**:5065–75.
14. Hatcher NG, Atkins Jr N, Annangudi SP, Forbes AJ, Kelleher NL, Gillette MU, et al. Mass spectrometry-based discovery of circadian peptides. *Proc Natl Acad Sci U S A* 2008;**105**:12527–32.
15. Hatcher NG, Sweedler JV. Aplysia bag cells function as a distributed neurosecretory network. *J Neurophysiol* 2008;**99**:333–43.
16. Hook V, Bark S, Gupta N, Lortie M, Lu WD, Bandeira N, et al. Neuropeptidomic components generated by proteomic functions in secretory vesicles for cell-cell communication. *AAPS J* 2010;**12**:635–45.
17. Horn TF, Engelmann M. In vivo microdialysis for nonapeptides in rat brain—a practical guide. *Methods* 2001;**23**:41–53.
18. Iannacone JM, Ren S, Hatcher NG, Sweedler JV. Collecting peptide release from the brain using porous polymer monolith-based solid phase extraction capillaries. *Anal Chem* 2009;**81**:5433–8.
19. Jimenez CR, van Veelen PA, Li KW, Wildering WC, Geraerts WP, Tjaden UR, et al. Neuropeptide expression and processing as revealed by direct matrix-assisted laser desorption ionization mass spectrometry of single neurons. *J Neurochem* 1994;**62**:404–7.
20. Jurchen JC, Rubakhin SS, Sweedler JV. MALDI-MS imaging of features smaller than the size of the laser beam. *J Am Soc Mass Spectrom* 2005;**16**:1654–9.
21. Khatib-Shahidi S, Andersson M, Herman JL, Gillespie TA, Caprioli RM. Direct molecular analysis of whole-body animal tissue sections by imaging MALDI mass spectrometry. *Anal Chem* 2006;**78**:6448–56.
22. Li L, Garden RW, Sweedler JV. Single-cell MALDI: a new tool for direct peptide profiling. *Trends Biotechnol* 2000;**18**:151–60.
23. Li L, Moroz TP, Garden RW, Floyd PD, Weiss KR, Sweedler JV. Mass spectrometric survey of interganglionically transported peptides in Aplysia. *Peptides* 1998;**19**:1425–33.

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24. Li L, Sweedler JV. Peptides in the brain: mass spectrometry-based measurement approaches and challenges. *Annu Rev Anal Chem (Palo Alto Calif)* 2008;**1**:451–83.
25. Morano C, Zhang X, Fricker LD. Multiple isotopic labels for quantitative mass spectrometry. *Anal Chem* 2008;**80**:9298–309.
26. Orlowska-Majdak M. Microdialysis of the brain structures: application in behavioral research on vasopressin and oxytocin. *Acta Neurobiol Exp (Wars)* 2004;**64**:177–88.
27. Ramos-Ortolaza DL, Bushlin I, Abul-Husn N, Annangudi SP, Sweedler J, Devi LA. Quantitative neuroproteomics of the synapse. *Methods Mol Biol* 2010;**615**:227–46.
28. Reed B, Bidlack JM, Chait BT, Kreek MJ. Extracellular biotransformation of beta-endorphin in rat striatum and cerebrospinal fluid. *J Neuroendocrinol* 2008;**20**:606–16.
29. Rubakhin SS, Greenough WT, Sweedler JV. Spatial profiling with MALDI MS: distribution of neuropeptides within single neurons. *Anal Chem* 2003;**75**:5374–80.
30. Rubakhin SS, Romanova EV, Nemes P, Sweedler JV. Profiling metabolites and peptides in single cells. *Nat Methods* 2011;**8**:S20–9.
31. Rubakhin SS, Sweedler JV. Quantitative measurements of cell-cell signaling peptides with single-cell MALDI MS. *Anal Chem* 2008;**80**:7128–36.
32. Southey BR, Amare A, Zimmerman TA, Rodriguez-Zas SL, Sweedler JV. NeuroPred: a tool to predict cleavage sites in neuropeptide precursors and provide the masses of the resulting peptides. *Nucleic Acids Res* 2006;**34**:W267–72.
33. Takats Z, Wiseman JM, Gologan B, Cooks RG. Mass spectrometry sampling under ambient conditions with desorption electrospray ionization. *Science* 2004;**306**:471–3.
34. Tucker KR, Serebryannyy LA, Zimmerman TA, Rubakhin SS, Sweedler JV. The modified-bead stretched sample method: development and application to MALDI-MS imaging of protein localization in the spinal cord. *Chem Sci* 2011;**2**:785–95.
35. Yin P, Hou X, Romanova E, Sweedle RJ. Neuropeptidomics: mass spectrometry-based qualitative and quantitative analysis. *Methods Mol Biol* 2011;**789**:223–36.

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Chapter 241

Peptide Degradation (Neprilysin and Other Regulatory Peptidases)

Anthony J. Turner and Natalia N. Nalivaeva

ABSTRACT

Turning off peptide signaling is principally mediated by a group of cell-surface peptidases whose active sites face the extracellular space (ectopeptidases). Two groups of metallopeptidases are the best characterized as peptide degrading enzymes: the neutral endopeptidase or neprilysin (NEP) family and the angiotensin-converting enzyme (ACE) family. NEP and its homologs are important in degrading both brain peptides (e.g. enkephalins, tachykinins) and cardioactive peptides (e.g. atrial natriuretic peptides). They also metabolize the Alzheimer amyloid β-peptide (Aβ). In the cardiovascular system, ACE and ACE2 act to counterbalance each other in controlling angiotensin peptide levels and may also participate in the metabolism of other peptides. Prolyl-directed peptidases such as dipeptidyl peptidase IV and prolyl oligopeptidase play highly specific roles in peptide metabolism. This chapter summarizes current developments in our understanding of the roles and the regulation of these key mediators of peptide signaling, their roles in disease and as potential therapeutic targets.

INTRODUCTION

Proteolysis, which regulates the production and removal of biologically active peptides, represents a key biological process underpinning events from fertilization, through development to cell death. The ubiquity and diversity of peptidases are indicated by the existence of some 28,000 specific peptidases and their homologs characterized in >6000 known organisms (http://merops.sanger.ac.uk/). In the human genome of approximately 24,000 protein-coding genes, 686 genes of putative peptidases are known alongside 423 of noncatalytic homologs. Many of these are orphan peptidases for which the biologically active peptide substrates have not yet been identified. Such a profusion of proteolytic events requires specifically controlled regulation. Hence, although genes for peptidases constitute about 2.8% of the human genome, a significantly higher number (7.4%) of genes encode protein inhibitors of peptidases.

The term "peptidase" refers to enzymes that hydrolyze peptide bonds and, as such, all known biologically active peptides represent potential substrates for these enzymes. According to the location of the peptide bond cleaved by the peptidases, they are conventionally subdivided into "endopeptidases" that cleave the bond within the polypeptide chain, or exopeptidases that release amino acids from the N- or C-terminus of the substrate. However, some peptidases, e.g. angiotensin-converting enzyme (ACE), can fulfill both endopeptidase and exopeptidase functions. The physiological role of peptidases is very diverse. In general, peptidases do not cleave their substrates at random but participate in very well-controlled, specific processing of peptide molecules. The term "limited proteolysis" was originally introduced by Linderstrøm-Lang to distinguish the restricted specificity of certain peptidases, particularly those involved in posttranslational modification of proprotein precursors, from the random proteolysis accompanying protein digestion and degradation.

All peptidases have been divided into six classes (metallo-, serine, cysteine, aspartic, threonine, and glutamate peptidases) with cysteine-, serine-, and metallopeptidases representing the major categories. A seventh class involving asparagine peptidases has recently been reported.[24] Aberrant functioning of these peptidase genes is implicated in such diverse pathologies as inflammatory and cardiovascular diseases, cancer, and neurodegeneration. Understanding the normal functional roles of peptidases and their regulation and restoration in disease is hence important for the design of novel and selective therapeutics.

This chapter is focused primarily on two major families of zinc peptidases participating in the specific degradation of biologically active peptides. The prototypes for these peptidases are neprilysin (neutral endopeptidase, NEP) and ACE. The roles of peptidases directed specifically toward prolyl residues are also reviewed. Table 1 summarizes the diversity of peptide substrates hydrolyzed by this cohort of enzymes.

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00241-4
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TABLE 1 Typical Biologically Active Peptides Degraded by Peptidases Discussed in this Chapter

Substrate	Peptidase
Adrenomedullin	NEP
Amyloid β-peptide	NEP, NEP II, ECE-1, IDE, ACE
Amyloid precursor protein intracellular domain (AICD)	IDE
Angiotensin I	ACE, NEP, ACE2, POP
Angiotensin II	ACE2, NEP, POP
Apelin	ACE2
Atrial natriuretic peptide	NEP, IDE
β-Endorphin	IDE, POP
Big endothelin-1	ECE-1, ECE-2, NEP, NEP II
Bradykinin	ACE, ECE-1, ECE-2, IDE, NEP, APP2, POP
Brain natriuretic peptide	IDE
Cholecystokinin-8	ACE, NEP
Corticotropin	ACE, NEP
Dynorphins	NEP, IDE, ECE-2
Endomorphin	DPP IV, NEP
Enkephalins	NEP, ACE
Endothelin-1	NEP
Gastrin	NEP
Ghrelin	ACE2
Glucagon-like peptide-1	DPP IV
Gonadoliberin-1	ACE, POP
Insulin	IDE
Insulin-like growth factor I	DPPIV
Insulin-like growth factor II	IDE
Luliberin	NEP, ACE
Neuropeptide Y	DPPIV, NEP, APP2
Neurotensin	ACE, ACE-2, ECE-1, ECE-2, NEP, POP
Oxytocin	NEP, POP
Somatostatin	NEP, ECE-1, IDE
Substance P	NEP, ACE, DPPIV, ECE-1, ECE-2, POP
VIP	NEP, ACE, IDE

NEUTRAL ENDOPEPTIDASE (NEPRILYSIN) FAMILY

Name and History

Neprilysin activity was first characterized from rat kidney brush border membranes as an enzyme hydrolyzing insulin B chain at neutral pH, and hence termed "neutral endopeptidase" (NEP), although it plays no role in the physiological insulin inactivation, which is mediated by another peptidase, insulin-degrading enzyme (IDE). NEP is a zinc metallopeptidase resembling the bacterial enzyme thermolysin, including its sensitivity to the inhibitor, phosphoramidon. NEP has a diverse range of biological roles and hence has been rediscovered in different proteolytic contexts. For example, after the discovery of the enkephalins, NEP was rediscovered in brain as a membrane enzyme hydrolyzing these opioid peptides and was termed enkephalinase. It is also the principal enzyme inactivating many other neuropeptides (e.g. substance P) and cardioactive peptides (atrial natriuretic peptide (ANP) family) and hence represents the first general "neuropeptidase." NEP plays an important role in the immune system where it is referred to as the common acute lymphoblastic leukemia antigen (CALLA or CD10), although its substrate(s) have not been identified. NEP also contributes to the metabolism of the Alzheimer amyloid β-peptide (Aβ) in the brain.[12] The human genome contains at least seven NEP-like enzymes, including the biosynthetic enzyme endothelin-converting enzyme (ECE-1). The family is even more abundant in Drosophila (24 predicted members) and *Caenorhabditis elegans* (22 members).[4] NEP is also known as MME, for membrane metalloendopeptidase, in the Human Gene Nomenclature database.

Properties

NEP is exclusively localized to the plasma membrane (type II integral membrane protein) with the active site facing the extracellular space (ectoenzyme) where it is poised to inactivate circulating peptides. It is a glycoprotein of approximately M_r 90,000. The enzyme is classified as a "gluzincin" peptidase with the typical His-Glu-Xaa-Xaa-His (HEXXH) zinc-binding motif. The glutamate in this motif (Glu^{584} in the human enzyme) is essential for catalysis and an additional glutamate (Glu^{646}) constitutes the third zinc ligand. NEP is an archetypal peptidase rather than proteinase because it generally only hydrolyzes peptides up to about 50 amino acids long. The 3D structure of the NEP catalytic domain in complex with its inhibitor phosphoramidon has been deduced aiding the design of novel inhibitors and providing a better understanding of its substrate specificity and catalytic mechanism.[20] The active site region is restricted in size preventing access of large peptides and hence explaining its oligopeptidase nature.

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In general, NEP hydrolyzes peptidases on the amino-terminal side of hydrophobic residues, e.g. the Gly[3]-Phe[4] bond of enkephalins, giving it a very broad specificity for known biologically active peptides although its physiologically validated substrates are more restricted. In addition to opioids, tachykinins, and the ANP family, it inactivates endothelins, bradykinin, and somatostatin. Other NEP substrates include glucagon, the vasoactive intestinal peptide family and adrenomedullin. NEP may also regulate bone metabolism through its presence on the surface of human osteoblasts and its ability to metabolize osteogenic peptides such as calcitonin and calcitonin gene-related peptide. Because inhibition of NEP can, in theory, potentiate the action of any of its peptide substrates, much emphasis has been directed toward developing novel and potent NEP inhibitors, with thiorphan originally being synthesized as an inhibitor of its enkephalin-hydrolyzing activity, and this is still the most commonly used NEP inhibitor. Many others have subsequently been developed as potential therapeutics in cardiovascular disease by prolonging the actions of ANP. The combined actions of NEP and ACE inhibitors have improved benefits over ACE inhibitors alone. The prodrug of thiorphan, acetorphan (racecadotril), is an effective antidiarrheal agent through its potentiation of the antisecretory effects of enkephalins in the intestine. However, the NEP substrate that has attracted by far the most interest over the last decade has been the Alzheimer amyloid-β peptide (Aβ).

Distribution/Localization

NEP and its homologs are widely distributed enzymes in the animal kingdom reflecting the general need for terminating the action of circulating regulatory peptides. Many invertebrate peptides are inactivated by NEP-like peptidases where it often plays a role in development and reproduction. In *C. elegans*, NEP seems to be an effector of locomotion. In mammals, the enzyme is particularly abundant in the kidney where its expression levels in brush border membranes are several orders of magnitude higher than in brain synaptic membranes. It is also highly expressed in the reproductive system (testis, ovary), although its functional role is unknown. Elsewhere NEP, although widespread, is expressed at much lower levels.

In the brain, NEP inactivates peptide neurotransmitters, especially opioid and tachykinin peptides, after their release into the synapse. Given the role of these peptides in pain mechanisms, NEP inhibitors have antinociceptive actions. However, most attention has been focused on the role that NEP plays, alongside other peptidases such as ECE, insulin-degrading enzyme, and possibly ACE, in detoxifying the Aβ peptide that predisposes to Alzheimer's disease (Fig. 1). The susceptibility of Aβ is perhaps not surprising given the

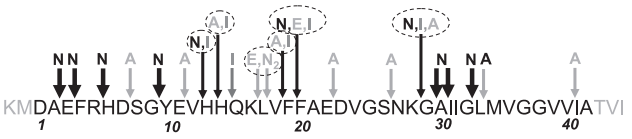

FIGURE 1 Main cleavage sites of amyloid-β peptide by peptidases. The Aβ-(1–42) sequence is shown in black with the flanking amino acids of the amyloid precursor protein (APP) shown in gray. The principal peptidases involved in Aβ peptide metabolism are indicated in black and the other activities in gray. N, neprilysin; N_2, neprilysin-2; A, angiotensin-converting enzyme; E, endothelin-converting enzyme-1; I, insulin-degrading enzyme. Letters in circles indicate peptidases that cleave Aβ at the same site.

specificity of NEP, as it is a highly hydrophobic peptide. Indeed, under normal conditions there is considerable evidence that Aβ itself may be a regulatory peptide, and it is only in pathological conditions when it accumulates excessively that it becomes toxic. This process is enhanced in aging because there is a well-documented age-related decline in NEP expression. The levels of brain Aβ are hence increased in NEP-deficient mice. NEP expression is also diminished after periods of ischemia or hypoxia. Infusion of an NEP inhibitor into rat brain leads to a significant accumulation of the more neurotoxic Aβ42 peptide, and hence, upregulation of NEP has been proposed as a potential therapeutic strategy in Alzheimer's disease.[12,18] NEP gene transfer can decrease amyloid-like pathology and cognitive decline in transgenic mice.[16] Given the potential of NEP inhibitors to increase brain Aβ levels and hence to induce Alzheimer's disease-like symptoms, interest in the development of novel NEP inhibitors as potential cardiovascular therapeutics has considerably diminished.

In the brain, NEP is mainly localized on neuronal cells, especially in the striatonigral pathway consistent with its role in turning off opioid peptide and tachykinin signaling, and it is also located in the hippocampus, where it functions to inactivate somatostatin and in cortical regions where it may also act to degrade Aβ . The enzyme is also present on activated astrocytes and on Schwann cells in the peripheral nervous system where it may function in nerve development and/or regeneration.

Unlike many proteases, NEP is not synthesized in an inactive proenzyme form and is not generally regulated by endogenous inhibitors although sialorphin, the peptide product of the *Vcsa1* gene, has been reported to inhibit the enzyme *in vivo*.[26] In most tissues, much of the NEP expression is constitutive but recent demonstration of epigenetic regulation of NEP has opened up the possibility of new approaches to control NEP levels in brain.[3,21] The Aβ peptide is formed by proteolytic processing of the amyloid precursor protein (APP). Another metabolite of APP is its intracellular domain (AICD), a 50–59 amino acid peptide, which can be translocated to the nucleus where it can bind to the NEP promoter (and promoters of some other genes) displacing

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histone deacetylases which act as transcriptional repressors, and hence upregulating gene expression.[3] This effect seems to be mediated preferentially by the neuronal isoform of APP (APP695).[2] Through a related mechanism, histone deacetylase inhibitors such as trichostatin A and valproic acid, can enhance NEP expression *in vitro* and *in vivo* and may therefore have therapeutic value in Alzheimer's disease.[3]

NEP is an important diagnostic marker in certain forms of childhood leukemia and has also been implicated in cancer progression, particularly of the prostate. NEP gene transcription is androgen-regulated in androgen-responsive tissues. Hence, the downregulation of NEP through hypermethylation of the promoter contributes to androgen-independent progression of prostate cancer because of a reduced ability to metabolize mitogenic peptides, particularly endothelin-1. NEP may also be important in the development of obesity and its expression levels associate with insulin resistance and the metabolic syndrome.[28]

Related Peptidases

Seven NEP-like proteins have been identified in the human genome.[4] Most of these are of unknown biological function, and their substrate specificity is unclear. The most close in similarity to NEP is a peptidase referred to as NEP II in the rat, "soluble secreted endopeptidase" (SEP) or NEP-like 1 (NL1) in the mouse, or MMEL2 (human). The best-characterized NEP homologs are the endothelin-converting enzymes (ECE-1, ECE-2). ECE-1 participates in the conversion of big endothelin to the vasoactive peptide, endothelin-1 (see the chapter on Cardiovascular Peptides and below). ECE-2 probably plays a role as a processing enzyme in the biosynthesis of regulatory peptides and can process such peptides at non-classical processing sites. Mice deficient in ECE-2 have learning and memory deficits and show abnormal responses to morphine and have altered peptide levels in the spinal cord.[17]

NEP II was first identified in mice deficient in ECE-1 as a potential endothelin-processing enzyme.[11] Although it is the most similar peptidase to NEP, it has many distinct properties; for example, it occurs as both a soluble, secreted enzyme and an alternatively spliced form retained in the endoplasmic reticulum. NEP II is particularly localized to neuronal populations in the brain and spinal cord and is also abundant in reproductive tissue—male mice deficient in NEP II show reduced fertility. Originally, NEP II was reported to have a broad repertoire of substrates, similar to those for NEP itself,[12] although its physiological role apart from male fertility, remains unresolved. However, rodent NEP II exhibits a much wider substrate specificity than the human enzyme.[33] For example, only substance P and angiotensin I are hydrolyzed at comparable rates by human NEP and NEP II.[33] The inhibitor thiorphan is also more potent toward NEP than NEP II. A physiological role for NEP II has emerged alongside NEP in the degradation of Aβ peptide. NEP II has now been shown to contribute significantly to Aβ degradation both *in vitro* and *in vivo* in gene knockout and transgenic studies (Fig. 1).[9]

ECE-1, like NEP, is a membrane-bound metalloprotease, which is found at the cell surface and is also present in the trans-Golgi network and endosomal system. The early history of ECE-1 discovery is summarized in Ref. 30. ECE-1 and NEP are both inhibited by phosphoramidon, but ECE-1 is relatively insensitive to the action of thiorphan. ECE-1 is undoubtedly the principal enzyme generating endothelin-1 from its inactive precursor, big endothelin. However, it is capable of hydrolyzing a wide range of biologically active peptides with a specificity broadly similar to that of NEP, although peptides smaller than hexapeptides (e.g. enkephalins) are hydrolyzed poorly, if at all. Among peptides hydrolyzed by ECE-1 are angiotensin I, atrial natriuretic peptide, bradykinin, calcitonin, calcitonin gene–related peptide (CGRP), neurotensin, somatostatin peptides, and substance P. A unique role for ECE-1 in peptide metabolism is its ability to degrade neuropeptides (e.g. CGRP, tachykinins, somatostatin peptides) within endosomes to control receptor recycling.[25] This property is consistent with an acidic pH optimum for the enzyme. ECE-1 can also hydrolyze the amyloid peptides $A\beta_{1-40}$ and $A\beta_{1-42}$ at three major cleavage sites (Fig. 1) and seems to have a physiological role in Aβ metabolism as shown in mice deficient in the peptidase.[7]

In addition to its cardiovascular role ECE-1 is important developmentally, especially for neural crest development, again through its ability to generate endothelin-1. ECE-1 has also been implicated in numerous cancers: thyroid, ovarian, endometrial, lung, colon, and especially prostate. Indeed, it has been shown that siRNA-mediated knockdown of ECE-1 reduces prostate cancer cell invasion in culture.[14]

THE ACE FAMILY (ACE AND ACE-2)

As described in the angiotensin chapter in the Cardiovascular Peptides section of the book, ACE was identified >50 years ago by Leonard Skeggs and colleagues as the enzyme responsible for converting angiotensin I into the vasoconstrictor peptide, angiotensin II (termed hypertensins I and II at that time). In parallel, it inactivates the vasodilator and inflammatory peptide bradykinin, which is the most favorable peptide substrate for ACE, being hydrolyzed approximately 25 times more efficiently than is angiotensin I. Hence, the inhibition of ACE has a powerful antihypertensive effect, and the enzyme has therefore been a major cardiovascular target for many years leading to the development of numerous potent and specific inhibitors (e.g. captopril, lisinopril). The catalytic activity of ACE is predominantly as a "peptidyl dipeptidase" or carboxydipeptidase, removing

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dipeptides from the C-terminus of a susceptible peptide substrate. However, ACE can act as an endopeptidase in the hydrolysis of some peptides (e.g. both substance P and luteinizing hormone-releasing hormone), although far less efficiently. ACE is a type I integral membrane protein and zinc metallopeptidase with the typical HEXXH zinc-binding motif, which is expressed on the surface of both endothelial and epithelial cells. Mammalian ACE exists as two distinct forms, arising from the use of alternative promoters. The simplest is the testis-specific form of ACE, which is essential for male fertility, and which carries a single zinc-binding and catalytic domain. Elsewhere in the body, the somatic form of ACE has arisen by gene duplication and has two homologous but not identical active sites (N-domain and C-domain sites) with an overall M_r of 150–180 kDa. Hence the two sites differ somewhat in substrate specificity and inhibitor sensitivity. For example, the hemoregulatory peptide Ac-Ser-Asp-Lys-Pro (AcSDKP) and angiotensin-(1-7) are preferred substrates of the N-domain catalytic site whereas angiotensin I is mainly metabolized by the C-domain. A soluble form of ACE exists in serum and other body fluids and is derived from the membrane form of ACE by proteolytic shedding (for a review on ACE see Ref. 13).

Elucidation of the structure of ACE eluded investigators for many years because of the heavy glycosylation of the glycoprotein hindering crystallization. It was not until 2003 that the first X-ray structures of ACE were determined for the testicular form with and without inhibitor (lisinopril) bound.[19] Analysis of the structures has revealed that ACE most closely resembles a neurotensin-degrading zinc endopeptidase known as neurolysin rather than NEP, or carboxypeptidase A, on whose structure the design of ACE inhibitors was originally based. Subsequent structure determination of the N-domain[1] has allowed comparisons of the two active sites and aided the development of domain-selective inhibitors.

An unexpected substrate identified for ACE has been the Aβ peptide with the enzyme apparently cleaving the Asp^7-Ser^8 bond of $A\beta_{1–40,}$[10] although this did not seem to fit with the known specificity of ACE. Subsequently, other sites of cleavage have been identified within Aβ, including the ability of ACE to convert $A\beta_{1–42}$ to $A\beta_{1–40}$, which is more consistent with its carboxydipeptidase specificity[35] (Fig. 1). This cleavage would be beneficial in removing the more toxic $A\beta_{1–42}$ species. This study also showed that treatment of transgenic mice that develop Alzheimer-like pathology with an ACE inhibitor significantly increased the deposition of brain $A\beta_{1–42}$. However, there is no epidemiological evidence that human patients on ACE inhibitor therapy are any more prone to the development of Alzheimer's disease.

The only known functional homolog of ACE in humans was discovered in 2000 through genomics-based approaches.[29] ACE2, like ACE, is a transmembrane glycoprotein but with only a single catalytic site equivalent to the C-domain of ACE. Although ACE2 is highly similar in sequence to ACE around the active site, it displays a somewhat different substrate specificity and is not sensitive to inhibition by ACE inhibitors. ACE2 behaves as a simple carboxypeptidase removing a single C-terminal amino acid from a susceptible substrate. Hence, angiotensin I is converted to angiotensin-(1–9) by ACE2 rather than to angiotensin II (angiotensin-(1–8)). However, ACE2 acts much more efficiently on angiotensin II converting it to the vasodilatory angiotensin-(1–7) and hence serving to counterbalance the actions of ACE (see the section on Cardiovascular Peptides in this Handbook). Angiotensin-(1–7) can also be formed directly from angiotensin I through the action of NEP. Of 126 biologically active peptides tested as ACE2 substrates, only 11 were hydrolyzed by the enzyme, the most efficient being angiotensin II, apelin-13, and dynorphin A-(1-13).[32] Apelin-13 may be a physiologically relevant ACE2 substrate. A number of potent and specific ACE2 inhibitors have been reported, of which the first were the non-peptide inhibitor, MLN-4760, and a peptide identified from phage library screening, DX600.

INSULIN-DEGRADING ENZYME (INSULYSIN, IDE)

Insulin-degrading enzyme (IDE, insulysin) is another zinc endopeptidase involved in the degradation of biologically active peptides although it differs substantially from the ACE and NEP families in location and catalytic mechanism. IDE was originally isolated as an enzyme regulating plasma levels of insulin, hence its name, and is therefore seen as of potential significance as a therapeutic target in diabetes. In addition to insulin, IDE can hydrolyze a number of biologically active peptides including insulin-like growth factor II, tumor growth factor-α, and atrial natriuretic peptide.[27] Another important peptide substrate of IDE is the amyloid precursor protein intracellular domain, AICD, which acts as a transcriptional regulator controlling the expression of NEP and some other genes as discussed above.[3] IDE also degrades somatostatin, a neuropeptide that declines in aging and Alzheimer's disease.[5] Unlike the other peptidases described above, IDE is mainly a soluble cytoplasmic enzyme although it also exists as a secreted form.[34] There is also a splice-variant of IDE targeted to mitochondria. The mechanisms responsible for the subcellular targeting and secretion of IDE remain unresolved. The secreted form of IDE seems to play a role in the metabolism of Aβ peptide, was the first such peptidase identified and remains as a well validated Aβ-degrading enzyme from both *in vitro* and *in vivo* studies, including the use of transgenic approaches.[18] Hence, IDE is also seen as a potential target for upregulation in the treatment of Alzheimer's disease.

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IDE differs from ACE and NEP in possessing an inverted zinc-binding motif (His-Xaa-Xaa-Glu-His) and shows unusual allosteric behavior with small peptides. The structural solution of IDE[27] has shown the active site to be enclosed within a flexible clamshell-like structure and ATP is required to facilitate the opening of the catalytic chamber. Until recently, there were no potent and specific IDE inhibitors to facilitate studies of the role of the enzyme in the metabolism of biologically active peptides but rationally designed inhibitors based on the structural information have now been produced which confirm the role of IDE in regulating insulin signaling.[15] Such inhibitors may well have potential for the treatment of type 2 diabetes.

PROLINE-DIRECTED PEPTIDASES

Numerous biologically active peptides are partially protected from exopeptidase degradation by the presence of highly conserved prolyl residues especially at or near to the peptide N- and C-termini. Hence, very specific peptidases are required to initiate their metabolism because the conformational constraint imposed by the cyclic imino structure of proline hinders proteolysis.[31] The Xaa-Pro sequence is present at the N-terminus of >100 biologically active peptides or proteins, including peptide hormones, neurotransmitters, immunopeptides, and kinins. The best-characterized prolyl peptidase is the cell-surface serine peptidase, dipeptidyl peptidase IV (DPP IV) which, as its name suggests, hydrolyzes a dipeptide from the N-terminus of peptides provided that the second residue is a proline (or less favorably an alanine or serine). DPP IV is therefore one of only a few enzymes that have specificity directed toward prolyl residues. DPP IV was originally described as a rat liver enzyme releasing naphthylamine from Gly-Pro-2-naphthylamide and postulated to be involved in collagen metabolism. However, it was unable to cleave collagen and its physiological role remained unclear. Subsequent studies showed it to be an abundant peptidase of the renal and intestinal brush borders although it is present on most cell types. It is identical with the T cell-activating antigen (CD26) and, on T cells, it associates with the immunodeficiency-related protein adenosine deaminase via a cluster of charged amino acid residues. It is a type II membrane protein with only a short cytoplasmic tail (six residues) with the bulk of the enzyme facing the extracellular space where it can participate in the metabolism of a diverse range of biologically active peptides. DPP IV is also found as a soluble protein in serum whose levels are altered in cancer and some other diseases. The soluble form is most probably shed from the membrane-bound form although the protease responsible for this process is unknown. The enzyme is discussed in further detail, especially in relation to the immune response in this Handbook (see the chapter in Immune/Inflammatory Peptides section).

DPP IV is a typical serine peptidase, and a member of the "prolyl oligopeptidase" family, possessing the catalytic triad residues Ser/Asp/His (in rat $Ser^{631}/Asp^{709}/His^{741}$) and the enzyme functions as a homodimer and can also form functional tetramers. The crystal structure reveals that each subunit consists of two domains, an α/β-hydrolase domain and an eight-bladed β-propeller domain[23] with the catalytic site located in a cavity formed between the two domains both of which participate in substrate/inhibitor binding. The human DPP IV gene consists of 26 exons and, unusually among serine proteases, the nucleotides that encode the active site sequence (Gly-Xaa-Ser-Xaa-Gly) are split between two exons. DPP IV may play a digestive role in the digestion and uptake of prolyl-containing peptides. Most interest has focused, however, on the role of the enzyme in metabolizing biologically active peptides. In this capacity, it can either generate active peptide from a precursor, convert one active peptide to another with different receptor specificity (e.g. peptide YY or neuropeptide Y), or inactivate peptides. It is the latter role that has allowed the development of DPP IV inhibitors as the first new therapeutic class of orally active antihyperglycemic drug for type 2 diabetes mellitus for a long time through the ability of DPP IV to inactivate incretin hormones (glucagon-like peptide-1; glucose-dependent insulinotropic peptide).[6] Almost 50 biologically active peptides have been identified as substrates for DPP IV most of which have the N-terminal Xaa-Pro sequence, but some have Xaa-Ala or less commonly Xaa-Ser.

Another cell-surface peptidase capable of hydrolyzing peptides with a penultimate N-terminal prolyl residue is aminopeptidase P (APP2 to distinguish it from a cytosolic prolyl aminopeptidase, APP1). The enzyme removes the N-terminal residue leaving a free prolyl residue at the N-terminus. Examples of good substrates include bradykinin and neuropeptide Y. The specific inhibitor, apstatin, can reduce blood pressure by potentiating the action of bradykinin. A prolyl residue in the third position of a substrate renders the peptide resistant to the action of DPP IV (e.g. bradykinin, Arg-Pro-Pro-…). APP2 is a zinc peptidase and is unusual among cell-surface peptidases in being anchored to the cell surface via a glycosyl-phosphatidylinositol membrane anchor.

Unlike DPP IV and APP2, which are exopeptidases, another prolyl peptidase functions as an endopeptidase and can hydrolyze a wide range of small (<30 amino acids) biologically active peptides, at least *in vivo*. This is a serine peptidase originally known as postproline cleaving enzyme or proline endopeptidase, which cleaves peptides on the carboxy-terminal side of prolyl residues. It is now known as prolyl oligopeptidase (POP) and was originally implicated in oxytocin metabolism. Other potential substrates include substance P, angiotensins, bradykinin, β-endorphins, neurotensin, and thyrotropin releasing hormone (TRH). To evaluate the role of POP, numerous potent and relatively specific

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small molecule inhibitors have been developed, which exhibit a number of interesting physiological properties, for example, in enhancing memory, which is perhaps consistent with vasopressin also being a substrate for the enzyme. There are many reports that POP may have a role in psychiatric and neurodegenerative diseases although it does not seem to play a role in Aβ metabolism.[22] The evidence for a physiological role for POP in peptide degradation is, in most cases, lacking (see Ref. 8 for review) and a topological problem is that POP is a cytosolic enzyme and therefore unlikely to interact with circulating peptides. Another related proline-specific peptidase (acylpeptide hydrolase), which can be secreted from neuronal cells, may however contribute to Aβ metabolism.

Two specific areas where POP may also participate are in inositol phosphate signaling, and in angiogenesis and hence progression of malignancies. POP is able to enhance inositol phosphate signaling and cell sensitivity to lithium acting via the enzyme inositol polyphosphate phosphatase and hence regulate gene expression. POP is also able to cleave thymosin-β4 to generate the hemoregulatory peptide AcSDKP, itself a substrate for the N-domain of ACE (see above). Both AcSDKP and POP activity are substantially increased in certain malignancies.

CONCLUSIONS

Unlike the high specificity of ligand–receptor interactions, peptidases that degrade biologically active peptides tend to have multiple substrates, and hence, relatively few are needed to control the circulating levels of the multiplicity of such peptides described in this Handbook. They mostly exist as cell-surface ectoenzymes, or in a soluble, secreted form. The properties of some of the key ones, especially of the neprilysin family, have been described in this chapter, as well as methods for manipulating their activity. Structures are now available for most of these peptidases allowing rational design of potent and specific inhibitors. The promiscuity of these peptidases might suggest that inhibiting their activity could have undesirable side effects. However, in practice, this does not seem to be the case, as best exemplified by ACE inhibitors. NEP inhibitors also have a role to play in many conditions, although their chronic use could potentially lead to a build-up of brain Aβ peptide. There still remain numerous orphan peptidases, however, for which their physiological peptide substrates remain to be identified. Comparative *in vivo* peptidomic approaches in animals deficient in a particular peptidase now provide a powerful tool to categorize physiological substrate profiles for individual enzymes. We can expect to have a much clearer picture of the roles of peptidases in the metabolism of biologically active peptides in the next few years.

REFERENCES

1. Anthony CS, Corradi HR, Schwager SL, Redelinghuys P, Georgiadis D, Dive V, et al. The N domain of human angiotensin-I-converting enzyme: the role of N-glycosylation and the crystal structure in complex with an N domain-specific phosphinic inhibitor, RXP407. *J Biol Chem* 2010;**285**:35685–93.
2. Belyaev ND, Kellett KA, Beckett C, Makova NZ, Revett TJ, Nalivaeva NN, et al. The transcriptionally active amyloid precursor protein (APP) intracellular domain is preferentially produced from the 695 isoform of APP in a β-secretase-dependent pathway. *J Biol Chem* 2010;**285**:41443–54.
3. Belyaev ND, Nalivaeva NN, Makova NZ, Turner AJ. Neprilysin gene expression requires binding of the amyloid precursor protein intracellular domain to its promoter: implications for Alzheimer disease. *EMBO Rep* 2009;**10**:94–100.
4. Bland ND, Pinney JW, Thomas JE, Turner AJ, Isaac RE. Bioinformatic analysis of the neprilysin (M13) family of peptidases reveals complex evolutionary and functional relationships. *BMC Evol Biol* 2008;**8**:16.
5. Ciaccio C, Tundo GR, Grasso G, Spoto G, Marasco D, Ruvo M, et al. Somatostatin: a novel substrate and a modulator of insulin-degrading enzyme activity. *J Mol Biol* 2009;**385**:1556–67.
6. Demuth H-U, McIntosh CH, Pederson RA. Type 2 diabetes-therapy with dipeptidyl peptidase IV inhibitors. *Biochim Biophys Acta* 2005;**1751**:33–44.
7. Eckman EA, Watson M, Marlow L, Sambamurti K, Eckman CB. Alzheimer's disease β-amyloid peptide is increased in mice deficient in endothelin-converting enzyme. *J Biol Chem* 2003;**278**:2081–4.
8. Garcia-Horsman JA, Männistö PT, Venäläinen JI. On the role of prolyl oligopeptidase in health and disease. *Neuropeptides* 2007;**41**:1–24.
9. Hafez D, Huang JY, Huynh AM, Valtierra S, Rockenstein E, Bruno AM, et al. Neprilysin-2 is an important β-amyloid degrading enzyme. *Am J Pathol* 2011;**178**:306–12.
10. Hu J, Igarashi A, Kamata M, Nakagawa H. Angiotensin-converting enzyme degrades Alzheimer amyloid β-peptide (Aβ); retards Aβ aggregation, deposition, fibril formation; and inhibits cytotoxicity. *J Biol Chem* 2001;**276**:47863–8.
11. Ikeda K, Emoto N, Raharjo SB, Nurhantari Y, Saiki K, Yokoyama M, et al. Molecular identification and characterisation of a novel membrane-bound metalloprotease, the soluble form of which hydrolyzes a variety of vasoactive peptides. *J Biol Chem* 1999;**274**:32469–77.
12. Iwata N, Tsubuki S, Takaki Y, Watanabe K, Sekiguchi M, Hosoki E, et al. Identification of the major Aβ1–42 degrading catabolic pathway in brain parenchyma: Suppression leads to biochemical and pathological deposition. *Nature Med* 2000;**6**:143–50.
13. Lambert DW, Clarke NE, Turner AJ. Not just angiotensinases: new roles for the angiotensin-converting enzymes. *Cell Mol Life Sci* 2010;**67**:89–98.
14. Lambert LA, Whyteside AR, Turner AJ, Usmani BA. Isoforms of endothelin-converting enzyme-1 (ECE-1) have opposing effects on prostate cancer cell invasion. *Br J Cancer* 2008;**99**:1114–20.
15. Leissring MA, Malito E, Hedouin S, Reinstatler L, Sahara T, Abdul-Hay SO, et al. Designed inhibitors of insulin-degrading enzyme regulate the catabolism and activity of insulin. *PLoS One* 2010:5:e10504.
16. Marr RA, Rockenstein E, Mukherjee A, Kindy MS, Hersh LB, Gage FH, et al. Neprilysin gene transfer reduces human amyloid pathology in transgenic mice. *J Neurosci* 2003;**23**:1992–6.

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17. Miller L, Hou X, Gagnidze K, Sweedler JV, Sweedler JV, Wetsel WC, et al. Mice deficient in endothelin-converting enzyme-2 exhibit abnormal responses to morphine and altered peptide levels in the spinal cord. *J Neurochem* 2011;**119**:1074–85.
18. Nalivaeva NN, Fisk LR, Belyaev ND, Turner AJ. Amyloid-degrading enzymes as therapeutic targets in Alzheimer's disease. *Curr Alzheimer Res* 2008;**5**:212–24.
19. Natesh R, Schwager SL, Sturrock ED, Acharya KR. Crystal structure of the human angiotensin-converting enzyme–lisinopril complex. *Nature* 2003;**421**:551–4.
20. Oefner C, D'Arcy A, Hennig M, Winkler FK, Dale GE. Structure of human neutral endopeptidase (neprilysin) complexed with phosphoramidon. *J Mol Biol* 2000;**296**:341–9.
21. Pardossi-Piquard R, Petit A, Kawarai T, Sunyach C, Alves da Costa C, Vincent B, et al. Presenilin-dependent transcriptional control of the Aβ-degrading enzyme neprilysin by intracellular domains of βAPP and APLP. *Neuron* 2005;**46**:541–54.
22. Petit A, Barelli H, Morain P, Checler F. Novel proline endopeptidase inhibitors do not modify Aβ40/42 formation and degradation by human cells expressing wild-type and Swedish mutated β-amyloid precursor protein. *Br J Pharmacol* 2000;**130**:1613–7.
23. Rasmussen HB, Branner S, Wiberg FC, Wagtmann N. Crystal structure of human dipeptidyl peptidase IV/CD26 in complex with a substrate analog. *Nat Struct Biol* 2003;**10**:19–25.
24. Rawlings ND, Barrett AJ, Bateman A. Asparagine peptide lyases: a seventh catalytic type of proteolytic enzymes. *J Biol Chem* 2011; **286**:38321–8.
25. Roosterman D, Cottrell GS, Padilla BE, Muller L, Eckman CB, Bunnett NW, et al. Endothelin-converting enzyme 1 degrades neuropeptides in endosomes to control receptor recycling. *Proc Natl Acad Sci U S A* 2007;**104**:11838–43.
26 Rougeot C, Messaoudi M, Hermitte V, Rigault AG, Blisnick T, Dugave C, et al. Sialorphin, a natural inhibitor of rat membrane-bound neutral endopeptidase that displays analgesic activity. *Proc Natl Acad Sci U S A* 2003;**100**:8549–54.
27. Shen Y, Joachimiak A, Rosner MR, Tang WJ. Structures of human insulin-degrading enzyme reveal a new substrate recognition mechanism. *Nature* 2006;**443**:870–4.
28. Standeven KF, Hess K, Carter AM, Rice GI, Cordell PA, Balmforth AJ, et al. Neprilysin, obesity and the metabolic syndrome. *Int J Obes (Lond)* 2011;**8**:1031–40.
29. Tipnis SR, Hooper NM, Hyde R, Karran E, Christie G, Turner AJ. A human homolog of angiotensin-converting enzyme. Cloning and functional expression as a captopril-insensitive carboxypeptidase. *J Biol Chem* 2000;**275**:33238–43.
30. Turner AJ, Murphy LJ. Molecular pharmacology of endothelin converting enzymes. *Biochem Pharmacol* 1996;**51**:91–102.
31. Vanhoof G, Goossens F, De Meester I, Hendriks D, Scharpé S. Proline motifs in peptides and their biological processing. *FASEB J* 1995;**9**:736–44.
32. Vickers C, Hales P, Kaushik V, Dick L, Gavin J, Tang J, et al. Hydrolysis of biological peptides by human angiotensin-converting enzyme-related carboxypeptidase. *J Biol Chem* 2002;**277**:14838–43.
33. Whyteside AR, Turner AJ. Human neprilysin-2 (NEP2) and NEP display distinct subcellular localisations and substrate preferences. *FEBS Lett* 2008;**582**:2382–6.
34. Zhao J, Li L, Leissring MA. Insulin-degrading enzyme is exported via an unconventional protein secretion pathway. *Mol Neurodegener* 2009;**4**:4.
35. Zou K, Yamaguchi H, Akatsu H, Sakamoto T, Ko M, Mizoguchi K, et al. Angiotensin-converting enzyme converts amyloid β-protein 1-42 (Aβ1-42) to (Aβ1-40), and its inhibition enhances brain Aβ deposition. *J Neurosci* 2007;**27**:8628–35.

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Chapter 242

Peptidomics of Mice Lacking Peptide Processing Enzymes

Jonathan H. Wardman and Lloyd D. Fricker

ABSTRACT

Neuropeptides and most other biologically active peptides are produced from precursors through limited proteolysis. The major endopeptidases implicated in neuropeptide biosynthesis include prohormone convertase 1 (PC1, also known as PC3) and prohormone convertase 2. After endopeptidase cleavage, most peptides require the action of carboxypeptidase E to remove the basic C-terminal residues. Each of these various peptide-processing enzymes has been studied by purification and characterization of the activity with a variety of peptide substrates. An additional approach to study these enzymes has involved the generation of mice lacking each enzyme, by gene disruption and/or by mutation, and then the analysis of this disruption on the peptidome of the mouse brain. These peptidomic approaches are described in this chapter.

INTRODUCTION

For an enzyme to be considered a peptide-processing enzyme, it must meet several criteria. First, the enzyme must be expressed in the tissues where the neuropeptide is produced, and within that tissue it must be present in the correct subcellular compartment. For neuropeptide biosynthesis, this means that the enzymes should be present within the neuroendocrine system and localized to the secretory pathway, specifically the immature and mature secretory granules. Second, the enzyme must have the correct properties when purified and tested with substrates. These properties include pH optima and ion regulation, and substrate specificity. Finally, conclusive proof that the enzymes function in peptide processing requires the demonstration that elimination of enzyme activity has an effect on peptide levels. Thus, analysis of peptides in knockout mice, or in mice that lack enzyme activity due to a genetic mutation, is a critical step in defining each enzyme's function.

The major processing enzymes in the production of most biologically active neuropeptides are the prohormone convertases (PCs, which are also known as proprotein convertases), and carboxypeptidase E (CPE). There are two PCs that play a major role in neuropeptide production; these are PC1 (which is also known as PC3, and abbreviated as PC1/3) and PC2 (described in detail in other chapters in this section of the book). Other PCs that contribute to the production of some neuropeptides include furin and related enzymes that are present in the trans-Golgi network. However, furin and most other trans-Golgi enzymes are excluded from the secretory granules where the majority of neuropeptide precursor processing takes place. For example, the trans-Golgi enzyme carboxypeptidase D can participate in the processing of some neuropeptide precursors, but most of the carboxypeptidase processing is believed to occur through the action of CPE, which is present in the maturing secretory granules. Some bioactive peptides require a C-terminal amide group for biological activity; this is catalyzed from precursors containing a C-terminal glycine residue via the action of peptidylglycine-alpha-amidating monooxygenase (PAM). Many bioactive peptide hormones require this modification, but fewer neuropeptides are C-terminally amidated.

Knockout mice for PC1/3 and PC2, CPE, and PAM have been created and characterized using a variety of techniques.[4,6,9,27] In addition, mice lacking CPE activity because of a naturally occurring mutation have been studied.[15] Of these mice, the PAM knockout mice are embryonic lethal, thus limiting the analysis; all others are viable. The individual phenotype of each knockout or mutant line is described below, in the section describing the results of the peptidomic analysis. Each of these enzymes is also the subject of a separate chapter in this section of the handbook.

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00242-6
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QUANTITATIVE PEPTIDOMIC TECHNIQUES

Peptidomics is defined as the analysis of peptides within a biological sample. In general, peptidomics approaches are similar to proteomics techniques, except that most peptidomic studies do not treat the samples with digestive enzymes that are usually included to make the analysis of proteins easier (by converting the proteins into peptides). By avoiding the digestive enzyme step, the endogenous forms of the peptides can be identified by mass spectrometry. Technically, it is not possible to detect all peptides that are present in a sample due to the limitations of the detection method. In most biological samples, the range of peptide levels from the most abundant to the least abundant can span many orders of magnitude, while the range of typical mass spectrometers is only a few orders of magnitude due to problems associated with background and ion suppression. In addition, some peptides do not ionize well. Peptides with no positively charged groups will not be detected in the positive-ion mode of the mass spectrometer, which is more commonly used and more sensitive than the negative ion mode. Some biologically active peptides, such as thyrotropin releasing hormone, are not detected by peptidomics studies, presumably because of the size (three amino acids).

Despite these limitations, peptidomics is ideally suited for the analysis of the peptidome of mice lacking peptide-processing enzymes. In particular, the use of quantitative peptidomics techniques to compare the relative levels of peptides in mutant mice versus wild-type littermates provides an ideal system to examine the biological role of the peptide-processing enzymes. Peptidomics studies can rapidly detect hundreds of peptides in a single experiment and generally reveal the precise forms of each peptide.[1,8,10,19,21] This is in contrast to radioimmunoassays (RIAs) that require considerable time for the generation and characterization of the antisera, and these antisera are rarely specific for a single form of a peptide and usually detect N- and/or C-terminally extended forms. Additional studies are required to verify that the peptide detected by RIA corresponds to a particular form of the peptide. By contrast, the mass of a peptide determined by modern mass spectrometers can be determined within a fraction of a Dalton, much less than the differences between forms of a peptide due to altered processing.

A variety of quantitative approaches have been used to compare the relative levels of peptides among two or more samples. One approach uses isotopic labels in which a peptide-modifying reagent is tagged with different numbers of stable isotopes. Our laboratory has tested a variety of reagents that modify amine groups; most peptides contain one or more free amines, and so this is a convenient way to label the most number of peptides. The ideal labels for quantitative peptidomics are those based on trimethylaminobutyric acid (TMAB), with the amine-reactive form of the label produced as the N-hydroxysuccinimide (NHS) ester (Fig. 1). It is possible to synthesize the TMAB tags in five isotopic forms that differ by 3 Da between each form; this enables comparison of up to five different samples in a single experiment.[14] This technique has been used to examine the peptide levels in mice lacking peptide-processing enzymes, relative to the levels of these peptides in control wild-type mice.[22–24]

CPE-DEFICIENT MICE

Mice with a naturally occurring point mutation within the CPE gene were initially detected in the 1970s, although it took decades before the genetic analysis was performed and the mutation identified. The mutant mouse was named *fat* because of the increased adiposity of these mice. Subsequent to the identification of *Cpe* as the gene containing the mutation, the mouse was renamed $Cpe^{fat/fat}$.[15] These mice also show greatly impaired reproduction and are generally sterile due to an inability to mate properly. Using RIAs, many peptides were found to be greatly affected by the mutation of CPE.[7] In most cases, the mature form of the peptide was present at much lower levels in the $Cpe^{fat/fat}$ mouse brain or pituitary, compared with that of wild-type mouse tissue. Exceptions to this are mainly the C-terminal prohormone peptides, which do not require CPE for biosynthesis. In a few situations, the peptide-processing intermediates were examined by RIA in the $Cpe^{fat/fat}$ mice and found to be greatly elevated. However, these studies were difficult to do because most antisera did not readily distinguish between the form of the peptide containing the C-terminal basic residue (i.e. the processing intermediate) and the form lacking this residue (i.e. the mature form). Therefore, peptidomics analysis of $Cpe^{fat/fat}$ mouse brain and pituitary is an ideal way to examine the relative levels of hundreds of neuropeptides and the peptide-processing intermediates.

The TMAB labels were used for peptidomic analysis of relative levels of peptides in $Cpe^{fat/fat}$ mice, compared with that in wild-type mice. In addition, to comparing the variability of these peptides among replicates of wild-type mice, controls were performed in which peptidomic analysis of groups of wild-type mice was compared with that of other wild-type mice. This analysis showed low variability of the peptides among control groups, in contrast to the difference between peptide levels in $Cpe^{fat/fat}$ mice versus wild-type mice.[23] To visualize these differences, summary plots of the peptidome were generated from the previously published data. For these plots, the raw data was plotted, with each dot corresponding to the ratio for a peptide observed in one of the experiments. This ratio was either the comparison of one wild-type mouse sample to another wild-type mouse sample (Fig. 2, top panels) or the comparison of one $Cpe^{fat/fat}$ mouse sample with a wild-type mouse

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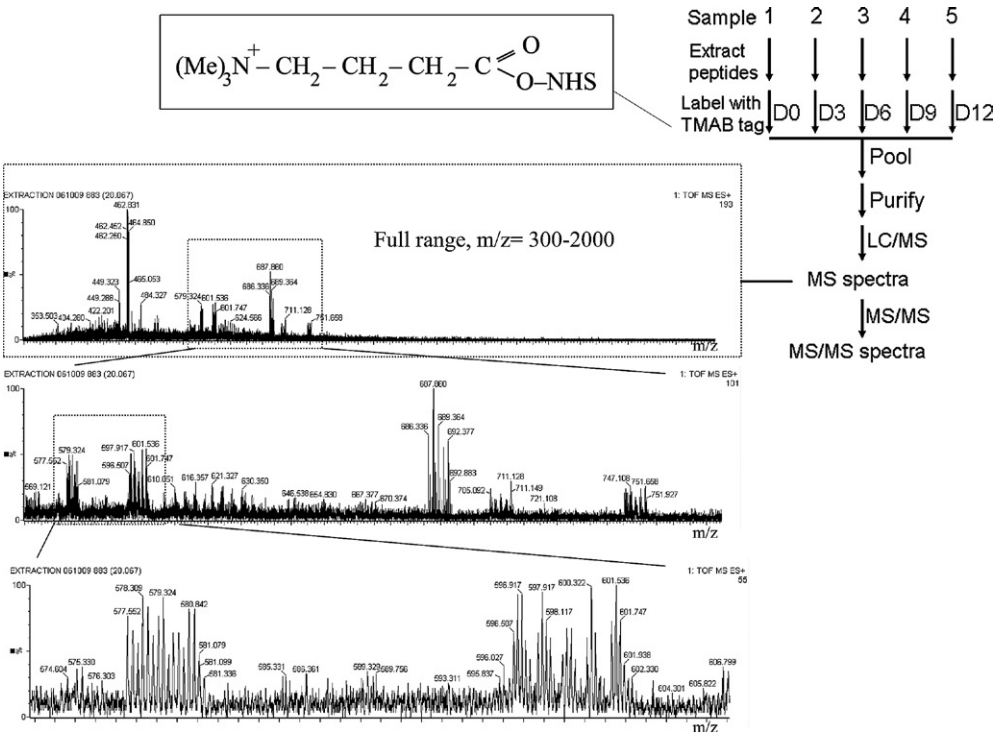

FIGURE 1 Quantitative peptidomic scheme using trimethylaminobutyric acid (TMAB) tags activated with *N*-hydroxysuccinimide (NHS). The synthesis of the TMAB labels and incorporation of these labels into the peptides have been recently described.[14] In general, the labeling is done early in the process of peptide extraction and purification so that losses of peptide during purification steps do not contribute to variation in the peptide levels between samples. Once peptides are labeled and pooled, the similar chemical nature of the different isotopic tags means that all forms will show equal recoveries on the subsequent purification steps. Analysis is typically performed by running the samples on liquid chromatography (HPLC) followed directly by on-line electrospray mass spectrometry. The resulting mass spectra show groups of peaks that differ by 3 Da, each representing a different form of the TMAB label. The intensity of each of these peaks reflects the levels of peptide in the original samples, thus providing a relatively rapid measurement.[14] The mass spectrometer also provides sequence information of peptides selected for fragmentation and further mass spectrometry of the product ions, which are referred to as MS/MS spectra. From these MS/MS spectra, it is often possible to determine the sequence of the peptide. Thus, the quantitative peptidomic approach provides relative quantification and identification of hundreds of peptides in a single sample. Figure copyright 2011, Lloyd Fricker, reprinted with permission.

sample (Fig. 2, bottom panels). Each experiment was run with multiple replicates, and with many brain regions; for original data, see supplemental tables with the original published report.[23] To generate the summary plots shown in Fig. 2, all ratio values for a particular comparison (i.e. wild-type versus wild-type, or *Cpe*$^{fat/fat}$ versus wild-type) were combined into a single column on a spreadsheet. If a peptide was found in multiple replicates, or in different brain regions, each time the peptide was found, it appeared in the spreadsheet. Then, the combined list of all ratio values was sorted from low to high and plotted. The *y*-axis is the ratio of each data point, while the *x*-axis represents the rank order of that peptide in the replicate.

The summary plots show the relative low variability when peptides are compared among different wild-type mice, with most of the ratio values around the theoretically expected 1.00 (Fig. 2, top). This is true both for peptides derived from secretory pathway proteins (i.e. neuropeptide precursors) as well as peptide derived from cytosolic or mitochondrial proteins (Fig. 2, top left versus top right). By contrast, the summary plots for peptides in the *Cpe*$^{fat/fat}$ mice, relative to the wild-type mice, show that the peptides derived from secretory pathway proteins are greatly affected by the absence of CPE activity (Fig. 2, bottom left). Approximately one-third of the peptides are not detected in the *Cpe*$^{fat/fat}$ mice (levels <10% of the wild-type level; this is the cut-off for the peptidomic analysis because of the detection limit for the typical peptide). These peptides represent CPE products that require this enzyme for the production of the majority of the peptide.[23] Another one-third of the peptides show ratios <1.0, but above the lower detection limit of 0.1; these peptides probably correspond to products of CPE that are also cleaved by CPD earlier in the secretory pathway.[23] Thus, the levels of these peptides decrease in the CPE-deficient mice, but they are still detected, reflecting the contribution from CPD. A large number of peptides are greatly elevated in the *Cpe*$^{fat/fat}$ mice, with ratios ≥10 indicating a 10-fold increase or more.

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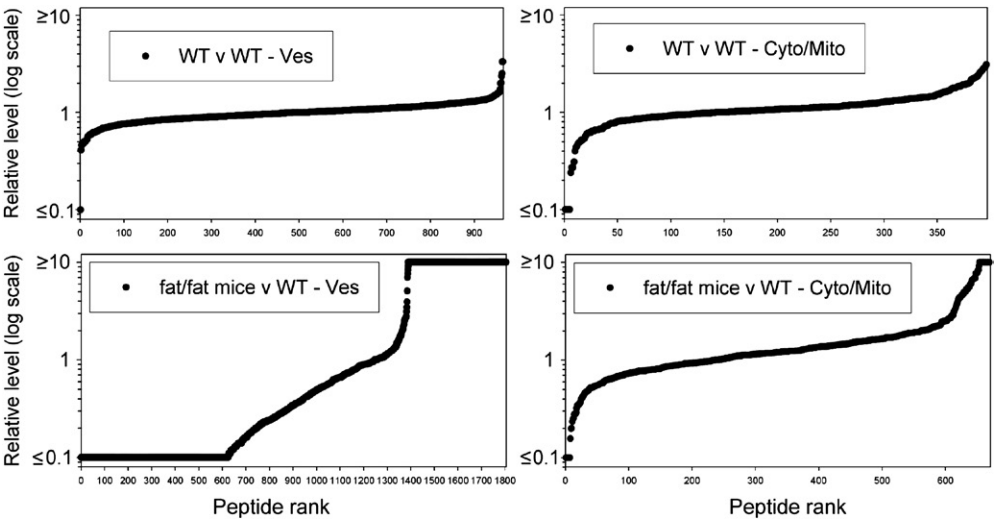

FIGURE 2 Summary plots of the peptidome of mouse brain. Top panels: Levels of peptides in one group of wild-type (WT) mice were compared with the levels of another group of WT mice to determine the general variability of the peptides among groups of mice. Bottom panels: Levels of peptides in *Cpe*$^{fat/fat}$ mice were compared with the levels in WT mice. Peptides were identified by MS/MS analysis. Plots of peptides from secretory pathway proteins (such as neuropeptide precursors) are shown in the left panels, and peptides derived from cytosolic or mitochondrial proteins are shown in the right panels. To generate these plots, the ratio of peptide levels, relative to the control group, were combined into a single column in a spreadsheet, sorted by relative order from lowest to highest, and then plotted. The *y*-axis represents the relative level (log scale), with the lowest values capped at 0.10 and the highest capped at 10 (i.e. any value <0.10 or >10 was plotted as 0.10 and 10, respectively). The *x*-axis for these plots represents the relative order of the peptide after sorting. Data were previously published.[13,23] Abbreviations: Ves, secretory pathway (vesicles); Cyto/Mito, cytosolic and/or mitochondrial. Copyright Lloyd Fricker, 2011; reprinted with permission.

Most of these peptides contain C-terminal Lys and/or Arg residues, and these peptides are not detected in the wild-type mice. Only a few peptides showed no change or a moderate increase in the *Cpe*$^{fat/fat}$ mice; these are generally C-terminal fragments of the prohormone precursor that do not require CPE for their biosynthesis.[23]

PC KNOCKOUT MICE

The prohormone convertases have been studied using many *in vitro* techniques, including purified recombinant enzymes, viral overexpression techniques, and several different neuroendocrine cell lines, including AtT-20 mouse adrenal corticotroph cells, PC12 rat pheochromocytoma cells, and others.[2,3,12,25,26] Despite the observation that both PC1/3 and PC2 cleave peptides at multibasic sites, each enzyme has its own preferences.[11,18,22] These peptidases are also expressed at varying levels in different neuroendocrine tissues, which can lead to tissue specific processing of a single precursor. Although *in vitro* enzymatic and cell biological methods are effective for understanding the basic properties of the prohormone convertases, studies on mice lacking the enzymes are needed to complement the *in vitro* studies.

The PC1/3 null mice exhibit severe developmental defects including abnormal growth, a phenotype probably resulting from incomplete processing of growth hormone-releasing hormone (GHRH).[27] PC1/3 mice are hyperproinsulinemic, though they exhibit normal glucose tolerance, likely due to the insulin-like effect of proinsulin.[27] Initial studies in the effects of the loss of PC1/3 on the mouse brain peptidome were performed using RIA. These studies reveal an essential role for PC1/3 in the processing of several mouse brain peptides, including those derived from the enkephalin precursor. These RIA studies reveal peptides processed specifically by PC1/3 for which PC2 could not compensate.[17]

Quantitative peptidomic techniques were used to compare the processing of peptides in the PC1/3 null mouse brain relative to their wild-type counterparts.[22] These studies used the stable isotopic TMAB tags for quantitative analysis and found that the lack of PC1/3 causes changes in the levels of many neuropeptide precursors. Among the peptides affected are some putative specific substrates of PC1/3, which show large decreases despite the presence of PC2. This study also reveals many peptides that are unchanged despite the lack of PC1/3, indicating that they are produced by another enzyme.[17] Further peptidomic studies performed using PC1/3 null mice examined the function of PC1/3 across several brain regions, including the hypothalamus, the amygdala, and the striatum, using multiple isotopic tags. One of the goals of the peptidomics study of PC knockout mice is to better understand the nature of the cleavage site of PC1/3 and to determine the amino acid sequences characteristic of specific PC1/3 substrates.[17] By observing the amino acids frequently found in the cleavage site of those peptides affected by the lack of PC1/3, and comparing those residues with those observed in peptides

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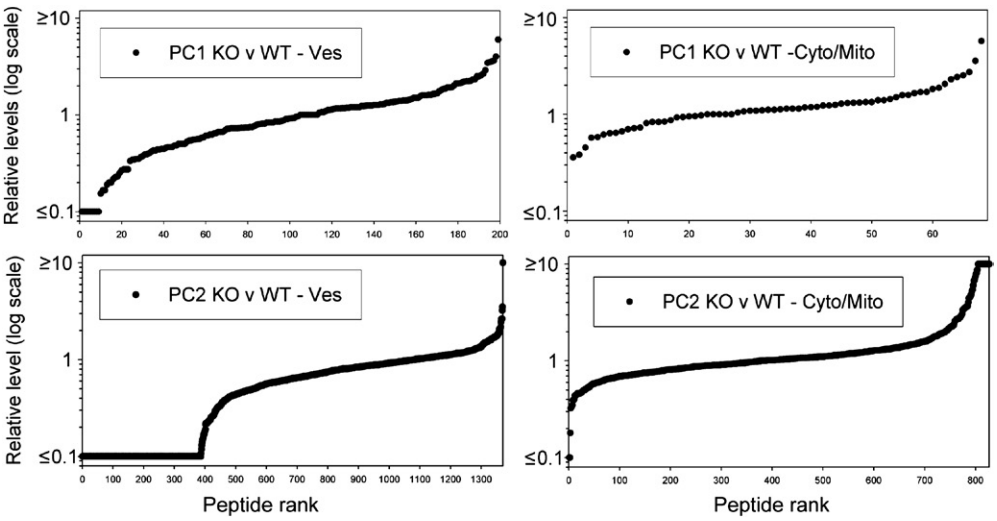

FIGURE 3 Summary plots of the peptidome of mouse brain, comparing PC1/3 knockout mice to WT mice (top panels) or PC2 knockout mice to WT mice (bottom panels). Both secretory pathway peptides (left) and peptides derived from cytosolic or mitochondrial proteins (right) are indicated. See Fig. 2 legend for explanation of the details of the plot and abbreviations. Data were previously published.[22,24] Copyright Lloyd Fricker, 2011; reprinted with permission.

specifically cleaved by PC2, it is possible to glean predictive sequence information for cleavage by each endopeptidase. This information in turn is useful in determining where and when in the secretory pathway the peptide is cleaved, both within the secretory pathway and among different cell types expressing different endopeptidase cohorts in the multiple brain regions examined.

These PC1/3 knockout studies reveal a few peptides that show a large decrease relative to wild-type mice (Fig. 3, upper left panel). These peptides that show a large decrease are presumably specific products of PC1/3 cleavage, as PC2 cannot make up for the lack of PC1/3 in these tissues. Many secretory pathway peptides are unaffected by the knockout of PC1/3, or only slightly affected, and this indicates that the enzyme does not have a unique role in the production of these peptides (Fig. 3, upper left panel). There are also several peptides that show an increase in the PC1/3 knockout animals, these probably being substrates of PC1/3 cleavage which increase because of the absence of PC1/3. Peptides derived from proteins found in the cytoplasm and mitochondria compartments show relatively low variability, with the majority of these peptides having a ratio value of approximately 1.00 (Fig. 3, top right panel), much like the wild-type vs. wild-type comparison (Fig. 2, top right panel).

PC2 null mice have also been studied to determine the specific contribution of this endopeptidase to peptide processing. The PC2 null mice exhibit retarded growth, despite being indiscernible from wild-type littermates at birth.[5] These mice also exhibit chronic fasting hypoglycemia and are deficient in circulating glucagon.[20] Quantitative peptidomic studies were performed with the PC2 null mice using the quantitative peptidomic technique previously described. In this case, hypothalami from wild type and PC2 knockout mice were compared using stable isotopic TMAB tags. Peptides from many secretory pathway neuropeptide precursors were identified.[16] Many of the peptides detected in wild-type mice are either undetectable in the PC2 knockout mice, or are present at levels ranging from 25 to 75% of wild-type levels (Fig. 3, lower left panel). The specifics of cleavage by PC2 were further characterized by comparing the residues most frequently found in the cleavage site of the incompletely processed peptides. The cleavage site residues in these substrate peptides must therefore be specifically cleaved by PC2, otherwise other prohormone convertases would have cleaved them in the absence of PC2. These comparative studies suggest that sequences with a Trp, Tyr, and/or Pro in the P1′ or P2′ position, or a basic residue in the P3 position, are particularly good PC2 substrates, versus other secretory pathway enzymes.[16] These studies were early indicators of the physiological specificity of propeptide cleavage by one endopeptidase versus another. Later, further quantitative peptidomic studies were performed with the PC2 null mice to better understand the physiological actions of PC2 in multiple brain regions, including the prefrontal cortex, hypothalamus, striatum, hippocampus, thalamus, and amygdala.[24] Like the previous studies, many of the peptides identified are undetectable in the tissues of the PC2 knockout mice, and another large percentage are significantly decreased. Only three peptides are increased significantly in the tissues of the knockout mice (Fig. 3, lower left panel).[24] This study serves to reinforce the broad role of PC2 in the processing of secretory pathway propeptides, but still maintains its redundancy with PC1/3 in many tissues. Cleavage

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site analysis reveals similar sequence preferences for PC2 over other secretory pathway peptidases as observed in the hypothalamus across all tissues examined in this study.[24]

CONCLUSIONS

Taken together, quantitative peptidomic studies provide an important level of analysis that complements the other approaches to study enzymes and their peptide substrates. By themselves, the studies examining peptides in tissues of mice lacking processing enzyme activity do not provide a complete picture because of several issues. If, for example, the levels of a peptide change in the knockout mice, one interpretation is that the missing enzyme is responsible for the production of that peptide. Alternatively, it is possible that the change in peptide results from an indirect effect, where the missing enzyme affects a system that in turn causes the peptide to change. Likewise, if a peptide does not change in the knockout mice, one interpretation is that the enzyme does not participate in the production of that peptide. Alternatively, it is possible that the enzyme does play a role in the production of the peptide, but because of redundancy in the system, the absence of any one enzyme does not show a measurable change. Thus, although the *in vivo* analysis is important, it needs to be carefully interpreted and considered together with the *in vitro* studies on the capability of the purified enzymes to cleave peptides. Despite the caveats, the use of quantitative peptidomics to detect, quantify, and identify the peptides is a great advantage over conventional approaches for the detection and quantification of neuropeptides (such as RIAs) because of the large number of peptides that can simultaneously be measured with peptidomics, and the accuracy of the identifications based on precise mass values that distinguish between related forms of peptides, as occurs when processing enzymes are absent.

REFERENCES

1. Baggerman G, Verleyen P, Clynen E, Huybrechts J, De Loof A, Schoofs L. Peptidomics. *J Chromatogr B Analyt Technol Biomed Life Sci* 2004;**803**:3–16.
2. Benjannet S, Rondeau N, Day R, Chretien M, Seidah NG. PC1 and PC2 are proprotein convertases capable of cleaving proopiomelanocortin at distinct pairs of basic residues. *Proc Natl Acad Sci USA* 1991;**88**:3564–8.
3. Breslin MB, Lindberg I, Benjannet S, Mathis JP, Lazure C, Seidah NG. Differential processing of proenkephalin by prohormone convertases 1(3) and 2 and furin. *J Biol Chem* 1993;**268**:27084–93.
4. Cawley NX, Zhou J, Hill JM, Abebe D, Romboz S, Yanik T, et al. The carboxypeptidase E knockout mouse exhibits endocrinological and behavioral deficits. *Endocrinology* 2004;**145**:5807–19.
5. Chabrol B, Martens K, Meulemans S, Cano A, Jaeken J, Matthijs G, et al. Deletion of C2orf34, PREPL and SLC3A1 causes atypical hypotonia-cystinuria syndrome. *J Med Genet* 2008;**45**:314–8.
6. Czyzyk TA, Ning Y, Hsu MS, Peng B, Mains RE, Eipper BA, et al. Deletion of peptide amidation enzymatic activity leads to edema and embryonic lethality in the mouse. *Dev Biol* 2005;**287**:301–13.
7. Fricker LD, Berman YL, Leiter EH, Devi LA. Carboxypeptidase E activity is deficient in mice with the fat mutation: effect on peptide processing. *J Biol Chem* 1996;**271**:30619–24.
8. Fricker LD, Lim J, Pan H, Che FY. Peptidomics: identification and quantification of endogenous peptides in neuroendocrine tissues. *Mass Spectrom Rev* 2006;**25**:327–44.
9. Furuta M, Yano H, Zhou A, Rouille Y, Holst JJ, Carroll R, et al. Defective prohormone processing and altered pancreatic islet morphology in mice lacking active SPC2. *Proc Natl Acad Sci USA* 1997;**94**:6646–51.
10. Hummon AB, Amare A, Sweedler JV. Discovering new invertebrate neuropeptides using mass spectrometry. *Mass Spectrom Rev* 2006;**25**:77–98.
11. Johanning K, Juliano MA, Juliano L, Lazure C, Lamango NS, Steiner DF, et al. Specificity of prohormone convertase 2 on proenkephalin and proenkephalin-related substrates. *J Biol Chem* 1998;**273**:22672–80.
12. Johanning K, Mathis JP, Lindberg I. Role of PC2 in proenkephalin processing: antisense and overexpression studies. *J Neurochem* 1996;**66**:898–907.
13. Lim J, Berezniuk I, Che FY, Parikh R, Biswas R, Pan H, et al. Altered neuropeptide processing in prefrontal cortex of Cpe fat/fat mice: implications for neuropeptide discovery. *J Neurochem* 2006;**96**:1169–81.
14. Morano C, Zhang X, Fricker LD. Multiple isotopic labels for quantitative mass spectrometry. *Anal Chem* 2008;**80**:9298–309.
15. Naggert JK, Fricker LD, Varlamov O, Nishina PM, Rouille Y, Steiner DF, et al. Hyperproinsulinemia in obese fat/fat mice associated with a point mutation in the carboxypeptidase E gene and reduced carboxypeptidase E activity in the pancreatic islets. *Nat Genet* 1995;**10**:135–42.
16. Pan H, Che FY, Peng B, Steiner DF, Pintar JE, Fricker LD. The role of prohormone convertase-2 in hypothalamic neuropeptide processing: a quantitative neuropeptidomic study. *J Neurochem* 2006;**98**:1763–77.
17. Pan H, Nanno D, Che FY, Zhu X, Salton SR, Steiner DF, et al. Neuropeptide processing profile in mice lacking prohormone convertase-1. *Biochemistry* 2005;**44**:4939–48.
18. Paquet L, Zhou A, Chang EY, Mains RE. Peptide biosynthetic processing: distinguishing prohormone convertases PC1 and PC2. *Mol Cell Endocrinol* 1996;**120**:161–8.
19. Sasaki K, Takahashi N, Satoh M, Yamasaki M, Minamino N. A peptidomics strategy for discovering endogenous bioactive peptides. *J Proteome Res* 2010;**9**:5047–52:Epub 2010/08/05. http://dx.doi.org/10.1021/pr1003455:PubMed PMID: 20681733.
20. Scamuffa N, Calvo F, Chretien M, Seidah NG, Khatib AM. Proprotein convertases: lessons from knockouts. *FASEB J* 2006;**20**:1954–63.
21. Svensson M, Skold K, Nilsson A, Falth M, Svenningsson P, Andren PE. Neuropeptidomics: expanding proteomics downwards. *Biochem Soc Trans* 2007;**35**:588–93.
22. Wardman JH, Zhang X, Gagnon S, Castro LM, Zhu X, Steiner DF, et al. Analysis of peptides in prohormone convertase 1/3 null mouse brain using quantitative peptidomics. *J Neurochem* 2010;**114**:215–25.
23. Zhang X, Che FY, Berezniuk I, Sonmez K, Toll L, Fricker LD. Peptidomics of Cpe(fat/fat) mouse brain regions: implications for neuropeptide processing. *J Neurochem* 2008;**107**:1596–613.

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24. Zhang X, Pan H, Peng B, Steiner DF, Pintar JE, Fricker LD. Neuropeptidomic analysis establishes a major role for prohormone convertase-2 in neuropeptide biosynthesis. *J Neurochem* 2010;**112**:1168–79.
25. Zhou A, Mains RE. Endoproteolytic processing of proopiomelanocortin and prohormone convertases 1 and 2 in neuroendocrine cells overexpressing prohormone convertases 1 or 2. *J Biol Chem* 1994;**269**:17440–7.
26. Zhou Y, Lindberg I. Purification and characterization of the prohormone convertase PC1 (PC3). *J Biol Chem* 1993;**268**:5615–23.
27. Zhu X, Zhou A, Dey A, Norrbom C, Carroll R, Zhang C, et al. Disruption of PC1/3 expression in mice causes dwarfism and multiple neuroendocrine peptide processing defects. *Proc Natl Acad Sci USA* 2002;**99**:10293–8.

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Chapter 243

Peptidomics Strategy for Discovering Endogenous Biologically Active Peptides

Kazuki Sasaki and Naoto Minamino

ABSTRACT

Assisted by nanoflow liquid chromatography, mass spectrometry (MS) permits a large-scale identification of endogenous peptides on a chromatographic timescale for an organism whose genome sequence is available. In analogy with other "-omics" disciplines, a systematic study of such peptides is collectively referred to as peptidomics. Although MS seems to be an appealing technique for identifying previously uncharacterized peptide hormones or neuropeptides, comprehensive peptidomics of brain or endocrine tissues does not lead to a fruitful outcome. The rapid metabolism of bioactive peptides should also be considered. This chapter describes the concept of "secretopeptidome" that we have proposed, and some of our peptidomic strategies to discover mammalian biologically active peptides through secretopeptidome mining. Functions of the novel bioactive peptides identified with this approach will also be discussed.

MASS SPECTROMETRY IN ENDOGENOUS PEPTIDE RESEARCH

Discovery of biologically active peptides, whatever approaches are taken, remains a great challenge in life science. Aside from an in silico search for peptides homologous to biologically active peptides, most studies have involved activity-guided purification using specific biological or biochemical assays in conjunction with liquid chromatography (LC), and Edman degradation for sequence determination and subsequent cDNA cloning with established molecular biology techniques. In the 1990s, mass spectrometry (MS) was adopted to profile peptides in biological samples, where in most cases only dominant peaks were identified on the basis of the measured mass values corresponding to known biologically active peptides.[6] The impact of MS to differentiate peptides by one mass unit was acknowledged but was not suitable to discovery-oriented approaches at that time.

The introduction of mass spectrometers capable of tandem mass spectrometry (MS/MS) inspired peptide researchers in many aspects. By using genome sequence data, MS/MS provides digital information that helps to identify a peptide sequence, along with the identification and localization of accompanying posttranslational modifications. There are obvious advantages of MS/MS over conventional methods; we need not remove peptide N-terminal blocking or purify a peptide of interest to near homogeneity. Earlier this century, the term peptidomics was advocated to study the full complement of endogenous peptides, or the peptidome.[5,25] Initial studies intended for biomedical or agrochemical applications have been published.[5,22,25,26] Peptidomics absolutely requires that every endogenous peptide be analyzed in its native form to understand how the peptide is generated through precursor processing and then degraded by proteases/peptidases. The analysis of peptides present in complex mixtures is promoted by LC–MS/MS, which has become a key technology indispensable to peptidomics. With the available genome information, LC–MS/MS elucidates hundreds of peptide sequences of a target organism on a chromatographic timescale. In current peptidomics laboratories worldwide, LC–MS/MS is conducted on a nanoflow LC column connected on-line with a high-performance tandem mass spectrometer. The state-of-the-art mass spectrometers enable mass measurement within a mass accuracy of 2 ppm in a routine LC–MS/MS experiment[23] (Table 1).

Early peptidomics studies concerned the identification of endogenous peptides in the rat and mouse hypothalamus and pituitary, but they suffered from an overwhelming number of peptide fragments derived from cytosolic proteins or major contaminating proteins like albumin and hemoglobins. Most of these peptides were produced as a result of protease activation that occurs postmortem and/or during tissue extraction and sample preparation.[15] Some solutions to this problem include heat denaturation such as focused

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00243-8
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TABLE 1 Peptides Observed as Intense Signals in an LC–MS/MS Analysis of the TT Secretopeptidome

Mr(obsd)	Mr(calc)	Mass error (ppm)	N-term	Sequence	C-term	Precursor
1243.561	1243.562	-0.36	QDEMRLELQR	SANSNPAMAPRE	RKAGCKNFFW	SST
1510.753	1510.753	0.32	Signal	FPKPGGSQDKSLHN	RELSAERPLN	SCG3
2451.066	2451.066	0.13	IGYGAPGKKR	DMSSDLERDHRPHYSMPQNAN	C-term	CT
1914.011	1914.011	-0.20	EEKRKRKK	NAPPEPYPPPRAAPAPTHY	RSPQPPPPAP	VGF
2435.071	2435.071	0.08	IGYGAPGKKR	DMSSDLERDHRPHYSMPQNAN	C-term	CT
3950.988	3950.988	0.12	Signal	APPGRPEAQPPPLSSEHKEPYAGDAVPGPKDGSAPEYRGA	RNSEPQDEGE	VGF
2321.028	2321.028	-0.10	IGYGAPGKKR	DMSSDLERDHRPHYSMPQNA	N(C-term)	CT
3666.827	3666.828	-0.13	Signal	APPGRPEAQPPPLSSEHKEPYAGDAVPGPKDGSAPEY	RGARNSEPQD	VGF
4960.486	4960.486	-0.02	M	Ac-SDKPDMAEIEKFDKSKLKKTETQEKNPLPSKETIEQEKQAGES	C-term	TMSB4X
4933.523	4933.523	0.05	M	Ac-ADKPDMGEIASFDKAKLKKTETQEKNTLPTKETIEQEKRSEIS	C-term	TMSB10
1278.671	1278.672	-1.05	LSRSGGYYKN	NFYPTNYGSKAF-NH2	GRRRDLQA(C-term)	CGRP
3931.966	3931.966	-0.02	Signal	FPKPGGSQDKSLHNRELSAERPLNEQIAEAEEDKI	KKTYPPENKP	SCG3
3406.686	3406.687	-0.05	RDFSPSSAKR	QQETAAAETETRTHTLTRYNLESPGPERYW	RASWGEFQAR	VGF
3389.659	3389.660	-0.35	RDFSPSSAKR	<QQETAAAETETRTHTLTRYNLESPGPERYW	RASWGEFQAR	VGF
2283.124	2283.123	0.45	GSQDKSLHNR	ELSAERPLNEQIAEAEEDKI	KKTYPPENKP	SCG3
1599.869	1599.870	-0.18	Signal	YPLPAGGGTVLTKMYP	RGNHWAVGHL	GRP
2642.338	2642.338	0.06	EGSRIIAQKR	ACDTATCVTHRLAGLLSRSGGVVKN	NFYPTNYG	CGRP
1752.775	1752.775	-0.30	AMAPRERK	AGCKNFFWKTFTSC	C-term	SST
2642.319	2642.319	-0.07	Signal	APFRSALESSPADPATLSEDEARLL	LAALYQDY	CT/CGRP
1844.887	2642.319	-0.36	EGSRIIAQKR	ACDTATCVTHRLAGLLS	RSGGYVKN	CGRP
5687.911	5687.908	0.48	Signal	ERPYFTNHFLYELHKGGEDKARQYAAEHGFGYRKLPFAEGLYHFYHNGLA	KAKRRRSLHH	PC2
4349.040	4349.042	-0.40	EIINSNQYKR	YPGQGSSEDDLQEEEQIEQAIKEHLNQGSSQETDKLAPYS	KRFPYGPPKN	SCG2
3547.634	3547.632	0.45	SSLDSPRSKR	CGNLSTCMLGTYTQDFNKFHTFPQTAIGVGAP-NH2	GKKRDMSSDL	CT
3903.001	3903.000	0.32	EGSRIIAQKR	ACDTATCVTHRLAGLLSRSGGVVKNNFYPTNYGSKAF-NH2	GRRRDLQA(C-term)	CGRP
8559.618	8559.617	0.17		intact ubiquitin	C-term	Ubiquitin
3705.834	3705.835	-0.15	GREAQARR	AQEEAEAEERRLQEQEELENYIEHVLLRRP	C-term	VGF
3531.633	3531.637	-1.31	LDSPRSKR	CGNLSTCMLGTYTQDFNKFHTFPQTAIGVGAP-NH2	GKKRDMSSDL	CT
4992.517	4992.518	-0.17	WLKKHDKK	GNKEDYDLSKMRDFINKQADAYYEKGILDKEEAEAIKRIYSSL	C-term	SCG3
6217.036	6217.040	-0.53	Signal	APFRSALESSPADPATLSEDEARLLLAALYQDYYQMKASELEQEQEREGSSLDSPRS	KRCGNLSTCM	CT
6056.045	6056.044	0.23	Signal	APFRSALESSPADPATLSEDEARLLLAALYQDYYQMKASELEQEQEREGSRIIAQ	KRACDTATCV	CGRP

LC–MS/MS for sequence identification was performed on an LTQ-OrbiTrap mass spectrometer, with a mass accuracy within 2 ppm. Peptides are listed in elution order. Mr(obsd), observed monoisotopic mass value of the peptide; Mr(calc), theoretical monoisotopic mass value of the indicated sequence. The N-(N-term) and C-terminal (C-term) flanking 10 amino acids (columns 4 and 6) are shown and marked as follows: closed boxes with white letters indicate typical PC1/3 or PC2 consensus cleavage sites containing consecutive basic residues or canonical C-terminal amidation motifs; dark gray boxes, PC1/3 or PC2 consensus cleavage sites containing basic residues at P4, P6, or P8 positions; pale gray boxes, sites having basic residues at P1 but not at P2, P4, P6, or P8 positions. "Signal" indicates that the peptide is adjacent to its signal sequence. "C-term" indicates that the peptide C-terminus is the end of the precursor. Ac-, N-terminal acetylation; -NH_2, C-terminal amidation; <Q, pyroglutamic acid. Oxidized methionines are underlined in column 5. CGRP, calcitonin gene-related peptide; CT, calcitonin; GRP, gastrin-releasing polypeptide; PC2, prohormone convertase 2; SCG2, secretogranin II; SCG3, secretogranin III; SST, somatostatin; TMSB4X, thymosin β-4 X-linked; TMSB10, thymosin β-10. "CT/CGRP" indicates that the peptide is shared by CT and CGRP precursors.

microwave irradiation of the rodent brain[4,26] or heat stabilization of tissues after sampling.[27] In so doing, nonsecretory peptides identified in the tissue peptidome were decreased in number.[8] Still, most of the identified peptides turned out to be fragmented peptides derived from highly expressed neuropeptide precursors, far from tracking the processing sites of known neuropeptides described to date. This results from the limited performance of MS and MS/MS technologies; relatively abundant and/or smaller peptides (<2000 Da) are more readily detected by MS, and larger ones (>3500 Da) are difficult to identify by MS/MS regardless of signal intensity. Owing to the advances in mass spectrometers, however, the upper mass ceiling that is accessible to MS/MS has now stretched well beyond 3500 Da to encompass larger peptides. In any case, it had become clear that additional strategies would be required to discover biologically active peptides. Given that such peptides are of a secretory nature, an important lesson from the early studies is that we should investigate analytes that are believed to be least contaminated with non-secretory peptides. From here on, we will describe in chronological order how we have improved our peptidomic strategy and the newly identified peptides over the course of our study.

PEPTIDOMIC IDENTIFICATION AND BIOLOGICAL VALIDATION OF NOVEL C-TERMINALLY AMIDATED PEPTIDES: NEUROENDOCRINE REGULATORY PEPTIDES (NERPs)

Nearly half of the biologically active peptides found in the nervous and endocrine systems possess a C-terminal alpha-amide group.[7] This posttranslational modification has long been considered a hallmark of such peptides, because for many of them it is important or essential for bioactivity.[10,19,28,32] In 1978, Tatemoto and Mutt developed a chemical derivatization method to identify C-terminally amidated peptides, leading to the discovery of galanin, peptide tyrosine tyrosine, neuropeptide Y, peptide histidine isoleucine,

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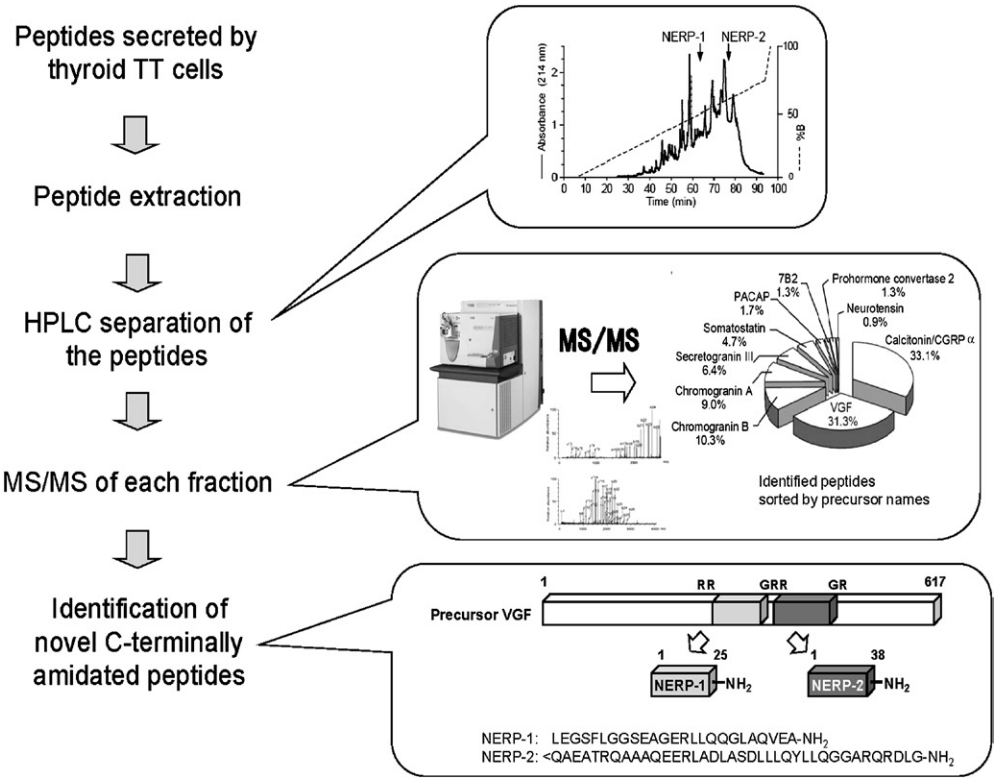

FIGURE 1 MS/MS identification of NERPs. Peptides secreted by the human medullary thyroid carcinoma TT cell line were separated by HPLC into 50 fractions, each of which was subjected to MS/MS. Two previously uncharacterized C-terminally amidated peptides, derived from the neurosecretory protein VGF, were identified and designated NERPs. Rat NERPs were inferred from the corresponding sequences and subsequently confirmed by mass characterization of peptides reactive to antibodies recognizing the amide groups.

and pancreastatin.[28] In invertebrates, the structure of a cardioexcitatory substance from a molluscan ganglion was determined to be the tetrapeptide FMRF-NH2.[19] Since then, many FMRFamide-related peptides have been isolated from various invertebrate species. The peptides that crossreact with an antiserum directed against FMRFamide have been purified from bovine brain extract.[32] With the advent of the postgenome era, an in silico search for unknown members of the RFamide family in the human database led to the discovery of two RFamide-related peptides.[10]

To identify unknown C-terminally amidated peptides independently of any specific amide structure, we used MS/MS techniques to analyze a complex mixture of peptides secreted by TT cells, a human thyroid medullary carcinoma cell line[31] (Fig. 1). A total of 233 peptides were identified, of which 15 underwent C-terminal amidation, including intact calcitonin (CT), intact calcitonin gene-related peptide (CGRP) and a series of N-terminally truncated forms of CGRP. In addition, we were able to identify two novel amidated peptides with monoisotopic masses of 2677.4 and 4062.2, both of which arise from distinct regions of the neurosecretory protein VGF. The former (VGF[281-306]-NH2) and the latter (VGF[310-347]-NH2) were designated neuroendocrine regulatory peptide (NERP)-1 and NERP-2, respectively.[31] Because peptidomics of rat brain fell short of identifying NERPs, the tissue extract was immunoprecipitated with antibodies recognizing each amide structure. By molecular characterization of the major immunoprecipitates, it was concluded that, according to the rat VGF sequence, NERP-1 and NERP-2 occur as 25- and 38-amino acid C-terminally amidated peptides, respectively.[31] For both the peptides, both N- and C-termininal ends are cleaved at the general motif $((K/R)-(X)_n-(K/R)\downarrow$, where $n=0$, 2, 4, or 6 and X is any amino acid other than Cys) recognized by prohormone convertase (PC)1/3 and PC2,[3] indicating that they represent *bona fide* endogenous peptides generated through VGF processing. Fricker and colleagues reported that PC2 is at least in part involved in the processing of NERP-1 (identical between rat and mouse) in their quantitative neuropeptidome analysis of PC2 knockout mice,[33] which is discussed in a different chapter of the Peptide Biosynthesis/Processing section of this book.

The *Vgf* gene was first cloned in rat pheochromocytoma PC12 cells stimulated with nerve growth factor.[12] It was also independently identified as a gene associated with human neuroblastoma differentiation.[17] The encoded protein is expressed in neurons and endocrine cells and composed

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of 615 amino acids in human and 617 amino acids in rodents.[13] It is stored in dense core granules and secreted through the regulated pathway.[13,21] Before the identification of NERPs, a couple of peptides recognized by antibodies raised against the C-terminus of rat VGF had been reported and shown to possess biological activity;[1,2,11,13,30] TLQP-62/VGF[556–617] and AQEE-30/VGF[588–617] enhance synaptic activity in a whole-cell patch clamp recording on rat hippocampal cells,[1] and AQEE-30 and LQEQ-19/VGF[599–617] enhance penile erection on injection into the paraventricular nucleus (PVN) of male rats.[13] Centrally administered AQEE-30 elicits a robust antidepressant response in mice.[11] Using the antibody raised against the N-terminus of TLQP-62, Bartolomucci and colleagues have identified TLQP-21/VGF[556–576], which increases energy expenditure by stimulating autonomic activation of adrenal medulla and adipose tissues.[2] By contrast, NERPs were identified by MS/MS without using antibodies, showing that peptidomics contributes to peptide research from a different standpoint.[31]

Because NERPs were discovered using a "peptide-first" approach, we began with immunohistochemical studies to identify NERPs expressing cells in the rat nervous system.[31] Cell bodies strongly immunostained with NERP antibodies were found in the supraoptic nucleus (SON) and the PVN, the hypothalamic nuclei where vasopressin neurons and oxytocin neurons reside. By immunofluorecence microscopy, NERPs were found to be colocalized with vasopressin rather than with oxytocin.[31] This colocalization was confirmed by immunogold electron microscopy at the secretory granule level.[31] In situ mRNA hybridization showed that VGF expression was concomitantly upregulated with vasopressin in rats not allowed access to water.[31] These findings suggest a functional relationship between NERPs and the antidiuretic hormone vasopressin. In rats receiving central injection of NERPs, NERPs in a dose-dependent manner suppress the increase in plasma vasopressin levels that is normally induced by central administration of hypertonic NaCl or angiotensin II.[31] The sustained increase in vasopressin levels caused by dehydration was also decreased by icv injection of NERP-1 or NERP-2. Further, NERPs antibodies significantly reverse the plasma vasopressin suppression induced by acute water loading, suggesting that NERPs function as endogenous peptides to regulate vasopressin secretion.[31] These inhibitory effects of NERPs on vasopressin secretion have also been confirmed on hypothalamic explants. C-terminal glycine-extended NERPs had no such activity. This suggests that the C-terminal amide groups are also important to NERPs, as reported in many biologically active peptides.[7,31]

NERP-2 immunoreactivity was also found in the lateral hypothalamus (LH).[29] The LH serves as a center for controlling feeding, as damage to the area causes reduced food intake. In the LH, orexin and melanin-concentrating hormone (MCH) are expressed in distinct neuronal populations.[20] We found that NERPs were colocalized with orexins, rather than MCH, suggesting a functional relationship between NERPs and orexins. In support of this, VGF mRNA was upregulated in the LH of rats fasted for 48 h but returned to basal levels after re-feeding.[29] Centrally administered NERP-2 induced Fos activation in a subset of LH orexin neurons.[29] It was also found that centrally administered NERP-2 stimulates feeding behavior, body temperature, and locomotor activity in a dose-dependent manner, the effects not observed with either NERP-1 or C-terminal glycine-extended NERP-2.[29] These effects were considered orexin-dependent because the enhanced food intake was canceled by the central administration of neutralizing antibody to orexins or selective antagonists to the orexin receptor. More specifically, the NERP-2-stimulated feeding did not occur in orexin-knockout mice.[29]

Vgf knockout mice exhibit reduced body weight gain and increased oxygen consumption as compared with that of wild-type littermates.[9] This phenotype suggests that VGF or VGF-derived peptides function in the regulation of energy homeostasis. To date, VGF-derived peptides that are shown to be involved in energy homeostasis are catabolic TLQP-21 and anabolic NERP-2. Given the complexity of VGF processing, the observed phenotype of the *Vgf* knockout mice cannot be explained solely by the two peptides that seem to centrally modulate metabolic functions in opposite directions. However, we should not jump to a conclusion that the principal role of NERP-2 in the central nervous system is to enhance feeding. This is inferred from the research history of orexin, which is implicated in NERP-2 functions; orexin, as its name implies, had received attention because of its orexigenic effect, but is now recognized as a neuropeptide that physiologically regulates sleep and wakefulness through interactions with the nervous systems that are closely related with emotion, reward and energy homeostasis.[20,29]

The distribution of NERPs in humans was studied by immunohistochemistry.[14] NERPs immunoreactivity was also found in the human SCN and PVN. In the islets of the pancreas, NERPs are colocalized extensively with insulin, partially with glucagon, and do not overlap with somatostatin. In addition, NERPS immunoreactivity is found in the thyroid CT-producing cells and the gastrin-producing cells of the gastric antrum.[14] Molecular characterization of NERPs in these locations has shown that the major forms of NERP-1 and -2 are identical to those originally identified from the TT culture supernatant.[14,31] These observations suggest as yet unknown biological functions of NERPs in the endocrine system.

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ELUCIDATING PRECURSOR CLEAVAGE SITES BY SECRETOPEPTIDOME ANALYSIS

The identification of NERPs made us realize two critical points in pursuing peptidomics-based discovery studies. First, although endoproteolytic and exoproteolytic cleavages cannot be distinguished with a given sequence alone, we need to opt for samples highly rich in secretory peptides that retain endoproteolytic cleavage sites. This is because large-scale identification of such peptides has the potential to reveal precursor processing sites, which in turn should be helpful to predict the generation of candidate bioactive peptides. Second, C-terminal amidation of a target peptide does not guarantee that it is biologically active. Actually, we had gone through many hit-or-miss experiments to elucidate the aforementioned biological actions of NERP.[31] We will describe a solution to the second point later, and we now turn to the first one.

Investigating a carefully isolated peptide source is all the more important, as LC–MS/MS is an unbiased analysis automatically conducted without knowing the nature of an analyte in advance. In this regard, peptides localized in the secretory granules of neurons and endocrine cells are believed to be the most ideal target, which can be quickly recovered in supernatant after an acute secretagog challenge in a noninvasive manner.[23] As proof-of-principle study, we investigated a complex mixture of peptides secreted by TT cells that received a secretagog challenge for 2 min.[23] A total of 400 peptides were identified, of which 395 (99%) arose from 20 secretory precursor proteins having signal peptides.[23] Of note, 19 precursors are known to be processed in the regulated secrsetory pathway and fell into three categories: (1) peptide hormone and neuropeptide precursors such as proCT, proCGRP, progastrin-releasing peptide, propituitary adenylate cyclase-activating polypeptide, prosomatostatin, proenkephalin A, proneuromedin U, proneuropeptide W, and proghrelin; (2) granin-like proteins such as chromogranins A, B, and C, secretogranin III, 7B2, proSAAS, and VGF; (3) enzymes involved in peptide hormone processing such as PC1/3, PC2, and peptidyl-glycine alpha-amidating monooxygenase.

We examined N-terminal and C-terminal cleavage sites of the 395 peptides to see if they fit the general motif recognized by PC1/3 and PC2[23] (Table 1). Such consensus sites were found in 299 peptides, which revealed many processing sites of known peptide hormone precursors. No less importantly, monobasic sites, defined here as the sites containing basic residues at P1 but none at P2, P4, P6, or P8 position, were found in 98 peptides, including those previously shown to be cleaved by PC1/3 or PC2.[23] Overall, these findings indicate that the complement of peptides harvested after a stimulatory challenge well reflects the precursor processing in the regulated secretory pathway, and suggest that comprehensive analysis of such peptides contributes to the understanding of precursor processing. Thus, we have advocated the term secretopeptidome, to distinguish from conventional peptidome studies (Fig. 2).[23] Using this scheme, peptide hormones and neuropeptides stored in secretory granules have been consistently recovered from several established cell lines of endocrine origin, including intact or N-terminally or C-terminally truncated peptides of proadrenomedullin, proopiomelanocortin, prourocortin III, progalanin, proluteinizing hormone-releasing hormone type II, proneurotensin, and proapelin.

The peptide processing map of precursor VGF is shown in Fig. 3, where the peptides identified in the TT secretopeptidome were aligned across the precursor sequence. Consisting of several clusters of many peptides sharing either N-termini or C-termini, this map highlights major endoproteolytic cleavage sites of the precursor. Although the cell-type-specific processing should be considered, processing sites deduced through the map are for the most part in agreement with the processing pattern of rat VGF that has been elucidated using conventional

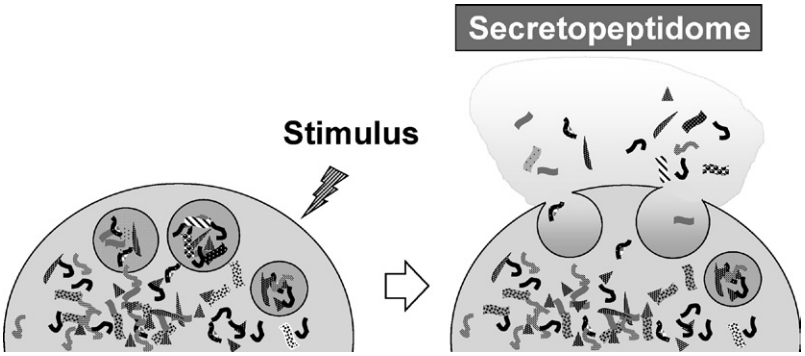

FIGURE 2 The secretopeptidome concept. Peptides stored in the secretory granules of neurons and endocrine cells can be harvested in culture supernatant on secretagog stimulation in a noninvasive manner. Accumulated information about the sequenced peptides is used to predict the generation of endogenous peptides as a result of endoproteolytic processing.

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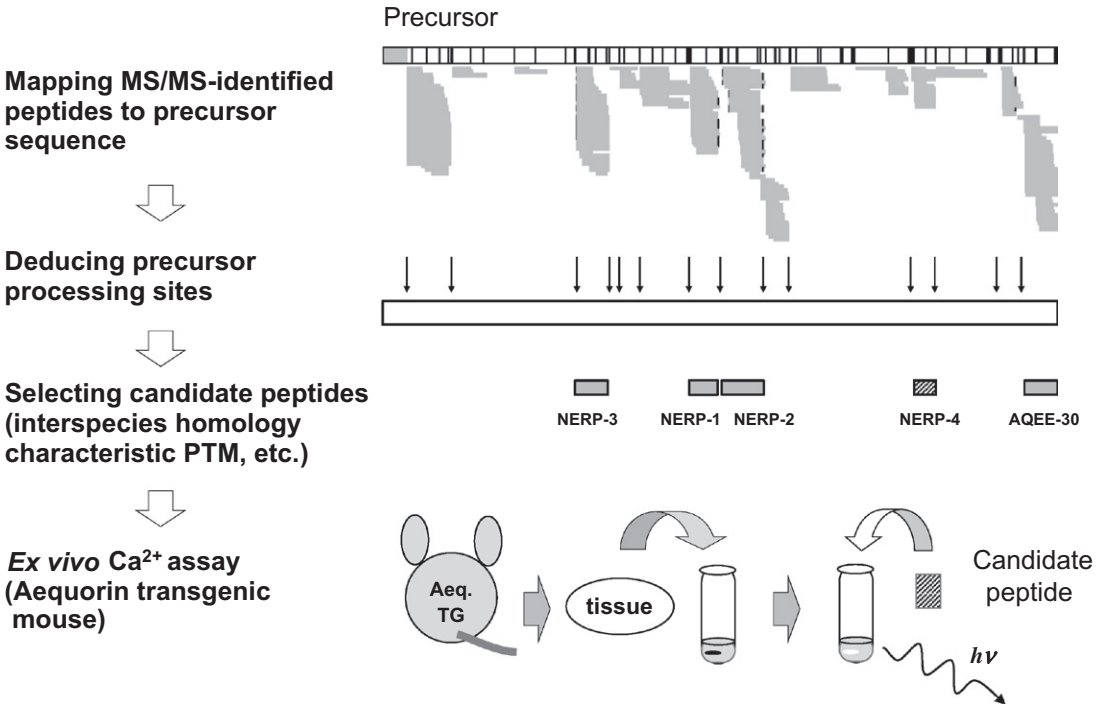

FIGURE 3 The workflow for a peptidomic-based strategy for discovering biologically active peptides. (Top) VGF processing deduced from identified peptides. The black lines in the white box indicate basic amino acid residues. Peptides identified by MS/MS are shown by gray bars, with N-terminal and C-terminal black boxes indicating pyroglutamination and amidation, respectively. (Middle) Deducing precursor processing sites and subsequent selection of candidate peptides. Arrows denote major endoproteolytic/processing sites deduced from the peptide sequences. (Bottom) tissues from transgenic mice expressing the calcium indicator protein aequorin are used, on which candidate peptides are tested on their ability to evoke calcium transient. PTM, posttranslational modification.

antibody-dependent biochemical methods in the brain and gut of rat.[16] Furthermore, we are made aware that even if peptides are quickly harvested (2 min after a stimulation challenge), they are attacked by numerous endopeptidases or exopeptidases. These findings also caution us about assessing peptide amounts by radioimmunoassays using a limited set of antibodies.

A UNIVERSAL PEPTIDOMICS STRATEGY FOR DISCOVERING ENDOGENOUS BIOLOGICALLY ACTIVE PEPTIDES

On the basis of findings obtained with secretopeptidome mining, we have proposed a universal approach to simultaneously screen for bioactive peptides and their target tissues.[24] This takes advantage of the common features of known peptide hormones and neuropeptides, i.e. strong interspecies homology and frequent use of calcium as a second messenger. The workflow for this strategy is illustrated in Fig. 3. As proof-of-principle study, >150 distinct VGF-derived peptides were identified by LC–MS/MS in the TT secretopeptidome and mapped to the precursor sequence to deduce endogenous peptide forms from the sequence ladders.[24] Peptides showing sequence identity between human and mammals (at least rodents) are selected and synthesized for candidate peptides. Candidates are tested on an ex vivo calcium assay using tissue pieces from transgenic mice expressing the calcium indicator protein aequorin.[24] Tissues are cut out as 1- to 2-mm^3 dices and incubated with coelenterazine, a type of luciferin, for 3 h, then sequentially challenged with medium, test peptide, known secretagogue, and finally with the detergent Triton X-100. Secretagogs used are ATP, angiotensin II, and bradykinin, but other peptides can be used. Triton X-100 is used to lyse cells and confirm the generation of functionally active aequorin complexed with coelenterazine. If a receptor for the target peptide uses calcium and is expressed at appreciable levels in a test tissue sample, a calcium transient is detected as emitted blue light on a luminometer. Because this calcium assay is performed on tissues consisting of heterogeneous cell types, we are more likely to get a hit as compared with cell-based assays. Another advantage is the broad applicability to various tissues or organs, including those not amenable to *in vitro* culture. This approach led to the discovery of human VGF[177-206] and [485-503], designated NERP-3 and NERP-4, which evoke a calcium response in the pituitary and hypothalamus.[24] The natural occurrence of these

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peptides was also confirmed by immunoprecipitation-mass spectrometry and the tissue neuropeptidome analysis of PC2 knockout mice.[33]

FOCUSED SECRETOPEPTIDOME MINING: DISCOVERY OF ANTIMICROBIAL PEPTIDE AMP-IBP5

Next, we conducted secretopeptidome mining to search for cationic peptides.[18] Many antimicrobial peptides (AMP) contain multiple internal basic residues, a physicochemical property necessary for them to interact with target microbe's negatively charged cell wall. A secretopeptidome from the human islet cell line QGP-1 was enriched for highly charged peptides using cation exchange chromatography. In this peptidomic survey, a novel intramolecular disulfide-linked 22-residue C-terminally amidated peptide was identified and designated as AMP-IBP5 (antimicrobial peptide derived from insulin-like growth factor-binding protein 5).[18] It was so named according to its potent antimicrobial activity against six of the eight microorganisms tested, which is equal to or even better than the representative mammalian AMPs β-defensin-2 and cathelicidin.[18] AMP-IBP5 is identical at the amino acid level between humans, rats, mice, pigs, and cows. Natural occurrence of this peptide as the originally isolated form has been confirmed by MS of major immunoreactive peptides from rat brain and intestine. Consistent with its identification as a C-terminally amidated peptide, AMP-IBP5 is flanked by the canonical amidation motif GRKR. It is presumed that the site GRKR↓G first undergoes endoproteolytic cleavage (=precursor processing) by PC1/3 or PC2, followed by carboxypeptidase E-mediated removal of RKR. As in many C-terminally amidated peptides, the exposed glycine serves as a donor of the amide group. The N-terminal cleavage site MVPR↓A has no basic residue at either P2, 4, 6, or 8 position and is regarded as atypical for PC1/3 or PC2 cleavage; however, the cleavage C-terminal to a single arginine by PC2 has been reported with the N-terminus of neuromedin C, MYPR↓G, using a quantitative peptidomics approach by comparing the neuropeptide profiles between PC2 knockout mice and wild-type mice.[33] Although we have evidence that several peptides are cleaved from IGFBP-5 at the consensus motif of PC1/3 and PC2, complete sequence identity between mammals is restricted to the AMP-IBP5 sequence.

IGFBP-5 is a well-known secreted protein regulating cellular availability of the binding partners' insulin-like growth factors. Our peptidomics study is the first to show that IGFBP-5 undergoes processing to generate a biologically active peptide.[18] The observation that secretagog stimulation causes a 5- to 10-fold increase in immunoreactive AMP-IBP5 in culture supernatant of two different endocrine cells suggests that AMP-IBP5 is also sorted into the regulated secretory pathway granules.[18]

The fact that the peptide has an intramolecular disulfide bond means that a pair of cysteine residues involved in IGFBP-5 disulfide formation has been experimentally identified. Like many proteins, the overall disulfide connectivity of parent IGFBP-5 remains to be elucidated. In current public databases, each of the two cysteines of IGFBP-5 has been assigned a different disulfide linkage on the basis of sequence homology to the IGFBP family members IGFBP-4 and -6. This means that AMP-IBP5 is in principle beyond the reach of in silico prediction that is dependent on the current databases. It is also noteworthy that because of the presence of multiple basic residues, the peptide escapes identification in current bottom-up proteomics, again illustrating the benefit of peptidomics.[18]

CONCLUSIONS

It is envisaged that forthcoming improvements to related technologies (bioinformatics, LC–MS/MS, sample preparation techniques) will enable in-depth secretopeptidome analysis and have a far-reaching impact on peptide biology. Whatever elegant approaches may be developed for peptide discovery, they will not detract from the value of peptidomics in peptide research.

ACKNOWLEDGMENTS

The authors thank M. Nakazato, H. Yamaguchi, and K. Toshinai (Miyazaki University) for functional studies of NERPs, T. Takao and Y. Satomi (Osaka University) for mass spectrometry techniques, S. Shioda and H. Kageyama (Showa University) for morphological studies, and M. Matsubara (National Cerebral and Cardiovascular Center) for preparing the figures.

REFERENCES

1. Alder J, Thakker-Varia S, Bangasser DA, Kuroiwa M, Plummer MR, Shors TJ, et al. Brain-derived neurotrophic factor-induced gene expression reveals novel actions of VGF in hippocampal synaptic plasticity. *J Neurosci* 2003;**23**:10800–8.
2. Bartolomucci A, La Corte G, Possenti R, Locatelli V, Rigamonti AE, Torsello A, et al. TLQP-21, a VGF-derived peptide, increases energy expenditure and prevents the early phase of diet-induced obesity. *Proc Natl Acad Sci U S A* 2006;**103**:14584–9.
3. Cameron A, Apletalina EV, Lindberg I. The enzymology of PC1 and PC2. In: Dalby RE, Sigman DS, editors. *The enzymes*. New York: Academic Press; 2002. p. 291–328.
4. Che FY, Lim J, Pan H, Biswas R, Fricker LD. Quantitative neuropeptidomics of microwave-irradiated mouse brain and pituitary. *Mol Cell Proteomics* 2005;**4**:1391–405.
5. Clynen E, Baggerman G, Veelaert D, Cerstiaens A, Van der Horst D, Harthoom L, et al. Peptidomics of the pars intercerebralis–corpus cardiacum complex of the migratory locust, *Locusta migratoria*. *Eur J Biochem* 2001;**268**:1929–39.

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6. Desiderio DM. Quantitative analytical mass spectrometry of endogenous neuropeptides in human pituitaries. *Life Sci* 1992;**51**:169–76.
7. Eipper BA, Stoffers DA, Mains RE. The biosynthesis of neuropeptides: peptide alpha-amidation. *Annu Rev Neurosci* 1992;**15**:57–85.
8. Fricker LD, Lim J, Pan H, Che FY. Peptidomics: identification and quantitation of endogenous peptides in neuroendocrine tissues. *Mass Spectrom Rev* 2006;**25**:327–44.
9. Hahm S, Mizuno TM, Wu TJ, Wisor JP, Priest CA, Kozak CA, et al. Targeted deletion of the Vgf gene indicates that the encoded secretory peptide precursor plays a novel role in the regulation of energy balance. *Neuron* 1999;**23**:537–48.
10. Hinuma S, Shintani Y, Fukusumi S, Iijima N, Matsumoto Y, Hosoya M, et al. New neuropeptides containing carboxy-terminal RFamide and their receptor in mammals. *Nat Cell Biol* 2000;**2**:703–8.
11. Hunsberger JG, Newton SS, Bennett AH, Duman CH, Russell DS, Salton SR, et al. Antidepressant actions of the exercise regulated gene VGF. *Nat Med* 2007;**13**:1476–82.
12. Levi A, Eldridge JD, Paterson BM. Molecular cloning of a gene sequence regulated by nerve growth factor. *Science* 1985;**229**:393–5.
13. Levi A, Ferri GL, Watson E, Possenti R, Salton SR. Processing, distribution, and function of VGF, a neuronal and endocrine peptide precursor. *Cell Mol Neurobiol* 2004;**24**:517–33.
14. Matsuo T, Yamaguchi H, Kageyama H, Sasaki K, Shioda S, Minamino N, et al. Localization of neuroendocrine regulatory peptide-1 and-2 in human tissues. *Regul Pept* 2010;**163**:43–8.
15. Minamino N, Tanaka J, Kuwahara H, Kihara T, Satomi Y, Matsubae M, et al. Determination of endogenous peptides in the porcine brain: possible construction of peptidome, a fact database for endogenous peptides. *J Chromatogr B Analyt Technol Biomed Life Sci* 2003;**792**:33–48.
16. Mishiro-Sato E, Sasaki K, Matsuo T, Kageyama H, Yamaguchi H, Date Y, et al. Distribution of neuroendocrine regulatory peptide-1 and -2, and proteolytic processing of their precursor VGF protein in the rat. *J Neurochem* 2010;**114**:1097–106.
17. Nagasaki K, Sasaki K, Maass N, Tsukada T, Hanzawa H, Yamaguchi K. Staurosporine enhances cAMP-induced expression of neural-specific gene VGF and tyrosine hydroxylase. *Neurosci Lett* 1999; **267**:177–80.
18. Osaki T, Sasaki K, Minamino N. Peptidomics-based discovery of an antimicrobial peptide derived from insulin-like growth factor-binding protein 5. *J Proteome Res* 2011;**10**:1870–80.
19. Price DA, Greenberg MJ. Structure of a molluscan cardioexcitatory peptide. *Science* 1977;**197**:670–1.
20. Sakurai T. The neural circuit of orexin (hypocretin): maintaining sleep and wakefulness. *Nat Rev Neurosci* 2007;**8**:171–81.
21. Salton SR, Ferri GL, Hahm S, Snyder SE, Wilson AJ, Possenti R, et al. VGF: a novel role for this neuronal and neuroendocrine polypeptide in the regulation of energy balance. *Front Neuroendocrinol* 2000;**21**:199–219.
22. Sasaki K, Sato K, Akiyama Y, Yanagihara K, Oka M, Yamaguchi K. Peptidomics-based approach reveals the secretion of the 29-residue carboxy-terminal fragment of the putative tumor suppressor protein DMBT-1 from pancreatic adenocarcinoma cell lines. *Cancer Res* 2002;**62**:4894–8.
23. Sasaki K, Satomi Y, Takao T, Minamino N. Snapshot peptidomics of the regulated secretory pathway. *Mol Cell Proteomics* 2009;**8**:1638–47.
24. Sasaki K, Takahashi N, Satoh M, Yamasaki M, Minamino N. A peptidomics strategy for discovering endogenous bioactive peptides. *J Proteome Res* 2010;**9**:5047–52.
25. Schrader M, Schultz-Knappe P. Peptidomics technologies for human body fluids. *Trends Biotechnol* 2001;**10**:S55–60.
26. Svensson M, Sköld K, Svenningsson P, Andren PE. Peptidomics-based discovery of novel neuropeptides. *J Proteome Res* 2003;**2**: 213–9.
27. Svensson M, Boren M, Sköld K, Fälth M, Sjögren B, Andersson M, et al. Heat stabilization of the tissue proteome: a new technology for improved proteomics. *J Proteome Res* 2009;**8**:974–81.
28. Tatemoto K, Mutt V. Chemical determination of polypeptide hormones. *Proc Natl Acad Sci U S A* 1978;**75**:4115–9.
29. Toshinai K, Yamaguchi H, Kageyama H, Matsuo T, Koshinaka K, Sasaki K, et al. Neuroendocrine regulatory peptide-2 regulates feeding behavior via the orexin system in the hypothalamus. *Am J Physiol Endocrinol Metab* 2010;**299**:E394–401.
30. Trani E, Giorgi A, Canu N, Amadoro G, Rinaldi AM, Halban PA, et al. Isolation and characterization of VGF peptides in rat brain. Role of PC1/3 and PC2 in the maturation of VGF precursor. *J Neurochem* 2002;**81**:565–74.
31. Yamaguchi H, Sasaki K, Satomi Y, Shimbara T, Kageyama H, Mondal MS, et al. Peptidomic identification and biological validation of neuroendocrine regulatory peptide-1 and -2. *J Biol Chem* 2007;**282**:26354–60.
32. Yang HY, Fratta W, Majane EA, Costa E. Isolation, sequencing, synthesis, and pharmacological characterization of two brain neuropeptides that modulate the action of morphine. *Proc Natl Acad Sci U S A* 1985;**82**:7757–61.
33. Zhang X, Pan H, Peng B, Steiner DF, Pintar JE, Fricker LD. Neuropeptidomic analysis establishes a major role for prohormone convertase-2 in neuropeptide biosynthesis. *J Neurochem* 2010;**112**:1168–79.

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Chapter 244

Peptidylglycine Amidating Monoxygenase (PAM)

KS Vishwanatha, Richard E. Mains and Betty A. Eipper

ABSTRACT

Amidation, a terminal modification in peptide biosynthesis, often increases the affinity of the peptide for its receptors and retards proteolytic degradation. Peptide amidation involves the sequential action of two enzymes. Peptidylglycine α-Hydroxylating Monooxygenase (PHM, EC 1.14.17.3), a copper, ascorbate and molecular-oxygen-dependent monooxygenase, catalyzes the stereospecific hydroxylation of the glycine α-carbon of the peptidylglycine substrate. Peptidyl-α-hydroxyglycine α-Amidating Lyase (PAL, EC 4.3.2.5) then catalyzes the cleavage of the C–N bond linking the α-hydroxyglycine to the penultimate amino acid, generating the α-amidated peptide and glyoxylate. In many primitive species, PHM and PAL are encoded by separate genes. In mammals, both enzymes are part of PAM (Peptidylglycine α-Amidating Monoxygenase), a bifunctional integral membrane protein. Genetic elimination of *Pam* causes embryonic lethality. Mice with a single *Pam* gene exhibit deficits in thermal regulation, anxiety-like behavior, and fear-based learning. PAM plays a role in maintaining copper homeostasis and in coordinating luminal and cytosolic events.

NAME AND HISTORY

About half of all known bioactive peptides terminate with a C-terminal α-amide. Amidated peptides are produced from peptidylglycine precursors by peptidylglycine α-amidating monooxygenase (PAM).[11] This posttranslational modification is often essential for receptor binding and may also increase peptide half-life in the circulation. Peptidylglycine-α-amidating monooxygenase (PAM) is required for the biosynthesis of all α-amidated peptides.[11] In the first step of the α-amidation reaction, the α-carbon of Gly is hydroxylated by peptidylglycine α-hydroxylating monooxygenase (PHM; EC 1.14.17.3) (Fig. 1). This reaction requires copper, molecular oxygen, and ascorbate. In the second step of the reaction, peptidyl-α-hydroxyglycine α-amidating lyase (PAL; EC 4.3.2.5) catalyzes an N-dealkylation reaction, generating amidated product and glyoxylate. At alkaline pH, the lyase reaction occurs spontaneously. The first *in vitro* assay for PAM was developed in 1982.[5] PAM was purified from bovine neurointermediate pituitaries in 1986, cloned in 1987, and first crystallized in 1997.[26]

A key step in the development of an assay for the biosynthesis of α-amidated peptides came from the sequencing of cDNAs encoding peptide precursors and the realization that α-amidated peptides were always derived from peptidylglycine precursors (Fig. 1). Amidated peptides play critical roles in *Planaria* and *Cnidarians*, creatures with a simple nerve net, as well as in mammals, flies and parasites like *Schistosoma*. Amidated peptides are produced in a similar manner in each of these organisms. A bioinformatically identified secretory protein whose predicted proteolytic cleavage sites generate a product with a C-terminal glycine is highly likely to generate a secreted product whose C-terminus is α-amidated.

The earliest studies of PAM revealed its similarities to dopamine β-monooxygenase (DBM, EC 1.14.17.1), the enzyme that converts dopamine into norepinephrine; the catalytic core of PHM [PHMcc; rat PAM(42–356)] is 28% identical to the corresponding region of DBM. Conservation of exon/intron junctions and eight of the ten Cys residues in PHMcc show their evolutionary relationship. The Cys residues, which form essential disulfide bridges, are conserved across species. PHM and DBM define a family of copper-dependent monooxygenases that includes at least one additional member, monooxygenase X (MOX), which resembles DBM more closely than PHM.[27]

FUNCTION OF PAM IN PEPTIDE BIOSYNTHESIS

PAM functions at a very late stage of the peptide biosynthetic pathway (Fig. 1). For most prohormones, endoproteolytic cleavage by a prohormone convertase followed by exoproteolytic trimming by carboxypeptidase E is required to produce a peptidylglycine substrate for PHM (Fig. 1). The subtilisin-like endoproteases Prohormone Convertase 1/3 (PC1/3) (Chapter 245 in this section of the book) and Prohormone Convertase 2 (PC2) (Chapter 246) are often

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00244-X
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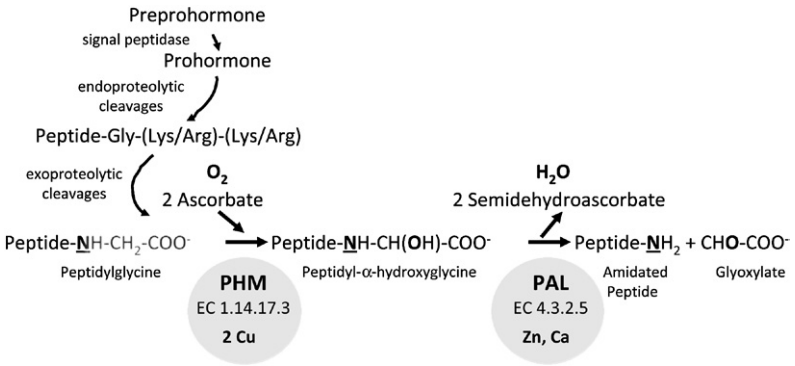

FIGURE 1 Peptide amidation is a two-step reaction. After cotranslational removal of the N-terminal signal sequence from the preprohormone, subsequent modifications occur in the lumen of the secretory pathway. The newly synthesized cargo moves through the Golgi complex and into immature secretory granules, which undergo a maturation process that involves luminal acidification. Peptidylglycine α-hydroxylating monooxygenase (PHM) catalyzes the stereospecific α-hydroxylation of the α-carbon of Gly residues at the C-terminus of peptides generated by endoproteoloytic and exoproteolytic cleavage of prohormones. Peptidyl α-hydroxyglycine α-amidating lyase (PAL) catalyzes cleavage of the C-N bond. Molecular oxygen, O_2; N of Gly α-NH_2 group, N.

responsible for producing reaction intermediates in which Lys or Arg is the C-terminal residue. Removal of C-terminal Lys and Arg residues is catalyzed by carboxypeptidase E, a carboxypeptidase B-like exopeptidase (Chapter 235). If a Gly residue is exposed after these endo- and exoproteases have acted, the active site of PHM can accommodate a wide variety of peptidylglycine substrates. Ubiquitin, a cytosolic protein not normally exposed to PAM, terminates with a C-terminal Gly and can be amidated by PAM.[21]

PAM substrates derived from prohormones do not generally become available until late in the biosynthetic pathway. Based on the ability to visualize α-amidated products in electron micrographs of corticotroph tumor cells, amidation occurs primarily in the low pH environment of immature secretory granules. Because copper is delivered to the lumen of the secretory pathway by the P-type ATPases ATP7A and ATP7B, which are localized to the trans-Golgi Network (TGN), it may be that PHM is first loaded with copper in this region of the cell.[23] If so, PHM would not be catalytically competent even if presented with a peptidylglycine substrate earlier in the secretory pathway. In many tissues, very little precursor (peptidylglycine) is detected in tissue lysates and the amidated product is by far the major form secreted. In a few cases, the peptidylglycine substrate for PHM can be detected in lysates and after secretion. For example, pyro-Glu-His-Pro-Gly, the precursor to TRH, is more prevalent than TRH (pyro-Glu-His-Pro-NH_2) in hypothalamus[4] and both oxytocin-Gly and gastrin-Gly are readily detected in serum.[27] It is interesting that –Pro-Gly (TRH) and –Gly-Gly (oxytocin) are poor substrates in test tube assays of PAM activity.

PROPERTIES OF THE PHM DOMAIN OF PAM

The catalytic cores of PHM and PAL have been crystallized,[26,27] revealing key features of their active sites (Fig. 2). Recombinant PHM and PAL secreted by stably transfected Chinese Hamster Ovary cells were used for these studies. PHM is the only member of the PHM/DBM/MOX family that has been crystallized. Crystallographic analysis of oxidized PHMcc, reduced PHMcc, oxidized PHMcc with bound substrate and reduced PHM with a slow substrate in the presence of molecular oxygen has revealed a great deal about the way in which molecular oxygen is used to catalyze the α-hydroxylation of glycine. In all the structures examined, PHMcc adopts the same overall structure, with only small differences in the conformation of several active site side chains and in the coordination geometry of the Cu-binding sites.

PHMcc has two domains, each about 150 residues long (Fig. 2). Despite having little sequence identity, the N- and C-terminal domains of PHMcc each contain a similar eight-stranded antiparallel β-sandwich. The two domains are connected by a three-residue linker. Each domain has a single Cu-binding site. The Cu-binding site in the N-terminal domain (Cu_H or Cu_A) is formed by three His residues (His^{107}-His^{108} and His^{172}). Three disulfide bonds anchor loops in domain I without linking the two major β-sheets. The Cu-binding site in the C-terminal domain (Cu_M or Cu_B) is formed by two His residues and a Met (His^{242}, His^{244}, Met^{314}). Two disulfide bonds connect the two sheets of the C-terminal β-sandwich.

Although connected by only a single strand, the N- and C-terminal domains are closely associated through a large, well-conserved hydrophobic interface. This interdomain interface is further stabilized by a hydrogen-bonded antiparallel β-sheet that links the two domains. A large, solvent-filled cleft separates the Cu_H site in N-terminal domain from the Cu_M site in the C-terminal domain. The two Cu ions, which must both participate in the reaction, are separated by an 11 Å cleft, with no evidence that they move closer together during the reaction.

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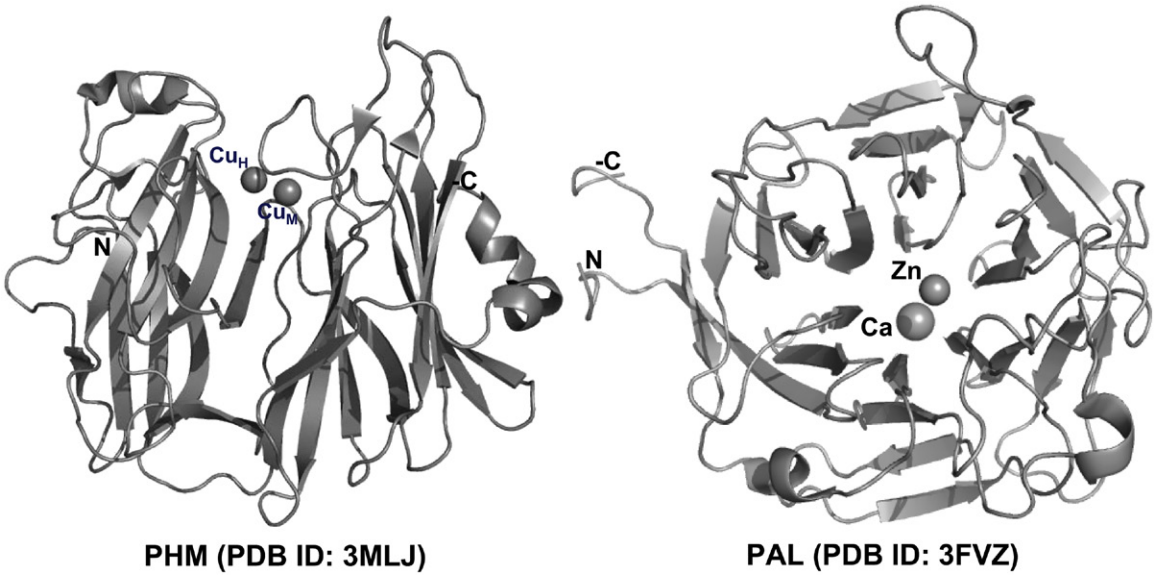

FIGURE 2 The catalytic cores of PHM and PAL. The structures determined for oxidized PHMcc without bound substrate[26] and PALcc without substrate[7] are shown. Bound metal ions are indicated. The N- and C-terminal ends of the polypeptide chains are indicated. The peptide substrate binds near Cu_M in PHMcc and near the Zn in PALcc. A linker region of variable length connects PHMcc to PALcc in PAM. In membrane PAM, a short linker region connects PALcc to an α-helical transmembrane domain.

The PHM Reaction

Much remains to be learned about the reaction catalyzed by PHM, but certain features are clear; kinetic studies indicate that the peptidylglycine substrate must bind before molecular oxygen can interact with PHM, perhaps limiting the production of reactive oxygen intermediates.[28] Molecular oxygen binds to the Cu_M site. The amino acid that will be α-amidated interacts with Met^{314} and several residues situated in the β-strand that extends beyond Met^{314}. Crystallization of reduced PHMcc with N-acetyl-diiodotyrosyl-D-threonine, which serves as a very slow substrate, clarifies two critical roles played by the C-terminal Gly. The α-carbon is positioned near the Cu_M site, placing the hydrogen to be extracted close to the molecular oxygen bound at this site; L-amino acids cannot occupy this position. In addition, the Gly carboxylate is bound to the guanidinium group of Arg^{240} in a bidentate salt bridge.

Although peptides terminating with hydrophobic amino acid amides are more common than peptides terminating with charged amino acid amides (-Lys-NH_2, -Arg-NH_2, -Asp-NH_2, -Glu-NH_2), PAM can catalyze the production of all 20 amino acid amides. The open nature of the active site is consistent with its ability to accommodate a variety of substrates. PHM catalyzes N-dealkylation, O-dealkylation and sulfoxidation reactions of nonpeptide substrates.[21] Purified recombinant PAM efficiently converts fatty acyl glycines, ranging in length from N-formyl to N-arachidonyl, into fatty acyl amides through an *N*-acyl-hydroxyglycine intermediate. PAM also catalyzes the conversion of nicotinuric acid into nicotinamide and glutathione into γ-Glu-Cys-NH_2.[21] Access of potential substrates to PAM is clearly critical in determining which of these reactions actually occur *in vivo*.

Ascorbate is the preferred reductant *in vivo*, but other single electron donors can support the α-hydroxylation reaction. PHM is absolutely dependent on copper; no other metal can replace it. For the reaction to proceed, both Cu_H and Cu_M must be reduced. PHM functions most effectively at pH 4.5 to pH 5.5, the pH observed in mature secretory granules.

PHM activity can be assessed with peptidylglycine substrates that are labeled with [^{125}I] or with a fluorescent tag; substrate and product are separated by RP-HPLC or by phase separation.[19] An oxygen electrode can be used to quantify PHM activity when large amounts of enzyme are available.[3,12] The manner in which the Cu_H and Cu_M sites interact with molecular oxygen to catalyze the α-amidation reaction remains a subject of intense interest and debate.[3,12,28]

PROPERTIES OF THE PAL DOMAIN OF PAM

The initial purification of peptide amidating activity from bovine neurointermediate pituitaries yielded soluble 45 kDa PHM. The existence of PAL became clear when the cDNA encoding PHM was found to encode a larger integral membrane protein and the properties of purified PHM were found to be distinctly different from those of the amidating activity in tissue lysates. A stable, protease resistant PAL catalytic core [PALcc; rat PAL(498–820)] was defined and expressed in CHO cells. Like PHMcc, disulfide bonds play

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a critical role in PALcc. A single Zn(II) is bound to three conserved His residues in the active site and a single Ca(II) plays an essential structural role[7] (Fig. 2). Chelators of divalent metals (e.g. EDTA, EGTA) inactivate PALcc.

Crystallization of PALcc reveals a six-bladed β-propeller, with its blades positioned around a central cavity (central pore).[7] Each blade contains four antiparallel β-strands, with the first strand at the center of the propeller and the fourth strand at the outer edge. The loops connecting strand 4 of one blade to strand 1 of the next blade and the loops connecting strands 2 and 3 of each blade form the "cup" of the β-propeller. As in other β-propeller-enzymes, the cup encompasses the active site Zn(II). Loops 2–3 of the first blade contains 20 residues, significantly longer than the equivalent loop in the other blades. This long first blade loop provides a tryptophan (Trp^{538}) that creates a hydrophobic pocket for the substrate and may also provide a surface that can interact with the PHM domain when both catalytic domains occur in a bifunctional PAM protein.

The PAL Reaction

PAL activity has been quantified using [^{125}I]-labeled α-N-acetyl-Tyr-Val-α-hydroxyglycine or α-OH-hippuric acid.[21] Purified PALcc exhibits an acidic pH optimum, although significant activity is observed at neutral pH. The K_m for α-N-acetyl-Tyr-Val-α-hydroxyglycine is 38 ± 13 μM, slightly higher than the K_m of PHM for α-*N*-acetyl-Tyr-Val-Gly (10 ± 2 μM). The active site of PALcc contains an essential Tyr residue, which is situated near the bound Zn. Unlike PHMcc, which can lose its bound Cu and can later be reconstituted with additional copper, the metals in PALcc seem to play an essential structural role.[7]

THE PAM GENE

In *Drosophila melanogaster*,[17] sea anemone (*Calliactis parasitica*), and *Schistosoma mansoni*, PHM and PAL are encoded by separate genes. The two *Drosophila* PAL genes are not always expressed with PHM, suggesting that PAL may function independently. The *Planarian* genome encodes PHM, but a PAL ortholog has not yet been identified.[1] The *Caenorhabditis elegans* genome encodes a bifunctional PAM and a monofunctional PAL. As in mammals, the pond snail (*Lymaea stagnalis*) and the frog (*Xenopus laevis*)[27] genomes encode integral membrane proteins with both PHM and PAL domains. In *Lymnaea*, a single type-1 membrane protein contains four independent, functional PHM domains followed by a single PAL domain.

In several species, tissue-specific alternative splicing generates multiple isoforms of PAM; the major splice variants of rat and human PAM are compared in Fig. 3. The rat PAM gene consists of 27 exons and encompasses >160 kb of genomic DNA.[24] Rat PAM-1, the longest isoform, is a

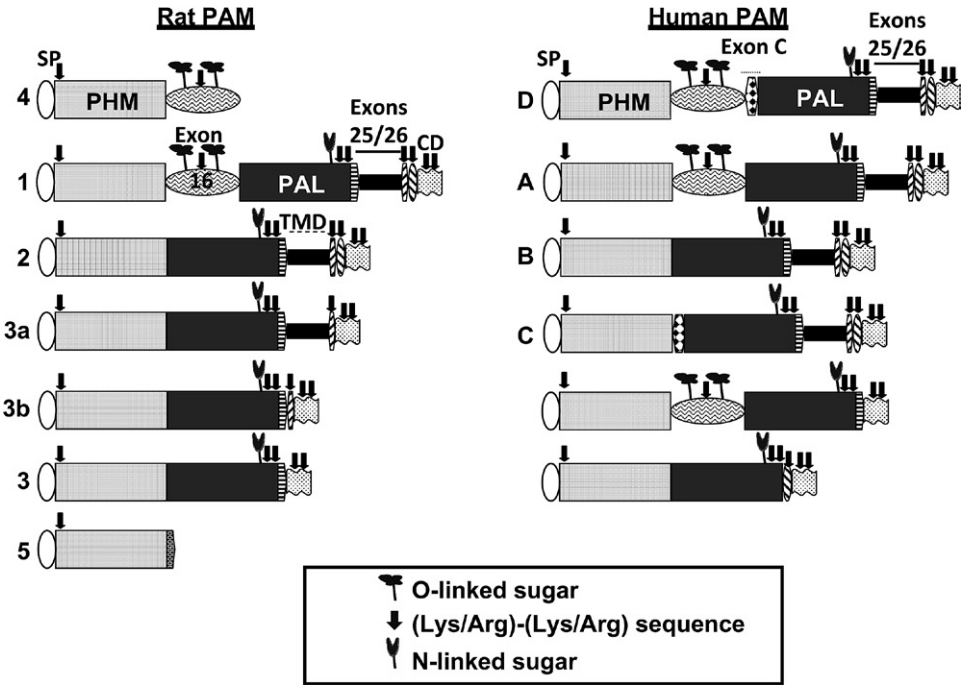

FIGURE 3 Isoforms of rat and human PAM. The splice variants of rat *Pam* and human *PAM* are shown. Inclusion of the exon that encodes the transmembrane domain generates a type-1 integral membrane protein. After removal of the signal sequence, tissue-specific cleavage at pairs of basic residues can generate soluble forms of PHM, PAL, and PAM, which are secreted along with the amidated peptides they create.

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976 amino acid protein with a molecular weight of 108,650. The N-terminal signal sequence is followed by a 10 amino acid proregion that assists in folding but is not essential. N-glycosylation at a single site in the PAL domain and O-glycosylation at multiple sites in the linker region between PHM and PAL (exon 16) result in a PAM-1 protein with an apparent molecular weight of about 120,000. The 86 amino acid cytosolic domain (CD) of PAM is unstructured, highly phosphorylated and essential for the trafficking of PAM in the biosynthetic and endocytic pathways.[29]

Alternative splicing of the PAM gene is tissue specific and developmentally regulated. Splicing determines whether PAM will be an integral membrane protein or a soluble protein (Fig. 3). Splice variants lacking the exon that encodes the transmembrane domain are soluble and will be secreted along with their peptide products during exocytosis. As discussed below, membrane PAM that appears on the cell surface after exocytosis undergoes endocytosis and can return to the TGN and re-enter secretory granules.

Like other proteins in the lumen of the secretory pathway, PAM proteins are subject to endoproteolytic cleavage in the linker regions that connect the catalytic cores to each other and to the transmembrane domain (Fig. 3). In rat PAM, exon 16 includes a pair of basic amino acids that can be cleaved by prohormone convertases, releasing soluble PHM and creating membrane PAL. Splice variants that lack exon 16 are resistant to endoproteolytic cleavages that separate PHM from PAL. That a region equivalent to exon 16 occurs in *Lymnaea* and in *Xenopus* suggests that this region serves an important function.

Orthologs of PAM, DBM, or MOX have not yet been detected in bacterial, archaeal, or yeast genomes, suggesting that this family of copper-dependent monooxygenases may have evolved with multicellular organisms.

Properties of the Bifunctional PAM Protein

The catalytic functions performed by PHM and PAL are performed by PAM, with slight differences in pH optimum and kinetic parameters. Despite attempts to show substrate channeling from PHM to PAL, there is no evidence to support this suggestion.[22] Evidence for very low levels of the peptidyl-α-hydroxyglycine intermediate produced by PHM in tissue lysates was recently obtained using mass spectroscopy.[35]

The structure of PAL latches its N- and C-termini in close proximity[7] (Fig. 2). In a bifunctional PAM protein, the linker that joins the catalytic cores of PHM and PAL would be expected to place the domains in close proximity. In membrane PAM, PALcc is followed by a membrane spanning α-helix. The "latch"-like arrangement of the N- and C-termini of PALcc places PHMcc close to the membrane, where it might be well positioned for receipt of electron equivalents from cytochrome b_{561} and Cu(I) from ATP7A or ATP7B.

As discussed below, the presence of the transmembrane domain places the C-terminus of PAM in the cytosol, where it can interact with cytosolic trafficking machinery in a manner that reflects the actions of cytosolic factors and the luminal milieu. Regulated intramembrane proteolysis in the endocytic pathway is believed to release a soluble fragment of PAM (sf-CD) that accumulates in the nucleus, where it affects gene expression.[13,29]

REGULATION OF PAM ACTIVITY

Expression of PAM mRNA is regulated in a tissue-specific manner. In the melanotropes of the intermediate pituitary, the expression of PAM falls in response to the inhibitory effects of dopaminergic agonists, as does expression of proopiomelanocortin. In the anterior pituitary, the expression of PAM is increased by hypothyroidism and decreased by hyperthyroidism. Electroconvulsive shock increases the expression of PAM and increased production of amidated peptides, along with expression of its target peptides in the hippocampus.

The ability of PAM to function is dependent on the availability of copper and ascorbate in the secretory pathway. It is not clear that the availability of either copper or ascorbate is normally used to regulate PAM function. Lumenal pH is believed to play an important role in PAM function, with little PHM activity at neutral pH and effects of pH on the conformation of PAM that are believed to affect trafficking. Cleavage of membrane PAM to create PHM increases its catalytic activity and is believed to play a role in the activation of PAM and increased production of amidated peptides in response to hypoxia.[31]

Distribution/Localization: Sites of PAM Expression

Sites of PAM expression have been mapped using *in situ* hybridization, enzyme activity, and antibodies. In the adult rat, PAM expression is the highest in atrial myocytes, pituitary endocrine cells, and in subsets of neurons. However, PAM expression is not limited to classical neuroendocrine tissues. PAM is also expressed in airway and olfactory epithelia, endothelial cells, chondrocytes of bronchial cartilage, smooth muscle cells, brain ependymal cells, and astrocytes.[27]

In the brain of adult rats, transcripts encoding PAM are found in virtually all major brain areas with the exception of the cerebellum. A broad distribution of PAM in the CNS is consistent with its role in the synthesis of all α-amidated neuropeptides and the presence of α-amidated neuropeptides in all major brain areas. High levels of PAM mRNA and immunoreactivity are found in the hypothalamus,

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hippocampus, and cortex. The magnocellular hypothalamic neurons of the SON and PVN, well known for their synthesis of vasopressin and oxytocin, exhibit the highest levels of PAM mRNA in the rat brain.[30]

During development, PAM transcripts first appear in decidualizing uterine endometrium and myometrial smooth muscle; later, PAM expression is apparent in the cardiogenic region. PAM expression in the central nervous system exhibits a complex developmental pattern. Non-neuronal cell populations that express especially high levels of PAM include limb mesoderm and the mesenchyme adjacent to nasal, maxillary, palatal, and dental epithelia during tissue fusion and remodeling. Expression of PAM is detectable in many neuroendocrine tumors, and amidated peptides may contribute to tumor growth as autocrine growth factors.[16]

In *Drosophila*, PHM is one of the genes expressed when DIMM, a basic-helix-loop-helix protein, turns on expression of key components of the regulated secretory pathway.[25] In *Planaria*, PHM is expressed in brain, eyes, and ventral nerve cord.[1] In *Cnidarians*, genes encoding the precursors to many amidated peptides are expressed in very discrete locations for defined periods of time; it is not yet clear whether the expression of PHM is coordinated with peptide precursor expression in this organism.[34]

DISTRIBUTION/LOCALIZATION: BIOSYNTHETIC AND ENDOCYTIC TRAFFICKING OF PAM

In cells that store peptides in secretory granules, PAM is largely localized to those granules. Cell-type-dependent cleavage of the different isoforms of PAM generates soluble PHM, soluble PAL, and soluble PAM, which are secreted along with the stored peptides (Fig. 4). In cells in which membrane PAM is a major product, PAM is inserted into the plasma membrane at the time of exocytosis. PAM is rapidly removed from the cell surface by endocytosis; very little PAM is found on the cell surface at steady state. PAM that enters the endocytic pathway can be returned to the TGN for reentry into secretory granules or it can be degraded. The endocytic trafficking of PAM is responsive to copper levels and involves regulated entry of PAM into the intraluminal vesicles of the multivesicular bodies (Fig. 4).

INHIBITORS OF AMIDATION

Because amidated peptides can serve as autocrine growth factors in lung cancer and contribute significantly to the acute inflammatory response in conditions such as

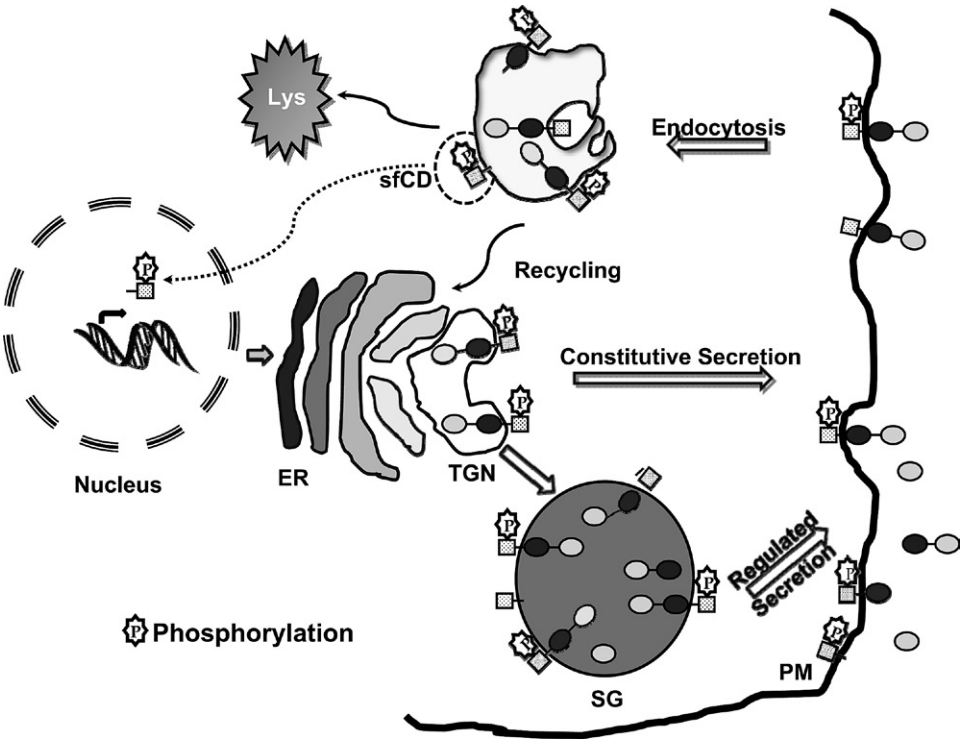

FIGURE 4 Biosynthetic and endocytic trafficking of PAM: generation of sf-CD. Membrane PAM enters regulated secretory granules when it exits the trans-golgi network (TGN). In the regulated secretory granules, PAM can undergo prohormone convertase-mediated cleavage, generating soluble PHM, membrane PAL, soluble PAL, soluble PAM, and transmembrane/CD fragments. On exocytosis, the soluble proteins are secreted, and the membrane proteins undergo endocytosis. Experiments with antibodies to the lumenal domains of PAM and surface biotinylation show that endocytosed PAM can return to secretory granules. It can also undergo regulated intramembrane cleavage, generating sf-CD, which accumulates in the nucleus. PAM-CD is unstructured and multiply phosphorylated. Both the biosynthetic and endocytic trafficking of PAM are regulated by CD phosphorylation and dephosphorylation.

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rheumatoid arthritis, PAM inhibitors have been sought.[16] In addition, PAM plays a role in the proliferation of androgen-independent prostate cancer. Homocysteine-extended peptides, acetopyruvate, pyruvate-extended amino acids, (4-methoxybenzoyloxy) acetate, *d*- and *l*-styrylglycine-extended peptides, and mimosine inhibit PAM with IC_{50} or K_i values of ≥100 μM.[20]

Both 4-phenyl-3-butenoic acid and 5(acetylamino)-4-oxo-6-phenyl-2-hexenoic acid inhibit PAM activity in serum.[2] Detailed studies of DBM facilitated the development of mechanism-based PHM inhibitors.[21] By the use of α-unsaturated thioacetic acid derivatives, such as *trans*-styrylthioacetic acid, as enzyme-activated inhibitors of horse serum PHM, it was shown that a radical rather than a reactive sulfoxide was formed as the PHM-inactivating transient species,[6] similar to that postulated for 4-phenyl-3-butenoic acid.

Hippurate analogs, *S*-(2-phenylthioacetyl)thioglycolate and *S*-(4-methylthiobenzoyl) thioglycolate, which have highest affinity for PAM, have been used as lead compounds in inhibiting the growth of human androgen-independent prostate cancer DU 145 cells.[21] Disulfiram, used as an alcohol deterrent, chelates copper and inhibits PHM, reducing the levels of amidated peptides in adult rat pituitary and cerebral cortex. *In vivo* studies of phenylbutenoic acid have shown a decrease in serum PHM activity and decreased inflammatory responses against carrageenan.[27]

Because PAL may not play an essential role in the production of amidated products, there have been fewer attempts to develop inhibitors of this enzyme. Potent inhibitors of PAL include pyruvate-extended *N*-acetyl amino acids and 2, 4-dioxo-5-acetamido-6-phenylhexanoate.[27]

PATHOPHYSIOLOGICAL IMPLICATIONS

Genetic Manipulation of PAM—Knockout Flies and Knockout Mice

Elimination of the PHM gene in *Drosophila* results in larval death;[17] in insects, >90% of the neuropeptides are α-amidated and non-amidated precursors accumulate in the absence of PHM. Mice lacking a functional PAM gene do not survive past embryonic day 15, exhibiting severe edema and malformed vessels.[9] The total absence of amidation activity in these embryos supports the conclusion that PAM is the sole source of amidation activity.

Perhaps more importantly, mice heterozygous for the PAM gene ($PAM^{+/-}$) are viable, fertile, and show no gross motor abnormalities. Tissues from $PAM^{+/-}$ mice have half the normal level of amidation activity. As they age, $PAM^{+/-}$ mice show signs of adiposity and impaired glucose utilization.[9] They are unable to maintain their body temperature when placed into a cold environment, largely because of their inability to vasoconstrict peripheral vessels.[4] $PAM^{+/-}$ mice exhibit an increase in anxiety-like behavior and a deficit in short- and long-term contextual and cued fear conditioning.[14] Although the levels of TRH-Gly were elevated in the hypothalami of $PAM^{+/-}$ mice, the effect was small and peptidomic analyses of the pituitaries of $PAM^{+/-}$ mice revealed the accumulation of peptidyglycine precursors without significant depletion of amidated products.[35] As discussed below, the deficits in $PAM^{+/-}$ mice are believed to reflect alterations in the ability of the PAM protein to fulfill its noncatalytic roles, including a role in copper homeostasis.

Genetics and Environment: Copper and Ascorbate

To function, PAM requires molecular oxygen, reduced ascorbate and copper. In humans, both ascorbate and copper must come from dietary sources; rodents can synthesize ascorbate. To be used by PAM, both ascorbate and copper must be delivered into the lumen of the secretory pathway, a process that requires the participation of multiple additional genes.

Members of the SVCT2 family of high-affinity Na^+-dependent ascorbate transporters are highly enriched in neurons, neuroendocrine cells, exocrine cells, osteoblasts, and endothelial cells.[27] These antiporters use the Na^+ gradient to concentrate ascorbate in the cytosol. In anterior pituitary endocrine cells, cytosolic ascorbate levels are 2–5 mM; secretory granule levels can be ten-fold higher, although the mechanism by which ascorbate gains entry to the secretory pathway remains unclear.

Ascorbate is converted into semidehydroascorbate when it donates a single electron to Cu(II), forming Cu(I); production of one mole of amidated peptide consumes one mole of ascorbic acid. Cytochrome b_{561}, an integral membrane protein, shuttles reducing equivalents from the cytosol across the secretory granule membrane. A close association of Cytochrome b_{561} and PAM is apparent in *Planaria*, with coexpression of these genes.[1] Mutation of *Drosophila* cytochrome b_{561} (*nemy*) results in defects in memory retention and an inability to amidate neuropeptides.[15]

Several agents capable of single electron transfer can reduce Cu(II) bound to PHM to Cu(I); however, reactions using ascorbic acid as the reductant result in the highest PHM activity in both *in vivo* and *in vitro* assays. This lack of reductant specificity in PHM is further supported by the observation that no ascorbate binding site was identified in crystals of PHM analyzed in the presence of high levels of ascorbate.[27]

As discussed above, copper plays an essential role in the ability of PHM to use molecular oxygen. Although many proteins bind copper, the number of cuproenzymes is small. Unlike ceruloplasmin, which binds copper tightly and is degraded in its apoform, copper is rather loosely bound to PHM. Copper is lost during the purification of PHM and

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can easily be replaced; the affinity of PHM for Cu(II) is estimated to be approximately 0.06 μM. The structure of PHM is sensitive to the presence of copper[26,32] and the endocytic trafficking of PAM is affected by the availability of copper.[10]

Delivery of copper to the secretory pathway involves uptake in the gut, transport through the circulation and across the blood–brain barrier, uptake across the plasma membrane, chaperone binding, transfer to a P-type ATPase, and transport into the secretory pathway. Although many of the key players in whole body copper homeostasis are known, much remains to be learned about their control.[18] Mutations in ATP7A, an X-linked gene, cause Menkes Disease. In the mottled-brindled mouse, a model of Menkes Disease, peptide amidation is severely compromised.[23,33] Although cytosolic copper is known to bind to a variety of chaperones, little is known about the handling of copper in the lumen of the secretory pathway.

LATER FINDINGS

Role of PAM as a Luminal Domain Sensor

Analysis of corticotroph tumor cells engineered to allow control of PAM expression reveals the ability of membrane PAM to alter cytoskeletal organization and diminish the endoproteolytic processing and regulated release of proopiomelanocortin products.[8] Kalirin, a Rho GDP/GTP exchange factor for Rac1 and RhoG, and Uhmk1, a Ser/Thr kinase that phosphorylates the CD of PAM, were identified through their ability to interact with the CD of PAM. Regulated intramembrane proteolysis of PAM by γ-secretase produces a soluble fragment of the cytosolic domain (sf-CD), which accumulates in the nucleus in a phosphorylation-regulated manner.[13] Microarray analysis reveals the ability of PAM to increase the expression of a subset of genes in AtT-20 corticotroph tumor cells; included in this set of PAM-regulated genes is aquaporin 1 (*Aqp1*), a protein known to play an essential role in secretory granule biogenesis.

Role of PAM in Copper Homeostasis

Several deficits observed in $PAM^{+/-}$ mice are reversed by supplementary dietary copper.[4] In addition, similar behavioral deficits appear in wild-type mice kept on a mildly copper-deficient diet. $PAM^{+/-}$ mice consuming copper-supplemented water regain the ability to maintain body temperature in the cold, and copper-deficient wild-type mice lose the ability to do so. Similarly, the increase in anxiety-like behavior observed in $PAM^{+/-}$ mice is eliminated when they are provided with copper-supplemented water, and copper-deficient wild-type mice show an increase in anxiety-like behavior. Q-PCR analysis of atrium and pituitary reveals tissue-specific effects of PAM heterozygosity on the expression of the plasma membrane copper transporter Ctr1, the cytosolic copper chaperone Atox1 and ATP7A. The fact that copper affects the trafficking of membrane PAM and the conformation of PHM may contribute to the altered copper homeostasis observed in $PAM^{+/-}$ mice.

Role of PAM in Hypoxia

Intermittent hypoxia, which occurs during sleep apnea, is known to affect the synthesis of neurotransmitters in the central and peripheral nervous systems; in particular, neuropeptide signaling is altered. Analysis of brainstem lysates of adult male rats exposed to intermittent or continuous hypoxia shows that intermittent, but not continuous hypoxia, results in increased PHM activity; levels of amidated substance P and amidated neuropeptide Y are also increased, without an increase in PAL activity.[31] The increase in PHM activity is the result of increased proteolytic processing of membrane PAM and is blocked by an antioxidant that prevents the generation of reactive oxygen species.

REFERENCES

1. Asada A, Orii H, Watanabe K, Tsubaki M. Planarian peptidylglycine-hydroxylating monooxygenase, a neuropeptide processing enzyme, colocalizes with cytochrome b561 along the central nervous system. *FEBS J* 2005;**272**:942–55.
2. Bauer JD, Sunman JA, Foster MS, Thompson JR, Ogonowski AA, Cutler SJ, et al. Anti-inflammatory effects of 4-phenyl-3-butenoic acid and 5-(acetylamino)-4-oxo-6-phenyl-2-hexenoic acid methyl ester, potential inhibitors of neuropeptide bioactivation. *J Pharmacol Exp Ther* 2007;**320**:1171–7.
3. Bauman AT, Jaron S, Yukl ET, Burchfiel JR, Blackburn NJ. pH Dependence of peptidylglycine monooxygenase. Mechanistic implications of Cu-methionine binding dynamics. *Biochemistry* 2006;**45**:11140–50.
4. Bousquet-Moore D, Prohaska JR, Nillni EA, Czyzyk T, Wetsel WC, Mains RE, et al. Interactions of peptide amidation and copper: novel biomarkers and mechanisms of neural dysfunction. *Neurobiol Dis* 2010;**37**:130–40.
5. Bradbury AF, Finnie MD, Smyth DG. Mechanism of C-terminal amide formation by pituitary enzymes. *Nature* 1982;**298**:686–8.
6. Casara P, Ganzhorn A, Phillippo C, Chanal MC, Danzin C. Unsaturated thioacetic acids as novel mechanism-based inhibitors of peptidylglycine alpha-amidating monooxygenase. *Bioorg Med Chem Let* 1996;**6**:393–6.
7. Chufan EE, De M, Eipper BA, Mains RE, Amzel LM. Amidation of bioactive peptides: the structure of the lyase domain of the amidating enzyme. *Structure* 2009;**17**:965–73.
8. Ciccotosto GD, Schiller MR, Eipper BA, Mains RE. Induction of integral membrane PAM expression in AtT-20 cells alters the storage and trafficking of POMC and PC1. *J Cell Biol* 1999;**144**:459–71.
9. Czyzyk TA, Ning Y, Hsu MS, Peng B, Mains RE, Eipper BA, et al. Deletion of peptide amidation enzymatic activity leads to edema and embryonic lethality in the mouse. *Dev Biol* 2005;**287**:301–13.

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10. De M, Ciccotosto GD, Mains RE, Eipper BA. Trafficking of a secretory granule membrane protein is sensitive to copper. *J Biol Chem* 2007;**282**:23362–71.
11. Eipper BA, Milgram SL, Husten EJ, Yun HY, Mains RE. Peptidylglycine alpha-amidating monooxygenase: a multifunctional protein with catalytic, processing, and routing domains. *Protein Sci* 1993;**2**:489–97.
12. Francisco WA, Blackburn NJ, Klinman JP. Oxygen and hydrogen isotope effects in an active site tyrosine to phenylalanine mutant of peptidylglycine alpha-hydroxylating monooxygenase: mechanistic implications. *Biochemistry* 2003;**42**:1813–9.
13. Francone VP, Ifrim MF, Rajagopal C, Leddy CJ, Wang Y, Carson JH, et al. Signaling from the secretory granule to the nucleus: Uhmk1 and PAM. *Mol Endocrinol* 2010;**24**:1543–58.
14. Gaier ED, Rodriguiz RM, Ma XM, Sivaramakrishnan S, Bousquet-Moore D, Wetsel WC, et al. Haploinsufficiency in peptidylglycine alpha-amidating monooxygenase leads to altered synaptic transmission in the amygdala and impaired emotional responses. *J Neurosci* 2010;**30**:13656–69.
15. Iliadi KG, Avivi A, Iliadi NN, Knight D, Korol AB, Nevo E, et al. Nemy encodes a cytochrome b561 that is required for Drosophila learning and memory. *Proc Natl Acad Sci USA* 2008;**105**:19986–91.
16. Iwai N, Martinez A, Miller MJ, Vos M, Mulshine JL, Treston AM. Autocrine growth loops dependent on peptidyl alpha-amidating enzyme as targets for novel tumor cell growth inhibitors. *Lung Cancer* 1999;**23**:209–22.
17. Jiang N, Kolhekar AS, Jacobs PS, Mains RE, Eipper BA, Taghert PH. PHM is required for normal developmental transitions and for biosynthesis of secretory peptides in Drosophila. *Dev Biol* 2000;**226**:118–36.
18. Kim BE, Turski ML, Nose Y, Casad M, Rockman HA, Thiele DJ. Cardiac copper deficiency activates a systemic signaling mechanism that communicates with the copper acquisition and storage organs. *Cell Metab* 2010;**11**:353–63.
19. Kolhekar AS, Mains RE, Eipper BA. Peptidylglycine alpha-amidating monooxygenase: an ascorbate-requiring enzyme. *Methods Enzymol* 1997;**279**:35–43.
20. Kulathila R, Merkler KA, Merkler DJ. Enzymatic formation of C-terminal amides. *Nat Prod Rep* 1999;**16**:145–54.
21. Merkler DJ, Asser AS, Baumgart LE, Carballo N, Carpenter SE, Chew GH, et al. Substituted hippurates and hippurate analogs as substrates and inhibitors of peptidylglycine alpha-hydroxylating monooxygenase (PHM). *Bioorg Med Chem* 2008;**16**:10061–74.
22. Moore AB, May SW. Kinetic and inhibition studies on substrate channelling in the bifunctional enzyme catalysing C-terminal amidation. *Biochem J* 1999;**341**(Pt 1):33–40.
23. Niciu MJ, Ma XM, El MR, Pachter JS, Mains RE, Eipper BA. Altered ATP7A expression and other compensatory responses in a murine model of Menkes disease. *Neurobiol Dis* 2007;**27**:278–91.
24. Ouafik LH, Stoffers DA, Campbell TA, Johnson RC, Bloomquist BT, Mains RE, et al. The multifunctional peptidylglycine alpha-amidating monooxygenase gene: exon/intron organization of catalytic, processing, and routing domains. *Mol Endocrinol* 1992;**6**:1571–84.
25. Park D, Hadzic T, Yin P, Rusch J, Abruzzi K, Rosbash M, et al. Molecular organization of Drosophila neuroendocrine cells by dimmed. *Curr Biol* 2011;**21**:1515–24.
26. Prigge ST, Kolhekar AS, Eipper BA, Mains RE, Amzel LM. Amidation of bioactive peptides: the structure of peptidylglycine alpha-hydroxylating monooxygenase. *Science* 1997;**278**:1300–5.
27. Prigge ST, Mains RE, Eipper BA, Amzel LM. New insights into copper monooxygenases and peptide amidation: structure, mechanism and function. *Cell Mol Life Sci* 2000;**57**:1236–59.
28. Prigge ST, Eipper BA, Mains RE, Amzel LM. Dioxygen binds end-on to mononuclear copper in a precatalytic enzyme complex. *Science* 2004;**304**:864–7.
29. Rajagopal C, Stone KL, Mains RE, Eipper BA. Secretion stimulates intramembrane proteolysis of a secretory granule membrane enzyme. *J Biol Chem* 2010;**285**:34632–42.
30. Schafer MKH, Stoffers DA, Eipper BA, Watson SJ. Expression of peptidylglycine α- amidating monoxygenase (EC 1.14.17.3) in the rat central nervous system. *J Neurosci* 1992;**12**:222–34.
31. Sharma SD, Raghuraman G, Lee MS, Prabhakar NR, Kumar GK. Intermittent hypoxia activates peptidylglycine alpha-amidating monooxygenase in rat brain stem via reactive oxygen species-mediated proteolytic processing. *J Appl Physiol* 2009;**106**:12–9.
32. Siebert X, Eipper BA, Mains RE, Prigge ST, Blackburn NJ, Amzel LM. The catalytic copper of peptidylglycine alpha-hydroxylating monooxygenase also plays a critical structural role. *Biophys J* 2005;**89**:3312–9.
33. Steveson TC, Ciccotosto GD, Ma XM, Mueller GP, Mains RE, Eipper BA. Menkes protein contributes to the function of peptidylglycine alpha-amidating monooxygenase. *Endocrinology* 2003;**144**:188–200.
34. Williamson M, Hauser F, Grimmelikhuijzen CJ. Genomic organization and splicing variants of a peptidylglycine alpha-hydroxylating monooxygenase from sea anemones. *Biochem Biophys Res Commun* 2000;**277**:7–12.
35. Yin P, Bousquet-Moore D, Annangudi SP, Southey BR, Mains RE, Eipper BA, Sweedler JV. Probing the production of amidated peptides following genetic and dietary copper manipulations. *PLoS One* 2011;**6**(12);28679.

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Chapter 245

Prohormone Convertase 1/3

Mirella Vivoli and Iris Lindberg

ABSTRACT

The prohormone convertase PC1/3 is a eukaryotic serine protease that initiates the neuroendocrine-specific proteolytic cleavage of peptide hormone and neuropeptide precursors. Within the regulated secretory pathway, PC1/3 is synthesized as a glycosylated and sulfated precursor which undergoes initial rapid propeptide cleavage followed by further C-terminal cleavages in the later stages of the secretory pathway, resulting in a variety of PC1/3 forms differing in size, charge, and activity. PC1/3 has a propensity to oligomerize, which reduces its activity. PC1/3 interacts with the neuroendocrine protein proSAAS, which represents a highly potent natural inhibitor of this enzyme. Gene disruption experiments, and the many naturally occurring human mutations, support the involvement of PC1/3 in pathological conditions such as obesity, diabetes, opportunistic diseases, and hypercholesterolemia, making PC1/3 a potentially strong target for drug design. Recent studies are directed toward the development of potent and specific inhibitors.

DISCOVERY AND GENERAL FEATURES OF PC1/3

Many secretory proteins and peptide precursors are subjected to intracellular limited proteolysis, a process essential for acquisition of bioactivity. The enzymes responsible for this intracellular cleavage have been characterized in molecular and functional terms and belong to an evolutionarily conserved family of serine proteases related to the bacterial enzyme subtilisin and the yeast processing protease kex2 or kexin (reviewed in Ref. 28). In humans, PC1/3 is encoded by the *PCSK1* gene located on chromosome 5 and consists of 14 exons. Because of the homology of their catalytic domains to that of the bacterial enzyme subtilisin, these enzymes have been termed subtilisin-like proprotein convertases (SPCs or PCs).[28] Thus far, seven mammalian PCs have been identified, and their nomenclature has been standardized as follows: PC1/3 (previously known as PC1, PC3 and sPC3); PC2; furin; PACE4; PC4; PC5/6; and PC7/8 (previously known also as sPC7, LPC, and PC8). All these enzymes specialize in the cleavage of basic residues within the general motif Arg-$(X)_n$-Arg↓ where n = 0, 2, 4, 6 and X is any amino acid except Cys or Pro. Two other enzymes, subtilisin/kexin-like isozyme-1 (also known as SKI-1 and site-1 protease) and PCSK9, which perform cleavages at hydrophobic residues (reviewed in Ref. 30), are also present within the secretory pathway. The conservation of catalytic domains among these enzymes suggests a common evolutionary origin for PC genes; over time, ancestors of these enzymes have probably undergone duplications, insertions, translocations, and/or deletions to generate the current family.

PC1/3 was cloned in 1991 as the third PC to be identified; as early as 1988, Hutton and colleagues described a proinsulin-processing enzyme in insulinoma granules, which they termed "Type I proinsulin converting endopeptidase," which was later identified as PC1/3.[28] Analysis of the tissue expression and cellular localization of the cloned enzyme confirmed that this enzyme is found in dense core secretory granules within neuroendocrine tissues where it plays a pivotal role in the processing of neuropeptide and endocrine precursors. In this chapter, we review essential information concerning PC1/3, focusing on the work of the last decade.

PEPTIDE BIOSYNTHESIS AND PROCESSING: PC1/3 FUNCTION

Peptide hormones and neuropeptides are initially generated as relatively large precursors that require activation through a series of posttranslational processing events occurring during transit through the regulated secretory pathway. These processing reactions begin in the lumen of endoplasmic reticulum, and continue within the various secretory pathway compartments and the maturing secretory granules. The initial processing reactions consist of the removal of signal peptide, core glycosylation (which is relatively infrequent), and proper folding. Proteolytic cleavage of precursors most often occurs at single/double basic residues, such as Lys–Arg and Arg–Arg, and is believed to begin in the trans-Golgi network; PC1/3 cleavage often initiates this proteolysis. Within the secretory granules, intermediate-sized and final peptide products are subjected to additional exoproteolytic cleavages at C-terminal basic residues by

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00245-1
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carboxypeptidase E.[13] In certain cases (depending on primary sequence), further terminal modifications can occur, such as C-terminal amidation, N-terminal acetylation, pyroglutamylation, phosphorylation, and/or sulfation; these modifications may be required to render the final peptide products biologically active (reviewed in Ref. 26). In contrast to the secretion of liver proteins into the bloodstream, which proceeds constitutively, the release of mature peptides from endocrine and neuroendocrine cells requires the presence of a cell-specific stimulus, such as depolarization (nerve cells) or high glucose levels (pancreatic beta cells). During a still poorly understood sorting process occurring in the trans-Golgi network, processed hormones and/or neuropeptides are condensed into immature secretory vesicles, which gradually become more acidic due to the presence of a proton pump within the membrane. This decrease in pH probably facilitates the final enzymatic steps of precursor maturation, as many of the posttranslational processing enzymes have an acidic pH optimum. Finally, after a stimulus event, a portion of docked mature vesicles fuses with the plasma membrane, resulting in the release of mature peptides into either the circulation or the synaptic cleft.

Work from several laboratories has confirmed that PC1/3 accomplishes the initial physiological processing of proopiomelanocortin (POMC), the common precursor for the melanocortin-related peptides ACTH/α-MSH, β-MSH, and γ-MSH and the opioid peptide β-endorphin. In anterior pituitary corticotrophs, which are highly enriched in PC1/3, POMC undergoes an ordered sequence of proteolytic cleavages beginning with PC1/3 action. This proteolysis yields ACTH, the N-terminal region of POMC, and β-lipotropin. In intermediate lobe melanotrophs, which contain both PC1/3 and PC2, different POMC products are generated via the sequential action of these enzymes (Fig. 1).[32] Differential tissue-specific processing by the two enzymes yields a variety of biologically active peptides involved in diverse cellular functions.

Many other peptide hormone and neuropeptide precursors are now known to be cleaved by PC1/3, including proinsulin, pancreatic and intestinal proglucagon, proghrelin, prosomatostatin, propancreatic polypeptide, progastrin, proparathyroid hormone, proenkephalin, prodynorphin, proluteinizing hormone-releasing hormone, and prorenin (reviewed in Ref. 6); most of these have been identified and quantified in PC1/3 knockout mouse brains subjected to quantitative peptidomics techniques[33] (see the Peptidomic chapters in this section of the book). It is probable that other precursor substrates remain to be discovered.

PROPERTIES OF PC1/3

General Properties

Similarly to other members of the eukaryotic subtilisin family, PC1/3 contains four domains: the propeptide, the catalytic domain, the P or Homo B domain, and the carboxy-terminal domain. Like subtilisin, this protease is activated through an autocatalytic cleavage of the N-terminal

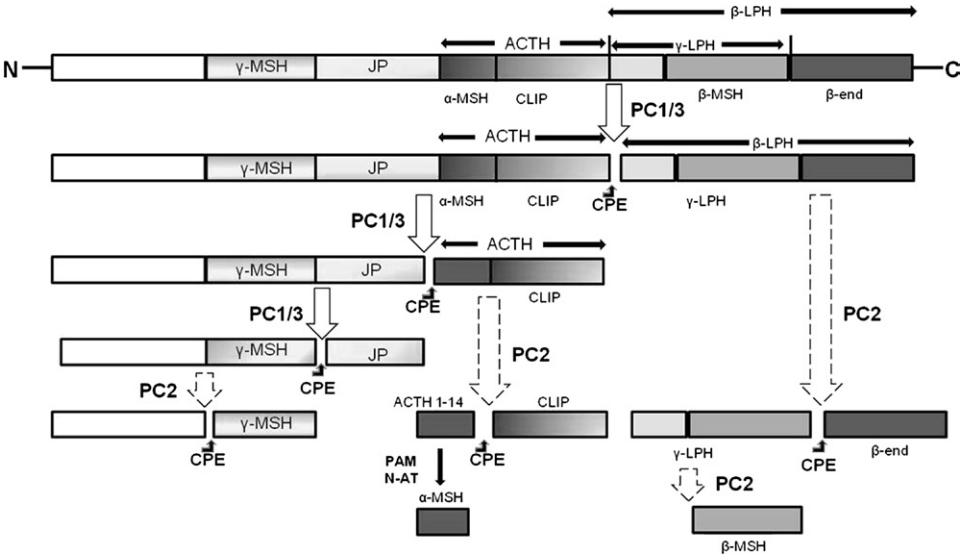

FIGURE 1 POMC processing by PC1/3 and PC2 in the pituitary. The POMC precursor, already modified by phosphorylation and glycosylation, is first cleaved by PC1/3 (shown with solid arrows), which results in a N-terminal segment containing the peptides γ-MSH (melanocyte-stimulating hormone), JP (*joining peptide*) and ACTH (adrenocorticotropic hormone), and β-LPH (β-lipotropin). After any internal proteolytic cleavage, C-terminal basic residues are removed by carboxypeptidase E (CPE; shown with black bent arrows). In tissues that contain PC2, such as the intermediate pituitary, further cleavage (shown with dashed arrows) generates ACTH 1–14, α-MSH, γ-MSH, γ-LPH, and β-endorphin (β-end). Additional terminal modification of ACTH 1–14 then occurs, consisting of CPE trimming, amidation, carried out by peptidyl α-amidating monooxygenase (PAM), and acetylation by an unknown N-acetyl transferase (N-AT).

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propeptide. As shown in Fig. 2 (top), the catalytic domain, featuring the serine–histidine–aspartic acid catalytic triad, which performs catalytic action, lies adjacent to this N-terminal domain. The P or Homo B domain of approximately 150 amino acids is the carboxy terminal to the catalytic domain; this domain is believed to assist in correct protein folding, to contribute to enzyme stability, and also to contribute to calcium and pH dependence.[6] Lastly, the carboxyl-terminal domain probably plays a role in cellular routing (reviewed in Ref. 6).

Both proPC1/3 and its prohormone substrates are subjected to similar posttranslational processing reactions and travel together through the regulated secretory pathway of neuroendocrine tissues. During translation, preproPC1/3 is inserted into the lumen of the endoplasmic reticulum, where the 27 residue signal peptide is removed. The resulting proPC1/3 zymogen of approximately 94 kDa then undergoes very rapid autocatalytic cleavage of the proregion, resulting in an 87-kDa form. The evidence for such a mechanism derives from several studies showing that mutations at the PC1/3 active site result in a failure of the enzyme to undergo both propeptide cleavage and oligosaccharide maturation; it is probable that this poorly folded precursor remains in the endoplasmic reticulum, destined for degradation.[16] PC1/3 (87 kDa), which has three Asn-X-Ser/Thr N-glycosylation sites, is then decorated by sugars and moves into the Golgi apparatus, where it is subjected to additional posttranslational modifications, such as terminal glycosylation and sulfation. Whether the cleaved propeptide (which is a potent inhibitor of the enzyme) remains associated with the mature enzyme during transport is unclear; this occurs for furin, which does not become fully active until it reaches acidic compartments in which it undergoes internal propeptide cleavage and dissociation (reviewed in Ref. 26). It is however clear that once 87-kDa PC1/3 arrives in the slightly acidic trans-Golgi network, it acquires the ability to initiate prohormone cleavage; cleavage continues in the immature and mature secretory granule (reviewed in Ref. 6). Additional proteolytic modification of PC1/3 takes place in the secretory granules; this consists of intermolecular proteolysis of the C-terminus and results in the formation of the 74 and 66 kDa truncated forms of PC1/3, as shown in Fig. 2 (top). The 66-kDa form exhibits much higher specific activity but also lesser stability (reviewed in Ref. 6). This truncation event is apparently extremely important to the physiology of PC1/3, as shown by the introduction of mutations that block the removal of the carboxy-terminal tail which

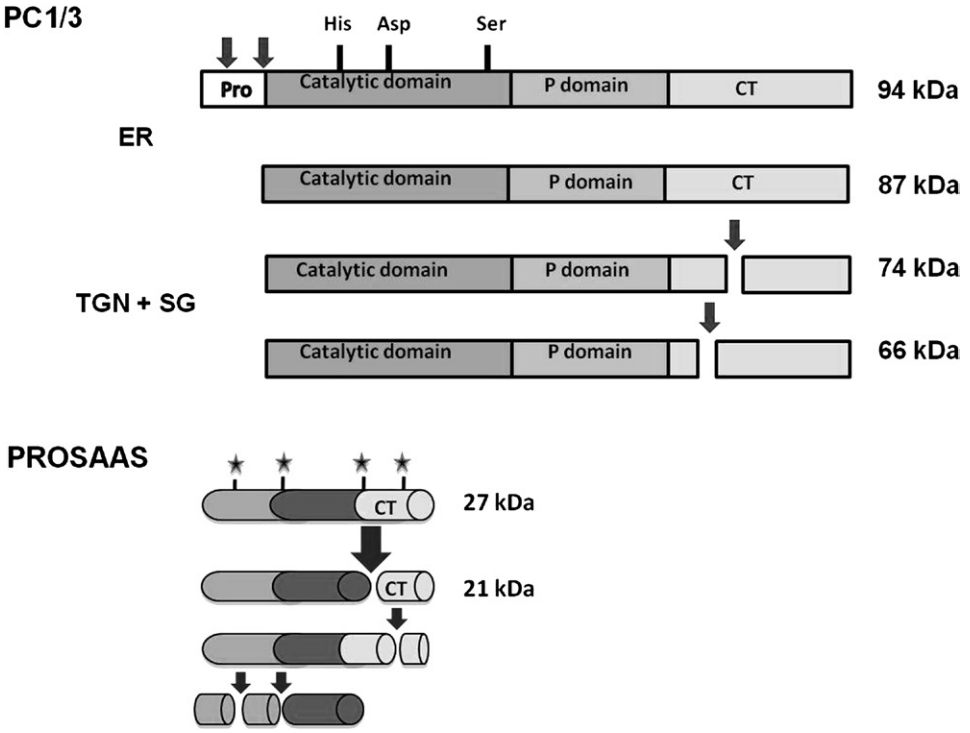

FIGURE 2 Schematic representation of the domains of PC1/3 (top) and proSAAS (bottom). Top: Pro, propeptide domain; catalytic domain, which contains the catalytic triad His, Asp and Ser; P domain (also known as "middle" or Homo B domain); and CT, the carboxyl-terminal domain. ER, endoplasmic reticulum; TGN, trans-golgi network; SG, secretory granules. Known cleavage sites are shown with arrows. Bottom: The 27-kDa form of the PC1/3 binding protein proSAAS, formed after cleavage of the signal peptide, is cleaved first at the furin cleavage site separating the 21 kDa from the CT peptide domains (shown with larger arrow); further cleavages then liberate potential bioactive peptides (shown with smaller arrows). As in Fig. 1, CPE is required to remove carboxy-terminal basic residues. Stars depict dibasic cleavage sites.

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prevent full enzyme activation (and also result in abnormal cellular localization).[24] The carboxy-terminal region behaves as a bimodal regulator of PC1/3 activity:[27] it can function either to stimulate the conversion of the 87-kDa form into the more active 66-kDa form, or to inhibit PC1/3 autocatalytic cleavage in the early secretory compartments. The carboxy-terminal domain also clearly stabilizes the enzyme, and plays a role in routing of the enzyme to the secretory granules.[24] One group has provided evidence to support transmembrane insertion of PC1/3,[23] whereas its categorization as a transmembrane protein[2] has been refuted by others.[31]

After the publication of the cDNA sequence of PC1/3, the production and characterization of recombinant PC1/3 became possible using overexpression methods. Indeed, recombinant PC1/3 is now obtainable from several sources, including methotrexate-amplified transfected CHO cells, transfected L-cells, vaccinia virus-mediated overexpression, and baculovirally mediated insect cell expression (reviewed in Ref. 6). Recombinant PC1/3 is easily assayed using the fluorogenic substrate pGlu-Arg-Thr-Lys-Arg-aminomethylcoumarin (AMC); recent results show that tissue PC1/3 can also be assayed if (1) tissues are homogenized in the presence of general protease inhibitors; (2) general protease inhibitors are present in the assay mixture to block the much more active cellular enzymes capable of cleaving the convertase substrate; and (3) the specific inhibitor LLRVKR is used to block nonspecific activity, which is then subtracted from the total. The assay is described in Ref. 17.

Substrate Specificity

Previous *in vitro* and *in vivo* studies support the idea that PC1/3 represents the first enzyme involved in the production of larger intermediates from precursor proteins (unlike PC2, whose action generates small peptides from those larger intermediates). Common procedures to analyze the substrate specificity in a cellular context are coexpression of PC cDNA with a target precursor; antisense studies; and/or gene knockout experiments. By contrast, *in vitro* experiments with purified enzymes generally use kinetic measurements in which the purified PC is incubated with either a recombinant precursor or a synthetic fluorogenic peptide substrate. A generally accepted general model of enzymatic cleavage (Schecter and Berger nomenclature) depicts substrate amino acids designated as P1, P2, P3, P4, etc. proceeding in the N-terminal direction from the scissile bond; similarly, the residues proceeding in the C-terminal direction from the scissile bond are designated P1′, P2′, P3′, P4′, etc. Both *in vitro* and *in vivo* studies show that PC1/3 processes prohormone and neuropeptide precursors C-terminally to a pair of basic residues (occupying the P1 and P2 positions), or, rarely, to a single arginine. A comparison of cleavage sites within peptide precursors, coupled with the known occurrence of given residues at different positions, enables certain conclusions to be made as to the preferred substrate specificity of PC1/3 (reviewed in Ref. 6). Briefly, an aliphatic residue, such as Leu, Ile, Val, Met, or Thr, almost never occurs at the P1′ position within substrates; it is also very rare to find a Pro in the P1′ or the P2 position, which impairs both recognition and cleavage; moreover, Arg, Lys, Asp, or Glu are also rarely present at position P1′ or P3′. In addition, Cys or His have thus far rarely been found in the close vicinity of the scissile bond, as shown in a human proalbumin synthetic peptide, where the presence of such amino acids prevents PC1/3 cleavage. This result is consistent with the idea that these residues are unfavorable when occurring close to a PC1/3 cleavage site.[21] Moreover, other *in vitro* studies using recombinant enzyme have also shown that PC1/3 prefers substrates with positively charged amino acids, particularly Arg, at positions P2, P4, P6, or P8. For example, the replacement of these arginines with Lys or Orn in the synthetic substrate acetyl-Arg-Ser-Lys-Arg-AMC results in decreased catalytic efficiency due to an increased K_m.[20] In the substrate human proparathyroid hormone substitution of the natural residue Arg at position P4 with Val does not affect cleavage rate; however, in human proalbumin, peptide substitution by Arg at the same subsite increases cleavage velocity by fivefold,[21] suggesting that substrate context affects the role played by Arg at the P4 position. The specificity of PC1/3 has been investigated *in vitro* using pGlu-Arg-Thr-Lys-Arg-AMC as fluorogenic substrate. Because its K_m value for PC1/3 is in the micromolar range (varying from 8 to 23 μM), catalytic cleavage of this substrate is clearly very inefficient compared with that of other proteolytic enzymes such as trypsin and even the yeast convertase kex2. K_m values observed with larger peptide substrates are also in the micromolar range, indicating that catalytic inefficiency is an intrinsic property of PC1/3 rather than a substrate effect. Interestingly, decreased affinity for shorter peptides has been observed, often occurring with an increase of V_{max} (which compensates for decreased affinity and results in similar overall catalytic efficiency).[5] These findings suggest that PC1/3-mediated processing is not greatly influenced by substrate length. Although this work begins to illuminate the substrate specificity of PC1/3, in the absence of the availability of a crystal structure, further studies are necessary to define the relative contribution of each residue lining the catalytic pocket to the minimal and maximal substrate recognition sites.

Regulation of Activity

The specific activity of PC1/3 is 50–80 times lower than that of PC2, suggesting considerable room for regulatory mechanisms. Despite the critical importance of PC1/3 in the

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bioactivation of neuropepetide precursors and prohormones, regulatory mechanisms affecting activity have not yet been completely elucidated. Similar to the other convertases, the enzymatic activity of PC1/3 is highly dependent on the presence of calcium and is inhibited by chelators such as EDTA (reviewed in Ref. 26); the known calcium gradient within the secretory pathway may thus control PC1/3 activity. Truncation of PC1/3 also clearly plays a role in controlling PC1/3 activity. Recombinant PC1/3 is obtained as three enzymatic forms differing in length at the C-terminus; as described above, removal of the carboxy-terminal fragment has a major impact on enzymatic properties, such as pH optimum, calcium dependence and enzyme stability (reviewed in Ref. 6). The pH optimum of the 87-kDa form is in the range of 5.5–6.5, and this form of the enzyme requires 2 mM concentrations of calcium for maximal activity. Truncation of 87-PC1/3 to the 66 kDa species increases the optimal calcium concentration by several fold and decreases the pH optimum from 5.5 to 5.0.[26]

PC1/3 biosynthesis and activity can also be regulated by a binding partner, called proSAAS, a 21-kDa granin-like neuroendocrine secretory protein.[11] ProSAAS contains two domains, a large N-terminal domain and a C-terminal peptide domain (Fig. 2, bottom); like the PC2 chaperone protein 7B2, these domains are separated by a furin cleavage site. The C-terminal peptide represents a potent endogenous PC1/3 inhibitor *in vitro*. Although the N-terminal domain of 7B2 is critical for the expression of PC2 activity (see the next chapter), the N-terminal domain of proSAAS has no effect on PC1/3 activity *in vitro*. However, the expression of proSAAS N-terminal domain reduces the secretion of PC1/3 in both CHO cells stably expressing PC1/3, as well as in transiently-transfected HEK cells.[11] This reduction in secretion rate is reminiscent of chaperone protein action; for example, BiP overexpression is well known to delay the secretion of molecules through the secretory pathway. Overexpression of proSAAS blocks the dynamic maturation of newly synthesized POMC to ACTH, but paradoxically has little effect on the total cellular content of ACTH.[11] More work remains to be done to fully understand the physiological role of proSAAS interactions with PC1/3. Interestingly, further cleavage of proSAAS to smaller peptides occurs in certain tissues.[29]

PC1/3 can also be transcriptionally regulated; several studies have shown that the PC1/3 promoter contains binding sites for both STAT3 (signal transducer and activator of transcription 3) and Nhlh2 (nescient helix-loop-helix 2, a member of the large family of basic helix-loop-helix transcription factors); both are involved in hypothalamic PC1/3 expression. These transcription factors may also coordinately regulate transcription of PC1/3 following leptin stimulation.[12] PC1/3 expression is also dependent on activation of the CREB/cAMP system.[9] Lastly, PC1/3 is regulated on a translational level: for example, in pancreatic islets, glucose regulates both the synthesis of PC1/3 and its substrate proinsulin.[1]

Hoshino et al.[18] showed that recombinant and natural PC1/3 forms exist as multiple populations, differing not only in charge but also in size. These species consist of monomers, oligomers, and aggregates; the more acidic forms of PC1/3 consist of defined oligomers and aggregates, whereas the more basic PC1/3 forms are monomers and dimers. A variety of experimental techniques, such as gel filtration, radiation inactivation, and dynamic light scattering, support the idea that one of the active forms of PC1/3 is an 87-kDa homodimer. In addition, certain small peptides terminating in pairs of basic residues were found to both stabilize the 66-kDa form of PC1/3, and behave as activators of 87-kDa PC1/3. Because the carboxy-terminal domain differentiates the 66 from the 87-kDa form of PC1/3, it is likely that this domain contributes to stimulation, most probably by altering the conformation of 87-kDa PC1/3. Taken together, these findings suggest that oligomerization and allosteric peptide activation represent important factors involved in the regulation of PC1/3 activity; further study is required to elaborate the precise binding sites for these events.

Zandberg et al.[34] reported that N-glycosylation of PC1/3 affects zymogen activation and trafficking and influences prohormone processing. However, the probability that N-glycosylation dynamically controls these processes *in vivo* seems slight.

DISTRIBUTION/LOCALIZATION

Analysis of the mRNA expression of PC1/3 in rat tissues and cell lines by Northern blotting shows that PC1/3 is expressed in many tissues and cells containing secretory granules, including pituitary, adrenal gland, pancreatic islets, endocrine cells within the small intestine, in the gastric mucosa, and within the large luteal cells of the ovary. Although PC1/3-like transcripts of various sizes have been detected by Northern blot analysis in lung, skeletal muscle, and heart, the ability of these various transcripts to express PC1/3 has not been established. Moreover, small amounts of PC1/3 are also expressed in various cells of the immune system. The enzyme is not found in liver, kidney, and spleen (reviewed in Ref. 26).

PATHOPHYSIOLOGICAL IMPLICATIONS OF PC1/3 VARIANTS

The varied physiological roles of PC1/3 have been revealed through the characterization of gene knockout phenotypes in mice and by the discovery of mutations in human patients. PC1/3 misregulation would be predicted to lead to multiple hormone and neuropeptide deficiencies. Indeed, knockout of the PC1/3 gene in mice results

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in severe developmental abnormalities, such as growth retardation and dwarfism, most probably because in mice (though not in humans, because of sequence variations), PC1/3 is involved in the processing of the growth hormone-releasing hormone (GHRH) precursor.[35] Other precursor proteins, such as pituitary POMC, pancreatic proinsulin and proglucagon, also undergo defective processing in tissues from this null mouse.[35] In 1997, the first human PC1/3 mutation was described;[10,20] this individual was shown to be a compound heterozygote for PC1/3, which resulted in a null phenotype. This phenotype was characterized by extreme obesity, intestinal dysfunction, and highly increased levels of circulating prohormones.[10] More recently, two other genetic abnormalities of PC1/3 have been described in humans: compound heterozygosity for PC1/3 mutations, including the G593R mutation which causes failure of maturation of the zymogen form of PC1/3 (proPC1/3) and its retention in the endoplasmic reticulum; and an A → C + 4 substitution in the donor splice site of intron 5, which results in exon skipping, frameshift, and introduction of a premature stop codon in the catalytic domain. These mutations result in defective absorption of monosaccharides and fat, indicating an important—but as yet, unclear—role for PC1/3 in intestinal function.[19] Another missense mutation, S370L, causes early-onset obesity and hyperphagia.[10] Strikingly, in mice, the N222D mutation is associated with obesity, hyperphagia, and increased metabolic efficiency in homozygotes;[22] the neighboring mutation N221D, which is associated with decreased PC1/3 activity, has also been reported to be linked to obesity.[4] Finally, in obese humans, common polymorphisms such as T690S and Q665E are associated with reduced postprandial fat oxidation; both of these polymorphisms are located in the C-terminal domain of PC1/3.[7] However, the identification of the specific PC1/3 substrates involved in the development of both obesity and intestinal dysfunction remains to be elucidated. Interestingly, an increasing number of genome wide association studies of PC1/3 show that changes in body weight, insulin sensitivity and processing, and blood glucose are associated with single nucleotide polymorphisms (SNPs) in PC1/3 in both European and Chinese populations.[10,20]

Peptidomic comparisons of PC1/3 substrates in PC1/3 null mouse and wild-type brains reveal few PC1/3 substrates involved in mouse development, but identify many peptides implicated in human obesity. Interestingly, a study of proSAAS knockout mice indicates that prenatal proSAAS expression is important in regulating prenatal processing of neuropeptides. By contrast, peptide levels seem normal in adult proSAAS knockout mice, suggesting that PC1/3 activity is not always affected by the absence of proSAAS.[25] Transgenic mice overexpressing proSAAS show an increase in body weight, but precursor processing by PC1/3 seems to be normal.[25]

Other important pathological conditions linked to PC1/3 expression include diabetes,[15] opportunistic diseases[8] and hypercholesterolemia, a high-risk condition for cardiovascular diseases.[2]

Owing to these clinical linkages, there is increasing interest in PC1/3 as a novel target for drug design. Such drugs will be of use not only for pathological intervention but also for biochemical studies to detail the role of PC1/3 in various peptidergic pathways. Indeed, a variety of convertase inhibitors (nearly all for furin) have been described during the past decade, representing proteins, peptides, and nonpeptide small molecules. Examples include the natural products of the andrographolide family and their succinoyl ester derivatives, certain metal complexes, dicoumarol and its derivatives (reviewed in Ref. 14) and 2,5-dideoxystreptamine derivatives. Because proteins or larger peptides are poorly permeable and often antigenic, small molecule inhibitors are more attractive as useful therapeutic targets; these often exhibit long-lasting metabolic and proteolytic stability, enhanced bioavailability, and easier syntheses.[3] However, only one report of a small molecule PC1/3 inhibitor has been published thus far, belonging to the andrographolide family.[3] Our ongoing work indicates that substituted dideoxystreptamine derivatives can function as low micromolar inhibitors (Vivoli et al., 2012, in *Mol Pharmacol*), though these first-generation inhibitors crossreact with furin to an unacceptable degree.

SUMMARY AND CONCLUSIONS

Both biochemical and *in vivo* studies have contributed immensely to our understanding of the physiological functions of PC1/3 and have shown that this enzyme is mostly localized within the Golgi apparatus and immature and dense core secretory granules of neural and endocrine cells. PC1/3 initiates the processing of many neuropeptide and peptide hormone precursors into their functional hormones, through proteolytic cleavage events occurring at pairs or single basic residues. More recent findings regarding the oligomerization and peptide activation of PC1/3 add to our knowledge of the regulation of PC1/3 activity, though additional biochemical characterization of regulatory events remains to be accomplished. The interesting phenotypes of the PC1/3 gene knockout and mutants in mice, and the discovery of increasing numbers of human patients with coding mutations in the PC1/3 gene both highlight the physiological importance of PC1/3. Indeed, humans with inactivating PC1/3 mutations exhibit high circulating levels of unprocessed prohormones, severe obesity, abnormal glucose homeostasis, and infertility of hypothalamic origin. However, more work remains to define the specific contribution of PC1/3 action to diseases such as obesity, endocrine dysfunction, and hypercholesterolemia. Efforts to obtain the crystal structure of PC1/3, made difficult by

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its propensity to oligomerize, will contribute greatly to the eventual successful therapeutic manipulation of this enzyme by specific inhibitors.

REFERENCES

1. Alarcon C, Lincolin B, Rhodes C. The biosynthesis of the subtilisin-related proprotein convertase PC3, but not that of PC2 convertase, is regulated by glucose in parallel to proinsulin biosynthesis in rat pancreatic islets. *J Biol Chem* 1994;**268**:4276–80.
2. Arnaoutova I, Smith AM, Coates LC, Sharpe JC, Dhanvantari S, Snell CR, et al. The prohormone processing enzyme PC3 is a lipid raft-associated transmembrane protein. *Biochemistry* 2003;**42**:10445–55.
3. Basak A. Inhibitors of proprotein convertases. *J Mol Med* 2005;**83**: 844–55.
4. Benzinou M, Creemers JW, Choquet H, Lobbens S, Dina C, Durand E, et al. Common nonsynonymous variants in PCSK1 confer risk of obesity. *Nat Genet* 2008;**40**:943–5.
5. Brakch N, Rholam M, Simonetti M, Cohen P. Favourable side-chain orientation of cleavage site dibasic residues of prohormone in proteolytic processing by prohormone convertase 1/3. *Eur J Biochem* 2000;**267**:1626–33.
6. Cameron A, Apletalina EV, Lindberg I. The enzymology of PC1 and PC2. *Enzymes* 2001;**XXII**:291–331.
7. Corpeleijn E, Petersen L, Holst C, Saris WH, Astrup A, Langin D, et al. Obesity-related polymorphisms and their associations with the ability to regulate fat oxidation in obese Europeans: the NUGENOB study. *Obesity (Silver Spring)* 2010;**18**:1369–77.
8. Decroly E, Vandenbranden M, Ruysschaert JM, Cogniaux J, Jacob GS, Howard SC, et al. The convertases furin and PC1 can both cleave the human immunodeficiency virus (HIV)-1 envelope glycoprotein gp160 into gp120 (HIV-1 SU) and gp41 (HIV-I TM). *J Biol Chem* 1994;**269**:12240–7.
9. Espinosa VP, Liu Y, Ferrini M, Anghel A, Nie Y, Tripathi PV, et al. Differential regulation of prohormone convertase 1/3, prohormone convertase 2 and phosphorylated cyclic-AMP-response element binding protein by short-term and long-term morphine treatment: implications for understanding the "switch" to opiate addiction. *Neuroscience* 2008;**156**:788–99.
10. Farooqi IS, Volders K, Stanhope R, Heuschkel R, White A, Lank E, et al. Hyperphagia and early-onset obesity due to a novel homozygous missense mutation in prohormone convertase 1/3. *J Clin Endocrinol Metab* 2007;**92**:3369–73.
11. Fortenberry Y, Hwang JR, Apletalina EV, Lindberg I. Functional characterization of ProSAAS: similarities and differences with 7B2. *J Biol Chem* 2002;**277**:5175–86.
12. Fox DL, Good DJ. Nescient helix-loop-helix 2 interacts with signal transducer and activator of transcription 3 to regulate transcription of prohormone convertase 1/3. *Mol Endocrinol* 2008;**22**:1438–48.
13. Fricker LD. *Methods for studying carboxypeptidase E. Peptidases and neuropeptide processing*. San Diego: Academic Press; 1995, pp. 237–250.
14. Fugere M, Day R. Cutting back on pro-protein convertases: the latest approaches to pharmacological inhibition. *Trends Pharmacol Sci* 2005;**26**:294–301.
15. Furuta M, Yano H, Zhou A, Rouille Y, Holst JJ, Carroll R, et al. Defective prohormone processing and altered pancreatic islet morphology in mice lacking active SPC2. *Proc Natl AcadSci USA* 1997;**94**:6646–51.
16. Goodman LJ, Gorman CM. Autoproteolytic activation of the mouse prohormone convertase mPC1. *Biochem Biophys Res Commun* 1994;**201**:795–804.
17. Helwig M, Vivoli M, Fricker LD, Lindberg I. Regulation of neuropeptide processing enzymes by catecholamines in endocrine cells. *Mol Pharmacol* 2011;**80**:304–13.
18. Hoshino A, Kowalska D, Jean F, Lazure C, Lindberg I. Modulation of PC1/3 activity by self-interaction and substrate binding. *Endocrinology* 2011;**152**:1402–11.
19. Jackson RS, Creemers JW, Farooqi IS, Raffin-Sanson ML, Varro A, Dockray GJ, et al. Small-intestinal dysfunction accompanies the complex endocrinopathy of human proprotein convertase 1 deficiency. *J Clin Invest* 2003;**112**:1550–60.
20. Jackson RS, Creemers JW, Ohagi S, Raffin-Sanson ML, Sanders L, Montague CT, et al. Obesity and impaired prohormone processing associated with mutations in the human prohormone convertase 1 gene. *Nat Genet* 1997;**16**:303–6.
21. Ledgerwood EC, Brennan SO, Birch NP, George PM. The specificity of the neuroendocrine convertase PC3 is determined by residues NH2- and COOH-terminal to the cleavage site. *Biochem Mol Biol Int* 1996;**39**:1167–76.
22. Lloyd DJ, Bohan S, Gekakis N. Obesity, hyperphagia and increased metabolic efficiency in Pc1 mutant mice. *Hum Mol Genet* 2006;**15**:1884–93.
23. Lou H, Smith AM, Coates LC, Cawley NX, Loh YP, Birch NP. The transmembrane domain of the prohormone convertase PC3: a key motif for targeting to the regulated secretory pathway. *Mol Cell Endocrinol* 2007;**267**:17–25.
24. Lusson J, Benjannet S, Hamelin J, Savaria D, Chretien M. The integrity of the RRGDL sequence of the proprotein convertase PC1 is critical for its zymogen and C-terminal processing and for its cellular trafficking. *Biochem J* 1997;**326**:737–44.
25. Morgan DJ, Wei S, Gomes I, Czyzyk T, Mzhavia N, Pan H, et al. The propeptide precursor proSAAS is involved in fetal neuropeptide processing and body weight regulation. *J Neurochem* 2010;**113**: 1275–84.
26. Muller L, Lindberg I. The cell biology of the prohormone convertases PC1 and PC2. (K Moldave, Ed) *Prog Nucleic Acids Res* 1999;**63**: 69–108.
27. Rabah N, Gauthier D, Dikeakos JD, Reudelhuber TL, Lazure C. The C-terminal region of the proprotein convertase 1/3 (PC1/3) exerts a bimodal regulation of the enzyme activity in vitro. *FEBS J* 2007;**274**:3482–91.
28. Rouille Y, Duguay SJ, Lund K, Furuta M, Gong Q, Lipkind G, et al. Proteolytic processing mechanisms in the biosynthesis of neuroendocrine peptides: the subtilisin-like proprotein convertases. *Front Neuroendocrinol* 1995;**16**:322–61.
29. Sayah M, Fortenberry Y, Cameron A, Lindberg I. Tissue distribution and processing of proSAAS by proprotein convertases. *J Neurochem* 2001;**76**:1833–41.
30. Seidah NG, Benjannet S, Hamelin J, Mamarbachi AM, Basak A, Marcinkiewicz J, et al. The subtilisin/kexin family of precursor convertases. Emphasis on PC1, PC2/7B2, POMC and novel enzyme SKI-1. *Ann NY Acad Sci* 1999;**885**:57–73.
31. Stettler H, Suri G, Spiess M. Proprotein convertase PC3 is not a transmembrane protein. *Biochemistry* 2005;**44**:5339–45.
32. Tanaka S. Comparative aspects of intracellular proteolytic processing of peptide hormone precursors: studies of proopiomelanocortin processing. *Zoolog Sci* 2003;**20**:1183–98.

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33. Wardman JH, Zhang X, Gagnon S, Castro LM, Zhu X, Steiner DF, et al. Analysis of peptides in prohormone convertase 1/3 null mouse brain using quantitative peptidomics. *J Neurochem* 2010;**114**:215–25.
34. Zandberg WF, Benjannet S, Hamelin J, Pinto BM, Seidah NG. N-Glycosylation controls trafficking, zymogen activation, and substrate processing of proprotein convertases PC1/3 and SKI-1. *Glycobiology* 2011.
35. Zhu X, Zhou A, Dey A, Norrbom C, Carroll R, Zhang C, et al. Disruption of PC1/3 expression in mice causes dwarfism and multiple neuroendocrine peptide processing defects. *Proc Natl Acad Sci U S A* 2002;**99**:10293–8.

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Chapter 246

Prohormone Convertase 2

Mirella Vivoli and Iris Lindberg

ABSTRACT

The prohormone convertase PC2 plays an important role in the production of bioactive peptides. This calcium-dependent serine proteinase exhibits several unique features: a substitution in the oxyanion hole, and the requirement for a binding protein, 7B2, for blockade of unproductive zymogen aggregation. Knockout mouse experiments have shown that PC2 is involved in the synthesis of >100 different peptides, with possibly the widest substrate specificity of all of the convertases. Cell transfection experiments confirm that PC2 mostly participates in the later stages of precursor maturation. In at least one case, that is, glucagon synthesis, PC2 activity is regulated by levels of 7B2; other regulatory mechanisms consist of translational and transcriptional controls for both PC2 and 7B2. PC2 variants have been associated with only a few diseases as yet, and more will likely be discovered within the coming years. Future challenges include the creation of specific inhibitors and a crystal structure for this important enzyme.

DISCOVERY AND GENERAL FEATURES OF PC2

Prohormone convertase 2 (PC2) was one of the earliest eukaryotic convertases to be discovered. Hutton and colleagues first showed the presence of two calcium-dependent enzymes in insulinoma granule extracts prepared from transplantable rat islet cell tumors in 1988 (see the preceding chapter for references). However, another seven years passed before the protein corresponding to this enzymatic activity was finally identified as the second member of a new family of eukaryotic subtilases (reviewed in Ref. 28). It is a happy coincidence that both the Hutton and Steiner (and then Seidah) groups gave this enzyme the same number, though in the former case, it stood for "Type-II proinsulin converting endopeptidase," and in the latter two cases, the number became a part of the acronym for "prohormone convertase 2," or PC2. In this chapter, we present a general overview of the literature on this enzyme, focusing on recent work and referencing reviews wherever possible.

PC2 is initially synthesized as a 75 kDa glycosylated and sulfated proenzyme, which undergoes autocatalytic intramolecular maturation to a 64-kDa enzyme within regulated secretory granules.[20] Like other convertases, proPC2 contains a propeptide, a catalytic domain, a P domain, and a carboxy-terminal tail domain (Fig. 1). The propeptide functions as an intramolecular chaperone and has weak inhibitory activity against the active enzyme. The catalytic domain contains a serine proteinase catalytic triad in the subtilase configuration; however, one highly unusual feature of PC2 as a subtilase is the presence of an aspartate, as opposed to an asparagine, in the oxyanion hole which stabilizes the transition state. The adjoining P domain, also known as the Homo B or "middle" domain, is believed to contribute to enzyme stability and to calcium and pH dependence, and is integral to function.[2] Domain swap experiments show that PC2 requires its own propeptide and P domain for proper synthesis; corresponding domains from other convertases cannot be substituted.[2] Lipkind et al. proposed an eight-stranded β-barrel structure for the P domain, based on predicted secondary structure;[19] this structure was confirmed on crystallization of the homolog furin.[9] One group has shown that the carboxy-terminal domain of PC2 is capable of redirecting heterologous proteins to secretory granules; however, the role of the C-terminus of PC2 for sorting of the enzyme remains controversial.[30] Others have reported that the last 25 amino acids of PC2 form an amphipathic α-helix capable of interacting with raft-resident lipids.[1]

In humans, PC2 is encoded by the gene *PCSK2*, located on chromosome 20 p11.2 and consisting of 12 exons. In

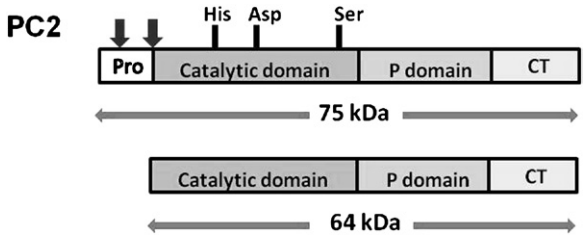

FIGURE 1 Schematic representation of PC2 domains: proPC2: *Pro*, propeptide domain; *catalytic domain*, containing the catalytic triad His, Asp and Ser; *P domain*; *CT*, carboxyl-terminal domain. The propeptide cleavage sites (primary and secondary) are shown with arrows.

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00246-3
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mice, the gene is located on chromosome 2, which also harbors the 7B2 gene.

PC2 FUNCTION IN PEPTIDE BIOSYNTHESIS AND PROCESSING

Where PC1/3 is present, PC2 performs the later steps of peptide precursor processing on PC1/3-generated intermediates, as shown in the previous chapter (Fig. 1, Chapter 1).[2] However, many tissues express little or no PC1/3 and only PC2 (e.g. much of the brain), indicating that previous cleavage by PC1/3 is not a prerequisite for PC2 cleavage. This was confirmed on blockade of initial processing sites within proenkephalin which results in the efficient use of nearby sites.[2] PC2 cleavage has been implicated in the synthesis of at least 115 different peptides from 28 different precursors.[35] Although many of these precursors have been described in an earlier review,[2] the peptide precursors that have been added to this list in the last ten years (resulting in part from peptidomic analysis of the PC2 knockout mouse) include procholecystokinin, protachykinins A and B, secretogranin II, promelanin-concentrating hormone, pronociceptin/orphanin, prothyrotropin-releasing hormone, and VGF.[35] A critical role for the PC2 homolog *amontillado* (*amo*) in cleavage of signaling proteins in *Drosophila* was shown by gene knockout, which resulted in embryonic lethality, defective larval growth, and difficulty in hatching[24] (hence the name *amontillado*, from Poe's short story about an entombment, "The Cask of Amontillado"). Interestingly, worms seem to be less affected by mutation of their PC2 homolog *egl-3*; they do not die, but do exhibit pronounced egg laying and mechanosensory defects, probably caused by the failure to synthesize specific neuropeptides.[12]

PROPERTIES OF PC2

General Enzymatic Properties and Cell Biology

PC2 is a calcium-dependent serine proteinase with a pH optimum of 5.0s.[2] Enzymatic activity requires a low pH; lack of acidification, for example, by blockade of the vesicular proton pump[11] results in impaired substrate cleavage. PC2 is secreted from constitutively-secreting cells as a zymogen, proPC2; unless its obligate binding protein 7B2 (not a contraction; its name is derived from a fraction designation) is coexpressed, proPC2 is incapable of productive maturation to an active species[2,17] (Fig. 2). Within cells containing a regulated secretory pathway, proPC2 is exported to the Golgi after binding with 7B2, but does not undergo propeptide removal until after it reaches the secretory granule compartment. Activation is autocatalytic, intramolecular, requires a low pH, and is not calcium dependent;[2] this late, acidic compartment propeptide removal distinguishes

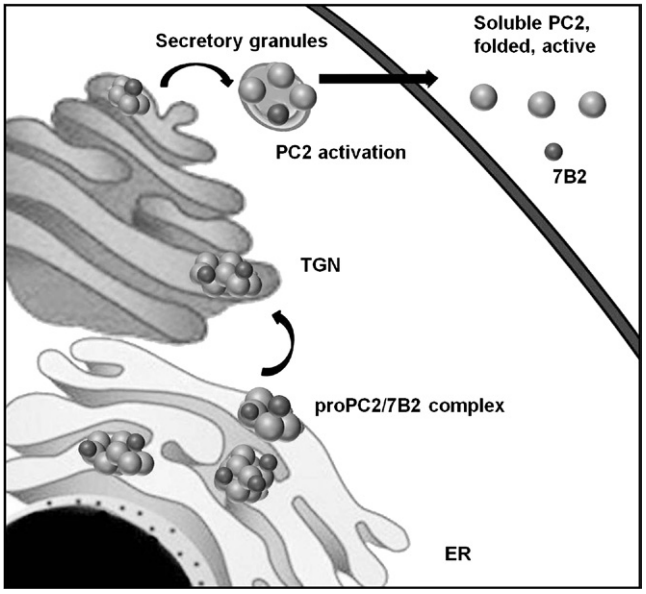

FIGURE 2 Maturation of proPC2 within the regulated secretory pathway. Following cotranslational cleavage of the signal peptide, slow folding of proPC2 occurs in the endoplasmic reticulum, which contains large quantities of this protein. The 27-kDa form of 7B2 binds to proPC2 in the endoplasmic reticulum, preventing oligomerization of the zymogen into inactivatable aggregates. In the Golgi apparatus, 7B2 is cleaved to its 21-kDa form by furin, decreasing its affinity for proPC2. Both proPC2 and 7B2 are then transferred to regulated secretory granules, where the low pH facilitates autocatalytic intramolecular maturation of proPC2 to PC2 (*note: 7B2 and PC2 are not drawn to scale*).

proPC2 activation from that of other convertases. After much work ruling out other mechanisms, it now seems that 7B2 functions mainly as a postfolding chaperone that blocks unproductive aggregation of proPC2 in various secretory compartments, thus preserving its ability to undergo activation.[17]

7B2, also known as secretogranin V and Sgne1 (secretory granule neuroendocrine protein 1), consists of two functional domains: an inhibitory C-terminal peptide and an N-terminal domain involved in proPC2 maturation; these are separated by a furin consensus cleavage site, as shown in Fig. 3.

In addition to the PC2 propeptide, two naturally occurring PC2 inhibitors are known, the 31 residue 7B2 carboxy-terminal peptide, which represents a nanomolar inhibitor of the enzyme *in vitro* (but see the discussion that follows); and the cystatin-related protein CRES,[3] present in testes (which however does not contain significant amounts of PC2). The enzyme is also inhibited by agents that chelate calcium, such as EDTA and EGTA, and by heavy metals such as zinc.[2] Synthetic inhibitors are just beginning to be developed; more recent results show that bicyclic guanidines and pyrrolidine bis-piperazines[14] and certain guanidinyl derivatives of dideoxystreptamine represent micromolar inhibitors of this enzyme (Vivoli et al., 2012, in *Mol Pharmacol*).

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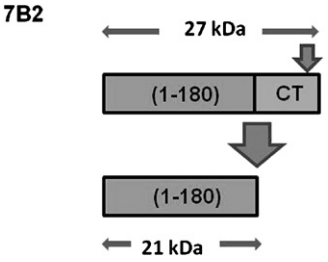

FIGURE 3 Schematic representation of 7B2: Cleavage at the furin site (indicated with a large arrow) results in the production of a 21-kDa domain, which is critical to proPC2 maturation; and a 31-residue "CT peptide," which represents a potent and highly specific inhibitor of active PC2. 7B2 inhibition is relieved by further cleavage within the CT peptide (indicated with small arrow) followed by CPE action.

Recombinant murine PC2, generated by dihydrofolate reductase-coupled overexpression in CHO cells, is about 80 times more active than PC1/3, with a specific activity of 30 μmol/h/mg protein.[2] Although this specific activity is comparable with that of the related enzyme furin, PC2 is much less active than the yeast enzyme kex2, which is itself orders of magnitude less active than trypsin. Because the residence time of peptide substrates with PC2 within secretory granules can be measured on the order of days, rapid enzyme action is not necessarily expected; however, pulse-chase studies in neuroendocrine cells and neuroendocrine cell lines indicate near-complete cleavage of peptide substrates within a matter of hours. Membrane tethering of PC2 with a portion of the peptidyl alpha-amidating enzyme results in alterations in several enzymatic properties, including increased activity,[2] possibly indicating constitutive inhibition by the carboxyl terminus similar to that observed for PC1/3 (see the preceding chapter).

Enzymatic activity for PC2 can be assayed in the laboratory in the extracts prepared from expressing tissues by blocking other, more active enzymes with a cocktail of protease inhibitors that do not affect PC2, such as pepstatin, TLCK, and E-64. Because the synthetic PC2 substrate pGlu-Arg-Thr-Lys-Arg-aminomethylcoumarin (AMC) is not specific for this (or any) convertase, PC2 activity must be given as the difference between activity found in the presence and the absence of micromolar concentrations of its specific inhibitor, the 7B2 carboxy-terminal peptide.[18]

Substrate Specificity

Catalysis by PC2 has been explored using a series of synthetic substrate analogs and by using internally quenched (IQ) peptides (reviewed in Cameron et al.[2]). Precursor processing has mostly been explored by transfection studies, though recombinant prohormones and chemically synthesized peptides have sometimes been used in *in vitro* experiments. The substitution of Ala in place of Pro at the position P1' (where the nomenclature of Schecter and Berger is used to denote positions prior to (P) and following (P') the scissile bond) in the dynorphin A 1-17 peptide enhanced cleavage by recombinant murine PC2, suggesting that Pro at this position hinders PC2 activity.[2] Moreover, the presence of charged residues such as Lys or Arg in the P2' position, again using dynorphin A 1-17 as a model substrate, hinders PC2 cleavage, whereas the presence of amino acids with aliphatic groups improved PC2 activity. Comparison of the various cleavage sites present in different prohormones and neuropeptide precursors suggests that sequences containing bulky groups such as Trp, Tyr, and/or Pro in the P1′ or P2′ positions are preferentially cleaved by PC2, and poorly if at all by other convertases.[35] The specificity of PC2 with regard to the P3 and P5 subsites has not been well defined; while a slight preference for an Arg or an acidic residue exists,[35] no clear preferences or restrictions have been shown as yet for these positions (see review in Cameron et al.[2]). Indeed, although it is clear that the substrate binding pocket of PC2 is more accepting than that of other convertases, further investigation is required to derive its precise binding specificity. Proghrelin, with a cleavage site of Leu-Asn-Pro-Arg-|Ala-Leu-Ala, is one of the few PC1/3 substrates that is completely resistant to cleavage by PC2.[22]

Regulation of Activity

The pH gradient within the secretory pathway is believed to regulate activation of the zymogen, contributing to the known late action of this enzyme within the secretory pathway, that is, in the mature secretory granule.[2] By contrast, PC1/3, with a higher pH optimum, is believed to initiate proteolytic action within the Golgi apparatus (see the previous chapter).

Like PC1/3, PC2 can be regulated by large quantities of catecholamines, such as those present within the adrenal chromaffin granule.[8] Whether this process also occurs in brain is not yet known. PC2 is also inhibited by its propeptide, though this inhibition is far weaker than the inhibition of other convertases by their propeptides (reviewed in Ref. 2). Other regulatory mechanisms for PC2 include translational[31] and transcriptional[21] controls.

Recent results strongly suggest that PC2 activity can be regulated by the small neuroendocrine protein 7B2 in at least some cases. As mentioned above, the carboxy-terminal 31 residues of this protein constitute a nanomolar inhibitor of this enzyme. When this peptide is overexpressed in neuroendocrine cells, it surprisingly does not reduce PC2-mediated cleavage;[6] therefore, the function of this carboxy-terminal peptide may instead be to prevent premature activity of PC2 within earlier portions of the secretory pathway.[6] However, 7B2 expression apparently can control PC2 activity via other means. For example, in strains of

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mice exhibiting higher concentrations of endogenous 7B2 because of a polymorphism in the 5' region, the cleavage of glucagon from its precursor is enhanced.[29] Recent results from our laboratory confirm that reduction of 7B2 levels by antisense introduction reduces the production of glucagon, but not that of α-MSH; both are known PC2 products (Helwig et al., 2011, in *J Biol Chem*). The mechanism for this differential effect of 7B2 on precursor processing is not yet clear but may have to do with differential susceptibility of the two precursors to PC2 action. It will be interesting to learn whether cleavage of other precursors is affected by 7B2 modulation.

DISTRIBUTION/LOCALIZATION OF PC2

PC2 is widely distributed within neural and endocrine systems and is rich within the pancreas, the pituitary, and the adrenal gland. PC2 is widely expressed in the brain, but is especially concentrated in the hypothalamus. In the rodent, it is most concentrated in the neurointermediate lobe of the pituitary (reviewed in Ref. 28). Small quantities are also present in human dermal fibroblasts[26] and in the immune system.[15] Within neuroendocrine cells, PC2 is most concentrated in the endoplasmic reticulum as proPC2, which contains large amounts of immature carbohydrate chains, while the mature enzyme resides mainly within secretory granules.[2] The reasons for the large stores of endoplasmic reticulum zymogen are unclear but suggest potential dynamic control of activity, possibly by 7B2.

PATHOPHYSIOLOGICAL IMPLICATIONS OF PC2 AND 7B2 VARIANTS AND NULL ANIMALS

Only a few diseases have been associated with PC2 polymorphisms to date. These include increased risk of stroke and type-2 diabetes for four SNPs (single nucleotide polymorphisms) in an African-American population.[16] 7B2 variants have also been associated with alterations in glucose levels; interestingly, these polymorphisms occur in the 5′ untranslated region, an area known to exert considerable control over expression.[5,27] In certain strains of mice, increased levels of 7B2 are associated with a lean phenotype, while the adrenalectomized 7B2 knockout becomes obese in certain backgrounds[5] (see the discussion that follows on strain specificity).

The PC2 knockout mouse exhibits a surprisingly mild phenotype in most background strains (reviewed in Ref. 28). It is hypoglycemic due to the total absence of glucagon (because proglucagon processing is highly dependent on PC2 action),[33] and this knockout also exhibits depressed nociception[4] and delayed intestinal motility.[7] When the C57Bl/6 PC2 knockout is placed on the 7B2 null 129Sv background and back-bred for five generations or more, it phenocopies the lethal 7B2 null mouse (see below) and dies between 5 and 9 weeks of age.[23] The C57Bl/6 PC2 knockout mouse is available from Jackson Laboratories.

As discussed above, proPC2 requires a chaperone protein, 7B2, for the expression of enzymatic activity. The original 7B2 null mouse, generated in the Leder laboratory on the 129S6/SvEvJ background[34] exhibits a very interesting phenotype of extremely high circulating corticosterone, which results in death by 5–8 weeks of age. This high steroid level arises as a consequence of lack of PC2 processing of ACTH in the intermediate lobe of the pituitary, causing ACTH hypersecretion followed by excessive adrenocortical stimulation. These high corticosterone levels, coupled with a total lack of glucagon production, result in massive liver steatosis and necrosis coupled with chronic severe hypoglycemia, and lead to death between 5 and 8 weeks.[25] Interestingly, not all 129Sv substrains recapitulate this lethal phenotype; when bred onto the Jackson Laboratories 129Sv substrain, the 7B2 null does not show any particular abnormalities, either as received from Jackson Laboratories (the knockout is commercially available from this supplier), or after further back-breeding onto the Taconic 129Sv background (M. Helwig and I. Lindberg, unpublished observations). Thus, the lethal phenotype must represent a combination of both the lack of 7B2 and of specific modifier genes present within the original null 129S6/SvEvJ background. The identification of these modifier genes represents an interesting area for future study, which may be highly relevant to human Cushing's disease, a disease of excessive steroid production.

Interestingly, 7B2 expression is downregulated by epigenetic control in human medulloblastoma and in various gliomas,[32] suggesting that the ability to form colonies in soft agar is associated with lowered expression of 7B2. Most neural and endocrine cell lines express 7B2; there is apparently only one neuroendocrine cell line, which totally lacks 7B2 expression, the SK-N-MC neuroblastoma cell line. Introduction of 7B2 by transfection results in the manifestation of PC2 activity (reviewed in Refs. 17, 28).

PC2 may also play a role in tumor development and progression: increased expression of PC2 in neuroendocrine tumors over surrounding normal tissue occurs in various tumors, such as small-cell lung carcinoma, pituitary adenomas, medullary thyroid carcinoma, and lung and pancreatic cancer (reviewed in Ref. 13). Further investigation is required to elucidate the importance of this enzyme in cancer biology, with a view to a possible therapeutic intervention.

New work on PC2 is focused on identifying novel substrates,[35] inhibitor discovery,[14] and identifying additional diseases resulting from PC2 insufficiency. Detailed phenotypic characterization of the PC2 null reveals problems with intestinal transport,[7] and subtle behavioral anomalies, such as impaired nociception.[4]

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SUMMARY AND CONCLUSIONS

The proprotein convertase PC2, one of the earliest convertases to evolve, plays an integral role in peptidergic signaling in organisms as diverse as flies and humans. More recent studies have elaborated the intricate interplay of PC2 and its binding protein 7B2 and suggest that there are unusual points of regulation for this enzyme in the production of active enzyme from zymogen via control of expression of its antiaggregant chaperone protein 7B2. These control mechanisms may differ from organism to organism, as the cell biology of PC2 is very different in insect cells than it is in higher organismss.[10] Unlike PC1/3 (see the previous chapter) few diseases associated with PC2 genetic variants have been identified as yet; whether this is a matter of experimental inattention, or whether PC2 represents a gene which is inherently more stable, remains to be established. A crystal structure for this enzyme, preferably in complex with 7B2, would provide much information on the molecular basis for its substrate specificity and the binding location of 7B2, as well as provide needed structural information for inhibitor design.

REFERENCES

1. Assadi M, Sharpe JC, Snell C, Loh YP. The C-terminus of prohormone convertase 2 is sufficient and necessary for raft association and sorting to the regulated secretory pathway. *Biochemistry* 2004;**43**:7798–807.
2. Cameron A, Apletalina EV, Lindberg I. The enzymology of PC1 and PC2. *The Enzymes* 2001;**XXII**:291–331.
3. Cornwall GA, Cameron A, Lindberg I, Hardy DM, Cormier N, Hsia N. The cystatin-related epididymal spermatogenic protein inhibits the serine protease prohormone convertase 2. *Endocrinology* 2003;**144**:901–8.
4. Croissandeau G, Wahnon F, Yashpal K, Seidah NG, Coderre TJ, Chretien M, et al. Increased stress-induced analgesia in mice lacking the proneuropeptide convertase PC2. *Neurosci Lett* 2006; **406**:71–5.
5. Farber CR, Chitwood J, Lee SN, Verdugo RA, Islas-Trejo A, Rincon G, et al. Overexpression of Scg5 increases enzymatic activity of PCSK2 and is inversely correlated with body weight in congenic mice. *BMC Genet* 2008;**9**:34.
6. Fortenberry Y, Liu J, Lindberg I. The role of the 7B2 CT peptide in the inhibition of prohormone convertase 2 in endocrine cell lines. *J Neurochem* 1999;**73**:994–1003.
7. Gagnon J, Mayne J, Chen A, Raymond A, Woulfe J, Mbikay M, et al. PCSK2-null mice exhibit delayed intestinal motility, reduced refeeding response and altered plasma levels of several regulatory peptides. *Life Sci* 2011;**88**:212–7.
8. Helwig M, Vivoli M, Fricker LD, Lindberg I. Regulation of neuropeptide processing enzymes by catecholamines in endocrine cells. *Mol Pharmacol* 2011;**80**:304–13.
9. Henrich S, Cameron A, Bourenkov GP, Kiefersauer R, Huber R, Lindberg I, et al. The crystal structure of the proprotein processing proteinase furin explains its stringent specificity. *Nat Struct Biol* 2003;**10**:520–6.
10. Hwang JR, Siekhaus DE, Fuller RS, Taghert PH, Lindberg I. Interaction of *Drosophila melanogaster* prohormone convertase 2 and 7B2. Insect cell-specific processing and secretion. *J Biol Chem* 2000;**275**:17886–93.
11. Jansen EJ, Hafmans TG, Martens GJ. V-ATPase-mediated granular acidification is regulated by the V-ATPase accessory subunit Ac45 in POMC-producing cells. *Mol Biol Cell* 2010;**21**:3330–9.
12. Kass J, Jacob TC, Kim P, Kaplan JM. The EGL-3 proprotein convertase regulates mechanosensory responses of *Caenorhabditis elegans*. *J Neurosci* 2001;**21**:9265–72.
13. Khatib AM, Siegfried G, Chretien M, Metrakos P, Seidah NG. Proprotein convertases in tumor progression and malignancy: novel targets in cancer therapy. *Am J Pathol* 2002;**160**:1921–35.
14. Kowalska D, Liu J, Appel JR, Ozawa A, Nefzi A, Mackin RB, et al. Synthetic small-molecule prohormone convertase 2 inhibitors. *Mol Pharmacol* 2009;**75**:617–25.
15. Lansac G, Dong W, Dubois CM, Benlarbi N, Afonso C, Fournier I, et al. Lipopolysaccharide mediated regulation of neuroendocrine associated proprotein convertases and neuropeptide precursor processing in the rat spleen. *J Neuroimmunol* 2006;**171**:57–71.
16. Leak TS, Keene KL, Langefeld CD, Gallagher CJ, Mychaleckyj JC, Freedman BI, et al. Association of the proprotein convertase subtilisin/kexin-type 2 (PCSK2) gene with type 2 diabetes in an African American population. *Mol Genet Metab* 2007;**92**:145–50.
17. Lee SN, Lindberg I. 7B2 prevents unfolding and aggregation of prohormone convertase 2. *Endocrinology* 2008;**149**:4116–27.
18. Li QL, Naqvi S, Shen X, Liu YJ, Lindberg I, Friedman TC. Prohormone convertase 2 enzymatic activity and its regulation in neuroendocrine cells and tissues. *Regul Pept* 2003;**110**:197–205.
19. Lipkind GM, Zhou A, Steiner DF. A model for the structure of the P domain in the subtilisin-like prohormone convertases. *Proc Natl Acad Sci USA* 1998;**95**:7310–5.
20. Muller L, Lindberg I. The cell biology of the prohormone convertases PC1 and PC2 In: Moldave K, editor. *Prog Nucleic Acid Res* 1999; **63**:69–108.
21. Oyarce AM, Hand TA, Mains RE, Eipper BA. Dopaminergic regulation of secretory granule-associated proteins in rat intermediate pituitary. *J Neurochem* 1996;**67**:229–41.
22. Ozawa A, Cai Y, Lindberg I. Production of bioactive peptides in an in vitro system. *Anal Biochem* 2007;**366**:182–9.
23. Peinado JR, Laurent V, Lee SN, Peng BW, Pintar JE, Steiner DF, et al. Strain-dependent influences on the hypothalamo-pituitary-adrenal axis profoundly affect the 7B2 and PC2 null phenotypes. *Endocrinology* 2005;**146**:3438–44.
24. Rayburn LY, Gooding HC, Choksi SP, Maloney D, Kidd 3rd AR, Siekhaus DE, et al. Amontillado, the drosophila homolog of the prohormone processing protease PC2, is required during embryogenesis and early larval development. *Genetics* 2003;**163**:227–37.
25. Sarac MS, Zieske AW, Lindberg I. The lethal form of Cushing's in 7B2 null mice is caused by multiple metabolic and hormonal abnormalities. *Endocrinology* 2002;**143**:2324–32.
26. Schiller M, Raghunath M, Kubitscheck U, Scholzen TE, Fisbeck T, Metze D, et al. Human dermal fibroblasts express prohormone convertases 1 and 2 and produce proopiomelanocortin-derived peptides. *J Invest Dermatol* 2001;**117**:227–35.
27. Schmidt G, Sirois F, Anini Y, Kauri LM, Gyamera-Acheampong C, Fleck E, et al. Differences of pancreatic expression of 7B2 between C57BL/6J and C3H/HeJ mice and genetic polymorphisms at its locus (Sgne1). *Diabetes* 2006;**55**:452–9.

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28. Seidah NG. The proprotein convertases, 20 years later. *Methods Mol Biol* 2011;**768**:23–57.
29. Tadros H, Schmidt G, Sirois F, Mbikay M. Regulation of 7B2 mRNA translation: dissecting the role of its 5′-untranslated region. *Methods Mol Biol* 2011;**768**:217–30.
30. Taylor NA, Jan G, Scougall KT, Docherty K, Shennan KI. Sorting of PC2 to the regulated secretory pathway in AtT20 cells. *J Mol Endocrinol* 1998;**21**:209–16.
31. Tschuppin G, Rhodes C. Specific co-ordinated regulation of PC3 and PC2 gene expression with that of preproinsulin in insulin-producing beta TC3 cells. *Biochem J* 1996;**313**:259–68.
32. Waha A, Felsberg J, Hartmann W, Hammes J, Knesebeck AV, Endl E, et al. Frequent epigenetic inactivation of the chaperone SGNE1/7B2 in human gliomas. *Int J Cancer* 2011.
33. Webb GC, Akbar MS, Zhao C, Swift HH, Steiner DF. Glucagon replacement via micro-osmotic pump corrects hypoglycemia and alpha-cell hyperplasia in prohormone convertase 2 knockout mice. *Diabetes* 2002;**51**:398–405.
34. Westphal CH, Muller L, Zhou A, Zhu X, Bonner-Weir S, Schambelan M, et al. The neuroendocrine protein 7B2 is required for peptide hormone processing in vivo and provides a novel mechanism for pituitary Cushing's disease. *Cell* 1999;**96**:689–700.
35. Zhang X, Pan H, Peng B, Steiner DF, Pintar JE, Fricker LD. Neuropeptidomic analysis establishes a major role for prohormone convertase-2 in neuropeptide biosynthesis. *J Neurochem* 2010;**112**:1168–79.

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Chapter 247

Proprotein Convertase Furin and Proprotein Convertase PC5/6

Nabil G. Seidah

ABSTRACT

The secretory proprotein convertases furin and proprotein convertase 5/6 (PC5/6), while often exhibiting similar preference for polypeptide precursor cleavage at pairs or single basic amino acids motifs *in vitro*, also have unique functions *in vivo*, especially during embryonic development. Thus, although furin knockout mice exhibit a heart looping defect and die at embryonic day 10.5, mice lacking PC5/6 expression show multiple body axis defects and die at birth. Thus, although both proteinases are essential, they do so by the efficient cleavage of distinct precursors. Furin has a ubiquitous tissue expression and is implicated in the processing of a very wide panel of substrates throughout life. By contrast, PC5/6 is somewhat more restricted in its expression, and so far, its only specific substrate is growth-differentiating factor 11 (GDF11). Tissue-specific knockout of each convertase unraveled both redundant and specific functions emphasizing that unique *in vivo* substrates are yet to be identified for both enzymes.

DISCOVERY AND NOMENCLATURE

Activation via processing of a wide variety of in active secretory precursors by cleavage at specific single or pairs of basic amino acids (aa) was proposed since the mid-1960s, based on work on the model precursors of Insulin and Melanotropin-Stimulating Hormone (α- and β-MSH) (for reviews see Refs. 23, 28). Virologists also noted that to acquire fusiogenic activity, many infectious retroviruses required host cell proteases to process their cell surface glycoproteins at single or paired basic residues.[11] However, the identification of the cognate proprotein convertases (PCs) had to await the mid 1980s and really started in 1990. The major breakthrough was the elucidation of the primary structure of the yeast subtilisin-like serine protease Kex2p. The latter was genetically shown to be implicated in the processing of pro-α-mating factor and opened the way to the identification of mammalian homologs in 1990 (for reviews see Refs. 23, 28). The mammalian furin,[34] and the two regulated convertases PC1/3[24] (originally called PC1 or PC3) and PC2[24,27] were all discovered in 1989–1990. This initial wave of discoveries was soon followed by the identification of PC4, PC5/6 (originally called PC5 or PC6), PACE4 and PC7.[23,28] The human genes encoding these basic aa-specific Proprotein Convertases Subtilisin/Kexin-like were given the names of *PCSK1* to *PCSK7*. The furin-encoding *PCSK3* gene is often called *FUR* for FES/FPS Upstream Region. In this chapter, emphasis will only be on furin and PC5/6, its functionally closest PC-family member, both of which cleave precursors within constitutive secretory pathways at the RX(K/R)R↓ consensus motif. The *PCSK3 (FUR) and PCSK5* genes map to human and mouse chromosomes 15, 9 and 7, 19, respectively.

FUNCTIONS IN PEPTIDE BIOSYNTHESIS/ PROCESSING

Furin

Zymogen Activation and Cellular Trafficking

Like most secretory precursor proteins that undergo multiple posttranslational processing events before attaining their final bioactive form, the processing enzyme furin (Peptidase S8 family) is also subject to various modifications before becoming fully competent to cleave precursor proteins. The first event occurs during the translation of its mRNA in the endoplasmic reticulum (ER), whereby the type-1 transmembrane containing mouse preprofurin precursor (aa 1–793) loses its signal peptide (aa 1–24) by the action of the signal peptidase that is N-glycosylated at three sites $Asn_{387,440,553}$. Within the ER, the resulting profurin (aa 25–793) folds under the guidance of its prosegment (aa 25–107) and autocatalytically cleaves itself at site 1 (S1) within the recognition sequence $\underline{K}R\underline{R}A\underline{KR}_{107}$↓ DV, to generate a heterodimer of the inhibitory prosegment (pro; aa 25–107) and mature furin (aa 108–793) (Fig. 1). This pro≡furin complex is enzymatically inactive. However, cleavage at S1 allows the exit of pro≡furin from the ER toward the Golgi apparatus, whereby furin undergoes trimming and maturation of its *N*-glycosyl moieties and phosphorylation of its cytosolic tail at $Ser_{772,774}$

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00247-5
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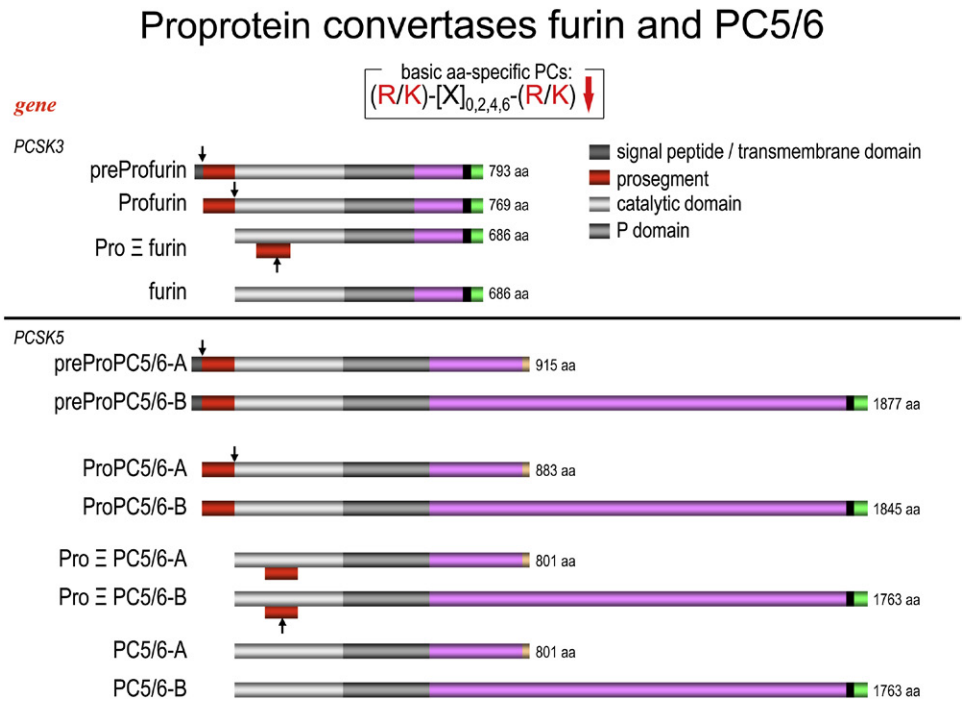

FIGURE 1 Schematic representation of the processing of preProfurin and preProPC5/6. The number of aas in each form is emphasized, as well as the gene symbol, and the different domains. The arrows point to sites of either signal peptidase cleavage or autocatalytic cleavages. See color plate 64.

by the protein kinase CK2. On reaching the *trans* Golgi network (TGN), the mildly acidic pH and Ca^{2+} conditions are now favorable for a second autocatalytic processing of pro≡furin at an internal S2 site $_{69}$**HR**GVT**KR**$_{75}$↓, thereby eliminating the inhibitory prosegment and unmasking active furin, which is then capable of cleaving substrates *in trans*. Interestingly, His_{69} of the prosegment acts as a pH sensor allowing cleavage at S2 only under acidic conditions.[7] In agreement with the requirement of calcium for furin activity, the crystal structure of furin revealed that it binds two Ca^{2+} ions.[8] Furin is mostly concentrated in the TGN, a subcellular localization requiring phosphorylation of its cytosolic tail. However, furin can cycle from the TGN to the cell surface and back through the endosomal pathway. This traffic is regulated by the state of phosphorylation of its cytosolic tail and its interaction with the cytosolic phosphofurin acidic cluster sorting protein 1 (PACS-1) which mediates TGN localization and connection to clathrin adapters.[33] *In vivo*, furin exists as di, mono and nonphosphorylated forms, the latter being the preferred form at the cell surface.

In vivo Mouse Knockout Models

Furin is expressed at embryonic day e7.5 in the extraembryonic endoderm and mesoderm and at the late somite stages; it is also expressed in the cardiovascular system. Inactivation of the *fur* locus by homologous recombination in mouse causes embryonic death shortly after e10.5, due to hemodynamic insufficiency and cardiac ventral closure defects.[20] Although the specific physiological substrates of furin in the embryonic cardiovascular system have not yet been identified, the striking overlap in the distribution of furin mRNA coincides with that of several molecules reported to play critical cardiac-specific roles during this developmental stage. Among these molecules, members of the transforming growth factor (TGF) β family, such as TGFβ1,[3] and bone morphogenetic protein BMP10 (BMP10)[31] are efficiently processed by furin. This suggests that lack of processing of TGFβ-like precursors involved in the early cardiac development could be responsible for the defects observed in furin-null embryos.

In addition to these cardiovascular defects, the chorion of the furin-null embryos fails to fuse with the allantois that becomes highly vacuolated around e9.5. Similar absence of chorioallantoic fusion was previously observed in embryos lacking both BMP5 and BMP7, as well as vascular cell adhesion molecule (VCAM-1) and α4-integrin, two cell surface adhesion molecules that bind each other and are required for cell–cell adhesion. The most damaging deficiency in furin-null mutant embryos is their failure to undertake ventral closure to form a looping heart tube and a coherent primitive gut. So far, the exact mechanism behind these phenotypes and the cognate *in vivo* furin substrates, have yet to be identified unambiguously. However, the lack of suitable and sensitive antibodies to detect the

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endogenous levels and forms of various substrates *in vivo* is hampering such an effort, and may be the use of quantitative mass spectrometry may help in this elucidation in the future.

An approach to reduce the complexity of the analysis *in vivo* furin functions is the use of tissue-specific knockout (KO) mice in which furin is inactivated only in a subset of tissues and cells. Such a strategy has indeed been very promising. A furin KO in liver and other tissues from adult mice, using the inducible Mx1-cre transgene, results in mice with no unusual phenotype, showing redundancy with other PCs, because typical furin substrates are still cleaved, albeit to a lesser extent.[19] By contrast, *in vivo* studies showed that in a spatiotemporal manner furin can uniquely process the Ac45 subunit of the vacuolar type H^+-ATPase in pancreatic β-cells.[13] Further, conditional deletion of furin in T cells allows for normal T-cell development but impairs the function of regulatory and effector T cells, which produce less TGFβ1.[18] Results obtained by furin deletion in salivary glands also suggest an important role for furin in enhancing the proto-oncogene PLAG1-induced salivary gland tumorigenesis in mice.[2] By using a hepatocyte-specific deletion of furin expression in liver, we showed that furin is the major PC implicated in the *in vivo* inactivation of the proprotein convertase PCSK9 by cleavage at the **R**FH**R**$_{218}$↓QA sequence.[5] In fact, primary hepatocytes derived from hepatocyte-specific furin KO mice[5] allowed us to unambiguously show that furin is the major processing enzyme of BMP10.[31] Finally, screening databases for potential furin-like substrates that exhibit a motif RxRR↓N similar to that of BMP10, reveal two new candidates, namely, the type II transmembrane growth factor ectodysplasin-A (*EDA*; **R**V**RR**$_{156}$↓**N**K), and the extracellular matrix collagen type V-alpha 1 (*Col5a1*; **R**T**RR**$_{1585}$↓**N**I), a minor component of collagen I fibrils. In e10.5 mice, *Col5a1* expression is found in the heart and dorsal aorta wall, and the *Col5a1* KO mice reveal that this protein is important in the development of functional connective tissues.

In vitro Properties of Soluble Furin and Information from Its Crystal Structure

A large body of data has been accumulated over the last 20 years on the *in vitro* enzymatic properties of soluble furin, a form lacking the transmembrane-cytosolic tail of the protease. The pH optimum of furin is approximately 7.0, but the enzyme is active at lower and higher pHs ranging from pH 6.0 to 8.5. Furin requires approximately 1 mM Ca^{2+} for optimal activity, the latter being completely inhibited by calcium chelators such as EDTA or EGTA. Heavy metal ions, such as Zn^{2+}, Hg^{2+}, and Cu^{2+} also inhibit furin activity, as well as the reducing agent DTT. Potent peptide-based furin inhibitors include those that contain the Arg-X-Lys/Arg-Arg-chloroalkanes, polyarginines, and the furin-prosegment. Several engineered inhibitors have been proposed such as the RXXR mutant of α1-antitrypsin (α1-PDX), and α1-proteinase inhibitor. However, so far nobody has found an endogenous secretory inhibitor specific for furin, except for the recently identified latent-TGFβ binding proteins (LTBPs), which albeit can also inhibit PC5/6 and PACE4.[30]

The consensus optimal recognition site for furin is Arg-X-Lys/Arg-Arg↓, containing a basic residue at P1, P2, and P4 positions. However, alternative motifs in which a P6 Arg can compensate for the absence of a basic residue at P4 or P2 have also been reported (**R**XX**R**↓ and **R**XXXX**R**↓). The residues at positions P1′, P2′, P3, and P5 and secondary structures such as β-turns also influence the efficacy of cleavage, in fact the **R**R**RR**↓EL sequence has been used to introduce optimal furin-like sites in specific regions of precursor proteins,[1] also exploiting the preference of furin for an acidic residue at P1′ and an aliphatic one at P2′.

The crystal structure of the soluble form of furin reveals a wide negatively charged catalytic pocket that is well suited to process secretory precursors at multibasic sites.[8] Homology modeling suggested a great similarity of this catalytic structure to that of the other basic aa-specific PCs that share >50% sequence identity.[9] The primary sequence and crystal structure of furin confirms it to be a type-I transmembrane subtilisin-like protease consisting of a signal peptide, a prosegment terminating at the autocatalytic Arg_{107} site (aa 25–107), a subtilisin-like catalytic domain containing the active sites Asp_{153}, His_{194}, and Ser_{368} and the oxyanion hole Asn_{295} (aa 108–445), a middle domain known as the P domain (aa 446–578), and a cysteine-rich domain (CRD; aa 556–705), followed by a C-terminal transmembrane anchor (aa 719–735) and cytosolic tail (aa 736–793) (Fig. 1). Furin is both *N*-glycosylated at $Asn_{387,440,553}$, and phosphorylated at $Ser_{772,774}$, resulting in an apparent mass on SDS-PAGE of approximately 100 kDa. The 2.6-Å crystal structure of soluble furin (lacking the CRD) and in complex with the inhibitor decanoyl-RVKR-chloromethane, revealed an 8-stranded jelly-roll P domain associated with the catalytic domain and compensating for the high negative field of the latter, thereby stabilizing the catalytic pocket.[8] Surface loops and highly negatively charged complementary substrate pockets are thought to explain the stringent requirement of furin for Arg at P1 and P4 and Lys at P2. However, the role of the CRD and its possible interaction with luminal or extracellular factor(s) that may regulate the function of furin are yet to be defined.

Furin Substrates: Activation or Inactivation?

Furin processes a very wide variety of precursors (Fig. 2) either in the TGN, cell surface or endosomes.[33] Although furin is mostly implicated in the processing of precursors secreted by constitutive pathway, such as growth factors and their receptors, adhesion molecules, blood coagulation

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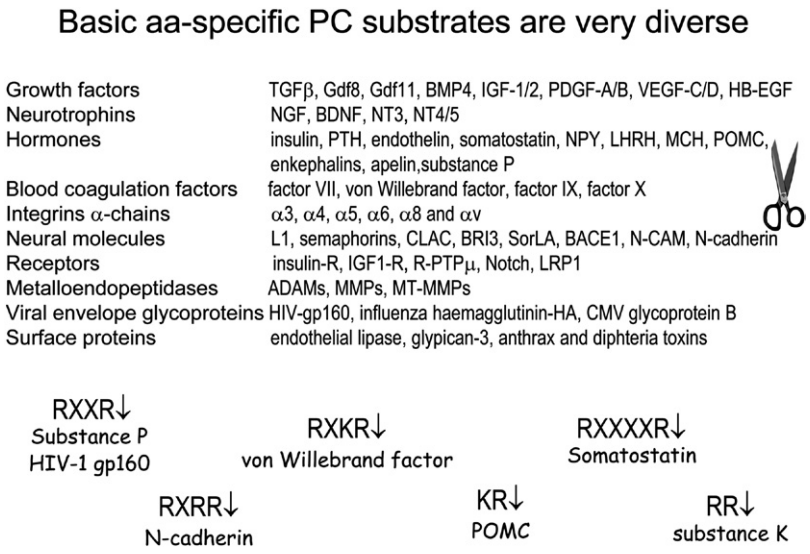

FIGURE 2 The types of precursor substrates of the basic aa convertases are shown, together with their functional annotations. The motifs recognized by these enzymes are emphasized.

factors, enzymes, and viral glycoproteins, it is also a major player in the processing of the regulated propeptide processing enzyme PC2, by cleavage of its chaperone 7B2 in the TGN.[17] The latter is needed for the productive zymogen activation of proPC2 into active PC2 in immature secretory granules. Further, furin not only has a pronounced effect on the regulated secretory pathway implicating dense-core granules, but also affects their acidification as it is critical in the *in vivo* activation of Ac45, an accessory subunit of the proton pump V-ATPase in pancreatic β-cells.[13]

In most situations, it was believed that furin would cleave precursor proteins at one or more sites resulting in active products. However, furin can also cleave certain precursors at strategic sites resulting in their inactivation. Although there are a number of such situations, we only present some of them. (i) Furin inactivates endothelial and lipoprotein lipases by cleavage at **R**N**KR**$_{330}$↓NS and **R**A**KR**$_{334}$↓SS, respectively. This results in an increase in circulating HDL.[10] (ii) The second example is the processing of PCSK9 by furin *in vivo* at the **R**FH**R**$_{218}$↓QA sequence, resulting in the loss of function of PCSK9 on the LDLR and increased levels of circulating LDL.[5] This is relevant physiologically, because a subset of patients suffering from hypercholesterolemia had an R218S mutation at the furin cleavage site, thus preventing its action and resulting in higher levels of active PCSK9 circulating. (iii) The liver peptide hepcidin is activated by low levels of furin by cleavage at a very favorable mutibasic R**RR**R**R**59↓DT site,[21] and inhibits iron absorption and recycling. Membrane HJV (m-HJV) acting as a coreceptor for BMPs has a key role in hepcidin regulation, and its inactivation causes severe iron overload. The shed form of m-HJV, known as soluble HJV (s-HJV) may down regulate hepcidin in a competitive way by interfering with BMP signaling. Furin is the convertase that generates s-HJV through the cleavage of m-HJV at a relatively less favorable site than hepcidin, that is, at **R**N**RR**335↓GA, resulting in the inactivation of HJV. Indeed, *in vivo* furin is upregulated by iron deficiency resulting in an increased level of s-HJV. Thus, while low levels of furin activate hepcidin to decrease iron uptake, on iron deficiency and hypoxia, the increased concentration of furin inactivates HJV to act as a counterbalance to increase iron absorption. (iv) Although furin activates a number of cell surface matrix metalloproteases, within the TGN it can also cleave the prodomain of proMMP-2 at **R**KP**R**72↓GA, leading to an inactive form of MMP-2. (v) Semaphorin-3B (sema3B) and semaphorin-3F (sema3F) are secreted tumor suppressors of lung cancer. Sema3F functions as an antiangiogenic factor that repels endothelial cells and compromises their proliferation/survival. However, sema3B is inactivated by furin-like PCs by cleavage at **R**F**RR**569↓QD, suggesting that upregulation of furin-like convertases in malignant cells may enable tumors to evade the antiangiogenic effects of sema3B.

Pathophysiological Implications

So far, no diseases have been found to implicate mutations in furin, possibly because of the lethality phenotype observed in KO mice. However, heterozygote mice are viable and show no overt phenotype(s), suggesting that only a small amount of furin is sufficient to perform its multiple functions. However, like PC1/3,[12] it is possible that the efficiency of cleavage of substrates that are weakly recognized by furin would be affected, especially in heterozygote mutants or in human mutants lacking one active furin allele. One example is the one caused by the natural SNP

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causing a frame shift mutation due to the addition of a `C` nucleotide to the ACC codon coding for Thr_{381} and replacing it by CACC for His_{381} and hence replacing conserved wildtype sequence IIALL**T**LEANK by IIALL**H**LPGGQ (rs35998150). This is expected to result in a complete loss of function of furin. The other 29 SNPs reported for human furin that result in point mutations have yet to be carefully analyzed (http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?geneId=5045).

Notwithstanding the effect of mutations in the furin coding sequence, furin is implicated in the processing of various growth factors and their receptors and furin upregulation may result in tumor growth and/or metastasis (for review see Ref. 22). Further, furin is implicated in the activation of cell surface viral glycoproteins resulting in enhanced viral infection and spread.[33] Therefore, a furin inhibitor may control these pathologies. The use of either an orally active nonpeptidic small molecule inhibitor or injectable biologics such as an antisense oligonucleotide, an antisense shRNA, or inhibitory monoclonal or single domain antibodies are but some of the available options.

Cancer patients are being recruited for treatment with an autologous vaccine that expresses rhGMCSF and bi-shRNAfurin (http://clinicaltrials.gov/ct2/show/NCT01061840). The GMCSF protein is a potent stimulator of the immune system, recruiting immune effectors to the site of intradermal injection and promoting antigen presentation. The furin bifunctional shRNA blocks furin production, resulting in a reduced processing of the proTGFβ1 and proTGFβ2 and by a feedback mechanism also decreases the levels of their mRNAs. The decrease in TGFβ1 and TGFβ2 proteins reduces their induced local immunosuppression and promotes tumor surface antigen and MHC protein display. We will have to await the results of such type of studies before drawing any conclusions.

However, the inhibition of furin may also cause problems, and could in some cases exacerbate the pathological condition. Thus, a targeted inhibitor to specific tissues may also be valuable. Finally, whether injection of active furin itself may be of value in some pathology is yet to be determined.

PC5/6

Zymogen Activation and Cellular Trafficking

After the discovery of the mammalian kexin/subtilisin-like convertases furin, PC1/3, PC2, and PC4, a new effort was made to detect other convertases in various tissues and cells. Using the PCR methodology as applied to mRNA isolated from rat and mouse testis, we were successful in isolating a novel convertase, which we termed proprotein convertase 5 (PC5).[14] At about the same time, another group led by K. Nakayama in Japan, also succeeded in isolating a similar cDNA from mouse brain and called it PC6. It turned out that PC5 and PC6 were identical, and the term PC5/6 is now mostly used. Alternative splicing of the mRNA from the single gene gives rise to two major forms of PC5/6, PC5/6-A and PC5/6-B, the former encodes the soluble protein of 915 aa that we isolated, and the latter a type-I membrane bound enzyme of 1877 aa.[25] This isoform, is generated from a single *PCSK5* gene presumably by alternative splicing of the PC5/6 heteronuclear RNA transcript.[15] Quantitative analysis of the tissue distribution of PC5/6 mRNA by qPCR revealed that the small intestine and kidney are the richest source of PC5/6-B, whereas all other tissues express mostly PC5/6-A, with the adrenal cortex especially rich in PC5/6-A transcripts.[4] Both transcripts share the first 20 exons encoding the signal peptide, prosegment, catalytic domain, P domain and the CRD. The 21st exon of PC5/6-A, coding for its last 38 residues, is replaced by 18 additional exons encoding the last 1000 residues for PC5/6-B.[16,26] Thus, the CRD of PC5/6-A contains 44 cysteine residues arranged in five tandem repeats of the consensus motif Cys-X_{2-3}-Cys-X_{3-4}-Cys-X_{2-7}-Cys-X_{5-10}-Cys-X_2-Cys-X_{9-13}-Cys-X_{3-5}-Cys-X_{7-16} (as it is also found in its closest homolog PACE4). The extended CRD of PC5/6-B contains 22 repeats of this cysteine-rich motif. The CRD binds both tissue inhibitors of metalloproteases (TIMPs) and heparin sulfate proteoglycans (HSPGs).[25]

PCs must go through an ordered process of autoactivation before they can cleave other protein substrates. After signal peptidase cleavage, human proPC5/6-A (aa 33–915) is *N*-glycosylated at 6 sites $Asn_{225,381,665,752,802,852}$ and proPC5/6-B (aa 33–1877) at 12 sites $Asn_{225,381,665,752,802,852,1014,1191,1290,1497,1985,1707}$. Both zymogens are then first cleaved at the S1 site **KK**R**T**K**R**$_{114}$↓DY in the ER, allowing them to exit this compartment as heterodimeric enzymatically inactive complexes pro≡PC5/6 and reach the TGN. Although furin is activated following a second autocatalytic cleavage of its prosegment leading to its dissociation from the catalytic subunit in the TGN,[7] the exact cellular location of PC5/6 activation, which also requires cleavage at an internal S2 site **R**TI**KR**$_{82}$↓SV of its prosegment is not so clear, as it may occur in both the TGN and at the cell surface. The complex pro≡PC5/6-A was detected bound to (HSPGs) at the cell surface.[25] These results indicated a novel pathway for PC5/6-A zymogen activation. Indeed, the loss of the prosegment coincides with the appearance of the PC5/6-A activity on various cell surface substrates (PCSK9, lefty, endothelial lipase and ADAMTS-4) was triggered after a 3-h cAMP treatment of cells by an as-yet undefined molecular mechanism. This mode of PC5/6-A activation is unique among the PCs and provides an additional level of cleavage regulation for cell surface substrates.

From the limited results available on the specificity of PC5/6 and its physiological substrates, it is expected that this convertase will have a major impact on cellular

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proliferation and on sexual differentiation. The richness of PC5/6-B within the digestive system[4] also implies involvement in food metabolism. Interestingly, PC5/6-A and PC5/6-B are not expected to act on similar precursors, because this C-terminal variation causes the two isoforms to either remain within the constitutive secretory pathway (PC5/6-B) or could partially enter dense-core secretory vesicles (PC5/6-A) to process precursors destined to the regulated secretory pathway. Indeed, deletion of the unique C-terminal 38 amino acid segment in PC5/6-A leads to its secretion from the constitutive secretory pathway, suggesting that it contains a powerful signal for sorting toward the granules. The role of the CRD domain in PC5/6 is not yet understood, but it could well be implicated in protein–protein interactions either at the cell surface, intracellularly or in the extracellular matrix.

As for furin and PC7, PC5/6-B also has a transmembrane domain and a cytosolic tail, and cycles from the cell surface back to the TGN through endosomes.[35] This regulated transit into multiple compartments is controlled by several sorting signals in their cytosolic tails and their interactions with specific sorting adapters.

In vivo Mouse KO Models

PC5/6 exhibits a distinct distribution within the brain and it is expressed early during development.[4] The complete KO of PC5/6 causes embryonic death at birth, with mice exhibiting multiple morphogenic defects probably related to impaired homeotic transformations.[6,32] Thus, newborns exhibit major defects in the anteroposterior axis with extrathoraxic and lumbar vertebrates (18 and 8 instead of 13 and 6, respectively), and a lack of tail.[6] This phenotype had been reported for mice lacking the TGFβ-like factor, growth differentiation factor 11, GDF11, also known as BMP11. Both GDF11- and PC5/6-deficient mice lack kidneys, although the phenotype was more penetrant in PC5/6 KO mice (100% agenesis versus 86% lacking one or two kidneys). We showed that GDF11 is a favorite substrate of PC5/6, in part due to an Asn residue at P1′, the first position after the cleavage site **RSRR**$_{296}$↓NL.

Although PC5/6-deficiency perfectly mimics the GDF11 one, it also results in other phenotypes, suggesting the lack of processing of other substrates: *Pcsk5*$^{-/-}$ newborns die earlier, in the first 2 h versus first 2 days after birth for *Gdf11*$^{-/-}$ mice, probably by asphyxiation (lung alveoli were collapsed). They also exhibit smaller size than WT, retarded ossification, severe hindlimb hypoplasia, abdominal herniation, and superficial and tissular hemorrhages, suggesting vascular fragility.[6] Magnetic resonance imaging revealed severe phenotypes reminiscent of those observed in patients exhibiting VACTERL (vertebral, anorectal, cardiac, tracheoesophageal, renal, limb) malformations.[32] Finally, exon sequencing of control and VACTERL patients link mutations in the human PC5 gene (*PCSK5*) to this syndrome.[32] Thus, PC5/6, at least in part via GDF11, coordinately regulates caudal Hox paralogs, to control anteroposterior patterning, nephrogenesis, skeletal, and anorectal development.

The above studies suggest a role for PC5/6 in cardiovascular disease. Because lethality at birth of mice lacking PC5/6 precluded elucidation of its function in the adult, we generated mice in which the gene of PC5/6 (*Pcsk5*) is specifically inactivated in endothelial cells (ecKO), which are viable and do not exhibit overt abnormalities. To uncover the function of PC5/6 in the cardiovascular system, the effect of ecKO was studied in aging mice. In 16- to 18-month-old ecKO mice, the left ventricle (LV) mass, media cross-sectional area of aorta and coronary arteries, and media-to-lumen ratio of mesenteric arteries are decreased. The LV present decreased diastolic function, and mesenteric arteries show decreased stiffness. Collagen is decreased in the LV myocardial interstitium and perivascularly in coronary arteries and aorta. Cardiovascular hypotrophy probably develops with aging, because no significant changes are observed in 2-month-old ecKO mice. Fibroblasts, as a source of collagen in myocardium and vasculature, may play a role in the decrease in collagen deposition. Fibroblasts co-cultured with ecKO endothelial cells show decreased collagen production, decreased insulin-like growth factor (IGF)-1/Akt/mTOR signaling, and enhanced autophagic activation. PC5/6 inactivation in endothelial cells results in cardiovascular hypotrophy associated with decreased collagen deposition, decreased LV diastolic function, and vascular stiffness, suggesting a trophic role of endothelial PC5/6 in the cardiovascular system, probably mediated by IGF-1/Akt/mTOR signaling and control of autophagy. In conclusion, our results suggest that there is a specific role for endothelial cell-derived PC5/6 in fibroblast function. Although endothelial cells express 15- to 20-fold more furin than PC5/6 mRNA, furin exhibits similar ex vivo cleavage specificity to PC5/6, and cannot compensate *in vivo* for the loss of endothelial PC5/6. This indicates that a PC5/6-specific substrate(s) is responsible for the observed phenotypes. Inactivation of PC5/6 in endothelial cells results in a hypotrophic phenotype in mice that affects the cardiovascular system through its effects on fibroblasts. Autophagy plays a critical role in this process, mediated at least in part by decrease in the activity of the IGF/Akt/mTOR axis. These novel findings provide insights into potential new mechanisms, whereby PC5/6 in endothelial cells may participate in cardiovascular pathophysiology and may delineate new targets for cardiovascular therapy.

In vitro Properties of PC5/6

The convertase PC5/6[14,16] localizes to the cell surface via its C-terminal CRD, which binds TIMPs and HSPGs, and in many cases inactivate HSPG-bound proteins such as

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endothelial and lipoprotein lipases,[10] and possibly adhesion molecules. The second cleavage of the prosegment, needed for zymogen activation, occurs at the cell surface, thereby limiting the functions of active PC5/6-A to the cell surface and/or extracellular matrix, thereby favoring substrates that are also bound to HSPGs. In the central nervous system, PC5/6-A can process the neural adhesion molecule L1 assisting in neuronal repair and migration. The specific physiological substrates of PC5/6 need to be unraveled *in vivo*, because in cellular experiments and *in vitro* many of the substrates processed by either enzyme were also cleaved by furin.

PC5/6-A has been prepared as a soluble 117-kDa product isolated from the medium of transfected cells with recombinant mouse or human PC5/6-A. A smaller, 69-kDa form of PC5/6 has also been detected on SDS-PAGE of a stable transfectant in AtT20 cells. Expression of PC5/6-A in baculovirus Sf9 cells or in Schneider 2 (S2) cells allowed its production at the level of 1 mg/l. The enzyme is most active at acidic pH 6.5 and is maximally activated by 5 mM Ca^{2+}. PC5/6 was able to cleave both monobasic and dibasic substrates without a strict requirement for a basic residue at P4. Although the PC5/6-B form of the convertase was predicted to be membrane bound like furin, soluble forms of both enzymes are released into the medium as a result of a cleavage at N-terminal to the transmembrane domain by an as yet unidentified "sheddase" or "secretase." The shed form of PC5/6-B exhibits an apparent mass on SDS-PAGE of approximately 170 kDa.

PC5/6 Substrates

PC5/6 is widely expressed in both endocrine and nonendocrine tissues, with high expression in the adrenal cortex, the gut, in testicular Sertoli cells[14] and in endothelial cells. These observations led to the analysis of the processing of human prorenin in the adrenals, pro-Mullerian inhibiting substance (proMIS) in Sertoli cells, and the receptor tyrosine phosphatase R-PTP-μ in endothelial cells. Interestingly, in both proMIS [**R**G**R**AG**R**↓S**K**] and R-PTP-μ [EE**RP**R**R**↓T**K**], the cleavage site contains a P2′ Lys, within the general sequence: **R**XX**R**↓S**K** or **R**XX**R**↓T**K**. PC5/6 is also responsible for the processing of a number of integrin α chains that exhibit a P1, P2 dibasic and an Ile at P4 but a His at P6, suggesting that this may also be a signature for PC5/6 processing. In addition, the TGFβ-like growth factor Lefty was also reported to be best processed by PC5/6-A, suggesting that PC5/6-A may have an important physiological role in the development of the heart and in endothelial cells. The human ortholog of the 915 amino acid PC5/6-A has been identified, and suggested to play a role in prorenin activation within the adrenal. However, KO studies revealed that PC5/6 is the major processing enzyme of proGDF11, cleaving at the distinguishing sequence **RSRR**$_{296}$↓NL, where Asn at the P1′ position is critical because if replaced by Asp, the site is now recognized by other convertases.[21] However, this motif may be specific to the GDF11 sequence, because other substrates with an Asn at P1′ are well cleaved by furin.[31]

Again, like the case of furin, PC5/6 may also be involved in the inactivation of substrates such as endothelial and lipoprotein lipases,[10] BMPs and adhesion molecules (Seidah et al., unpublished observations).

Pathophysiological Implications

We showed a downregulation of PC5/6 mRNA in human colon tumors at various stages.[29] Because PC5/6 is very rich in intestine, we evaluated its role in tumorigenesis by crosses with an $Apc^{Min/+}$ mouse model, which develops numerous adenocarcinomas along the small intestine and fewer in the colon. Because PC5/6-deficient mice die at birth, we generated mice lacking or not PC5/6-specifically in enterocytes (Villin-cre transgene) and analyzed the number and size of the tumors. The lack of PC5/6 in enterocytes results in a significantly higher tumor number in the duodenum, and a premature mortality of $Apc^{Min/+}$ mice, suggesting that intestinal PC5/6 is protective toward tumorigenesis, especially in mouse duodenum, and possibly in human colon.[29]

Increasing evidence suggests that extracellular accessory protein Vpr could contribute to HIV pathogenesis through its effect on bystander cells. Soluble forms of Vpr have been detected in the sera and cerebrospinal fluids of HIV-1-infected patients, and *in vitro* studies have implicated extracellular Vpr as an effector of cellular responses, including G2 arrest, apoptosis and induction of cytokines and chemokines production, presumably through its ability to transduce into multiple cell types. We provided evidence indicating that soluble forms of Vpr are present in the extracellular medium of HIV-1-producing cells. Release of Vpr in the extracellular medium is associated with HIV-1-mediated cytopathicity. Interestingly, Vprt undergoes proteolytic processing at a very well-conserved PC cleavage site, RQRR$_{88}$↓ located within the functionally important C-terminal Arg-rich domain of the protein. Vpr processing occurs extracellularly upon close contact to cells and most involve a cell surface-associated PC. Consistently, PC inhibitors suppress Vpr processing, while expression of extracellular matrix-associated PC5/6 and PACE4 enhances Vpr cleavage. PC-mediated processing of extracellular Vpr leads to the production of a truncated Vpr product that is defective in the induction of cell cycle arrest and apoptosis when expressed in human cells. Collectively, these results suggest that cell surface processing of extracellular Vpr by PC5/6 and/or PACE4 might regulate the levels of active soluble Vpr.

These results suggest that inhibition of PC5/6 may not be recommended, because in all the situations mentioned

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above either during the development or in the adult, PC5/6 acts as a protective protease. However, preliminary evidence suggests that in some specialized situations, increased levels of PC5/6-A are associated with increased tumor metastasis (Seidah et al., unpublished observations). If PC5/6 inhibitors could be targeted to the tumor, this may then be useful in some therapy against cancer metastasis.

CONCLUSIONS

Although there is no doubt that furin and PC5/6 play major roles in various homeostatic processes within and outside the cell, they have both redundant and specific roles that are not interchangeable with other PCs. This is especially evident during embryonic development, as both enzymes are essential for life and the loss of their expression results in unique phenotypes. In mice, although the loss of furin expression leads to early developmental death at embryonic day 10.5 because of the lack of heart looping morphogenesis, the KO of *Pcsk5* results in lethality at birth with various body axis malformations and cardiovascular defects. The former may be related in part to the absence of BMP10 processing in the absence of furin,[31] the latter in part results from the lack of processing of proGDF11 by PC5/6.[6] However, this does not necessarily mean that the same substrates are important in the adult, and probably other unique substrates for each enzyme will be uncovered from tissue-specific mouse KO studies, or from human genetic mutations. The latter approach is still in its infancy, as only few mutations related to congenital heart disease have been related to PC5/6 function[32] resulting in either one of the congenital malformation syndromes: VACTERL (Vertebral, Anorectal, Cardiac, Tracheo-Esophageal, Renal, Limb malformations), CRS (Caudal Regression Syndrome), or CS (Currarino Syndrome; also known as Sacral Agenesis syndrome). We identified four patients with nonsynonymous variants in PC5/6-B (two with E1575K; one with E1256K, one with P1228S). In 13 patients with CRS, but with a VACTERL score <3, we identified one patient with a nonsynonymous change (heterozygous V1250I). E1256K, V1250I, and P1228S were not present in the relevant exons of ethnically matched controls, indicating that they are not common polymorphisms.[32] Before we could assign these mutations to a loss of function, we still need to carefully test these mutants for their effects on the ability of PC5/6-B to process proGDF11.[6] The future will surely tell whether the control of the activity of either furin or PC5/6 could find applications in the treatment of certain pathologies, including viral or pathogenic infections and cancer/metastasis. However, more probably we may find that the expression levels of these enzymes may be correlated with the progression of certain diseases and correlated with their predictive outcome.

ACKNOWLEDGMENTS

The author would like to acknowledge the tremendous help of all the past and present members of the laboratory who have actively participated in all phases of the work leading to this synopsis of the functions of furin and PC5/6. This work was supported by a CIHR Canada chair # 216684, a CIHR grant # MOP 44363, and a Strauss foundation grant.

REFERENCES

1. Benjannet S, Rhainds D, Hamelin J, Nassoury N, Seidah NG. The proprotein convertase PCSK9 is inactivated by furin and/or PC5/6A: functional consequences of natural mutations and post-translational modifications. *J Biol Chem* 2006;**281**(41):30561–72.
2. De Vos L, Declercq J, Rosas GG, Van Damme B, Roebroek A, Vermorken F, et al. MMTV-cre-mediated fur inactivation concomitant with PLAG1 proto-oncogene activation delays salivary gland tumorigenesis in mice. *Int J Oncol* 2008;**32**(5):1073–83.
3. Dubois CM, Blanchette F, Laprise MH, Leduc R, Grondin F, Seidah NG. Evidence that furin is an authentic transforming growth factor-beta1-converting enzyme. *Am J Pathol* 2001;**158**(1):305–16.
4. Essalmani R, Hamelin J, Marcinkiewicz J, Chamberland A, Mbikay M, Chretien M, et al. Deletion of the gene encoding proprotein convertase 5/6 causes early embryonic lethality in the mouse. *Mol Cell Biol* 2006;**26**(1):354–61.
5. Essalmani R, Susan-Resiga D, Chamberland A, Abifadel M, Creemers JW, Boileau C, et al. In vivo evidence that furin from hepatocytes inactivates PCSK9. *J Biol Chem* 2011;**286**(6):4257–63.
6. Essalmani R, Zaid A, Marcinkiewicz J, Chamberland A, Pasquato A, Seidah NG, et al. In vivo functions of the proprotein convertase PC5/6 during mouse development: GDF11 is a likely substrate. *Proc Natl Acad Sci U S A* 2008;**105**(15):5750–5.
7. Feliciangeli SF, Thomas L, Scott GK, Subbian E, Hung CH, Molloy SS, et al. Identification of a pH sensor in the furin propeptide that regulates enzyme activation. *J Biol Chem* 2006;**281**(23):16108–16.
8. Henrich S, Cameron A, Bourenkov GP, Kiefersauer R, Huber R, Lindberg I, et al. The crystal structure of the proprotein processing proteinase furin explains its stringent specificity. *Nat Struct Biol* 2003;**10**(7):520–6.
9. Henrich S, Lindberg I, Bode W, Than ME. Proprotein convertase models based on the crystal structures of furin and kexin: explanation of their specificity. *J Mol Biol* 2005;**345**(2):211–27.
10. Jin W, Fuki IV, Seidah NG, Benjannet S, Glick JM, Rader DJ. Proprotein convertases are responsible for proteolysis and inactivation of endothelial lipase. *J Biol Chem* 2005;**280**(44):36551–9.
11. Klenk HD, Garten W. Host cell proteases controlling virus pathogenicity. *Trends Microbiol* 1994;**2**(2):39–43.
12. Lloyd DJ, Bohan S, Gekakis N. Obesity, hyperphagia and increased metabolic efficiency in Pc1 mutant mice. *Hum Mol Genet* 2006;**15**(11):1884–93.
13. Louagie E, Taylor NA, Flamez D, Roebroek AJ, Bright NA, Meulemans S, et al. Role of furin in granular acidification in the endocrine pancreas: identification of the V-ATPase subunit Ac45 as a candidate substrate. *Proc Natl Acad Sci USA* 2008;**105**(34):12319–24.
14. Lusson J, Vieau D, Hamelin J, Day R, Chretien M, Seidah NG. cDNA structure of the mouse and rat subtilisin/kexin-like PC5: a candidate proprotein convertase expressed in endocrine and nonendocrine cells. *Proc Natl Acad Sci U S A* 1993;**90**(14):6691–5.

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15. Nakagawa T, Hosaka M, Torii S, Watanabe T, Murakami K, Nakayama K. Identification and functional expression of a new member of the mammalian Kex2-like processing endoprotease family: its striking structural similarity to PACE4. *J Biochem (Tokyo)* 1993;**113**(2):132–5.
16. Nakagawa T, Murakami K, Nakayama K. Identification of an isoform with an extremely large Cys-rich region of PC6, a Kex2-like processing endoprotease. *FEBS Lett* 1993;**327**(2):165–71.
17. Paquet L, Bergeron F, Boudreault A, Seidah NG, Chretien M, Mbikay M, et al. The neuroendocrine precursor 7B2 is a sulfated protein proteolytically processed by a ubiquitous furin-like convertase. *J Biol Chem* 1994;**269**(30):19279–85.
18. Pesu M, Watford WT, Wei L, Xu L, Fuss I, Strober W, et al. T-cell-expressed proprotein convertase furin is essential for maintenance of peripheral immune tolerance. *Nature* 2008;**455**(7210):246–50.
19. Roebroek AJ, Taylor NA, Louagie E, Pauli I, Smeijers L, Snellinx A, et al. Limited redundancy of the proprotein convertase furin in mouse liver. *J Biol Chem* 2004;**279**(51):53442–50.
20. Roebroek AJ, Umans L, Pauli IG, Robertson EJ, van Leuven F, Van de Ven WJ, et al. Failure of ventral closure and axial rotation in embryos lacking the proprotein convertase Furin. *Development* 1998;**125**(24):4863–76.
21. Scamuffa N, Basak A, Lalou C, Wargnier A, Marcinkiewicz J, Siegfried G, et al. Regulation of prohepcidin processing and activity by the subtilisin-like proprotein convertases Furin, PC5, PACE4 and PC7. *Gut* 2008;**57**(11):1573–82.
22. Scamuffa N, Siegfried G, Bontemps Y, Ma L, Basak A, Cherel G, et al. Selective inhibition of proprotein convertases represses the metastatic potential of human colorectal tumor cells. *J Clin Invest* 2008;**118**(1):352–63.
23. Seidah NG. The proprotein convertases, 20 years later. *Methods Mol Biol* 2011;**768**:23–57.
24. Seidah NG, Gaspar L, Mion P, Marcinkiewicz M, Mbikay M, Chretien M. cDNA sequence of two distinct pituitary proteins homologous to Kex2 and furin gene products: tissue-specific mRNAs encoding candidates for pro-hormone processing proteinases. *DNA Cell Biol* 1990;**9**(10):789.
25. Seidah NG, Mayer G, Zaid A, Rousselet E, Nassoury N, Poirier S, et al. The activation and physiological functions of the proprotein convertases. *Int J Biochem Cell Biol* 2008;**40**(6–7):1111–25.
26. Seidah NG, Prat A. Precursor convertases in the secretory pathway, cytosol and extracellular milieu. *Essays Biochem* 2002;**38**:79–94.
27. Smeekens SP, Steiner DF. Identification of a human insulinoma cDNA encoding a novel mammalian protein structurally related to the yeast dibasic processing protease Kex2. *J Biol Chem* 1990;**265**(6): 2997–3000.
28. Steiner DF. The proprotein convertases. *Curr Opin Chem Biol* 1998;**2**(1):31–9.
29. Sun X, Essalmani R, Seidah NG, Prat A. The proprotein convertase PC5/6 is protective against intestinal tumorigenesis: in vivo mouse model. *Mol Cancer* 2009;**8**:73.
30. Sun X, Essalmani R, Susan-Resiga D, Prat A, Seidah NG. Latent TGF-beta binding proteins-2 and -3 inhibit the proprotein convertase 5/6A. *J Biol Chem* 2011;**286**:29063–73.
31. Susan-Resiga D, Essalmani R, Hamelin J, Asselin MC, Benjannet S, Chamberland A, et al. Furin is the major processing enzyme of the cardiac-specific growth factor bone morphogenetic protein 10. *J Biol Chem* 2011;**286**(26):22785–94.
32. Szumska D, Pieles G, Essalmani R, Bilski M, Mesnard D, Kaur K, et al. VACTERL/caudal regression/currarino syndrome-like malformations in mice with mutation in the proprotein convertase Pcsk5. *Genes Dev* 2008;**22**(11):1465–77.
33. Thomas G. Furin at the cutting edge: from protein traffic to embryogenesis and disease. *Nat Rev Mol Cell Biol* 2002;**3**(10):753–66.
34. Van de Ven WJ, Voorberg J, Fontijn R, Pannekoek H, van den Ouweland AM, van Duijnhoven HL, et al. Furin is a subtilisin-like proprotein processing enzyme in higher eukaryotes. *Mol Biol Rep* 1990;**14**(4):265–75.
35. Xiang Y, Molloy SS, Thomas L, Thomas G. The PC6B cytoplasmic domain contains two acidic clusters that direct sorting to distinct trans-Golgi network/endosomal compartments. *Mol Biol Cell* 2000;**11**(4):1257–73.

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Chapter 248

Proprotein Convertases PC4, PACE4, and PC7

Nabil G. Seidah

ABSTRACT

The unique secretory basic amino acid-specific proprotein convertases PC4, PACE4, and PC7 do not seem to have common substrates, and they have very different *in vivo* physiological functions. The loss of expression of either of these convertases is not essential, as all three knockout mice are viable. PC4 is exclusively expressed in male testis within germ cells and also in females within gonadotropin-activated macrophage-like ovarian cells. Male mice lacking PC4 are severely subfertile. PACE4 is widely expressed in many tissues, and in the adult brain, it is expressed in both neuronal and glial cells. In *Pcsk6* knockout mice, a proportion of embryos lacking this enzyme develop complex craniofacial malformations, establishing an important role for PACE4 in patterning the early mouse embryo. PC7 is mostly found at the cell surface and reaches this destination by both the conventional and unconventional secretory pathways. In cells, it indirectly induces the processing of proEGF, which is the only known physiological and specific target of PC7.

DISCOVERY AND NOMENCLATURE

The mammalian furin and the two regulated convertases PC1/3 and PC2 were all discovered in 1989–1990. This initial wave of discoveries was soon followed by the identification of PC4, PC5/6, PACE4, and PC7.[24] The human genes encoding the seven basic-aa specific Proprotein Convertases Subtilisin/Kexin-like were given the names of *PCSK1* to *PCSK7* (mouse *Pcsk1* to *Pcsk7*). In this review, emphasis will only be on PC4, PACE4, and PC7, each exhibiting unique physiological functions and cleaving very different substrates. The corresponding human and mouse genes map to chromosomes 19, 15, 11 and 10, 7, 9, respectively.

FUNCTIONS IN POLYPEPTIDE BIOSYNTHESIS/PROCESSING

PC4

In 1991, during our search by means of RT-PCR for other members of the PC-family in mRNA extracts of various tissues, we identified in rat testis three different approximately 600-bp cDNAs potentially coding for three different PCs, now known as PC4, PC5/6, and PACE4.[24] Shortly thereafter, an article appeared that reported the complete cDNA sequence of PACE4 from a human hepatoma HepG2 cell line and an osteosarcoma cDNA library.[12] We therefore first concentrated on the characterization of PC4.[27]

PC4 is expressed exclusively in male testicular germline pachytene spermatocytes and round spermatids, suggesting that it may play a specific physiological function in reproduction. In agreement, PC4 is detected in the acrosomal granules of round spermatids, in the acrosomal ridges of elongated spermatids, and on the sperm plasma membrane overlying the acrosome.[11] In female mice, PC4 is expressed in macrophage-like cells of the ovary, and its levels are downregulated in activated macrophages, such as in inflammation. PC4 is also expressed in human placenta. However, immunocytochemical results show that the PC4 protein is expressed at later stages of spermiogenesis and can even be detected in mature spermatozoa.

The structure of the catalytic domain of PC4 is closer to that of furin than that of any other proprotein convertase. Alignment of human, mouse, rat, and xenopus PC4 reveals that these are highly homologous except that each exhibits a specific C-terminal segment (from residue 593 onward). Interestingly, human and xenopus PC4 contains a single Cys-rich motif with six Cys within their unique C-terminal segment: Cys-X_2-Cys-X_3-Cys-X_2-Cys-X_7-Cys-X_2-Cys-X_{12}, similar to the ones found in furin, PC5/6, and PACE4.

Zymogen Activation and Cellular Trafficking

Results show that the bulk of PC4 remains as an intracellular zymogen (human aa 26–755), presumably trapped in the endoplasmic reticulum (ER), where it interacts with the general molecular chaperone glucose-regulated protein 78/Immunoglobulin heavy-chain binding protein (GRP78/BiP).[10] These results suggest that, unlike other members of the convertase family, proPC4 cannot efficiently self-activate in somatic

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00248-7
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cells. These cells may lack the intracellular environment and the interacting molecules specific to testicular germ cells where this enzyme is normally expressed. Nevertheless, we have been able to isolate small amounts of recombinant, active PC4 after overexpression of its vaccinia virus recombinant in the constitutively secreting green monkey kidney epithelial BSC40 cells.[1] The zymogen human proPC4 is autocatalytically cleaved in the ER at the S1 site **R**RVKR$_{113}$↓SV. Different from most other convertases, no evident secondary S2 site is found. The usual autocatalytically cleavable S2 site found in furin ($_{69}$**H**RGVTKR$_{75}$↓SL), with the characteristic His$_{69}$ acting as a pH sensor, is replaced in PC4 by a PC-uncleavable sequence $_{75}$**H**RGVVQQ$_{81}$SL. This suggests that the prosegment (human aa 26–113) and the active enzyme separate early on in the secretory pathway may be without the need of a secondary S2 cleavage, as is the case of PC7.[22] Even though most of the PC4 remains intracellular, possibly resulting from the presence of a transmembrane domain (human aa 709–729), some are secreted as a soluble, Ca^{2+}-dependent enzyme exhibiting a neutral pH optimum, and can cleave *in vitro* fluorogenic substrates such as pGlu-Arg-Thr-Lys-Arg NHMec [XRTKR↓X]. The secreted enzyme consisted of two protein bands migrating at approximately 62, approximately 53 kDa on SDS-PAGE, which are attributed to C-terminal truncated PC4 forms. This enzyme displays strong protease activity toward fluorogenic Boc-RVRR-MCA and various intramolecularly quenched peptides derived from PC4 substrates.

In vivo Mouse Knockout (KO) Models

It took much effort from M. Mbikay to obtain during a sabbatical year at the Jackson laboratories mice that lacked PC4 expression.[16] This article that appeared on June 24, 1997, was the first report on any convertase KO mouse. The *in vivo* fertility of homozygous mutant males is severely impaired in these mice, without any evident spermatogenic abnormality.[16] *In vitro*, PC4 deficiency seems to cause reduced fertilization and early embryonic death.[16] A comparison of wild-type and *Pcsk4*-null sperm for their abilities in capacitation, acrosome reaction, and egg binding *in vitro* was undertaken.[11] The results reveal that *Pcsk4*-null sperm undergo capacitation at a faster rate; they are induced to acrosome react by lower concentrations of zona pellucida; and their egg-binding ability is only half that of wild-type sperm. These sperm physiologic anomalies probably contribute to the severe subfertility of *Pcsk4*-deficient male mice. Accordingly, these results strongly suggest that PC4 will play an important role in fertility and that functional redundancy mediated by any other PC is quite limited in the testis. PC4 thus seems to be a crucial enzyme for reproduction. Alterations of PC4 expression or activity could be the underlying cause of some unexplained cases of human infertility. Conversely, inactivation of this protease represents a potential strategy for nonhormonal contraception

PC4 Substrates

Many C-terminally[27] and N-terminally modified isoforms are predicted from the isolated cDNAs, probably arising from an alternative splicing of the gene. PC4 is a very special prohormone convertase whose C-terminus is species specific, with human and xenopus forms possibly being type-1 transmembrane containing proteins.[27] This may be necessary to ensure no crossspecies fertilization. PC4 has only been characterized in rat and mouse testis, and it is not known whether invertebrates express this enzyme.

So far, very little is known about the natural substrates of PC4. Vaccinia virus expression of PC4 allows the isolation of an active form of the enzyme of ~53 kDa. *In vitro* analysis using synthetic peptide substrates demonstrated a possible role of PC4 in the maturation of proIGF-1 and proIGF-2 and propituitary adenylate-cyclase-activating polypeptide (proPACAP). These results suggest that PC4 prefers to cleave sequences containing the motif **K**XX**R**↓ with a P4 Lys (where X denotes any other residue). Cellular coexpression studies confirm that PC4 is a genuine and sole proPACAP convertase in the testis and ovary of mice.[13] From the autocatalytic cleavage site required for zymogen activation of PC4, and by analogy to other convertases, it was predicted that PC4 will cleave precursors at the consensus sequence of (Lys/Arg)-Arg↓ [KR↓X; RR↓X] or (Arg/Lys)-(Xaa)$_n$-Arg↓, where $n = 2$, 4 or 6. *In vitro* studies with purified enzyme conclude that the most probable sequence motif for recognition by PC4 is KXKXXR↓ or KXXR↓, where X is any aa other than cysteine and that PC4 prefers a proline at P3, P5 and/or P2′ positions. PC4 is a good candidate processing enzyme for the growth factors IGF-1 and -2, and several ADAM (A Disintegrin And Metalloprotease) proteins such as ADAM-1, -2, -3, and -5.[2] Finally, potent PC4-inhibitory activity with K_i in low nanomolar range is observed with the tetrapeptide chloromethyl ketones, decanoyl-RVKR/K-chloromethyl ketone. Optimized PC4 inhibitors, possibly derived from those reported in flavonoids may one day serve as male contraceptives.

Pathophysiological Implications

Intrauterine fetal growth restriction is a leading cause of perinatal mortality. There is an unusual property of PC4 in the processing of proIGF-2, which is an important regulator of fetoplacental growth. Thus, PC4 cleaves proIGF-2 to generate the intermediate processed form, IGF-2$_{1–102}$ and, subsequently, mature IGF-2$_{1–67}$, thereby regulating fetoplacental growth.[21] In the future, SNPs variants PC4 that could affect its activity may explain some of the pathophysiology of fetoplacental growth restriction http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?geneId=54760. Thus, although many of these coding variants are already known, they await biochemical confirmation of their effects,

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especially for the mutants R202C (close to the active site His_{199}), G302S (close to the oxyanion hole Asn_{300}), C338Y, Y561C, and C649F, which are expected to result in a loss-of-function of PC4, probably resulting from misfolding of the zymogen and/or retention in the ER.

PACE4

The proprotein convertase PACE4 was discovered in 1991,[12] shortly after the identification of PC1/3, PC2, and furin. The convertases PACE4 and PC5/6 form a class of their own based on their primary structures and their ability to bind the cell surface via their C-terminal Cys-rich domains, which bind tissue inhibitors of metalloproteases (TIMPs) and heparin sulfate proteoglycans (HSPGs).[29] Quantitative real-time PCR (qPCR) analysis of mRNA expression revealed that PACE4 is expressed in many tissues, and in mouse, it is rich in liver, pituitary, adipose tissue, heart, pancreas, digestive system, and brain cerebellum, with very little expression in male reproductive organs such as testis but significantly expressed in female ovary (Fig. 1). The present results strongly suggest unique tissue-specific functions of PACE4.[8] Thus, in the brain, it is found in both neuronal and glial cells. However, in areas that are rich in neuropeptides such as cortex, hippocampus, and hypothalamus, mRNA levels of PACE4 are low or undetectable. In regions, such as the amygdaloid body and thalamus, there is a distinct distribution of PACE4 mRNAs. The medial habenular and cerebellar Purkinje cells express very high levels of PACE4 mRNA, as do anterior pituitary cells.[8] The specific physiological substrates of PACE4 need to be unraveled *in vivo*, because in cellular experiments and *in vitro* many of the substrates processed by PACE4 are also cleaved by Furin, PC5/6, and/or PC7.

Zymogen Activation and Cellular Trafficking

PCs must go through an ordered process of autoactivation before they can cleave other protein substrates. Following signal peptidase cleavage of the unusually long signal peptide of preproPACE4 (human 63 aa), proPACE4 (human aa 64–969) is N-glycosylated at three sites $Asn_{259,913,942}$. Like all PCs, the prosegment (human aa 64–149) acts as an intramolecular chaperone and inhibitor of PACE4. It is first autocatalytically cleaved in the ER at the S1 site $\underline{K}R\underline{R}V\underline{KR}_{149}$↓QV. This generates an enzymatically inactive heterodimer complex (pro≡PACE4) of the prosegment (aa 64–149) with mature PACE4 (aa 150–969), which can

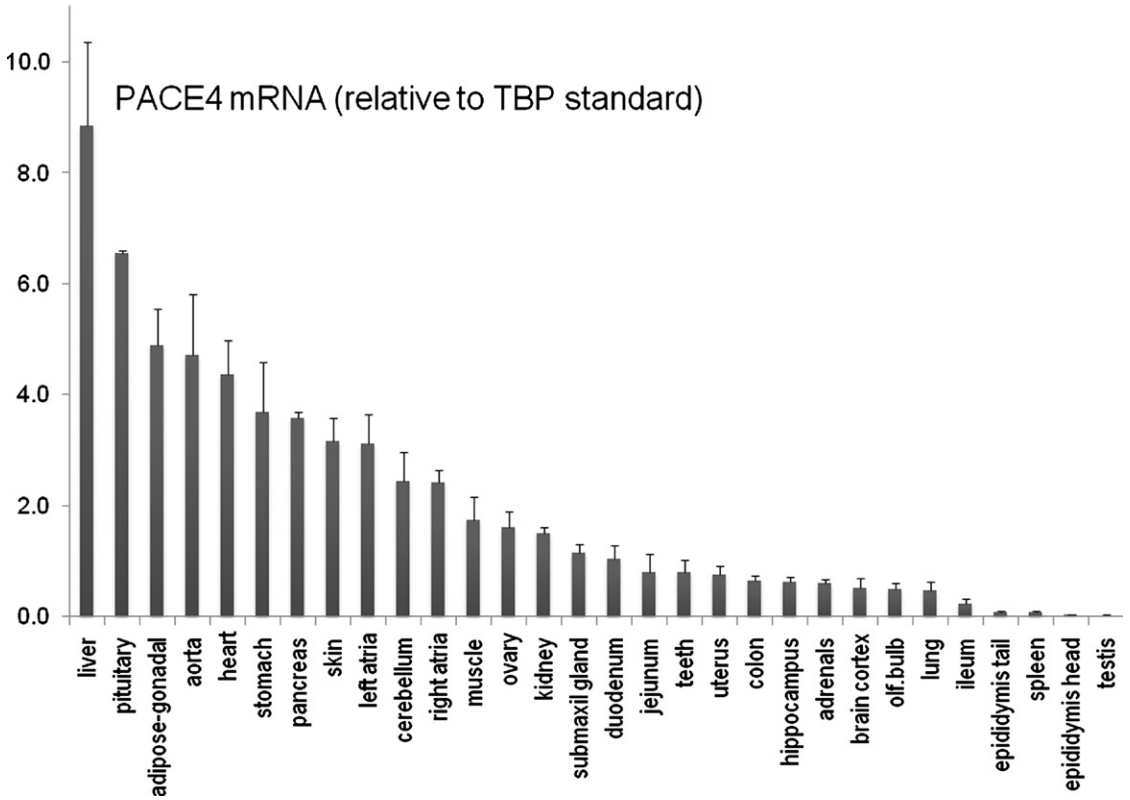

FIGURE 1 Quantitative reverse transcriptase PCR analysis of the mRNA expression of PACE4 in mouse tissues. Each measurement was done in triplicate, and the standard error from the mean is shown above each bar. The levels are normalized to the mRNA values of the housekeeping standard TATA-box binding protein (TBP).

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then exit from the ER and reach the trans-Golgi network (TGN), undergoing trimming and remodeling of its sugar moieties along the way. The usual autocatalytically cleavable S2 site found in furin ($_{69}$**H**RGVTKR$_{75}$↓SL), with the characteristic His$_{69}$ acting as a pH sensor, is replaced by $_{111}$**H**SKTFKR$_{117}$↓ST in PACE4. However, where and when the secondary autocatalytic cleavage at the S2 site occurs is still not completely understood. The presence of the probable pH sensor His$_{111}$ suggests that it would occur in an acidic environment, where the His$_{111}$ would be protonated (e.g. TGN and/or recycling endosomes). Indeed, evidence has been presented that except for PC5/6, and different from the other convertases, PACE4 seems to be in large part activated at the cell surface while in contact with HSPGs.[15] This limits the functions of active PACE4 to the cell surface and/or extracellular matrix (ECM), thereby favoring substrates that are also bound to HSPGs. This is especially evident in the cellular processing of the TGFβ-like bone morphogenetic protein BMP10, which is completely inhibited by a noncell permeable hexa-Arg inhibitor.[31]

Within the catalytic domain, human, rat, and mouse sequences exhibit 98% sequence identity and contain the characteristic subtilisin-like serine proteases His*, Asp*, and Ser* catalytic triad and the oxyanion hole Asn. The catalytic domain (human aa 150–454) is then followed by a P-domain that seems to function as a stabilizer of the catalytic pocket via neutralization of its highly negatively charged environment, needed to attract positively charged substrates. The P-domain is followed by a cysteine-rich domain (CRD; aa 695–930) that contains 44 cysteine residues arranged in five tandem repeats of the consensus Cys-X$_{2-3}$-Cys-X$_{3-4}$-Cys-X$_{2-7}$-Cys-X$_{5-10}$-Cys-X$_2$-Cys-X$_{9-13}$-Cys-X$_{3-5}$-Cys-X$_{7-16}$ motif (as it is also found in its closest homolog PC5/6). The CRD binds both TIMPs and HSPGs[29] at the cell surface or in the ECM.[15] We showed that the extracellular activity of PACE4 is probably regulated by endogenous inhibitors known as latent-TGFβ binding proteins (LTBPs 2,3).[30]

In vivo Mouse Pcsk6 KO Models

In a mixed genetic background, the complete KO of the PACE4 gene *Pcsk6* in mice resulted in a 75% viable phenotype with bone morphogenesis defects.[6] About 25% of homozygous knockout embryos die prenatally and, at a later stage of development, between E13.5 and E15.5. However, the severity and penetrance of this phenotype may partly depend on the background of the KO mouse, as it seems less severe in C57BL/6 pure background mice (D. Constam, personal communication). Similar to furin KO mice (*fur*$^{-/-}$), and PC5 KO mice (*Pcsk5*$^{-/-}$)[9,32] the embryos have cardiac malformations that probably lead to death. Defects are also found in other internal organs including the liver, lungs, pancreas, spleen, and gut. These left/right axis defects are preceded at embryonic day 8.5 by an abnormal expression of the axis determining TGFβ-like growth factors Nodal and Lefty, which are both PC-substrates. These genes are normally asymmetrically expressed and involved in determining the left–right axis, but in *Pcsk6*$^{-/-}$ embryos expression can be absent or bilateral. Some embryos deficient in PACE4 show severe craniofacial defects such as cyclopism and truncation of the head, showing that PACE4 activity is necessary for anterior patterning. So far, in *Pcsk6*$^{-/-}$ mice, the identity of the substrate(s), which if left unprocessed would result in the observed complex craniofacial malformations, including cyclopia,[6] is/are yet to be defined.

In vitro Properties of PACE4

It is clear that the substrate specificity is more limited than that of furin. It can cleave substrates such as pGlu-Arg-Thr-Lys-Arg-MCA to release the fluorescent AMC close to neutral pHs. It does require 1 mM Ca^{2+} for optimal activity, and the latter is inhibited by EGTA. Aside from the ability of PACE4 to cleave substrates at pairs of basic residues within the consensus R-X-K/R–R↓SF (http://merops.sanger.ac.uk/cgi-bin/pepsum?id=S08.075;type=P; based on 17 peptide cleavages), PACE4 can also process precursors at monobasic residues that lack an Arg at P4. Thus, within the constitutive secretory pathway, PACE4 efficiently processes prosomatostatin at RLELQR↓SA (P1 and P6 Arg) generating somatostatin 28, or at APRERK↓AG (P1 Lys and P4 Arg) producing somatostatin 14.[5]

Ex vivo PACE4 Substrates

From the Merops database (http://merops.sanger.ac.uk/cgi-bin/substrates?id=S08.075), ex vivo some of the type of cellular precursors best cleaved by PACE4 include ADAM-TS4 (RAKR↓FA), aerolysin (RRAR↓SV), BMP4 (RAKR↓SP), Diphtheria toxin (RVRR↓SV), HIV gp160 (REKR↓AV), Insulin receptor (RKRR↓SL), endothelial lipase precursor (RNKR↓NS), angiopoietin-like 3 (RAPR↓TT), MMP11 (RNKR↓FV), neurotrophin-3 precursor (RRKR↓YA), LRP1 (RHRR↓QI), PDGF-B (RGRR↓SL), and von Willebrand factor (RSKR↓SL).

In the adult, PACE4 plays an important role in myogenic differentiation through its association with the IGF-2 pathway. Thus, although PC4 process proIGF-2 in testis, PACE4 seems to be its cognate convertase in muscle. It was also reported that PACE4 could process the TGFβ like substrate Nodal and that its intracellular traffic is dictated by the 18-kDa EGF-containing GPI-anchored proteoglycan Cripto that directs its traffic through an unconventional secretory pathway directly from the ER to the cell surface, and sorting to detergent resistant membrane microdomains.[4] Cripto is the first receptor that binds both a PC and its substrate (Nodal), thereby enhancing the processing step.

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Pathophysiological Implications

PACE4 is a protective gene, because its specific absence results in a decreased extracellular inactivation of the HIV-1 accessory protein Vpr.[34] However, this does not exclude that under certain pathological conditions (e.g. metastatic cancer), overexpression of PACE4 may result in enhanced pathology. A similar type of situation may exist in osteoarthritis, where PACE4 is overexpressed, and its inactivation may relieve the associated pain symptoms. Indeed, proteolytic degradation of the major cartilage macromolecules, aggrecan and type II collagen, a key pathological event in osteoarthritis, is performed by ADAMTS-4,5, matrix metalloproteases that are proteolytically activated by PACE4 processing of their inhibitory prodomains in cartilage.[14,33]

More recently, it has been realized that PACE4 may play an important role in cancer development and possibly associated metastasis. For example, there is high expression of PACE4 in all different clinical stages of human prostate tumor tissues. Gene silencing studies targeting PACE4 in the DU145 prostate cancer cell line produced cells with slower proliferation rates, reduced clonogenic activity, and inability to grow as xenografts in nude mice. The direct role of PACE4 in prostate cancer is most probably through the upregulated processing of growth factors, or through the aberrant processing of growth factors, leading to sustained cancer progression, suggesting that PACE4 holds a central role in prostate cancer.[7] If PACE4 inhibitors or silencers could be targeted to the tumor, this may then be useful in some therapy against cancer and possibly its associated metastasis.

Finally, because PACE4 mRNA levels are consistently upregulated in prostate tumors and inhibition of PACE4 activity is associated with lower tumor growth, this suggests that this convertase could play an important role in either prostate tumorigenesis and/or its associated metastatis. It is therefore possible that PACE4 activity may be associated with enhanced tumorigenesis and conversely that loss-of-function mutations of PACE4 protease may be associated with a good long-term prognosis. In that context, we screened the publicly available missense or nonsense Single Nucleotide Polymorphism (SNP) data for the presence of SNPs (http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?geneId=5046) associated with PACE4 exons. Accordingly, we found a limited number of natural coding mutations that are predicted as most damaging ones found in the PACE4 SNP database, two of which are rs45587040[Q569X, C-terminal aa 569–969 truncated; 48% prevalence] and rs75922759 [C845W, disrupts the CRD]. It would be interesting to search for such mutations in prostate cancer or osteoarthritis patients and to correlate these to the outcome or progression of the pathology.

PC7

Zymogen Activation and Cellular Trafficking

The type-I membrane-bound PC7 is the most ancestral member of the mammalian family, exhibiting the closest relationship to yeast kexin.[28] Biochemical and enzymatic characterization of the novel proprotein convertase rat PC7 (rPC7) was carried out using vaccinia virus recombinants overexpressed in mammalian BSC40 cells. ProPC7 is synthesized as a glycosylated proenzyme (101 kDa) and processed into mature rPC7 (89 kDa) in the ER which is then Cys-palmitoylated (Cys-palm) at the cytosolic tail Cys_{699} and Cys_{704} (Fig. 2A).[22,28] No endogenously produced, soluble forms of this membrane-anchored protein were detected, revealing that it is not shed into the medium.

The inhibitory prosegment of PC7[3,35] is primarily secreted alone as a noninhibitory protein via the conventional, Golgi-dependent, secretory pathway.[22] Mature PC7 is partially sulfated and thus reaches the cell surface via the conventional route. However, a fraction of PC7 reaches the cell surface through a brefeldin A- and COPII-independent unconventional secretory pathway. The latter trafficking may explain the rapid (<10 min) transit of a fraction of PC7 from the ER to the cell surface. Electron microscopy further confirmed the localization of PC7 to the cell surface of HEK293 cells. PC7 also localizes within subcellular compartments distal to the TGN and can be stored in undefined vesicular stacks close to the Golgi apparatus. Within the cytosolic tail, only two cysteines (Cys_{699} and Cys_{704}) are palmitoylated, but this modification does not affect the choice of trafficking pathway. Swapping the transmembrane-cytosolic tail (TMCT) sequences of the convertases Furin and PC7 revealed that the transmembrane domain of PC7 regulates the sorting of PC7 toward the unconventional secretory pathway. Thus, PC7 is distinct from other proprotein convertases in its zymogen activation, subcellular localization, and trafficking.[22]

Interestingly, although overexpression of the prosegments of Furin, PC5/6 and PC7 results in potent inhibitors of substrate cellular processing,[19,35] only the prosegment of PC7 is secreted into the medium.[35] Finally, the prosegment C-terminal $\underline{RAKR}_{140}$ motif of r-PC7 is critical for its convertase inhibitory activity.[3] Altogether, these results point out the particularities of PC7 in its zymogen activation and subcellular localization.

In vitro Properties of PC7

Because PC7 is membrane bound and never shed,[18] to isolate an active soluble form of PC7, we introduced a stop codon close to the beginning of transmembrane segment, herein called PC7sol.[18,28] Thus, the soluble, *in vitro* active form produced spans the segment ending at Gly-Tyr-Ser_{622}.[18,28] We thus obtained both vaccinia virus and baculovirus

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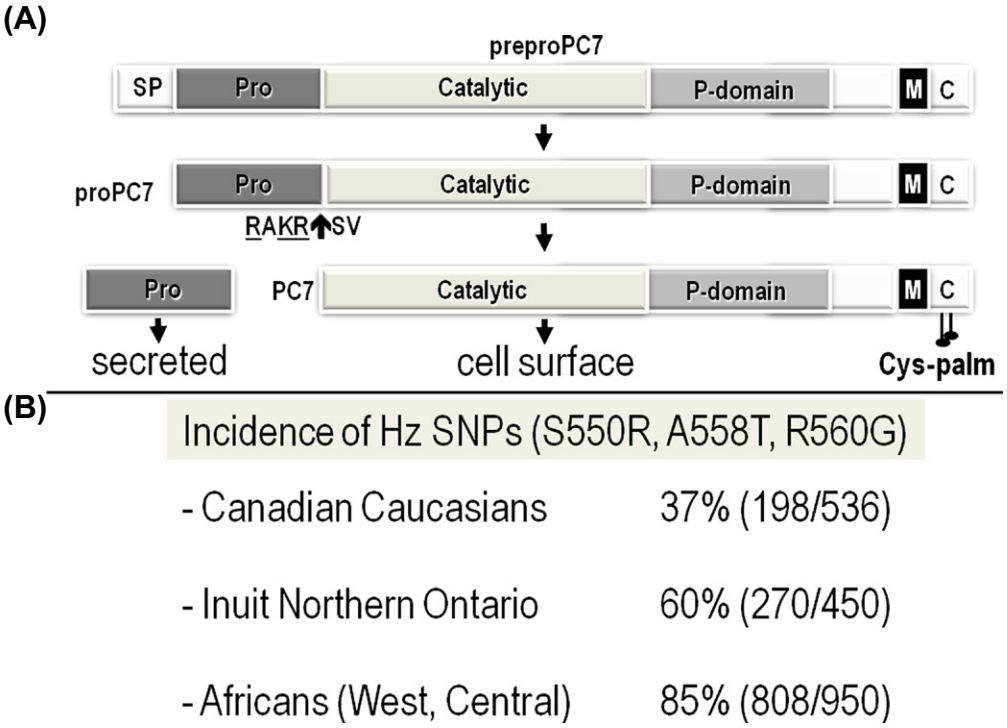

FIGURE 2 (A) Schematic representation of the autocatalytic processing of proPC7 into PC7 at **R**A**KR**$_{140}$↓SV and its posttranslational Cys-palmitoylation (at Cys_{699} and Cys_{704}; Cys-palm) in its cytosolic tail. The various domains are emphasized, including the signal peptide (SP), prosegment, catalytic and P-domains, the transmembrane domain (M) and the cytosolic tail (C). Although the cleaved prosegment is secreted alone, the active membrane-bound PC7 localizes to the cell surface. (B) incidence of heterozygote *PCSK7* coding SNPs (S550R, A558T, R560G) in three ethnic populations.

recombinants and showed that both systems yield active enzyme, in the range of 1–3 mg/l. Schneider 2 (S2) cells also produce active soluble human PC7. The activity is routinely measured using a fluorogenic substrate pyro-Glu-Arg-Thr-Lys-Arg-methyl-coumaryl-7-amide (pERTKR-MCA).

Enzymatic activity assays of PC7sol using fluorogenic substrates indicated that the enzyme exhibits a neutral pH optimum, and a Ca^{2+} dependence, and cleavage specificity largely similar to that of furin, with a general recognition sequence of either (K/R)-R↓ or (R/K)-$(X)_n$-R↓ where $n = 2$, 4 or 6.[25,26] However, with some substrates, cleavage specificity more closely resembles that of yeast kexin, suggesting a differential processing of proprotein substrates by this novel convertase. We examined the PC7sol- and human furin-mediated cleavage of synthetic peptides containing the processing sites of three proteins known to colocalize in situ with PC7. Although both enzymes correctly process the proparathyroid hormone tridecapeptide KSVKKR↓SVSEIQL and the heptadecapeptide YETLRRRVKR↓SLVVPTD of proPC4, neither enzyme cleaves *in vitro* a hexadecapeptide spanning the N-terminal cleavage site of proepidermal growth factor (proEGF): HLREDDHHYSVR-NSDS.

Several investigations aimed at defining the sequence recognition of PC7 and its redundancy with other convertases suggested that although less efficient than Furin, PC7 specifically cleaves overexpressed substrates at Arg↓ residues both *in vitro* and in cell lines. Thus, although Furin and PC7 have been proposed as the major gp160 processing convertases, rat liver microsomal gp160 processing activity is essentially different from Furin, and only partially overlaps with PC7. Density gradient studies reveal that PC7 resides in lighter subcellular fractions than Furin.

Ex vivo PC7 Substrates

Specific PC7 substrates are very difficult to find. We showed that only the full-length PC7, the most ancient and conserved basic amino acid-specific PC-family member, induces the processing of proEGF into an approximately115-kDa transmembrane form (EGF-115) at an unusual $VHPR_{290}$↓ motif.[23] Because site directed mutagenesis revealed that Arg_{290} is not critical, the generation of EGF-115 by PC7 is probably indirect. This was confirmed by testing a wide range of protease inhibitors, which revealed that the production of EGF-115 is most probably achieved via the activation by PC7 of a latent serine and/or cysteine protease(s). EGF-115 is more abundant at the cell surface than proEGF and is associated with a stronger EGF receptor (EGFR) activation, as evidenced by the higher levels of phosphorylated ERK1/2. This suggests that the generation of EGF-115 represents a regulatory mechanism of juxtacrine EGFR activation. Thus, PC7 is distinct from the other PCs in its ability to

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enhance the activation of the cell surface EGFR. This is the first reported PC7-specific processing reaction.

Another possible PC7-specific processing reaction seems to be the shedding of the type-2 membrane-bound transferrin receptor (TfR) into a secreted soluble form TfRsol. Indeed, a recent Genome Wide Association Study (GWAS) revealed that a PC7-specific SNP (rs236918) was very positively genetically associated with lower levels of circulating human TfRsol.[20] These authors suggest that PC7 is probably the sheddase of the TfR, at least in human. This will need biochemical validation.

In vivo Models

So far, no report appeared on the phenotypic analysis of *Pcsk7*$^{-/-}$ mice, which are viable and healthy and live a normal life span. Our own preliminary data suggest that complete loss of PC7 expression leads to an anxiolytic phenotype implicating amygdala and/or hippocampus dysregulation within the central nervous system (CNS). We are in the process of identifying the underlying molecular causes of this subtle abnormality.

Northern blot analyses reveal a wide expression of PC7 mRNA in all rat tissues and cell lines analyzed.[28] Quantitative real time-PCR (qPCR) analysis of PC7 expression in adult mouse tissues shows that colon, kidney, duodenum, and heart are the richest sources of PC7 mRNA.[22] These results suggest that PC7 may have multiple physiological functions, some of which may be redundant with other convertases. It is thus interesting to note that the observed CNS phenotype in *Pcsk7*$^{-/-}$ mice would not have been predicted from the levels of expression of PC7 in various tissues, as amygdala and hippocampus are not the richest sources of this convertase in the adult animal.[22,28] It is therefore conceivable that the molecular foundation for the observed anxiolytic phenotype may be already in place during embryonic development and that in the adult PC7 may have other functions.

To identify mutations within the coding region of *PCSK7* in human, we analyzed the relevant SNPs database http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?geneId=9159. To our surprise, we found 41 SNPs with aa changes. The predicted three most damaging, yet rare ones (<0.1% frequency), are: D188G (disrupts the conserved active site pocket around the active site Asp_{187}*), R316C (replaces the highly conserved Arg_{316} in all PCs by Cys, which may cause unfolding of the protein), L506P and frameshift afterward leading to a new 64 extra aa C-terminal domain followed by a stop codon (disrupts the P-domain and the whole C-terminus of PC7, likely causes unfolding of the protein).

Interestingly, we also found a more frequent damaging mutation R560G (often associated with A558(T/V) and/or S550R) in >1936 individuals of different ethnicity (Fig. 2B). The R560G mutation affects an absolutely conserved Arg_{560} in all convertases and the R560G mutant exhibits complete loss of activity, as confirmed biochemically in various cells using the proEGF target of PC7 (Seidah NG et al., in preparation). In this large cohort, we found that the heterozygote R560G mutation is very prevalent in Africans (85%), and its incidence diminishes in Aboriginal Inuits of Northern Ontario (60%) and Canadian Caucasians (37%). Amazingly, we never found any homozygote R560G mutation in any of the individuals analyzed. This curious result prompted us to look into more details at the PCSK7 gene. Indeed, multiple human genomic sequences revealed that exons 13–17 are inverted and duplicated in humans with the duplication residing 60 kb downstream to the complete gene of PCSK7, as originally reported for one *PCSK7* gene.[17] Deeper sequence analysis revealed that the R560G mutation is only found in the duplicated portion of *PCSK7* and not in the original complete gene. This explains the absence of homozygote mutation, because even if a homozygote R560G mutation was found, it will appear as heterozygote because the full gene is not mutated. Furthermore, analysis of the R560G mutation in other species revealed that it is found only in monkeys but not in any other species including rodents (Seidah NG, unpublished observations). This suggests that the R560G mutation probably occurred after exon 13–17 duplication and inversion. The fact that the incidence of the R560G mutation diminishes in ethnicities away from Africans, strongly supports the bottleneck effect that occurred approximately 100,000 years ago, when humanoids migrated out of Africa into the Middle East and Asia.

Pathophysiological Implications

So far, no pathology has been reported implicating PC7, except for its high expression in (https://www.oncomine.org/resource/login.html;jsessionid=EDAC840EAC27B5867C651747898FE3D1) hepatocellular carcinoma, colon adenocarcinoma, and B-cell lymphoma, and much reduced mRNA levels in many other types of cancers, for example, those originating in bladder, brain, breast, kidney, lung, prostate, and uterus.

CONCLUSIONS

Although PC4, PACE4, and PC7 are basic-aa specific proprotein convertases, they exhibit a widely different repertoire of substrates, and phenotypes observed in mice lacking these proteinases. The future development of specific inhibitors against each of these convertases will likely be used as male contraceptives, reduction of pain in osteoarthritis, and as novel anxiolytics, respectively.

ACKNOWLEDGMENTS

The author would like to acknowledge the tremendous help of all the past and present members of the laboratory who have actively

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participated in all phases of the work leading to this synopsis of the functions of PC4, PACE4, and PC7. This work was supported by a CIHR Canada chair # 216684, a CIHR grant # MOP 44363, and a Strauss foundation grant.

REFERENCES

1. Basak A, Toure BB, Lazure C, Mbikay M, Chretien M, Seidah NG. Enzymic characterization in vitro of recombinant proprotein convertase PC4. *Biochem J* 1999;**343**(Pt 1):29–37.
2. Basak S, Chretien M, Mbikay M, Basak A. In vitro elucidation of substrate specificity and bioassay of proprotein convertase 4 using intramolecularly quenched fluorogenic peptides. *Biochem J* 2004;**380** (Pt 2):505–14.
3. Bhattacharjya S, Xu P, Zhong M, Chretien M, Seidah NG, Ni F. Inhibitory activity and structural characterization of a C-terminal peptide fragment derived from the prosegment of the proprotein convertase PC7. *Biochemistry* 2000;**39**(11):2868–77.
4. Blanchet MH, Le Good JA, Mesnard D, Oorschot V, Baflast S, Minchiotti G, et al. Cripto recruits Furin and PACE4 and controls Nodal trafficking during proteolytic maturation. *EMBO J* 2008;**27** (19):2580–91.
5. Brakch N, Galanopoulou AS, Patel YC, Boileau G, Seidah NG. Comparative proteolytic processing of rat prosomatostatin by the convertases PC1, PC2, furin, PACE4 and PC5 in constitutive and regulated secretory pathways. *FEBS Lett* 1995;**362**(2):143–6.
6. Constam DB, Robertson EJ. SPC4/PACE4 regulates a TGFbeta signaling network during axis formation. *Genes Dev* 2000;**14**(9):1146–55.
7. D'Anjou F, Routhier S, Perreault JP, Latil A, Bonnel D, Fournier I, et al. Molecular validation of PACE4 as a target in prostate cancer. *Transl Oncol* 2011;**4**(3):157–72.
8. Dong W, Marcinkiewicz M, Vieau D, Chretien M, Seidah NG, Day R. Distinct mRNA expression of the highly homologous convertases PC5 and PACE4 in the rat brain and pituitary. *J Neurosci* 1995;**15**(3 Pt 1):1778–96.
9. Essalmani R, Zaid A, Marcinkiewicz J, Chamberland A, Pasquato A, Seidah NG, et al. In vivo functions of the proprotein convertase PC5/6 during mouse development: Gdf11 is a likely substrate. *Proc Natl Acad Sci U S A* 2008;**105**(15):5750–5.
10. Gyamera-Acheampong C, Sirois F, Denis NJ, Mishra P, Figeys D, Basak A, et al. The precursor to the germ cell-specific PCSK4 proteinase is inefficiently activated in transfected somatic cells: evidence of interaction with the BiP chaperone. *Mol Cell Biochem* 2011;**348** (1–2):43–52.
11. Gyamera-Acheampong C, Tantibhedhyangkul J, Weerachatyanukul W, Tadros H, Xu H, Van de Loo JW, et al. Sperm from mice genetically deficient for the PCSK4 proteinase exhibit accelerated capacitation, precocious acrosome reaction, reduced binding to egg zona pellucida, and impaired fertilizing ability. *Biol Reprod* 2006;**74**(4):666–73.
12. Kiefer MC, Tucker JE, Joh R, Landsberg KE, Saltman D, Barr PJ. Identification of a second human subtilisin-like protease gene in the fes/fps region of chromosome 15. *DNA Cell Biol* 1991;**10**(10):757–69.
13. Li M, Mbikay M, Arimura A. Pituitary adenylate cyclase-activating polypeptide precursor is processed solely by prohormone convertase 4 in the gonads. *Endocrinology* 2000;**141**(10):3723–30.
14. Malfait AM, Arner EC, Song RH, Alston JT, Markosyan S, Staten N, et al. Proprotein convertase activation of aggrecanases in cartilage in situ. *Arch Biochem Biophys* 2008;**478**(1):43–51.
15. Mayer G, Hamelin J, Asselin MC, Pasquato A, Marcinkiewicz E, Tang M, et al. The regulated cell surface zymogen activation of the proprotein convertase PC5A directs the processing of its secretory substrates. *J Biol Chem* 2008;**283**(4):2373–84.
16. Mbikay M, Tadros H, Ishida N, Lerner CP, De Lamirande E, Chen A, et al. Impaired fertility in mice deficient for the testicular germ-cell protease PC4. *Proc Natl Acad Sci U S A* 1997;**94**(13): 6842–6.
17. Meerabux J, Yaspo ML, Roebroek AJ, Van de Ven WJ, Lister TA, Young BD. A new member of the proprotein convertase gene family (LPC) is located at a chromosome translocation breakpoint in lymphomas. *Cancer Res* 1996;**56**(3):448–51.
18. Munzer JS, Basak A, Zhong M, Mamarbachi A, Hamelin J, Savaria D, et al. In vitro characterization of the novel proprotein convertase PC7. *J Biol Chem* 1997;**272**(32):19672–81.
19. Nour N, Basak A, Chretien M, Seidah NG. Structure–function analysis of the prosegment of the proprotein convertase PC5A. *J Biol Chem* 2003;**278**(5):2886–95.
20. Oexle K, Ried JS, Hicks AA, Tanaka T, Hayward C, Bruegel M, et al. Novel association to the proprotein convertase PCSK7 gene locus revealed by analysing soluble transferrin receptor (sTfR) levels. *Hum Mol Genet* 2011;**20**(5):1042–7.
21. Qiu Q, Basak A, Mbikay M, Tsang BK, Gruslin A. Role of pro-IGF-II processing by proprotein convertase 4 in human placental development. *Proc Natl Acad Sci U S A* 2005;**102**(31):11047–52.
22. Rousselet E, Benjannet S, Hamelin J, Canuel M, Seidah NG. The proprotein convertase PC7: unique zymogen activation and trafficking pathways. *J Biol Chem* 2011;**286**(4):2728–38.
23. Rousselet E, Benjannet S, Marcinkiewicz E, Asselin MC, Lazure C, Seidah NG. The proprotein convertase PC7 enhances the activation of the EGF receptor pathway through processing of the EGF precursor. *J Biol Chem* 2011;**286**:9185–95.
24. Seidah NG. The proprotein convertases, 20 years later. *Methods Mol Biol* 2011;**768**:23–57.
25. Seidah NG, Chretien M. Eukaryotic protein processing: endoproteolysis of precursor proteins. *Curr Opin Biotechnol* 1997;**8**(5):602–7.
26. Seidah NG, Chretien M. Proprotein and prohormone convertases: a family of subtilases generating diverse bioactive polypeptides. *Brain Res* 1999;**848**(1–2):45–62.
27. Seidah NG, Day R, Hamelin J, Gaspar A, Collard MW, Chretien M. Testicular expression of PC4 in the rat: molecular diversity of a novel germ cell-specific Kex2/subtilisin-like proprotein convertase. *Mol Endocrinol* 1992;**6**(10):1559–70.
28. Seidah NG, Hamelin J, Mamarbachi M, Dong W, Tardos H, Mbikay M, et al. cDNA structure, tissue distribution, and chromosomal localization of rat PC7, a novel mammalian proprotein convertase closest to yeast kexin-like proteinases. *Proc Natl Acad Sci U S A* 1996;**93**(8):3388–93.
29. Seidah NG, Mayer G, Zaid A, Rousselet E, Nassoury N, Poirier S, et al. The activation and physiological functions of the proprotein convertases. *Int J Biochem Cell Biol* 2008;**40**(6–7):1111–25.
30. Sun X, Essalmani R, Susan-Resiga D, Prat A, Seidah NG. Latent TGF-beta binding proteins-2 and -3 inhibit the proprotein convertase 5/6A. *J Biol Chem* 2011.
31. Susan-Resiga D, Essalmani R, Hamelin J, Asselin MC, Benjannet S, Chamberland A, et al. Furin is the major processing enzyme of the cardiac-specific growth factor bone morphogenetic protein 10. *J Biol Chem* 2011;**286**(26):22785–94.

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32. Szumska D, Pieles G, Essalmani R, Bilski M, Mesnard D, Kaur K, et al. VACTERL/caudal regression/currarino syndrome-like malformations in mice with mutation in the proprotein convertase Pcsk5. *Genes Dev* 2008;**22**(11):1465–77.
33. Tortorella MD, Arner EC, Hills R, Gormley J, Fok K, Pegg L, et al. ADAMTS-4 (aggrecanase-1): N-terminal activation mechanisms. *Arch Biochem Biophys* 2005;**444**(1):34–44.
34. Xiao Y, Chen G, Richard J, Rougeau N, Li H, Seidah NG, et al. Cell-surface processing of extracellular human immunodeficiency virus type 1 Vpr by proprotein convertases. *Virology* 2008;**372**(2):384–97.
35. Zhong M, Munzer JS, Basak A, Benjannet S, Mowla SJ, Decroly E, et al. The prosegments of furin and PC7 as potent inhibitors of proprotein convertases. In vitro and ex vivo assessment of their efficacy and selectivity. *J Biol Chem* 1999;**274**(48):33913–20.

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Chapter 249

Proprotein Convertases SKI-1/S1P and PCSK9

Nabil G. Seidah

ABSTRACT

SKI-1/S1P and PCSK9 are the last members of the proprotein convertase family. The former localizes to the *cis/medial* Golgi and is implicated in the processing of several membrane-bound transcription factors, e.g. SREBPs that regulate cholesterol and fatty acid synthesis and metabolism, ATF6 that affects the endoplasmic reticulum stress response, and other CREB-like proteins. Other substrates include proBDNF, prosomatostatin, the RGMa that regulates axonal connections, and the α/β-subunit precursor of GlcNAc-1-Ptase needed to phosphorylate mannose residues for protein export to lysosomes. It is also needed for infectivity of arenaviruses via the activation of their cell-surface glycoproteins. Mice lacking SKI-1/S1P die during very early stages of embryogenesis. PCSK9 only cleaves itself and then acts as a receptor binding protein. It is implicated in the induced intracellular degradation of the LDLR, VLDLR, and ApoER2 via its ability to bind these receptors and drag them to endosomes/lysosomes. Mice and humans lacking PCSK9 live and exhibit a hypocholesterolemia phenotype.

DISCOVERY AND NOMENCLATURE

The generation of bioactive proteins and peptides by limited proteolysis of inactive precursors is an evolutionarily ancient mechanism determining the level and duration of specific bioactivities. It was realized since the early 1970s that limited cellular processing of precursors occurs at single or pairs of basic amino acids (aa) and also at sites that are not occupied by basic residues. The search for the enzyme(s) responsible for such cleavages led to the identification of a novel pyrolysin-like homolog of subtilisin (in peptidase subfamily S8A) known either as subtilisin kexin-isozyme-1 (SKI-1),[30,31] or site-1 protease (S1P).[9] These studies revealed that SKI-1/S1P is a major processing enzyme of the endoplasmic reticulum/Golgi (ER/Golgi) lumen that acts on sterol regulatory element-binding proteins (SREBPs), and thereby regulates cholesterol and fatty acid biosynthesis. It is also critical for the N-terminal processing of probrain derived neurotrophic factor (proBDNF)[30] and prosomatostatin, resulting in products that are sorted to the regulated secretory pathway of endocrine and neural cells. Homology searches reveal that SKI-1/S1P is an enzyme highly conserved in vertebrate and invertebrate organisms.

FUNCTIONS IN POLYPEPTIDE BIOSYNTHESIS/PROCESSING

SKI-1/S1P

Zymogen Activation and Cellular Trafficking

Biosynthetic analysis of full-length SKI-1/S1P (gene *MBTPS1*) shows that proSKI-1/S1P (~150 kDa) is a membrane-bound zymogen that is progressively autocatalytically processed at three N-terminal prosegment sites (RKVF$_{133}$↓RSLK$_{137}$↓YA followed by RRLL$_{186}$↓RA) within the ER/*cis*-Golgi to finally generate the membrane-bound active SKI-1/S1P (98 kDa; aa 187–1052). The full-length prosegment (14 kDa; human aa 18–186) is both a chaperone and an inhibitor of the enzyme, which is autocatalytically cleaved into inactive 10- and 8-kDa fragments.[8] The mature protein has five N-glycosylation sites at Asn$_{236,305,515,728,939}$. These glycosylation sites are critical for the function of the enzyme and if N-glycosylation is inhibited by 5-thiomannose-containing disaccharides, it blocks the intracellular activity of SKI-1/S1P on SREBPs.[35]

Subcellular localization studies revealed that SKI-1/S1P can be found in the *cis/medial* Golgi and in endosomes/lysosomes, but not at the cell surface.[23] Thus, different from furin, proprotein convertase 7 (PC7) and PC5/6-B, which cycle from the cell surface to the trans Golgi network (TGN), SKI-1/S1P does not reach the cell surface, and probably transits directly from the TGN to endosomes/lysosomes.[23] In addition, careful analysis of the intracellular forms of SKI-1/S1P revealed a much shorter (~8.5 kDa) alternate translation product that may act as a dominant negative form of the enzyme.[23]

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00249-9
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SKI-1/S1P Substrates In Vitro

Because SKI-1/S1P is membrane bound, to obtain sufficient amounts of an active secreted enzyme, we engineered a soluble form called BTMD-SKI-1/S1P (before transmembrane domain), by placing a stop codon upstream of the C-terminal transmembrane segment. Thus, the soluble, *in vitro* active approximately 98-kDa form produced spans the segment ending either at $YNQE_{996}$ or at the shed site $QKLL_{953}$.[8] We thus obtained both a vaccinia virus recombinant and stable transfectants in CHO or HEK293 cells and showed that both systems yield an approximately 98-kDa active enzyme, in the concentration range of 100 μg/l. A larger milligram/liter quantity of active soluble SKI-1/S1P was also isolated from a baculovirus recombinant in insect cells.

Biochemical and enzymatic characterization of SKI-1/S1P reveal it to be a serine protease, because mutation of the active-site Ser abolished its autoactivation and its exit from the ER.[8,9,30] Further, *in vitro* assays with peptides mimicking either the SKI-1/S1P prosegment cleavage sites or that of the Lassa virus (LAV) glycoprotein reveal it to be maximally active around neutral pH.[2,30] The best *in vitro* SKI-1/S1P substrate is that which mimics the processing of the LAV glycoprotein precursor (GP0) to form the proteins GP1 and GP2 that are responsible for viral fusion with the host cell membrane. The favored quenched fluorogenic RRLL-containing substrate is Abz-Asp-Ile-Tyr-Ile-Ser-Arg-Arg-Leu-Leu↓Gly-Thr-Phe-Thr-(3-nitro)Tyr-Ala-$CONH_2$.[2] The 4-, 7- and 8-mer fluorogenic substrates RRLL-MCA, YISRRLL-MCA and IYISRRLL-MCA are also very efficiently cleaved by SKI-1/S1P.[20]

Potent inhibitors of SKI-1/S1P include the cell permeable decanoyl-RRLL-chloromethyl ketone,[20] a transfectable cDNA coding for the N-terminal prosegment R134E mutant of SKI-1/S1P,[24] or an α1-antitrypsin variant containing the RRVL sequence in the reactive site loop,[24] and a Pfizer pharmaceuticals nonpeptidyl small molecule aminopyrrolidine-amide inhibitor known as PF-429242.[12] The latter inhibits LAV glycoprotein processing.[33] The general serine protease inhibitor 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF) or decanoyl-RRLL-chloromethyl ketone also blocks the activity of SKI-1/S1P in cells and during primary bone mineralization.[11]

The compilation of all the known SKI-1/S1P-substrates suggests that the major recognition motif of SKI-1/S1P is: **R**-X-(**hydrophobic**)-Z↓,[20] with the basic P4 and hydrophobic P2 positions being the most critical ones (Fig. 1). Based on this conclusion, our results show that we could inhibit the cellular SKI-1/S1P activity using either an overexpression of an R134E P4 mutant of the prosegment of SKI-1/S1P, or alpha-1 antitrypsin variants such as those containing either an RRVL or RRYL sequences at the reactive center loop of this serpin.[24] The crystal structure of SKI-1/S1P is not yet defined, as it would really improve our understanding of the structure of this critical protease.

SKI-1/S1P Substrates Ex Vivo

In general, SKI-1/S1P processes precursors containing basic and hydrophobic/aliphatic residues at P4 and P2, respectively, with a relatively relaxed acceptance of amino acids at P1 and P3 (Fig. 1). SKI-1/S1P shows a preference for cleavage at luminal sites of membrane-bound transcription factors spanning the membrane either once (ATF6, Luman, Oasis, CREB-H, CREB-4) or twice (SREBP-1 and -2). The cleavage of these transcription factors is usually followed by a second cleavage by site-2 protease (S2P), a metalloprotease in the *medial* Golgi, to release a cytosolic fragment that translocates into the nucleus and activates the transcription of multiple mRNAs to produce proteins/enzymes that regulate many processes in the cell such as cholesterol and fatty acid biosynthesis (SREBPs), the tissue-specific ER stress response regulation (ATF6, CREB3, CREB3L1, CREB4, and CREB-H) (Fig. 1). Amazingly, SKI-1/S1P also regulates the sorting of proteins to lyosomes from the Golgi via the cleavage activation of the α/β-subunit precursor of the GlcNAc-1-phosphotransferase (GlcNAc-1-Ptase)[16] (Fig. 1). However, novel unsuspected functions of SKI-1/S1P are yet to be discovered, as this protease is also responsible for the generation of the N-terminal prosomatostatin peptide Antrin ($prosomatostatin_{1-10}$ fragment), and for the processing of the prodomain of proBDNF.[30] Sortilin binds to numerous ligands and plays a major role in mannose 6-phosphate receptor-independent transport of lysosomal hydrolases using motifs in intracellular luminal domains that mediate trafficking from the Golgi and late endosomes. Thus, the cleavage of prodomain of proBDNF

Substrate	P8	P6	P4	P2		P2′	P4′
(h) Pro-SKI-1 site B	R	K	V	F	**R**	S	**L**	K ↓	Y	A	E	S
site B′	V	T	P	Q	**R**	K	**V**	F ↓	R	S	L	K
site C	R	H	S	S	**R**	R	**L**	L ↓	R	A	I	P
(h) SREBP-2		S	G	S	G	**R**	S	**V**	L ↓	S	F	E	S
(h) SREBP-1		H	S	P	G	**R**	N	**V**	L ↓	G	T	E	S
(h) ATF6		A	N	Q	R	**R**	H	**L**	L ↓	G	F	S	A
(h) Luman		G	V	L	S	**R**	Q	**L**	R ↓	A	L	P	S
(m) OASIS (CREB3L1)		Q	M	P	S	**R**	S	**L**	L ↓	F	Y	D	D
(h) CREB-H		R	V	F	S	**R**	T	**L**	H ↓	N	D	A	A
(h) CREB-4		G	V	T	S	**R**	N	**I**	L ↓	T	H	K	D
(h) GlcNAc-1-Ptase		K	N	T	G	**R**	Q	**L**	K ↓	D	T	F	A
(h) proBDNF		K	A	G	S	**R**	G	**L**	T ↓	S	L	A	D
(r) Somatostatin		D	P	R	L	**R**	Q	**F**	L ↓	Q	K	S	L
Lassa (LAV)		I	Y	I	S	**R**	R	**L**	L ↓	G	T	F	T
CCHFV		S	S	G	S	**R**	R	**L**	L ↓	S	E	E	S
LCMV		K	F	L	T	**R**	R	**L**	A ↓	G	T	F	T

FIGURE 1 Comparative cleavage sites of SKI-1/S1P substrates, showing the conservation of and Arg at P4 and a hydrophobic/aliphatic residue at P2 as the major determinants of enzyme recognition.

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by SKI-1/S1P may modify its subcellular sorting and degradation in lysosomes that depends on sortilin binding to the prodomain. Finally, both SKI-1 and furin are involved in axonal growth, via cleavage of the repulsive guidance molecule (RGMa), which is essential for neogenin-mediated outgrowth inhibition.[32]

In Vivo Mouse Knockout Models (For Review see Ref. 31)

In situ hybridization data obtained in a day 2 postnatal rat also provide evidence of a widespread if not ubiquitous distribution of SKI-1/S1P mRNA.[30] Thus, SKI-1/S1P mRNA is detected in liver, the anterior and intermediate lobes of the pituitary as well as in the pituicytes of the pars nervosa, developing skin, striated muscles, cardiac muscles, bones and teeth, brain, and many internal organs. Strong hybridization signals are detected in the retina, cerebellum, pituitary, submaxillary, thyroid and adrenal glands, molars, thymus, kidney, and intestine. In the central nervous system, SKI-1/S1P mRNA labeling is mostly confined to neurons.

The gene name for SKI-1/S1P derives from an acronym to membrane-bound transcription factor site-1 protease (human *MBTPS1*, mouse *Mbtps1*) precursor, sometimes called *PCSK8*. The gene coding for SKI-1/S1P maps to human and mouse chromosomes 16q24 and 8, respectively. Complete gene deletion in mouse leads to death at the blastocyst stage (embryonic day 3.5) with the absence of inner cell mass formation. Complete knockdown of SKI-1/S1P mRNA in zebrafish is also lethal, revealing a disorganization of chondrocytes and showing that SKI-1/S1P is critical in cartilage formation.

The importance of SKI-1/S1P in the processing of SREBPs and in turn in the regulation of cholesterol and fatty acid metabolism was confirmed using conditional knockout studies using the Mx1-cre transgene. Thus, although a complete silencing of SKI-1/S1P results in an embryonic lethal phenotype, the elimination of approximately 70–90% of SKI-1/S1P protein expression in liver (but only ~50% of the active enzyme) results in a drop of about 50% in the level of circulating cholesterol and triglycerides. In addition, very low levels of nuclear SREBPs and reduced mRNA of their target genes are observed. In these mice, the liver biosynthesis of cholesterol and fatty acids declines by approximately 75%. Low-density lipoprotein (LDL) receptor mRNA declines by 50%, as does the clearance of LDL from plasma, but plasma cholesterol falls, suggesting that LDL production is reduced. In addition, tissue-specific knockout of the SKI-1/S1P gene in cartilage reveals that this protease is also essential for endochondral bone formation in mice.

A study of the mRNAs affected by overexpression of the R134E-prosegment inhibitor of SKI-1/S1P in HepG2 cells,[6] or the small-molecule inhibitor PF-429242[12] using μ-array analyses reveals that SKI-1/S1P inhibition reduces lipogenic enzyme gene expression and lipid synthesis in cultured cells and experimental animals. These results raise the possibility that SKI-1/S1P inhibitors, such as the non-peptidyl PF-429242,[12] might be effective lipid-lowering agents, but careful monitoring is advised for any side effects implicating other pathways affected by lowering the SKI-1/S1P activity.

Pathophysiological Implications

SKI-1/S1P plays a major role in the processing of surface GP0 of infectious arenaviruses such as LAV (Fig. 1), which is endemic in West Africa and is estimated to affect some 100,000 people annually. Other GP0s of arenaviruses activated by SKI-1/S1P include crimean Congo hemorrhagic fever virus (CCHFV), and lymphocytic choriomeningitis virus (LCMV) (Fig. 1). The present results suggest that arenavirus GP0s evolved to mimic SKI-1/S1P autoprocessing sites, probably ensuring efficient cleavage and perhaps avoiding competition with SKI-1/S1P's cellular substrates, which are less efficiently cleaved. No vaccines or antivirals are available against such deadly viruses, emphasizing the need for a potent SKI-1/S1P inhibitor to fight such deadly infections.

Mucolipidosis II is a severe lysosomal storage disorder caused by defects in the α and β subunits of the hexameric GlcNAc-1-Ptase complex essential for the formation of the mannose 6-phosphate targeting signal on lysosomal enzymes. The observation that SKI-1/S1P activates the α/β-subunit precursor of the GlcNAc-1-Ptase is physiologically pertinent in the biogenesis of lysosomes. In fact, loss of SKI-1/S1P expression, or by extension its inhibition, may result in lysosomal dysfunction, because instead of sorting via the mannose-6-phosphate receptor to lysosomes, proteins lacking mannose 6-phosphate will then be secreted into the extracellular matrix.[16]

Analysis of the SNPs associated with the human gene coding for SKI-1/S1P revealed many heterozygote mutations (http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?geneId=8720). Of these, 10 SNPs are deemed deleterious, including C38S, Y54C, S71X, R130Q (eliminates B' autocatalytic site), R191C, G220E (eliminates Gly_{220} important for active-site Asp* in the conserved DTG_{220} sequence in all subtilases), T413I (disrupts the Thr_{413} important for the active-site Ser* in the conserved $GT_{413}S$* sequence in all subtilases), S724C, S954C, T1049-frame shift (will extend the cytosolic tail from VHPPKT-PSV_{1052}-stop to VVPPKT-$LRCDRQPG_{1057}$-stop). However, the frequencies of these mutations are rather rare, and thus, their correlation with a specific phenotype in human will be difficult. Further, based on the liver-specific knockout of SKI-1/S1P, it is clear that a reduction of >80% of the activity of SKI-1/S1P is needed to observe a phenotype, suggesting that a heterozygote loss-of-function (LOF) SNP may seem to be a silent mutation.

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PCSK9

Complications resulting from cardiovascular disorders are the main cause of death worldwide. Elevated plasma cholesterol levels result in excess cholesterol deposition in arterial vessel walls and are a major risk factor for atherosclerosis and premature death by coronary artery disease. In the blood, cholesterol is transported in lipoprotein particles, approximately 70% of which in human are LDL. LDL is constantly cleared by internalization into cells by the LDL receptor (LDLR), which binds and internalizes LDL via its unique apolipoprotein B (apoB) protein. Mutations in LDLR or APOB genes are major causes for the frequent autosomal dominant genetic disorder known as familial hypercholesterolemia. Although hypercholesterolemia affects 1 in 20 subjects, inherited autosomal dominant hypercholesterolemia (ADH; OMIM #143890), which results in even higher levels of cholesterol, only occurs at a frequency of 1 in 500. To date, it has been linked to heterozygous dominant mutations occurring in the genes encoding the LDLR (67%), apoB (14%) or PCSK9 (~2%).[31] However, approximately 17% of ADH-affected patients have no mutations in these three genes, indicating that other genes remain to be identified. Thus, the rare occurrence of dominant heterozygote mutations in *PCSK9* is approximately 1 in 25,000 (2% of 1:500). Patients affected by this type of familial hypercholesterolemia are typically characterized by plasma LDL-cholesterol (LDLc) greater than the 95th percentile, and the presence of tendon xanthomas and premature atherosclerosis.

Originally named NARC-1 for "neural apoptosis-regulated convertase," PCSK9 was first discovered and characterized in my laboratory.[28] In collaboration with C. Boileau and M. Abifadel in Paris, we established the association between single-point mutations in the *PCSK9* gene and autosomal dominant hypercholesterolemia in two French families.[1] Thus, *PCSK9* is the third gene associated with familial hypercholesterolemia,[1,31] with *LDLR* and *APOB* as the other two. Later, Cohen et al. showed that nonsense PCSK9 mutations are associated with hypocholesterolemia in approximately 2% of black subjects.[4] Up to approximately 7% of black Africans living around the equator exhibit the loss of one allele of PCSK9. In summary, point mutations in PCSK9[31] are associated with either familial hypercholesterolemia (gain-of-function (GOF) of PCSK9) or hypocholesterolemia (LOF of PCSK9). Two women lacking functional PCSK9 exhibited an approximately 85% reduction in circulating LDLc. *Pcsk9*$^{-/-}$ mice are also viable and exhibit an approximately 80% drop in circulating LDLc,[34] emphasizing the therapeutic potential of a PCSK9 inhibitor/silencer.

Zymogen Activation and Cellular Trafficking

The discovery of PCSK9, the 9th member of the PC family[28,31] as a third protagonist in ADH shed light on the unsuspected regulation of the LDLR levels by this protein. PCSK9 is mostly expressed in hepatocytes and small intestinal enterocytes.[28] By an as yet unknown mechanism(s), and independent of its enzymic activity,[14] PCSK9 enhances the degradation of cell-surface LDLR in endosomes/lysosomes, resulting in increased circulating LDLc. Surprisingly, although PCSK9 undergoes an autocatalytic cleavage of its N-terminal prosegment that remains associated with PCSK9 and keeps it in an inhibited state, it has no other substrates but itself. Statins, the best cholesterol-lowering drugs, reduce cholesterol synthesis by inhibiting the rate-limiting HMG-CoA reductase. The resulting cellular cholesterol depletion leads to transcription of genes involved in cholesterol metabolism, including those of PCSK9 and LDLR. While upregulation of LDLR reduces circulating LDLc, that of PCSK9 counterbalances it through degradation of the LDLR. PCSK9 inhibition is thus a promising complement to statin therapy to lower LDLc.

PCSK9 (692 aa in human) comprises a signal peptide (aa 1–30) followed by a prosegment (Pro; aa 31–152), catalytic (aa 153–407), hinge region (HR; aa 408–452), and C-terminal Cys-His-rich domain (CHRD; aa 453–692) segments. After translocation into the ER, the prosegment is autocatalytically cleaved at the $VFAQ_{152}$↓SIP site.[3] In all the other PCs, the prosegment is an essential intramolecular chaperone and inhibitor, which is usually removed intracellularly to yield a fully active protease.[29] Different from other PCs, PCSK9 is secreted as a stable, enzymatically inactive, noncovalent complex [Pro≡PCSK9].[3,28,31] In accordance, enhanced degradation of the LDLR[3,17,19] induced by PCSK9 does not require its catalytic activity.[14] In human and mouse plasma, both full-length PCSK9 (aa 152–692) and a truncated form PCSK9-ΔN_{218} (aa 219–692) can be detected. The latter, which has no activity on LDLR, is probably generated by Furin, because it efficiently cleaves PCSK9 ex vivo at $RFHR_{218}$↓ and mice lacking furin in hepatocytes no longer exhibit the presence of this cleaved form in plasma.[10] Interestingly, the human GOF R218S, F216L and R215H mutations associated with hypercholesterolemia, prevent such a cleavage and presumably result in increased levels of active PCSK9. To optimize PCSK9 inactivation by Furin, we designed a PCSK9-$RRRR_{218}$EL mutant, which results in the secretion of only the inactive PCSK9-ΔN_{218}. We also contributed to the set-up of an ELISA assay of circulating human PCSK9, revealing a good correlation between the levels of PCSK9 and LDLc in human plasma.[7] Sequencing of *PCSK9* exons from individuals at the extremes of the PCSK9 distribution provides a database of PCSK9 mutations resulting in valuable tools in structure–function analyses. Indeed, we have identified a novel LOF variant, R434W, associated with low levels of circulating PCSK9 and LDLc.[7] This mutation, which occurs in an exposed loop of the hinge region, does not prevent LDLR binding, but drastically reduces the

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ability of PCSK9 to enhance the degradation of the LDLR.[7] A database of all PCSK9 mutations is online (http://www.ucl.ac.uk/ldlr/LOVDv.1.1.0/index.php?select_db=PCSK9).

The crystal structure of PCSK9 reveals three separate domains: the prosegment and catalytic domain in tight complex, and the spatially separated CHRD.[5] In all three crystal structures, aa 31–60 of the prosegment and portions of the CHRD are unresolved, indicating their unstructured nature. Biochemical and cocrystal structure studies reveal that aa 153–156 and 367–381 directly interact with the EGF-A domain of the LDLR. The most severe mutation associated with hypercholesterolemia, D374Y, is within aa 367–381 and results in an approximately 25-fold higher affinity of PCSK9 toward LDLR.[5] The shallow binding surface on PCSK9 is distant from its catalytic site, and the EGF-A domain of LDLR makes no contact with either the prosegment or the CHRD.

The Prosegment of PCSK9

Even though the prosegment does not bind the EGF-A domain of LDLR, it negatively regulates this interaction. The removal of its N-terminal acidic stretch (aa 31–53), which exhibits Tyr_{38} sulfation[28] and Ser_{47} phosphorylation, enhances the binding of PCSK9 to LDLR by up to approximately 7-fold. Whether this unstructured acidic stretch,[5] binds another domain of PCSK9 and/or interacts with another protein is yet to be defined.

The HR-CHRD of PCSK9

The HR is an exposed loop structure connecting the catalytic domain and the CHRD.[7] The latter is composed of a six β-strand structure repeated 3-times and hence forming 3-subdomain modules M1, M2, and M3.[5] In the reported crystal structures, disordered segments include aa 573–584 (in M2), 660–667 (in M3), and the C-terminal aa 683–692 exhibiting Ser_{688} phosphorylation. Many GOF (R469W, E482G, R496W, F515L, and H553R) and LOF (Q554E and the new one R434W)[7] mutations within the HR-CHRD were identified, but their underlying mechanisms are unknown.

The Cellular Biology of PCSK9 (For Review see Ref. 27)

PCSK9 Targets

More than 20 years' experience with PCs led us to predict that PCSK9 should have more than one target. We thus first tested PCSK9 ex vivo activity on other members of the LDLR-like protein family. The closest members to LDLR, i.e. very LDLR (VLDLR) and ApoER2, are degraded faster in the presence of PCSK9, in a cell-type dependent fashion.[21] This was confirmed *in vivo*, as circulating PCSK9 enhances the degradation of the VLDLR in adipose tissues.[26] We also showed that PCSK9 enhances the degradation of the major hepatitis C virus (HCV) receptor, the tetraspanin protein CD81.[15]

PCSK9-Enhanced Degradation of the LDLR

The cell-surface localization of PCSK9 is dependent on the presence of the LDLR. The two proteins also colocalize in early and late endosomes. We previously developed an approach in which the fusion of a secretory protein of interest with the transmembrane domain and cytosolic tail (TM-CT) of the lysosomal protein Lamp1 results in an efficient degradation of its partners. This strategy was applied to PCSK9 to better target its partners, including the LDLR, to degradative compartments. Thus, fusion of PCSK9 to the TM-CT of Lamp-1 (PCSK9-Lamp1) results in superactive forms of PCSK9 capable of depleting cells from its targets[21] including LDLR, VLDLR, and ApoER2.[21]

Extracellular versus Intracellular Pathways

The extracellular pathway is defined by the ability of extracellular PCSK9 to target the LDLR. Indeed, incubation of cells with PCSK9, but not PCSK9-ΔCHRD, enhances the degradation of cell-surface LDLR in endosomes/lysosomes. Because the PCSK9-ΔCHRD still interacts with the LDLR and is internalized, the CHRD is probably essential for the trafficking of [PCSK9≡LDLR] to endosomes/lysosomes. Internalization of cell-surface LDLR requires the adaptor protein ARH that binds its cytosolic tail on the cytoplasmic side of clathrin heavy chain-coated vesicles. In accordance, many pharmaceutical companies developed clinically relevant monoclonal antibodies that inhibit the PCSK9 interaction with LDLR. These mAbs are now in clinical trials (Fig. 2). However, we more recently showed the existence of an intracellular pathway. First, PCSK9 can degrade the LDLR *in vivo* and ex vivo in the absence of ARH. Second, siRNAs knockdown of both a and b chains of clathrin light chains, which exclusively blocks the intracellular pathway by preventing the trafficking from the TGN to lysosomes, resulted in a drastically decreased LDLR degradation.[22]

HR-CHRD Binding Proteins

In view of the critical importance of the HR-CHRD for targeting [PCSK9≡LDLR] to lysosomes, we hypothesized that it binds directly or indirectly a membrane-associated protein that would sort the complex to lysosomes. Accordingly, a Far-Western screen of endogenous interactors of PCSK9 revealed that annexin A2 (AnxA2) binds the HR-CHRD, and inhibits the ability of PCSK9 to enhance the degradation of the LDLR. AnxA2 lacks a signal peptide but is found at the cell surface of endothelia, keratinocytes, epithelial and tumor cells. The N-terminal repeat domains (R1-R7) of the LDLR itself can bind the CHRD, especially

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Type of agent	Company	Stage	Comments
mAb-REGN727	SA-Regeneron	Ph 2	30-65% LDL-C↓ – 12 wks
mAb-REGN727 + 80 mg Atorvastatin	SA-Regeneron	Ph 2	>65% LDL-C↓ *vs* 10% Atorvastatin alone
mAb-AMG145	Amgen	Ph 1	Completion: Mar-2012
mAb-RN316	Pfizer-Rinat	Ph 2	Completion: Jan-2012
mAb	Merck	In develop.	WO-patent appl. 2010
Adnectin	BMS-Adnexus	Pre-Clinical	*In preparation* for Ph 1
siRNA	Alnylam-Novartis	Pre-Clinical	Cationic lipidoid formulation
LNA-SPC5001	Santaris-Novartis	Ph 1	Preclin. 74% LDL-C↓
Small molecule	Shifa Biomedical Corp	Pre-Clinical	*In preparation* for Ph 1

FIGURE 2 Tabulation of the various ongoing clinical trials testing the effects of inhibitory PCSK9 mAbs or adnectins (engineered inhibitory fragment of fibronectin), or silencing antisense oligonucleotides (RNAi or locked nucleic acid, LNA). The phase 1 (Ph 1) and phase 2 (Ph 2) trials are indicated.

at acidic pHs, suggesting that at neutral pHs, and via its catalytic domain, PCSK9 first binds the EGF-A domain of the cell-surface LDLR, and then once the complex is internalized in acidic endosomes, the CHRD can then bind the repeat domains of the LDLR. However, how does this process lead to sorting of endosomes to lysosomes is still a mystery, especially because ex vivo PCSK9 can degrade an LDLR lacking a cytosolic tail,[13] which normally would bind cytosolic motor proteins implicated in subcellular movement of organelles.

In Vivo Mouse Pcsk9 Knockout Models

Knockout (KO; *Pcsk9*$^{-/-}$) mice exhibit higher levels of LDLR protein in liver and 42% less circulating total cholesterol, with an approximately 80% drop in LDLc.[25,34] By contrast, transgenic mice overexpressing PCSK9 exhibit 5- to 15-fold higher levels of LDLc.[34] We also developed mice carrying conditional floxed alleles, in which the proximal promoter and exon 1 of *Pcsk9* are flanked by *loxP* sites (*Pcsk9*$^{f/f}$). In mice expressing the Cre recombinase under the control of the albumin promoter, *Pcsk9* was specifically inactivated in hepatocytes by *loxP* sites recombination (*Pcsk9*$^{f/f}$ *Alb-cre*). Total KO and liver-specific KO (LivKO) mice exhibited 42% and 27% less circulating total cholesterol, respectively, indicating that hepatic PCSK9 is responsible for approximately two-thirds of the phenotype. This suggests that the role of PCSK9 in cholesterol homeostasis is primarily mediated by its activity on LDLR, because the liver accounts for approximately 70% of the body LDLc clearance. Analysis of [*Pcsk9*$^{f/f}$ *Alb-cre*] livers shows that PCSK9 expression is restricted to hepatocytes, from where circulating PCSK9 mostly originates. We generated double KO mice lacking both PCSK9 and the LDLR (dKO; *Pcsk9*$^{-/-}$ *Ldlr*$^{-/-}$) and showed that their plasma lipid profile is identical to that of *Ldlr*$^{-/-}$ mice, confirming that PCSK9 activity on LDLR mediates most of its role in cholesterol homeostasis. PCSK9 also binds VLDLR and apoER2[21] closely related to LDLR. Indeed, VLDLR proteins accumulate in adipose tissue of PCSK9 KO mice, resulting in marked adipocyte hypertrophy.[26] PCSK9 is thus pivotal in fat metabolism: it maintains high circulating cholesterol levels via hepatic LDLR degradation, but it also limits visceral adipogenesis likely via adipose VLDLR regulation.

Pathophysiological Implications

Circulating PCSK9

Human plasma contains approximately 100–200 ng/ml of PCSK9.[7] Its physiological role remains undefined, as well as that of its truncated form PCSK9-ΔN_{218} that represents approximately 50% of the PCSK9 species in mouse plasma. The analysis of transgenic lines that overexpress low or high levels of mouse PCSK9 in the liver indicates that only supraphysiological levels of circulating PCSK9 (~30-fold higher) increase circulating cholesterol (+60%); a 3-fold increase has no significant impact on circulating cholesterol. Moreover, results from transgenic mice expressing very high levels of human PCSK9 in kidney or liver, or continuous infusions of recipient wildtype (WT) mice with recombinant human PCSK9 indicated that micrograms per milliliter amounts of circulating PCSK9 are required to significantly affect liver LDLR protein levels. Transgenic expression in kidney (32-fold the endogenous liver levels) leads to 100% loss of LDLR protein in liver, but only 50% loss in kidney. Thus, even at high levels, circulating PCSK9 reduces primarily liver LDLR with little effect on extrahepatic tissues, e.g. adrenals. By contrast, our genetic models show that at physiological levels circulating PCSK9 is responsible for the downregulation of VLDLR protein levels in mouse adipose tissues, and its absence gives an adipocyte hypertrophy phenotype.[26]

Partial Hepatectomy (PHx)

To better understand the role of PCSK9 in the liver, its major site of expression, we challenged this tissue by performing

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sham or PHx operations in WT and KO mice. Hepatectomized KO, but not WT, mice develop lesions, still visible 10 days after the liver had recovered its original mass. In addition, the proliferation of KO hepatocytes is delayed.[34] Critically low levels of cholesterol may impede efficient liver regeneration. Indeed, HMG-CoA reductase mRNA levels are increased in KO regenerating livers only (2.5-fold at 72 h post-PHx). Also, when fed a high cholesterol diet one week before PHx, *Pcsk9*$^{-/-}$ mice no longer exhibit necrotic lesions.

Total Lack of PCSK9 May Affect β-cell Function and Predispose to Diabetes

It was originally observed that the pancreatic insulin-producing β-TC3 cells express high levels of PCSK9.[28] LDLR is also highly expressed in insulin-producing pancreatic islet β-cells, possibly affecting the function of these cells. We showed that compared with control mice, PCSK9-null male mice over 4 months of age carry more LDLR and less insulin in their pancreas; they were hypoinsulinemic, hyperglycemic, and glucose-intolerant; their islets exhibit signs of malformation, apoptosis, and inflammation. Collectively, these observations suggest that PCSK9 may be necessary for the normal function of pancreatic islets.[18]

Ongoing Clinical Trials Using PCSK9 Inhibitors/Silencers

In the USA alone, some 63 million individuals have elevated levels of LDLc. Of these, 12 million patients do not reach target LDLc levels with the available medications. Clearly, an alternative therapy is urgently needed. Many polyclonal antibodies against PCSK9 have been reported. Some of these are produced from a fragment of PCSK9 produced in bacteria, and others used the whole protein produced in HEK293 cells, or even via injection of a plasmid coding for human PCSK9. Further, some monoclonal antibodies against PCSK9 block its extracellular activity on LDLR by either interfering with the PCSK9 direct interaction with the EGF-A domain of the LDLR or by modifying the conformation of the CHRD. Another approach to inhibit the function of PCSK9 is to reduce its mRNA levels by injecting antisense oligonucleotides, e.g. as therapeutic RNAi, or 13-mer locked nucleic acids (Fig. 2). So far, no small molecule has been found to inhibit the function of PCSK9. The use of monoclonal antibodies and antisense oligonucleotides as injectable compounds has now entered in phase I and II clinical trials (Fig. 2). Preliminary Phase II trial results for REGN727—a human anti-PCSK9 monoclonal antibody codeveloped by Regeneron and Sanofi—indicate that it effectively and safely reduces the levels of low-density lipoprotein-cholesterol (LDLc) in patients with hypercholesterolemia undergoing statin therapy. Inhibiting PCSK9 function is expected to be specific and to have lower side effects than statins, which have a more extended repertoire of consequences, aside from only reducing LDLc production and increasing its uptake by liver. Accordingly, a combination therapy consisting of a statin and a PCSK9 inhibitor may be beneficial, as it would reduce the effective dose of the former. We will have to await clinical efficacy and side effects before we can assess the tolerability of this promising approach to silence the function of PCSK9.

CONCLUSIONS

Since 1999 when SKI-1/S1P was discovered, it became evident that some of the proprotein convertases would be implicated in lipid and sterol metabolism. However, little did we know that aside from these functions, SKI-1/S1P is also implicated in a wide variety of other cellular pathways that regulate ER-stress, chondrocyte differentiation, bone mineralization, brain development, and neuronal queuing, and lysosomal function. Highly infectious and deadly arenaviruses also exploit the host cell's ability to activate their surface glycoproteins. Whether inhibitors of SKI-1/S1P will one day reach the clinic to treat specific disorders is yet to be proven for this versatile and highly conserved PC.

It is amazing that PCSK9, the last member of the PC family, only needs its enzymatic activity to autocatalytically process its prosegment in ER, which remains tightly associated with the catalytic subunit, resulting in an inactive protease. The absence of enzymatic activity may well explain the dominant pathological consequences of the lack or excess of PCSK9, which may result from modified stoichiometric levels of protein–receptor complexes, such as PCSK9-LDLR. The development of specific inhibitors/modulators of PCSK9 may find future applications in the control of some pathology, e.g. hypercholesterolemia and associated metabolic syndrome including type-2 diabetes and atheroscleorosis. The difficult, but possible, identification of an orally active PCSK9 inhibitor may one day be the next future statin-equivalent drug.

ACKNOWLEDGMENTS

The author wishes to acknowledge the tremendous help of all the past and present members of the laboratory who have actively participated in all phases of the work leading to this synopsis of the functions of SKI-1/S1P and PCSK9. This work was supported by a CIHR Canada chair # 216684; CIHR grants # MOP 93792, 102741; and a Strauss foundation grant.

REFERENCES

1. Abifadel M, Varret M, Rabes JP, Allard D, Ouguerram K, Devillers M, et al. Mutations in PCSK9 cause autosomal dominant hypercholesterolemia. *Nat Genet* 2003;**34**:154–6.
2. Basak A, Chretien M, Seidah NG. A rapid fluorometric assay for the proteolytic activity of SKI-1/S1P based on the surface glycoprotein of the hemorrhagic fever Lassa virus. *FEBS Lett* 2002;**514**:333–9.

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3. Benjannet S, Rhainds D, Essalmani R, Mayne J, Wickham L, Jin W, et al. NARC-1/PCSK9 and its natural mutants: zymogen cleavage and effects on the low density lipoprotein (LDL) receptor and LDL cholesterol. *J Biol Chem* 2004;**279**:48865–75.
4. Cohen J, Pertsemlidis A, Kotowski IK, Graham R, Garcia CK, Hobbs HH. Low LDL cholesterol in individuals of African descent resulting from frequent nonsense mutations in PCSK9. *Nat Genet* 2005;**37**:161–5.
5. Cunningham D, Danley DE, Geoghegan KF, Griffor MC, Hawkins JL, Subashi TA, et al. Structural and biophysical studies of PCSK9 and its mutants linked to familial hypercholesterolemia. *Nat Struct Mol Biol* 2007;**14**:413–9.
6. De Windt A, Rai M, Bernier L, Thelen K, Soini J, Lefebvre C, et al. Gene set enrichment analysis reveals several globally affected pathways due to SKI-1/S1P inhibition in HepG2 cells. *DNA Cell Biol* 2007;**26**:765–72.
7. Dubuc G, Tremblay M, Pare G, Jacques H, Hamelin J, Benjannet S, et al. A new method for measurement of total plasma PCSK9: clinical applications. *J Lipid Res* 2010;**51**:140–9.
8. Elagoz A, Benjannet S, Mammarbassi A, Wickham L, Seidah NG. Biosynthesis and cellular trafficking of the convertase SKI-1/S1P: ectodomain shedding requires SKI-1 activity. *J Biol Chem* 2002;**277**:11265–75.
9. Espenshade PJ, Cheng D, Goldstein JL, Brown MS. Autocatalytic processing of site-1 protease removes propeptide and permits cleavage of sterol regulatory element-binding proteins. *J Biol Chem* 1999;**274**:22795–804.
10. Essalmani R, Susan-Resiga D, Chamberland A, Abifadel M, Creemers JW, Boileau C, et al. In vivo evidence that furin from hepatocytes inactivates PCSK9. *J Biol Chem* 2011;**286**:4257–63.
11. Gorski JP, Huffman NT, Chittur S, Midura RJ, Black C, et al. Inhibition of proprotein convertase SKI-1 blocks transcription of key extracellular matrix genes regulating osteoblastic mineralization. *J Biol Chem* 2011;**286**:1836–49.
12. Hawkins JL, Robbins MD, Warren LC, Xia D, Petras SF, Valentine JJ, et al. Pharmacologic inhibition of site 1 protease activity inhibits sterol regulatory element-binding protein processing and reduces lipogenic enzyme gene expression and lipid synthesis in cultured cells and experimental animals. *J Pharmacol Exp Ther* 2008;**326**:801–8.
13. Holla OL, Strom TB, Cameron J, Berge KE, Leren TP. A chimeric LDL receptor containing the cytoplasmic domain of the transferrin receptor is degraded by PCSK9. *Mol Genet Metab* 2010;**99**:149–56.
14. Horton JD, Cohen JC, Hobbs HH. Molecular biology of PCSK9: its role in LDL metabolism. *Trends Biochem Sci* 2007;**32**:71–7.
15. Labonte P, Begley S, Guevin C, Asselin MC, Nassoury N, Mayer G, et al. PCSK9 impedes hepatitis C virus infection in vitro and modulates liver CD81 expression. *Hepatology* 2009;**50**:17–24.
16. Marschner K, Kollmann K, Schweizer M, Braulke T, Pohl S. A key enzyme in the biogenesis of lysosomes is a protease that regulates cholesterol metabolism. *Science* 2011;**333**:87–90.
17. Maxwell KN, Breslow JL. Adenoviral-mediated expression of Pcsk9 in mice results in a low-density lipoprotein receptor knockout phenotype. *Proc Natl Acad Sci U S A* 2004;**101**:7100–5.
18. Mbikay M, Sirois F, Mayne J, Wang GS, Chen A, Dewpura T, et al. PCSK9-deficient mice exhibit impaired glucose tolerance and pancreatic islet abnormalities. *FEBS Lett* 2010;**584**:701–6.
19. Park SW, Moon YA, Horton JD. Post-transcriptional regulation of low density lipoprotein receptor protein by proprotein convertase subtilisin/kexin type 9a in mouse liver. *J Biol Chem* 2004;**279**:50630–8.
20. Pasquato A, Pullikotil P, Asselin MC, Vacatello M, Paolillo L, Ghezzo F, et al. The proprotein convertase SKI-1/S1P: *in vitro* analysis of lassa virus glycoprotein-derived substrates and ex vivo validation of irreversible peptide inhibitors. *J Biol Chem* 2006;**281**:23471–81.
21. Poirier S, Mayer G, Benjannet S, Bergeron E, Marcinkiewicz J, Nassoury N, et al. The proprotein convertase PCSK9 induces the degradation of low density lipoprotein receptor (LDLR) and its closest family members VLDLR and ApoER2. *J Biol Chem* 2008;**283**:2363–72.
22. Poirier S, Mayer G, Poupon V, McPherson PS, Desjardins R, Ly K, et al. Dissection of the endogenous cellular pathways of PCSK9-induced LDLR degradation: evidence for an intracellular route. *J Biol Chem* 2009;**284**:28856–64.
23. Pullikotil P, Benjannet S, Mayne J, Seidah NG. The proprotein convertase SKI-1/S1P: alternate translation and subcellular localization. *J Biol Chem* 2007;**282**:27402–13.
24. Pullikotil P, Vincent M, Nichol ST, Seidah NG. Development of protein-based inhibitors of the proprotein of convertase SKI-1/S1P: processing of SREBP-2, ATF6, and a viral glycoprotein. *J Biol Chem* 2004;**279**:17338–47.
25. Rashid S, Curtis DE, Garuti R, Anderson NN, Bashmakov Y, Ho YK, et al. Decreased plasma cholesterol and hypersensitivity to statins in mice lacking Pcsk9. *Proc Natl Acad Sci U S A* 2005;**102**:5374–9.
26. Roubtsova A, Munkonda MN, Awan Z, Marcinkiewicz J, Chamberland A, Lazure C, et al. Circulating proprotein convertase subtilisin/kexin 9 (PCSK9) regulates VLDLR protein and triglyceride accumulation in visceral adipose tissue. *Arterioscler Thromb Vasc Biol* 2011;**31**:785–91.
27. Seidah NG. PCSK9 as a therapeutic target of dyslipidemia. *Expert Opin Ther Targets* 2009;**13**:19–28.
28. Seidah NG, Benjannet S, Wickham L, Marcinkiewicz J, Jasmin SB, Stifani S, et al. The secretory proprotein convertase neural apoptosis-regulated convertase 1 (NARC-1): liver regeneration and neuronal differentiation. *Proc Natl Acad Sci U S A* 2003;**100**:928–33.
29. Seidah NG, Mayer G, Zaid A, Rousselet E, Nassoury N, Poirier S, et al. The activation and physiological functions of the proprotein convertases. *Int J Biochem Cell Biol* 2008;**40**:1111–25.
30. Seidah NG, Mowla SJ, Hamelin J, Mamarbachi AM, Benjannet S, Toure BB, et al. Mammalian subtilisin/kexin isozyme SKI-1: a widely expressed proprotein convertase with a unique cleavage specificity and cellular localization. *Proc Natl Acad Sci U S A* 1999;**96**:1321–6.
31. Seidah NG, Prat A. The proprotein convertases are potential targets in the treatment of dyslipidemia. *J Mol Med* 2007;**85**:685–96.
32. Tassew NG, Charish J, Seidah NG, Monnier PP. SKI-1 and Furin generate multiple RGMa fragments that regulate axonal growth. *Develop Cell* 2012;**22**:391–402.
33. Urata S, Yun N, Pasquato A, Paessler S, Kunz S, de la Torre JC. Antiviral activity of a small-molecule inhibitor of arenavirus glycoprotein processing by the cellular site 1 protease. *J Virol* 2011;**85**:795–803.
34. Zaid A, Roubtsova A, Essalmani R, Marcinkiewicz J, Chamberland A, Hamelin J, et al. Proprotein convertase subtilisin/kexin type 9 (PCSK9): hepatocyte-specific low-density lipoprotein receptor degradation and critical role in mouse liver regeneration. *Hepatology* 2008;**48**:646–54.
35. Zandberg WF, Benjannet S, Hamelin J, Pinto BM, Seidah NG. N-Glycosylation controls trafficking, zymogen activation, and substrate processing of proprotein convertases PC1/3 and SKI-1. *Glycobiology* 2011.

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Chapter 250

Tyrosylprotein Sulfotransferases

Jens R. Bundgaard and Jens F. Rehfeld

ABSTRACT

A tyrosylprotein sulfotransferase (TPST) catalyzes the transfer of a sulfonyl group from the cosubstrate 3′-phosphoadenosine-5′phosphosulfate (PAPS) to a tyrosyl residue of a protein or peptide. This O-sulfation is a common irreversible posttranslational modification of proteins traversing the *trans* Golgi network during biosynthesis. It affects up to 1% of tyrosyl residues in proteins of secretory cells. Two isoforms of TPST are present in all animal cells and a single one in lower eukaryotes and plants. In mammals both the isoforms seem to be ubiquitously expressed although the levels vary between tissues. Tyrosyl O-sulfation substantially alters the properties of the side chain from a hydrophobic to a more hydrophilic form. Accordingly, tyrosine sulfate typically affects protein–protein interactions. This has been shown in for instance receptor–ligand and enzyme–substrate interactions. The biological significance is underlined by the observation that mice lacking both isoforms die shortly after birth and deficiency in single isoforms display specific phenotypes.

The sulfotransferases include a variety of enzymes belonging to several classes, and they perform different cellular functions. The soluble sulfotransferases catalyze sulfation of molecules such as estrogens, phenols, and other small molecules, and they are expressed mainly in the liver. Another class is membrane anchored sulfotransferases that catalyzes the sulfation of carbohydrates and tyrosyl residues of larger proteins or peptides. They are ubiquitously expressed in accordance with their diversity of substrates. The latter class of enzymes, denoted as the tyrosylprotein sulfotransferases (TPST, EC 2.8.2.20), are the subject of this chapter.

Tyrosine sulfation was first recognized as a modification of fibrinopeptide B in the 1950s and was long considered to be an exceptional posttranslational modification. With the structural identification of the two gastrointestinal hormones gastrin and cholecystokinin (CCK) in the 1960s and the demonstration of tyrosine sulfate in both peptide hormones,[9,10,24] interest in the modification grew. In the 1980s, the regulatory role of tyrosine phosphorylation was unfolded and this spurred the interest in tyrosine sulfate as a posttranslational modification regulated in a corresponding way to that of tyrosine phosphate by kinases and phosphatases. However, because tyrosine sulfate turned out to be an irreversible posttranslational modification, interest declined, and it was not until the late 1980s that it was recognized that tyrosine sulfation is a widespread modification of not only secretory proteins but also of some lysosomal proteins.[11,12] It was then estimated, that up to 1% of all tyrosyl residues in the eukaryotic proteome of secretory cells are tyrosine sulfated. Nevertheless, the number of mammalian proteins that were proven targets of tyrosine sulfation remained less than a hundred, and today, it is still expected that tyrosine sulfation has escaped identification in a number of proteins. In the late 1980s, purification of the enzymes was initiated, and *in vitro* assays of tyrosine sulfation were established using enriched enzymatic activity obtained from purified Golgi membrane fractions, suggesting that the tyrosylprotein sulfotransferase was a membrane bound protein. These studies revealed the first characteristics of TPST in terms of substrate specificities and enzymatic properties. Moreover, pulse-chase labeling experiments suggested that tyrosine sulfation occurred as a late event in protein secretion, advocating that TPST activity was localized to the *trans* Golgi Network. Purification of the enzyme activity proved to be difficult, but in 1998, Moore et al. managed to purify a rat liver TPST and, based on deduced amino acid sequences, to clone a cDNAs encoding the human TPST-1.[26] The identification of a human isoenzyme, TPST-2, rapidly followed.[1,27] This increased the insight into enzyme structures and properties significantly. Both TPST genes are now known to be widely expressed in tissues, suggesting that early studies on enzyme activities were performed in a mixed pool of enzymes. Subsequent identification of TPST genes showed that the TPSTs are present also in fruit fly, zebra fish, and worms, although only a single form of TPST is present in lower eukaryotes. There is also a TPST in the plant *Arabidopsis*,[18] and thus TPST

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00250-5
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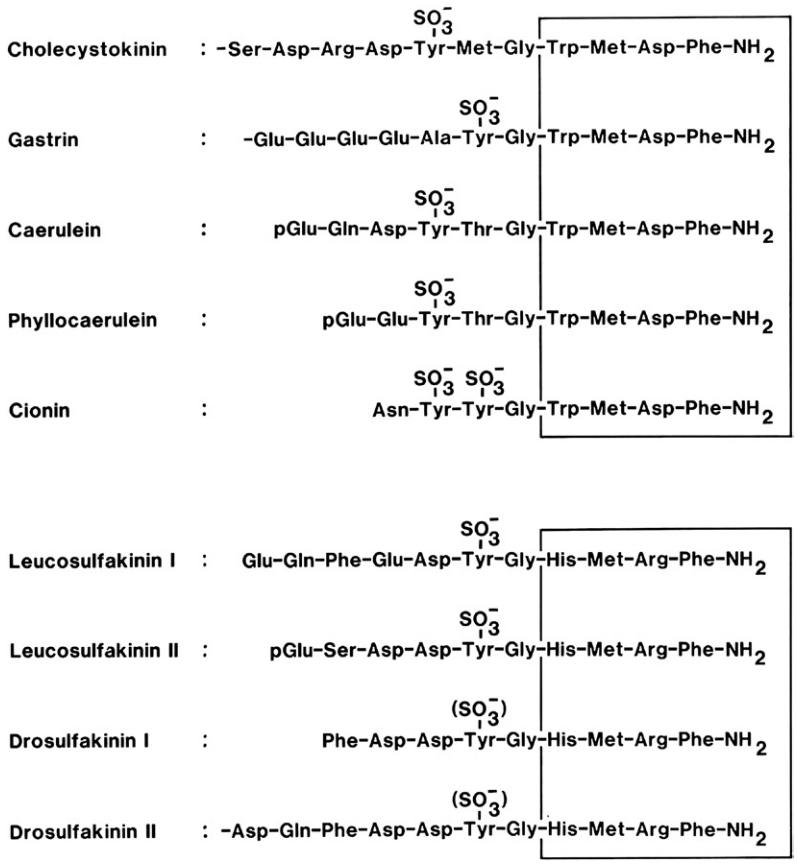

FIGURE 1 A comparison of the individual structures of the bioactive sites of members of the gastrin/CCK peptide hormone family (upper half) and the sulfakinins (lower half) showing the sulfated tyrosyl residues.

might be present in most, if not all multicellular organisms. *Arabidopsis* TPST is structurally very different from the known TPSTs and forms a new class of TPST that must have evolved independently from the mammalian TPSTs.

FUNCTION IN CELLULAR SYNTHESIS AND PROCESSING OF PEPTIDES

Tyrosine sulfation is a posttranslational modification that occurs in the vertebrate and protochordate bioactive peptides CCK, gastrin, cionin, caerulein, leucosulfakinin, and drosulfakinin. It has also been reported in several members of the granin family, for instance secretogranin II and chromogranin B, which are important factors in propeptide expression. Some enzymes that are active in propeptides activation are also targets of tyrosine sulfation, among these are the prohormone convertases 1/3 (PC1/3), PC2, and peptidylglycine alpha-amidating monooxygenase (PAM) described in separate chapters in this section of the book. Tyrosine sulfation also occurs in membrane receptors, particularly among chemokine receptors and endocrine receptors like the TSH receptor. Tyrosine sulfation is a physiologically irreversible posttranslational modification that introduces a charged hydrophilic moiety to an otherwise hydrophobic residue. The reported number of tyrosine-sulfated proteins and peptides is limited, and the reported functional effects even more so, although an increasing number of studies suggest a functional significance of the modification. One of the earliest descriptions of the biological functions of tyrosine sulfation concerned CCK that requires tyrosine sulfation to exert its defining cholecystokinetic, that is, gallbladder emptying effect. In peptide biosynthesis, the role of tyrosine sulfation has been intensively studied in the family of gastrin/CCK peptides (Fig. 1). Tyrosine sulfation affects many aspects of the biology and pathology of gastrin/CCK peptides, from the biosynthesis to the receptor activation. It has a pronounced influence on the accuracy of gastrin and CCK measurements by immunoassays in diagnostics. Gastrin and CCK share a C-terminal, carboxyamidated pentapeptide sequence that mediates a common affinity to the CCK-2 receptor. The sulfated tyrosyl residues are located in neighboring positions on the amino terminal side of the active site and the distance of the site defines the ability to bind to the CCK-1 receptor.

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The tyrosine sulfate in CCK-8 is necessary for interactions with the CCK-1 receptor but not the CCK-2 receptor. CCK and gastrin both bind to the CCK-2 receptor, whereas only sulfated CCK is a ligand for the CCK-1 receptor. Thus, tyrosine sulfation governs the specific actions of CCK. Although nonsulfated proCCK products have been reported, the majority of CCK peptides are sulfated. The homologous peptide gastrin displays a more complex sulfation pattern. Thus, progastrin-derived peptides vary grossly in stoichiometry of sulfation, depending on the site of expression. The majority of gastrin peptides are produced in the antral G-cells and is here partially sulfated, depending on the species. In man, half of the antral gastrins are tyrosine sulfated. Although the interaction with the CCK-2 receptor may be enhanced by sulfation, the modification is not decisive for gastrin activity. Other possible roles of gastrin sulfation have been reported. Sulfation of progastrin seems to increase the proteolytical cleavage the Lys–Lys residues,[3] an endoproteolytical cleavage by PC2, which processes gastrin-34 to gastrin-17. The stoichiometry of tyrosine sulfation varies with the different processing intermediates of human progastrin according to the mode of secretion. Thus, incompletely processed peptides that are secreted via a constitutive-like secretory pathway are more sulfated than the corresponding completely processed forms secreted via the regulated pathway.[2] This suggests that TPST activity might continue in an intracellular compartment of the secretory pathway later than the *trans* Golgi network.

ENZYME PROPERTIES

The mammalian TPSTs are type II integral membrane proteins of about 54 kDa that contain a small N-terminal cytoplasmic domain and a larger catalytic domain that is glycosylated and contains several disulfide bridges (Fig. 2). They catalyze the transfer of a sulfonate group from the universal sulfate donor molecule 3′-phosphoadenosine-5′phosphosulfate (PAPS) to the hydroxyl group of the target tyrosine. In accordance with their intracellular localization in the *trans* Golgi network, both TPST-1 and TPST-1 have optimal activities at slight acidic pHs of 6.5 and 6.0, respectively.[22] It has been suggested that TPSTs might form dimers or oligomers, but this is unclear, and it is not known whether the enzymes might form heterodimers of TPST-1 and TPST-2.[8] Several studies on the enzymatic properties of TPST have been performed. However, the early studies were performed using membrane fractions enriched in activity of both isoforms, and the individual properties were not revealed, because both isomers are almost ubiquitously present in all cells and tissues. Both isoenzymes are stimulated by Mn^{2+} ions, inhibited by Ca^{2+} ions and the substrate affinities are in the orders of micromolar to millimolar. The affinity of TPSTs toward the cosubstrate PAPS is in the micromolar range and was recently determined to be 8,4 micromolar for TPST-2. A major difference between the isoforms is that TPST-2 is stimulated by Mg^{2+} ions, whereas TPST-1 is not.

An intriguing question in TPST biochemistry concerns the substrate specificities of the isoenzymes. The presence of specific phenotypes in mice deficient in either TPST isoforms suggests that there are different targets of the isoenzymes, although it could be result of difference in expression of the isoforms. Because the number of known tyrosine-sulfated proteins is low compared with the relatively high abundance of sulfated tyrosyl residues in secretory cells, much attention has been given to the identification of new tyrosine sulfation substrates. Based on the known sulfation sites, Huttner et al. suggested a consensus sequence for the prediction of sulfation targets.[16] This consensus sequence emphasizes the presence of acidic residues on the N-terminal side of the tyrosyl residue, in particular in the −1 position. Thus, most known substrates, have an aspartate or a glutamate in this position. This is also the case in plants, although the homology between plant TPST and mammalian TPST is limited. In the case of the gastrin/CCK peptide family, CCK has an aspartate in the −1 position and is completely sulfated, whereas gastrin has an alanine in the −1 position, and is only partially sulfated (Fig. 1). But then, gastrin has a stretch of five glutamates in positions −2 to −6, which is believed to compensate for the lack of an acidic residue in the −1 position. However, gastrin and its precursors are expressed in various tissues under normal and pathological conditions and display large variations in the stoichiometry of sulfation. When the neutral alanine in the −1 position of gastrin is substituted for an aspartyl residue by mutagenesis and expressed in cell lines, the resulting peptides are completely sulfated, suggesting that an acidic residue in this position is important for sulfation.[3] Nevertheless, other substitutions in the acidic stretch N-terminally of the tyrosine displayed smaller influence on sulfation, even when basic residues were introduced.[4] Gastrin sulfation has been examined in both TPST-1 and TPST-2 deficient mice, and in both cases, about half of the gastrin expressed in the antrum is sulfated, which corresponds to the level in normal mice (unpublished results). Thus, both isoenzymes potentially sulfate gastrin and it is generally believed that TPST-1 and TPST-2 have overlapping substrate specificities.

Although the presence of acidic residues around the sulfation site seems to be the single most important determinant of tyrosine sulfation, it is also indicated there should be no structures that could introduce steric hindrance around the sulfation site, that is, glycosylation sites, disulfide bridges, or tertiary structures. Based on the structures of known sulfation sites, several computer algorithms have been proposed for prediction of protein sulfation with varying success and these have not until now been used for a general search of sulfated proteins.[23,34]

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Plant TPST is a type I membrane integral protein of 62 kDa that has no significant homology to the mammalian TPSTs (Fig. 2). Like the mammalian TPSTs, it is localized to the Golgi network of *Arabidopsis* cells. There have been no reports on the enzymatic properties of the plant TPST so far.

DISTRIBUTION

All higher eukaryote genomes examined contain two TPST genes, with the only exception of the fruit fly. When expressed sequence tags (ESTs) are examined, both the TPST-1 and TPST-2 genes appear to be widely expressed in all tissues. Nevertheless, there is a striking variation in EST counts between different tissues suggesting a variation in expression levels. This has been confirmed by TPST-1 and TPST-2 mRNA quantification in human tissues.[22] TPST-1 is expressed at higher levels than TPST-2 in neuronal tissue such as the cerebellum and in the reproductive organs. In contrast, TPST-2 is expressed at higher levels in particularly the liver and salivary glands. Thus, TPST seems to be ubiquitously expressed in all examined tissue in man and in all cells of higher eukaryotes.

PATOPHYSIOLOGICAL IMPLICATIONS

Over the years, a variety of important biological functions of tyrosine sulfated have been reported in several different species, some of which have been clarified on a molecular level. The first examples were that tyrosine sulfation is a prerequisite for CCK binding to the CCK-1 receptor and that the leech anticoagulant protein inhibitor, hirudin, depends on tyrosine sulfation to interact with human thrombin.[6] In fact, tyrosine sulfation plays a role in several coagulation factors, including Factor VIII, which depends on tyrosine sulfation in its interaction with von Willebrand factor.[20] Since then, many similar important functions involving direct protein–protein interactions have been reported. For instance, the chemokine receptor 5 (CCR5) is tyrosine sulfated, and sulfation is necessary for HIV-1 entry of cells by CCR5 and CD4.[7] Another example involves the cell adhesion molecule, p-selectin glycoprotein ligand-1, PSGL-1, which is a surface protein of the leukocytes that binds in a tyrosine sulfation dependent interaction to p- and s-selectin in an inflammatory response.[28] Tyrosine sulfation of PSGL-1 was also recently reported necessary for entry and replication of Enterovirus 71 in the leukocyte.[25]

After the cloning of the two mice TPSTs, generation of TPST-deficient mice gave distinct clues to important physiological functions. First, despite there being a considerable overlap in substrate specificity of TPST-1 and TPST-2, both TPST knockout mouse strains display phenotypes of reduced growth. A possible explanation of this could be a reduction of tyrosine sulfation of a TSH receptor leading to impaired responsiveness to TSH, a situation reported in the *grt* mice, a natural mouse strain having a mutation in the TPST-2 gene.[15] In addition, detailed studies have shown that the TPST-2 knockout mouse displays primary hypothyroidism,[32] but in addition, homozygous males also turn out to be infertile. This may be caused by the loss of ADAM6 and ADAM3 on epididymal sperm.[21] Alternatively, or in addition to this, Mfge8 is a protein present on mouse sperm cells that is specifically sulfated by TPST-2 and which is involved in fertilization.[14] Mice with disrupted Mfge8 gene have reduced fertility.

Similarly, tyrosine sulfation modulates the interactions between p-selectin and PSGL-1. Generation of TPST-deficient mice also allows for elaborate studies of the role of PSGL-1 sulfation on leukocyte rolling *in vivo* using transplanted hematopoietic progenitors from TPST-deficient animals. Experiments performed in *Ldlr* deficient mice result in a decrease of atherosclerosis in these mice, showing a direct link between tyrosine sulfation and atherosclerosis.[33]

Studies resulting from the breeding of a mouse strain lacking both the TPST-1 and TPST-2 genes underline the general biological importance of tyrosine sulfation. Thus, most of the pups die within the first two days of birth

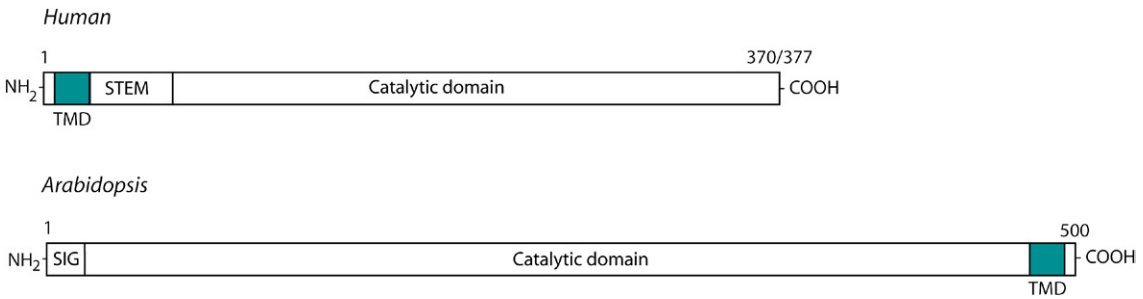

FIGURE 2 Structures of the two different types of TPSTs. The human TPST represents the two isoforms found in animals whereas *Arabidopsis* TPST represents the TPST found in plants. Note the fundamental differences in the types of the enzymes, which have very little homology in primary structure. Animal TPSTs are type II integral membrane proteins in contrast to the plant enzyme, which is of type I. Numbers refer to amino acid numbers, TMD denotes the transmembrane domain and SIG the signal peptide.

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because of failure of lung expansion leading to asphyxiation.[32] In the same mouse strain, tyrosine sulfation plays important functions in morphogenesis of outer segments of the retina, as well as synaptic function by an unknown mechanism.[31]

Implications of tyrosine sulfation have been most intensively investigated in mammalian biological systems. However, there have been notable important functions reported in other species. In the frog *Xenopus laevis*, tyrosine sulfation plays an important role during development. Here, the Wnt11 and Wnt5a proteins form complexes that are dependent on sulfated tyrosyl residues, and the complex formation is required for formation of the dorsal axis during development.[5] In *Caenorhabditis elegans*, tyrosine sulfation affects the formation of cuticles by regulating the secretion of a collagen protein called ROL-6. In plants, tyrosine sulfation also plays a pivotal role on plant development. In *Arabidopsis,* a group of proteins denoted root meristem growth factors (RGF) are targets of tyrosine sulfation, and these proteins play important roles in the formation and maintenance of the root stem cell niche.[35] Thus, deficiency in TPST activity in *Arabidopsis* leads to severe impairment of root growth and general developmental defects.

IMMUNOCHEMICAL AND DIAGNOSTIC IMPLICATIONS

In studies of tyrosine sulfation, biochemical as well as pathophysiological, an antibody specific for sulfated tyrosyl residues would be helpful, much in the same way as antibodies for phosphorylated tyrosine have proven its value.[17] For unknown reasons, it has, however, with a single exception been impossible to raise such antibodies.[13,19] This is a paradox because tyrosine sulfation as such profoundly influences the antibody binding of peptides. Hence, a study of seventeen different antisera raised against nonsulfated gastrin showed that tyrosine sulfation induced a hundredfold variation in the binding to these gastrin antibodies.[29]

Immunoassays are decisive tools for plasma measurement of secretory proteins and peptides in several endocrinological and cancer diseases. Because of the above-described influence of tyrosine sulfation on antibody binding, it is essential to know the extent to which an actual diagnostic immunoassay will measure sulfated peptides or proteins. The significance is revealed in a comparison of diagnostic gastrin immunoassays (ELISA (enzyme-linked immunosorbent assays) and RIA (radioimmunoassays)). The study shows that more than half of the commercially available diagnostic gastrin kits are inaccurate, often because tyrosine sulfation results in falsely high plasma concentrations in patients suspected of harboring gastrin-producing carcinomas.[30]

ADDITIONAL FINDINGS

Two reports on tyrosine sulfation may have important implications on the future studies of TPST activity. One is the discovery of another TPST enzyme in plants, which has limited similarity to both the animal TPSTs and the animal soluble sulfotransferases that catalyze sulfate conjugation to steroid hormones, bile salts, and other molecules. This has obvious implications in plant studies, because lack of plant enzymes displays altered phenotypes even though only two protein substrates are known. But having three variations of enzymes catalyzing similar chemical reactions, it might also be possible to refine our knowledge of the function of these enzymes in general and contribute to our insight into the phylogenetic evolution. The other finding concerns one of the major questions about tyrosine sulfation that remains to be answered: how many proteins are substrates of the animal TPSTs? It has always been expected that tyrosine sulfate in many more proteins awaited discovery. Consequently, much effort has been made to improve analytical methods and the provision of tools to predict the presence of the modifications. Several computer prediction models have been reported over the years, which have been more or less successful and with the report of an improved algorithm it will be interesting to examine possible new sulfation substrates.[34] To assist identification of new tyrosine-sulfated proteins, a monoclonal antibody that is specific for sulfotyrosine already has proved valuable for this purpose. Because tyrosine sulfate mediates protein–protein interactions, proteins acting as receptors or ligands will be of particular interest. Inhibitors of TPSTs are already available, although delivery across the cellular membrane poses a challenge, but maybe one day, tyrosine sulfation and the TPSTs might become a therapeutic target.

REFERENCES

1. Beisswanger R, Corbeil D, Vannier C, Thiele C, Dohrmann U, Kellner R, et al. Existence of distinct tyrosylprotein sulfotransferase genes: molecular characterization of tyrosylprotein sulfotransferase-2. *Proc Natl Acad Sci USA* 1998;**95**(19):11,134–9.
2. Bundgaard JR, Rehfeld JF. Distinct linkage between post-translational processing and differential secretion of progastrin derivatives in endocrine cells. *J Biol Chem* 2008;**283**(7):4014–21.
3. Bundgaard JR, Vuust J, Rehfeld JF. Tyrosine O-sulfation promotes proteolytic processing of progastrin. *EMBO J* 1995;**14**(13):3073–9.
4. Bundgaard JR, Vuust J, Rehfeld JF. New consensus features for tyrosine O-sulfation determined by mutational analysis. *J Biol Chem* 1997;**272**(35):21,700–5.
5. Cha SW, Tadjuidje E, White J, Wells J, Mayhew C, Wylie C, et al. Wnt11/5a complex formation caused by tyrosine sulfation increases canonical signaling activity. *Curr Biol* 2009;**19**(18):1573–80.
6. Chang JY. The functional domain of hirudin, a thrombin-specific inhibitor. *FEBS Lett* 1983;**164**(2):307–13.

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7. Farzan M, Mirzabekov T, Kolchinsky P, Wyatt R, Cayabyab M, Gerard NP, et al. Tyrosine sulfation of the amino terminus of CCR5 facilitates HIV-1 entry. *Cell* 1999;**96**(5):667–76.
8. Goettsch S, Badea RA, Mueller JW, Wotzlaw C, Schoelermann B, Schulz L, et al. Human TPST1 transmembrane domain triggers enzyme dimerisation and localisation to the Golgi compartment. *J Mol Biol* 2006;**361**(3):436–49.
9. Gregory H, Hardy PM, Jones DS, Kenner GW, Sheppard RC. The antral hormone gastrin. Structure of gastrin. *Nature* 1964;**204**:931–3.
10. Gregory RA, Tracy HJ. The constitution and properties of two gastrins extracted from hog antral mucosa. *Gut* 1964;**5**:103–14.
11. Hille A, Braulke T, von FK;, Huttner WB. Occurrence of tyrosine sulfate in proteins—a balance sheet. 1. Secretory and lysosomal proteins. *Eur J Biochem* 1990;**188**(3):577–86.
12. Hille A, Huttner WB. Occurrence of tyrosine sulfate in proteins—a balance sheet. 2. Membrane proteins. *Eur J Biochem* 1990;**188**(3): 587–96.
13. Hoffhines AJ, Damoc E, Bridges KG, Leary JA, Moore KL. Detection and purification of tyrosine-sulfated proteins using a novel anti-sulfotyrosine monoclonal antibody. *J Biol Chem* 2006;**281**(49):37,877–87.
14. Hoffhines AJ, Jen CH, Leary JA, Moore KL. Tyrosylprotein sulfotransferase-2 expression is required for sulfation of RNase 9 and Mfge8 in vivo. *J Biol Chem* 2009;**284**(5):3096–105.
15. Hosoda Y, Sasaki N, Agui T. Female infertility in grt mice is caused by thyroid hormone deficiency, not by insufficient TPST2 activity in the reproductive organs. *J Vet Med Sci* 2008;**70**(10):1043–9.
16. Huttner WB. Tyrosine sulfation and the secretory pathway. *Annu Rev Physiol* 1988;**50**:363–76.
17. Kehoe JW, Velappan N, Walbolt M, Rasmussen J, King D, Lou J, et al. Using phage display to select antibodies recognizing post-translational modifications independently of sequence context. *Mol Cell Proteomics* 2006;**5**(12):2350–63.
18. Komori R, Amano Y, Ogawa-Ohnishi M, Matsubayashi Y. Identification of tyrosylprotein sulfotransferase in Arabidopsis. *Proc Natl Acad Sci USA* 2009;**106**(35):15,067–72.
19. Lassen KS, Bradbury AR, Rehfeld JF, Heegaard NH. Microscale characterization of the binding specificity and affinity of a monoclonal antisulfotyrosyl IgG antibody. *Electrophoresis* 2008;**29**(12):2557–64.
20. Leyte A, van Schijndel HB, Niehrs C, Huttner WB, Verbeet MP, Mertens K, et al. Sulfation of Tyr1680 of human blood coagulation factor VIII is essential for the interaction of factor VIII with von Willebrand factor. *J Biol Chem* 1991;**266**(2):740–6.
21. Marcello MR, Jia W, Leary JA, Moore KL, Evans JP. Lack of tyrosylprotein sulfotransferase-2 activity results in altered sperm–egg interactions and loss of ADAM3 and ADAM6 in epididymal sperm. *J Biol Chem* 2011;**286**(15):13,060–70.
22. Mishiro E, Sakakibara Y, Liu MC, Suiko M. Differential enzymatic characteristics and tissue-specific expression of human TPST-1 and TPST-2. *J Biochem* 2006;**140**(5):731–7.
23. Monigatti F, Gasteiger E, Bairoch A, Jung E. The Sulfinator: predicting tyrosine sulfation sites in protein sequences. *Bioinformatics* 2002;**18**(5):769–70.
24. Mutt V, Jorpes JE. Structure of porcine cholecystokinin-pancreozymin. 1. Cleavage with thrombin and with trypsin. *Eur J Biochem* 1968;**6**(1):156–62.
25. Nishimura Y, Wakita T, Shimizu H. Tyrosine sulfation of the amino terminus of PSGL-1 is critical for enterovirus 71 infection. *PLoS Pathog* 2010;**6**(11):e1001174.
26. Ouyang Y, Lane WS, Moore KL. Tyrosylprotein sulfotransferase: purification and molecular cloning of an enzyme that catalyzes tyrosine O-sulfation, a common posttranslational modification of eukaryotic proteins. *Proc Natl Acad Sci USA* 1998;**95**(6):2896–901.
27. Ouyang YB, Moore KL. Molecular cloning and expression of human and mouse tyrosylprotein sulfotransferase-2 and a tyrosylprotein sulfotransferase homologue in *Caenorhabditis elegans*. *J Biol Chem* 1998;**273**(38):24,770–4.
28. Pouyani T, Seed B. PSGL-1 recognition of P-selectin is controlled by a tyrosine sulfation consensus at the PSGL-1 amino terminus. *Cell* 1995;**83**(2):333–43.
29. Rehfeld JF, De Magistris L, Andersen BN. Sulfation of gastrin: effect on immunoreactivity. *Regul Pept* 1981;**2**(5):333–42.
30. Rehfeld JF, Gingras MH, Bardram L, Hilsted L, Goetze JP, Poitras P. The Zollinger–Ellison syndrome and mismeasurement of gastrin. *Gastroenterology* 2011;**140**(5):1444–53.
31. Sherry DM, Murray AR, Kanan Y, Arbogast KL, Hamilton RA, Fliesler SJ, et al. Lack of protein-tyrosine sulfation disrupts photoreceptor outer segment morphogenesis, retinal function and retinal anatomy. *Eur J Neurosci* 2010;**32**(9):1461–72.
32. Westmuckett AD, Hoffhines AJ, Borghei A, Moore KL. Early postnatal pulmonary failure and primary hypothyroidism in mice with combined TPST-1 and TPST-2 deficiency. *Gen Comp Endocrinol* 2008;**156**(1):145–53.
33. Westmuckett AD, Moore KL. Lack of tyrosylprotein sulfotransferase activity in hematopoietic cells drastically attenuates atherosclerosis in Ldlr−/− mice. *Arterioscler Thromb Vasc Biol* 2009;**29**(11):1730–6.
34. Yang ZR. Predicting sulfotyrosine sites using the random forest algorithm with significantly improved prediction accuracy. *BMC Bioinformatics* 2009;**10**:361.
35. Zhou W, Wei L, Xu J, Zhai Q, Jiang H, Chen R, et al. Arabidopsis Tyrosylprotein sulfotransferase acts in the auxin/PLETHORA pathway in regulating postembryonic maintenance of the root stem cell niche. *Plant Cell* 2010;**22**(11):3692–709.

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Chapter 251

Classical Neurotransmitters and Neuropeptides

Tomas Hökfelt, Sven Ove Ögren and Zhi-Qing David Xu

ABSTRACT

Neuropeptides as transmitters in the nervous system have been seriously considered for approximately four decades. Starting at a modest level, their number has steadily increased, now encompassing >100 in mammals. In parallel, an equally rich occurrence has been seen in other species. A correspondingly large number of receptors have been discovered, almost always of the 7-transmembrane, G protein-coupled type. The diversity of the neuropeptide system is impressive, and neuropeptides quantitatively outnumber classic (and all other) transmitters such as monoamines and amino acids. It has been recognized that other classes of chemicals, such as large proteins (neurotrophins), small fatty acids (prostaglandins), and "gases" (nitric oxide), also act as neuronal messengers. These different molecules may be produced in, and released from, the same neuron–transmitter coexistence. This insight has provided a new perspective on signaling in the nervous system in health and disease as being complex, but perhaps also offering new openings for understanding and treating disease.

SOME HISTORICAL NOTES ON NEUROTRANSMITTERS

It was around 60 years ago that the first neurotransmitter was identified in the central nervous system—acetylcholine (ACh). Working on the spinal cord Eccles et al.[7] provided "strong evidence … indicating that at the synapses formed by the collaterals of motor axons on the interneurones transmission is mediated by acetylcholine." In a 38-page landmark article, the authors showed that activation of the Renshaw cells occurs via the release of ACh from collaterals of motor axons. Eccles et al.[7] in their summary wrote "In conformity with Dale's principle[6] that the same chemical transmitter is released from all the synaptic terminals of a neurone, pharmacological investigation has indicated that acetylcholine mediates the excitation of Renshaw cells by impulses in the collaterals of motor axons just as it mediates the excitation of muscle fibers at the peripheral terminals of the same axons." This is a sentence that has been much discussed more recently in relation to transmitter coexistence and corelease, not at least with regard to the large group of neuropeptide transmitters. Interestingly, it was Sir John Eccles who provided the first evidence for chemical transmission in the central nervous system, despite the fact that he had been a strong advocate for electrical communication.

Having been involved in neuroscience research for some 50 years, it is remarkable to have witnessed how new, proposed transmitter candidates regularly were met with skepticism, often been subject to strong criticism. In some cases, I felt sorry for the colleagues who had to take tough flak, especially because, eventually, they turned out to be correct. Not only in the beginning but also later,[2] there was doubt about peptides – and some of this may still persist. After one of my early lectures on neuropeptides, a leading enzyme biochemists voiced the view that all these small peptides were just breakdown products of a larger protein(s), without a specific function. I was pleased to see that a year later that particular laboratory was busy studying exactly those peptides. But that is science, I guess.

Chemical transmission, nonsynaptic or synaptic, is a fundamental process in normal nervous system function. However, there is also increased interest in the synapse in pathology. For example, in both autism and Rett's syndrome[35] as well as Alzheimer's disease and schizophrenia,[33] there is today increasing emphasis on the involvement of deranged synaptic transmission. As we now know, many molecules of different types are involved in neuronal (and glial) signaling (Table 1). At this point, it may not be relevant to discuss in depth, if they should be called, and meet stringent criteria for, a neurotransmitter. Other names have also been used: messenger molecule, signaling/transmitter substance, modulator, and more. The distinction between these molecules has become difficult, because they in addition to propagating the message across the synaptic cleft/nonsynaptic space, also can exert other functions, for example, acting as growth factors—even glutamate can

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00251-7
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TABLE 1 Different Categories of "Transmitter Substances"

Amino acids (GABA, glutamate, glycine)
Biogenic monoamines [dopamine. noradrenaline, adrenaline, serotonin (5-hydroxytryptamine), histamine]
Acetylcholine (first central transmitter)
Neuropeptides (substance P, cholecystokinin, LHRH, TRH, enkephalins, NPY, orexins/hypocretins, and many more)
"Gaseous" molecules (nitric oxide, carbon monoxide) ("on demand")
Others [arachidonoyl ethanolamide (AEA, anandamide), 2-arachoidonoyl glycerol (2-AG), ATP, adenosine, eicosanoids, leukotrienes, neurotrophic factors]

have trophic effects. Moreover, a messenger may have different functions during development and adult life; in some cases, they are highly expressed in the fetal nervous system followed by postnatal downregulation, and then reactivation can occur under certain conditions, for example, nerve injury.

NEUROPEPTIDE RESEARCH—THE BEGINNINGS

Early in the 1970s, an intense research on neuropeptides took off, fueled by many seminal discoveries including (i) most of the hypothalamic releasing and inhibiting hormones are peptides (Roger Guillemin, Andrew Schally, and coworkers). This was in good agreement with preceding studies by Vincent du Vigneaud, showing that vasopressin and oxytocin are nonapeptides, and extensive analyses on neurosecretory systems, for example, the magnocellular hypothalamic neurons, by Ernst and Berta Scharrer, Wolfgang Bargmann, and many others; (ii) substance P, discovered in 1931 (Ulf von Euler and John Gaddum), was chemically identified as an 11 amino acid peptide by Susan Leeman and coworkers; (iii) the first endogenous ligands for the morphine receptors are pentapeptides, leucine- and methionine-enkephalin, reported by John Hughes, Hans Kosterlitz and coworkers; (iv) the gut peptide cholecystokinin (CCK) (Erik Jorpes and Viktor Mutt) and the vasodilatory peptide, vasoactive intestinal polypeptide (VIP) (Sami Said and Viktor Mutt) were found in the brain, as shown for CCK-8 by Graham Dockray and Jens Rehfeld's groups and for VIP by Larsson, Rehfeld, and associates; (v) the exciting behavioral work carried out by David de Wied and colleagues and Abba Kastin and associates, showing interesting central effects of peripherally administered peptides such as vasopressin, oxytocin, and alpha-melanocyte-stimulating hormone, a field that today goes through an astounding revival;[12] and last but not least (vi) the isolation of dozens of peptides from frog skin by Vittorio Erspamer and his team. The rate of discovery of neuropeptides accelerated, and still during the first decade of this millennium new members of the family have been added. Thus, we now face at least a hundred different neuropeptides, many of them belonging to chemically, or functionally, distinct families—clearly the largest group of messenger molecules, in both the central and peripheral nervous systems.[3]

NEUROPEPTIDES AT KAROLINSKA

After Ulf von Euler, working with Sir John Gaddum in the U.K., had discovered substance P in 1931, and his student Bengt Pernow had written his thesis on this peptide in the early 1950s, it took another 20 years until this peptide was revived at Karolinska Institutet, together with discoveries of new peptides in other laboratories. Thus, the first half of the 1980s was a remarkable period in the group of Viktor Mutt, Professor in the Department of Biochemistry at Karolinska Institutet. Within a couple of years, Viktor and his Japanese graduate student Kazuhiko Tatemoto isolated/discovered, based on a novel principle, four important peptides [Peptide Histidine Isoleucine (PHI), Peptide Tyrosine Tyrosine (PYY), Neuropeptide Tyrosine (NPY), and galanin (see below)], which were the basis of Kazuhiko's PhD Thesis in 1982, and which were going to keep many laboratories around the world busy until this day and for days to come. As indicated above, Viktor had realized the danger of giving peptides a "functional name," so these peptides were named after their peptide content, for example, NPY [neuropeptide with C- and N-terminal tyrosine (Y), plus more Ys] and galanin (N-terminal glycine and C-terminal alanine). Also Abba Kastin and collaborators wrote about "the danger of constricted nomenclature."

NEUROTRANSMITTER STORAGE—SYNAPTIC VERSUS DENSE CORE VESICLES

Early electron microscopic studies showed the presence of at least two types of storage vesicles in neurons/nerve endings [see Ref. 14]: (i) synaptic vesicles (SVs) (diameter ~500 Å) and (ii) large dense core vesicles (LDCVs) (diameter ~1000 Å) (Fig. 1).

The synaptic vesicles were always believed to harbor classical transmitters such as ACh. Neuropeptides are, as shown with immunohistochemistry, exclusively stored in large dense core vesicles (LDCVs),[30] a view supported by subcellular fractionation studies.[9] The peptide folliculin-like 1 in SV-like vesicles seems to represent the first exception to this rule.[23] Histochemical analysis using $KMnO_4$ fixation provided evidence that some classic transmitters such as noradrenaline (NA) and 5-hydroxytryptamine (5-HT) could

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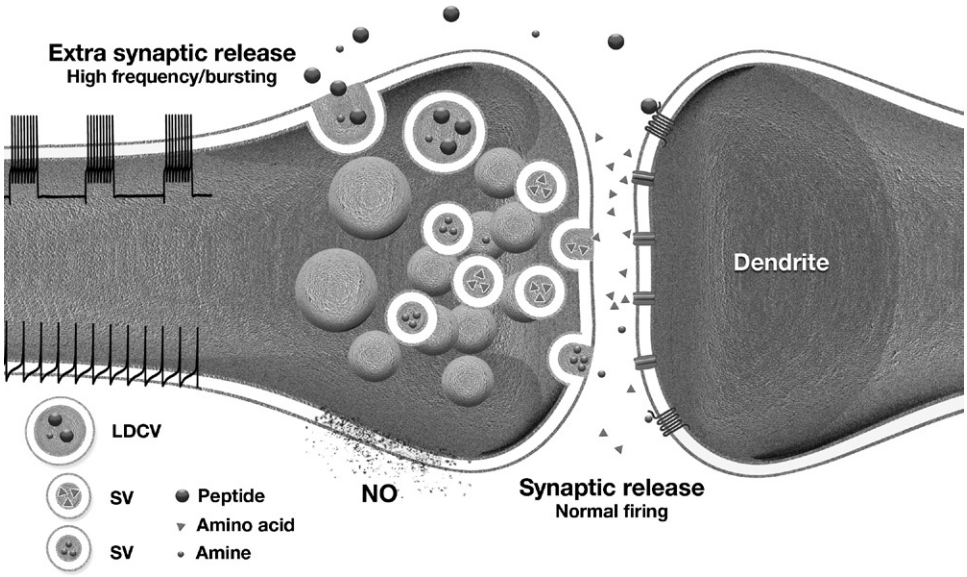

FIGURE 1 Schematic drawing of a synapse. A presynaptic nerve terminal (left) harbors different types of vesicles: Small synaptic vesicles (SVs) storing either an amino acid or, for example, an amine like serotonin. Large dense core vesicles (LDCVs) mainly store neuropeptides, but in some cases, a monoamine may also be present. Release from SVs occurs at low, normal firing rate, whereas exocytosis from LDCVs may occur in response to burst or high frequency firing. This release is extrasynaptic. Nitric oxide (NO) is not stored but synthesized and released on demand. Postsynaptic receptors on the dendrite are of two types, ligand gated channels and seven TM GPCRs. Receptors for neuropeptides are mostly extrasynaptic. (Drawing by Mattias Karlén).

be stored not only in SVs (then often called small dense core vesicles) but also in LDCVs.[19] Whether or not amino acid transmitters such as glutamate in addition are present in LDCVs is still unclear.[30]

NEUROTRANSMITTER COEXISTENCE

In 1976, Geoffrey Burnstock[4] wrote an influential review article suggesting reexamination of the "one-neuron-one-transmitter" concept, pointing in particular to the wide distribution of neuropeptides in the nervous system. This was in line with early evidence for multiple transmitters in isolated (large) invertebrate neurons.[22,31] Moreover, results from mononeuron cultures[10] showed a developmental switch in autonomic neurons from a noradrenergic to a cholinergic phenotype with a transient synthesis and release both NA and ACh. A further type of transmission was termed NANC (nonadrenergic, noncholinergic), the prime transmitter candidate being ATP, so called purinergic transmission with corelease of NA [see Ref. 5]. The first direct evidence for presence of a peptide and a classic transmitter in the same neuron was then observed in guinea pig sympathetic ganglia, where the tridecapeptide somatostatin was found in noradrenergic neurons,[21] histochemical findings that were rapidly followed by functional explorations,[25,26] and led to some further discussion on the "one-neuron-one-transmitter" hypothesis (see above).

A further modification, and in fact distinctly opposite to Dale's statement, were findings in Aplysia, showing that two different peptides can to be directed into different processes of a neuron,[34] thus not having the same transmission mechanism, in any case not the same transmitter, at all processes. Another interesting concept that has emerged is that peptides (and classic transmitters) can be released from soma and dendrites, and that dendrites and nerve endings of a neuron can operate separately and independently in releasing a messenger substance [see Ref. 24].

More recent work has shown many examples of coexistence of classic transmitters, including GABA with dopamine or 5-HT, glutamate with dopamine, NA or 5-HT and even two amino acids, excitatory glutamate with inhibitory GABA.[16] Here, the discovery of the vesicular glutamate transporters (VGLUTs) [see Refs 8, 28] has played an important role by providing tools to unequivocally identify glutamatergic neurons in the microscope.

PLASTICITY IN NEUROPEPTIDE EXPRESSION

Of particular interest, the expression of genes for peptides and sometimes also for peptide receptors is highly dynamic, for example, in response to stimuli such as nerve injury, electrical stimulation, seizure activity, or pharmacological manipulations. This reflects that each neuropeptide molecule released has to be replaced by ribosomal protein synthesis, which is preceded by a rapid increase in transcript levels. Thus, by monitoring transcript levels, one can indirectly get information of degree of activity of neurons and peptide release. The newly synthesized peptide has

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then to be centrifugally transported to the site of release from nerve terminals, dendrites, or cell soma. However, induction, even de novo synthesis of neuropeptides, may also occur in response, for example, to nerve injury. This elaborate mechanism for transmitter replacement may seem highly uneconomic but could reflect that peptide signaling probably has an ancient origin, in fact is present in organisms harboring the most primitive nervous system, such as coelenterates (Fig. 2).[15] This distinctly contrasts the complex machinery for fast transmission existing in the mammalian nervous system.

NEUROPEPTIDE RECEPTORS

Until Nakanishi and collaborators cloned the first neuropeptide receptor from the gut, a substance K receptor,[29] it was somewhat uncertain how neuropeptides exert their actions in the nervous system. The substance K receptor turned out to be a 7-transmemembrane (7-TM), G-protein-coupled receptor (GPCR), and subsequent work has shown that virtually all other, several hundreds or so, neuropeptide receptors are of that type. An exception: the peptide Phe-Met-Arg-Phe-NH_2 (FMRFamide) induces a fast excitatory depolarizing response via direct activation of an amiloride-sensitive sodium channel.[13] Several peptide receptors are expressed at low levels. Thus it is probable that neither in situ hybridization nor immunohistochemistry can visualize such low abundancy transcripts/proteins, even if powerful antibodies can produce impressive stainings. This view is strongly underlined by studies on hypothalamic, warm-sensitive neurons reporting hundreds of receptor transcripts in a single cell, in several cases evidencing functionality by electrophysiological analysis.[1] These results should be taken in consideration when discussing, for example, neurotransmitter–receptor mismatch[17] and volume transmission.[11]

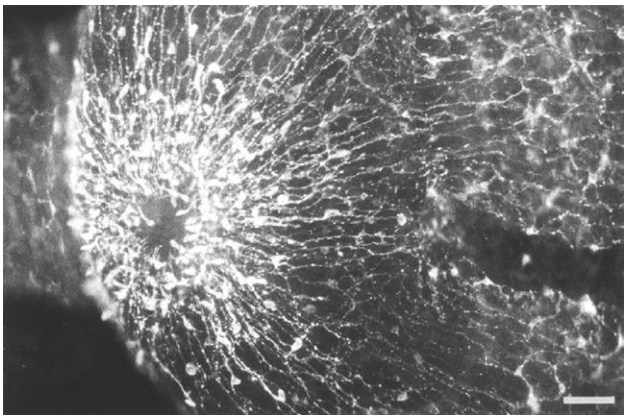

FIGURE 2 Immunofluorescence micrograph showing the RFamide peptide in the nervous system in the hypostome of *Hydra attenuata* (courtesy of Professor Cornelius Grimmelikhuijzen).

NEUROPEPTIDE RECEPTORS AND DRUG DEVELOPMENT

More than half of all drugs prescribed today are acting via seven transmembrane GPCRs, which also are targets for neuropeptides. The large number of receptors, their wide distribution in discrete systems, many directly associated with diseases, have provided a basis for drug development.[20] New drugs seem highly needed in view of the immense costs of, for example, mental disorders. Table 2 shows many candidate neuropeptide ligands that have been considered as potential antidepressants.[32] In our laboratory, focus has been on the 29 amino acid peptide galanin, and Fig. 3 illustrates a possible scenario how a galanin antagonist could exert an antidepressant action via locus coeruleus neurons costoring NA and galanin.[27]

An important aspect is that neuropeptide systems are prone to species variations.[2] Thus, results from animal experiments have to be validated through studies on human tissue to be a solid basis for developing drugs for treatment of disease. Also, the concept of cotransmission is associated with both advantages and problems, as indicated below:

1. Neuropeptides are comparatively large molecules and have difficulties in passing through the blood–brain barrier (BBB), reaching receptors in the brain and spinal cord only to a limited extent. Thus, small, BBB-penetrating, nonpeptide molecules are needed for systemic treatment of brain diseases. Many drugs have shown efficacy in animal experiments, but clinical tests have then not rarely failed, due to serious side effects.
2. If several transmitters are released from the same nerve ending, it may not be sufficient to block one receptor to obtain the expected effect. For example, even if animal work clearly indicated that substance P antagonists would be analgesic, this was not achieved in clinical trials.[18] It is possible that excitatory transmitters, such as glutamate, CGRP and others, coreleased from central sensory nerve endings, conveyed the pain message, even if NK1 receptors were blocked.
3. Intervention with antagonists may be preferred, since
   - **(i)** peptide transmission is mostly silent under normal conditions, and antagonists will therefore only affect deranged (upregulated) systems. This should result in fewer side effects;
   - **(ii)** agonists, acting on receptors in the entire body, should have more side effects. This is the case with morphine, which in addition to its well established and unsurpassed antinociception can produce serious side effects.

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CONCLUDING REMARKS

In summary, the following features seem to characterize neuropeptide systems:

1. Neuropeptides are ribosomally synthesized as large precursor molecules (MW ~ 25,000) in soma/dendrites and stored in LDCVs. The bioactive molecule is cut out by enzymes, convertases. By contrast, classic transmitters are mainly stored in SVs, although amines such as noradrenaline and serotonin can also be present in LDCVs.
2. Neuropeptides are believed to be extrasynaptically released, from nerve endings and soma/dendrites. By diffusion, they can reach distant receptors, so called volume transmission,[11] probably one explanation for "mismatch" between distribution of ligand and receptor.[17] Termination of action occurs via degradation by extracellular peptidases and "dilution."

TABLE 2 Brain Neuropeptide Receptors and Antidepressant-like Effects: Nonpeptide Ligands

Peptide	Type of drug	Drugs developed
MCH	MCH_1 antagonists	ATC0175; SNAP-7941
Substance P	NK_1 – antagonists	MK0869 (Aprepitant)
CRF	CRF antagonists	DMP696; R121919
NPY	Y1 agonists	Not available
	Y2 antagonist(?)	Not available
Galanin	GalR3 antagonists	SNAP37899
		SNAP398299
	GalR2 agonist	Not available
Nociceptin/orphanin FQ	NOP antagonist	J-113997

Modified from Ref. 32.

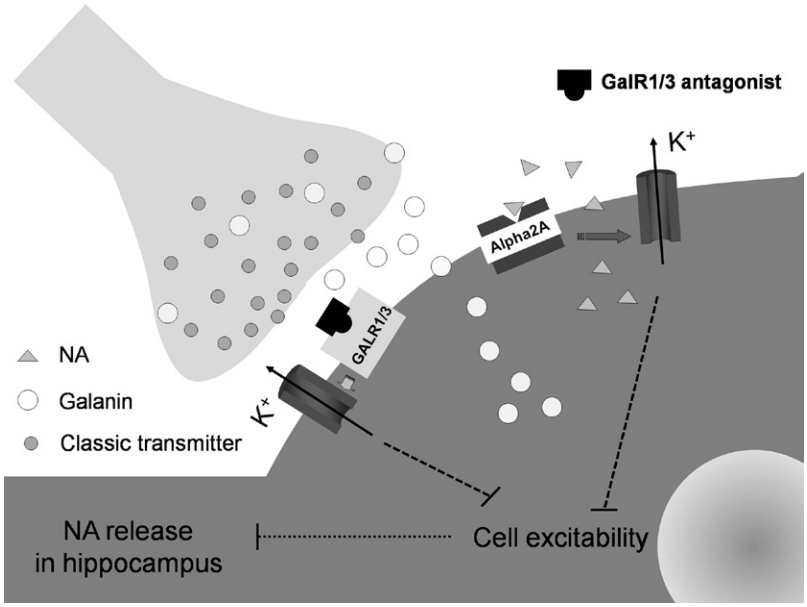

FIGURE 3 Schematic drawing of a noradrenergic neuron in the locus coeruleus costoring galanin and an adjacent nerve ending with a classic transmitter and galanin. LC neuron has both alpha2A receptors and galanin R1/3 receptors. It is well known that noradrenaline, presumably released from soma and dendrites can activate alpha2A receptors causing potassium outflow, hyperpolarization and decreased cell excitability. Galanin, released from soma/dendrites and possible nerve endings, can activate galanin R1/3 receptors similarly, causing potassium outflow and hyperpolarization. This signaling can be blocked by a galanin R1/3 antagonist, attenuating hyperpolarization and hypothetically preventing decrease in excitability and resulting in increased noradrenaline release in the forebrain and an antidepressant effect.

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3. Released molecules have to be replaced by new synthesis preceded by increase in transcript followed by intraaxonal transport to nerve endings, and/or to dendrites. Contrasting classic transmitters, there is no reuptake mechanism at the cell membrane or at the membrane of the storage vesicles.
4. Neuropeptides coexist with other molecules, classic transmitters (e.g. ACh, dopamine, noradrenaline, adrenaline, GABA, glutamate), nitric oxide and others. It has been shown that at least some neurons have a combination of several of them (e.g. serotonin + glutamate + substance P + nitric oxide). Neuropeptides are thus not the main messengers but have auxiliary, often modulating functions.
5. Neuropeptides act at very low concentrations, in the nanomolar range, and they virtually always, as many classic transmitters, act via seven TM, GPCRs.
6. The multiplicity of receptors offers unique and important openings for the development of specific agonists and antagonists as potential candidates for treatment of various diseases. Clinical trials of drugs targeting neuropeptide mechanisms have been initiated and, even if not yet reaching prescription drugs, there are many promising candidates that very well may take the decisive step into the clinic in a not too distant future. Close contenders are a calcitonin-gene-related peptide antagonist for treatment of migraine, and orexin/hypocretin antagonists as sleeping pills.

ACKNOWLEDGMENTS

This work has been supported by the Swedish (Medical) Research Council, and grants from The Marianne and Marcus Wallenberg Foundation, the European Community BIOLOGICALLY ACTIVE PEPTIDES (LHSM-CT-2003-503474) and Bristol-Myers-Squibb (Unrestricted Neuroscience Grant).

REFERENCES

1. Bartfai T, Buckley PT, Eberwine J. Drug targets: single-cell transcriptomics hastens unbiased discovery. *Trends Pharmacol Sci* 2011.
2. Bowers CW. Superfluous neurotransmitters? *Trends Neurosci* 1994;**17**:315–20.
3. Burbach JP. Neuropeptides from concept to online database. *Eur J Pharmacol* 2010;**626**:27–48www.neuropeptides.nl.
4. Burnstock G. Do some nerve cells release more than one transmitter? *Neuroscience* 1976;**1**:239–48.
5. Burnstock G. Physiology and pathophysiology of purinergic neurotransmission. *Physiol Rev* 2007;**87**:659–797.
6. Dale HH. *Transmission of effects from nerve-endings (Lecture)*. Oxford University Press; 1952.
7. Eccles JC, Fatt P, Koketsu K. Cholinergic and inhibitory synapses in a pathway from motor axon collaterals to motoneurones. *J Physiol* 1954;**126**:524–62.
8. Fremeau Jr RT, Voglmaier S, Seal RP, Edwards RH. VGLUTs define subsets of excitatory neurons and suggest novel roles for glutamate. *Trends Neurosci* 2004;**27**:98–103.
9. Fried G, Terenius L, Hökfelt T, Goldstein M. Evidence for differential localization of noradrenaline and neuropeptide Y in neuronal storage vesicles isolated from rat vas deferens. *J Neurosci* 1985;**5**:450–8.
10. Furshpan EJ, MacLeish PR, O'Lague PH, Potter DD. Chemical transmission between rat sympathetic neurons and cardiac myocytes developing in microcultures: evidence for cholinergic, adrenergic, and dual-function neurons. *Proc Natl Acad Sci USA* 1976;**73**:4225–9.
11. Fuxe K, Dahlstrom AB, Jonsson G, Marcellino D, Guescini M, Dam M, et al. The discovery of central monoamine neurons gave volume transmission to the wired brain. *Prog Neurobiol* 2010;**90**:82–100.
12. Goodson JL, Thompson RR. Nonapeptide mechanisms of social cognition, behavior and species-specific social systems. *Curr Opin Neurobiol* 2010;**20**:784–94.
13. Green KA, Falconer SWP, Cottreil GA. The neuropeptide Phe-Met-Arg-Phe-NH_2 (FMRF amide) directly gates two ion channels in an identified helix neurone. *Pflügers Arch—Europ J Physiol* 1994;**428**:232–40.
14. Grillo MA. Electron microscopy of sympathetic tissues. *Pharmacol Rev* 1966;**18**:387–99.
15. Grimmelikhuijzen CJ, Darmer D, Schmutzler C, Carstensen K, Moosler A, Nothacker HP, et al. The peptidergic nervous system of coelenterates. *Prog Brain Res* 1992;**92**:137–48.
16. Gutierréz R. *Co-existence and co-transmission of classical neurotransmitters. Ex uno plures*. New York: Springer; 2009.
17. Herkenham M. Mismatches between neurotransmitter and receptor localizations in brain: observations and implications. *Neuroscience* 1987;**23**:1–38.
18. Hill R. NK1 (substance P) receptor antagonists—why are they not analgesic in humans? *Trends Pharmacol Sci* 2000;**21**:244–6.
19. Hökfelt T. In vitro studies on central and peripheral monoamine neurons at the ultrastructural level. *Z Zellforsch Mikrosk Anat* 1968; **91**:1–74.
20. Hökfelt T, Bartfai T, Bloom F. Neuropeptides: opportunities for drug discovery. *Lancet Neurol* 2003;**2**:463–72.
21. Hökfelt T, Elfvin LG, Elde R, Schultzberg M, Goldstein M, Luft R. Occurrence of somatostatin-like immunoreactivity in some peripheral sympathetic noradrenergic neurons. *Proc Natl Acad Sci USA* 1977;**74**:3587–91.
22. Kupfermann I. Functional studies of cotransmission. *Physiol Rev* 1991;**71**:683–732.
23. Li KC, Zhang FX, Li CL, Wang F, Yu MY, Zhong YQ, et al. Follistatin-like 1 suppresses sensory afferent transmission by activating Na+, K+-ATPase. *Neuron* 2011;**69**:974–87.
24. Ludwig M, Leng G. Dendritic peptide release and peptide-dependent behaviours. *Nat Rev Neurosci* 2006;**7**:126–36.
25. Lundberg JM. Pharmacology of cotransmission in the autonomic nervous system: integrative aspects on amines, neuropeptides, adenosine triphosphate, amino acids and nitric oxide. *Pharmacol Rev* 1996;**48**:113–78.
26. Lundberg JM, Hökfelt T. Coexistence of peptides and classical transmitters. *Trends Neurosci* 1983;**6**:325–33.
27. Ma X, Tong YG, Schmidt R, Brown W, Payza K, Hodzic L, et al. Effects of galanin receptor agonists on locus coeruleus neurons. *Brain Res* 2001;**919**:169–74.
28. Masson J, Sagne C, Hamon M, El Mestikawy S. Neurotransmitter transporters in the central nervous system. *Pharmacol Rev* 1999;**51**:439–64.

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29. Masu Y, Nakayama K, Tamaki H, Harada Y, Kuno M, Nakanishi S. cDNA cloning of bovine substance-K receptor through oocyte expression system. *Nature* 1987;**329**:836–8.
30. Merighi A. Costorage and coexistence of neuropeptides in the mammalian CNS. *Prog Neurobiol* 2002;**66**:161–90.
31. Miller M. Colocalization and cotransmission of classical neurotransmitters: an invertebrate perspective. In: Gutierréz R, editor. *Coexistence and co-transmission of classical neurotransmitters ex uno plures*. New York: Springer; 2010. p. 243–61.
32. Ögren SO, Kuteeva E, Hökfelt T, Kehr J. Galanin receptor antagonists: a potential novel pharmacological treatment for mood disorders. *CNS Drugs* 2006;**20**:633–54.
33. Penzes P, Cahill ME, Jones KA, VanLeeuwen JE, Woolfrey KM. Dendritic spine pathology in neuropsychiatric disorders. *Nat Neurosci* 2011;**14**:285–93.
34. Sossin WS, Sweet-Cordero A, Scheller RH. Dale's hypothesis revisited: different neuropeptides derived from a common prohormone are targeted to different processes. *Proc Natl Acad Sci USA* 1990;**87**:4845–8.
35. Zoghbi H. Postnatal neurodevelopmental disorders: meeting at the synapse? *Nat Neurosci* 2011;**14**:285–93.

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Chapter 252

Evolution in Action: Skin Peptides

J. Michael Conlon

ABSTRACT

Peptides with antibacterial and antifungal properties are present in skin secretions of nonmammalian vertebrates that include Agnatha (hagfish and lampreys), Elasmobranchii (sharks and rays), Teleostei (bony fish), and Amphibia and constitute a component of the organism's system of innate immunity. Host-defense peptides produce cell death either by disrupting the integrity of the cell membrane or by translocating across the membrane without producing lysis and binding to intracellular targets. In the first category, there has been little or no evolutionary pressure to conserve amino-acid sequences that define antimicrobial activity but, in the great majority of cases, the peptides are cationic, contain a relatively high proportion of hydrophobic amino acids, and one or more domains with the ability to adopt an amphipathic α-helical conformation on binding to the cell membrane. The second category, of particular importance in the host-defense systems of teleost fish, includes strongly cationic peptides derived from the proteolytic cleavage of histones, particularly histones H1, 2A, and 2B, and ribosomal proteins.

INTRODUCTION

Peptides with potent antibacterial and antifungal activities play an important role in the system of innate immunity that predates adaptive immunity and constitutes the first-line defense against invading pathogens for all classes of vertebrate species. As might be expected, the major sites of synthesis of these peptides are those tissues that interface directly with the environment, such as skin, intestine, and lung/gill. A comparison of amino-acid sequences reveals evolutionary pressure to conserve the primary structures of the antimicrobial peptides has been extremely weak, and it is not possible to identify invariant domains that are associated with biological activity. This observation is consistent with the mechanisms by which the peptides produce cell death, which most commonly involve a nonspecific interaction with the bacterial cell membrane or with intracellular targets rather than binding to a specific receptor (see Cationic Antimicrobial Peptides chapter in the Bacterial/Antibiotic Peptide section of the book).

However, vertebrate host-defense peptides are associated with certain common physicochemical features. The majority of known antimicrobial peptides are cationic due to the presence of multiple arginine and lysine residues. Of the antimicrobial peptides listed in the http://aps.unmc.edu/AP/main.php database, approximately 90% are cationic with an average net charge of +4.4. Although there is no single mechanism by which cationic antimicrobial peptides produce bacterial cell death, the initial interaction of the peptide with the cell membrane involves binding of the polar residues in the peptide to the negatively charged phospholipid head groups in the lipid bilayer. Several structure–activity studies involving both naturally occurring antimicrobial peptides and synthetic model peptides show that increasing cationicity increases antimicrobial potency until a certain optimal molecular charge is reached. The peptides that act by membrane disruption generally have an overall hydrophobic content >40%, but effects of hydrophobicity on antimicrobial activity are less pronounced as electrostatic interactions of the peptide with the charged bacterial cell membrane predominate. Peptides with a very high proportion of hydrophobic amino acids are usually cytotoxic to mammalian cells such as human erythrocytes.[10]

Although the β-strand structural motif is found in several vertebrate antimicrobial peptides such as the defensins (see the Defensins chapter in the Bacterial/Antibiotic Peptides section of the book), the conformational feature that predominates among the host-defense peptides found in epidermal secretions is the amphipathic α-helix in which there is segregation of the polar residues on one face of the helix and the hydrophobic residues on the opposite face. The example of pseudin-2 from the paradoxical frog *Pseudis paradoxa* (Hylidae) is presented in Fig. 1 as a Schiffer–Edmundson helical wheel. The charged residues (Lys^6, Lys^7, Glu^{14}, and Lys^{17}) are located on one face of the helix and the hydrophobic residues (Leu^2, Leu^5, Val^8, Phe^9, and Ile^{16}) on the opposite face. The peptides generally exist in a random coil conformation in aqueous solution but adopt the helical conformation on

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00252-9
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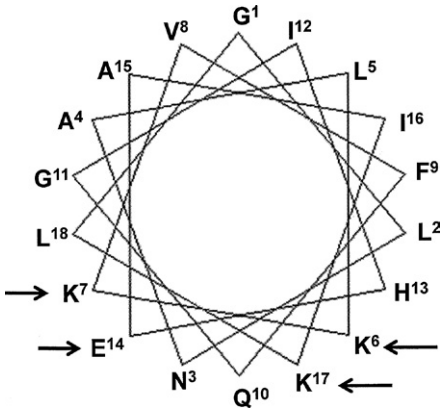

FIGURE 1 A Schiffer–Edmundson wheel representation of the N-terminal domain (residues 1–18) of pseudin-2 illustrating the amphipathic nature of the α-helical conformation. The polar residues are indicated by the arrows.

binding to the bacterial cell membrane. Structure–activity studies show that both helicity and amphipathicity correlate directly with hemolytic activity and small increases in the hydrophobic moment (a semiquantitative measure of the amphipathicity of a α-helical peptide) may produce major changes in activity.[10] Because they are readily incorporated into a helix, leucine, alanine, and lysine frequently occur in skin antimicrobial peptides but many contain an essential proline residue that produces a kink or bend in the molecule that facilitates membrane penetration.

Histones and peptides derived from histones by proteolytic cleavage constitute a second category of host-defense peptides found in epidermal secretions. Traditionally, post-translationally modified histones are known as major components of the nucleosome structure in eukaryotic cells that regulates of gene transcription. However, unmodified or minimally modified histones are also present in mitochondria and cytosolic granules. These proteins display biological activities different from those associated with chromatin and "extranuclear" histones play important roles in host defense, particularly in teleost fish. Their mechanism of action has not been elucidated but involves ATP-dependent translocation across the bacterial cell membrane without producing lysis and inhibiting cellular function by binding to intracellular DNA and/or RNA targets.[20]

The major antimicrobial peptides present in skin secretions and/or skin extracts of species belonging to the Agnatha (hagfish and lampreys), Elasmobranchii (sharks, rays, and skates), Teleostei (bony fish), and Anura (frogs and toads) are shown in Tables 1–3. Although many peptides with antimicrobial activity, such as the β-defensin-like crotamines and cathelicidin-related peptides, have been identified in the venoms of snakes (see Snake Peptides chapter in Venom Peptides section of the book), host-defense peptides in the skin secretions of reptiles and birds have not yet been described.

AGNATHA

The Agnatha, or jawless fishes, represent the first vertebrate stock whose line of evolution diverged from that leading to gnathostomes at least 550 million years ago. The fossil record indicates that agnathans were numerous during the late Silurian/early Devonian (390–420 million years ago) but only the lampreys (Petromyzontiformes) and hagfishes (Myxinoformes) have survived until the present day. The system of adaptive immunity that characterizes the higher vertebrates is not fully developed in the Agnatha so that host-defense peptides of the innate immune system are of particular importance.

Three potent, broad-spectrum antimicrobial peptides (HFIAP-1, -2, and -3), identified as ancient orthologs of the mammalian cathelicidin family, are present in hagfish intestinal tissue but, as yet, there is no evidence for their occurrence in skin secretions. However, a 12-amino-acid peptide, termed myxinidin, is present in the voluminous epidermal mucus produced by the Atlantic hagfish *Myxine glutinosa*.[35] The peptide shows potent growth-inhibitory activity against a wide range of bacteria and yeast pathogens. The microbicidal activity of myxinidin is retained in the presence of sodium chloride at concentrations up to 0.3 M and the peptide shows no hemolytic activity against mammalian red blood cells at concentrations up to 500 μg/ml.

An arginine/cysteine-rich peptide, termed lamprey corticostatin-related peptide (LCRP), is present in extracts of the skin and brain of the sea lamprey *Petromyzon marinus*.[11] A structurally similar peptide is present in the brain of the river lamprey *Lampetra fluviatilis* (J.M. Conlon unpublished data). The presence of six cysteine residues in LCRP indicates limited structural similarity to mammalian corticostatins and defensins (see the Defensins chapter in the Bacterial/Antibiotic Peptides section). In particular, the peptides contain a polyarginine sequence at the N-terminus of the molecule that is believed to be important in mediating both the antimicrobial actions and the inhibitory effect on ACTH-stimulated steroidogenesis of the corticostatins. The peptide from *P. marinus* shows potent growth-inhibitory activity against the Gram-negative bacterium *Escherichia coli* [Minimum Inhibitory Concentration (MIC) = 10 μM] but was less active against Gram-positive species.

ELASMOBRANCHII

The Elasmobranchii, represented in the present day by sharks, rays, and skates, diverged from the line of evolution leading to mammals at least 500 million years ago. The innate immune system of elasmobranchs is best known for hepatic production of the broad-spectrum antibacterial aminosterol, squalamine that is also active against fungi and protozoa. However, extracts of the skin of the fermented

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TABLE 1 Antimicrobial Peptide Isolation from Skin Secretions and/or Skin Extracts of Nontetrapod Vertebrates

Peptide	Species	Primary structure
Agnatha		
Myxinidin	*Myxine glutinosa*	GLHDILKYGKPS
LCRP	*Petromyzon marinus*	CPCGRRRCCVRGLNVYCCF
LCRP	*Lampetra fluviatilis*	CPCGRKRCCVRGLNVYCCA
Elasmobranchii		
Kenojeinin I	*Raja kenojei*	GKQYFPKVGGRLSGAPLAAKTHRRLKP.NH_2
Teleostei		
Piscidin 1	*Morone chrysops* x *M. saxatilis*	FFHHIFRGIVHVGKTIHRLVTG
Piscidin 4	*Morone chrysops* x *M. saxatilis*	FFRHLFRGAKAIFRGARQGXRAHKVVSRYRNRDV PETDNNQEEP
Epinecidin-1	*Epinephelus coioides*	GFIFHIIKGLFHAGKMIHGLV.NH_2
Pardaxin	*Pardachirus marmoratus*	GFFALIPKIISSPLFKTLLSAVGSALSSSGDQE
Pleurocidin	*Pleuronectes americanus*	GWGSFFKKAAHVGKHVGKAALTHYL
Pelteobagrin	*Pelteobagrus fulvidraco*	GKLNLFLSRLEILKLFVGAL
SAMP H1	*Salmo salar*	Ac.AEVAPAPAAAAPAKAPKKKAAAKPKKAGPS
HSDF-1	*Oncorhynchus kisutch*	Ac.AEVAPAPAAAAPAKAPKKKAAAKPKK
Hipposin	*Hippoglossus hippoglossus*	Ac.SGRGKTGGKARAKAKTRSSRAGLQFPVGRVHRLLRK GNYAHRVGAGAPVYL
Parasin I	*Parasilurus asotus*	SGRGKTGGKARAKAKTRSS
Oncorhyncin II	*Oncorhynchus mykiss*	KAVAAKKSPKKAKKPATPKKAAKSPKKVKKPAAAAKKAAK-SPKKATKAAKPKAAKPKAAKAKKAAPKKK
Oncorhyncin III	*Oncorhynchus mykiss*	PKRKSATKGDEPARRSARLSARPVPKPAAKPKKAAAPKKA-VKGKKAAENGDAKAEAKVQAAGDGAGNAK

Ac represents N-terminal acetylation

TABLE 2 Skin Antimicrobial Peptides from Frogs of the Archaeobatrachia

Anuran family	Representative genera	Peptide family
Leiopelmatidae	*Ascaphus*	Ascaphin
Alytidae	*Alytes*	Alyteserin-1
		Alyteserin-2
Bombinatoridae	*Bombina*	Bombinin
		Bombinin H
Pipidae	*Xenopus*	Magainin
	Silurana	Peptide glycine-leucine amide (PGLa)
		Caerulein-precursor fragment (CPF)
		Xenopsin-precursor fragment (XPF)

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TABLE 3 Skin Antimicrobial Peptides from Frogs of the Neobatrachia

Anuran family	Representative genera	Peptide family
Myobatrachidae	*Uperoleia*	Uperin
	Crinia	Signiferin
		Riparin
Hylidae: Phyllomedusinae	*Agalychnis*	Dermaseptin, Dermatoxin
	Phasmahyla	Hyposin, Phylloseptin, Phylloxin, Plasticin
	Phyllomedusa	
Hylidae: Pelodryadinae	*Litoria*	Aurein, Caerin
		Citropin, Dahlein
		Fallaxidin, Maculatin
Hylidae: Hylinae	*Pseudis*	Pseudin
	Hyla	Hylaseptin
	Hypsiboas	Raniseptin
Leptodactylidae	*Leptodactylus*	Ocellatin
		Plasticin
Hyperoliidae	*Kassina*	Kassinatuerin-1
Dicroglossidae	*Hoplobatrachus*	Tigerinin
Ranidae	*Amolops*	Brevinin-1, Brevinin-2
	Glandirana	Esculentin-1, Esculentin-2
	Hylarana	Ranatuerin-1, Ranatuerin-2
	Lithobates	Temporin, Palustrin-2
	Odorrana	Japonicin-1, Japonicin-2
	Pelophylax	Nigrocin-2, Ranacyclin
	Rana	

skate *Raja kenojei* contain a 28-amino-acid antimicrobial peptide, termed kenojeinin I, whose primary structure shows no significant similarity with any other known antimicrobial peptide. This strongly cationic peptide (molecular charge +9) shows potent growth activity against *E. coli*, and against the Gram-positive bacterium *Bacillus subtilis* and the yeast *Saccharomyces cerevisiae*.[4]

TELEOSTEI

The bony fish represent a particular successful branch of the vertebrate family tree with in excess of 20,000 species. It is speculated that the explosive radiation of present-day teleost fish is a consequence of whole genome duplication that occurred in the ancestral fish lineage immediately before the emergence of this vertebrate class (320–350 million years ago). The last decade has seen a marked increase in the number of antimicrobial peptides isolated from skin mucus and/or skin extracts from teleost fish and the peptides have excited interest for their therapeutic and commercial potential.

Piscidins are linear, amphipathic, antimicrobial peptides that were first identified in mast cells of the hybrid striped bass (*Morone chrysops* female x *M. saxatilis* male) but have a wide distribution in teleost fish species from the superorder Acanthopterygii.[32] Piscidins 1–3 are 22 amino acids long, whereas piscidin 4 comprises 44 residues and may contain a hydroxylated tryptophan at position 20.[25] The piscidins show potent, broad-spectrum, antibacterial activity against many fish and human pathogens and markedly reduce the infectivity of channel catfish virus.[3] Piscidin 1 adopts a α-helical structure from Phe^2 to Thr^{21} in the presence of SDS micelles.[23] The piscidin prepropeptide consists of three domains: a signal peptide (22 amino acids), a mature peptide (22 amino acids), and a C-terminal prodomain (35

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amino acids). The piscidin gene consists of three introns and four exons with a TATA box and several consensus-binding motifs for transcription factors found in the region 5′ to the transcriptional start site. The gene encoding a 21-amino-acid piscidin 1-related peptide, termed epinecidin-1, is present in the genome of the grouper *Epinephelus coioides*.[27] Like the piscidins, epinecidin-1 has the propensity to adopt an α-helical conformation and shows potent broad-spectrum antibacterial activity and cytotoxic activity against tumor cells. RT-PCR reveals high concentrations of epinecidin-1 mRNA in skin as well as intestine and head kidney. Epinecidin-1 treatment is effective in promoting fish survival after the injection of the pathogen *Vibrio vulnificus* in tilapia (*Oreochromis mossambicus*) and grouper.

Pardaxin, a 33-amino-acid pore-forming polypeptide isolated from defensive secretions of the Red Sea Moses sole *Pardachirus marmoratus*[26] is one of most intensively studied fish antimicrobial peptides because of its ionophore neurotoxin properties and its potential as a shark repellant. The peptide adopts the helix-hinge-helix structure that is a common structural motif found both in antibacterial peptides that can act selectively on bacterial membranes (e.g., cecropin), and in cytotoxic peptides that can lyse both mammalian and bacterial cells (e.g., melittin). Pardaxin possesses potent, broad-spectrum antibacterial activity (comparable to magainin, cecropins, and dermaseptins) by a mechanism that involves total disruption of the bacterial cell wall, but a significantly reduced hemolytic activity toward human red blood cells compared with melittin.[26]

A 25-residue linear broad-spectrum antimicrobial peptide, termed pleurocidin is present in the skin mucous secretions of the winter flounder *Pleuronectes americanus*.[7] The peptide has the propensity to adopt an amphipathic α-helical conformation, and there is some structural similarity with members of the dermaseptin family from the skins of phyllomedusid frogs (see the chapter on Dermaseptins in the Amphibian Peptides section of the book). The pleurocidin gene comprises four exons. Its upstream region demonstrates consensus-binding sequences for transcription factors found in host-defense genes in mammals, including sequences identical to the NF-IL6 and α- and γ-interferon response elements.[6] Subsequently, four genomic sequences encoding pleurocidin-related peptides have been identified in the genomes of the winter flounder and other flatfish species.[12] Northern and RT-PCR analyses of RNA from multiple tissues show that two of the pleurocidin genes are expressed predominantly in the skin whereas two other genes are expressed mainly in the intestine. RT-PCR assays of total RNA from larvae of different ages indicate that the pleurocidin gene is first expressed at 13 days posthatch in winter flounder. The 20-amino-acid peptide pelteobagrin is present in the skin mucus of the yellow catfish *Pelteobagrus fulvidraco* and shows broad-spectrum antibacterial and antifungal activity but low hemolytic activity.[33] This peptide shows little structural similarity to other vertebrate antimicrobial peptides.

Traditionally, histones are known as major components of the nucleosome structure in eukaryotic cells and are functionally classified into two groups: linker histones (histone H1) and core histones (histone H2A, H2B, H3, and H4). Posttranslationally unmodified, or minimally modified, histones and fragments derived from histones with antimicrobial properties are present in the epidermal mucus of a range of teleost species and their concentration increases after bacterial challenge.

A 30-amino-acid N-terminally acetylated peptide derived from the N-terminal part of histone H1 is the dominant antimicrobial peptide in skin mucus from Atlantic salmon (*Salmo salar*).[24] The peptide, termed salmon antimicrobial peptide (SAMP H1) is active against both Gram-negative and Gram-positive bacteria. The N-terminal acetyl group is not necessary for activity but the peptide exists in an inactive form that can be converted to the active form by treatment with peptidyl-proline cis–trans-isomerase, indicating that conformation of the proline peptide bonds are an important determinant of activity. Comparison of the active and inactive forms by CD spectroscopy suggests that the active form is more condensed and rigid than the inactive form which has a more nonstructured conformation. Intact histone H1 and a 26-amino-acid N-terminally acetylated peptide representing the (1–26) fragment of histone H1, termed histone-derived fragment 1 (HSDF-1), are present in the mucus of bacterial lipopolysaccharide-challenged coho salmon *Oncorhynchus mykiss*.[30] Although inactive itself against the salmon pathogens *Vibrio anguillarum* and *Aeromonas salmonicidia*, HSDF-1 potentiates the antimicrobial action of pleurocidin and lysozyme. A 69-amino-acid C-terminal fragment of histone H1, termed oncorhyncin II, is present in epidermal mucus of the rainbow trout *Oncorhynchus mykiss*.[15] With a molecular charge of +30, oncorhyncin II is the most cationic antimicrobial peptide identified to date. The peptide has MIC values in the submicromolar range against both Gram-positive and Gram-negative bacteria and it does not display significant hemolytic activity toward trout erythrocytes.

A potent broad-spectrum antimicrobial peptide, termed hipposin, is present in skin mucus of Atlantic halibut *Hippoglossus hippoglossus*. The 51-amino-acid peptide is N-terminally acetylated and represents the N-terminal domain of histone H2A.[2] CD analysis reveals that hipposin is nonstructured in aqueous solution but adopts an amphipathic, α-helical conformation in the presence of dodecylphosphocholine micelles and negatively charged liposomes. A smaller 19-amino-acid antimicrobial peptide, termed parasin I, also derived from histone H2A is present in the skin mucus of the catfish *Parasilurus asotus* after induction by epidermal injury.[28] Parasin I shows strong antimicrobial activity, about 12–100 times more potent than magainin-2,

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against a wide spectrum of microorganisms without any hemolytic activity. The peptide is generated by the action of cathepsin D activated by a matrix metalloproteinase 2. This activated form of cathepsin D then cleaves the Ser19–Arg20 bond of histone H2A to produce parasin I. Immunohistochemical analysis reveals that unacetylated histone H2A, the precursor of parasin I, and procathepsin D are present in the cytoplasm of epithelial mucous cells and that parasin I is produced on the mucosal surface after epidermal injury. Parasin I forms an amphipathic α-helical structure (residues 9–17) flanked by two random coil regions (residues 1–8 and 18–19) in helix-promoting environments. Structure–activity studies show that a basic residue at the N-terminus of the peptide is essential for membrane-binding activity and the α-helical structure is necessary for membrane-permeabilizing activity.[22]

Skin exudates of the rainbow trout *Oncorhynchus mykiss* contain a potent antimicrobial protein that was identified as intact histone H2A, acetylated at the N-terminus.[14] The protein exhibits rapid (loss of viability after 30 min) antibacterial activity against Gram-positive species with MIC values in the submicromolar range. Histone 2A has no hemolytic activity toward trout erythrocytes at concentrations <0.3 μM and its antibacterial activity probably does not involve the formation of transmembrane pores. Trout skin secretions also contain a 6.7-kDa peptide, termed oncorhyncin III, which is a cleavage product of the nonhistone chromosomal protein H6 (residues 1–66) that probably contains two methylated residues or one double methylation.[16] The purified peptide exhibits potent growth-inhibitory activity against both Gram-positive and Gram-negative bacteria, with MIC values in the submicromolar range. Scanning electron microscopy reveals that oncorhyncin III does not cause direct disruption of bacterial cells, suggesting that its mechanism of action involves translocation across the cell membrane and binding to intracellular targets.

Histone H2B, together with ribosomal protein L40, ribosomal protein L36A and ribosomal protein L35, contribute to the observed antibacterial and antifungal activities present in an extract of epidermal mucus of the Atlantic cod *Gadus morhua*.[1] Histone H2B is also present in acid extracts of channel catfish *Ictalurus punctatus* skin and is inhibitory to *Aeromonas hydrophila* and *Saprolegnia* spp., which are important bacterial and fungal pathogens of fish.[31] The 40S ribosomal protein S30 is present in skin secretions and epithelial cells of the rainbow trout *Oncorhynchus mykiss*.[17] The peptide is active at submicromolar concentrations against the marine bacterium *Planococcus citreus* suggesting that, in addition to its conventional function in the ribosomal complex, it may play a role in protection against intracellular or extracellular pathogens.

AMPHIBIA

For at least part of their life cycle, anurans are of necessity confined to a warm and moist environment that is conducive to the growth of potentially harmful bacteria and fungi. It is not surprising, therefore, that these animals have developed over the course of evolution a system of host defense that will protect the skin of the organism from invasion by pathogenic microorganisms. The extent to which the synthesis of dermal antimicrobial peptides is necessary to the frog's survival strategy or confers an evolutionary advantage to the species is unclear. The distribution of dermal antimicrobial peptides among anuran families is sporadic, and it is a common misconception that all frogs synthesize and release these peptides into their skin secretions. Among the phylogenetically more ancient Archaeobatrachia, peptides with the ability to inhibit growth of bacteria and fungi have been identified in species belonging to the families Leiopelmatidae, Alytidae, Bombinatoridae, and Pipidae. In contrast, such antimicrobial peptides have not been detected in those species examined to date that belong to the families Pelobatidae and Scaphiopodidae.[8] Among the more highly derived Neobatrachia, antimicrobial peptides have been identified in the skins of species belonging to the families Dicroglossidae, Hylidae, Hyperoliidae, Leptodactylidae, Myobatrachidae, and Ranidae. Such peptides have not been detected in those species examined to date that belong to the families Bufonidae, Ceratophyridae, Eleutherodactylidae, Microhylidae, and Pyxicephalidae.[8]

The frog skin antimicrobial peptides may be grouped together in peptide families on the basis of limited similarities in amino-acid sequence. Skin secretions from a single species frequently contain several members of a particular family that are presumed to have arisen from multiple duplications of an ancestral gene. The molecular heterogeneity of the peptides within a particular family is considerable with a peptide from one species rarely being found with an identical amino-acid sequence in another, even when those species are quite closely related phylogenetically. This variation in primary structure is reflected in a wide variation in antimicrobial potencies and specificities for different microorganisms. This multiplicity may provide a broader spectrum of defense against the range of pathogenic microorganisms encountered in the environment but firm evidence to support this claim is lacking. The distribution of antimicrobial peptides among genera classified in the Archaeobatrachia is shown in Table 2 and the distribution in genera classified in the Neobatrachia is shown in Table 3. Peptides belonging to the bombinin, dermaseptin, and temporin families and peptides from Australian frogs are reviewed in detail in the Amphibian Peptides section of the book.

With very few exceptions, the frog skin antimicrobial peptides are cationic, generally with a molecular charge between +1 and +4. The peptides exist in a random coil

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conformation in aqueous solution but adopt an amphipathic α-helical conformation in a membrane-mimetic solvent such as 50% trifluoroethanol (TFE)–water or in the environment of a phospholipid or sodium dodecylsulfate vesicle.[8] Detailed tertiary structures have been established by NMR for a wide range of frog skin antimicrobial peptides from different families that include magainin-2, PGLa, peptide XT-7 (Pipidae); alyteserin-1c (Alytidae); bombinin H2, maximin-4 (Bombinatoridae); caerin 1.1, citropin 1.1, dermaseptin B2 and S9, fallaxidin 4.1a, frenatin 3, maculatin 1.1, uperin 3.6 (Hylidae); brevinin-2, nigrocin-2, ranalexin, ranatuerin-2CSa, temporin-SH (Ranidae). All peptides contain either a single α-helical domain spanning most of the molecule, for example, peptide XT-7 from *Silurana tropicalis*,[34] two α-helical domains joined a flexible loop region, for example, brevinin-2 from *Glandirana emeljanovi*,[29] or three α-helical domains, for example, esculentin-1from *Rana esculenta* (reclassified as a hybrid between *Pelophylax lessonae and P. ridibundus*).[18] Ascaphin-8 from the N. American tailed frog *Ascaphus truei* (Leiopelmatidae), the most primitive extant anuran that is sister-group to the clade of all other living frogs, adopts an amphipathic α-helical conformation in 50% TFE water demonstrating that this structural feature arose early in anuran evolution.[10] Although amphipathicity characterizes the α-helical domain in most cases, this feature is not essential for antimicrobial activity, particularly against Gram-negative bacteria. For example, dermaseptin S9 shows broad-spectrum antibacterial activity despite the fact that spatial segregation of hydrophobic and hydrophilic/charged residues on opposing faces along the long axis of a helix is lacking (see the chapter on Dermaseptins in the Amphibian Peptides section of the book).

The plasticins are a group of glycine-leucine-rich antimicrobial peptides first identified in the skin secretions of frogs belonging to the genera *Agalychnis* and *Phyllomedusa* in the subfamily Phyllomedusinae[13] and subsequently in frogs of the genus *Leptodactylus* (Leptodactylidae).[9] The plasticins contain multiple copies of the motif GXXXG where X is any amino acid. This structural feature (or the related GXXXA/S and A/SXXXG) occurs in the bombinins and temporins and in PGLa peptides from frogs belonging to the Xenopodinae. The plasticins show greater conformational flexibility than most frog skin antimicrobial peptides. Plasticin-DA1 from *Agalychnis dacnicolor*, for example, adopts a predominantly helical conformation when bound to anionic 1,2-dimyristoylphosphatidyl glycerol phospholipid vesicles but a β-sheet structure when bound to zwitterionic dimyristoylphosphatidylcholine vesicles.[13] CD spectroscopy has shown that plasticin-L1 from *Leptodactylus laticeps* behaves in a similar fashion adopting a disordered or random coil conformation in water but a well-defined β-sheet structure in methanol and a helical structure in 50% TFE water.[9]

As with teleost fish, "extranuclear" histones and fragments of histones play a role in the system of host defense of some frogs. The best characterized amphibian histone-derived peptides are the potent antimicrobial peptides buforin 1, comprising residues (1–39) of histone H2A, and buforin 2 comprising residues (16–36) of buforin 1. These amphipathic α-helical peptides are present in an extract of stomach tissue of the toad *Bufo bufo gargarizans* but are probably not released into skin secretions in high concentrations.[21] Those Afro-Asian treefrogs belonging to the family Rhacophoridae examined to date do not produce cationic α-helical antimicrobial peptides in their skin secretions, but intact histone H2B with growth-inhibitory properties against *E. coli* is present in skin secretions of Schlegel's green tree frog *Rhacophorus schlegelii*.[20] Like the buforins, the mechanism of action does not involve lysis of the bacterial cell membrane but may involve generation of an N-terminal histone H2B fragment by outer membrane proteinase T (OmpT) that penetrates the cell membrane with subsequent inhibition of cell functions by binding to DNA or RNA targets. Similarly, cationic α-helical antimicrobial peptides are also not detected in an extract of the skin of the Japanese tree frog *Hyla japonica*, but an antibacterial post-translationally modified form of histone H4 is present that is strongly hemolytic against human erythrocytes.[19]

CONCLUSIONS

The emergence in all regions of the world of strains of pathogenic bacteria and fungi with resistance to commonly used antibiotics constitutes a potentially serious threat to public health and has necessitated a search for novel types of antimicrobial agent, with appropriate pharmacokinetic and toxicological properties, to which the microorganisms have not been exposed. The http://aps.unmc.edu/AP/main.php antimicrobial peptide database lists well over ten times as many amphibian peptides than those isolated from fish so that marine organisms represent an underexploited resource for discovery of novel peptides with the potential for development into therapeutically valuable anti-infective agents. The Acipenseriformes (sturgeons and paddlefish), Polypteriformes (bichirs and reedfish), and Dipnoi (lungfish) are yet to be examined as a source of antimicrobial peptides. Similarly, after 100,000 years of human history, mankind is finally obliged to replace the "hunter gatherer" phase of exploitation of marine resources as a food source by aquaculture. In such facilities, fish are more prone to infection by pathogenic microorganisms and the development disease-resistant transgenic fish expressing antimicrobial peptide genes, such as the pleurocidin and cecropin genes, is underway.

REFERENCES

1. Bergsson G, Agerberth B, Jörnavall H, Gudmundsson GH. Isolation and identification of antimicrobial components from the epidermal mucus of Atlantic cod (*Gadus morhua*). *FEBS J* 2005;**272**:4960–9.

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2. Birkemo GA, Lüders T, Andersen Ø, Nes IF, Nissen-Meyer J. Hipposin, a histone-derived antimicrobial peptide in Atlantic halibut (*Hippoglossus hippoglossus* L.). *Biochim Biophys Acta* 2003;**1646**:207–15.
3. Chinchar VG, Bryan L, Silphadaung U, Noga E, Wade D, Rollins-Smith L. Inactivation of viruses infecting ectothermic animals by amphibian and piscine antimicrobial peptides. *Virology* 2004;**323**:268–75.
4. Cho SH, Lee BD, An H, Eun JB. Kenojeinin I, antimicrobial peptide isolated from the skin of the fermented skate, *Raja kenojei*. *Peptides* 2005;**26**:581–7.
5. Cho JH, Park IY, Kim HS, Lee WT, Kim MS, Kim SC. Cathepsin D produces antimicrobial peptide parasin I from histone H2A in the skin mucosa of fish. *FASEB J* 2002;**16**:429–31.
6. Cole AM, Darouiche RO, Legarda D, Connell N, Diamond G. Characterization of a fish antimicrobial peptide: gene expression, subcellular localization, and spectrum of activity. *Antimicrob Agents Chemother* 2000;**44**:2039–45.
7. Cole AM, Weis P, Diamond G. Isolation and characterization of pleurocidin, an antimicrobial peptide in the skin secretions of winter flounder. *J Biol Chem.* 1997;**272**:12008–13.
8. Conlon JM. Structural diversity and species distribution of host-defense peptides in frog skin secretions. *Cell Mol Life Sci* 2011;**68**:2303–15.
9. Conlon JM, Abdel-Wahab YH, Flatt PR, Leprince J, Vaudry H, Jouenne T, et al. A glycine–leucine-rich peptide structurally related to the plasticins from skin secretions of the frog *Leptodactylus laticeps* (Leptodactylidae). *Peptides* 2009;**30**:888–92.
10.. Conlon JM, Galadari S, Raza H, Condamine E. Design of potent, non-toxic antimicrobial agents based upon the naturally occurring frog skin peptides, ascaphin-8 and peptide XT-7. *Chem Biol Drug Des* 2008;**72**:58–64.
11. Conlon JM, Sower SA. Isolation of a peptide structurally related to mammalian corticostatins from the lamprey *Petromyzon marinus*. *Comp Biochem Physiol B Biochem Mol Biol* 1996;**114**:133–7.
12. Douglas SE, Gallant JW, Gong Z, Hew C. Cloning and developmental expression of a family of pleurocidin-like antimicrobial peptides from winter flounder, *Pleuronectes americanus* (Walbaum). *Dev Comp Immunol* 2001;**25**:137–47.
13. El Amri C, Lacombe C, Zimmerman K, Ladram A, Amiche M, Nicolas P, et al. The plasticins: membrane adsorption, lipid disorders, and biological activity. *Biochemistry* 2006;**45**:14285–97.
14. Fernandes JM, Kemp GD, Molle MG, Smith VJ. Anti-microbial properties of histone H2A from skin secretions of rainbow trout, *Oncorhynchus mykiss*. *Biochem J* 2002;**368**:611–20.
15. Fernandes JM, Molle G, Kemp GD, Smith VJ. Isolation and characterisation of oncorhyncin II, a histone H1-derived antimicrobial peptide from skin secretions of rainbow trout, *Oncorhynchus mykiss*. *Dev Comp Immunol* 2004;**28**:127–38.
16. Fernandes JM, Saint N, Kemp GD, Smith VJ. Oncorhyncin III: a potent antimicrobial peptide derived from the non-histone chromosomal protein H6 of rainbow trout, *Oncorhynchus mykiss*. *Biochem J* 2003;**373**:621–8.
17. Fernandes JM, Smith VJ. A novel antimicrobial function for a ribosomal peptide from rainbow trout skin. *Biochem Biophys Res Commun* 2002;**296**:167–71.
18. Kang SJ, Son WS, Han KD, Mishig-Ochir T, Kim DW, Kim JI, et al. Solution structure of antimicrobial peptide esculentin-1c from skin secretion of *Rana esculenta*. *Mol Cells* 2010;**30**:435–41.
19. Kawasaki H, Iwamuro S, Goto Y, Nielsen PF, Conlon JM. Characterization of a hemolytic protein, identified as histone H4, from the skin of the Japanese tree frog *Hyla japonica* (Hylidae). *Comp Biochem Physiol B Biochem Mol Biol* 2008;**149**:120–5.
20. Kawasaki H, Koyama T, Conlon JM, Yamakura F, Iwamuro S. Antimicrobial action of histone H2B in *Escherichia coli*: evidence for membrane translocation and DNA-binding of a histone H2B fragment after proteolytic cleavage by outer membrane proteinase T. *Biochimie* 2008;**90**:1693–702.
21. Kim HS, Park CB, Kim MS, Kim SC. cDNA cloning and characterization of buforin I, an antimicrobial peptide: a cleavage product of histone H2A. *Biochem Biophys Res Commun* 1996;**229**:381–7.
22. Koo YS, Kim JM, Park IY, Yu BJ, Jang SA, Kim KS, et al. Structure–activity relations of parasin I, a histone H2A-derived antimicrobial peptide. *Peptides* 2008;**29**:1102–8.
23. Lee SA, Kim YK, Lim SS, Zhu WL, Ko H, Shin SY, et al. Solution structure and cell selectivity of piscidin 1 and its analogues. *Biochemistry* 2007;**46**:3653–63.
24. Lüders T, Birkemo GA, Nissen-Meyer J, Andersen Ø, Nes IF. Proline conformation-dependent antimicrobial activity of a proline-rich histone h1 N-terminal peptide fragment isolated from the skin mucus of Atlantic salmon. *Antimicrob Agents Chemother* 2005;**49**:2399–406.
25. Noga EJ, Silphaduang U, Park NG, Seo JK, Stephenson J, Kozlowicz S. Piscidin 4, a novel member of the piscidin family of antimicrobial peptides. *Comp Biochem Physiol B Biochem Mol Biol.* 2009;**152**: 299–305.
26. Oren Z, Shai Y. A class of highly potent antibacterial peptides derived from pardaxin, a pore-forming peptide isolated from Moses sole fish *Pardachirus marmoratus*. *Eur J Biochem* 1996;**237**:303–10.
27. Pan CY, Chen JY, Cheng YS, Chen CY, Ni IH, Sheen JF, et al. Gene expression and localization of the epinecidin-1 antimicrobial peptide in the grouper (*Epinephelus coioides*), and its role in protecting fish against pathogenic infection. *DNA Cell Biol* 2007;**26**:403–13.
28. Park IY, Park CB, Kim MS, Kim SC. Parasin I, an antimicrobial peptide derived from histone H2A in the catfish, *Parasilurus asotus*. *FEBS Lett* 1998;**437**:258–62.
29. Park S, Son WS, Kim YJ, Kwon AR, Lee BJ. NMR spectroscopic assessment of the structure and dynamic properties of an amphibian antimicrobial peptide (Gaegurin 4) bound to SDS micelles. *J Biochem Mol Biol* 2007;**40**:261–9.
30. Patrzykat A, Zhang L, Mendoza V, Iwama GK, Hancock RE. Synergy of histone-derived peptides of coho salmon with lysozyme and flounder pleurocidin. *Antimicrob Agents Chemother* 2001;**45**:1337–42.
31. Robinette D, Wada S, Arroll T, Levy MG, Miller WL, Noga EJ. Antimicrobial activity in the skin of the channel catfish *Ictalurus punctatus*: characterization of broad-spectrum histone-like antimicrobial proteins. *Cell Mol Life Sci* 1998;**54**:467–75.
32. Silphaduang U, Noga EJ. Peptide antibiotics in mast cells of fish. *Nature* 2001;**414**:268–9.
33. Su Y. Isolation and identification of pelteobagrin, a novel antimicrobial peptide from the skin mucus of yellow catfish (*Pelteobagrus fulvidraco*). *Comp Biochem Physiol B Biochem Mol Biol* 2011;**158**:149–54.
34. Subasinghage AP, Conlon JM, Hewage CM. Development of potent anti-infective agents from *Silurana tropicalis*: conformational analysis of the amphipathic, alpha-helical antimicrobial peptide XT-7 and its non-haemolytic analogue [G4K]XT-7. *Biochim Biophys Acta* 2010;**1804**:1020–8.
35. Subramanian S, Ross NW, MacKinnon SL. Myxinidin, a novel antimicrobial peptide from the epidermal mucus of hagfish *Myxine glutinosa* L. *Mar Biotechnol (NY)* 2009;**11**:748–57.

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Chapter 253

Peptide Chronomics

Franz Halberg, Weihong Pan, Earl E. Bakken and Germaine Cornélissen

ABSTRACT

Chronomics is the mapping of broad time structures (chronomes) in us and around us. It consists of endpoints of rhythms, trends, and deterministic chaos, and any residual variation. Trends, rhythms, and deterministic chaos are resolvable as a function of the length and density of a time series. Peptide chronomics is complicated by persistent biological effects after the original peptide has been degraded, and some of the secondary effects could be dissimilar or opposite to the native peptide. In regard to production and blood/tissue concentrations, 8-h rhythms are detected for endothelin, substance P, and neuropeptide Y, whereas circadian rhythms are seen for vasoactive intestinal peptide and leptin and most other variables. Chronomics mapping has been performed for gastrin and prolactin, among others. Exogenous peptides show differential effects at different circadian times of administration. Assessing circadian and other rhythms is more informative than analyses of means from single spot checks. A better understanding and use of peptide chronomics should improve current clinical practice.

PREAMBLE

In seemingly rigorous tests comparing two concomitantly sampled groups at some convenient time, we obtained, in our own replications, some very contradictory results. Many of the discrepancies may have originated from the fact that random sampling does not reflect general changes encompassed by chronomics. This is summarized in an extensive review of examples collected since the early 1950s.[9] Intergroup difference in phase has resulted in erroneous inferences from spot checks of caloric restriction effects on the incidence of breast cancer; this probably involves the rhythmicity of the hypothalamic–pituitary (ACTH)–adrenocortical network. The importance of rhythms lies in the assessment of intervention effects, notably in relation to stress or allergy. Another source of confusion is explained by an intergroup difference in the period of rhythms in blinded mice as compared with controls that led to the concept of free-running rhythms. In each case, information on time structure is the indispensable control information.

The goal of sampling across time span and cosinor analysis is to avoid such blunders while getting a usually better average. In addition to the mean, some dynamic parameters are included in the analysis. This includes the period (τ), an amplitude (A), acrophase (ϕ) at each τ, and a waveform [the (A,ϕ) pairs of harmonics of the fundamental τ] (Fig. 1). By quantifying these parameters, we attempt to find potentially useful new facts beyond the limited information available from spot check measurements of peptides.

INTRODUCTION

Peptides exert circadian time-dependent biological functions. However, the interpretation and statistical analysis of data in most studies do not usually take account of broad chronomes. Peptide chronomics addresses the production, distribution, actions, and turnover of peptides. Our goal is to use chronomics to characterize peptide production and actions in a way that better reflects the multitude interactions and complexity of the biological system.

Chronomes are broad time structures, whereas chronomics is the mapping of chronomes that involves description of rhythms, trends, and chaos. These parameters are resolved as a function of the length and density of a time series. Fig. 2 shows the three major components of the time structure: (1) multifrequency rhythms; (2) trends, when the data are long enough; and (3) probabilistic or other chaos, when the data are dense enough.[9] The analysis of chronomics contrasts with purely static interpretations of measurements. It has immediate clinical implications pertinent to human health. A single "snapshot," interpreted without any temporal reference, may not suffice for a medical diagnosis, notably of wellness. It has long been known that the values vary greatly along several time scales in health and disease, with age, geography, and/or lifestyle. However, even today, physicians typically rely on measurements at one or very few arbitrary time points for diagnosing gross clinical–chemical pathology based on spot checks at an unspecified time. This approach is time honored but ignores timing. Chronomics has the advantage of offering more accurate diagnoses and treatment.[3]

Here, we address the relationships of chronomic (time structural) components among several biological variables involving peptides. We propose that statistical analysis should

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00253-0
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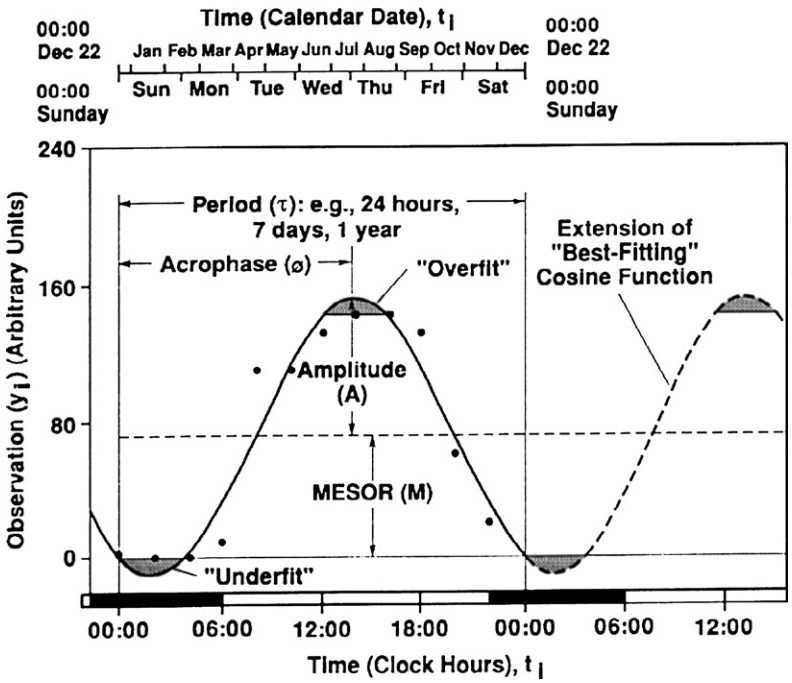

FIGURE 1 Estimation of parameters M, A, and ϕ by least-squares fit of cosine model with fixed period. $Y_i = M + A\cos(\omega t_i + \phi) + e_i$; where $\omega = 2\pi/\tau$, t_i=time; Y_i=observation at t_i and e_i=error at t_i; assumed as a first approximation to have the same independent normal distribution with mean zero and unknown variance σ^2, regardless of time; F statistic from zero-amplitude test compares sum of squares (SS) accounted for by fitted model either with the residual SS (original or pooled cosinor) or with the SS due to pure error, uncontaminated by lack of fit (unpooled cosinor).

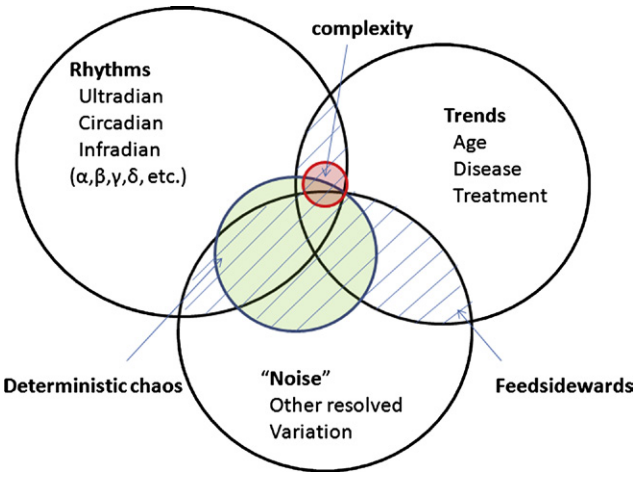

FIGURE 2 Measurable time structure (chronome) of a variable. The chronome (derived from *chronos*, *time*, *and nomos*, *rule)* represents quantitatively the measurable time structure of any variable, biological or environmental. In biology only, the ending "-ome" can also stand for "chromosome," to convey the genetic basis of (habitat and broader cosmos influenced) multifrequency rhythms, which are the major elements of chronomes, along with developmental and other age trends, and chaos, all interacting as feedsideward among different frequencies in us and in our environment. Modified from Ref. 9.

consider as many time-dependent issues as possible. We will summarize methods used to explore chronomics in the past few decades.[2,7] Peptide transcriptome analyses involving clock gene regulations are described elsewhere.[5,28]

AVOIDING BLUNDERS

The usual understanding of normal range does not take into account any rules of variation within this range. This is further complicated by free-running schedules without circadian cues, such as the erratic patterns of sleep and core body temperature in unsighted individuals. Two circadian rhythms or any other rhythms with the same frequency may happen to be out of phase, or may differ in frequency, and these observations are frequently overlooked. Blunders of interpretation are then unavoidable but are readily corrected by abandoning the misleading concept of homeostasis and by estimating parameters of the underlying rhythms. The solution of these puzzles in Minnesota led to chronobiology as a *sine qua non* to avoid a mistaken interpretation. Unrecognized differences in frequency can also play havoc, yielding faulty interpretations until resolved. By contrast, when the rhythms are recognized, a new rule (nomos) of behavior in time (chronos) may emerge as chronomics.[13]

CHRONOMICS SPAWNED BY CHRONOBIOLOGY MAPS DYNAMICS

Chronobiology is the study of mechanisms underlying chronomes, structures in time, found in organisms, in populations, and in the environment. The development of chronomics from chronobiology can be compared with that of genomics from genetics. Genetic focus on factors underlying

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inter-organismic diversity in space needs the complementary chronobiologic realization of intra-individual and intra-population diversity in time. Genetics led to genomics, the mapping of genomes; chronobiology led to chronomics, the mapping of chronomes that were found to be near matches of environmental chronomes near and far.[10]

ENDPOINTS

Computer-based chronomics provides, by cosine fitting, the probability of the occurrence of a cycle with a given anticipated τ length and of its uncertainties, and inferential statistical estimations of the parameters. The parameters include 95% confidence intervals for each, the τ (if there are replications of a cycle in the data analyzed), the A, a measure of extent of change; the acrophase, ϕ, a measure of timing, and the waveform given by the (A,ϕ) pairs of the harmonics of a given τ. Chronomics also assesses trends, for example, by the coefficients of polynomials fitted to the data. When time series are dense, with observations numerous enough, nonlinearity and endpoints such as an approximate entropy or a correlation dimension can also be determined, preferably analyzed further by curve fitting.[20] Trends are often superposed on cycles, and if the data are long enough, whether in a population or in the individual, they may turn out to be just parts of rhythms with a period, τ, longer than the data span.[14] Circadecadal cycles characterize individuals (whose vital functions are monitored for up to three or more decades). In populations, rhythms, again including circadecadals, are found in endpoints that are unique for the individual such as birth or death.

SUSCEPTIBILITY RHYTHMS

For a few variables, chronomics has already mapped a very broad spectrum of rhythms with τ covering cycles, for example, in a second, in fractions of a minute, a day, a year, and a decade or still longer spans in populations. There is a cycle stage-dependent set of responses in each of these rhythms. Not all serial data to be collected can be sampled over many multifrequency cycles; usually, the data cannot be collected densely enough to document all aspects of the stage dependence of effects of stimuli on the cycles involved. Single experiments performed along a system time, such as a day, reveal a dependence of responses, as shown for the case of ACTH[6] and TSH. This applies to peptide drugs. A promising molecule may be discarded because it is tested at the wrong time. Testing for timing should precede the regular three phases of drug testing and has hence been called a phase-zero design.[8]

ATLAS

Insufficient sampling may lead to bioaliasing. Thus, there is a need for an atlas of maps for international use. This will help to gain a proper time horizon, so that the whole mapped spectrum can be consulted instead of repeating the work of mapping in each study. Results can then consider the context of a much broader time horizon. This will include other cycles already mapped for the same variable and the rhythm investigated. In dense and long time series, chaos and/or trends that are organized by rhythms may also be resolved.[20]

BEGINNINGS OF OPPORTUNISTIC MAPPING ALONG THE SCALE OF YEARS

Chronomics may find associations of certain circannual rhythmic patterns of a peptide. We will first summarize the ultradian and infradian rhythms of endothelin-1 (ET-1, Fig. 3) and ACTH (Fig. 4). ET-1 has an 8-h and a 3.5-day cycle in human blood,[15,25,26] with eight-hourly components in other circulating peptides such as substance P and neuropeptide Y.[19] Extracircadian rhythms of ET-1 are also found in the population densities of endotheliocytes, the cells producing ET-1.[17]

These findings in blood and pinnal ear tissue contrast with earlier findings of circadians in circulating ET-1.[1] Indeed, some circadian rhythms may undergo changes with solar cycle stage in frequency to the point that the same variable a few years apart, can be primarily circadian[1] vs. primarily

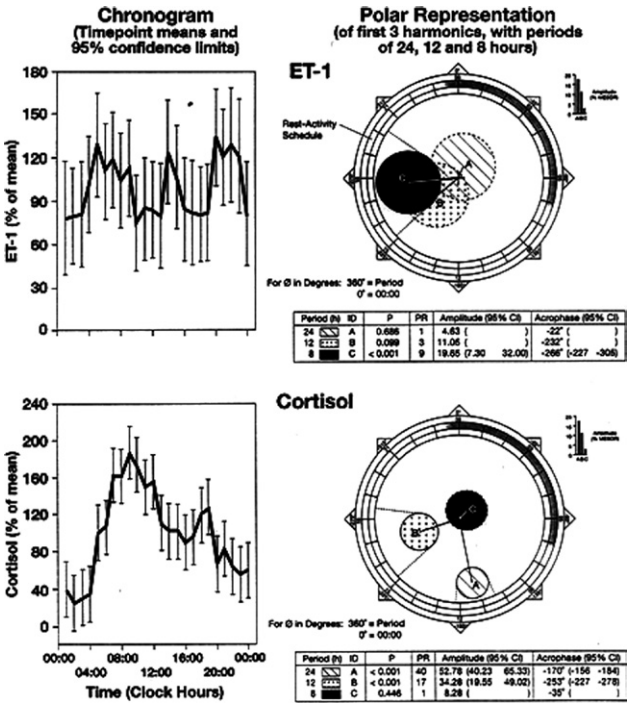

FIGURE 3 Circaoctohoran endothelin-1 (ET-1) vs. circadian cortisol in clinically healthy students, showing coexisting 8- and 24-h patterns in the same circulation. Data were obtained from two women and seven men, 22–27 years of age. Note that 8-h component is most significant for ET-1 ($p<0.001$), but it is not detected for cortisol ($p>0.4$).

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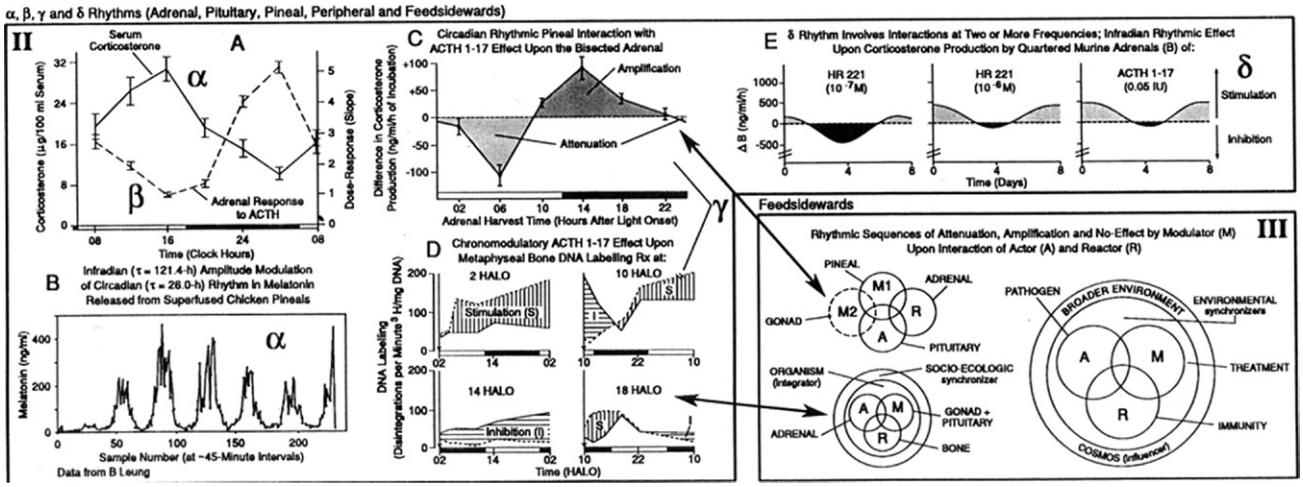

FIGURE 4 Endogenous time structure (chronome) shown by spontaneous (α), reactive (β), and modulatory (γ, δ) rhythms. The components of the chronome are internally coordinated through feedsideward in a network of spontaneous (α), reactive (β), and modulatory (γ, δ) rhythms. A: Serum corticosterone has a spontaneous rhythm (α) that is in antiphase with a reactive rhythm (β). B: Melatonin release has a spontaneous rhythm. C–E: Examples of ACTH and corticosterone rhythms in different *in vitro* studies. Reproducible sequences of attenuation, no-effect, and amplification, the time-qualified feedsidewards, may replace time unqualified feedbacks and feedforwards. Modified from Ref. 9.

extracircadian.[13,15,17,25,26] In view of the ubiquity of circadians, which is now widely accepted, a line of thought that allows for their disappearance seems unreasonable, just as half a century ago the opposite, namely, the possibility of a circadian rhythmic RNA and DNA synthesis, seemed unreasonable since nucleic acids were then regarded as the most constant material in the organism. Ascertaining stable vs. transient features of the maps here presented is also a task for the future.

In young healthy adults, there is a morning cortisol peak, but the circadian variation of ET-1 does not necessarily present itself as a circadian rhythm. Rather, the 8-h ultradian rhythm is most pronounced.[15] This circaoctohoran rather than a circadian variation also contrasts with a circadian rhythm of vasoactive intestinal peptide (VIP). The circadian rhythm of VIP shows a nocturnal peak about 8 h before the acrophase of cortisol. Like ET-1, substance P (SP) and neuropeptide Y (NPY) can have a circaoctohoran variation. Overall, the results indicate that maps of circadian and extracircadian patterns provide a broader picture of true variations, which may guide screening, diagnosis, and a better understanding of mechanisms underlying the etiology of various diseases.[19]

When the circadian rhythm of circulating concentrations of atrial natriuretic peptide (ANP) is determined after a week of synchronization with the environmental lighting schedule, a mean circadian acrophase of ANP is seen around 4 am. The ANP rhythm is in antiphase with those of blood pressure (BP) and heart rate, and seems to be anticipatory in its circadian periodic rise to awakening. This is more than what could be accounted for by postural changes.[22] It is known that ANP can affect BP by several mechanisms, including modification of renal function and vascular tone, counteraction of the renin–angiotensin–aldosterone system, and action on brain regulatory sites. The antiphase relationship between the circadian rhythms of ANP and BP is seen not only in health but also in essential hypertension. The loss in the nocturnal decrease of BP is accompanied by a comparable loss in the nocturnal surge of ANP in hypertensive renal failure and hypotensive heart failure. In the latter condition, BP and ANP variabilities correlate significantly both before and after therapy-induced functional recovery, independently of the mean BP. Overall, ANP is a modulator of the circadian rhythm of BP.[23]

In another study of ANP, VIP, and beta-endorphin in young and aged healthy subjects on controlled diets and structured sleep time (recumbency), ANP is higher in the elderly, whereas VIP, beta-endorphins, and cortisol are lower. The results indicate an age-dependent change in the secretion of these peptides.[4]

Circadian (daily) rhythms are present in almost all plants and animals. In mammals, a brain clock located in the hypothalamic suprachiasmatic nuclei maintains synchrony between environmental light/dark cycles and physiology and behavior. This has been greatly modified by modern living in the past century, by electric lighting, around-the-clock lifestyle, transmeridian air travel, and so on. Conceivably, environmental factors not only induce acute circadian disruption of physiology and behavior but also lead to chronic changes of circadian cellular mechanisms that impact on mental and physical health. In a mouse study with shortened light/dark cycle to 20-h, there is an accelerated weight gain and obesity, concurrent with changes in metabolic hormones. Circadian-disrupted mice have shorter dendritic length and decreased complexity of neurons in the prelimbic prefrontal

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cortex, a brain region important in executive function and emotional control. Changes in brain architecture coincide with decreased cognitive flexibility and changes in emotionality.[16] The findings are profound and strongly support the necessity to characterize peptide chronomics.

Leptin is a hormone mainly secreted by adipose tissue and has pleiotropic effects on many organ systems. Leptin concentration has a circadian rhythm with a nadir preceding the active dark phase in mice.[21] Circadian dependence of leptin effects on food intake, locomotor activity, glycemia, and plasma cortisol levels has been studied in goldfish (Carassius auratus). When leptin is injected intraperitoneally at the time of photophase feeding (10:00), there is a reduction in food intake and locomotor activity and an increase in glycemia. Leptin injected at the scotophase (22:00) or in fish maintained at constant lighting does not have an effect irrespective of the feeding schedule. The results suggest that leptin actions could be dependent on the feeding time and light/dark cycle as synchronizers.[27] The results are highly pertinent to human obesity, which has a higher prevalence in shift workers and in patients with nocturnal eating disorders.

As yet, there are no densely longitudinally sampled and replicated rhythms of about 10, about 20 and about 50-years mapped for peptides, but a circadecadal is seen for a steroidal metabolic variable coordinated by the peptide ACTH.[14] These rhythms are tentative not only because of the sparsity of sampling but also because each inferentially statistically validated cycle, however ample its underlying sampling, is first mapped as a candidate rhythm and needs a separate confirmation. About 30-day cycles, primarily in women, are complemented by data on circatrigintans in men. Then, there are half-yearly cycles, some changing with geographic (geomagnetic) latitude. A circannual rhythm characterizes the (peptide) vasopressin-containing nuclei in the hypothalamus.[24]

There is no single peptide variable in which we can quantify, with uncertainties of parameters, ultradians with a τ in the second, minute, or a few-hour domain and approximately 10-year rhythms, as can be done for heart rate and in the infradian as well as circadian range for 17-ketosteroid excretion. The need for mapping is introduced today to show that we should seek proper reference values for tomorrow. Chronomics, the systematic mapping of chronomes (time structures) based on long and dense serial peptide data, is undoubtedly complementary to genomics and proteomics.

CHRONOMICS IN MEN

One set of maps on peptide chronomics stems from clinically healthy and/or symptom-free men studied around-the-clock for 24 h on five occasions (always in May) at 5- to 10-year intervals over a 29-year span (1969–1998). This was part of a study of biological rhythms in more than 140 variables, in checking vital signs, blood and urine. The number of subjects and age range per study were as follows: Study 1: $n=13$, ages 22–27 years; Study 2: $n=14$, ages 23–51 years; Study 3: $n=11$, ages 41–60 years; Study 4: $n=11$, ages 46–72 years; Study 5: $n=10$, ages 52–77 years. Four of the original subjects participated in each of the 5 studies. During the study, participants were free to move about during the day without napping, with bed rest between 23:00 and 07:00, being briefly awakened for the 01:00 and 04:00 samplings. Meals were served at 16:30, 07:30, and 13:30. No food or liquids, except for water, were allowed between meals.

Measurements were obtained at 3-h intervals beginning at 19:00, with subsequent sampling beginning at 22:00, 01:00, 04:00 07:00, 10:00, 13:00, and 16:00 (and at 19:00 in Study 2). At each test time, the subjects voided their bladder for a urine sample, followed by measurement of vital signs before drawing of blood samples. Since variables were added or eliminated from the study over the years, not every variable was measured in each study or from each subject. Therefore, as few as 10 and up to 46 time series for some of the variables are available for analyses, yielding the candidate rhythms (Table 2 in Ref. 11). Changes with age notwithstanding, we are making a transition from candidate to replicated rhythms. This was actually first shown by the simple but very useful data transformation as percentage of series mean.[12] The original data are averaged and expressed as percentages of the series mean to obtain relative values. After these time-macroscopic steps, an approach by cosinor follows, which yields parameters with their uncertainties. Maps such as those of Fig. 5 are desirable for many peptides not analyzed in this figure and for many frequencies beyond the circadian. An example of gastrin chronomics is shown in Fig. 5.

A good example of the application of peptide chronomics occurs with prostate cancer risk assessment. As prostatic cancer shows geographic differences in morbidity and mortality, the extent of circannual variation also changes as a function of risk and/or cancer. In blood sampled with serial independence as to subjects in the morning at different times of the year, a prominent circannual rhythm in thyroid-stimulating hormone (TSH) of healthy subjects at low risk of prostatic cancer is lost in prostatic cancer, and even in men at high risk of prostatic cancer (Table 3 in Ref. 11). For prolactin, a circannual rhythm becomes demonstrable in the case of prostatic cancer, while it is not demonstrable with serially independent sampling in healthy men of low or high prostatic cancer risk (Table 4 in Ref. 11).

CHRONOMICS IN WOMEN

Another illustrative example of the application of chronomics is shown in a study of women. The Kyushu–Minnesota study involved sampling of three age groups of women in Minnesota, each group consisting of two subgroups, one at high, the other at low familial risk of developing breast

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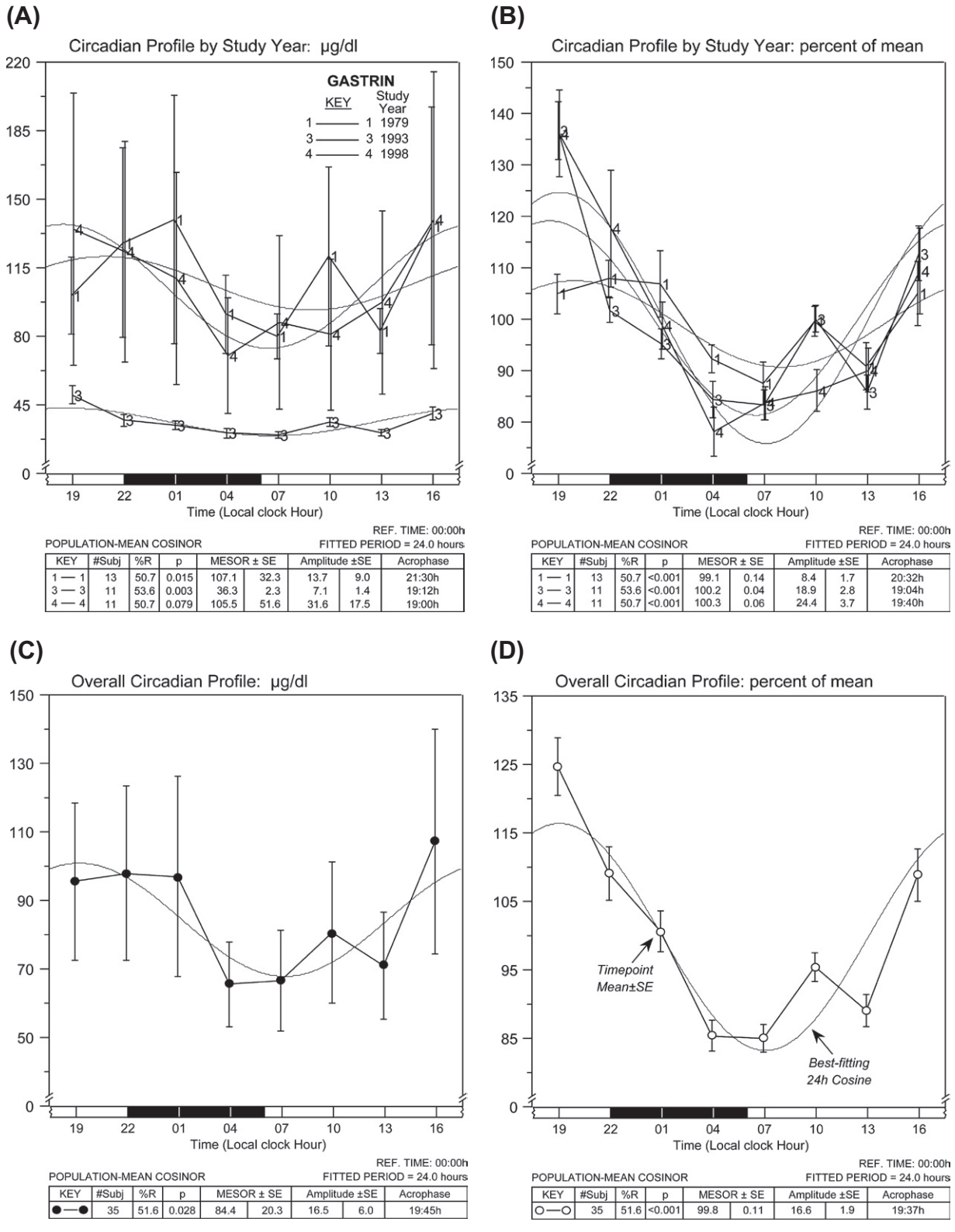

KEY	#Subj	%R	p	MESOR ± SE		Amplitude ±SE		Acrophase
1 — 1	13	50.7	0.015	107.1	32.3	13.7	9.0	21:30h
3 — 3	11	53.6	0.003	36.3	2.3	7.1	1.4	19:12h
4 — 4	11	50.7	0.079	105.5	51.6	31.6	17.5	19:00h

KEY	#Subj	%R	p	MESOR ± SE		Amplitude ±SE		Acrophase
1 — 1	13	50.7	<0.001	99.1	0.14	8.4	1.7	20:32h
3 — 3	11	53.6	<0.001	100.2	0.04	18.9	2.8	19:04h
4 — 4	11	50.7	<0.001	100.3	0.06	24.4	3.7	19:40h

KEY	#Subj	%R	p	MESOR ± SE		Amplitude ±SE		Acrophase
●—●	35	51.6	0.028	84.4	20.3	16.5	6.0	19:45h

KEY	#Subj	%R	p	MESOR ± SE		Amplitude ±SE		Acrophase
○—○	35	51.6	<0.001	99.8	0.11	16.6	1.9	19:37h

FIGURE 5 Gastrin was measured around the clock for 24 h in 11–13 men, followed-up longitudinally in three studies in 1979, 1988, and 1993. The large variability (A) can be reduced by expressing the data as a percentage of the 24h mean (B). Averaging across studies at each circadian stage is associated with large error bars obscuring the circadian variation (C). This is corrected by averaging the relative values at each circadian stage (D). From studies by E. Kanabrocki.

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cancer, and of three age groups of women at low risk in Japan. Two dozen, mostly endocrine variables were sampled around the clock, at four stages of the menstrual cycle in the two menstruating groups, in four seasons. From this international study, circulating prolactin was analyzed as three-hourly means of determinations at 20-min intervals. Geographic, ethnic and/or other differences were taken into consideration. A prolaction peak >50 ng/ml was seen in Japanese women at night; this was hardly more than half that value in Minnesotans. There was also a very marked within-day variation, overwhelming all other sources of variability. With harmonic interpolation, a second peak in the late afternoon could also be found for the Japanese subjects and has been discussed in the context of the literature. We emphasize that the smooth curves only describe results from a given season. But first, let us point out the errors that can occur if such rhythms are ignored.

This dense (20-min) sampling suggests both a larger circadian amplitude and a higher rhythm-, or rather chronome-adjusted mean or MESOR of the circadian rhythm in blood plasma prolactin in Japanese as compared with North American women ($P<0.002$ from Hotelling T^2 test). Judged on the basis only of daytime samples, erroneous conclusions may be drawn. Circadian acrophases of plasma prolactin of adolescent, young adult, and postmenopausal women from Japan and Minnesota in each of the four seasons show a relatively high degree of synchronization. The vectorial cosinor summary achieves at a glance, objectively and quantitatively, what a display of all original data cannot readily convey.

The difference of plasma prolactin between clinically healthy Japanese and Minnesotan women is seen again in a replication of part of the study in March 1978 (15 whites of mixed ethnic background [18–24 years old] in Minnesota and 20 Japanese [~20 years old] in Kyushu, Japan). Time-macroscopy by a chronogram is complemented by time-microscopy in a cosinor. The 95% error ellipses do not overlap the pole. The circadian amplitude (in nanograms per milliliter) is 5.4 (1.2–9.6) for Minnesotans and 19.3 (12.1–26.6) for Japanese.

We then applied circannual chronopathology to breast cancer risk assessment. An obliteration of the circannual but not of the circadian rhythm in prolactin is seen by an overlap of the pole by the 95% error ellipse for data on fibrocystic mastopathy as compared with clinical health for women sampled in Florence, Italy. Although the circadian amplitude (expressed in nanograms per milliliter or as percentage of MESOR) is not affected in the presence of benign breast disease, the circannual amplitude is drastically reduced. The alteration of a circannual prolactin rhythm may be a harbinger of increased breast cancer risk in Europe as in the USA.

A negative correlation between the total relative breast cancer risk evaluated from epidemiologic criteria and the circannual prolactin amplitude seems to corroborate the finding that an elevation of breast cancer risk is associated with a decrease in circannual amplitude (based on the least-squares fit of 365.25-day cosine curve to circadian MESORs assessed in each of the four seasons). There is positive correlation between epidemiologically assessed breast cancer risk and the circannual amplitude of TSH. Clinical hypothyroidism has empirically been associated with breast cancer risk. The circannual stage dependence of the correlation seems pertinent and may prove to be useful for treating an elevated cancer risk rather than the disease, to the extent that the interaction of rhythms with different frequencies can be untangled.

Thus, TSH and prolactin show opposite behavior along the 1-year scale in cancers of both breast and prostate rather than responding in the same fashion as occurs after acute stimulation with the hypothalamic tripeptide TSH-releasing hormone (TRH). It is also noteworthy that the circannual relations of plasma TSH and prolactin to the risk of breast cancer are opposite to those with respect to prostatic cancer (and as noted, opposite to each other in each cancer).

DISCUSSION

The new finding of spectral components longer than a year deserves emphasis.

Such components are the transyears, some, the near-transyears, exceeding the 1-year length by only a few weeks; others, far-transyears, exceeding the year length by a few months. Both transyear and far-transyear patterns are found in 17-ketosteroid excretion, a variable influenced by peptides, among other variables, but are not yet mapped for peptides. A chaotic regime has been proposed in accounting for a modulation by neuropeptides of information processing at the level of a single neuron;[18] apart from modeling, the use of algorithms for the analysis of probabilistic chaos in time series collected with appropriate density and length will constitute another challenge for students of peptide dynamics.

Alchemy seemed to be less complex than chemistry; nonetheless, we are now in an age of clinical and other chemistry and automation runs smoothly. We successfully diagnose gross deviations from a normal range of peptide values with time-unspecified samples! These spot checks are the routine of our day for sampling blood for peptides in the clinic and in research. The task of budding chronodesms, time-specified tolerance, and prediction intervals, not only for interpreting single values but also for time-structural characteristics, still lies ahead for peptides. But biological variability has to be known not only to reduce the width of the reference interval for single samples beyond that achieved by reducing the sources of technical variation, and not only because what may be acceptable at one time can be unacceptable at another, but also to avoid blunders and, equally importantly, to make new discoveries.

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A great opportunity for applications of peptide chronomics lies in the pharmaceutical industry. A peptide drug may act at one time but not at another, and testing across different circadian times allows the fit of a cosine curve and the detection of optimal timing. It is important to remember that peptides in organisms are not constants; they are variables. Indeed, the alternative to a putative homeostasis is to study the dynamics in the normal range and thus eventually to treat risk elevation, rather than waiting for actual disease to occur, whether we are dealing with severe vascular disease, cancer, or the basic science of our relation to our cosmos.

ACKNOWLEDGMENTS

The authors thank many colleagues and friends for their excellent contribution to the large body of work, in particular Drs. Eugene Kanabrocki, Robert B. Sothern, Erhard Haus, Samnuel Zinker, Rita Jozsa, Robert Tarquini, Federico Perfetto, Cristina Maggioni, and Othild Schwartzkopff.

REFERENCES

1. Artigou JY, Salloum J, Carayon A, Lechat P, Maistre G, Isnard R, et al. Changes in plasma endothelin during coronary spasm. *Arch Mal Coeur Vaiss* 1993 Nov;**86**(11):1581–6.
2. Bingham C, Arbogast B, Guillaume GC, Lee JK, Halberg F. Inferential statistical methods for estimating and comparing cosinor parameters. *Chronobiologia* 1982 Oct;**9**(4):397–439.
3. Cornelissen G, Halberg F. Broadly pertinent chronobiology methods quantify phosphate dynamics (chronome) in blood and urine. *Clin Chem* 1992 Mar;**38**(3):329–33.
4. Cugini P, Lucia P, Di PL, Re M, Canova R, Gasbarrone L, et al. The circadian rhythm of atrial natriuretic peptide, vasoactive intestinal peptide, beta-endorphin and cortisol in healthy young and elderly subjects. *Clin Auton Res* 1992 Apr;**2**(2):113–8.
5. Dequeant ML, Glynn E, Gaudenz K, Wahl M, Chen J, Mushegian A, et al. A complex oscillating network of signaling genes underlies the mouse segmentation clock. *Science* 2006 Dec;**314**(5805):1595–8.
6. Gunther R, Herold M, Halberg E, Halberg F. Circadian placebo and ACTH effects on urinary cortisol in arthritics. *Peptides* 1980;**1**(4):387–90.
7. Halberg F. Chronobiology. *Annu Rev Physiol* 1969;**31**:675–725.
8. Halberg F, Bingham C, Cornelissen G. Clinical trials: the larger the better? *Chronobiologia* 1993 Jul;**20**(3-4):193–211.
9. Halberg F, Cornelissen G, Katinas G, Syutkina EV, Sothern RB, Zaslavskaya R, et al. Transdisciplinary unifying implications of circadian findings in the 1950s. *J Circadian Rhythms* 2003 Oct;**1**(1):2.
10. Halberg F, Cornelissen G, Otsuka K, Watanabe Y, Katinas GS, Burioka N, et al. the International BIOCOS Study Group Cross-spectrally coherent ~10.5- and 21-year biological and physical cycles, magnetic storms and myocardial infarctions. *Neuro Endocrinol Lett* 2000;**21**(3):233–58.
11. Halberg F, Cornelissen-Guillaume GG, Kanabrocki E, Sothern RB, Haus E, Zinker S, et al. Peptide chronomics. In: Kastin AJ, editor. *Handbook of biologically active peptides*. Amsterdam: Elsevier; 2006. p. 1529–64.
12. Halberg F, Engel R, Treloar AE, Gully RJ. Endogenous eosinopenia in institutionalized patients with mental deficiency. *AMA Arch Neurol Psychiatr* 1953;**69**:462–9.
13. Halberg FE, Cornelissen G, Otsuka K, Schwartzkopff O, Halberg J, Bakken EE. Chronomics. *AMBiomed Pharmacother* 2001;**55**(Suppl. 1):153s–90s.
14. Halberg F, Cornelissen G, Watanabe Y, Otsuka K, Fiser B, Siegelova J, et al. Near 10-year and longer periods modulate circadians: intersecting anti-aging and chronoastrobiological research. *J Gerontol A Biol Sci Med Sci* 2001 May;**56**(5):M304–24.
15. Herold M, Cornelissen G, Loeckinger A, Koeberle D, Koenig P, Halberg F. About 8-hour variation of circulating human endothelin-1. *Peptides* 1998;**19**(5):821–5.
16. Karatsoreos IN, Bhagat S, Bloss EB, Morrison JH, McEwen BS. Disruption of circadian clocks has ramifications for metabolism, brain, and behavior. *Proc Natl Acad Sci U S A* 2011 Jan;**108**(4): 1657–62.
17. Katinas GS, Halberg F, Cornelissen G, Hawkins D, Bueva MV, Korzhevsky DE, et al. About 8- and approximately 84-h rhythms in endotheliocytes as in endothelin-1 and effect of trauma. *Peptides* 2001 Apr;**22**(4):647–59.
18. Komendantov AO, Kononenko NI. Deterministic chaos in mathematical model of pacemaker activity in bursting neurons of snail, *Helix pomatia*. *J Theor Biol* 1996 Nov;**183**(2):219–30.
19. Lockinger A, Koberle D, Konig PS, Saria A, Herold M, Cornelissen G, et al. Neuropeptide chronomics in clinically healthy young adults: circaoctohoran and circadian patterns. *Peptides* 2004 Apr;**25**(4):533–42.
20. Otsuka K, Cornelissen G, Halberg F. Circadian rhythmic fractal scaling of heart rate variability in health and coronary artery disease. *Clin Cardiol* 1997 Jul;**20**(7):631–8.
21. Pan W, Kastin AJ. Diurnal variation of leptin entry from blood to brain involving partial saturation of the transport system. *Life Sci* 2001;**68**:2705–14.
22. Portaluppi F, Montanari L, Bagni B, Degli UE, Trasforini G, Margutti A. Circadian rhythms of atrial natriuretic peptide, blood pressure and heart rate in normal subjects. *Cardiology* 1989;**76**(6):428–32.
23. Portaluppi F, Vergnani L, degli Uberti EC. Atrial natriuretic peptide and circadian blood pressure regulation: clues from a chronobiological approach. *Chronobiol Int* 1993 Jun;**10**(3):176–89.
24. Portela A, Cornelissen G, Halberg F, Halberg F, Halberg J, Hofman MA, et al. Metachronanalysis of circannual and circasemiannual characteristics of human suprachiasmatic vasopressin–containing neurons. *In Vivo* 1995 Jul;**9**(4):347–58.
25. Tarquini B, Cornelissen G, Perfetto F, Tarquini R, Halberg F. About-half-weekly (circasemiseptan) component of the endothelin-1 (ET-1) chronome and vascular disease risk. *Peptides* 1997;**18**(8):1237–41.
26. Tarquini B, Perfetto F, Tarquini R, Cornelissen G, Halberg F. Endothelin-1's chronome indicates diabetic and vascular disease chronorisk. *Peptides* 1997;**18**(1):119–32.
27. Vivas Y, Azpeleta C, Feliciano A, Velarde E, Isorna E, Delgado MJ, et al. Time-dependent effects of leptin on food intake and locomotor activity in goldfish. *Peptides* 2011 May;**32**(5):989–95.
28. Wang W, Ghosh BK, Pakrasi HB. Identification and modeling of genes with diurnal oscillations from microarray time series data. *IEEE/ACM Trans Comput Biol Bioinform* 2011 Jan;**8**(1):108–21.

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Chapter 254

Peptides and Behavior

Susan Rotzinger

ABSTRACT

The role of peptides in modulating a wide range of behaviors has been recognized for many years. It is increasingly apparent that peptides influence behavior in unique ways that are state dependent, coordinated, and redundant. In addition, peptides show unique dose–response characteristics and can have long-lasting effects on behavior. Peptides modulate behavior as part of a concerted and coordinated effect on physiology, neuroendocrine, and behavioral output to facilitate goal-directed behavior and support homeostasis. Peptides modulate behavior through their interactions with other peptides and neurotransmitter systems in the brain and represent a promising new class of therapeutics for neuropsychiatric disorders. This chapter reviews some general features of peptide modulation of behavior.

INTRODUCTION

With >100 different neuropeptides in the mammalian brain,[18] it is not surprising that peptides play a role in a large number of behavioral, emotional, and cognitive processes. It is also to be expected that with such a number of peptides, there will be many different peptides that modulate the same types of behaviors and that many individual peptides will modulate more than one type of behavior. Peptides can exert both peripheral and central effects. Peptides act through the hypothalamic–pituitary axes to initiate endocrine responses throughout the periphery. Most peptides and their receptors are also found in the brain at extrahypothalamic sites where they can exert effects on cognition, emotion, social, and motivational processes.

Many reviews of peptides and behavior have previously been published.[35] An exhaustive review of all peptides and behavioral effects is beyond the scope of this chapter. Instead, the focus will be on general concepts relating to the effects of peptides on behavior. The second section will cover the current understanding of some of the major peptide systems that impinge on a few of the most important behavioral processes. The third section will provide a brief discussion of the potential therapeutic aspects of peptide-based compounds.

GENERAL FEATURES OF PEPTIDE MODULATION OF BEHAVIOR

Peptides Are Modulatory

In examining the large body of data concerning the behavioral effects of peptides, one principle that emerges is that the influence of a peptide on behavior is dependent on the current physiological, neuroendocrine, and behavioral state of the organism, as well as the environmental conditions. In animal models of anxiety, for example, the effects of a peptide or peptide receptor ligand often depend on the animal in which they are tested. For example, the V1b antagonist SSR149415 shows antidepressant-like effects, but only in the genetically selected Flinders Sensitive Line, but not in Flinders Resistant Line.[23] Similarly, CRH_1 receptor antagonists have anxiolytic effects but often only in rats selectively bred for high anxiety behavior.[28] Intracerebroventricular (icv) infusions of oxytocin decreases anxiety in female rats selectively bred for high anxiety but not in their male counterparts.[31] Conversely, oxytocin increases anxiety in the female rats bred for low anxiety but again had no effect in the males. Thus, oxytocin increases anxiety in low anxiety rats, but it decreases it in high anxiety rats. The peptide teneurin C-terminal associated peptide (TCAP) has also shown differential effects on anxiety in the acoustic startle test depending on the baseline anxiety of the animal.[28]

In experimental models, test conditions will also affect the behavioral outcome of peptide manipulation. SSR149415 shows modest anxiolytic-like effects in classical tests of anxiety such as the elevated plus maze and light–dark test but shows more clear-cut anxiolytic actions in more traumatic models such as the social defeat test.[8] Cholecystokinin (CCK) ligands also show this property. For example, the CCK2 antagonist L-365,260 does not affect baseline acoustic startle responses, but does attenuate fear-potentiated acoustic startle.[29] Many examples of this principle can be found in which paradigms that involve more intense stress are required to observe a behavioral effect.[28]

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00254-2
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Peptides can also have sexually dimorphic effects on behavior. Intranasal arginine vasopressin (AVP) differentially affects social communication processes in men and women.[32] Men and women were administered intranasal AVP and then were shown pictures of same-sex models expressing various facial expressions and asked to rate the approachability/friendliness of the faces. AVP promotes agonistic responses in men, and affiliative responses in women, although both the groups reported an increase in anxiety on a self-report measure.

Environmental stimuli can also play an important role in the modulatory effects of peptides on behavior. Galanin is an orexigenic peptide, but its effects on feeding are dependent on the fat and carbohydrate content of the diet.[20]

These studies emphasize the modulatory role that neuropeptides play in behavior, particularly under challenging conditions of stress.[12]

Peptides Have Coordinated Effects on Behavioral and Physiological Processes

Peptides modulate behavior in a concerted and coordinated way, modulating several parallel behavioral processes in addition to the neuroendocrine and physiological properties required to support the ongoing behavior. Corticotropin releasing hormone (CRH) is the classic example of a peptide that activates a concert of neuroendocrine, autonomic, and behavioral responses to stress. Hypothalamic CRH activates adrenocorticotropin (ACTH) release from the pituitary and subsequently corticosteroid release from the adrenal cortex. In addition to these hypothalamic–pituitary adrenal (HPA) axis functions, CRH and its receptors are distributed throughout many limbic brain regions that modulate behavioral processes. CRH activates behavioral processes that would facilitate responding to a stressor, such as increasing locomotion, anxiety, arousal, and suppressing feeding. CRH has also been implicated in learning and memory processes, cognition, and addictions.

AVP also has both HPA and extrahypothalamic effects on behavior and physiology and works in a coordinated fashion with CRH. AVP plays a critical role in regulating blood pressure and water balance. However, it also modulates emotional and social behavior. In an interesting examination of the coordinated effects of stress on physiology and behavior, rats were rendered hypernatremic and exposed to a psychogenic stress.[15] Hypernatremic rats had decreased HPA and cardiovascular responses to stress, as well as increased circulating oxytocin levels, and decreased anxiety behavior in the social interaction test. The authors suggest that hypernatremia could initiate a coordinated set of behaviors to support engaging in drinking behavior, which for animals may necessitate increased social interaction and decreased anxiety behavior, such as would be required to approach a watering hole in the wild.

Oxytocin has peripheral effects on uterine contractions during childbirth and facilitates ejection of milk. Not surprisingly, oxytocin also plays a role in maternal behaviors, which in rats include nest building, and pup grooming, licking, and retrieval. Oxytocin also facilitates social behavior and reduces aggression. Its uses range clinically from induction of labor, to potential roles in social anxiety disorder and autism.

Neuropeptide Y (NPY) also affects a broad range of behaviors, most notably feeding and water consumption. However, consistent with this proconsummatory action, NPY also plays a role in many other behaviors that can be considered important to supporting this role, such as locomotor behavior, circadian rhythms, temperature regulation, cardiopulmonary function, learning and memory, pain perception, and anxiety. NPY also interacts with central reward pathways to increase the rewarding properties of food, and may play a role in addiction.

Peptides Have Unique Dose–Response Relationships

Peptides and peptide receptor antagonists often show nonlinear dose–response relationships, commonly an inverse-U shaped function.[3,13] Many examples of this can be found in the behavioral literature. For example, 1 μg of alpha-helical $CRH_{9\text{-}41}$ is effective in antagonizing stress-induced reductions in exploration in the elevated plus maze in rats, whereas higher doses of 5 and 25 μg have no effect.[11] Similarly, NPY shows an anxiolytic profile in the elevated plus maze at 700 pmol, but at 7 pmol is anxiogenic.[21] This is probably attributed to the differential activation of NPY receptors at different doses, having an anxiogenic effect via Y2 receptors, and anxiolytic via Y1.

The Tyr-MIF-1 family of peptides also shows unique dose–response characteristics, with low doses being more effective than high doses in the rat forced swim test.[14] In clinical trials, low doses (60–75 mg) of MIF-1 but not higher doses (150–750 mg) are associated with improvement in depressive symptoms.[25]

Unique dose–response relationships for peptides may also result from transport across the blood brain barrier. For example, low leptin levels trigger an increase in feeding, but high circulating leptin in obese individuals does not suppress feeding and high leptin levels aggravate weight gain.[7] However, this phenomenon can largely be explained by saturation of the transport system for leptin.[13]

Peptides May Have Long-Lasting Effects on Behavior

The half-life of a peptide in the blood does not necessarily correlate with the duration of its effect in the body.[13] This may result from a number of factors, including transport

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mechanisms. However, the action of neuropeptides also involves longer-term changes in gene expression and function of local circuits. Peptides can act on G protein-coupled receptors, neighboring cells, and over circuits through effects on gene expression and synaptogenesis. They can also prime activity-dependent release.[18] "Priming" makes vesicles more ready for release, thus enhancing the capacity for local interactions. Peptide-mediated reprogramming of a neuronal system could be the substrate for prolonged behavioral effects.

NPY injected into the basolateral amygdala (BLA) has an anxiolytic effect in the social interaction test which persists for 8 weeks after the end of a series of five NPY injections.[30] Nemifitide, a peptide analog of MIF-1 shows antidepressant effects in the rat forced swim test that continues for 5 days after the completion of a 14-day treatment regimen, an effect that is not seen with tricyclic antidepressants.[24] Repeated TCAP administration shows long-lasting effects on the acoustic startle response in rats.[28]

Consistent with the long-term reorganization of circuits associated with the effects of neuropeptides on behavior is the finding that repeated dosing with neuropeptides is often required to observe a behavioral effect. For example, acute injections of the V1b antagonist SSR149415 are inactive in the rodent forced swim and tail suspension tests, whereas repeated injections show an effect.[23] Repeated injections of oxytocin for 8–10 days show an enhanced antidepressant effect over acute dosing in preclinical models.[1] An acute icv dose of oxytocin has no effect on anxiety-like behavior, but 6 days of treatment results in a decrease in anxiety in female, but not male rats bred for high anxiety behavior.[31]

PEPTIDE MODULATION OF SPECIFIC BEHAVIORS

Peptides play a role in modulating virtually every behavioral process, and integrating behavior with neuroendocrine responses. It is not possible to cover all behaviors in detail in this chapter, and therefore the focus will be on the three most extensively studied classes of peptide-mediated behavior, namely, social, consummatory, and mood related behaviors.

Social Behavior

Social behavior, including parental and reproductive behavior, is critical to the ability to successfully interact with members of one's species, obtain food and mates, and avoid predation.[22] It is therefore not surprising that many different peptides influence social behavior. Oxytocin and vasopressin have been the most extensively studied peptides in this area, are highly conserved and modulate social and reproductive behavior across many species. Many excellent reviews of their role in social behavior are available.[27] Expression of oxytocin and vasopressin receptors outside of the hypothalamus is both sex and species specific, and is believed to be important in defining differences in behavioral effects across species. AVP has a more important role in male behavior and aggression, whereas oxytocin is more closely linked to female behavior. Oxytocin is a key peptide for maternal behavior, and both oxytocin and AVP are important in pair bonding, mate preference, and social recognition memory. In preclinical models, oxytocin generally has positive effects on social behavior, and can restore stress-suppressed social behavior.[28] AVP and oxytocin modulate social behavior in opposite directions and have been suggested to be part of a circuit modulating anxiety especially in response to social defeat in rodents.[17] Modulation of oxytocin and vasopressin systems are targets for intervention in a number of disorders involving social cognition such as schizophrenia, autism, social anxiety disorder, and depression.[4,9]

Social behavior is also facilitated to a large degree by peptide interactions with central reward pathways, such as the mesolimbic dopamine system. AVP facilitates social attachment through interactions with nucleus accumbens dopamine systems. Intranucleus accumbens injections of morphine increase social play in rats, suggesting that mu opioid receptors may be important for the rewarding aspects of social behavior.[33] Many other opiate peptides also play a role in social behavior.[2]

Peptides that decrease anxiety also facilitate social behavior in the social interaction test in rodents. NPY, CRH_1 antagonists, galanin antagonists, and NK-1 antagonists all decrease anxiety in the social interaction test in rodents, and facilitate social interaction.[28]

CONSUMMATORY BEHAVIOR

Feeding behavior is normally tightly controlled by central nervous system mechanisms that balance nutrient and energy requirements of the organism. Many different peptides play a central role in these processes, such as adiposity signals such as leptin and insulin that detect the status of energy reserves, and other peptides that are activated by these signals to increase or decrease the feeding behavior and corresponding alterations in energy expenditures.

Leptin is an adiposity signal and its levels generally correlate directly with body fat. Leptin regulates melanocortin signaling in the hypothalamus through distinct populations of neurons to increase or decrease food intake.

CCK is a satiation factor produced by the intestinal cells in response to meal ingestion. CCK reduces meal size, and CCK-1 antagonists can increase meal size. Other gut peptides that reduce meal size are gastrin releasing peptide, neuromedin B, enterostatin, somatostatin, glucagons-like peptide-1, apolipoprotein A-IV, and peptide YY. Ghrelin is

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a gut peptide that stimulates food intake whether peripherally or centrally administered.

Urocortin inhibits food intake, increases anxiety, and improves performance on some learning and memory tasks. Urocortin interacts extensively with leptin, NPY, orexins, and CRH in its modulation of feeding behavior.

Galanin is an orexigenic peptide, and injection into the hypothalamus stimulates feeding without affecting other feeding-related behaviors such as drinking, locomotion, or grooming.[20] It plays a role in diverse physiological functions such as nociception, arousal/sleep, cognition, feeding, energy balance, thermoregulations, osmotic and water balance, and reproduction.

NPY is involved in a wide range of behaviors, including food intake, anxiety and depression, reward, pain, circadian rhythms, as well as neuroendocrine and cardiorespiratory behaviors. Central injections of NPY stimulate feeding and water intake.

In addition to caloric and nutritional value, feeding behavior is intertwined with the rewarding and emotional aspects of food intake, especially in humans.[34] The reward system plays an important role in feeding behavior, and dopaminergic cells in the VTA have receptors for many peptides involved in feeding such as NPY, leptin, ghrelin, insulin, orexin, and CRH.[34] Similarly, melanocortins regulate feeding and other rewarding and addictive behaviors through interactions with mesolimbic neurons.[5] Orexigenic peptides may also play a role in addiction that the consumption of drugs of abuse and of palatable foods is stimulated by similar mechanisms. Opiates also affect feeding behavior, probably through mu opiate interactions with central reward pathways.[2] Not surprisingly, opiates play a role in the rewarding effects of drugs of abuse of all classes. Opiates also interact with other peptides and neurotransmitters to modulate sexual behavior. Central CCK function has also been implicated in sexual behavior and other reward processes,[29] and effects on feeding may also be influenced by central reward pathways.

Anxiety and Depression

Many different peptides have the potential to influence mood, and indeed, the list of peptides that have shown effectiveness in animal models of anxiety and depression is extensive.[28] There is abundant evidence for the role of HPA dysregulation in mood disorders, and in many psychiatric disorders. Prolonged stress and anxiety are closely linked to mental illness. Therefore, it is probable that any peptide that is involved in the modulation of stress responses could have potential to influence the mood. Most peptides that have anxiogenic properties also show prodepressant effects in animal models, whereas those with anxiolytic effects generally show antidepressant-like effects. The effects of neuropeptide receptor manipulation on anxiety and depression have been reviewed[28] and are briefly summarized here.

The CRH family of peptides in humans consists of CRH, urocortin, urocortin 2 and urocortin 3. Antidepressant and anxiolytic effects of CRH1 antagonists have been noted in behavioral tests that involve either rats selectively bred for high depression or anxiety-like behavior, or under enhanced stress conditions during testing.[28] Urocortin acts on a broad range of behaviors from anxiety, learning and memory to food intake.[26] CRH_1 antagonists, and urocortin (Ucn) 2 and 3 show antidepressant activity in preclinical models of depression. Ucn1 is anxiogenic, whereas Ucn2 and 3 can show both anxiolytic and anxiogenic properties.

NPY is highly implicated in mood regulation. NPY immunoreactivity is expressed throughout cortical and subcortical regions of the rat brain and in key behavioral centers such as the hippocampus, nucleus accumbens, amygdale, and the bed nucleus of the stria terminalis. Preclinical models have shown an antidepressant-like profile after central NPY administration, as well as an anxiety-reducing effect. The anxiety regulating effects of NPY are thought to depend on activity in the amygdala. NPY itself is anxiolytic, whereas blockade of NPY receptors has an anxiogenic effect.[28] NPY compounds have therapeutic potential for obesity, depression, anxiety, addiction, and posttraumatic stress disorder (PTSD).

Clinical trials with MIF-1 have shown rapid antidepressant effects at low doses with good tolerability. MIF-1 has an antidepressant effect that has been shown in several small clinical trials, including a double-blind, placebo-controlled, crossover study in which depressed individuals receiving 10 mg/day of subcutaneous MIF-1 showed remission of symptoms after 5 days.[6]

Arginine vasopressin (AVP) acts peripherally to regulate water balance but has many central neuropeptide effects on behavior mediated through V1a and V1b receptors that are distributed in brain regions associated with regulation of mood and emotion, such as the lateral septum, amygdala, bed nucleus of the stria terminalis (BNST), hippocampal formation, and cortical regions.[17] Vasopressin is also highly implicated in anxiety related behavior, and AVP receptor antagonists are anxiolytic and antidepressant. V1b antagonists have shown antidepressant potential in the rat forced swim test.[8,23]

Similarly, oxytocin receptor agonists are anxiolytic and show antidepressant effects in some models. Intraperitoneal injections of oxytocin can have antidepressant-like effects in the forced swim test.[1] Intranasal oxytocin also has antidepressant potential in humans and shows promise in social anxiety disorder.[10]

Substance P is the preferred ligand for the NK-1 receptor, which is abundantly expressed in limbic regions. Although Substance P was originally implicated in pain and nociception, it is also clear that it plays a role in stress

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and mood regulation. Substance P is anxiogenic and has pro-depressant properties, whereas NK1 receptor antagonists have been tested extensively in animal models of depression, and NK1 antagonists are currently in clinical trials for their antidepressant potential, as well as a possible role in PTSD.

Galanin and Gal3 antagonists have anxiolytic effects in rodent models. Galanin shows prodepressant effects in the forced swim test, probably through GalR1 and GalR3 receptors, whereas galanin antagonists show an antidepressant effect. In rat models of depression, galanin administration seems to enhance depression-like behavior. Central administration of galanin increased immobility in the rat forced swim test.[16] However, the depressive-like effect seems to be specific to the GAL1 and GAL3 subtypes, whereas GAL2 stimulation results in an antidepressant profile. The effects of galanin on anxiety-like behavior seem to depend heavily on the specific test conditions and administration conditions. It seems that galanin has a more modulatory role in anxiety and not a directionally specific anxioltyic or anxiogenic property.

CCK is abundant in the brain and is distributed throughout limbic brain regions and cortex. CCK has well-characterized effects on anxiety and panic behavior in both animals and humans.[29] CCK is anxiogenic, which has been mostly attributed to the CCK2 receptor. CCK2 antagonists are generally anxiolytic, whereas CCK peptides and CCK2 agonists are anxiogenic. CCK2 antagonists have shown efficacy in animal models of depression.

PEPTIDE THERAPEUTICS

Neuropsychiatric disorders represent an area of great potential for peptide-based therapeutics. Their coordinated effects on multiple behavioral and physiological systems provide distributed control over affected networks and may bring more integrated modulation of dysregulated systems for complex disorders such as mood and anxiety disorders in which multiple physiological systems are affected. For example, in depression, in addition to mood dysregulation, there are abnormalities in motivation, reward processes, circadian rhythms, feeding behavior, sexual behavior, and cognitive processes. CRH, SP, NPY, AVP, oxytocin have all been implicated in the disorders of social behavior, mood, and feeding. Thus, peptide modulation is poised to represent a truly novel therapeutic option for future drug development which may more effectively target core symptoms in addition to other related dysfunctional systems.

The development of peptide-based therapeutics for CNS diseases has been hampered by issues relating to poor bioavailability, low stability, and difficulties in crossing the blood brain barrier. However, new research is addressing these issues.[19] Oral delivery of peptides is problematic because of poor absorption and high enzymatic degradation, but many alternative routes of administration exist and are in development such as across nasal, buccal, and other mucosa. Improvements in bioavailability and uptake can be achieved through the inhibition of enzymatic degradation, or chemical modification to improve uptake so that peptide therapeutics can be delivered to the brain and exert their multiple effects. Other methods of facilitating peptide delivery to the CNS are in development.

Peptides have unique properties, including their unique dose–response characteristics, and their modulatory, coordinated effects on multiple systems. Individual differences in behavior and physiology may be a key factor influencing the outcome of peptide therapeutics. The move toward personalized medicine in psychiatry and other CNS therapeutic areas has grown in popularity as a result of the difficulty in matching the correct treatment to the correct patient, and because of the heterogeneity of clinical presentation. As flexible modulators of neurotransmitters, immune function, and neurotrophic factors, peptide therapeutics stand to provide new avenues for therapeutic development.

CONCLUSION

Peptides represent a fascinating class of compounds that modulate behavior in concert with the appropriate physiological and neuroendocrine support. Social behavior, consummatory behavior, and stress modulation are three key areas for survival and are thus modulated by many different peptides. The unique properties of peptides should not be a deterrent to peptide-based therapeutics but rather should be exploited for the unique opportunities associated with peptides.

REFERENCES

1. Arletti R, Bertolini A. Oxytocin acts as an antidepressant in two animal models of depression. *Life Sci* 1987;**41**:1725–30.
2. Bodnar RJ. Endogenous opiates and behavior: 2010. *Peptides* 2011.
3. Calabrese EJ, Baldwin LA. Peptides and hormesis. *Crit Rev Toxicol* 2003;**33**:355–405.
4. Caldwell HK, Wersinger SR, Young 3rd WS. The role of the vasopressin 1b receptor in aggression and other social behaviours. *Prog Brain Res* 2008;**170**:65–72.
5. Davis JF, Choi DL, Shurdak JD, Krause EG, Fitzgerald MF, Lipton JW, et al. Central melanocortins modulate mesocorticolimbic activity and food seeking behavior in the rat. *Physiol Behav* 2011;**102**:491–5.
6. Ehrensing RH, Kastin AJ, Wurzlow GF, Michell GF, Mebane AH. Improvement in major depression after low subcutaneous doses of MIF-1. *J Affect Disord* 1994;**31**:227–33.
7. Gautron L, Elmquist JK. Sixteen years and counting: an update on leptin in energy balance. *J Clin Invest* 2011;**121**:2087–93.
8. Griebel G, Simiand J, Serradeil-Le Gal C, Wagnon J, Pascal M, Scatton B, et al. Anxiolytic- and antidepressant-like effects of the non-peptide vasopressin V1b receptor antagonist, SSR149415, suggest an innovative approach for the treatment of stress-related disorders. *Proc Natl Acad Sci U S A* 2002;**99**:6370–5.

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9. Guastella AJ, Einfeld SL, Gray KM, Rinehart NJ, Tonge BJ, Lambert TJ, et al. Intranasal oxytocin improves emotion recognition for youth with autism spectrum disorders. *Biol Psychiatry* 2010;**67**:692–4.
10. Guastella AJ, Howard AL, Dadds MR, Mitchell P, Carson DS. A randomized controlled trial of intranasal oxytocin as an adjunct to exposure therapy for social anxiety disorder. *Psychoneuroendocrinology* 2009;**34**:917–23.
11. Heinrichs SC, Menzaghi F, Pich EM, Baldwin HA, Rassnick S, Britton KT, et al. Anti-stress action of a corticotropin-releasing factor antagonist on behavioral reactivity to stressors of varying type and intensity. *Neuropsychopharmacology* 1994;**11**:179–86.
12. Hokfelt T, Broberger C, Xu ZQ, Sergeyev V, Ubink R, Diez M. Neuropeptides – an overview. *Neuropharmacology* 2000;**39**:1337–56.
13. Kastin AJ, Pan W. Concepts for biologically active peptides. *Curr Pharm Des* 2010;**16**:3390–400.
14. Kastin AJ, Scollan EL, Ehrensing RH, Schally AV, Coy DH. Enkephalin and other peptides reduce passiveness. *Pharmacol Biochem Behav* 1978;**9**:515–9.
15. Krause EG, de Kloet AD, Flak JN, Smeltzer MD, Solomon MB, Evanson NK, et al. Hydration state controls stress responsiveness and social behavior. *J Neurosci* 2011;**31**:5470–6.
16. Kuteeva E, Hokfelt T, Wardi T, Ogren SO. Galanin, galanin receptor subtypes and depression-like behaviour. *EXS* 2010;**102**:163–81.
17. Litvin Y, Murakami G, Pfaff DW. Effects of chronic social defeat on behavioral and neural correlates of sociality: vasopressin, oxytocin and the vasopressinergic V1b receptor. *Physiol Behav* 2011;**103**:393–403.
18. Ludwig M. Are neuropeptides brain hormones? *J Neuroendocrinol* 2011;**23**:381–2.
19. Malavolta L, Cabral FR. Peptides: important tools for the treatment of central nervous system disorders. *Neuropeptides* 2011;**45**:309–16.
20. Merchenthaler I. Galanin and the neuroendocrine axes. *EXS* 2010;**102**:71–85.
21. Nakajima M, Inui A, Asakawa A, Momose K, Ueno N, Teranishi A, et al. Neuropeptide Y produces anxiety via Y2-type receptors. *Peptides* 1998;**19**:359–63.
22. Norman GJ, Hawkley LC, Cole SW, Berntson GG, Cacioppo JT. Social neuroscience: the social brain, oxytocin, and health. *Soc Neurosci* 2011.
23. Overstreet DH, Griebel G. Antidepressant-like effects of the vasopressin V1b receptor antagonist SSR149415 in the Flinders Sensitive Line rat. *Pharmacol Biochem Behav* 2005;**82**:223–7.
24. Overstreet DH, Hlavka J, Feighner JP, Nicolau G, Freed JS. Antidepressant-like effects of a novel pentapeptide, nemifitide, in an animal model of depression. *Psychopharmacology* 2004;**175**:303–9.
25. Pan W, Kastin AJ. From MIF-1 to endomorphin: the Tyr-MIF-1 family of peptides. *Peptides* 2007;**28**:2411–34.
26. Pan W, Kastin AJ. Urocortin and the brain. *Prog Neurobiol* 2008;**84**:148–56.
27. Pfaff DW, Kordon C, Chanson P, Christen Y. *Hormones and social behavior. Research and perspectives in endocrine interactions*. Berlin Heidelberg: Springer-Verlag; 2008.
28. Rotzinger S, Lovejoy DA, Tan LA. Behavioral effects of neuropeptides in rodent models of depression and anxiety. *Peptides* 2010;**31**:736–56.
29. Rotzinger S, Vaccarino FJ. Cholecystokinin receptor subtypes: role in the modulation of anxiety-related and reward-related behaviours in animal models. *J Psychiatry Neurosci* 2003;**28**:171–81.
30. Sajdyk TJ, Johnson PL, Leitermann RJ, Fitz SD, Dietrich A, Morin M, et al. Neuropeptide Y in the amygdala induces long-term resilience to stress-induced reductions in social responses but not hypothalamic-adrenal-pituitary axis activity or hyperthermia. *J Neurosci* 2008;**28**:893–903.
31. Slattery DA, Neumann ID. Chronic icv oxytocin attenuates the pathological high anxiety state of selectively bred Wistar rats. *Neuropharmacology* 2010;**58**:56–61.
32. Thompson RR, George K, Walton JC, Orr SP, Benson J. Sex-specific influences of vasopressin on human social communication. *Proc Natl Acad Sci U S A* 2006;**103**:7889–94.
33. Trezza V, Damsteegt R, Achterberg EJ, Vanderschuren LJ. Nucleus accumbens mu-opioid receptors mediate social reward. *J Neurosci* 2011;**31**:6362–70.
34. Vucetic Z, Reyes TM. Central dopaminergic circuitry controlling food intake and reward: implications for the regulation of obesity. *Wiley Interdiscip Rev Syst Biol Med* 2010;**2**:577–93.
35. Yu Y, Jawa A, Pan W, Kastin AJ. Effects of peptides, with emphasis on feeding, pain, and behavior A 5-year (1999–2003) review of publications in Peptides. *Peptides* 2004;**25**:2257–89.

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Chapter 255

Peptides and Sleep

A. Steiger

ABSTRACT

A bidirectional interaction exists between the electrophysiological activity of sleep as recorded by sleep electroencephalogram (EEG) and nocturnal hormone secretion. Various neuropeptides participate in sleep regulation. A reciprocal interaction of growth hormone-releasing hormone (GHRH) and corticotropin-releasing hormone (CRH) plays a key role in sleep regulation. GHRH promotes sleep and growth hormone and inhibits cortisol, whereas CRH exerts opposite effects. Age and gender seem to modulate these effects. Also ghrelin and galanin promote sleep, whereas somatostatin impairs sleep. Neuropeptide Y regulates sleep onset. VIP is involved in the temporal organization of sleep. Pituitary adenylate cyclase-activating polypeptide modulates sleep homeostasis. Orexin/hypocretin is an important sleep-regulating factor. Its lack results in narcolepsy.

INTRODUCTION

Human sleep is characterized by the cyclic occurrence of periods of non-rapid eye movement (non-REM) sleep and rapid eye movement (REM) sleep and by distinct patterns of hormone secretion. During the first non-REM sleep period, the major portion of slow wave sleep (SWS) and in electroencephalogram (EEG) spectral analysis the major portion of slow wave activity (SWA) occur. During the first half of the night, the growth hormone (GH) surge preponderates, whereas corticotropin (ACTH) and cortisol levels are low. It is vice versa during the second half of the night; ACTH and cortisol concentrations are high, whereas GH release is low. This pattern points to (1) a reciprocal interaction of the hypothalamic–pituitary–somatotropic (HPS) and the hypothalamic–pituitary–adrenocortical (HPA) systems and (2) the existence of common regulators of the sleep EEG and the nocturnal hormone secretion. As described in detail below, a reciprocal interaction of the key hormones of the HPS and HPA systems, GH-releasing hormone (GHRH) and corticotropin-releasing hormone (CRH) seem to play a major role in sleep regulation.

HYPOTHALAMIC–PITUITARY–SOMATOTROPIC (HPS) SYSTEM

Basic Activity

In humans, the major GH peak is found closer to sleep onset. This GH surge is associated with the first SWS period. The GH surge is widely sleep-dependent and is suppressed during sleep deprivation, whereas there is a weak circadian component in the regulation of GH release.[30] As early as during the third decade of our life span, parallel decrease in SWS, SWA, and GH secretion begin.[3]

Hypothalamic GHRH mRNA depends on a circadian rhythm. In rats, its highest concentration occurs at the onset of the light period when sleep propensity reaches its maximum. Hypothalamic GHRH has sleep-related variations with low levels in the morning, increases in the afternoon, and decreases at night.[18] In patients with isolated GH deficiency, SWS is lower than in normal controls. Excessive GH levels are found in patients with acromegaly. One year after tumor removal, REM sleep and SWS time increase in these patients.[1]

Growth Hormone-Releasing Hormone

GHRH is an important endogenous sleep-promoting substance. In mice, the GHRH gene is found in the region linked to SWA. Intracerebroventricular (icv) GHRH increases SWS in rats and rabbits. The same effect occurs after the injection of GHRH into the medial preoptic area in rats or after iv administration to rats.[18,27]

Similarly, after repetitive hourly iv injections of GHRH around sleep, onset SWS and GH increase whereas cortisol is blunted in young men. Mimicking of the pulsatile endogenous release seems to be crucial because sleep remains unchanged after GHRH infusion. Sleep promotion in young males resumes after GHRH by iv and intranasal administration. The effects of GHRH on human sleep were examined in three states with a change of the GHRH/CRH ratio in favor of CRH. (1) After repetitive iv GHRH between 04:00 and 07:00 h, sleep is not affected. Only a weak

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00255-4
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sleep-promoting effect of GHRH occurs in the elderly. (2) The effect of iv GHRH in drug-free depressed patients of both sexes (19–76 years) and in matched controls shows a sexual dimorphism in the response to GHRH. In male patients and control subjects, GHRH decreases ACTH and cortisol, whereas these hormones are enhanced in normal and depressed females. Similarly, non-REM sleep increases and wakefulness decreases in males, whereas sleep-impairing effects occur in women.[27] These results point to a reciprocal antagonism of GHRH and CRH in men, whereas a synergism of GHRH and CRH is suggested in women. In rats, non-REM sleep decreases and sleep latency increases after GHRH receptor antagonists. Non-REM sleep is reduced after administration of antibodies to GHRH.[18]

In the rat preoptic hypothalamus, GABAergic neurons in the median preoptic nucleus and the ventrolateral preoptic nucleus (VLPO) are potential targets of the sleep-regulatory actions of GHRH.[20] Multilateral application of low doses of GHRH to the surface of the somatosensory cortex of rats diminishes SWA ipsilaterally, whereas after higher dosages SWA increases. Multilateral application of a GHRH antagonist onto the somatosensory cortex surface of rats decreases SWA ipsilaterally. Similarly injection of GHRH receptors small interfering RNA is followed by the suppression of SWA for up to 24 h.[13]

Sleep deprivation is a powerful stimulus for sleep. GHRH seems to mediate this effect. It is antagonized by GHRH antibodies in rats and by microinjections of a GHRH antagonist into the area preoptica of rats. After sleep deprivation, a depletion of hypothalamic GHRH and low hypothalamic GHRH contents occur, whereas hypothalamic GHRH mRNA increases in rats. This high rate of release is thought to stimulate transcription. The increase in hypothalamic GHRH mRNA concentration is associated with decreases in hypothalamic somatostatin. GHRH receptor mRNA and GHRH binding decline in the rat hypothalamus after sleep deprivation, whereas pituitary GHRH receptors remain unchanged.[18]

The effect of GHRH on recovery sleep after sleep deprivation was tested in a sample of healthy volunteers of a wide age range and of both sexes. The increase of non-REM and the decrease of wakefulness after sleep deprivation are augmented by GHRH. Interestingly this effect is shared by CRH.[25]

In mice, viral infections enhance non-REM sleep. After influenza A infection, non-REM sleep increases in wild-type mice. However, in the GHRH-receptor-deficient *lit/lit* mice, non-REM sleep and SWA decrease after influenza inoculation. Non-REM sleep decreases after negative feedback inhibition of GHRH by GH in humans, cats, and rats or after higher dosages of icv insulin-like growth factor-(1 IGF-1). However, GH antagonism impairs sleep.[18,27] In sum, non-REM sleep is promoted by high GHRH activity at least in male humans and animals, whereas non-REM sleep declines when GHRH activity is reduced.

Somatostatin

Selective increases of REM sleep have been reported in rats after icv somatostatin. Systemic and icv administration of the somatostatin analog octreotide in rats decreases non-REM sleep and GH.[2] Similarly, SWS declines, and wakefulness increases in young men after subcutaneous octreotide administration. Octreotide is long acting and more potent than somatostatin is. This explains why repetitive iv somatostatin impairs sleep in normal elderly subjects, but not in young men.[27] A reciprocal interaction of GHRH and somatostatin in sleep regulation similar to their opposite effects on GH release is probable.

Ghrelin

Similar to GHRH, repetitive iv ghrelin enhances SWS and GH in young men. In contrast to GHRH, which blunts cortisol release in young men, ACTH and cortisol increase after ghrelin administration. Also in mice, ghrelin promotes non-REM sleep. An intact GHRH receptor is the prerequisite for this effect, because ghrelin does not modulate sleep in mice with nonfunctional GHRH receptors.

In addition to its stimulating effect on GH and cortisol, ghrelin elevates the nocturnal release of prolactin in young healthy male volunteers. Furthermore, in healthy young men receiving ghrelin, the concentrations of luteinizing hormone (LH) and testosterone decrease during the early hours of the night. After ghrelin administration, both a delay and a suppression of the amplitude of LH pulses occur. After ghrelin administration in young healthy female and male volunteers, nocturnal thyroxine (T_4) concentrations are elevated followed by marked decline of thyroid-stimulating hormone (TSH). These findings suggest that ghrelin suppresses the hypothalamus–pituitary–thyroid (HPT) axis in humans which counteracts its energy-saving effects.

In a series of human studies, the role of the time of administration, sex, and age on the effects of ghrelin on sleep were studied. Sleep EEG is not changed after pulsatile administration of ghrelin during the early morning hours. In young healthy female volunteers, pulsatile ghrelin injections around sleep onset do not affect sleep EEG, whereas the changes of cortisol and GH are similar to those in young men. This sexual dimorphism is also found in older healthy women and men. Although sleep is promoted in healthy male volunteers who are ≥60 years, sleep EEG remains unaffected in women of the same age range.

A bolus injection of 100 μg of ghrelin at 22:00 h prompted distinct hunger in a single healthy volunteer. This effect differs from sleep promotion after 4 × 50 μg of ghrelin

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starting at the same time. The development of hunger after ghrelin administration may explain the contrasting findings of a series of studies of Szentirmai and colleagues, in the rat. Wakefulness and food intake increase after intracerebroventricular and intrahypothalamic ghrelin administration. In some of these experiments, the feeding period is followed by increases of SWA. This change may mirror a delayed sleep-promoting action of ghrelin. Similarly ghrelin knockout mice sleep less than the wild type do. Also, this finding is compatible with the sleep-promoting effect of ghrelin.

The relationships among plasma ghrelin and hypothalamic ghrelin content and sleep and feeding were investigated in rats. There is a major influence of feeding on the diurnal rhythm of ghrelin. Variations in hypothalamic ghrelin suggest an association with the sleep–wake activity. One study examined interactions of sleep and ghrelin levels in young men who were semirecumbent during 24 h. When they were allowed to sleep, ghrelin increased by sleep onset. This is followed by a decline throughout the night. The nocturnal rise is blunted during sleep deprivation. Ghrelin levels were determined between 20:00 and 07:00 h in normal female and male control subjects who were active during daytime. In males, ghrelin increases continuously between 20:00 and 23:00 h. In females, however, ghrelin levels at 20:00 h are already in the same range as they are during the sleeping period. In a sample of >1000 subjects, short sleep-time is associated with higher ghrelin levels. Similarly, the comparison between extended (12 h) and restricted (4 h) sleep time during 2 days each shows increases in ghrelin after sleep restriction at daytime.[28]

GH Secretagogs

Before the discovery of ghrelin, synthetic GH secretagogs were available. Several studies investigated the effects of these substances on human sleep. Oral administration of the GH secretagog MK-677 for 1 week prompts a distinct sleep-promoting effect in healthy young male volunteers, whereas a weak effect is observed in elderly subjects. This study shows that oral administration of a peptide is capable of promoting sleep. After repetitive intravenous administration of GH-releasing peptide 6 (GHRP-6), non-REM sleep stage 2 increases. Similar to the effects of ghrelin in male subjects GH, ACTH, and cortisol are elevated. In a set of studies, the intranasal, oral, and sublingual administration of GHRP-6 was tested. By these routes of administration the effects of the peptide on the sleep EEG and on hormone secretion are less distinct and partly different from those after intravenous injection. In contrast to the sleep-promoting effects of GHRP-6 and ghrelin, hexarelin prompts a decrease of SWS, whereas the pattern of endocrine effects after hexarelin resemble the endocrine changes after ghrelin and GHRP-6 in that there is a marked stimulation of GH. The decrease of SWS after hexarelin may be related to negative feedback inhibition of endogenous GHRH. After a single dose of GHRP-2 during the third period of REM sleep, sleep remains unchanged. The lack of effects in this study may be related to the method that uses only a single injection of the substance.[28]

Animal Models of HPS System Changes

In the giant transgenic mice, GH is permanently elevated. During the light period, non-REM sleep is higher and REM sleep is almost doubled in these mice. After sleep deprivation, these mice sleep more than normal mice do. In dwarf rats, with deficits in the central GHRHergic transmission and reduced hypothalamic GHRH, non-REM sleep is reduced compared with that in the wild type. In dwarf homozygous *(lit/lit)* mice with a nonfunctional GHRH receptor, non-REM sleep and REM sleep are lower than that in control mice. Chronic infusion of GH leads to normalization of REM sleep but not of non-REM sleep. Ghrelin, GHRH, and octreotide exert no effect on sleep EEG in dwarf mice. These results suggest that (1) GHRH deficiency is linked with decreases in non-REM sleep; (2) decreases in GH lead to decreases in REM sleep; (3) the actions of GHRH, ghrelin, and octreotide on sleep EEG require intact GHRH receptor signaling.[18]

HYPOTHALAMIC PITUITARY ADRENOCORTICAL SYSTEM

Basic Activity

In rats, CRH gene transcription levels increase during the dark period, when the animals are active, and decrease throughout the light period. In humans, both the nadir and the major portion of the secretion of ACTH and cortisol occur during sleep. Between 02:00 and 03:00 h, the first pulse of cortisol occurs. It is followed by further pulses until awakening.[32] Although ACTH is the prime regulator of nocturnal cortisol secretion in humans, the secretion of ACTH and cortisol may dissociate[4] [for reviews, see Ref. 27].

Sleep in Disorders with Pathological Changes of HPA Activity

Characteristic sleep-EEG findings in depressed patients are disturbed sleep continuity, a decrease of non-REM sleep, and REM sleep disinhibition. Well documented endocrine changes include signs of (1) HPA overactivity and (2) HPS dysfunction. Most sleep-endocrine studies in depressed patients report elevated cortisol and ACTH, in comparison with normal controls. GH is blunted in most but not in all studies.[27]

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These findings suggest a causal relationship between shallow sleep, low GH, and HPA hyperactivity in depression. Further, there are similarities in the sleep-endocrine changes during depression and during normal aging. Linkowski et al.[15] compared HPA hormones longitudinally between acute depression and recovery. These authors reported a decrease of ACTH and cortisol 24 h after recovery. Furthermore, Linkowski et al. reported a normalization of REM sleep after recovery. Because some of the patients received antidepressants, which suppress REM sleep, it is difficult to differentiate between the effects of drugs and of remission. In another study, an intraindividual comparison was performed in depressed patients who were drug-free at least 14 days before each examination. This study confirmed a decrease of cortisol after recovery. However, the sleep-EEG changes and low GH concentrations persist in the recovered patients.[27] Similar to the findings in depression, 24-hour ACTH and cortisol are higher in young insomniacs than in controls.[31]

Effects of Changes of Sleep–Wake Behavior on HPA Hormones

In rats, after 72 h of sleep deprivation, CRH levels increase in the striatum, limbic areas, and pituitary, whereas hypothalamic CRH decreases. CRH binding decreases in the striatum and in the pituitary. When rats are sleep deprived, during intervals up to 92 h, ACTH and corticosterone plasma levels increase from 24 h of sleep deprivation and decrease during the recovery period. The expectation of waking up at a certain time induces a marked increase in ACTH before the end of sleep. The anticipatory increase in ACTH may facilitate spontaneous waking.[27]

Corticotropin-Releasing Hormone

After icv CRH, SWS decreases in rats[8] and rabbits.[19] Even after 72 h of sleep deprivation, CRH reduces SWS in rats. Further, sleep latency and REM sleep increase. Similarly after repetitive iv CRH around sleep onset in young men SWS and REM sleep decrease. Furthermore, GH is blunted and cortisol increases. A dose of CRH that does not affect sleep in young men, impairs sleep in middle-aged men.[27] Obviously, the vulnerability of sleep to CRH increases during aging.

After two different CRH antagonists, α-helical CRH and astressin, wakefulness declines. By contrast, another study reported an effect of α-helical CRH only in stressed animals. In these rats, REM sleep increases and decreases to values of the nonstressed condition after the substance. In sleep-deprived rats, α-helical CRH diminishes the REM sleep rebound during recovery sleep. Stress acting via CRH is believed to induce the REM sleep rebound after sleep deprivation. In the rat, amygdala kindling at light onset decreases SWS and REM sleep and enhances corticosterone. Icv astressin or alpha-helical CRH antagonizes the decrease of SWS after kindling. After a 4-week trial with a CRH receptor-1-antagonist, the characteristic sleep-EEG changes in patients with depression are counteracted. The number of awakenings and REM sleep density decreases and SWS increases. These results suggest that CRH is involved in the pathophysiology of sleep-EEG changes during depression and that CRH-1-receptor antagonism helps to treat symptoms of depression including impaired sleep.[27]

Vasopressin

Icv vasopressin increases wakefulness in rats. After acute infusion of the peptide to humans, stage 2 sleep increases and REM sleep decreases. Chronic intranasal vasopressin for 3 months improves sleep in elderly subjects.[27]

CORTICOTROPIN

Infusions of ACTH suppress REM sleep in normal subjects, whereas cortisol and GH increase. The synthetic ACTH (4–9) analog ebiratide shares several behavioral effects of ACTH whereas it has no influence on peripheral hormone secretion. Accordingly, after repetitive iv ebiratide administration, GH and cortisol remain unchanged in young men. A set of sleep-EEG changes is found after ebiratide corresponding to a general central nervous system (CNS) activation.[27]

Animal Models of HPA System Changes

In the Lewis rat, the synthesis and the release of CRH is reduced in comparison with other rat strains. Lewis rats spend more time in SWS and less time awake than the intact strains. After icv CRH, waking increases similarly in Lewis and Sprague–Dawley rats. A role of CRH in the maintenance of wakefulness and sleep-disturbing effects of CRH is confirmed by this study. Similarly, spontaneous wakefulness of rats decreases after CRH antisense.[27]

Sleep of conditional mouse mutants overexpressing CRH in the total CNS or selectively in the forebrain including limbic structures was investigated in comparison to sleep in control animals. In both strains of homozygous CRH-overexpressing mice, elevated REM sleep occurs constantly during 24 h. A weak decrease of non-REM sleep during the night is observed only in the strain overexpressing CRH in the entire CNS. REM sleep increases after 6 h of sleep deprivation during recovery sleep and also in heterozygous CRH-overexpressing mice. This effect is reversed in heterozygous and homozygous CRH-overexpressing mice as well by treatment with a CRH-1 receptor antagonist. No

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change of peripheral HPA hormones occurs at baseline or after sleep deprivation. Because the peripheral HPA axis is not modulated in these animals, the sleep-EEG changes, particularly the elevation of REM sleep, probably results from elevated forebrain CRH through activation of CRH-1 receptors.[12]

HYPOTHALAMIC–PITUITARY–THYROID SYSTEM

The release of TSH follows a circadian rhythm. The lowest TSH levels are found during daytime. TSH increases during the night and reaches its maximum by midnight. Pulsatile iv thyrotrophin-releasing hormone (TRH) prompts decreases of sleep efficiency and earlier occurrence of the cortisol morning increase in young male subjects.[27]

OTHER PEPTIDES

After icv cortistatin, non-REM sleep increases in rats distinctly.[6]

Delta-Sleep Inducing Peptide (DSIP)

Delta-sleep inducing peptide (DSIP) was first detected in the cerebral venous blood of rabbits that had been subjected to sleep by electric stimulation of the intralaminal thalamic area. Promotion of SWS after DSIP was reported in several species, although not all preclinical studies reproduced these effects. Only one study has investigated the effects of DSIP on sleep EEG in normal men and found only minor effects. Studies on the efficiency of DSIP in the treatment of insomnia produced conflicting results. DSIP-like immunoreactivity was compared in young men between baseline sleep, sleep deprivation, and recovery sleep. Plasma DSIP-like immunoreactivity decreased at the transition from wakefulness to sleep in both evening sleep and morning recovery sleep. These results suggest that DSIP is induced by mechanisms that are involved in the initiation of sleep.[27]

Galanin

Sleep in rats remains unchanged after icv galanin, whereas REM sleep deprivation induces galanin gene expression. After repetitive iv galanin in young men, SWS and REM sleep periods increase.[27] A cluster of GABAergic and galaninergic neurons was identified in the ventrolateral preoptic area, which may stimulate non-REM sleep.[23] Iv galanin or placebo were given to patients with depression during a steady state of antidepressive therapy with trimipramine. After galanin administration, REM sleep latency increases and the severity of depression decreases.[27]

Neuropeptide S

Neuropeptide S (NPS) is believed to elevate wakefulness and to participate in the regulation of anxiety. NPS acts via the NPS receptor. NPS mRNA is widely localized in the brain including the amygdala and the midline thalamic nuclei. Central administration of NPS to rats increases wakefulness and decreases SWS and REM sleep in a dose-dependent fashion.[21]

Neuropeptide Y (NPY)

After icv neuropeptide Y (NPY), EEG spectral activity changes in rats similarly to the effects of benzodiazepines.[9] The prolongation of sleep latency by CRH is antagonized dose dependently by NPY in rats.[10] Although these findings point to a sleep-promoting effect of NPY, intracerebroventricular and lateral hypothalamic administration of NPY to rats suppressed non-REM sleep and REM sleep during the first hour after the injection.[29] However, also human studies suggest sleep-promoting effects of NPY. In young men, repetitive iv administration of NPY decreases sleep latency, the first REM sleep period and cortisol and ACTH levels, and increases stage 2 sleep and sleep period time. In depressed patients of both sexes, with a wide age range and age-matched controls, the sleep latency decreases and prolactin increases after NPY administration, whereas cortisol and ACTH levels and the first REM sleep period remain unchanged.[27] These results suggest that NPY participates in sleep regulation, particularly in the timing of sleep onset as an antagonist of CRH acting via the $GABA_A$ receptor.

Pituitary Adenylate Cyclase Activating Polypeptide (PACAP)

Icv pituitary adenylate cyclase-activating polypeptide (PACAP) enhances REM sleep in rats.[11] Repetitive iv injections of PACAP to young male healthy volunteers prompt a significant decrease in the theta frequency range during the first 4-h interval and in the spindle frequency range during the second 4 h interval. The decline of the time constant τ (tau) of the physiological delta power increases during the night. This observation suggests a decrease of the dynamics of homeostatic sleep regulation. Furthermore, nocturnal prolactin secretion tends to decrease trend during the first four hours of the night.[16]

Substance P

Microinjection of substance P into the ventrolateral preoptic area of rats enhances SWS. This effect is blocked by a phospholipase C inhibitor and by 3-mercaptopropionic acid, an

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inhibitor of GABA synthesis and release.[33] In young men, repetitive iv substance P increases wakefulness and REM sleep latency.[14]

Vasoactive Intestinal Polypeptide (VIP)

Icv vasoactive intestinal polypeptide (VIP) enhances REM sleep. VIP given to rats during the dark period enhances non-REM sleep and REM sleep. Also, VIP microinjections into the pontine reticular tegmentum enhance REM sleep in rats. Immunoneutralization of prolactin inhibits the REM sleep promotion by systemic VIP in rats. Stimulation of prolactin seems to be involved in the promotion of REM sleep after VIP. In young men, two doses of iv VIP prompt different effects. After the lower dose prolactin decreases, sleep EEG remains unchanged. By contrast, after the administration of a higher dose of VIP, prolactin increases. Further, the non-REM sleep – REM sleep cycles are decelerated. Each of the non-REM sleep and REM sleep periods is prolonged, the cortisol nadir seems advanced and the GH surge is blunted. It is believed that VIP exerts a specific effect on the temporal organization of the sleep cycles and the sleep-related hormone secretion. VIP seems to affect the circadian clock, resulting in longer sleep cycles and the early occurrence of the cortisol nadir. The blunted GH surge may arise from the advanced elevated HPA activity.[27]

OREXINS/HYPOCRETINS

In 1998, two groups discovered a pair of neuropeptides in the lateral hypothalamus, which they named orexins[22] or hypocretins.[7] Animal models showed that a lack of orexins or their receptors causes symptoms of narcolepsy. In narcolepsy, several abnormalities of the sleep–wake organization and the sleep structure occur. This includes cataplexy, a sudden and transient episode of loss of muscle tone, triggered by emotions, excessive daytime sleepiness, and in the sleep EEG sleep onset REM periods. The latter means the occurrence of REM sleep during the first 10 min after sleep onset. A cataplexy-like behavior occurs in knockout mice, which do not produce orexins. A genetic form of narcolepsy was found in dogs related to mutation of the gene for the orexin-B receptor. Only few orexin neurons are found in the brains of patients with narcolepsy.[17] Orexin A levels are low in patients with narcolepsy, whereas plasma concentrations are in the normal range.[5] Saper et al.[24] suggest that the role of the orexins is to stabilize the sleep–wake pattern. This occurs by preventing unwanted transitions between sleep and wake stages. In patients with narcolepsy, a lack of orexins or orexin receptors leads to many unintended transitions between the vigilance states and to the intrusion of fragments of REM sleep into wakefulness. Siegel[26] hypothesized that orexin facilitates "motor activity tonically and physically in association with motivated behaviors and to coordinate their facilitation with the activation of attention and sensory systems." After orexin A administration, cataplexy is reduced, and the sleep–wake pattern is normalized in a dog model of narcolepsy.[23]

CONCLUSIONS

The results reviewed in this chapter show that various neuropeptides exert specific effects on the sleep EEG in several species including humans. Figure 1 provides a model of peptidergic sleep regulation in humans.

It is well documented that a reciprocal interaction of the neuropeptides GHRH and CRH plays a key role in sleep regulation. GHRH promotes sleep, at least in males, and stimulates GH, whereas CRH enhances vigilance and the secretion of ACTH and cortisol and impairs sleep. Changes in the CRH:GHRH ratio in favor of CRH contribute to shallow sleep, elevated cortisol, and blunted GH during depression and aging. GHRH participates in sleep promotion after sleep deprivation. There may be CRH-like effects of GHRH in women. Several findings, particularly the effects of CRH-1 receptor antagonism in patients with depression, suggest that CRH promotes REM sleep, whereas other studies do not support this view. NPY is another endogenous antagonist of CRH. Its major role seems to be the timing of sleep onset. Besides CRH, somatostatin is a sleep-impairing peptide. At least in males' GHRH and somatostatin exert opposite actions on GH and on sleep. Besides GHRH, galanin, and ghrelin promote SWS. Intact GHRH receptors are the prerequisite for sleep promotion by ghrelin. In contrast to GHRH, ghrelin stimulates HPA hormones in males and may act as an interface between the HPA and HPS systems. Galanin is colocalized with GABA in the ventrolateral preoptic nucleus. Many hypothalamic GHRH responsive neurons are GABAergic. Galanin, ghrelin, and GHRH may either act in a synergistic fashion, or these peptides may be part of a cascade resulting in the promotion of non-REM sleep. GABAergic neurons probably mediate the effects of these peptides. In young normal men, after administering VIP, the non-REM sleep/REM sleep cycle is decelerated, probably by action on the suprachiasmatic nucleus. PACAP seems to be involved in the regulation of sleep homeostasis. Orexins/hypocretins are important factors of sleep–wake regulation. They lack results in narcolepsy.

ACKNOWLEDGMENTS

Studies in the authors' laboratory were supported by grants from the Deutsche Forschungsgemeinschaft (Ste 486/1-2, 5-1 to 5-4).

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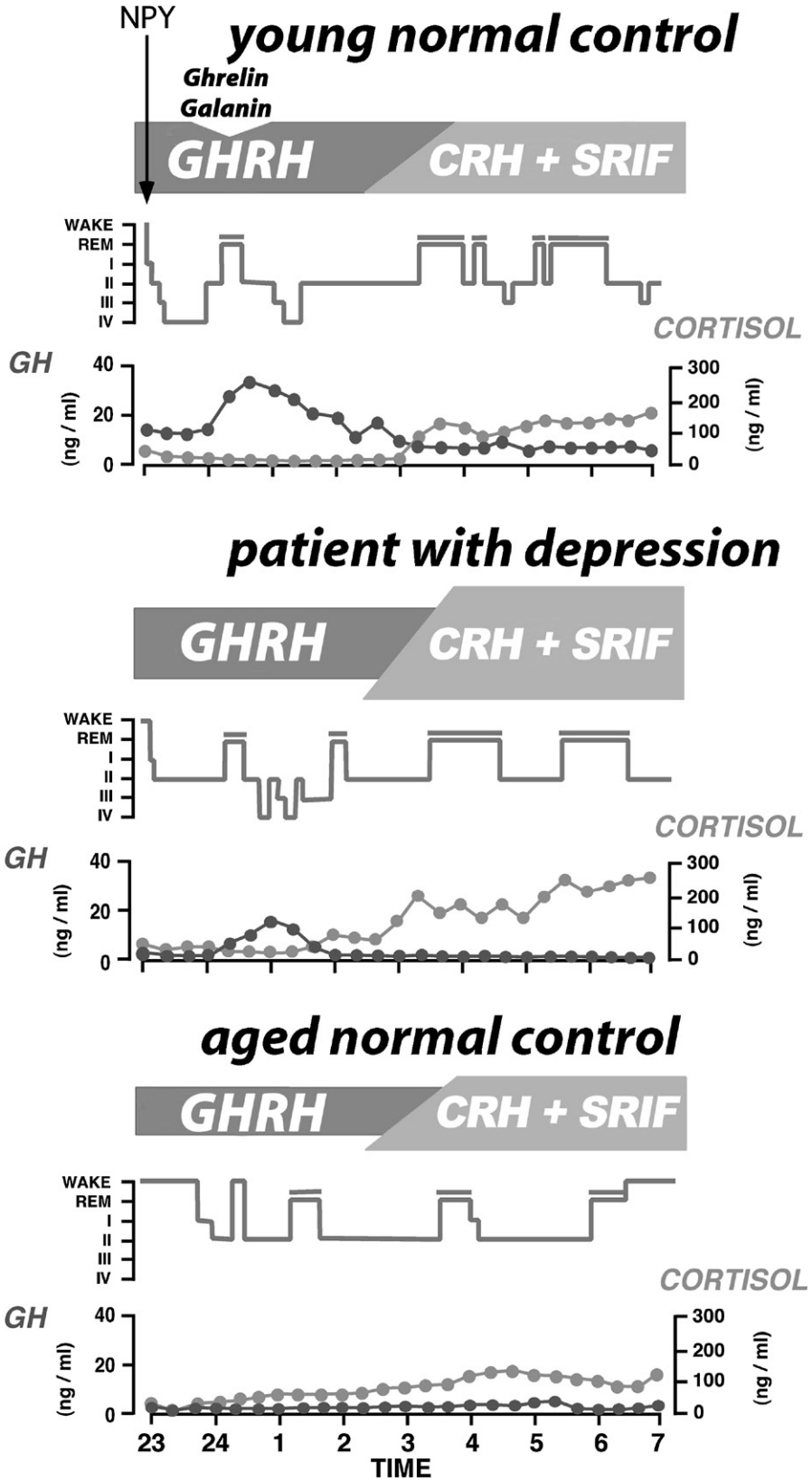

FIGURE 1 Model of peptidergic sleep regulation, CRH, corticotrophin-releasing hormone; GHRH, growth hormone-releasing hormone; NPY, neuropeptide Y; SRIF, somatostatin. Characteristic hypnograms and patterns of cortisol and GH secretion are shown in a young and in an elderly control subject and in a depressed patient. It is believed that GHRH is released during the first half of the night, whereas CRH preponderates during the second half of the night. GHRH contributes to the high amounts of GH and SWS after sleep onset, whereas CRH is linked with cortisol release und REM sleep in the morning hours. Neuropeptide Y (NPY) is a signal for sleep onset. In addition to GHRH, galanin and ghrelin are sleep-promoting factors, whereas somatostatin is a sleep-impairing factor. During depression (CRH overactivity) and during normal aging, similar changes of sleep-endocrine activity occur. It is believed that changes in the GHRH/CRH balance in favor of CRH play a key role in these alterations. Nervenarzt (1995), 66: 15–27, Schlafendokrinologie, Axel Steiger, Fig. 2, Copyright Springer-Verlag 1995, with kind permission of Springer Science and Business Media.

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REFERENCES

1. Åström C, Trojaborg W. Effect of growth hormone on human sleep energy. *Clin Endocrinol* 1992;**36**:241–5.
2. Beranek L, Hajdu I, Gardi J, Taishi P, Obál Jr F, Krueger JM. Central administration of the somatostatin analog octreotide induces captopril-insensitive sleep responses. *Am J Physiol Regul Integr Comp Physiol* 1999;**277**:R1297–304.
3. Bliwise DL. Sleep in normal aging and dementia. *Sleep* 1993;**16**:40–81.
4. Born J, Fehm HL. Hypothalamus-pituitary-adrenal activity during human sleep: a coordinating role for the limbic hippocampal system. *Exp Clin Endocrinol Diabetes* 1998;**106**:153–63.
5. Dalal MA, Schuld A, Haack M, Uhr M, Geisler P, Eisensehr I, et al. Normal plasma levels of orexin A (hypocretin-1) in narcoleptic patients. *Neurology* 2001;**56**:1749–51.
6. de Lecea L, Criado JR, Prospero-Garcia O, Gautvik KM, Schweitzer P, Danielson PE, et al. A cortical neuropeptide with neuronal depressant and sleep-modulating properties. *Nature* 1996;**381**:242–5.
7. de Lecea L, Kilduff TS, Peyron C, Gao XB, Foye PE, Danielson PE, et al. The hypocretins–hypothalamus-specific peptides with neuroexcitatory activity. *Proc Natl Acad Sci U S A* 1998;**95**:322–7.
8. Ehlers CL, Reed TK, Henriksen SJ. Effects of corticotropin-releasing factor and growth hormone-releasing factor on sleep and activity in rats. *Neuroendocrinology* 1986;**42**:467–74.
9. Ehlers CL, Somes C, Lopez A, Kirby D, Rivier JE. Electrophysiological actions of neuropeptide Y and its analogs: new measures for anxiolytic therapy? *Neuropsychopharmacology* 1997;**17**:34–43.
10. Ehlers CL, Somes C, Seifritz E, Rivier JE. CRF/NPY interactions: a potential role in sleep dysregulation in depression and anxiety. *Depress Anxiety* 1997;**6**:1–9.
11. Fang J, Payne L, Krueger JM. Pituitary adenylate cyclase activating polypeptide enhances rapid eye movement sleep in rats. *Brain Res* 1995;**686**:23–8.
12. Kimura M, Müller-Preuss P, Lu A, Wiesner E, Flachskamm C, Wurst W, et al. Conditional corticotropin-releasing hormone overexpression in the mouse forebrain enhances rapid eye movement sleep. *Mol Psychiatry* 2010;**15**:154–65.
13. Liao F, Taishi P, Churchill L, Urza MJ, Krueger JM. Localized suppression of cortical growth hormone-releasing hormone receptors state-specifically attenuates electroencephalographic delta waves. *J Neurosci* 2010;**30**:4151–9.
14. Lieb K, Ahlvers K, Dancker K, Strohbusch S, Reincke M, Feige B, et al. Effects of the neuropeptide substance P on sleep, mood, and neuroendocrine measures in healthy young men. *Neuropsychopharmacology* 2002;**27**:1041–9.
15. Linkowski P, Mendlewicz J, Kerkhofs M, Leclercq R, Golstein J, Brasseur M, et al. 24-hour profiles of adrenocorticotropin, cortisol, and growth hormone in major depressive illness: effect of antidepressant treatment. *J Clin Endocrinol Metab* 1987;**65**:141–52.
16. Murck H, Steiger A, Frieboes RM, Antonijevic IA. Pituitary adenylate cyclase activating peptide affects homeostatic sleep regulation in healthy young men. *Am J Physiol Endocrinol Metab* 2007;**292**:E853–7.
17. Nishino S. Hypothalamus, hypocretins/orexin, and vigilance control. *Handb Clin Neurol* 2011;**99**:765–82.
18. Obál Jr F, Krueger JM. GHRH and sleep. *Sleep Med Rev* 2004;**8**:367–77.
19. Opp M, Obál Jr F, Krueger JM. Corticotropin-releasing factor attenuates interleukin 1-induced sleep and fever in rabbits. *Am J Physiol Regul Integr Comp Physiol* 1989;**257**:R528–35.
20. Peterfi Z, McGinty D, Sarai E, Szymusiak R. Growth hormone releasing hormone activates sleep regulatory neurons of the rat preoptic hypothalamus. *Am J Physiol Regul Integr Comp Physiol* 2010;**298**:R147–56.
21. Reinscheid RK. Neuropeptide S: anatomy, pharmacology, genetics and physiological functions. *Results Probl Cell Differ* 2008;**46**:145–58.
22. Sakurai T, Amemiya A, Ishii M, Matsuzaki I, Chemelli RM, Tanaka H, et al. Orexins and orexin receptors—a family of hypothalamic neuropeptides and G protein-coupled receptors that regulate feeding behavior. *Cell* 1998;**92**.
23. Saper CB, Chou TC, Scammell TE. The sleep switch: hypothalamic control of sleep and wakefulness. *Trends Neurosci* 2001;**24**:726–31.
24. Saper CB, Scammell TE, Lu J. Hypothalamic regulation of sleep and circadian rhythms. *Nature* 2005;**437**:1257–63.
25. Schüssler P, Yassouridis A, Uhr M, Kluge M, Weikel JC, Holsboer F, et al. Growth hormone-releasing hormone and corticotropin-releasing hormone enhance non-rapid-eye-movement-sleep after sleep deprivation. *Am J Physiol Endocrinol Metab* 2006;**291**:E549–56.
26. Siegel JM. Hypocretin (orexin): role in normal behavior and neuropathology. *Annu Rev Psychol* 2004;**55**:125–48.
27. Steiger A. Neurochemical regulation of sleep. *J Psychiatr Res* 2007;**41**:537–52.
28. Steiger A, Dresler M, Schüssler P, Kluge M. Ghrelin in mental health, sleep, memory. *Mol Cell Endocrinol* 2011;**340**:88–96.
29. Szentirmai E, Krueger JM. Central administration of neuropeptide Y induces wakefulness in rats. *Am J Physiol Regul Integr Comp Physiol* 2006;**291**:R473–80.
30. Van Cauter E, Kerkhofs M, Caufriez A, Van Onderbergen A, Thorner MO, Copinschi G. A quantitative estimation of growth hormone secretion in normal man: reproducibility and relation to sleep and time of day. *J Clin Endocrinol Metab* 1992;**74**:1441–50.
31. Vgontzas AN, Chrousos GP. Sleep, the hypothalamic-pituitary-adrenal axis, and cytokines: multiple interactions and disturbances in sleep disorders. *Endocrinol Metab Clin North Am* 2002;**31**:15–36.
32. Weitzman ED. Circadian rhythms and episodic hormone secretion in man. *Annu Rev Med* 1976;**27**:225–43.
33. Zhang G, Wang L, Liu H, Zhang J. Substance P promotes sleep in the ventrolateral preoptic area of rats. *Brain Res* 2004;**1028**:225–32.

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Chapter 256

Peptides and Stress

Annika Thorsell

ABSTRACT

Many disorders prevalent today stem from chronic activation of our stress response system, a system biologically intended to be used in acute, short-lasting, stress exposure. The regulation of the general stress response, the hypothalamic–pituitary–adrenal axis and sympathetic nervous system activation, is well known. However, many neuropeptides participate in the central regulation of other components of the behavioral and endocrine stress response. The chapter will focus on how the neuropeptides regulate stress-related phenomena (behavioral and endocrine responses), as well as on how the neuropeptides themselves are regulated (expression pattern, activity and actions) by the individual's exposure to stress. The most commonly known neuropeptide involved in centrally mediated stress regulation is corticotropin-releasing hormone (CRH), an endogenous anxiogenic neuropeptide. The most common anxiolytic neuropeptide is neuropeptide Y (NPY). In this chapter, only their involvement in central stress- and anxiety-related events will be covered. In addition, the following neuropeptides will be discussed: the urocortins, substance P, neuropeptide S, and vasopressin.

INTRODUCTION

Neuropeptides are involved in central regulation and mediation of numerous behaviors. Their relative abundance and regional expression patterns, as well as their structural and functional diversity, may indicate an involvement in the fine tuning of centrally regulated behaviors and responses. Neuropeptides tend to exert a long-lasting modulatory effect on the small-molecule neurotransmitters with which they colocalize, by regulating the response times of second messenger and intracellular signaling systems. Thus, neuropeptides are important for adaptation of the nervous system to changes in physiological conditions. To understand the place for neuropeptides in the regulation of complex mechanisms such as stress and stress-related events, a background on the concepts involved in the general stress response as well as the endocrine mechanisms will be presented here. After that, a short, summarized description of each neuropeptide is given and the role of the neuropeptide in stress-related events described.

THE "STRESS" CONCEPT

A "stress" or "stressor" can be defined as "anything that disrupts the optimal balance of the body." In turn, the stress response is defined as the responses made by the body to restore this optimal, or homeostatic, balance. Stress as a term refers to processes involving perception, appraisal, and response to threatening, challenging and/or possibly harmful stimuli. There exists a division of stressful stimuli into emotional, or psychological, stimuli and physiological stimuli. Examples of stimuli that are considered emotional/psychological include social interactions/conflicts, inescapable situations, death of a loved one and so forth. Physiological stimuli can be hypo/hyperthermia, an immune challenge, starvation, and also drug withdrawal.

Under acute conditions, stress invokes a response that may be beneficial for survival, for example, fleeing a dangerous situation, and the systems activated then are necessary to provide the means to avoid being attacked (or ending up as someone's dinner). However, when activated chronically, stress responses can cause damage and be the basis for, as well as accelerate, disease conditions. This is because of the issue of chronically activating endocrine factors and central neurotransmitter systems that are not evolutionary intended to be switched "on" continuously. The changed balance induced by the internal stress response can be related by the term "allostasis," and this refers to the process of reestablishing homeostasis in response to a challenge. As mentioned above, homeostasis refers to the consistency of internal parameters within a normal/optimal range, while allostasis describes the body's way to keep stable outside the normal range by changing internal systems to match external demands. Allostasis links the brain with the endocrine system and our immune system to coordinate appropriate responses to an (external) stressor.

Cannon (1927) pioneered the concept of a specific site regulating emotional responses. Later work by Papez (1937) and Klüver and Bucy (1937) confirmed a role for, among other regions, the amygdala in emotional regulation. Studies in human subjects, and in animals, have since shown that activation of the amygdala is crucial for the mediation of

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00256-6
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behavioral and emotional responses associated with anxiety. Lesioning of the amygdala in turn leads to decreased levels of experimental anxiety and decreased sensitivity to stress. A role has been proposed for the amygdala to integrate inputs from the varying brain regions to produce a coherent behavioral response to fear and anxiety. Many of the neuropeptides described in this chapter exercise their anxiety-related (be it either anxiogenic or anxiolytic) properties through actions within the amygdala complex. However, other anatomical targets are also involved and these include, for example, the hypothalamus, the parabrachial nucleus, the hippocampus, and other limbic structures.

The endocrine response to stress consists mainly of the hypothalamic–pituitary–adrenal (HPA) axis. The paraventricular nucleus (PVN) of the hypothalamus contains neuroendocrine neurons that synthesize and secrete vasopressin and corticotropin-releasing hormone (CRH). CRH and vasopressin are released from neurosecretory nerve terminals at the median eminence. CRH is transported to the anterior pituitary through the portal blood vessel system of the pituitary stalk and vasopressin is transported by axonal transport to the posterior pituitary. There, CRH and vasopressin act synergistically to stimulate the secretion of stored adrenocorticotropic hormone (ACTH) from corticotroph cells. CRH released from the hypothalamus acts to release ACTH (corticotropin) from the anterior lobe of the pituitary. ACTH in turn activates the release of glucocorticoids (cortisol in humans; corticosterone in rodents) from the adrenal gland cortices. Glucocorticoids in turn act back on the hypothalamus and pituitary (to suppress CRH and ACTH production) in a negative feedback cycle. The HPA axis is controlled by complex regulatory mechanisms. Numerous factors such as CRH, vasopressin, oxytocin, angiotensin II, and conceivably other peptidergic hormones may stimulate the release of the stress hormone ACTH. On the other hand, glucocorticoids inhibit the secretion of ACTH by acting at the hypothalamic and/or pituitary level. The release of ACTH is therefore the final outcome of the interactions between the hypothalamus, the adrenal gland, and possibly other organs. The multimolecular nature of the factors responsible for the control of the pituitary–adrenal axis gives rise to the speculation that various factors could have specific roles in various stress situations. They provide a highly sensitive mechanism regulating very finely the stress hormone in response to a whole variety of endogenous and exogenous stimuli. Depending on the type of stress, they may therefore singly or in combination affect the amount and duration of ACTH and steroid secretion. The released glucocorticoids may then produce their numerous downstream effects.

The clinical implications of a dysregulated stress response are many. Together with genetic predisposing factors, the chronic activation and dysregulation of the stress response may underlie some of the pathophysiology of anxiety- and stress-related disorders, as well as depressive illnesses. Anxious individuals emotionally anticipate an aversive situation. Approximately one in four individuals will experience an anxiety-related disorder during their lifetime. This together with well-known issues such as side effects and limited efficacy for existing anxiolytics makes the need for new treatments urgent. Neuropeptide systems may offer opportunities for such treatment development. Neuropeptides have been suggested to only be released if the firing of a neuron exceeds a certain level, i.e. frequency-dependent release. Furthermore, neuropeptides, when compared with classical neurotransmitters, can act over longer distances and may thus be used to recruit large neuronal populations to produce a concerted physiological effect. Additionally, the accessibility of neuropeptides is higher because of limited reuptake mechanisms as compared with that of classical neurotransmitters.

Neuropeptides such as neuropeptide Y (NPY) and CRH have been implicated not only in the acute regulation of stress/anxiety-related behaviors but adaptations and changes in these neuropeptide systems may also participate in the regulation of behavior and endocrine responses during chronic stress. NPY is an endogenous anxiolytic neuropeptide, whereas CRH has anxiogenic properties on central administration. Changes in these neuropeptide systems may contribute to disease states and give us indications for putative treatment targets for stress/anxiety disorders and alcohol/drug dependence. In this review, we briefly present these two systems and review their involvement in mediating the responses to acute and chronic stressors, as well as their possible roles in the development and progression of stress/anxiety disorders. In addition other neuropeptide systems involved in the central regulation of stress-related responses and behaviors will be presented, including the urocortins, substance P (SP), neuropeptide S, and (arginine) vasopressin/oxytocin. The urocortins are peptides activating the CRH-R1 and CRH-R2 receptors, while SP may be considered as "CRH light" in the sense that many of its actions and effects are similar to the effects of CRH. Vasopressin coexists and coregulates the effects of CRH on the HPA axis, and has stress effects of its own.

CRH

CRH is the major neurohormone regulating the vertebrate endocrine stress response. CRH is a 41 amino acid peptide that is part of a peptide family also including urocortin, urocortin II and urocortin III. The human and rat CRH-peptide sequence are identical and only differ from the ovine in seven positions, indicating an overall conservation of peptide sequence and structure between species. The effects of these peptides are mediated via two G protein (Gs)-coupled receptors: CRH-R1 and CRH-R2. Agonist activation of these receptors leads to increased intracellular concentrations of

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cyclic AMP (cAMP). CRH-R1 is the primary receptor for CRH and participates in mediation of the behavioral stress response.

The distribution of CRH within and beyond the hypothalamus provides an anatomical context for the observation that CRH can simultaneously activate and coordinate metabolic, circulatory, and behavioral responses that are adaptive in "stressful" situations. CRH not only stimulates the HPA axis, but it also influences several aspects of CNS function which may be of relevance to psychiatric illnesses. Within the hypothalamus CRH is primarily found within the PVN. Other regions with predominant CRH-containing neurons are the lateral bed nucleus of the stria terminalis and the central division of the central nucleus of the amygdala. To a smaller degree, there are CRH cells in the lateral hypothalamus, prefrontal, and cingulate cortex. In brainstem regions, CRH cells are clustered near the locus coeruleus (Barrington's nucleus), the parabrachial region, and regions of the solitary nucleus. The regions mentioned are involved in behavioral stress responses and regulation of autonomic function.

CRH participates in the mediation of the autonomic, neuroendocrine, and behavioral responses to stress. The action of CRH can be potentiated by vasopressin, oxytocin, epinephrine, norepinephrine, VIP, and angiotensin II. Intracerebroventricular administration of CRH in the rat produces prolonged elevations of plasma epinephrine, norepinephrine, it elevates mean arterial pressure and heart rate, increases locomotor activity and decreases feeding and sexual behavior.

On central administration, CRH induces anxiogenic (i.e. it increases anxiety levels and anxiety-related behavioral measures) responses in several animal behavioral tests such as the novel open-field, acoustic startle, and operant conflict tests. Transgenic mice overexpressing CRH show increased anxiety-like behaviors, and these behaviors may be reversed by administration of CRH-R1 receptor antagonists. CRH is released within the amygdala during periods of stress exposure and increases in anxiety-like responses after restraint stress are dependent on the activation of CRH-R1 receptors within the amygdala. Many stressors induce CRH release and CRH mRNA expression within the amygdala, for example, drug withdrawal, restraint stress, and neonatal stress exposure.

Whether the centrally mediated, anxiogenic behavioral effects of CRH are dependent on the HPA axis, or completely independent thereof requires further discussion. Lesions of the central nucleus of the amygdala, but not of the hypothalamic PVN, disrupt CRH-potentiated fear responses, and peripheral blockade of ACTH/glucocorticoids does not affect the behavioral response. Central administration of CRH in dexamethasone-treated rats still elicits an anxiogenic effect. However, adrenalectomy abolishes the locomotor activity-inducing effects of central CRH administration. Further, in nonhuman primate models of early adverse events chronic elevation of cerebrospinal fluid (CSF) CRH is seen, and patients with posttraumatic stress disorder (PTSD) have significant elevation of CSF CRH, indicating significant activation of the extrahypothalamic CRH system in these conditions.

Given the above studies, the administration of CRH-R1 receptor antagonists may offer an effective treatment for stress-induced anxiety disorders. Indeed, treatment with CRH-R1 receptor antagonists does result in decreased levels of experimental anxiety in rats with a "high anxiety" phenotype, in mice after exposure to a chronic mild stress paradigm, as well as in human subjects diagnosed with major depression or PTSD. Further, a CRH-R1 antagonist was effective in blocking alcohol withdrawal-induced anxiety on the elevated plus-maze as well as stress-induced reinstatement of alcohol seeking. Similar to other potent stressors, acute alcohol withdrawal induces anxiety-like responses that are correlated with increased CRH levels in the central nucleus of the amygdala and in the bed nucleus of the stria terminalis, sites mediating the behavioral responses to stress. Accordingly, anxiogenic effects of alcohol withdrawal are reversed by CRH-R1 antagonism. It should be noted that limited to no effect of CRH-R1 antagonists on administration to naive/unstressed animals was seen, suggesting that the CRH system may have limited tone under baseline (nonchallenged) conditions.

NEUROPEPTIDE Y

Neuropeptide Y is a 36 amino acid peptide abundantly expressed throughout the mammalian central nervous system, with high levels in regions regulating stress- and anxiety-related behaviors. The mature NPY peptide is derived from a protein precursor that also contains a signal peptide and a C-terminal flanking peptide, CPON. The signal peptide is cleaved on entry of the NPY prepropeptide into the lumen of the endoplasmatic reticulum. The generated NPY propeptide migrates into specific large dense core vesicles which are present in dendrites, cell bodies, and axons. Phylogenetic studies have revealed NPY to be well conserved during evolution implying important functional roles of the peptide. Consequently, there is very high between-species homology, and, for example, the peptide sequence between human and rat NPY is identical.

In the CNS of rats, high levels are found in brain regions such as the hypothalamus, in particular the arcuate and the PVN, nucleus accumbens, the periaqueductal gray, and septum. Moderate expression levels are detected in the amygdala, the hippocampal formation, the thalamus, and the basal ganglia. Although tissue concentrations of the peptide correlate well with fiber density and terminal networks, this is not true for cell-body density. NPY is expressed both in interneurons and long projection neurons.

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To date, all NPY receptors cloned belong to the superfamily of G protein-coupled receptors, but differ in their ligand affinity profiles. NPY receptors are coupled via Gi/o proteins to several downstream signaling pathways, including inhibition of adenylyl cyclase, activation of mitogen-activated protein kinase, regulation of intracellular calcium (Ca^{2+}) concentrations and activation of G protein-coupled, inwardly rectifying potassium (K^+) channels. Currently, there are four subtypes of NPY receptors known to mediate biological responses: Y1, Y2, Y4, and Y5. The predominantly postsynaptic Y1 receptor requires the intact NPY sequence for recognition and activation and has been proposed as the subtype mediating the antianxiety actions of NPY. The presynaptic Y2 receptor subtype is in addition activated by C-terminal fragments of NPY, such as NPY13–36 and NPY3–36. The Y4 receptor has low affinity for NPY and is primarily the target for pancreatic polypeptide (PP), another member of the PP family of peptides. The Y5 receptor was initially believed to be the receptor regulating the effect of NPY on feeding behavior and Y5 receptor antagonists have been evaluated for putative obesity treatment, possibly in combination with compounds aimed at other NPY receptors such as the Y1.

After its isolation, NPY has been extensively studied with particular attention to anxiety/stress-related behavior and feeding. Already in the late 1980s, mounting evidence was presented to indicate anxiolytic/sedative properties of NPY. These studies included the synchronization of electroencephalographic activity, the prevention of stress-induced ulcers, and suppression of locomotor activity. It has since been repeatedly shown that central administration of exogenous NPY produces an anxiolytic phenotype in numerous animal models of experimental anxiety. Antianxiety effects of NPY are dependent on the activation of Y1 receptors, proposedly within the amygdala. Intracerebroventricular injections of NPY or Y1 receptor agonists, but not Y2 receptor agonists, are anxiolytic in behavioral models of experimental anxiety.

In agreement with antianxiety effects observed after the central administration of exogenous NPY, a role for endogenous NPY in the control of stress- and anxiety-related behaviors is suggested by several findings. Acute physical restraint, which promotes experimental anxiety, suppresses NPY mRNA and peptide levels within the amygdala and cortex. By contrast, repeated exposure to the same stressor once daily for 10 days leads to a complete behavioral and endocrine habituation, accompanied by an upregulation of amygdala NPY expression. Further, chronic overexpression of NPY generates a stress-insensitive and "anxiolytic" phenotype. Genetic variations in human NPY expression affect stress response and emotion. We have therefore proposed that an upregulation of NPY expression may contribute to the behavioral adaptation to stress. This extends a proposed hypothesis that endogenous NPY may act to "buffer" behavioral effects of stress-promoting signals within the central nervous system. In the periphery, it would seem the opposite is valid with NPY exacerbating the cardiovascular and metabolic adverse effects of chronic stress exposure.

EVIDENCE FOR INTERACTIONS BETWEEN CRH AND NPY SYSTEMS

NPY and CRH are present in many regions of the brain that are relevant for the behavioral and emotional response to stressful stimuli and/or fear/anxiety-producing events. In 1994, Heilig et al. proposed opposing roles for NPY and CRH within the amygdala to maintain emotional balance after stress exposure. NPY was suggested to be released subsequently to increases in amygdalar CRH producing an opposing action on the stress system, facilitating return to a homeostatic (or allostatic) balance.

Numerous studies have indicated anxiolytic and anxiogenic effects of NPY and CRH, respectively, on administration into the CNS. In addition, studies have revealed overlapping biological substrates, primarily within the amygdala, for these effects. Both CRH and NPY receptors are found within the amygdala. The basolateral nucleus (BLA) is of particular interest because CRH, as well as urocortin I, elicit long-term anxiety-like responses on administration into the BLA. However, the opposite behavioral response is seen on BLA administration of NPY. Furthermore, pretreatment with NPY into the BLA before administration of urocortin blocks the anxiogenic effect of the CRH-R1/2 receptor activation on anxiety-related behavior. This indicates a direct interaction of CRH and NPY systems within the BLA to modify anxiety-related behavior. The molecular mechanism for this interaction has been proposed to be differential regulation of cAMP levels. In addition, at the level of the hypothalamus, dose-dependent increases in NPY overflow from the PVN were observed after the administration of CRH. Further investigation into the clinical implications for these interactions is needed.

The opposing roles of CRH and NPY in the regulation of stress/anxiety-related behaviors and responses may open the way for putative combination treatments aimed at targets within these systems.

UROCORTINS

As previously noted, there are additional members to the CRH family of peptides, the urocortins. Urocortin-1 was introduced in 1995 as the mammalian ortholog of urotensin. Because urocortin-1 exhibited greater affinity for and activation of CRH-R2 receptors than did CRH, it was hypothesized to be a natural CRH-R2 receptor ligand. Genomic sequence mining revealed the presence of another lineage of peptides, urocortin-2 and urocortin-3 in 2001, and they were recognized for their structural relation to both CRH

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and urocortin-1 preproteins. They were named "urocortins" because of the predominant affinity of predicted mature peptides for the CRH-R2 receptor.

The urocortin peptides are phylogenetically distinct, although they share moderate sequence identity with one another and with CRH. The CRH/urocortin peptide family resulted from multiple gene duplication events during evolution before the divergence of modern vertebrate fish from tetrapods. In addition, each peptide has a unique anatomical distribution under the control of different genes. Consequently, despite a structural family resemblance, the natural functions of urocortins and CRH in stress responses *in vivo* may differ significantly.

In the mammalian brain, the most abundant expression of urocortin-1 has been reported in neurons located in the midbrain in the Edinger–Westphal (EW) nucleus. The EW is compact, cholinergic, preganglionic parasympathetic brain structure primarily involved in oculomotor adaptation; however, the EW is much more complex and the existence of distinct neuron populations within the EW has been postulated. Certain acute, stressful stimuli (e.g. lipopolysaccharide administration and restraint) will activate the EW urocrtin-1 system, whereas chronic stress may result in a partial habituation of urocortin-1 mRNA responses to stress. The possibility of a long-term, inverse relation between CRH and EW urocortin-1 systems in homeostatic stress responses has been proposed because CRH-activating events seemingly decrease the urcortin-1 system activity and vice versa. The urocortin-1 EW sends ascending projections to the lateral septum and to the bed nucleus of the stria terminalis, the globus pallidus, the hypothalamus, thalamus, and periaqueductal gray. Descending urocortin-1-projections go to, among other regions, the substantia nigra, the dorsal raphe nucleus, and the parabrachial nucleus.

Urocortin-2 mRNA is localized in the suproptical nucleus and magnocellular subdivision of the PVN as well as in brainstem motor neurons and the spinal cord. Urocortin-2 also has marked expression in the arcuate nucleus of the hypothalamus and the locus coeruleus.

Urocortin-3 is synthesized in among other regions of the median preoptic nucleus of the hypothalamus and the dorsomedial amygdala. In addition, urocortin-3-like immunoreactive fibers of unknown origin project heavily to the ventromedial hypothalamus and the arcuate nucleus, and medial amygdala urocortin-3 neurons project to the ventral premammillary nucleus.

One of the most prominent physiological functions of CRH is activation of the HPA axis. For the urocortins, peripheral administration of urocortin-2 or urocortin-3 does not increase corticosterone secretion, consistent with the relative absence of their preferred CRH-R2 receptor on ACTH-secreting pituitary corticotroph cells. However, both central and peripheral administration of urocortin-1 activates the HPA axis, supposedly via a CRH-R1-dependent mechanism. However, urocortin-1-deficient mice exhibit normal basal and stress-induced HPA hormone levels indicating that urocortin-1 is not a physiological regulator of the HPA axis. Female mice lacking urocortin-2 or the CRH-R2 receptors exhibit greater peak corticosterone and ACTH concentrations at the circadian light/dark phase transition. In addition, urocortin-2 and urocortin-3 mRNA is increased in the parvocellular PVN after restraint stress, and hypothalamic urocortin-2 expression is increased by the administration of glucocorticoids. Arginine vasopressin (AVP) expression within the hypothalamus is increased in urocortin-2 knockout, which is known to augment ACTH secretion. Thus, urocortin-2 may via CRH-R2 receptors modulate basal HPA axis circadian amplitude via an AVP-dependent mechanism.

The anxiogenic-like properties of central urocortin-1 infusion, mediated at least partly by CRH-R1 receptors, have been shown in several animal models of anxiety-like behavior, including the open field, elevated plus-maze, light-dark box, and social interaction. Quite unlike CRH-R1 receptor agonists, central administration of urocortin-2/3 does not consistently have anxiogenic-like effects in rats. On the contrary, central administration of urocortin-3 acutely produced and anxiolytic-like phenotype in the elevated plus-maze as well as the light/dark box test. Central administration of urocortin-2 also lacks anxiogenic-like effects in the rat open field and elevated plus-maze.. Rather, urocortin-2 has a delayed, anxiolytic-like effect under high baseline anxiety conditions in the plus-maze and counteracts the anxiogenic-like and locomotor activating effects of CRH in the open field.

In summary, although CRH-R1 receptor activation has known anxiogenic-like effects, conclusions regarding anxiety-related effects of central urocortin administration are not yet possible and may be brain site-specific. Moreover, the endogenous anxiety-related roles of urocortins remain similarly unclear as shown by the differential anxiety-like behavior in different lines of urocortin-1 knockout mice. In addition, urocortin-2 deficient mice also do not exhibit altered anxiety-like behavior in the plus-maze, light/dark box, or conditioned fear tests. Whether these results result from compensatory mechanisms commonly seen in constitutive knockouts, or other factors, remains to be seen.

SUBSTANCE P

Substance P (SP) was originally discovered in 1931 by Ulf von Euler and John H. Gaddum as a tissue extract that caused intestinal contraction *in vitro*. It is one of the most abundant neuropeptides in the brain and was one of the earliest to be purified and sequenced. The tachykinins are bioactive peptides defined by a common carboxy-terminal amino acid sequence. SP and a second tackykinin, neurokinin A, are both encoded by the preprotackykinin A gene. The preprotachykinin A gene has four different splice variants, all

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of which encode SP but only two also encode neurokinin A. The third tackykinin, neurokinin B, is encoded by a separate gene preprotackykinin B. These three peptides act on three receptor subtypes, NK-1, -2, and -3. The preferential, although perhaps not exclusive, receptor for SP is NK-1, for neurokinin A is the NK-2, and for neurokinin B the NK-3 receptor subtype (Regoli et al., 1994). However, it is important to keep in mind that there is a notable crosstalk among the three receptor subtypes because of the limited selectivity of the neurokinins. Binding of SP to NK-1 results in internalization by a clathrin-dependent mechanism to acidified endosomes where the SP/NK-1 complex disassociates. SP is subsequently degraded and the NK-1 receptor is reexpressed on the cell surface. As a neuropeptide, SP coexists with other fast neurotransmitters within axons including glutamate, GABA, serotonin, and acetylcholine depending on its location in the brain. Further, SP may be found in neurons containing norepinephrine and serotonergic neurons express the NK-1 receptor. Indeed, SP regulates both enkephalin and serotonin neurotransmission in regions important for mood and pain such as the periaqueductal gray and the dorsal raphe nucleus. In the human brain, about ½ of the serotonin-containing neurons in the dorsal raphe nucleus coexpress SP. However, the extracellular efflux of serotonin in the cerebral cortex has been shown to be not affected by the increased firing rate of dorsal raphe nucleus neurons resulting from genetic deletion or pharmacological blockade of NK-1 receptors. Blockade of an inhibitory SP pathway such as the one originating in the habenula may instead be responsible for this.

The NK-1 and NK-3 receptor subtypes are widely expressed in the brain, while the NK-2 subtype is only observed in areas such as the prefrontal cortex, hippocampus, thalamus, and septum. For the SP peptide, high levels have been detected in areas such as the cingulate cortex, caudate putamen, nucleus accumbens, hippocampus, septum, amygdala, periaqueductal gray, parabrachial nucleus, locus coeruleus, and in the dorsal raphe, among others. Many of these areas are involved in the modulation and regulation of stress responses and anxiety-related behaviors. In general, there is good overlap between the expression of SP and the NK-1 receptor, with the exception of the substantia nigra.

There is much evidence indicating a role of the SP-NK-1 system in stress-related behaviors. For example, the SP concentrations are altered in regions relevant to stress-related behavior after aversive or stressful stimuli; exposure to foot shock increases SP levels in the septum and dentate gyrus, restraint stress induces SP expression in the periaqueductal gray, and so does isolation stress. After maternal separation in guinea pigs internalization of the NK-1 receptor is seen in the amygdala indicating previous SP release and receptor binding, and subsequent receptor/peptide complex internalization. Furthermore, central administration of SP is anxiogenic in a number of animal models of anxiety, and induces behaviors and a cardiovascular response similar to that seen after exposure to stressful stimuli.

Genetic deletion or pharmacological blockade of NK-1 receptors may lead to an attenuated behavioral stress response. Deletion or inactivation of the NK-1 gene leads to an anxiolytic-like and antidepressant-like behavioral phenotype in mice neurokinin. Neonatal stress responses, such as vocalizations, are inhibited in two independent lines of NK-1 knockout mice after separation stress. In addition, a less aggressive phenotype occurs in NK-1 knockout mice as well as an antidepressant-like phenotype in the forced swim test.

Pharmacological blockade of the NK-1 receptor has been made possible in the last decade through the development of highly selective NK-1 antagonists. With the high-affinity human NK-1 receptor antagonist L-760735 neonatal stress responses, such as vocalizations, are inhibited dose-dependently and enantioselectively, mimicking the results seen after genetic deletion. The L-760735 compound also causes a dose-related inhibition of aggressive behavior in a hamster resident-intruder test. And the depressant effect of pharmacological blockade of the NK-1 receptor has been shown both in experimental preclinical studies and in clinical studies using patients with major depression and high anxiety.

Regional specificity in the behavioral responses to SP has been shown through direct local administration of SP into regions such as the amygdala. The amygdala is a crucial component in the mediation of extrahypothalamic behavioral stress responses. Injection of SP into the medial amygdala produces an anxiogenic behavioral profile in the elevated plus-maze test, and this can be blocked by pretreatment with an NK-1 receptor antagonist. Administration of the antagonist alone in addition blocks the anxiogenic effect of an immobilization stress but does not by itself affect behavioral measures in the elevated plus-maze. Although the amygdala seemingly mediates only the anxiogenic effect of SP, the lateral septum may be involved in the mediation of both anxiety-related and depression-like behaviors. This was indicated by a significant antidepressant-like effect of an NK-1 receptor antagonist, L-822,429, as well as an anxiolytic-like effect after administration into the lateral septum.

There is very limited evidence for the effect of anxiolytic or antidepressant treatments on SP and/or NK-1 expression levels or activity. In rats, a single dose of diazepam, a benzodiazepine anxiolytic, reduces SP levels in the hippocampus and the periaqueductal gray while the level was increased in the substantia nigra. Results with antidepressant treatments are ambiguous and have yielded mixed and contradictory results.

There is growing evidence that SP is involved in the regulation of the HPA axis through actions in the hypothalamus and the adrenal glands. SP-containing fibers heavily innervate the PVN of the hypothalamus, and there are also NK-1 receptors present within this structure. Presumably, SP stimulates

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the secretion of cortisol/corticosterone during stress exposure or when the HPA axis is disturbed. During normal conditions however, SP acts as a preventive agent for any excess secretion of glucocorticoids. Endogenous SP does not inhibit the initial magnitude of the stress response but reduces the duration of the response, proposedly through a central action on NK-1 receptors. This suggests that SP may be an important central agent controlling the transition between acute and chronic stress. In addition, an intraglandular modulatory role of SP has been suggested, and SP mRNA and SP immunoreactivity have been detected in the anterior pituitary as well as the adrenal glands of several mammalian species. Immunocytochemistry shows the presence of both cell and nerve fibers containing SP immunoreactivity in the anterior pituitary. Further, SP-positive nerve terminals are distributed around parenchymal cells and blood vessels of the adenohypophysis of monkeys, dogs, guinea pigs, and rats. SP immunoreactivity has been shown by RIA in the adrenal glands, both in nerve fibers and medullary chromaffin cells, of several mammalian species, including human, cow, dog, guinea pig, rabbit, mouse, and rat. The SP-containing nerve fibers penetrate the capsule and the cortex to innervate the medulla in both human and rat adrenal. The evidence suggests that the role of tachykinins, and especially of SP, could become very relevant under paraphysiological (e.g. physical or inflammatory stresses) or pathological conditions (e.g. ACTH-secreting pituitary tumors), when an excess of steroid-hormone production has to be counteracted. Stress-induced expression of c-fos is lower in NK-1 knockout mice than their wildtype controls, indicating an involvement of the SP/NK-1 system in hypothalamic activation in response to stress exposure.

NEUROPEPTIDE S

Neuropeptide S, a 20 amino acid peptide named after its highly conserved N-terminal serine, is a recently identified neuropeptide with anxiolytic and activity-promoting properties. It was revealed through reverse pharmacology to be the ligand for a former orphan G protein-coupled receptor (PGR14; GPR154; VRR1). Activation of the NPS receptor induces elevation of intracellular Ca^{2+} and cAMP, implying coupling of the NPS receptor to both Gq and Gs proteins and a resulting increased cellular excitability. Human NPSR1 mRNA encodes eight splice variants only two of which, hNPSR1-A and hNPSR1-B, are functional receptors. In rodents, only one single NPSR exists and it shows high similarity with hNPSR1-A.

As with many other neuropeptides, NPS has strong sequence conservation across species and is evolutionarily old. The N-terminal amino acid in all animal species is a serine, and the N-terminal sequence Ser-Phe-Arg-Asn-Gly-Val-Gly is identical in all species, possibly indicating a representation of the peptides bioactive core. The NPS peptide precursor (ppNPS; 89 amino acids) contains a hydrophobic signal peptide and a pair of basic residues in addition to the mature NPS sequence. Although the mRNAs for NPS and its receptor are expressed in many tissues, including the thyroid gland and brain, in the brain, NPS expression is mainly limited to three brainstem nuclei – and area adjacent to the locus coeruleus, the lateral parabrachial nucleus, and the principle trigeminal nucleus. Additionally, a few scattered NPS-expressing neurons have been detected in the medial amygdala and dorsomedial hypothalamus. Most of the NPS-expressing cells in the area adjacent to the locus coeruleus and the principle trigeminal nucleus are glutamatergic neurons. Within the area adjacent to the locus coeruleus there is also expression of NPS in cholinergic neurons. In the lateral parabrachial nucleus NPS is coexpressed with CRH.

In contrast to ppNPS, the NPS receptor is widely expressed in the central nervous system. High levels of NPSR mRNA expression can be detected in the thalamus, hypothalamus, cortex, olfactory nuclei, amygdala, and subiculum. The expression pattern for the NPS receptor is indicative of the confirmed role for the NPS/NPSR system in arousal, anxiety, and regulation of food intake. Central administration of NPS increases locomotor activity in both naive and habituated mice. Furthermore, it was shown in multiple behavioral models of anxiety-related behavior that central administration of NPS is anxiolytic. Additional evidence for a role of NPS in the regulation of stress-related behaviors and measures come from a study indicating that central administration of NPS significantly reduces the hyperthermic response induced by stress. On the contrary, NPS does not seem to have an antidepressant-like effect, as measured in the mouse tail suspension test.

With regards to interactions with other neuropeptide systems, central NPS administration activates c-fos expression in hypocretin-1/orexin-A (Hcrt-1/Ox-A) immunoreactive neurons in the lateral hypothalamus and in the perifornical area. In addition, NPS is colocalized with CRH in the parabrachial nucleus. CRH acts within the dorsal aspect of the brachium and the locus subcoeruleus to increase plasma ACTH and AVP through actions within the medial parabrachial nucleus. Intracerebroventricular administration of NPS stimulates the HPA axis activity and leads to increased plasma levels of ACTH and corticosterone in rats. Neither icv nor iPVN administration of NPS stimulated the release of either LH or TSH, suggesting a direct and specific effect of NPS on the HPA axis. In addition, treatment of pituitary segments with NPS did not alter ACTH release, suggesting that NPS does not have a direct effect on the pituitary gland. It is therefore probable that the effects of NPS on the HPA axis are mediated via the hypothalamus through the release of CRH and AVP.

Also of interest is that NPS induces not only locomotor activity but also anxiety-like behavior, as measured in the light-dark box test, and cocaine-seeking behavior (reinstatement) through a mechanism dependent on the CRH-1 receptor. Thus, NPS on one hand is anxiolytic and

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on the other hand it is activity-inducing, activates the HPA axis, and induces reinstatement of drug-seeking, probably through a CRH-dependent mechanism.

ARGININE VASOPRESSIN

Vasopressin is reviewed here because of its prominent effects on the HPA axis. Already in the 1890s scientists showed that the extracts of pituitary when administered altered blood pressure. However, isolation and identification of the peptides/hormones vasopressin and oxytocin would take almost half a century. In the 1950s, amino acid sequences, structures, and synthesis of the peptides were elucidated. Both vasopressin and oxytocin are nine amino acid peptides, nonapeptides. Oxytocin and vasopressin are ancient neuropeptides that are highly conserved between species. Additionally, vasopressin is evolutionarily well conserved as even the primitive organism hydra expresses a vasopressin-like peptide and, vasotocin, the evolutionary progenitor of the vertebrate vasopressin can be found in birds and reptiles.

As for most neuropeptide receptors, the vasopressin receptors, V1 (with subtypes V1a and V1b, central nervous system expression) and V2 (predominantly peripherally expressed in liver and kidney), are seven transmembrane G protein-coupled receptors. Activation of V2 increases cAMP, and this mediates the classical diuretic effect of vasopressin. V1a and V1b activate phospholipase C activity.

The majority of vasopressin within the CNS is located within the magnocellular neurons of the supraoptic nucleus and the PVN. There are projections from the PVN to primarily the brainstem and spinal cord, but there is scant evidence for any extrahypothalamic projections from the supraoptic nucleus. Vasopressin fibers innervate the hippocampus and subiculum, the locus coeruleus, the solitary tract nucleus, and the spinal cord, among other regions. Further, vasopressin is expressed in the bed nucleus of the stria terminalis, in parvocellular neurons of the suprachiasmatic nucleus, and the medial amygdala.

Vasopressin is primarily known for its role in fluid metabolism; however, vasopressin also regulates the HPA axis and stress-related behaviors. In response to a stressor, vasopressin is released from the median eminence into the pituitary portal circulation, thereby potentiating the effect of CRH on ACTH release. The relative contributions of CRH, vasopressin, as well as oxytocin, to the control of ACTH secretion seemingly varies with different types of stress. In general, vasopressin stimulates ACTH secretion in all species studied. Oxytocin also stimulates ACTH release in rats but is inhibitory in primates. The involvement of AVP and OT in the control of ACTH secretion may have important implications for physiological and pathological conditions associated with activation of the HPA axis.

In response to acute and chronic stress, release of vasopressin in the PVN increases. Stress exposure can also increase V1b in the pituitary. Antagonism of the V1b-receptor reduces ACTH secretion and V1b knockout mice have significantly lower ACTH and corticosterone levels compared with that of wildtype mice after certain stress exposures.

High densities of vasopressinergic fibers and V1a-receptors are found within the septum and septal infusion of antisense oligodeoxynucleotide to the V1a-receptor mRNA, as well as with a V1a-receptor antagonist, d(Ch2)5Tyr(Me)AVP, significantly reduces anxiety-related behavior, as measured on the elevated plus-maze. Additionally, overexpression of the V1a-receptor gene in the lateral septum significantly increases anxiety-related behavior and V1a-receptor knockout males display significantly less anxiety-related behavior than wildtype males on a variety of tasks, further implicating V1a in controlling the anxiety response V1a. Differences in strain background and technique may account for these differences. Interestingly, V1a knockout females do not display anxiolytic behaviors. Also, contradictory results have been presented indicating an anxiolytic-like effect of central and peripheral administration of vasopressin.

Oral administration of SSR149415, a V1b-receptor antagonist, prevents the vasopressin-induced rise in plasma ACTH levels. Additionally, peripheral administration of SSR149415 is anxiolytic in various classical and atypical models of anxiety. Local infusion of SSR149415 into the basolateral amygdala, but not central or medial nuclei, produces anxiolytic-like effects in performance on the elevated plus-maze.

CONCLUDING REMARKS

There are many more stress-related peptides than those mentioned here. For example, galanin, ghrelin, nociception/orphanin Q, the orexins, melanin-concentrating hormone, are some of the ones not included in this chapter. As with all the peptides discussed in this chapter, other chapters in the book discuss these peptides.

In conclusion, this review briefly discusses the importance of neuropeptide systems in the regulation of stress- and anxiety-related behaviors and responses. The "dominant players," CRH and NPY, have been well characterized with regards to their individual and overlapping contribution to stress/anxiety-related behaviors and responses. The other members of the CRH family, the urocortins, are still being studied and evaluated. SP is predominantly known for its effects on nociception but is emerging as a possible future dominant player in stress- and anxiety-related behaviors and disorders. In general, it seems neuropeptide systems may offer more attractive treatment targets than the classical neurotransmitter systems because of the limited tonic activity of neuropeptide systems. Lack of tonic activity may generate treatments that have efficacy only during times of specific neuronal activity and may thus lead to state-specific treatments as well as a reduction in the appearance of side effects.

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Chapter 257

Peptides and Temperature

Miklós Székely, Márta Balaskó and Erika Pétervári

ABSTRACT

The body's overall energy balance includes thermal balance and involves regulatory peptides representing the actual feeding status (hunger/satiety) or the nutritional status (body weight/composition). Acting directly or through vagal afferents, peripheral peptides (e.g. leptin, cholecystokinin, ghrelin, glucagon-like peptide-1) modify the expression/action of peptides in hypothalamic or brainstem nuclei (melanocortins, cocaine-amphetamine-regulated transcript, corticotropin-releasing factor, neuropeptide-Y, orexins, melanin-concentrating hormone, etc.), and influence the function of brain peptidergic systems. Catabolic peptide interactions suppress food intake, enhance metabolic rate, and eventually body temperature; these may be adaptive changes to acute/chronic calorie excess (postprandial hyperthermia, particularly in overfeeding). To retain calories in acute/chronic fasting, anabolic peptide activities result in hunger, hypometabolism, and (especially at subthermoneutral environments) in temperature decline. Apart from securing energy balance, body weight, and the adaptation to caloric availability, actions of these and other peptides participate in thermal adaptation, fever, and stress responses, and they influence nonexercise activity.

INTRODUCTION

Metabolic rate, on the one hand, is in equilibrium with food intake to maintain stability of body weight, while on the other hand, it has to be in equilibrium with heat loss to secure homeothermy. Each component of the overall energy balance has relative independence, allowing for alterations in body weight or body temperature. An increasing number of results show that besides the role of monoamines that have been widely accepted as regulators of individual components of energy balance for over half a century, regulatory peptides may also make an outstanding contribution to the regulation of the overall balance and can also influence body temperature. Depending on the mechanism of action, this influence and the physiological importance of various peptides may be different.

BODY TEMPERATURE AND ITS REGULATION

In homeothermic species, core temperature is regulated at different levels, and with varying accuracy, depending on the species. The regulatory arch consists of (1) thermosensors, (2) regulatory nuclei of the central nervous system (CNS, mainly hypothalamic and brainstem nuclei), which influence (3) thermoeffectors for heat production, heat loss, and thermoregulatory behavior. Heat production (metabolic rate) provides the link between regulations of body weight and body temperature. Each component of the arch of thermoregulation can be influenced by nonthermal factors, e.g. thermosensors by local anesthetics, effectors by the state and amount of heat producing tissues/organs (muscle, brown adipose tissue) and substrate availability, and the central regulation by many hormones, neurotransmitters, and neuromodulators, including regulatory peptides.

In humans and in experimental rodents, body temperature may be altered passively, when the exogenous/endogenous thermal load (e.g. extreme cold/heat, exhausting exercise, postprandial state) cannot be completely counterbalanced by the thermoregulatory effectors, or it may be altered actively in a centrally regulated process (e.g. fever). Skin vasodilatory substances may induce passive hypothermia (e.g. in cold environments alcohol acts partly this way), while other effects (e.g. generalized convulsions) may elevate body temperature passively. Peptides may also contribute to such passive changes in body temperature by acting directly on the effector systems (e.g. skin vessels, brown fat). In the past decades, however, more and more important roles were found for peripheral and central peptides that influence functions at higher CNS levels (brainstem, hypothalamus, etc.) and participate in many processes affecting metabolic and thermal balance in centrally regulated ways. Many relevant studies on the regulation of the overall energy balance were analyzed in a recent review.[29] This chapter deals with the thermoregulatory effects of some of the most important peptides in this field.

PARTICIPANTS OF ENERGY BALANCE REGULATION

Decerebrate rats with a functional brainstem are capable of regulating short-term ingestive behavior and satiety.[4] The nucleus of the solitary tract (NTS) with the area postrema

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00257-8
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serves as a portal for assessing and integrating visceral afferent signals (vagal signals, nutrients), and the dorsal motor nucleus of the dorsal vagal complex (DVC) provides outbound signals toward the neurons of the rostral medullary raphe to influence efferent responses. However, although this system can adapt to the actual feeding status (satiety/hunger), it cannot coordinate long-term adaptation (e.g. adaptation to starvation, to high-fat diet, or to changes in nutritional status) to maintain standard body weight. Cognitive, rewarding, aversive, and emotional responses are also absent in these rats. In addition to the indispensable hindbrain, the hypothalamus, and higher regulatory centers may modify the activities of the brainstem.

Similar mechanisms are involved in the regulation of body temperature.[3] Although the circadian rhythm of body temperature may also be maintained at the brainstem level,[14] the brainstem may not be sufficient for normal adaptation to alterations of environmental temperature or to nutritional status, to emotional status, etc. These necessitate the contribution of higher centers. Neurons of the dorsomedial hypothalamus, paraventricular nucleus (PVN), the ventrolateral medulla, and NTS convey information to the inhibitory rostral raphe pallidus in the brainstem; disinhibition leads to enhanced brown fat thermogenesis, skin vasoconstriction, and temperature elevation. Peptides are involved at several levels of this complex regulatory system and help adaptation to various situations. Thus, peripheral peptides, e.g. cholecystokinin (CCK), can influence NTS activity, and leptin can bind to its receptors on neurons of the arcuate nucleus (ARC), PVN, and the retrochiasmatic area. The ARC contains neurons expressing proopiomelanocortin (POMC), cocaine-amphetamine-regulated transcript (CART), neuropeptide-Y (NPY), and agouti-related peptide (AgRP), whereas the second-order neurons of the lateral hypothalamus contain melanin-concentrating hormone (MCH), orexins, and those of the PVN contain corticotropin-releasing hormone (CRH). Neurons of the PVN and elsewhere project to the DVC and further to the brown adipose tissue (BAT); they influence sympathetic tone and modify the expression and function of uncoupling protein-1 (UCP-1). It has not been clarified as to how the central influences alter the expression of other UCP-s in other tissues (e.g. UCP-2 and UCP-3 in muscles).

Peptides vs. Temperature: Acute/Chronic Exposure to Various Environmental Temperatures

A cold environment-induced heat loss should be compensated by increased metabolic rate and/or tendency to reduce further loss of heat in order to maintain stability of core temperature. At thermoneutrality, the metabolic rate is minimal (basal/resting metabolic rate: BMR), and no heat loss mechanism is activated. In a warm environment, heat loss should be increased and the metabolic rate reduced to or near BMR level. Results are scarce regarding peptidergic regulation of changes in heat loss, but results are abundant regarding the role of peptides in regulation of metabolic rate (role of brown fat,[6] nonshivering, and activity-related thermogenesis). Hypothermic effects of any peptide can only be observed at subthermoneutral or cold ambient temperatures (only toxic effects suppress metabolism below BMR; therefore, only these can cause temperature decrease at relatively warm environments), whereas peptide-induced temperature elevations may develop more easily at any environment (except in extreme cold).

Leptin, Insulin

Leptin primarily represents the nutritional status (adiposity), and it influences both food intake and energy expenditure. The peptide is derived mainly from white adipose tissue. By a receptor-mediated saturable transport system, it can enter the CNS and bind to its receptors in the ARC (similarly to insulin), with projections to the PVN,[5] the lateral hypothalamic area, the median preoptic area, and also to the DVC and spinal cord. It normally activates those neurons of the ARC, which produce anorexigenic peptides of the POMC and CART systems. It also binds to and suppresses the ARC cells producing orexigenic NPY and AgRP, with projections to the perifornical, paraventricular, dorsomedial, ventromedial, lateral hypothalamic, and median preoptic nuclei. The POMC-melanocortin system acts to stimulate, whereas the NPY system acts to minimize the sympathetic drive to BAT, thereby influencing thermogenesis in this tissue.[29] Intracerebroventricular (icv) infusion of leptin to young rats results in temperature elevation, particularly during the nadirs of the circadian rhythm.[25] Both the absence of leptin (ob/ob mice) and abnormalities of the peptide (Zucker fa/fa rats) or its receptors (db/db mice, Koletsky f/f rats) are accompanied with defective metabolic response and hypothermia on exposure to a subthermoneutral environment.[27] In ob/ob mice, leptin repletion elevates the low body temperature. With aging and in diet-induced obesity, leptin resistance develops,[22] and the thermal response to exogenous leptin becomes attenuated both in old rats (Fig. 1) and in diet-induced obese 6-month-old ones.[25] In contrast to lean controls, in severely obese (leptin resistant) subjects, cold exposure does not easily enhance metabolic rate. Rather, it promotes vasoconstriction in the insulating fat layer. In acute cold exposure or during cold adaptation, plasma leptin concentrations decrease in normal rats and mice, allowing for an increase in food intake; the simultaneous increase in heat production must be independent of leptin.

Direct administration of leptin to the hindbrain also induces hyperthermia, in a cooperative interaction with thyrotropin-releasing hormone (TRH) and by activation of

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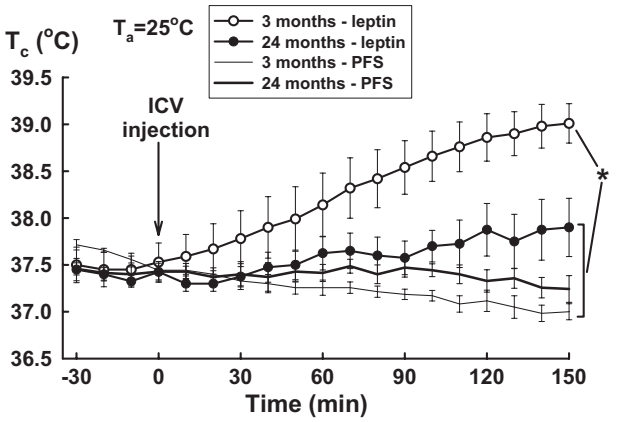

FIGURE 1 The effect of icv injection of leptin (4 μg) vs. pyrogen-free saline on core temperature in 3-month-old (○ vs. thin line) and 24-month-old (● vs. thick line) male Wistar rats at 25 °C environmental temperature (unpublished data of the authors).

the hindbrain melanocortin system. Normally, the forebrain actions of leptin (mainly by projections from the PVN) are superimposed on and regulate the integrative functions at hindbrain level;[15] melanocortin production in the brainstem increases, and this can excite the locally dense melanocortin receptors. Leptin also has effects on peripheral white adipose tissue, but this is only indirectly related to the thermoregulatory changes evoked by the peptide.

Apparently, the activity of leptin is influenced mainly by nutritional status (adiposity), which develops in a long-term process, and the peptide acts as a peripheral catabolic substance in the first line of defense for maintaining energy balance also on the long run. This includes the stability of body temperature, but the effects are not directed primarily to the regulation of body temperature. In physiological amounts, leptin-induced metabolic effects do not necessarily lead to temperature changes (resulting from the simultaneous rise in heat loss). Not all of the leptin effects are exerted directly; the activity of a number of other regulatory peptides is influenced by but not fully dependent on leptin.

In the regulation of energy balance, insulin has a similar role has as leptin; it provides information about the general status of energy availability in the body. Hypothalamic insulin receptors exhibit similar localization and roles as leptin,[5,19] and they are also present in the preoptic area. Preoptic injection of insulin-like growth factor-1 elicits dose-dependent hyperthermia, except in transgenic mice lacking neuronal insulin receptors. In humans, intranasal insulin enhances postprandial hyperthermia.

Melanocortins, AgRP

On leptin's binding to its receptors or as a local effect of nutrients, the appropriate cells of the ARC enhance the production of POMC and its derivatives, mainly alpha-melanocyte stimulating hormone (alpha-MSH) as an endogenous agonist of the melanocortin system. On similar stimuli, melanocortins are also produced in the brainstem, where their receptors are particularly numerous. In the NTS, CCK can also activate POMC neurons. In the absence of melanocortin-3 and -4 receptors (MC3R and MC4R), leptin cannot exert its activities, while leptin-deficient animals are capable of responding to melanocortin agonists, indicating that melanocortins act downstream to the point of leptin actions. Leptin-independent mechanisms (driven mainly by nutrients and their metabolites) may contribute to the activation of melanocortins. Melanocortins act as catabolic peptides at their receptors (mainly MC4R) on the second-order neurons in the PVN and other nuclei: They suppress food intake and enhance energy expenditure. The icv administration of melanocortin agonists enhances metabolic rate and leads to hyperthermia. The hyperthermic effect depends on the initial body temperature,[2] and because it is not accompanied by skin vasoconstriction, it cannot be regarded as a regulated hyperthermia or fever. In rats, a melanocortin antagonist (SHU9119) given icv reduces core body temperature.[1] Either insufficient activation of the melanocortin system (e.g. resulting from a defective leptin effect), or the absence of MC4R (in knockout mice) leads to failure of upregulation of UCP-1 and heat production of BAT on cold exposure[33] leading to a decline in core temperature, while at the usual (not cold) laboratory temperatures in such animals, the ingested energy accumulates and obesity develops. The leptin-melanocortin circuitry is presumed to play an essential role in cold adaptation[33] by its influence on BAT function. However, the role of melanocortins may be even more essential in the short-term adaptive changes of metabolism in response to feeding status (central effects of nutrients, CCK, glucagon-like peptide) and in indirect metabolic reactions to the nutritional status (conveying central leptin, insulin actions). Melanocortins also participate in the mediation of the effects of other peptides in the adaptation process, and they influence peripheral substrate use.

The endogenous melanocortin antagonist (inverse agonist) AgRP and several exogenous antagonists of the system decrease the resting metabolic rate in rats and diminish the cachexia-related elevation of metabolism in tumor-bearing mice. AgRP also suppresses the elevation of metabolic rate and body temperature induced by beta-3 agonist stimulation of BAT thermogenesis. As compared with wild-type mice, AgRP deficient mice exhibit higher metabolic rate and body temperature together with an age-related lean phenotype.[34]

In sum, the melanocortin system is probably the principal central catabolic system in the short-term (partly in the long-term) regulation of overall energy balance. It acts in a complex relationship with its endogenous antagonist, the AgRP peptide, as well as other peptides of the regulation of energy balance. The system plays an important role in metabolic adaptations to the actual feeding

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status, to the nutritional status, and to the predominant nutrients.

CART

Cocaine-amphetamine regulated transcript (CART) is a CNS peptide that was originally described as an anorexigenic peptide causing anorexia and high-energy expenditure, resembling the effects of melanocortins. In line with this idea, the peptide was shown to be coexpressed mainly with POMC in cells of the ARC and retrochiasmatic area. Its production is activated by leptin, and it contributes not only to anorexia but also to stimulation of the sympathetic system and BAT thermogenesis.[7] Results obtained later suggest a somewhat different role for CART. The absence of MC4R prevents leptin's ability to increase UCP-1 in BAT and white adipose tissue (but not its effect to suppress food intake). The absence of MC3R prevents leptin-induced anorexia (but not its effect to increase UCP-1 in BAT and fat deposition in white adipose tissue), while the absence of CART has no influence on either effect of leptin. Moreover, a 7-day long treatment of rats with CART injections into the ARC enhances food intake and also enhances UCP-1 mRNA expression in BAT and the thermogenic responses to a beta-3 agonist.[10] The finding that on food deprivation CART overexpressing rats lost more weight than the controls did also indicates that CART has a hypermetabolic effect. Chronic overexpression of CART (by recombinant adenoassociated virus technique) in rats enhances food intake and increases body weight. The hypermetabolic and hyperphagic dual effect suggests a role for CART in special physiological situations, e.g. in cold adaptation.[10] Indeed, after chronic cold exposure, hypothalamic CART mRNA expression is elevated. Acute cold exposure also increases CART mRNA levels in the PVN.[21]

However, in contrast to the effects of direct intrahypothalamic injections, hindbrain (fourth cerebral ventricle) injections of CART have hypophagic and hypothermic effects; decerebration or GLP-1 receptor antagonist prevents the hypothermia. These indicate a role for extrahypothalamic sites of CART action.[24] Such CART actions were suggested to be distinct from coordinated regulatory patterns of energy balance, but they are believed to show a role for CART either in other processes mediated by GLP-1, or a role in hypothermic neuroprotection.

TRH

Apart from regulating thyroid function, TRH has a direct impact on energy balance. Production of the peptide in the PVN is influenced by melanocortins and CART from the ARC. TRH acts also on the thermosensitive cells of the preoptic/anterior hypothalamic region, the activation of which leads to an increase in core temperature.[11]

CRH, Urocortins

CRH-containing cells of the CNS are located mainly in the PVN and the ventromedial nucleus. The CRHR1 and CRHR2 receptors are sensitive not only to CRH but also to various urocortins and other ligands (e.g. sauvagine). Urocortin is produced mainly in the neurons of the Edinger–Westphal nucleus and in midbrain sympathetic neurons adjacent to it, but the brainstem, hypothalamus (supraoptic and lateral nuclei), and substantia nigra also contain this peptide. CRH is a regulator of the hypothalamus–pituitary–adrenal axis; it is important in stress reactions, in anxiety (mainly via CRHR1), and it also has various neurotransmitter or modulator roles. Cold exposure may represent a special form of stress: in cold-adapted rats CRH elicits an enhanced ACTH response, and it has metabolic actions. Cold exposure increases the hypothalamic mRNA expression of both CRH and CRHR2, but not CRHR1. Although CRH may participate in stress-induced hyperthermia (conjointly with orexin), many metabolic and thermoregulatory effects of CRH or urocortins seem to be independent of stress.[17] CRH suppresses food intake, increases oxygen consumption, and elevates body temperature in rodents, although urocortin-1 is a more potent ligand. These actions of the peptides are similar as those of melanocortins, but in MC4R knockout obese mice CRH and urocortin-1 still attenuate food intake, indicating that the CRH action is downstream to the point of action of melanocortins. Minute amounts of intraperitoneal urocortin also cause satiety.

The mediation of hyperthermia is complex. Urocortin-1-induced hyperthermia (via BAT activation) seems to be mediated by CRHR2. Urocortin-2 and -3 (and partly CRH) are less potent, but they also elevate body temperature by acting at CRHR2 in a fever-like fashion by increasing BAT thermogenesis and by causing skin vasoconstriction. Urocortins have been suggested to play a role in the response to cold; the thermogenic response is smaller in urocortin-1 knockout mice. In CRHR2-deficient mice, high-fat feeding or repeated cold stress results in high calorie intake and hypermetabolism with a lean body, presumably resulting from the relative hyperactivity of CRHR1; unfortunately, skin vasomotor change or other form of heat loss was not analyzed. The issue of the role of CRH is further complicated by some reports on a hypothermia-inducing effect of the peptide.

NPY

Peripherally produced NPY, although it acts synergistically with the sympathetic fibers, influences mainly the vasomotor system and no results are known regarding its effects on thermoregulation. In the CNS, NPY production is under the feedback regulation of leptin and ghrelin; leptin inhibits while ghrelin activates the NPY-producing neurons of

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the ARC.[8] The NPY thus produced acts at receptors of the second-order neurons in the PVN and perifornical hypothalamus.[5] Early experiments showed that, when applied into these nuclei or administered icv to rats, this peptide induces food intake at the same time it blocks BAT activity and metabolic rate, and induces hypothermia (without concurrent rise in skin temperature) at subthermoneutral environments, e.g. at usual laboratory temperatures. However, it may elevate core temperature at suprathermoneutral environments. An icv injection of an NPY Y1-receptor agonist induces and its antagonist prevents torpor-like hypothermia in Siberian hamsters. Similar application of an agonist of the Y5-receptor also causes dose-dependent hypothermia, whereas a Y5-receptor antagonist increases energy expenditure. Centrally injected NPY Y1-receptor antisense elicits hyperthermia. NPY knockout mice have higher oxygen consumption and UCP-1 expression during fasting than the wild-type controls. This indicates that the thermoregulatory/metabolic effects of NPY have an adaptive role during fasting. The orexigenic and hypometabolic effects of the peptide are not interconnected; perifornical NPY injections elicit food intake only, but not the thermal effects. Apparently, the effect of this peptide on food intake may be more important than its influence on body temperature; the metabolic effects have an importance in the course of adaptation to low food intake.

In acute cold exposure, the NPY content in the arcuate and dorsomedial nuclei decreases. Chronic cold exposure presents a controversial issue: to adapt to the cold, the orexigenic but not the hypothermia-inducing effects of NPY should be enhanced. According to experimental results, during cold adaptation the hypothalamic NPY level does not change, although the orexigenic responsiveness to NPY and the spontaneous food intake increases. In cold-adapted animals, the basal core temperature is somewhat lower, and the responsiveness to acute cold exposure may increase significantly.[31]

Orexin-A

In addition to the first recognized effects of orexins, i.e. their role in increasing the arousal state, orexin-A has also been described as a peptide that contributes to the regulation of feeding behavior and energy balance. Orexins are produced mainly by neurons of the lateral/posterior hypothalamic and perifornical areas. Its type-1 and type-2 receptors can be found in many areas of the CNS. Peripheral metabolic factors (glucose). leptin, CCK, and ghrelin may influence the production/activity of orexin neurons via vagal afferents and the NTS (hypoglycemia is stimulatory). On central administration, orexin-A was reported to elicit short-lasting food intake, increase spontaneous activity and elevate core temperature. However, the results on the influence on core temperature are controversial. According to various reports, the orexin-A-dependent increased arousal status is coupled with higher body temperature.[35] This is suggested to be connected with increased sympathetic tone. Orexins activate CRH-containing neurons, and they are important in the stress-induced increase in metabolic rate, but orexins may be more important in non-exercise activity-induced thermogenesis. By contrast, other reports have shown that icv orexin-A may induce a short-lasting fall in core temperature in semirestrained animals, which is later followed by an increase. The hypothermia was prevented by NPY antiserum.[29] In orexin knockout mice, resting body temperature (nadirs of circadian rhythm during sleep) is elevated.[13] In rats, the orexin-A receptor antagonist SB-334867-A increases BAT thermogenesis.[32]

MCH

In the CNS, the expression of MCH is most abundant in the neurons of the lateral hypothalamus, where it is mostly coexpressed with orexin. These neurons project to the DVC and have interaction with afferent impulses reaching the NTS. Strong hyperphagia and a fall in core temperature develop after MCH administration into the fourth or the lateral cerebral ventricle: metabolic rate and UCP-1 expression in the BAT decreases.[23] Chronic treatment elicits obesity and hypothermia in mice. MCH or MCH-receptor knockout mice are lean, hypophagic, hyperactive, hypermetabolic, and have high body temperature. An icv infusion of the MCH antagonist SNAP-7941 increases brown fat thermogenesis in rats.[32] Also, in rats, MCH expression increases on acute cold exposure, probably as part of an adaptive metabolic change. On refeeding (after a short-term fasting) or after CCK treatment, the expression of MCH and its receptor in the afferent vagus is downregulated. The high hypothalamic MCH content of ob/ob mice[23] contributes to their tendency for hypothermia.

CCK

Although the so far analyzed peptides are connected mainly with nutritional status, the activity of CCK and other peptides of the gastrointestinal tract is influenced mainly (though not exclusively) by the actual feeding status.

As hormones of gastrointestinal regulation, CCK peptides have been known for a century. The recognition that CCK is present also in the brain and may have a principal role in satiety, and that it is important in energy balance dates back for only three decades, but our knowledge in this field is still growing. It is clear that energy balance and body temperature can be influenced by either peripheral or central actions of CCK.[30] The CCK-1 (CCK-A) receptors of the peptide are located mainly in the periphery, mainly on the afferent vagus, some in the CNS (hippocampus, NTS, hypothalamus, etc.), while the CCK-2 type (CCK-B) receptors are present throughout the CNS. In subthermoneutral environments, peripherally applied fragments of the peptide induce hypothermia through action on the afferent vagus; capsaicin-induced desensitization of the vagus prevents

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CCK-hypothermia.[26] This indicates that the hypothermia develops on the basis of a reflex and not on local action of the peptide. CCK-1 receptor defective OLETF (Otsuka Long Evans Tokushima Fatty) rats do not respond to CCK administration, their circadian core temperature rhythm is similar to controls; the only difference they have is that their responses to changes in environmental temperature are slow. Although most of the peripheral CCK binds to receptors on the afferent vagus in complex interaction with other peptides that also bind at this site, some CCK binds to the ARC and nuclei of the brainstem (NTS) or other parts of the CNS (e.g. supraoptic nucleus, PVN). In OLETF rats, CCK cannot inhibit the anabolic activities of the ARC, and thus, the hypothalamic NPY content is higher than in controls, contributing to their obesity. Centrally applied CCK also exerts anorexigenic effects, but it elevates body temperature[30] in a way corresponding to fever (increase in metabolic rate together with skin vasoconstriction). This idea will be dealt with in the section on fever. The central effects of CCK better represent a catabolic-type activity.

Ghrelin

This orexigenic peptide originates predominantly from the stomach. Its production is enhanced by fasting, weight loss, and hypoglycemia, and it is inhibited by feeding or hyperglycemia. Apart from its growth-hormone secretagog function, ghrelin activates the receptors of NPY/AgRP producing neurons of the ARC (which inhibit the POMC-producing neurons), and also activates receptors of the PVN where NPY/AgRP are released, thereby increasing food intake.[5] CCK-1 and ghrelin receptors are colocalized on vagal afferent neurons where they are in antagonistic interaction. The effect of ghrelin on ARC is also attenuated by CCK pretreatment. Apart from enhancing NPY activity and the indirect (NPY-dependent) ways to induce hypothermia, ghrelin binding axonal terminals are located in the vicinity of thermosensitive neurons in the median preoptic area of the hypothalamus, and this provides an anatomical basis for direct thermoregulatory effects of the peptide. The sympathetic activity and BAT function are suppressed and hypothermia is induced by icv infusion of ghrelin. By contrast, in transgenic rats in which the ghrelin receptor is suppressed, BAT function is activated.[12] Deletion of ghrelin and its receptor is followed by increased energy expenditure.[12] In mice fasting in a cool environment a torpor-like hypothermia can be induced which is deepened by peripheral ghrelin (via NPY-associated pathways).

GLP-1

On food ingestion, GLP-1 is produced in the gut. It causes satiety and temperature elevation, and in animals, it acts even after decerebration or subdiaphragmatic vagotomy, indicating that the hormone acts directly in the lower brainstem. Intravenous or icv administration of GLP-1 increases metabolic rate and body temperature. Accordingly, a role for GLP-1 in postprandial hypermetabolism is suggested. However, in other experiments fourth cerebroventricular injections of GLP-1 receptor agonist or antagonist[20] have been reported to induce hypothermia or hyperthermia, respectively.

Miscellaneous Peptides

In addition to its known endocrine functions in salt/water balance, the *renin–angiotensin* system (RAS) participates in the regulation of energy balance. In a transgenic mouse model, the brain RAS activity induces a marked increase in energy expenditure and body temperature, which exceeds the simultaneously increased calorie intake and results in a lean body. Both food intake and energy expenditure increases in transgenic rats overexpressing the human renin gene, but the ratio changes in the opposite way, and obesity develops.

Vasopressin causes hypothermia by acting at V1 receptors.

An icv injection of *neurotensin* decreases food intake and also induces hypothermia in mice and rats. Neurotensin 1-receptor knockout mice are hyperthermic and slightly heavier than the controls.

Amylin injected icv causes dose-dependent hyperthermia. Similar results have been reported for *substance P*.

Brain-derived neurotrophic factor (BDNF) suppresses food intake and increases body temperature. The effects are prevented by a CRH antagonist, indicating that BDNF acts via the activation of the CRH system.

Infused icv, *ciliary neurotrophic factor* (CNTF) suppresses food intake and elevates body temperature even in high-fat diet-induced obese mice.

Nociceptin/orphanin FQ infusion icv leads to obesity resulting from high food intake and low energy expenditure, with low body temperature.

The *RF amide peptide QRFP* is an endogenous ligand of the orphan G protein-coupled receptor GPR103. An icv infusion of QRFP43, an active sequence of the peptide, causes hyperphagia, weight gain (particularly in mice kept on a high-fat diet), and a decrease in the UCP-1 of BAT with a decrease in body temperature.

The newly discovered *C-type natriuretic peptide* (CNP) possibly contributes to the regulation of energy balance: CNP-knockout mice are of smaller size, consume less food, and have a higher metabolic rate and hyperthermia.

An icv injection of a stable (not receptor-specific) *somatostatin* analog modulates the action of several transmitters, all together increasing food intake and elevating body temperature.

Fever and Hypothermia in Systemic Inflammation, Peptides

The role of some peptides can be seen not only in basic responses to thermal challenges but also in experimental fevers as fever-promoting or -antagonizing substances.

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Because of leptin-induced hyperthermia, the question repeatedly arises: "Does leptin participate in febrigenesis?" Leptin's action is presumably mediated by interleukins that do participate also in febrigenesis. Still, in obese Zucker or OLETF rats, the febrile response to lipopolysaccharide (LPS) is similar to that in controls, and in diet-induced obesity (presumably coupled with leptin resistance), the fever is even more pronounced. Accordingly, leptin may have a different role in the LPS-response: after hypothermia induced by large LPS doses the recovery is faster in control than in leptin-receptor-deficient Koletsky rats.[27]

Although peripherally applied CCK causes hypothermia, icv administration is followed by fever-like coordinated (high heat production, low heat loss) temperature elevation, which is not sensitive to antagonists of prostaglandin synthesis. A centrally applied CCK-2 receptor antagonist partially inhibits LPS-induced fever.[30] The LPS fever is also diminished in CCK-2 receptor knockout mice. Administration of a GLP-1 antagonist enhances the LPS-induced fever.[20]

Fasting attenuates LPS-induced fever. This may be connected with fasting-induced changes in peptide activities. In starving rats, the attenuated fever can be at least partly normalized by leptin. The fasting-induced elevation in ghrelin levels possibly contributes to the suppression of experimental fevers. In interleukin-1-induced fever, hypothalamic NPY release decreases, probably contributing to both the febrile anorexia and the elevation of body temperature. By contrast, during fasting high NPY levels may contribute to attenuation of the febrile response.

After 1980, many peptide hormones, including melanocortins, vasopressin, and CRH, were regarded as endogenous antipyretics.[9] Interestingly, not all of them caused only hypothermia. Several studies investigated the role of melanocortins in LPS fever. Although this peptide itself elevates metabolic rate and core temperature, it also increases heat loss, and the latter action possibly explains the antipyretic effect.[2] Vasopressin-induced antipyresis has been confirmed several times, without clarifying the precise mechanism of action. Similarly, the role of CRH has not been clarified.

Peptides and Adaptation to Food Restriction vs. Feeding

Food restriction is usually accompanied by a decrease in temperature, which eventually may be severe (particularly in cold), whereas food consumption is followed by a postprandial increase in body temperature (unless a high simultaneous heat loss can prevent it, e.g. in a very cold environment). Several mechanisms are believed to participate in these changes.

Fasting tends to suppress core temperature in humans and experimental animals. This is not simply a result of substrate deficiency. In fasting cold-adapted rats, the core temperature is lower than in the fed counterparts, but an icv injection of prostaglandin E elevates the temperature to the same level (the increase is relatively greater in the fasting group). On acute cold exposure, fasting rats can still elevate metabolic rate, although only for a shorter period. During complete fasting, in rodents, the nadirs but not the acrophases of the circadian temperature rhythm decrease until the very end. Accordingly, regulatory changes are responsible for the suppression of temperature during fasting. Regulatory peptides may have a role in this process. In food-deprived or calorie-restricted animals, the plasma leptin concentration decreases, hypothalamic melanocortin expression decreases, too, whereas NPY/AgRP expression increases. Suppressed CCK- and enhanced ghrelin-secretion are characteristic for the fasting state. All these changes are factors that indicate the presence of adaptive energy saving mechanisms and help in lowering the resting body temperature.

In leptin-deficient ob/ob mice that fasted at a room temperature of 22 °C, even torpor develops with a core temperature of 24 °C, and this can be reversed by leptin treatment. Similarly, in db/db mice, the hypothermia induced by fasting or cold exposure is prevented by BDNF via activating the sympathetic system and inducing UCP-1 gene expression in BAT. Because of results showing leptin-dependent reversal of large dose LPS-induced hypothermia, leptin has been suggested to counteract other conditions of negative energy balance, like fasting-induced hypothermia.[28] Indeed, in 6-month-old calorie-restricted rats icv leptin infusion induces much greater hyperthermia than in controls or obese ones of the same age.[25]

After food intake (postprandial state), the heat production and resting body temperature rise, especially after previous fasting. In a cold environment, this heat is used as part of the thermoregulatory excess heat production and the increase in core temperature may be missing. The normal elevation partly results from the caloric content of the food or the specific dynamic action of the nutrients. However, after previous fasting, even calorie-free feeding can evoke a temperature increase, that is, the increase has regulatory components originating from stretching of the gastrointestinal system. Gastric stretch evokes a rise in CCK and a fall in ghrelin-secretion via the vagal afferents that can influence the function of the brainstem and the hypothalamus, including peptidergic regulation (e.g. activation of the melanocortin system, inhibition of NPY). Perivagal capsaicin desensitization can block some forms of the postprandial hyperthermia (e.g.the form evoked by calorie-free food).[18] The nutrients (glucose, fatty acids) may act directly at the area postrema and/or the ARC, and they also modify peptidergic functions.

Peripheral nutrients and metabolites can also influence the energetic effects of peptides. Urocortin crosses the

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blood–brain barrier (BBB) more easily in mice pretreated with either peripheral glucose or insulin, and this contributes to the development of satiety. Urocortin also enhances the influx of leptin through the BBB.[17] Glucose-transporter type-2 (Glut-2) of the ARC leads to the enhancement of leptin sensitivity in POMC and NPY neurons; the Glut-2 defective mice become hypothermic in a cold environment, and in their circadian rhythm the temperature nadirs are extremely low.[16]

In rats of high-fat diet-induced obesity, leptin resistance develops and icv or peripheral injection of leptin does not induce hyperthermia. Diet-induced obesity leads also to ghrelin resistance in NPY/AgRP neurons,[5] and the fasting-induced hyperphagia is attenuated as well as the cold responsiveness. Hindbrain leptin injections remain effective and cause hyperthermia even in diet-induced obese animals. In the hindbrain leptin acts via the local melanocortins and melanocortin sensitivity is maintained despite obesity. In these rats, the melanocortin antagonist SHU9119 can prevent the leptin-induced hyperthermia.[24] POMC defective mice do not decrease food intake even if maintained on a high-fat diet, and, unlike control mice, they do not increase either physical activity or energy expenditure. In obesity resistant mice a high-fat diet enhances spontaneous activity and muscle thermogenesis: this is how obesity is prevented.

ACKNOWLEDGMENT

The authors received financial support, while compiling this chapter, by grants from the Hungarian Scientific Research Fund OTKA PD-84241 (to EP) and by research funds of the University of Pécs, Medical School (34039/KA-OTKA/11-01 to MB).

REFERENCES

1. Adage T, Scheurink AJ, de Boer SF, de Vries K, Konsman JP, Kuipers F, et al. Hypothalamic, metabolic, and behavioral responses to pharmacological inhibition of CNS melanocortin signaling in rats. *J Neurosci* 2001;**21**:3639–45.
2. Balaskó M, Garami A, Soós S, Koncsecskó-Gáspár M, Székely M, Pétervári E. Central alpha-MSH, energy balance, thermal balance, and antipyresis. *J Therm Biol* 2010;**35**:211–7.
3. Berthoud H-R. The caudal brainstem and the control of food intake and energy balance. In: Stricker EM, Woods SC, editors. *Handbook of behavioral neurobiology*. New York: Plenum; 2004. p. 195–240.
4. Blessing WW. *The lower brainstem and bodily homeostasis*. New York: Oxford University Press; 1997, 323–372.
5. Briggs DI, Andrews ZB. Metabolic status regulates ghrelin function on energy homeostasis. *Neuroendocrinology* 2011;**93**:48–57.
6. Cannon B, Nedergaard J. Brown adipose tissue: function and physiological significance. *Physiol Rev* 2004;**84**:277–359.
7. Elias CF, Lee C, Kelly J, Aschkenasi C, Ahima RS, Couceyro PR, et al. Leptin activates hypothalamic CART neurons projecting to the spinal cord. *Neuron* 1998;**21**:1375–85.
8. Kalra SP, Kalra PS. Neuropeptide Y: a physiological orexigen modulated by the feedback action of ghrelin and leptin. *Endocrine* 2003;**22**:49–56.
9. Kluger MJ. Fever: role of pyrogens and cryogens. *Physiol Rev* 1991;**71**:93–127.
10. Kong WM, Stanley S, Gardiner J, Abbott C, Murphy K, Seth A, et al. A role for arcuate cocaine and amphetamine regulated transcript in hyperphagia, thermogenesis, and cold adaptation. *FASEB J* 2003;**17**:1688–90.
11. Lechan RM, Fekete C. The TRH neuron: a hypothalamic integrator of energy metabolism. *Prog Brain Res* 2006;**153**:209–35.
12. Mano-Otagiri A, Iwasaki-Sakino A, Nemoto T, Ohata H, Shuto Y, Nakabayashi H, et al. Genetic suppression of ghrelin receptors activates brown adipocyte function and decreases fat storage in rats. *Regul Pept* 2010;**160**:81–90.
13. Mochizuki T, Klerman EB, Sakurai T, Scammel TE. Elevated body temperature during sleep in orexin knockout mice. *Am J Physiol* 2006;**291**:R533–40.
14. Moriya T, Aida R, Akiyama M, Doi M, Hayasaka N, Nakahata N, et al. The dorsomedial hypothalamic nucleus is not necessary for food-anticipatory circadian rhythms of behavior, temperature or clock gene expression in mice. *Eur J Neurosci* 2009;**29**:1447–60.
15. Morton GJ, Blevins JE, Williams DL, Niswender KD, Gelling RW, Rhodes CJ, et al. Leptin action in the forebrain regulates the hindbrain response to satiety signals. *J Clin Invest* 2005;**115**:703–10.
16. Mounien L, Marty N, Tarussio D, Metref S, Genoux D, Preitner F, et al. Glut2-dependent glucose-sensing controls thermoregulation by enhancing the leptin sensitivity of NPY and POMC neurons. *FASEB J* 2010;**24**:1747–58.
17. Pan W, Kastin AJ. Urocortin and the brain. *Prog Neurobiol* 2008;**84**:148–56.
18. Pétervári E, Ember Á, Gőbel G, Pákai E, Székely M. Signaling postprandial hyperthermia: a role for cholecystokinin. *J Therm Biol* 2004;**29**:797–803.
19. Porte Jr D, Baskin DG, Schwartz MW. Leptin and insulin action in the central nervous system. *Nutr Rev* 2002;**60**:S20–9.
20. Rinaman L, Comer J. Antagonism of central glucagon-like peptide-1 receptors enhances lipopolysaccharide-induced fever. *Auton Neurosci* 2000;**85**:98–101.
21. Sánchez E, Fekete C, Lechan RM, Joseph-Bravo P. Cocaine- and amphetamine-regulated transcript (CART) expression is differently regulated in hypothalamic paraventricular nucleus of lactating rats exposed to suckling or cold stimulation. *Brain Res* 2007;**1132**: 120–8.
22. Scarpace PJ, Matheny M, Shek EW. Impaired leptin signal transduction with age-related obesity. *Neuropharmacology* 2000;**39**: 1872–9.
23. Segal-Lieberman G, Bradley RL, Kokkotou E, Carlson M, Trombly DJ, Wang X, et al. Melanin-concentrating hormone is a critical mediator of the leptin-deficient phenotype. *PNAS* 2003;**100**:10085–90.
24. Skibicka KP, Alhadeff AL, Grill HJ. Hindbrain cocaine- and amphetamine-regulated transcript induces hypothermia mediated by GLP-1 receptors. *J Neurosci* 2009;**29**:6973–81.
25. Soos S, Balasko M, Jech-Mihalffy A, Szekely M, Petervari E. Anorexic *vs.* metabolic effects of central leptin infusion in rats of various ages and nutritional states. *J Mol Neurosci* 2010;**41**:97–104.
26. South EH. Cholecystokinin reduces body temperature in vehicle- but not capsaicin-pretreated rats. *Am J Physiol* 1992;**263**:R1215–21.

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27. Steiner AA, Dogan MD, Ivanov AI, Patel S, Rudaya AY, Jennings DH, et al. A new function of the leptin receptor: mediation of the recovery from lipopolysaccharide-induced hypothermia. *FASEB J* 2004;**18**: 1949–51.
28. Steiner AA, Romanovsky AA. Leptin: at the crossroads of energy balance and systemic inflammation. *Prog Lipid Res* 2007;**46**:89–107.
29. Szekely M, Petervari E, Balasko M. Thermoregulation, energy balance, regulatory peptides: recent developments. *Front Biosci (Schol Ed)* 2010;**2**:1009–46.
30. Szelényi Z. Cholecystokinin: role in thermoregulation and other aspects of energetics. *Clin Chim Acta* 2010;**411**:329–35.
31. Uzsoki B, Fekete Á, Pétervári E, Balaskó M, Székely M. Enhanced responsiveness to central prostaglandin E or neuropeptide Y in cold-adapted rats. *J Therm Biol* 2001;**26**:499–504.
32. Verty AN, Allen AM, Oldfield BJ. The endogenous actions of hypothalamic peptides on brown adipose tissue thermogenesis in the rat. *Endocrinology* 2010;**151**:4236–46.
33. Voss Andreae A, Murphy JG, Ellacott KLJ, Stuart RC, Nilni EA, Cone RD, et al. Role of the central melanocortinergic circuitry in adaptive thermogenesis of brown adipose tissue. *Endocrinology* 2007;**148**:1550–60.
34. Wortley KE, Anderson KD, Yasenchak J, Murphy A, Valenzuela D, Diano S, et al. Agouti-related protein-deficient mice display an age-related lean phenotype. *Cell Metab* 2005;**2**:421–7.
35. Yoshimichi G, Yoshimatsu H, Masaki T, Sakata T. Orexin-A regulates body temperature in coordination with arousal state. *Exp Biol Med* 2001;**226**:468–76.

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Chapter 258

Pheromone Peptides

Miriam Altstein

ABSTRACT

Pheromones are chemical messages known to elicit changes in behavioral reactions that are followed by physiological alterations in conspecific receiver organisms. Pheromones comprise a wide range of compounds, of varied chemical nature, that range from small-molecule volatile compounds to high-molecular-weight, water-soluble, nonvolatile peptides and proteins. Although volatile pheromone molecules have been studied extensively, much less is known about the chemical nature of the equivalent nonvolatile water-soluble cues. In this chapter, we summarize the state of our knowledge of pheromone peptides and a few proteins, in a variety of species belonging to various phyla and classes.

INTRODUCTION

Pheromones are chemical messages that are secreted from an individual and that elicit a behavioral reaction or an endocrine change in another individual of the same species. In the receiving organism, pheromones can elicit an immediate behavioral reaction, such as alarm, defense, aggregation, attraction, kin and colony recognition, marking of territories and egg-deposition sites, mating behavior, recruitment, and thermoregulation. They also can induce complex behavioral and physiological alterations—such as development of a particular caste, or sexual maturation—via long-term endocrine changes.

Chemical communication among organisms is a highly sophisticated procedure that involves the regulation of many genes in the donor, and initiation of complex processes in the recipient, in which interpretation of the individual chemical constituents or "signals" depends on the particular combination, ratios, concentrations, and order of presentation of signal molecules, and also on the physiological state of the receiver. It is believed that, during the course of evolution, pheromones of different organisms have become very distinctive, to avoid ambiguity and to ensure that the channels of communication through which they exert their activity do not overlap, which causes even greater complexity.

Chemically, pheromones range from small-molecule volatile compounds to high-molecular-weight, water-soluble, nonvolatile peptides and proteins. Usually, small molecules are used for communication, which requires rapid dispersal of the signal, whereas the larger molecules of less volatile compounds tend to function in attraction and stimulation, for which prolonged exposure is necessary. Although airborne signal molecules have been identified and studied extensively, and are being applied commercially to disrupt mating and to monitor insect pests, much less is known about the chemical nature of the equivalent water-borne cues.

Pheromone peptides are widely distributed throughout the prokaryotic and eukaryotic organisms; they serve to mediate a variety of functions in Gram-positive and Gram-negative bacteria, fungi, ciliates, and in many different animals that belong to the mollusk, annelid, arthropod, and vertebrate (amphibians and mammals) phyla. The very extensive distribution of this means of signaling, among so many, widely diverse organisms, hints at the evolutionary importance of this mode of communication.

Pheromone peptides have been studied for many years, and in several systems, the nature of the peptides, their origins, and their modes of action are quite well understood. In other systems, however, our understanding is still very nascent, and the chemical, molecular, physiological, and biological aspects remain to be explored. Interestingly, in the few years that have passed since the publication of this chapter in the 1st edition of this book, >30 review articles have been published on pheromone peptides in organisms belonging to the bacterial, fungal, and animal kingdoms (most of them on bacterial and mammalian peptide pheromones). A vast amount of knowledge has accumulated on some of these signaling molecules, covering aspects such as: identification of the chemical nature of these peptides; discovery of new peptide pheromones and identification of their biosynthetic pathways, their molecular cloning and their structure/activity relationship (SAR); their inter-molecular interactions; their mechanisms of production and reception, as well as their modes of action at the cellular level; signal transduction pathways that follow peptide pheromone binding to the receptor; intracellular communication at the genome level; and the neural circuitry leading to specific pheromonal outputs and physiological effects. These "signal transduction" and "intracellular communication" studies attracted most

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00258-X
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attention from many research groups, and thus emerged as the most studied topic across the various phyla, leaving an overall impression that the field had made immense progress and had moved from "preliminary-discovery" studies that merely documented the involvement of peptide pheromones in conspecific species to studies that focused on in-depth understanding of the molecular and cellular mechanisms that underlie peptide pheromone reception and activity. This chapter presents a short summary of the state of our knowledge on pheromone peptides and a few proteins, in a variety of species. The limited scope of the chapter does not enable a detailed description of each system, and the reader is referred to the references (mostly recent review articles) in each section, for further information. A summary of the peptides mentioned in this chapter is presented in Table 1.

BACTERIAL PHEROMONE PEPTIDES

One of the most complex and diverse systems of pheromone peptides is found in bacteria. Both unicellular organisms and individual cells of metazoans have evolved complex signaling mechanisms by which they respond to the environment and communicate with one another. These mechanisms involve short peptides that can be secreted as unmodified structures or as posttranslationally modified peptides. These peptides are sensed by target organisms via cognate sensors, with each signal molecule initiating a complex multilevel response pathway. There are two basic types of bacterial communication systems: those in which the signal is detected solely by organisms other than the emitter, and those in which the signal is sensed by the emitting organism as well.

TABLE 1 Major Pheromone Peptides or Protein Families in Bacteria, Fungi and the Animal Kingdom

Pheromone Peptide or Protein Family	Organism or Group Studied: (Order/Genus/Species)	Function	Ref.
Bacterial Pheromone Peptides			
Mating pheromones			
cCF10, cAD1, cPD1, cAM373, cOB1	*Enterococcus faecalis*	Cellular aggregation and plasmid transfer	Dunny and Johnson[13], Thoendel and Horswill[29], Wardal, Sadowy, and Hryniewicz[31]
Quorum-sensing peptides			
Gram-positive peptides			
Autoinducing peptide (AIP)	*Staphylococcus aureus*	Virulence peptides	Thoendel and Horswill[29]
Autoinducing peptide (AIP)	*Lactobacillus plantarum*	Virulence peptides	Thoendel and Horswill[29]
Gelatinase biosynthesis activating pheromone (GBAP)	*Enterococcus faecalis*	Virulence peptides	Thoendel and Horswill[29]
PapR	*Bacillus cereus*	Virulence peptides	Thoendel and Horswill[29]
Cytolysin subunit ($CylL_s''$)	*Enterococcus faecalis*	Virulence peptides	Shankar et al.[26]
Competence-stimulating peptides (CSP-1, CSP-2)	*Streptococcus pneumoniae*	Genetic competence	Thoendel and Horswill[29]
Competence-stimulating factor (CSF, PhrC)	*Bacillus subtilis*	Genetic competence	Thoendel and Horswill[29]
Competence factor ComX	*Bacillus subtilis*	Genetic competence	Thoendel and Horswill[29]
Nisin	*Lactococcus lactis*	Production of bacteriocins	Kleerebezem et al.[18]
Subtilin	*Bacillus subtilis*	Production of bacteriocins	Kleerebezem et al.[18]
Plantaricin A (PlnA) peptides	*Lactobacillus plantarum*	Production of bacteriocins	Diep et al.[11]
BlpC (also termed SpiP) peptides	*Streptococcus pneumoniae*	Production of bacteriocins	Lyon and Novick[23]
Gram-negative peptides			
Double Gly (G-G) containing peptides	Various bacterial spp.	Quorum sensing	Thoendel and Horswill[29]

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TABLE 1 Major Pheromone Peptides or Protein Families in Bacteria, Fungi and the Animal Kingdom—*Cont'd*

Pheromone Peptide or Protein Family	Organism or Group Studied: (Order/Genus/Species)	Function	Ref.
Fungal Pheromone Peptides			
α-factor	*Saccharomyces cerevisiae*	Mating initiation	Naider and Becker[24]
a-factor	*Saccharomyces cerevisiae*	Mating initiation	Anderegg et al.[2]
Ciliates Pheromone Peptides			
Er-1, Er-2, Er-7, Er-10, Er-11, Er-20, Er-21	*Euplotes* spp. and other hypotrechs	Sexual reproduction	Luporini et al.[22]
G3, G4	*Euplotes* spp. and other hypotrechs	Sexual reproduction	Luporini et al.[22]
Mollusk Pheromone Peptides			
Attractins, enticin, temptin, seductin,Alb-1	*Aplysia* spp.	Egg laying, mating, aggregation	Cummins, Degnan, and Nagle,[8] Cummins et al.[9]
ILME (Ile-Leu-Met-Glu)	*Loligo pealeii, Sepia officinalis*	Transport of oocytes in the genital tract	Susswein and Nagle[28]
Sperm attractin peptide (SepSAP)	*Sepia officinalis*	Sperm attraction and facilitation of external fertilization	Susswein and Nagle[28]
Annelid Pheromone Peptides			
Nereithione	*Nereis succinea*	Control of reproductive behavior, mate recognition, and gamete releases	Hardege et al.[15]
Arthropod Pheromone Peptides			
Insect peptides			
Sex peptide (accessory gland protein—Acp70A)	*Drosophila melanogaster*	Variety of reproductive and postmating functions	Chapman and Davies[6]
DUP99B	*Drosophila melanogaster*	Variety of reproductive and postmating functions	Kubli[19]
Pyrokinin/Pheromone biosynthesis activating neuropeptide (PK/PBAN) family	Various moth spp. (see list in Ref. [1] Table 1)	Sex-pheromone biosynthesis	Altstein and Hariton[1]
Crustacean peptides			
Settlement pheromones (e.g. arthropodin)	Barnacles (e.g. *Balanus amphitrite*)	Colony aggregation and settlement	Forward[14], Ziegler and Forward[35]
Larval-release pheromones	Various decapod crustaceans (brachyuran crabs)	Synchronization of larval release	Forward[14], Ziegler and Forward[35]

Continued

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TABLE 1 Major Pheromone Peptides or Protein Families in Bacteria, Fungi and the Animal Kingdom—*Cont'd*

Pheromone Peptide or Protein Family	Organism or Group Studied: (Order/Genus/Species)	Function	Ref.
Vertebrate Pheromone Peptides			
Amphibian peptides			
Sodefrin	*Cynops pyrrhogaster*; *Triturus carnifex*	Female attraction	Woodley[32]
Silefrin	*Cynops ensicauda*	Female attraction	Woodley[32]
Aonirin	*Cynops pyrrhogaster*	Female attraction	Woodley[32]
Splendipherin	*Litoria splendida*	Female attraction	Woodley[32]
Courtship pheromones (PRF, PMF, SPLF)	*Plethodon jordani*	Shortening of courtship process	Woodley[32]
Leptodactylus aggression-stimulating peptide (LASP)	*Leptodactylus fallax*	Stimulation of male aggression	King et al.[17]
Mammalian peptides			
Aphrodisin	Rodents	Induction of copulatory behavior	Briand, Trotier, and Pernollet[5]
Major urinary proteins (MUPs)	Rodents	Communication of individual identity and ownership via scent marks	Touhara[30]
Major histocompatibility complex, (MHC,) class I peptides	Rodents	Communicate individual identity and mediate pregnancy blockage or spontaneous abortion	Touhara[30]
Tear exocrine-gland-secreting peptide 1 (ESP1)	Rodents	Communication of individual identity and ownership via scent marks	Touhara[30]
Vasopressin and oxytocin	Rodents and sheep	Assistance in processing of pheromonal cues associated with social discrimination	Bielsky and Young,[4] Higashida et al.[16]

The first type is typified by the mating pheromones of the enterococcal bacteria, e.g. *Enterococcus faecalis*: cCF10 and cAD1, which are hepta- and octapeptides with sequences LVTLVFV and LFSLVLAG, respectively. Among these bacteria, a potential recipient releases a small peptide that is taken up by a potential donor, in which it elicits a plasmid-coded response that leads to cellular aggregation and transfer of the plasmid from donor to recipient. Production of the mating-pheromonal peptide in the donor is dependent on the close proximity of recipient cells. The enterococcal pheromone is processed from a chromosomally encoded lipoprotein precursor. Most strains produce multiple pheromones with mutually differing spectra of activities, but each plasmid responds specifically to its cognate pheromone with extremely high specificity and sensitivity. Details on the synthesis of the enterococcal mating pheromone molecules, the components of the regulatory machinery that interact specifically with the peptides, the molecular basis for their exquisite sensitivity and specificity, and the regulatory circuits controlling the multiple levels of regulation of intracellular communication have been reviewed recently.[13,29,31]

The second group of bacterial pheromone peptides comprises the autoinduction or quorum-sensing (QS) systems, which are widespread among bacteria, in which they elicit population-wide responses (cell-cell or cell-environment responses) to low-molecular-weight signaling molecules, depending on cell-population density. The structures of the signaling molecules differ between Gram-negative and

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Gram-positive bacteria: in the latter, the mating pheromones and most QS signals are peptides that are sensed by transmembrane receptor components that activate an intracellular response pathway.

Examples of QS-induced processes in Gram-positive bacteria include virulence, genetic competence, and production of antimicrobial peptides. Virulence is induced in *Staphylococcus aureus* and *Lactobacillus plantarum* by autoinducing peptides (AIPs)—a 7- to 9- and a 5-residue cyclic peptide, respectively,—and in *E. faecalis* and *Bacillus cereus* by gelatinase biosynthesis activating pheromone (GBAP)—an 11 residue cyclic peptide—and PapR (a heptapeptide with the sequence ADLPFEF[29]), respectively. Genetic competence is induced in *Streptococcus pneumoniae* and *Bacillus subtilis* by competence-stimulating peptides CSP and competence-stimulating factor (CSF), which are 17 amino acid peptides with the sequence EMRLSKFFRDFILQRKK and a pentapeptide with the sequence ERGMT, respectively.[29] *B. subtilis* 168-type strain produces another competence pheromone: a posttranslation modified 10 residue linear peptide containing a farnesyl group, termed Com-X (ADPITRQWGD).[29]

Production of antimicrobial peptides, known as bacteriocins, also involves a QS mode of regulation. Bacteriocins are divided into Class I posttranslationally modified peptides, often referred to as lantibiotics, and Class II, which are not posttranslationally modified; their production can be regulated in a cell-density-dependent manner by the antimicrobial peptides themselves, as with nisin and subtilin production in *Lactococcus lactis* and *B. subtilis*, respectively, or by signaling molecules that are distinct from the antimicrobial peptides and which include the plantaricin A (PlnA) or BlpC peptides—also termed SpiP peptides—that regulate bacteriocin production in *L. plantarum* and *S. pneumoniae*, respectively.[11,23] Another example of QS is the regulation of cytolysin production in *E. faecalis*, in which a subunit of the bacteriocin, $CylL_s$ regulates the transcription of the toxin.[26] The mechanisms regulating each of the above QS systems are very complex and differ from one another with respect to secretion and sensing of the autoinducer, and in the regulatory cascade that results from its perception. Detailed reviews on QS peptides in Gram-positive bacteria and their biosynthetic pathways have been published recently (see Refs 11–13, 29 and references therein).

In Gram-negative bacteria, most QS signals are *N*-acyl homoserine lactones, which are internalized by diffusion and bind to an intracellular receptor molecule to activate the response.[29] However, recent studies have revealed the existence of signaling peptides in Gram-negative bacteria too. Studies based on an in silico strategy to screen the Gram-negative bacterial genome for the presence of leader double-glycine sequences, which are conserved leader motif sequences of many Gram-positive pheromone peptides, have revealed the presence of genes coding for putative peptides that contain such motifs and that also show structural similarity to bacteriocins and pheromone peptides of Gram-positive bacteria. The role of such peptides in signal transduction, and the mechanisms by which they might exert their activity in Gram-negative bacteria are unclear at present.

FUNGAL PHEROMONE PEPTIDES

Although diverse fungi generate chemical pheromone gradients during mating, the pheromone peptides of yeasts are among the best documented fungal pheromones. Two pheromone peptides that initiate mating in the yeast *Saccharomyces cerevisiae*—the α-factor and the a-factor, which contain 13 and 12 amino acids, respectively, are currently known. The peptides are secreted by donor cells and act on the recipient via G-protein-coupled receptors (GPCRs). The mating pheromones are absolutely indispensible for triggering the mating cycle, and cells that cannot produce these molecules or that lack their cognate receptors are sterile. The α-factor is a tridecapeptide pheromone (WHWLQLKPGQPMY) synthesized constitutively by *S. cerevisiae* mating type α cells, and it acts on *S. cerevisiae* mating type ***a*** cells. The peptide was isolated and characterized by Stotzler and Duntze[27] and later found to be encoded by two genes: *MFα1* and *MFα2*.[20] The **a**-factor of *S. cerevisiae* is a dodecapeptide pheromone (YIIKGVFWDPAC[farnesyl]-OCH_3)[2] that requires posttranslational modification with a farnesyl isoprenoid and carboxymethyl group to enable the expression of its full biological activity. Biosynthesis of these mating pheromones has been studied extensively and used as a model for posttranslational processing, modification, and secretion of mammalian peptide hormones and proteins. The cellular events and signal transduction pathways stimulated by these pheromones were recently found to affect the cell cycle, and they are currently used as a model to study this complex system in eukaryotic cells (for review see Ref. 7). The SAR studies and the extensive biological, biochemical, molecular, and biophysical analyses of the α-factor peptide provided a model of its structure and of its interaction with its receptor (for an overview see Ref. 24). Recently, GPCRs that bind peptide pheromones that mediate mating in filamentous fungi were identified, and their signaling was characterized.[21] The pheromones, their receptors, the downstream intracellular signal transduction pathway by which the yeast cells respond to the presence of mating peptide pheromone in their vicinity, the importance of chemical gradients in yeast chemotropism, and the peptide's mode of action at the cellular level have been examined thoroughly and are summarized in reviews.[3,7,21]

CILIATES

Ciliates can exhibit both asexual and sexual reproduction. Asexual reproduction occurs by binary fission whereas sexual reproduction involves conjugation, i.e. mating, of two cells that divide to form four new cells. Ciliate sexual mating involves chemical signaling that is mediated by a

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complex pheromonal system of small protein pheromones, containing 75 and 130–150 amino acid residues: E*r*-1, E*r*-2, E*r*-7, E*r*-10, E*r*-11, E*r*-20, E*r*-21, G3, and G4. Formation of such mating pairs enables some species to avoid mating between siblings, and promotes mating with foreigners, and thereby retains cell-type-specific signals for distinguishing self from nonself. Most ciliates' pheromones were characterized in *Euplotes* spp. and other hypotrechs. Details of the structures of the various polypeptides, their putative receptors, and their possible modes of interaction and function are detailed in Ref. 22

MOLLUSK PHEROMONE PEPTIDES

Pheromone peptides and proteins have also been implicated in the control of a number of behaviors in mollusks—from egg laying to mate attraction—but only a few have been characterized. Most studies of mollusk pheromone peptides were carried out on *Aplysia*, and the two groups of pheromones that were identified so far were mostly found during these studies. These groups comprise a contact pheromone whose nature has not yet been determined, which triggers a synchronous discharge of neuroendocrine bag cells which, in turn, secrete a family of peptides that promote egg release and mate-attracting pheromones. The latter comprise a family of water-borne pheromonal protein attractants, of which the major is attractin, a 58 residue glycosylated protein that is released from the albumen gland during egg laying and that acts in concert with other water-borne protein pheromones—enticin, temptin, and seductin (which are, respectively, 69-, 103-, and 192-residue proteins found in the albumen gland)—to stimulate mate attraction by recruiting other *Aplysia* to the freshly laid eggs, thereby stimulating other *Aplysia* to increase egg laying and to form mating aggregations (for review see Refs. 8, 9). Recently, another protein—Alb-1, a 101 residue protein—has been identified and cloned from the albumen gland; it is believed to play a pheromonal role, together with the above-mentioned four peptides,[8] and has been suspected to be associated with factors from non-laying *Aplysia* that might act in conjunction with attractin to stimulate enhanced mate attraction.[9] Other pheromone peptides are: a tetrapeptide (Ile-Leu-Met-Glu, termed ILME) that is eluted from egg masses of the cephalopod *Sepia*, and that is thought to stimulate the transport of oocytes in the genital tract; and a *Sepia* sperm-attracting peptide, termed SepSAP, that is released from oocytes during egg laying and that facilitates external fertilization by attracting spermatozoa.[28] Evidence for the pheromonal role of the above peptides and proteins in various *Aplysia* and *Sepia* species, their expression and localization, biochemical and molecular characterization, structure and SAR, release, inter-molecular interactions and bioactivity is presented in Refs. 8–10.

ANNELID PHEROMONE PEPTIDES

Females of the ragworm, *Nereis succinea* use a tetrapeptide, cysteinyl-glutathione (CSSG) as a mate-recognition and gamete-release pheromone during reproduction. The tetrapeptide pheromone (termed nereithione) controls reproductive behavior and release of gametes. The pheromone is released into water by swimming ripe females, and it induces males to swim faster and to release sperm. The role of peptide-based pheromones in these worms and the time course of their appearance as related to the worms' sexual maturation have recently been reviewed.[15]

ARTHROPOD PHEROMONE PEPTIDES

Insects

One of the largest and best documented groups of pheromones comprises those of insects. Chemical cues are major sources of information used by most insects to interpret environmental stimuli; they lead to the initiation of gregarious behavior, formation of aggregations at food sites, dispersal behavior during predator attack, synchronized sexual maturity, mating attraction, etc. However, none of the pheromones that elicit these functions is of a peptidic nature. There are only two examples of peptides (and proteins) being related to pheromonal activity in insects. One involves *Drosophila melanogaster*, in which the peptides act as primer pheromones that cause physiological changes associated with reproductive activity in the fly. The other involves peptides that originate in the central nervous system (CNS) of Lepidopteran species and that stimulate sex-pheromone biosynthesis in female moths.

The first system involves proteins and peptides that are transferred, together with sperm and seminal fluid, by males; they act within and outside the reproductive tract of their female targets, and elicit a wide variety of short-term and long-term responses such as increased oogenesis and ovulation, decreased female receptivity, enhanced sperm storage, and formation of the mating plug.[33] The very first seminal fluid peptide to be identified was the "sex peptide" (SP) or accessory gland peptide (Acp70A). From the 1960s through the 1980s, the nature of Acp70A was gradually revealed, and the *Acp70A* gene that encodes the 36 amino acid Acp70A peptide was identified (for an overview see Ref. 6 and references therein). In around 2000, another closely related peptide (DUP99B)—a homolog of SP—was revealed.[19] Within females, SP and its closely related homolog DUP99B are thought to elicit postmating responses through a specific GPCR, termed SPR,[34] which triggers these responses mainly by modulating neuronal activity in the subsets of the *fruitless* and ventral nerve cord neurons.[19] Detailed SAR studies have associated some of the SP functions with specific parts of the molecules,[19] and

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detailed cellular studies uncovered a neuronal mechanism by which SP exerts its control over reproductive behavior.[33] The ongoing study of seminal fluid molecules is revealing that they have an unexpected variety of functions and, furthermore, that some of the genes that encode these molecules show evidence of extremely rapid evolutionary change. These findings suggest that seminal fluid molecules might be strong targets for natural or sexual selection. At present, homologous recombination and RNA interference, along with microarrays and yeast two-hybrid systems, are being used to gain better insight into the functions of these molecules and their receptors. Information on the currently known functions of the seminal fluid proteins and peptides of *D. melanogaster*, and the approaches used to study their role in the fruit fly's reproductive and postmating functions have been published.[33] Interestingly, numerous attempts to identify SP orthologs in other insects revealed their presence in the mosquito *Anopheles gambiae*.

The second system involves a sub-esophageal ganglion neuropeptide—pheromone biosynthesis activating neuropeptide (PBAN)—that acts via a GPCR mechanism to initiate sex-pheromone biosynthesis in the pheromone glands of female moths. Studies of the regulation of sex-pheromone biosynthesis in moths have revealed that this function can be elicited by additional neuropeptides, all of which share the common C-terminal pentapeptide FXPRL-amide (in which X = S, T, G, or V) and belong to a large family of peptides: the pyrokinin (PK)/PBAN family. In the past two decades extensive studies were carried out on the chemical, cellular, and molecular aspects of PBAN and the other peptides of the PK/PBAN family, aiming to understand the mode of their action on sex-pheromone biosynthesis (for an overview see Ref. 1 and references therein). A detailed review of some of the above topics is presented in the chapter on "The FXPRL-amide Pyrokinin/PBAN Family" by Altstein et al. in the Invertebrate Peptide section of this book.

Crustaceans

Another group of pheromone peptides in arthropods comprises those of the crustaceans. Crustacean pheromone peptides have been implicated in two different behavioral patterns: gregarious settlement of barnacles, which involves "settlement pheromones"; and synchronization of larval release, which involves larval-release pheromones—also termed "pumping pheromones"—which are released from newly hatched embryos and induce stereotypical larval-release behavior in crab (*Rhithropanopeus harrisii*) or lobster (*Panulirus argus*) females. This larval-release behavior involves rapid abdominal extensions and pleopod pumping, which help to break open the egg membrane and results in synchronous release of the larvae. The settlement pheromones are peptides released from intact living barnacles, whereas the larval-release pheromones are released from the brood. Knowledge of the biological activity and SAR of synthetic settlement pheromones and of larval-release pheromones is quite elusive at present, and most studies were carried out with crude extracts or synthetic analogous peptides, because the natural pheromone peptides have not yet been fully identified. In light of current knowledge, it is well accepted that all known crustacean release pheromone peptides are based on serine protease degradation products that have basic carboxyl termini, which are essential for bioactivity. Recent studies have revealed that the released peptide pheromones are dipeptides and tripeptides that have a neutral amino acid, i.e. Gly, at the amino terminus, a basic amino acid, i.e. Arg, at the carboxy terminus, and a basic-basic dipeptide (Lys-Arg). The current state of knowledge has recently been summarized.[14,35]

VERTEBRATE PHEROMONE PEPTIDES

Amphibians

In recent years, it has became apparent that although communication among amphibians depends primarily on auditory signals, pheromonal signaling is used by both males and females for mating attraction; it is widespread in salamanders and newts, and may also be important in some frogs and toads. These pheromones are typically peptides and proteins, and several of them have been behaviorally, biochemically and molecularly identified. The best-characterized amphibian pheromones are those that influence male–female interaction. The first pheromone identified in amphibians was the female attractant sex pheromone—sodefrin—of the newt, *Cynops pyrrhogaster*, and a cDNA comparable with the cDNA encoding *Cynops* sodefrin was also isolated from another newt, *Triturus carnifex*. In 2000, a second sex pheromone was isolated from another *Cynops* species, *C. ensicauda* and was termed silefrin. *Cynops* sodefrin and silefrin are decapeptides with amino acid sequences SIPSKDALLK and SILSKDAQLK, respectively, they are secreted from the abdominal glands of males via the cloacae, their synthesis is regulated by prolactin and androgens, and each pheromone attracts only conspecific females. In 2007, a third decapeptide—aonirin—was isolated from the abdominal gland of the male *C. pyrrhogaster*, which is found in the Nara region of Japan (for review see Ref. 32). Another sex pheromone, isolated from the frog, *Litoria splendida*, is a 25-residue peptide—splendipherin[32]—which is produced by a gland in the head of the male during the mating season. Splendipherin attracts conspecific females and has no effect on males or on other species.

In recent years, many studies focused on the courtship pheromones of the salamander. Currently, it is well established that the salamander pheromone is a complex blend of compounds secreted by the male submandibular (mental)

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gland. Several such pheromones—plethodontid receptivity factor (PRF), plethodontid modulating factor (PMF), and sodefrin precursor-like factor (SPLF)—have been isolated, molecularly and biochemically characterized, and tested for effects on female behavior. During courtship, salamanders transfer mental-gland pheromones via two main modes: the olfactory mode, in which pheromones from the mental gland of the male reach the female's nasal cavity; and the transdermal mode, in which the male pheromone is deposited directly onto the skin of the female and diffuses into the peripheral vasculature.

Male–male pheromonal signaling has also been reported in amphibians. In 2005, an amphibian pheromonal peptide—leptodactylus aggression-stimulating peptide (LASP)—has been characterized and isolated from male norepinephrine-stimulated skin secretions.[17] LASP exhibited a chemoattractive effect on males, in which it stimulated aggressive behaviors that resulted in the emergence of dominant animals that subsequently attracted females to nesting sites.

Most amphibian pheromones are detected by the vomeronasal organ (VNO) and the main olfactory epithelium (MOE), which form a unique chemosensory system. Chemosensory neurons present in those organs express VNO receptors or olfactory receptors. In recent years, detailed studies were carried out on these pheromones, on the anatomy of the receptive organs, and on the molecular, genomic and physiological characteristics of the chemosensory receptors. To date, no chemosensory ligands have been matched to VNO receptors or olfactory receptors; this indicates the need for further examination of the issue, which could also provide an insight into the nature and evolution of vertebrate pheromonal communication (for detailed review on amphibian pheromones, their receptors and the chemosensory organs, please refer to the review in Ref. 32 and references therein, and to the chapter by Kikuyama in the Amphibian Peptides section of this book).

Mammals: Rodents

Needless to say, the most complex, interesting, but as yet unidentified signaling molecules are those of mammals. All mammals emit chemical cues into the environment, via urine, saliva, and/or divers secreted fluids. So far, only a few mammalian pheromones, especially those of rodents, have been identified, and examination of their chemical nature has revealed a wide diversity of compounds, that range from small organic molecules to large proteins. Several proteinaceous pheromonotropic families are described below; they all play essential roles in sex discrimination and attraction between males and females, and thereby promote mate choice and successful reproduction. Among them are: aphrodisin (for review, see Ref. 5); mouse urinary proteins (MUPs); major histocompatibility complex (MHC) class I peptides; and the tear exocrine-gland-secreting peptide 1 (ESP1) (for a detailed review, see Ref. 30 and references therein).

Aphrodisin is a protein that belongs to the lipocalin family; it is found in hamster vaginal secretions, and when it is detected by the male accessory olfactory system copulatory behavior is induced. At present, it is not clear whether aphrodisin itself performs the pheromonal function or whether it is simply a carrier for hydrophobic small pheromone molecules that have not yet been identified. Studies on this topic focus on the aphrodisin structure, its biological properties, and the associated signal transduction processes in the VNO.[5]

Other members of the lipocalin family are the MUPs, of 19 kD, found in sexually mature male mice, rats, and some other rodents. These proteins are believed to be responsible for the binding and release of low-molecular-weight pheromones, thereby providing a slow-release mechanism for volatile components of scent marks. However, the proteins may function as chemosignaling molecules in their own right, filling one or more roles in communication of individual identity, territorial behavior, and ownership. Male MUPs seem to promote, among other actions, aggressive behavior between males. Other pheromonotropic compounds present in male mouse urine are the MHC class I peptides—SYFPEITHI and AAPDNRETF—which have also been shown to convey information about individual identity and to mediate pregnancy blockage or spontaneous abortion, i.e. the Bruce effect. Recently, a male-specific peptide—ESP1—has been found to be secreted in the tears of the extraorbital lacrimal gland of male mice. This peptide is a member of the ESP gene family and seems to be involved in sexual behavior associated with the conveyance of information on strain and species, similar to the function of MUPs. A few recent reviews[25,30] summarize the current understanding of the structure and function of urinary and other proteins, and speculate on their roles as supporters or as key participants in the elaboration of the complex chemosensory properties of a rodent scent mark.

As in amphibians, peptide and protein pheromones in rodents—MPUs, MHC peptides, and ESPs——are believed to be recognized by specific transmembrane receptors—members of the VIR and V2R family—that are expressed in the VNO on sensory neurons; however, this issue is still somewhat controversial. Some chemosensory peptidic pheromonal signals, i.e. MHC peptides in rodents are conveyed via MOE. Much of the research on the molecular mechanisms underlying vomeronasal and MOE sensing of peptide pheromones has been conducted with mice and has focused on the structure of the two areas and their receptors, the search for natural ligands, signal processing and signal transduction from the chemosensory organs to the CNS (for review see Refs 25, 30 and references therein).

The VNO in mammals has received a lot of attention within a broader aspect in the past few years, and studies of

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this secondary olfactory system yield much new information such as the existence of a large number of VNO receptor genes. This unexpected receptor diversity indicates that there probably is a wide variety of chemosensory signals sensed by these receptors, about which little is known at present and which needs to be further explored. Although peptide pheromones and VNO play a most important role in communication between individuals of mammalian—rodent—and amphibian species, it seems that there was a tendency toward loss of VNO function and elimination of peptide pheromones at this point in evolution, as indicated by the absence of peptide or protein pheromones, and also of the VNO receptor gene families that mediate these cues, in primates, including humans.[30] The current consensus is that the VNO is not functional in adult humans. It is possible, however, that pheromone communication does exist in humans and is processed by the receptor neurons in the MOE. This issue also needs further exploration.

Last but not least are the neurohypophysial peptides—vasopressin (AVP) and oxytocin (OT)—that do not serve directly as pheromonal cues in mammalian species, but are rather critical to the mammalian ability to process such cues appropriately in the olfactory circuit and throughout the brain. In a recent review by Higashisa et al.[16] and an earlier review by Bielsky and Young,[4] the roles of OT and AVP in rodent social interaction, social recognition, pair bonding, and maternal behavior are discussed.

SUMMARY

There is no doubt that the wide variety of pheromone peptides described above highlight their crucial role in many and varied behavioral patterns that are associated mostly, but not exclusively, with courtship and mating, or transfer of genetic material. Despite the vast amount of information that is already available to us, and the immense progress that was made in the past few years the study of pheromone peptides still requires further exploration, and it seems that we have only just begun to understand some of the languages of a few of the "inhabitants" of this fascinating world. As our studies advance along the evolutionary tree we find that the chemical cues responsible for mating become more and more complex, and it is reasonable to assume that social and sexual behavior in vertebrates is too complicated to be controlled solely by the few components or mechanisms so far identified, and that it requires the integration of multiple sensory cues within the neural network of the brain, all of which are still to be explored. Further, comparison among the peptide or protein pheromones that are used for sociosexual communication by the various species reveals no homology, which suggests that each peptide has undergone an independent molecular evolutionary process, and hints at the possibility that there are many more novel peptide pheromones, associated with many and varied species, that need to be discovered in the future. Understanding of the interplay between behavioral and biochemical factors in the deposition and reception of scents, identification of key signal molecules and elucidation of their chemical nature, and exploitation of their modes of action and regulation in donor and recipient organisms present a challenge that is currently being evaluated in depth, and, as peptide signaling systems continue to be discovered, there is a growing need to understand the details of these communication mechanisms.

In light of current knowledge, it is already obvious that pheromones of a peptidic nature play an important role in species-specific communication, either directly or via neural processing of the olfactory signal. Their high solubility, specificity, and variability, which are achieved through the existence of multiple genes encoding for peptide variants, ensure their highly efficient pheromonal activity. The currently available novel biochemical, molecular and chemical analytical methods and approaches will enable us further to identify new peptide pheromones, to study their biosynthetic pathways, to dissect their sequences of behavioral events, and to study their mechanisms of action in the donors and recipients at the cellular and molecular levels. It is anticipated that such studies will widen our understanding of the extraordinary diversity that surrounds us, and will stimulate further studies that may lead to the unraveling of the chemical signaling mediated by peptides, and thereby lead to practical applications, such as development of new therapies against bacterial infections or of behavior-modifying compounds for agrochemical, aquacultural, and medical applications.

REFERENCES

1. Altstein M, Hariton A. Rational design of insect control agents: the PK/PBAN family as a study case. In: Ishaaya I, Horowitz R, editors. *Biorational control of arthropod pests*. NY: Springer; 2009. p. 49–80.
2. Anderegg RJ, Betz R, Carr SA, Crabb JW, Duntze W. Structure of *Saccharomyces Cerevisiae* mating hormone A-factor—Identification of S-farnesyl cysteine as a structural component. *J Biol Chem* 1988;**263**:18236–40.
3. Arkowitz RA. Chemical gradients and chemotropism in yeast. *Cold Spring Harb Perspect Biol* 2009 Aug;**1**(2).
4. Bielsky IF, Young LJ. Oxytocin, vasopressin, and social recognition in mammals. *Peptides* 2004;**25**:1565–74.
5. Briand L, Trotier D, Pernollet JC. Aphrodisin, an aphrodisiac lipocalin secreted in hamster vaginal secretions. *Peptides* 2004;**25**:1545–52.
6. Chapman T, Davies SJ. Functions and analysis of the seminal fluid proteins of male *Drosophila melanogaster* fruit flies. *Peptides* 2004;**25**:1477–90.
7. Correia I, onso-Monge R, Pla J. MAPK cell-cycle regulation in *Saccharomyces cerevisiae* and *Candida albicans*. *Future Microbiol* 2010 Jul;**5**(7):1125–41.
8. Cummins SF, Degnan BM, Nagle GT. Characterization of Aplysia Alb-1, a candidate water-borne protein pheromone released during egg laying. *Peptides* 2008 Feb;**29**(2):152–61.

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9. Cummins SF, Nichols AE, Schein CH, Nagle GT. Newly identified water-borne protein pheromones interact with attractin to stimulate mate attraction in Aplysia. *Peptides* 2006 Mar;**27**(3):597–606.
10. Cummins SF, Xie F, Misra M, Amare A, Jakubowski JA, de Vries MR, et al. Recombinant production and structural studies of the Aplysia water-borne protein pheromone enticin indicates it has a novel disulfide stabilized fold. *Peptides* 2007 Jan;**28**:94–102.
11. Diep DB, Mathiesen G, Eijsink VGH, Nes IF. Use of lactobacilli and their pheromone-based regulatory mechanism in gene expression and drug delivery. *Curr Pharm Biotechnol* 2009 Jan;**10**(1):62–73.
12. Dunny GM. The peptide pheromone-inducible conjugation system of *Enterococcus faecalis* plasmid pCF10: cell-cell signalling, gene transfer, complexity and evolution. *Philos Trans R Soc B Biol Sci* 2007 Jul;**362**(1483):1185–93.
13. Dunny GM, Johnson CM. Regulatory circuits controlling enterococcal conjugation: lessons for functional genomics. *Curr Opin Microbiol* 2011 Apr;**14**(2):174–80.
14. Forward RB. Larval biology of the crab *Rhithropanopeus harrisii* (Gould): a synthesis. *Biol Bull* 2009 Jun;**216**(3):243–56.
15. Hardege JD, Bartels-Hardege H, Muller CT, Beckmann M. Peptide pheromones in female *Nereis succinea*. *Peptides* 2004;**25**:1517–22.
16. Higashida H, Lopatina O, Yoshihara T, Pichugina YA, Soumarokov AA, Munesue T, et al. Oxytocin signal and social behaviour: comparison among adult and infant oxytocin, oxytocin receptor and CD38 gene knockout mice. *J Neuroendocrinol* 2010 May;**22**(5):373–9.
17. King JD, Rollins-Smith LA, Nielsen PF, John A, Conlon JM. Characterization of a peptide from skin secretions of male specimens of the frog, *Leptodactylus fallax* that stimulates aggression in male frogs. *Peptides* 2005;**26**:597–601.
18. Kleerebezem M, Quadri LEN, Kuipers OP, deVos WM. Quorum sensing by peptide pheromones and two-component signal-transduction systems in Gram-positive bacteria. *Mol Microbiol* 1997;**24**:895–904.
19. Kubli E. Sexual behaviour: a receptor for sex control in Drosophila females. *Curr Biol* 2008 Mar;**18**(5):R210–2.
20. Kurjan J, Herskowitz I. Structure of a yeast pheromone gene (MF alpha): a putative alpha-factor precursor contains four tandem copies of mature alpha-factor. *Cell* 1982 Oct;**30**:933–43.
21. Li L, Wright SJ, Krystofova S, Park G, Borkovich KA. Heterotrimeric G protein signaling in filamentous fungi. *Annu Rev Microbiol* 2007;**61**:423–52.
22. Luporini P, Vallesi A, Miceli C, Bradshaw RA. Chemical signaling in ciliates. *J Eukaryot Microbiol* 1995 May;**42**(3):208–12.
23. Lyon GJ, Novick RP. Peptide signaling in *Staphylococcus aureus* and other Gram-positive bacteria. *Peptides* 2004;**25**:1389–403.
24. Naider F, Becker JM. The alpha-factor mating pheromone of *Saccharomyces cerevisiae*: a model for studying the interaction of peptide hormones and G protein-coupled receptors. *Peptides* 2004;**25**:1441–63.
25. Rodriguez I, Boehm U. Pheromone sensing in mice. In: Korsching S, Meyerhof W, editors. *Chemosensory systems in mammals, fishes, and insects*. 47th ed. Berlin/Heidelberg: Springer; 2009. p. 1–20.
26. Shankar N, Coburn P, Pillar C, Haas W, Gilmore M. Enterococcal cytolysin: activities and association with other virulence traits in a pathogenicity island. *Int J Med Microbiol* 2004;**293**:609–18.
27. Stotzler D, Duntze W. Isolation and characterization of four related peptides exhibiting alpha factor activity from *Saccharomyces cerevisiae*. *Eur J Biochem* 1976 May;**65**:257–62.
28. Susswein AJ, Nagle GT. Peptide and protein pheromones in molluscs. *Peptides* 2004;**25**:1523–30.
29. Thoendel M, Horswill AR. Biosynthesis of peptide signals in Gram-positive bacteria. *Ad Appl Microbiol* 2010;**71**:91–112.
30. Touhara K. Sexual communication via peptide and protein pheromones. *Curr Opin Pharmacol* 2008 Dec;**8**(6):759–64.
31. Wardal E, Sadowy E, Hryniewicz W. Complex nature of enterococcal pheromone-responsive plasmids. *Pol J Microbiol* 2010; **59**(2):79–87.
32. Woodley SK. Pheromonal communication in amphibians. *J Comp Physiol A Neuroethol Sens Neural Behav Physiol* 2010 Oct;**196**(10):713–27.
33. Yang CH, Rumpf S, Xiang Y, Gordon MD, Song W, Jan LY, et al. Control of the postmating behavioral switch in drosophila females by internal sensory neurons. *Neuron* 2009 Feb;**61**(4):519–26.
34. Yapici N, Kim YJ, Ribeiro C, Dickson BJ. A receptor that mediates the post-mating switch in Drosophila reproductive behaviour. *Nature* 2008 Jan;**451**:33–7.
35. Ziegler TA, Forward RB. Larval release behaviors in the Caribbean spiny lobster, *Panulirus argus*: role of peptide pheromones. *J Chem Ecol* 2007 Sep;**33**(9):1795–805.

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Chapter 259

Prebiotic Peptides

Bernd M. Rode and Kristof Plankensteiner

ABSTRACT

Based on modern geochemistry view of the atmospheric and geological conditions on the primordial earth ~3.8–4 billion years ago, the most realistic scenario for the formation of amino acids and peptides in chemical evolution is discussed, including possible reasons for sequence preferences in early proteins and for biohomochirality. Arguments for a "peptide/protein world" as primary origin of life, preceding RNA/DNA-based evolution, are presented.

INTRODUCTION

Looking at the astonishing variety and complexity of peptides and proteins in today's living organisms, the question about their origin and subsequent evolution is one of the most fundamental ones for chemists and biologists. Providing the pathways to these compounds long before life in its modern form developed, chemical evolution has become a most challenging research topic after Oparin's hypothesis[23] of a "primordial soup," containing numerous organic compounds synthesized by nature under the conditions of the primitive earth some 3.8 billion years ago, when the earth had cooled down sufficiently to allow the existence of liquid water. As the key role of peptides for the origin of life[21,23] is increasingly better understood, the question of how amino acids and their first polymers came into being became crucial. Was the formation of the first prebiotic peptides a matter of chance with arbitrary results, or did chemistry and the given scenario point the way to specific, almost compulsory syntheses? The main difficulty of "prebiotic research" is our limited knowledge about this scenario, i.e. the conditions prevailing on the primitive earth. Fifty years ago, these conditions were, in the literal sense, still mostly "*terra incognita*," but since then, specific and interdisciplinary research in physics, chemistry, and earth sciences has widely improved our knowledge.

With this knowledge, it has become possible to achieve quite trustworthy reconstructions of the processes forming amino acids and subsequently linking them to peptides, almost ubiquitously and within a relatively wide variation of environmental conditions. As we cannot get any direct access to remnants of that time, the only chance to obtain conclusive results is the attempt to experimentally "repeat" the evolutionary processes under laboratory conditions mimicking the primordial scenario. Any of these experiments can be scrutinized, therefore, by challenging the availability of necessary components, environmental conditions and stability of products in the most likely scenario predicted by geochemical sciences for the primitive earth.

THE PRIMITIVE EARTH SCENARIO

According to present geochemical knowledge, Terra should have cooled down below the boiling point of water about 4 billion years ago, forming the first stable hydrosphere in its history. At that time, because of the weak gravitational field, the earth had already lost its primary atmosphere of hydrogen and helium, and a secondary atmosphere was formed by gases of volcanic origin,[9,13,14,18,19,23,28] which were stable enough to withstand the constant UV radiation of the sun and thus mainly consisted of CO_2, N_2, and water vapor, with smaller amounts of SO_2, possibly also some CO and H_2S and traces of noble gases.[12,23,30] Even certain amounts of oxygen, formed by decomposition of CO_2 and water, should have been present.[3,8,11,23,28,29] Because of the high temperature, weather phenomena such as thunderstorms and rain must have been much more violent than today. For the same reason, evaporation and recondensation in coastal regions of the primitive ocean and lakes must have been common and frequent processes. Many minerals—partly still identifiable in precambrian rocks—could have served as reactants and catalysts for chemical reaction, together with inorganic salts dissolved in the sea and lakes, where they could reach considerable concentrations.

THE FORMATION OF AMINO ACIDS

In 1953, Stanley Miller initiated the quickly expanding field of experimental simulations of prebiotic chemistry by his famous experiment investigating the possible formation of organic molecules in an assumed primitive atmosphere consisting of methane, hydrogen, ammonia, and water, under

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00259-1
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the influence of electric discharges.[23] Soon some amino acids were identified among the products. However, after it had been realized that this reducing atmosphere could not have existed on the primordial earth, the experiment had to be repeated varying atmospheric composition and sources of energy.[6,20,23] It was shown only more recently[21,23] that amino acids can readily be formed in a neutral or even mildly oxidizing atmosphere of the type previously outlined as the most probable geochemical scenario of the primitive earth. Up to now numerous amino acids, such as Gly, Val, Ala, Ser, Pro, His, and Lys have been identified as products,[23] and the fragments found by means of Proton Transfer Reaction Mass Spectroscopy in the plasma of the artificial "lightning" in these experiments[22] point at a variety of synthetic pathways to these and other types of precursor molecules of today's biochemistry.

THE FORMATION OF PEPTIDES

As the synthesis of a variety of amino acids on the primitive earth thus seems to have been an easy and common process, the subsequent major evolution problem is the synthesis of peptides from these amino acids in aqueous solution. To form a peptide bond, water has to be removed, which makes the process thermodynamically very unfavorable. A second problem for peptide synthesis in water is the kinetic barrier, because amino acids are rather unreactive concerning a nucleophilic substitution by an amino nitrogen at the carbonyl carbon. Therefore, the reaction partners would have to be activated somehow to lower the activation energy of the reaction.[23]

When prebiotic chemistry research aimed at the formation of peptides, the main concern, therefore, was to find ways to overcome both thermodynamic and kinetic obstacles. Evaporation of water, condensation reagents, and catalysts were the main tracks along which solutions were searched. Fox and Harada proposed melts of dried amino acids to form peptides.[8,21,23] At the melting temperatures of amino acid mixtures, all formed water evaporates and is thus removed from the reaction. However, a large excess of acidic and/or basic amino acids is necessary to allow polymerization and to avoid decomposition. As these amino acids are rarely synthesized in atmospheric processes and the temperature range between melting and decomposition is very narrow, the prebiotic feasibility of this reaction seems highly questionable. Further, a more thorough analysis of the resulting polymers (initially termed "proteinoids") showed that they are polymers that only occasionally contained peptide bonds.[23]

Another approach to prebiotic peptide formation was the use of condensation agents to facilitate the reactions between amino acids.[21,23] Substances used in these experiments included cyanamides, cyanates, trimetaphosphate, ATP, GTP, CTP, UTP, linear and cyclic inorganic polyphosphates, and imidazoles, all of which must have been rather scarce or unstable in a primordial earth setting, because of rapid hydrolytic decomposition and/or precipitation as insoluble salts. There is, therefore, only a very minor probability that these types of peptide synthesis have contributed to chemical evolution.

Under assumed hydrothermal vent conditions,[23] amino acids can also condense to peptides, but this scenario would be restricted to specific locations in the deep sea and especially high partial pressures of carbon monoxide, and thus its relevance for large-scale prebiotic peptide formation is rather questionable.

A further possibility is peptide formation on the surface of minerals like clays, silica, or alumina.[21,23] In principle, it is possible to form peptides from adsorbed amino acids, but the yields are very low and the applicability apparently restricted to very few amino acids such as Gly and Ala. However, these adsorption processes are of great importance for prebiotic peptide formation since peptides formed in other processes can be protected from decomposition[1] on the surface of such minerals, and further, short peptides can be elongated to longer chains by this mechanism.[21,23] For example, the Salt-induced peptide formation (SIPF) reaction[23] discussed in detail below, which efficiently forms peptides, perfectly harmonizes with clay catalyzed peptide elongation in a one-pot reaction.[21,23]

This SIPF reaction is a special case among the many proposals for peptide formation under primordial earth conditions.[23] It allows for peptide formation in aqueous solution directly from amino acids or amino acids and short peptides, as long as sodium chloride, Cu(II) ions, and thermal energy are available. Sodium chloride has always been ubiquitously present[9,23] in the oceans, in salt lakes, and in lagoons and puddles along the sea shore. In concentrations >3 M that can be reached either in large volume salt lakes or lagoons (simulated by the constant volume type[21,23] of SIPF reaction with constantly high sodium chloride concentrations) or in evaporating small puddles and small lagoons (simulated by evaporation cycle experiments[21,23] where the active concentration is reached by evaporation and subsequent redilution), sodium ions do not have a saturated first hydration shell and can, therefore, act as a dehydrating agent to overcome the thermodynamical barrier of peptide formation in aqueous solution.[23] Computational investigations of sodium chloride solutions,[21,23] showing this effect in detail, were the basis for the discovery of the SIPF reaction. Subsequent simulations and experiments showed that complexation of amino acids to metal ions, in particular to Cu(II),[23] can activate them and thus lower the activation energy for the peptide formation process. As precambrian rock formations contain large amounts of Cu(II)-containing minerals,[17,23] the availability of these ions on the primitive earth seems secured,

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and the proper oxidation state could have been reached easily under the assumed atmospheric conditions.[23] Since the late 1980s the SIPF reaction has been and still is being investigated in many areas,[21,23] continuously revealing new favorable aspects and proving its very general applicability for prebiotic peptide formation under the conditions of the primordial earth scenario outlined above.[21]

The ideal temperature for the SIPF reaction is between 60 and 90 °C,[21,23] a very realistic condition for recently condensed oceans on the cooling surface of the earth. The reaction prefers the biologically relevant α-amino acids compared with amino acids containing a more distant amino group.[21,23] Amino acids such as glycine and histidine and also small linear or cyclic peptides like diglycine and diketopiperazine show a yield-increasing catalytic effect in the reaction,[21,23] which can produce peptides even with otherwise almost nonreactive amino acids, thus resulting in peptide formation with all amino acids investigated so far. The catalytic mechanism[23] apparently involves intermediary formation of a peptide with the catalyst, followed by condensation with another educt amino acid and the release of the catalyst by hydrolysis to the product peptide and the catalyst.

Quite striking evidence for the prebiotic relevance of the SIPF reaction resulted from a comparison of the specific dipeptide yields of the reaction to the frequency of the respective peptide linkages in membrane proteins of archaea and procaryonta, some of the oldest still existing organisms on earth: a correlation of the preferred sequences was observed that could hardly have occurred only by chance,[21,23] indicating an evolutionary pathway from the SIPF process to the proteins of the first living organisms. It is interesting that the (in)famous protein-like prions show an even higher degree of similarity with the most preferred SIPF sequences (Gly-Gly > Gly-Leu > Ala-Ala > Ala-Gly > Ala-Val > Pro-Pro > Pro-Gly > Val-Val > Gly-Ala > Val-Gly > Leu-Gly > His-Gly > Leu-Leu > Val-Ala > Gly-His > Val-Lys > Lys-Val), in particular the characteristic dominance of the Gly-Gly linkage, thus indicating an origin of the prions at a very early stage of evolution.[24]

As mentioned previously, the oligopeptides formed in the SIPF reaction could have been stabilized and elongated to longer chains with the help of clay minerals such as montmorillonite or hectorite in the same environment.[21,23] Thus the role of clay minerals in peptide evolution could have been a quite significant one, not so much for the primary formation of peptides from amino acids, but for the further development of larger and more complex peptides and for their "survival" against hydrolytic attack.

Another quite important feature of the SIPF reaction refers to chirality-related aspects; during the reaction, optical purity of the amino acids is almost fully conserved,[21,23]

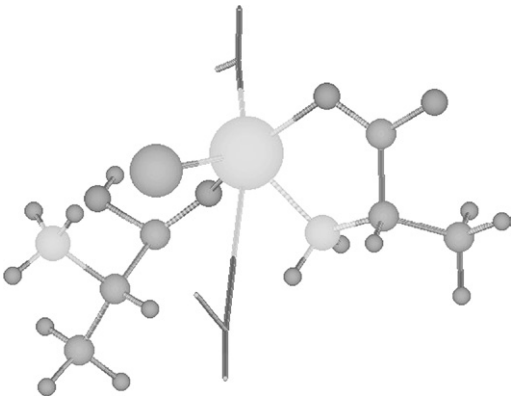

FIGURE 1 Ab initio geometry optimized structure of the tetrahedrally distorted SIPF complex $[CuCl(alaH_2)(H_2O)_2]^+$.[21,23]

especially at low concentrations as would be expected in a very dilute prebiotic "soup." This preservation of chirality, albeit quite important, is not the only characteristic of the reaction with respect to optical activity. The active complex in the SIPF reaction[21,23] is a copper(II) complex with one amino acid coordinated as a chelate, another amino acid or oligopeptide bound end-on via its carboxylic group, a chloride ion, and two water molecules at elongated distance (Fig. 1).

Quantum chemical calculations have predicted a pseudotetrahedral arrangement of the amino acid and chloride ligands,[21] thus leading to a central chirality at the Cu(II) ion that seems to amplify the small inherent chirality of copper due to the parity violating effect, which because of its Z^5 to Z^6-dependence (Z being the atomic number), is considerably larger than in organic compounds. This chirality effect can lead in some cases, especially with simple aliphatic amino acids such as Ala and Val, to a preference of L-amino acids over D-amino acids in the SIPF reaction[4,21,23] and might be, together with yet unknown amplification mechanisms, a basis for the processes having led to the phenomenon of biohomochirality. Yield differences according to the chirality of the educt amino acids, differences in the complex formation constants of L- and D-alanine, and CD spectroscopy have supplied independent indications toward this particular stereoselectivity in the SIPF reaction in favor of L-amino acids.[4,23]

A 'PEPTIDE WORLD' AS THE ORIGIN OF LIFE?

Any discussion on the "origin" of life naturally involves a definition of a "living system." It has become a kind of convention to characterize "life" by the essential properties metabolism, replication, propagation of information, and the possibility of mutation, allowing evolution to adapted

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and more complex forms. Although a kind of metabolism can be assigned to many chemical systems, the other characteristics can be achieved according to our contemporary chemical knowledge by two types of "biomolecules" only, namely, peptides/proteins and nucleic acids, which are the key players in all of today's living organisms. Nucleic acids are the "modern" information carrying molecules and replicators, but peptides and proteins could have preceded them in that task, as peptides can also work as carriers of information and can replicate,[23] albeit not as efficiently as nucleic acids. Under the harsh conditions of the primordial earth, however, evolution of life-like processes based on peptides would have had many favorable aspects. Besides various problems with the formation of nucleic acids under primordial earth conditions[5,7,26] the first argument to support this statement is the chemical and physical (in)stability of nucleic acids; in a hot salty ocean, irradiated by the UV light of the sun, nucleic acids, and their building blocks decompose very rapidly[10,16,23,27] and thus would not have had time to pass on their information and to chemically evolve. Peptides, however, could be formed much more efficiently[21,23] and, once formed, would be much more stable[2,15,23] and well protected from UV damage by dissolved ions and other organic molecules in the prebiotic "soup" that absorbed a great part of UV radiation in the wavelength region below 220 nm that would have been harmful to them.[23] Further, peptides are far less prone to errors caused by mutations, since their informational content and their functionality are less harmed by an exchange of amino acids in the sequence. For example, a hydrophobic amino acid would mainly be replaced by another hydrophobic amino acid, which in most cases would not have a strong influence on the characteristics of the peptide, while the replacement of one or several nucleobases by another of the four possibilities can have dramatic effects, even leading to a total loss of function. Without the help of sophisticated enzymatic repair mechanisms (which demands the existence of sophisticated proteins!) nucleic acid evolution would quickly reach a dead end. Peptides can deal with many more errors by themselves. Based on information theory, Freeman Dyson's model of chemical evolution[23] has predicted that, given the low autocatalytic efficiency of the first prebiomolecules, four basic building blocks, as in the case of nucleic acids, would be too few to reach a state where the development of autocatalytic biochemical cycles would be possible; at least 8–10 would be required. This figure is realistic for early peptides, even considering a still limited number of amino acids formed during prebiotic chemical evolution. Finally, follow-up investigations on Eigen's RNA hypercycles[23] quickly showed through computational simulations[23] that such cycles would quickly be terminated by various catastrophic conditions, thus not providing a possible development of living systems.

Therefore, an initial "Peptide/Protein World,"[21] as opposed to an "RNA World" scenario, seems a much more probable starting point on the way to life on earth. Amino acids and peptides would have been formed first and then have chemically evolved to assume the function of primary information carriers and replicators. Nucleic acids could probably only have been synthesized and have chemically evolved after some kind of protection against environmental influences had become available, most probably in the form of peptide/protein membranes or shells.[25] Inside this protected environment, further evolution of the nucleic acids to nucleosides and nucleotides and the necessary enzymatic repair mechanisms could take place, passing over the functions of the first peptides and proteins to RNA and DNA and ultimately leading to the genetic apparatus of today's organisms. This later involvement of RNA/DNA in evolution has actually been anticipated by numerous scientists in the past 3 decades,[21,23] but only the recent development in peptide research has shown a plausible and more convincing alternative for the evolution of life on our planet.

REFERENCES

1. Bernal JD. *The physical basis of life*. London: Routledge and Kegan Paul; 1951.
2. Blank JG, Miller GH, Ahrens MJ, Winans RE. Experimental shock chemistry of aqueous amino acid solutions and the cometary delivery of prebiotic compounds. *Orig Life Evol Biosph* 2001;**31**:15–51.
3. Carver JH. Prebiotic atmospheric oxygen levels. *Nature* 1981;**292**: 136–8.
4. Egel R, Lankenau DH, Mulkidjanian AY. *Origins of life: the primal self-organization*. Heidelberg: Springer; 2011, p. 107–127.
5. Eschenmoser A. Chemical etiology of nucleic acid structure. *Science* 1999;**284**:2118–24.
6. Fox SW, Dose K. *Molecular evolution and the origin of life*. San Francisco: Freeman; 1972.
7. Gibson LJ. Did life begin in an "RNA world?" *Origins* 1993;**20**: 45–52.
8. Grandstaff DE. Origin of uraniferous conglomerates at Elliot lake, Canada and Witwatersrand, South Africa: implications for oxygen in the Precambrian atmosphere. *Precambrian Res* 1980;**13**:1–26.
9. Holland HD. *The chemistry of the atmosphere and oceans*. New York: Wiley; 1978.
10. Isbell HS, Frush HL, Wade CWR, Hunter CE. Transformations of sugars in alkaline solutions. *Carbohydr Res* 1969;**9**:163–75.
11. Kasting JF. The evolution of the prebiotic atmosphere. *Orig Life Evol Biosph* 1984;**14**:75–82.
12. Kasting JF. Earth's earliest atmosphere. *Science* 1993;**259**:920–6.
13. Kasting JF. Archaean atmosphere and climate. *Nature* 2004;**432**. doi: 10.1038/nature03166.
14. Kasting JF, Ackerman TP. Climatic consequences of very high carbon dioxide levels in the earth's early atmosphere. *Science* 1986;**234**:1383–5.
15. Lai MC, Topp EM. Solid-state chemical stability of proteins and peptides. *J Pharm Sci* 1999;**88**:489–500.
16. Lindahl T. Instability and decay of the primary structure of DNA. *Nature* 1993;**362**:709–15.

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17. Nutman AP, McGregor VR, Friend CRL, Bennett VC, Kinny PD. The Itsaq gneiss complex of southern West Greenland; the world's most extensive record of early crustal evolution (3900–3600 Ma). *Precambrian Res* 1996;**78**:1–39.
18. Ohmoto H, Watanabe Y. Archaean paleosols and archaean air. *Nature* 2004;**432**. doi: 10.1038/nature03168.
19. Ohmoto H, Watanabe Y, Kumazawa K. Evidence from massive siderite beds for a CO_2-rich atmosphere before ~1.8 billion years ago. *Nature* 2004;**429**:395–9.
20. Oró J. Synthesis of organic molecules by physical agencies. *J Br Interplanet Soc* 1968;**21**:12–25.
21. Plankensteiner K, Reiner H, Rode BM. From earth's primitive atmosphere to chiral peptides—the origin of precursors for life. *Chem Biodiv* 2004;**1**:1308–15.
22. Plankensteiner K, Reiner H, Rode BM, Mikoviny T, Wisthaler A, Hansel A, et al. Discharge experiments simulating chemical evolution on the surface of Titan. *Icarus* 2007;**87**:616–9.
23. Rode BM, Fitz D, Jakschitz T. The first steps of chemical evolution towards the origin of life. *Chem Biodiv* 2007;**4**:2674–702.
24. Rode BM, Flader W, Sotriffer C, Righi A. Are prions a relic of an early stage of peptide evolution? *Peptides* 1999;**20**:1513–6.
25. Santoso S, Hwang W, Hartman H, Zhang S. Self-assembly of surfactant-like peptides with variable glycine tails to form nanotubes and nanovesicles. *Nano Lett* 2002;**2**:687–91.
26. Shapiro R. The improbability of nucleic acid synthesis. *Orig Life Evol Biosph* 1984;**14**:565–70.
27. Shapiro R. Prebiotic cytosine synthesis: a critical analysis and implications for the origin of life. *Proc Natl Acad Sci USA* 1999;**96**:4396–401.
28. Sleep NH. Palaeoclimatology: archaean palaeosols and archaean air. *Nature* 2004;**432**. doi: 10.1038/nature03167.
29. Towe KM. Precambrian atmospheric oxygen and banded iron formations: a delayed ocean model. *Precambrian Res* 1983;**20**:161–70.
30. Walker JCG. Carbon dioxide on the early earth. *Orig Life Evol Biosph* 1985;**16**:117–27.

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Chapter 260

Viral Fusion Peptides

Avraham Ashkenazi and Yechiel Shai

ABSTRACT

A viral fusion peptide is a segment of a viral fusion protein partly responsible for the ability of the protein to induce virus–cell membrane fusion. Despite its importance, there is no precise definition for a fusion peptide. Generally, it is hydrophobic, rich in Gly and Ala residues, and located at the N-terminus of the fusion protein. Studies using synthetic fusion peptide reveal that it binds and inserts into membranes, disrupts membranes, induces vesicle aggregation, creates pores, and mixes lipids between various populations of vesicles. Importantly, synthetic fusion peptides inhibit virus–cell fusion possibly because of their interaction with the native fusion peptide. Emerging studies on distal virus families suggest that this segment is not the sole factor in promoting fusion. Other regions within the fusion protein synergize with the fusion peptide to induce fusion, thus drawing a more complex picture for the mechanism of virus–cell membrane fusion.

INTRODUCTION

Mechanism of Membrane Fusion

Membrane fusion is a fundamental event that is critical to many biological systems such as entry of enveloped viruses to their host cells, intracellular vesicle fusion, and cell-to-cell fusion in fertilization and development. These processes are mediated by many transmembrane proteins that remarkably share similar motifs.[6,27,30]

The fusion of two stable biological membrane bilayers into one is a complex event that requires physical forces. In many biological systems, membrane merger can be divided into two steps: the formation of a hemifusion intermediate and the fusion pore.[13,30] Both steps are governed by the membrane lipids and proteins. First, the contacting "cis" monolayer leaflets are merged, but the "trans" distal leaflets remain intact. The structures of these intermediates are often called the ''stalks'' that further progress to form a hemifusion diaphragm. At this stage, lipid mixing occurs only between the cis monolayer leaflets with no content mixing. The hemifusion state is a key step because a productive fusion requires transformation of the hemifusion intermediate into an expanding fusion pore. Posthemifusion is the rate-limiting step of the fusion reaction, and theoretical analysis confirms that fusion pore expansion is more energetically demanding than hemifusion.[5] Overcoming the energy barriers is related to the membrane lipids and proteins that effect the tensions and stresses of the membranes by mechanisms that are not yet fully understood.[10]

Viral Membrane Fusion

A basic phenotypic distinction for animal viruses is whether or not they are sheathed in a membrane envelope. Progeny envelope viruses acquire a cell-derived membrane following budding from infected cells.[29,30] As expected, these membranes are enriched in viral fusion proteins, which are virally encoded. It is these fusion proteins that are responsible for carrying out the task of merging the viral envelope with the target cell plasma membrane to transfer the genome-containing nucleocapsid into the host cytosol. Viral fusion proteins can be further categorized into two classes, I and II. Signature features of class I fusion proteins include (i) trimeric organization in a metastable state on the virion surface, which is retained while folding to a more stable conformation during fusion, (ii) predominantly α-helical six-helix bundle (SHB) low-energy conformation with hallmark internal trimeric parallel coiled coil, (iii) membrane active fusion peptide usually located at the N-terminus, and buried in the trimer interface in the metastable state.[1,3,4,9,31] Examples include Influenza, Parainfluenza, HIV, SIV, Ebola, SARS, Measles, and Sendai.

Class II fusion proteins have a somewhat different organization, with the following key features: (i) dimeric organization in the metastable state, which reassembles to a low-energy trimer during fusion, (ii) predominantly β-sheet structural organization of both dimeric and trimeric states, (iii) an internal loop fusion peptide, which is sequestered in the metastable dimer. Enveloped viruses characterized as class II, based on their fusion protein, include Tick-Borne Encephalitis, Dengue, and Semliki Forest viruses. Despite their obvious structural differences, it is evident that both Class I and II viruses share a similar fusion mechanism based on target membrane insertion of the hydrophobic

Handbook of Biologically Active Peptides. http://dx.doi.org/10.1016/B978-0-12-385095-9.00260-8
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fusion peptide, followed by refolding of the fusion protein to a low-energy conformation which directs fusion.[6]

Structural Organization of Class I Fusion Proteins

The essential commonality is the mechanism by which the transmembrane (TM) subunit directs membrane merger via a set of conformational changes. It is precisely at this level that Influenza sets the stage for our mechanistic understanding of Class I viral fusion, based on both precedent and the fact that there is a more complete set of high-resolution structures of its fusion protein in different conformations associated with different stages of fusion.[5,6] However, although high-resolution structures of fusion proteins serve as the basis for models describing viral fusion, these structures are of soluble fragments in solution environments. The most interesting information lies at the membrane–protein–water interface, an environment that is technically challenging to reproduce, especially for studies at the atomic level.

Being a single pass TM protein, the Class I fusion protein consists of Ecto-, TM-, and Endo-domains. Widely held models identify the ectodomains as being primarily responsible for fusion based on both conformational changes as well as direct membrane interactions.[4,6] For many Class I fusion proteins, the ectodomain is organized in a conserved manner (Fig. 1). The membrane active and hydrophobic fusion peptide is located at the N-terminus and is abutted by a N-terminal heptad repeat (NHR). A loop region separates the NHR from a C-terminal heptad repeat (CHR), which sits adjacent to the TM domain. Remarkably, different Class I fusion proteins, with decidedly different sequences, fold into a conserved low-energy SHB conformation through their cognate NHR and CHR regions. These SHB's are characterized by three NHR regions forming a parallel trimeric coiled coil, with three cognate α-helical CHR regions packing against the coiled coil in an antiparallel manner (Fig. 2C). Their highly helical character is a hallmark of Class I fusion proteins, and the low-energy SHB conformation is believed to represent a final, end-state fusion conformation. SHBs can form in solution from free cognate NHR and CHR peptides, and high-resolution crystal structures have been determined for Class I viral fusion proteins, including Influenza,[31] HIV-1,[3,28] SIV, Ebola, SARS, and Parainfluenza.

The Definition of Viral Fusion Peptides

There are many parameters that characterize fusion peptide segments from viral fusion proteins. There is no general rule that allows identification of a fusion peptide. Some of the following parameters can match one family of peptides and not the other. The fusion peptide is often hydrophobic, rich in Gly residues and located at the N-terminal end of the fusion protein. In some cases mutations in the fusion peptide region leads to loss of activity. Many viral fusion peptides adopt an α-helical structure when inserted into a

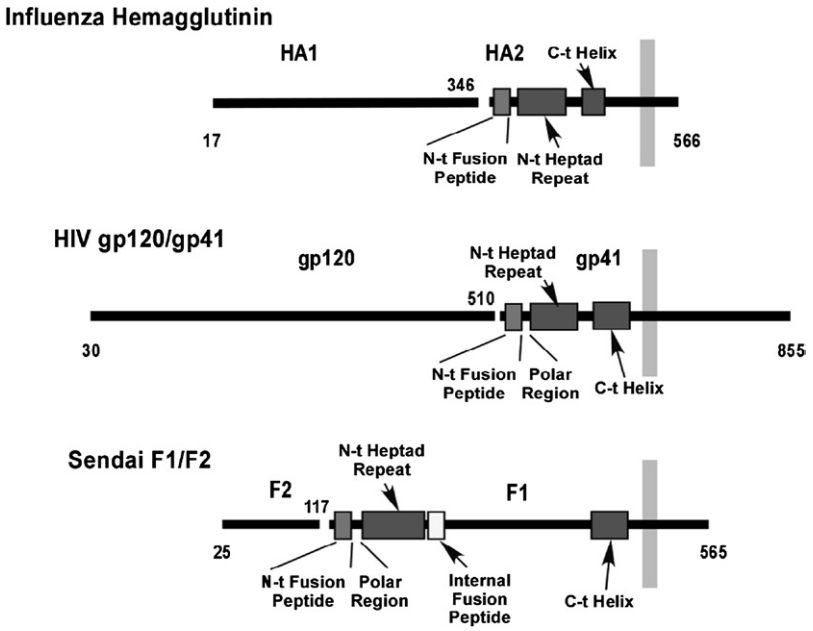

FIGURE 1 Schematic representation of the ectodomain organization of several class I fusion proteins: Influenza, HIV, and Sendai viruses. The boxes represent: the N-terminal fusion peptide; the N-terminal heptad repeat; and the C-terminal helix (heptad repeat). The bright vertical strip represents the viral envelope. In Sendai F1, the additional bright box represents the putative internal fusion peptide. Note that the residue numbers in the three proteins correspond to the mature polypeptides, after processing of the signal sequences.

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membrane. The insertion is often at an oblique angle. Some of them favor a β-sheet in the membrane, and as for internal fusion peptides, they do not have stable secondary structures. A common property of a number of fusion peptides is that they lower the bilayer of phosphatidylethanolamine to hexagonal phase transition temperature, indicating that they promote negative curvature. This property is well correlated with conditions that lead to membrane fusion. Additionally, lowering membrane rupture tension is another important characteristic that is attributed to several fusion peptides. The fusion peptide does not function as an isolated unit, but is rather assembled into an organized complex in which several fusion peptide segments can act in a coordinated fashion. In addition, there are other regions of the fusion protein outside of the fusion peptide that can also participate in accelerating fusion.[10]

METHODS TO ANALYZE FUSION PEPTIDE STRUCTURE AND FUNCTION

Conformation of Fusion Peptides within the Membrane

A detailed X-ray or nuclear magnetic resonance (NMR) structure of a segment of the ectodomain of a viral fusion protein can be obtained as a crystal in the absence of membranes. In addition, NMR spectroscopy can be used to obtain the conformation of a small synthetic fusion peptide, either in the presence or absence of membranes. A general assessment of the secondary structure of a fusion peptide can be made using less specific spectroscopic techniques, based on the properties of the amide chromophores, such as circular dichroism (CD) or infrared spectroscopy. In addition to the secondary structure, polarized attenuated total

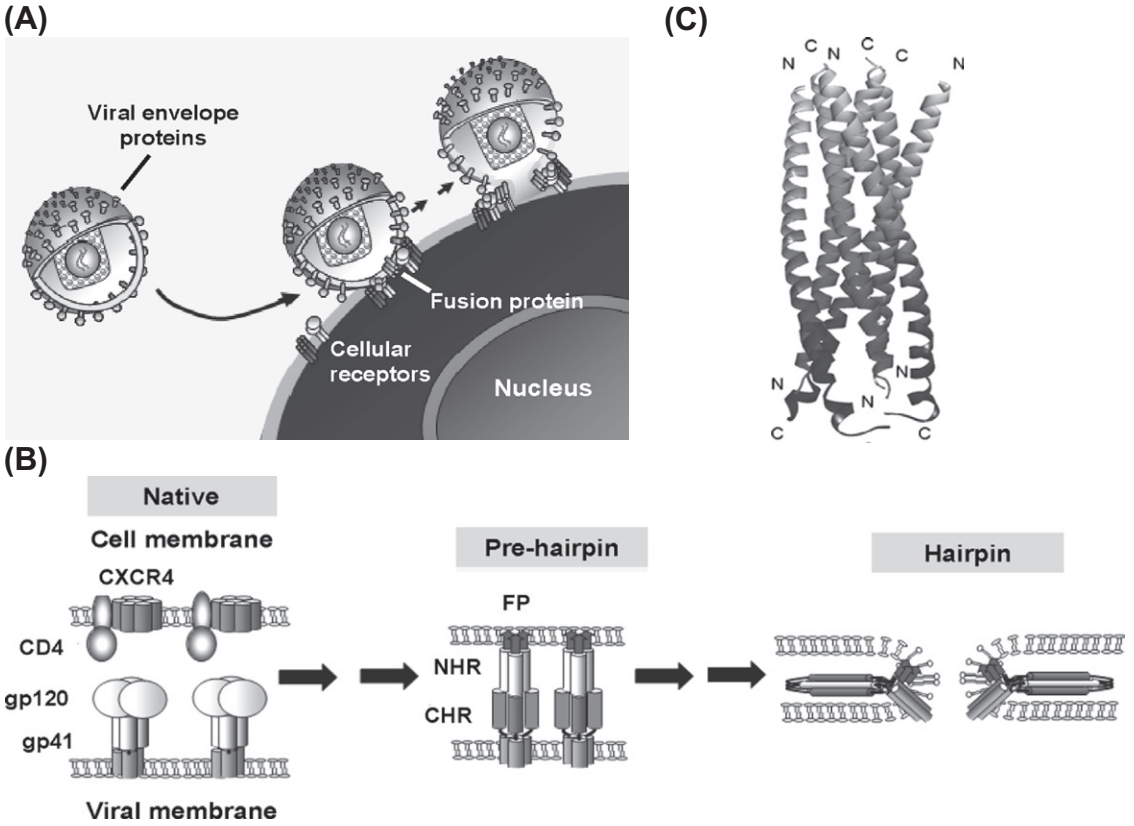

FIGURE 2 (A) Illustration of entry of an enveloped virus into its host cell. The first step in viral infection (e.g. HIV or Sendai) is fusion between the membranes of the virus and the host cell, leading to the insertion of the viral genetic material. The surface subunit of the envelope protein facilitates the interaction with cellular receptors. This interaction induces conformational changes that lead to the exposure of the transmembrane fusion protein. Additional conformations of the fusion protein subsequently lead to membrane fusion. (B) The proposed model of HIV fusion mediated by gp41, its transmembrane fusion protein. First, the envelope protein is in its metastable native state on the virion surface. In this state gp41 is considered to be sheltered by gp120. Second, binding of gp120 to CD4 and coreceptor (such as CXCR4) involves conformational changes in both gp120 and gp41 resulting in the prehairpin conformation. At this point, gp41 is exposed and extended leading to the insertion of the fusion peptide (FP) into the host cell membrane. Third, additional conformational changes generate the hairpin conformation. This conformation comprises a SHB that is created by three NHR regions into which three CHR regions pack in an antiparallel manner. (C) A side view of the recent hairpin structure of HIV-1 gp41[2] (PDB ID: 2x7r) comprising the trimeric inner coiled coil of the N-helices and the three packing C-helices. C, carboxyl terminus; N amino terminus. Note that the structure lacks the fusion peptide and the transmembrane domain.

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reflectance Fourier transform infrared spectroscopy (ATR-FTIR) can be used to determine the orientation of a helical peptide in a membrane.[14,25]

Functional Role of a Fusion Peptide in Membrane Fusion

Mutations in the Fusion Protein and Hydrophobic Affinity

Various experimental approaches have been used to study the functional role of fusion peptides in the membrane fusion process. For example, the interaction between fusion proteins and membranes has been studied using hydrophobic photoaffinity labeling. This technique was used to show that the fusion peptide of Influenza hemagglutinin (HA) penetrates deeply into the target membrane. Mutational studies reveal that substitution of critical residues in fusion peptides of, for example, Influenza HA, SIV gp32, HIV gp41,[12] and VSV G protein, abolish membrane fusion. Nevertheless, in addition to the local effects on the fusion peptide, single mutations may sometimes affect the overall folding of the fusion protein and thus make the analysis difficult.

Using Synthetic Fusion Peptides

Because membrane fusion is a complex process and involves a wide range of biochemical and biophysical interactions, it is difficult to address the specific role of fusion peptides in the context of the full-length envelope proteins. In an attempt to overcome this, researchers have studied the interactions between liposomes or cells and synthetic peptides, corresponding to regions of the intact protein. The relevance of this approach was demonstrated by (i) effects of mutations in synthetic peptides, corresponding to segments of the fusion protein, are mostly similar to those in the full-length protein,[12,17–19,23] and (ii) factors, such as pH or the addition of inhibitory agents, which affect the infectivity of the virus *in vivo*, also affect the activity of the synthetic peptides *in vitro*.

Measurement of the interactions between synthetic fusion peptides and the membrane is commonly achieved by fluorescent studies. For example, (i) the binding affinity constant of a fusion peptide to a membrane with specific lipid composition is calculated from a titration curve of liposomes to a solution of peptide, labeled with a fluorescent probe which is known to be quenched inside the membrane (such as NBD), and (ii) the lipid mixing activity of a fusion peptide is often determined by introducing the peptide to a solution which comprises two populations of liposomes: the first one is fluorescently labeled with two probes that are quenched in close proximity (such as rhodamine and NBD), and the second one is unlabeled liposomes at 10-fold higher concentration. The induction of lipid mixing by the peptide would induce fusion between the unlabeled and labeled liposome populations, leading to dequenching of the fluorescent signal.

The use of peptidic fragments from a membrane protein that interfere with its function is an emerging approach for understanding protein function. Synthetic fusion peptides in some cases inhibit viral fusion, and models have been suggested to describe where they interact with the fusion protein and at which stage during the fusion event.[9,17]

FUSION PEPTIDES IN RETROVIRUSES

HIV-Mediated Membrane Fusion

Human immunodeficiency virus (HIV) is the most known membrane enveloped retrovirus, which mainly infects cells from the immune system: T-cells and macrophages. It is the cause of acquired immunodeficiency syndrome (AIDS). The first step in HIV infection is fusion between the membranes of the virus and the host cell, leading to the insertion of the viral genetic material into the cytoplasm (Fig. 2A). After reverse transcription, the viral cDNA is integrated into the chromosomes of the cell. The membrane fusion step is mediated by the viral envelope glycoprotein. Like other class I fusion proteins, the HIV envelope protein adopts trimeric organization on the viral surface. It consists of two noncovalently associated subunits (Fig. 1): The gp120 subunit enables cell receptors and coreceptors binding, whereas the gp41 TM subunit endows the physical membrane fusion.[13]

The model of HIV membrane fusion asserts the conversion between at least three major envelope protein conformations (Fig. 2B). First, the envelope is in its metastable native state on the virion surface.[9] In this state, gp41 is considered to be sheltered by gp120. Second, binding of gp120 to CD4 and a coreceptor (such as CXCR4 and CCR5) involves conformational changes in both gp120 and gp41 resulting in the prehairpin conformation.[9,11] At this point, gp41 is exposed and extended leading to the insertion of the fusion peptide into the host cell membrane.[10] Third, additional conformational changes generate the hairpin conformation.[6,13] This conformation comprises a trimeric central coiled coil that is created by three NHR regions, into which three CHR regions pack in an antiparallel manner (Fig. 2C). This structure is usually referred to as the SHB or "core" structure, and it is suggested to be essential for complete membrane merger and pore formation.

The HIV Fusion Peptide

Functional Role

The assumptions that the fusion peptide serves as a membrane anchor, whereas folding between NHR and CHR regions draws together the virion and cell are straightforward

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enough. Yet, they fail to describe how fusion occurs. This brings us to an interesting though little understood aspect of the gp41 fusion protein; its ability to directly destabilize membranes. The tendency for peptides or regions of a protein to bind or otherwise interact with membranes is based on their regions of hydrophobicity.[32] Early on, the fusion peptide was believed to be membrane active based on high hydrophobocity in its 16 N-terminal residues.[30] In addition, the heptad repeats with leucine/isoleucine zippers, which create amphipathic helices, were also purported to be membrane active through their hydrophobic interfaces.[25] Although HIV undergoes extensive mutation because of the low fidelity of its polymerase and rapid turnover, the sequences comprising the hydrophobic fusion peptide and the hydrophobic leucine zipper residues involved in both coiled coil and SHB formation are highly conserved.[8] Indeed, studies with free peptide fragments reveal that the fusion peptide binds and inserts into membranes and disrupts membrane vesicles.[17,24,33] In addition, it disrupts membranes causing vesicle aggregation, pore formation, mixing of lipids between populations of vesicles, as well as vesicle fusion.[7,17,19,20,24,33,34]

A polar amino acid substitution at position 2 of the fusion peptide of HIV-1 gp41 (V2E) results in an envelope glycoprotein that dominantly interferes infection mediated by the wild-type HIV-1 envelope glycoprotein. This mutant elicits a dominant interfering effect even in the presence of excess wild-type glycoprotein, which suggests that a higher order envelope glycoprotein complex is involved in membrane fusion.[12] The fusogenic properties of the intact gp41 and its V2E mutant could be further modeled with synthetic peptides. This synthetic peptide model was used to explore the mechanism in which the V2E mutant interferes with the fusion peptide activity. The dominant form of both peptides is a dimer but the wild-type peptide also forms higher order oligomers. The inability of the V2E mutant to form higher order oligomers may be correlated with its loss of activity. Further, the coassembly of the two peptides in their membrane-bound state suggests that the inhibition of membrane fusion exhibited by the V2E mutant occurs via its association with the wild type. This inhibition may occur either because the V2E mutant decreases the portion of higher order oligomers of wild type or because the V2E mutant forms nonfunctional higher order oligomers with the wild-type peptide. Because the V2E mutant gp41 elicits a dominant interfering effect even in the presence of excess wild-type glycoprotein, the second possibility is a more probable mechanism.[17]

Structural Dilemma

Despite more than a decade of effort put in by many research groups, the structural organization of the HIV fusion peptide region remains debated. Class I fusion peptides are somewhat loosely defined, but it is generally accepted that they include the following traits: (i) high hydrophobicity, (ii) membrane interacting; free fragments disrupt model membranes, (iii) N-terminus of fusion protein, (iv) rich in Ala and Gly residues, and (v) conserved sequences with mutations leading to loss of function.[10,29] For HIV, the N-terminal 16 residues fit these criteria. The HIV fusion protein, gp41, is expressed along with the gp120 surface subunit as the gp160 precursor, which is posttranslationally cleaved to generate the free gp41 N-terminus. This free N-terminus is probably immediately sequestered at the gp41 trimer interface as in Influenza.[31] Hence, the membrane disrupting fusion peptide region is held like an attack dog on a leash, away from membranes until specific activation and release. Toxicity is directly linked to the fusion peptide at the free, exposed N-terminus, and probably results from membrane disruption effects on expressing cells. Given the absence of biophysical data describing the fusion peptide in context of gp41, a wealth of results exists concerning fusion peptide fragments amenable to chemical synthesis. It must be kept in mind that when using such a reductionist approach to study a specific region of a protein, the probability of artifact increases. From the outset, and continuing until the present, structural characterization of free fusion peptide fragments in membrane and membrane-mimetic environments shows that the fusion peptide is structurally plastic. Inherent structural plasticity in the fusion peptide may result from enrichment in Gly and Ala residues. Reports of predominantly α-helical conformation[15,18] contend with those of those predominantly β-sheet structure,[20,24,33] and share space in the database with reports of helical or β-structure, which is dependent on environment. Caution must be exercised on extrapolation to *in vivo* structure in function because these studies are of fragments representing specifically the fusion peptide.

During the different stages of fusion, the fusion peptide is expected to interact with the gp41/gp120 trimer in the metastable state,[31] probably is solvent exposed at least briefly after triggering or activation, and then is expected to interact with and insert into the target cell membrane.[30] The fusion proteins of Influenza and Sendai have been crystallized in both their metastable[31] and fusion active (SHB) conformations,[35] with their N-terminal regions included for both. In each case, the fusion peptide is a helical extension of the helical coiled coil NHR in the fusion active conformation.[35] However, because crystallization and data acquisition conditions lack the presence of a membrane or otherwise membrane-mimetic environment, the α-helical fusion peptide conformation in both cases may represent a transient solvent exposed interaction. All current high-resolution structures of the HIV fusion protein lack the fusion peptide region.[3,28] A clear understanding of the structure of the HIV fusion peptide, in the context of its gp41 fusion protein in both solution and a membrane environment, promises to illuminate the poorly understood process of membrane merger directed by gp41. If the HIV fusion

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peptide is helical, is it continuous with the adjacent NHR? If it is β-structured, what is the organization and how does this transition to the parallel trimeric coiled coil NHR? The structural analysis and membrane activity of the N-terminal fusion peptide in context with its adjacent coiled coil provided insights into these questions.[25] The results suggest that the HIV fusion peptide structure differs from that of Influenza and Parainfluenza in the fusion active conformation. A β-structured fusion peptide may be unique to HIV as a Class I fusion protein.

FUSION PEPTIDES IN PARAMYXOVIRUSES

Paramyxoviruses-Mediated Membrane Fusion

The family of paramyxoviruses includes the causing agents of several common childhood diseases, such as mumps, measles, and respiratory infections, as well as more serious pathogens that attack the central nervous system. One of the two common envelope proteins, named HN, N, or G, depending on the genus, plays the role of "docking" during the fusion process, most commonly by binding to sialic acid-containing receptors in the target cell.[1,22] F, the other protein common to all paramyxoviruses, is responsible for mediating the actual membrane fusion step. After protease activation, F is formed by two disulfide-linked subunits, F1 (the TM subunit) and F2, the surface (SU) subunit. Binding of HN to the cell receptors has been postulated to trigger a conformational change in F1–F2 that starts the fusion process.[16] The way in which HN modifies F1–F2 conformation is still not clearly known, although it has been proposed to involve direct contacts between both proteins. Indeed, HN interacts with F1–F2, either in the endoplasmic reticulum or on the surface of infected cells.[1,16]

As in other class I fusion proteins (and as described above for HIV gp41), the F1 subunit contains two heptad repeat regions, one (named NHR) is located close to the N-terminal fusion peptide, whereas the other (named CHR) precedes the TM domain (Fig. 1). Also common to other class I fusion proteins, peptides derived from the NHR and CHR regions form a stable complex in solution, in which three NHR peptides fold into an internal trimeric coiled coil, surrounded by three CHR helices, a structure presumably formed at a late stage during the fusion process.[4,22]

Paramyxovirus F1 Protein Has Two Fusion Peptides

The fusion peptide located at the N-terminus of F1 was initially believed to be solely responsible for the actual merging of the viral and cell membranes. However, it is unlikely that the whole membrane fusion process is mediated by such a short segment in a protein consisting of >560 residues. Additionally, it has been shown in Influenza virus, HIV-1 and Sendai virus that regions other than the N-terminal fusion peptide bind to membranes, suggesting that these regions have possible roles during the fusion process. Accordingly, screening of the Sendai F1 ectodomain for regions homologous to previously known fusion peptides renders, in addition to F1 N-terminus, a segment (corresponding to amino acids 214–226) that is strikingly similar to HIV-1 and Respiratory Syncytial virus (RSV) fusion peptides (Fig. 3). Experimental evidence for the participation of this internal region in Sendai virus cell entry comes from protein dissection studies in which short peptides encompassing this region induce membrane fusion of large unilamellar vesicles.[14,21] Elongation of the peptide results in higher fusion activity, strongly supporting a similar role for this sequence in the full-length protein. Further, the peptides are potent inhibitors of Sendai virus infection of red blood cells, presumably by blocking conformational changes in the F protein. Interestingly, mutations that abolish this inhibitory activity also reduce the ability of the peptides to induce fusion of vesicles. Further, peptides from the same region of the Measles virus F1 protein behave similarly, despite having low-sequence similarity at their C-terminus, a region poorly conserved among paramyxovirus F proteins.[26] This suggests that the presence of two fusogenic regions might be a general feature of paramyxovirus F proteins.[22,26]

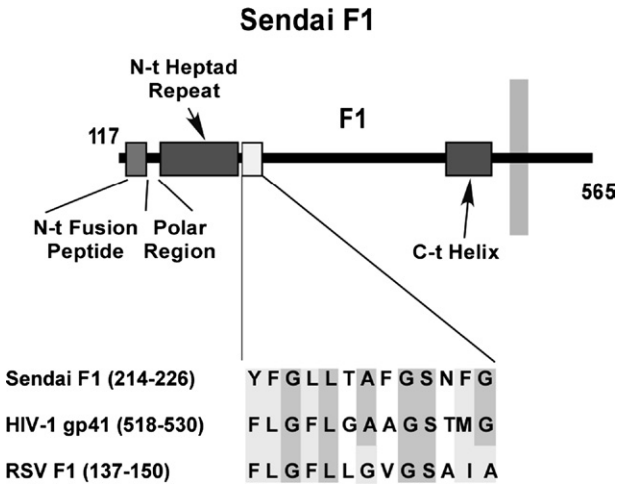

FIGURE 3 Schematic representation of Sendai F1 protein indicating the region that corresponds to the putative internal fusion peptide. As observed, this segment is highly similar to the N-terminal fusion peptides of HIV-1 gp41 (a retroviral protein) and Respiratory Syncytial virus (RSV) F1 (a paramyxoviral protein).

CONCLUSIONS AND PROSPECTS

A major segment within the fusion protein, which is important to its function, is the fusion peptide. The assumptions that the fusion peptide serves as a membrane anchor,

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whereas folding between NHR and CHR regions draws together the virion and cell membranes, are too simple to describe how fusion occurs.

Early studies predicted that a coiled coil structure adjacent to the N-terminal fusion peptide is a common theme in viral fusion protein architecture. The fusogenic interplay between these two subdomains is consistent among different viral families. The HA2 and F-protein subunits of Influenza and Sendai virus fusion proteins, respectively, function similarly to the gp41 subunit of the HIV fusion protein. The NHR of HA2, F protein, and gp41 bind membranes. The NHR acts synergistically with the fusion peptide in inducing membrane fusion for the Sendai F protein and the HIV-1 gp41. In recent years, a more complex picture is emerging for membrane fusion in which multiple regions from the viral fusion protein interact with membranes and synergize with the fusion peptide to induce membrane fusion. Future experiments are needed to determine the role of each region within the fusion protein in facilitating the membrane fusion reaction.

ACKNOWLEDGMENTS

The authors thank Winfried Weissenhorn for supplying the recent 3D structure of gp41. Yechiel Shai has The Harold S. and Harriet B. Brady Professorial Chair in Cancer Research. This study was supported by the Israel Science Foundation and The Minerva Foundation.

REFERENCES

1. Baker KA, Dutch RE, Lamb RA, Jardetzky TS. Structural basis for paramyxovirus-mediated membrane fusion. *Mol Cell* 1999;**3**:309–19.
2. Buzon V, Natrajan G, Schibli D, Campelo F, Kozlov MM, Weissenhorn W. Crystal structure of HIV-1 gp41 including both fusion peptide and membrane proximal external regions. *PLoS Pathog* 2010;**6**:e1000880.
3. Chan DC, Fass D, Berger JM, Kim PS. Core structure of gp41 from the HIV envelope glycoprotein. *Cell* 1997;**89**:263–73.
4. Chen L, Gorman JJ, McKimm-Breschkin J, Lawrence LJ, Tulloch PA, Smith BJ, et al. The structure of the fusion glycoprotein of Newcastle disease virus suggests a novel paradigm for the molecular mechanism of membrane fusion. *Structure* 2001;**9**:255–66.
5. Chernomordik LV, Kozlov MM. Protein-lipid interplay in fusion and fission of biological membranes. *Annu Rev Biochem* 2003;**72**: 175–207.
6. Colman PM, Lawrence MC. The structural biology of type I viral membrane fusion. *Nat Rev Mol Cell Biol* 2003;**4**:309–19.
7. Curtain C, Separovic F, Nielsen K, Craik D, Zhong Y, Kirkpatrick A. The interactions of the N-terminal fusogenic peptide of HIV-1 gp41 with neutral phospholipids. *Eur Biophys J* 1999;**28**:427–36.
8. Dong XN, Xiao Y, Dierich MP, Chen YH. N- and C-domains of HIV-1 gp41: mutation, structure and functions. *Immunol Lett* 2001;**75**: 215–20.
9. Eckert DM, Kim PS. Mechanisms of viral membrane fusion and its inhibition. *Annu Rev Biochem* 2001;**70**:777–810.
10. Epand RM. Fusion peptides and the mechanism of viral fusion. *Biochim Biophys Acta* 2003;**1614**:116–21.
11. Finzi A, Xiang SH, Pacheco B, Wang L, Haight J, Kassa A, et al. Topological layers in the HIV-1 gp120 inner domain regulate gp41 interaction and CD4-triggered conformational transitions. *Mol Cell* 2010;**37**:656–67.
12. Freed EO, Delwart EL, Buchschacher Jr GL, Panganiban AT. A mutation in the human immunodeficiency virus type 1 transmembrane glycoprotein gp41 dominantly interferes with fusion and infectivity. *Proc Natl Acad Sci U S A* 1992;**89**:70–4.
13. Gallo SA, Finnegan CM, Viard M, Raviv Y, Dimitrov A, Rawat SS, et al. The HIV Env-mediated fusion reaction. *Biochim Biophys Acta* 2003;**1614**:36–50.
14. Ghosh JK, Peisajovich SG, Shai Y. Sendai virus internal fusion peptide: structural and functional characterization and a plausible mode of viral entry inhibition. *Biochemistry* 2000;**39**:11581–92.
15. Gordon LM, Curtain CC, Zhong YC, Kirkpatrick A, Mobley PW, Waring AJ. The amino-terminal peptide of HIV-1 glycoprotein 41 interacts with human erythrocyte membranes: peptide conformation, orientation and aggregation. *Biochim Biophys Acta* 1992;**1139**:257–74.
16. Hsu M, Scheid A, Choppin PW. Activation of the Sendai virus fusion protein (f) involves a conformational change with exposure of a new hydrophobic region. *J Biol Chem* 1981;**256**:3557–63.
17. Kliger Y, Aharoni A, Rapaport D, Jones P, Blumenthal R, Shai Y. Fusion peptides derived from the HIV type 1 glycoprotein 41 associate within phospholipid membranes and inhibit cell-cell fusion. Structure-function study. *J Biol Chem* 1997;**272**:13496–505.
18. Martin I, Schaal H, Scheid A, Ruysschaert JM. Lipid membrane fusion induced by the human immunodeficiency virus type 1 gp41 N-terminal extremity is determined by its orientation in the lipid bilayer. *J Virol* 1996;**70**:298–304.
19. Nieva JL, Nir S, Muga A, Goni FM, Wilschut J. Interaction of the HIV-1 fusion peptide with phospholipid vesicles: different structural requirements for fusion and leakage. *Biochemistry* 1994;**33**:3201–9.
20. Peisajovich SG, Epand RF, Pritsker M, Shai Y, Epand RM. The polar region consecutive to HIV-1 fusion peptide participates in membrane fusion. *Biochemistry* 2000;**39**:1826–33.
21. Peisajovich SG, Samuel O, Shai Y. Paramyxovirus F1 protein has two fusion peptides: implications for the mechanism of membrane fusion. *J Mol Biol* 2000;**296**:1353–65.
22. Peisajovich SG, Shai Y. New insights into the mechanism of virus-induced membrane fusion. *Trends Biochem Sci* 2002;**27**:183–90.
23. Pritsker M, Rucker J, Hoffman TL, Doms RW, Shai Y. Effect of nonpolar substitutions of the conserved Phe^{11} in the fusion peptide of HIV-1 gp41 on its function, structure, and organization in membranes. *Biochemistry* 1999;**38**:11359–71.
24. Rafalski M, Lear JD, DeGrado WF. Phospholipid interactions of synthetic peptides representing the N-terminus of HIV gp41. *Biochemistry* 1990;**29**:7917–22.
25. Sackett K, Shai Y. The HIV fusion peptide adopts intermolecular parallel beta-sheet structure in membranes when stabilized by the adjacent N-terminal heptad repeat: a 13C FTIR study. *J Mol Biol* 2005;**350**:790–805.
26. Samuel O, Shai Y. Participation of two fusion peptides in measles virus-induced membrane fusion: emerging similarity with other paramyxoviruses. *Biochemistry* 2001;**40**:1340–9.
27. Skehel JJ, Wiley DC. Coiled coils in both intracellular vesicle and viral membrane fusion. *Cell* 1998;**95**:871–4.
28. Weissenhorn W, Dessen A, Harrison SC, Skehel JJ, Wiley DC. Atomic structure of the ectodomain from HIV-1 gp41. *Nature* 1997;**387**:426–30.

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29. White J, Kielian M, Helenius A. Membrane fusion proteins of enveloped animal viruses. *Q Rev Biophys* 1983;**16**:151–95.
30. White JM. Membrane fusion. *Science* 1992;**258**:917–24.
31. Wilson IA, Skehel JJ, Wiley DC. Structure of the haemagglutinin membrane glycoprotein of influenza virus at 3 A resolution. *Nature* 1981;**289**:366–73.
32. Wimley WC, White SH. Experimentally determined hydrophobicity scale for proteins at membrane interfaces. *Nat Struct Biol* 1996;**3**:842–8.
33. Yang J, Gabrys CM, Weliky DP. Solid-state nuclear magnetic resonance evidence for an extended beta strand conformation of the membrane-bound HIV-1 fusion peptide. *Biochemistry* 2001;**40**:8126–37.
34. Yang J, Prorok M, Castellino FJ, Weliky DP. Oligomeric beta-structure of the membrane-bound HIV-1 fusion peptide formed from soluble monomers. *Biophys J* 2004;**87**:1951–63.
35. Yin HS, Paterson RG, Wen X, Lamb RA, Jardetzky TS. Structure of the uncleaved ectodomain of the paramyxovirus (hPIV3) fusion protein. *Proc Natl Acad Sci U S A* 2005;**102**:9288–93.

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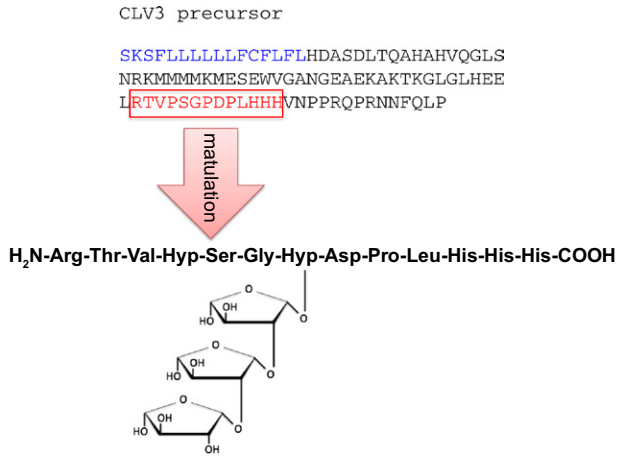

PLATE 1. Structure of CLV3 preprotein and matured peptide. ***(Chapter 1, see page 2).***

PLATE 2. Phenotypes of the ida mutant. (A) In wildtype Arabidopsis plants, the floral organs (petals, sepals, and filaments) abscise shortly after the flower is pollinated (arrow head). (B) In the inflorescence deficient in abscission (ida) mutant, the floral organs remain attached indefinitely to the receptacle of the growing fertilized silique (arrow). ***(Chapter 5, see page 24).***

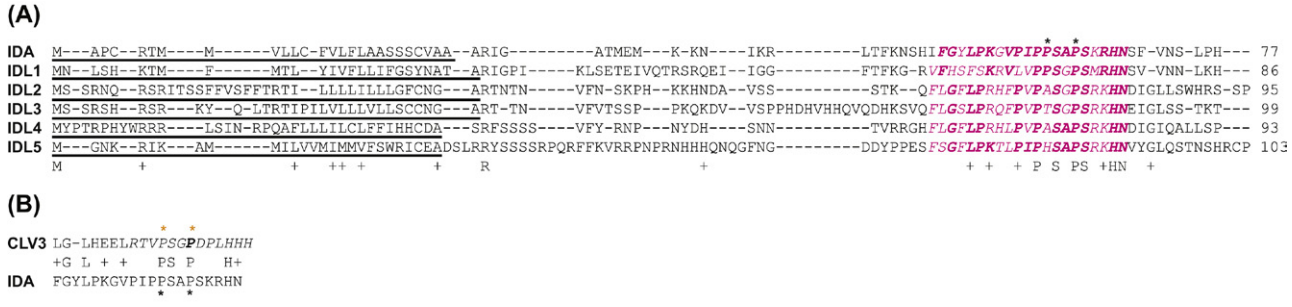

PLATE 3. Alignment of IDA and IDL proteins and similarities to the mature CLV3 Peptide. (A) The IDA and IDL proteins have predicted N-terminal signal peptides (underlined) and a conserved C-terminal EPIP domain (italics in magenta). Amino acids (aa) in the EPIP domains of the IDL proteins identical to the IDA-EPIP domain are in bold italics, asterisks indicate prolines, which are probably hydroxyprolinated in IDA. Identical aa are indicated by letters and conserved aa by (+). (B) The mature CLV3 (MCLV3) peptide, indicated in italics, and some of the upstream aa have similarities to the EPIP domain of IDA. Identical aa are indicated by letters and conserved aa by (+). The MCLV3 peptide has two hydroxyprolines, indicated by orange asterisks, where one is posttranslationally modified with triarabinoside (bold italics). The predicted hydroxyprolines of the IDA-EPIP domain are indicated with black asterisks. ***(Chapter 5, see page 25).***

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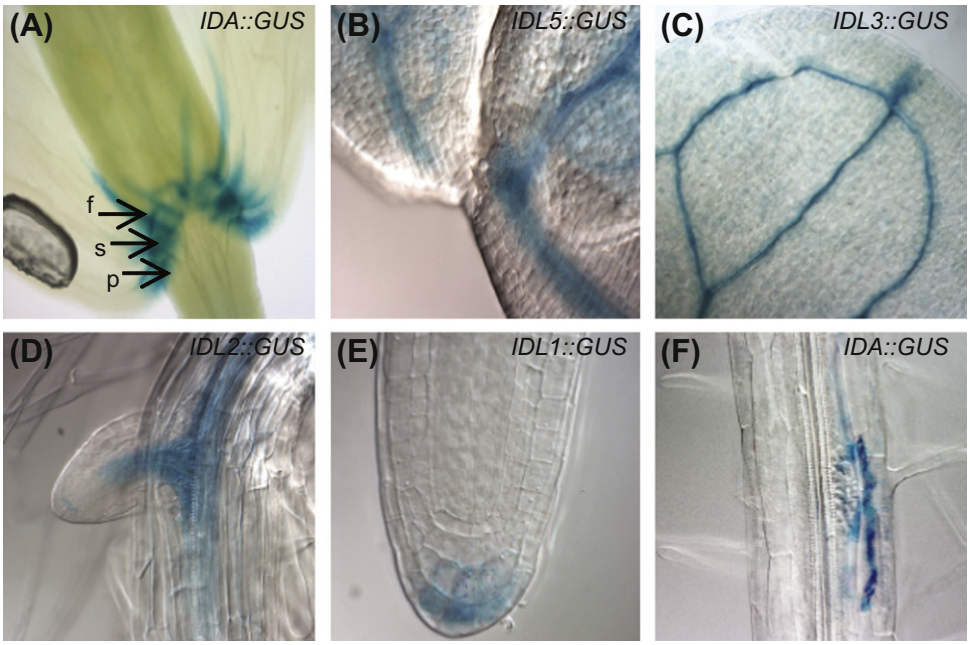

PLATE 4. Promoter:GUS expression of IDA and IDL genes. (A) IDA is expressed in the filament (f), sepal (s), and petal (p) abscission zones during the abscission process. (B) IDL5 expression in the vascular tissue shortly after germination. (C) IDL1, IDL2, IDL3, and IDL4 are all expressed in the vascular tissue of the expanding cotyledon, here IDL3 is shown as a representative example. (D) IDL2, IDL3, and IDL4 are all expressed in the vascular tissue of the main root, and the expression accumulates around the lateral root; here, IDL2 is shown as a representative example. (E) IDL1 expression in the columella cells at tip of the main root. (F) IDA is also expressed in the cortex and epidermal cells of the main root during lateral root emergence. ***(Chapter 5, see page 27).***

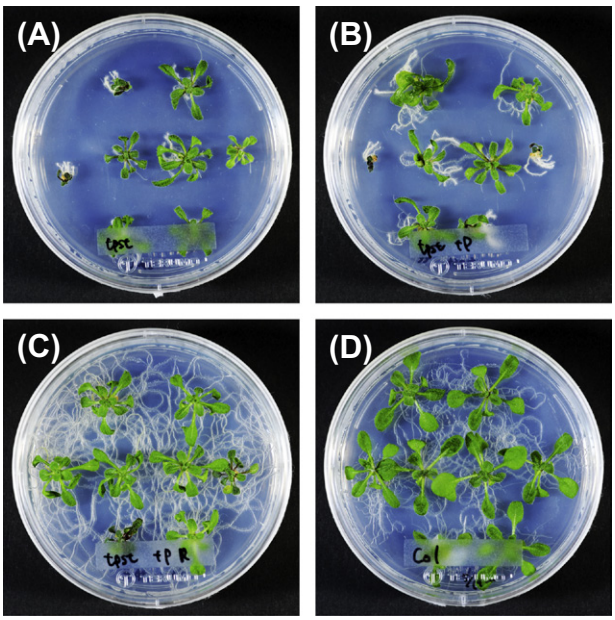

PLATE 5. Effect of RGF1 on root phenotypes of tpst-1. Photographs of tpst-1 seedlings grown in the absence (A) or the presence of PSK (100 nM) (B) or both PSK (100 nM) and RGF1 (100 nM) (C) 16 days after germination. PSK is required for full activity of RGF1. (D) Wild-type seedlings as a control. ***(Chapter 10, see page 51).***

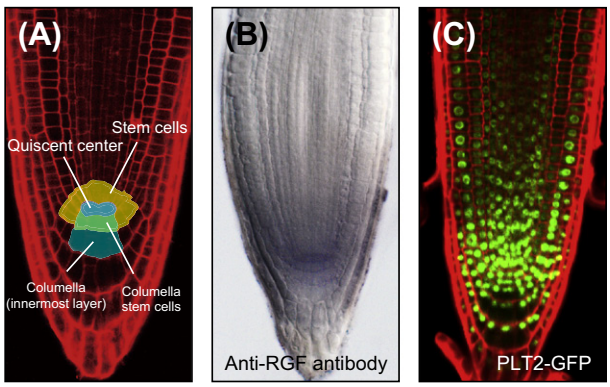

PLATE 6. RGF peptides define expression levels and patterns of PLTs. (A) Schematic representation of the representative cell types present in the Arabidopsis root meristem. (B) Distribution of RGF peptides in the root meristem visualized by whole-mount immunostaining using anti-RGF antibody. (C) Expression pattern of PLT2-GFP protein showing gradient distribution with maxima in the stem cell area. ***(Chapter 10, see page 52).***

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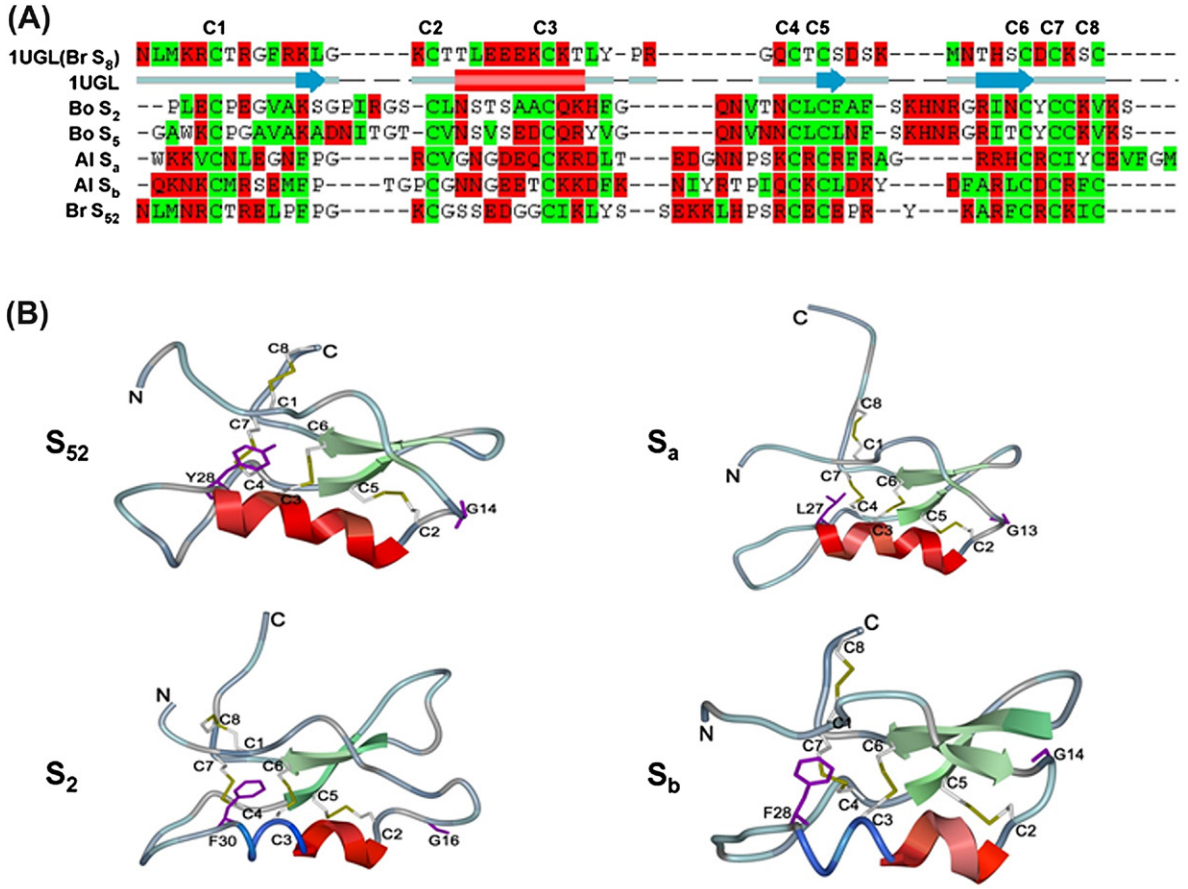

PLATE 7. Predicted conservation of secondary structure in SCR variants. (**A**) Structure-based sequence alignment of SCR variants from B. rapa (Br) S52, B. oleracea (Bo) S2 and S5, and A. lyrata (Al) Sa and Sb. The NMR structure (model 1) of the B. rapa S8 variant13 (PDB code: 1UGL) was used as template. The secondary structure of the experimental model was computed by the method of Kabasch and Sanders.5 The rectangle in the "1UGL" line depicts the α-helix and the arrows show the β-strands. Hydrophilic residues are shaded in dark gray and hydrophobic residues are shaded in light gray. The conserved C1–C8 residues are shown above the alignment. (**B**) Three-dimensional (3D) models of the SCR variants encoded by the Brassica S52 and S2 and the A. lyrata Sa, and Sb haplotypes. These predicted models were generated with the program MODELLER5 based on the S8 SCR NMR structure (PDB code: 1UGL) determined by Mishima et al.13 and the alignments given in A. The 3D models show the N/C termini and the location of the conserved cysteine residues (C1–C8) and glycine (G) in the G x C2 motif. Lines connecting pairs of cysteines depict the disulfide bridges. The α-helix is shown as a dark gray coiled ribbon and β-sheets are depicted by light gray arrows. The aromatic residue in the C3xxxY/F motif found in most SCRs is shown in stick representation for SCR variants of S52 (Y28), S2 (F30), and Sb (F28). SCRa contains a lysine (L27) at the corresponding position. ***(Chapter 12, see page 62).***

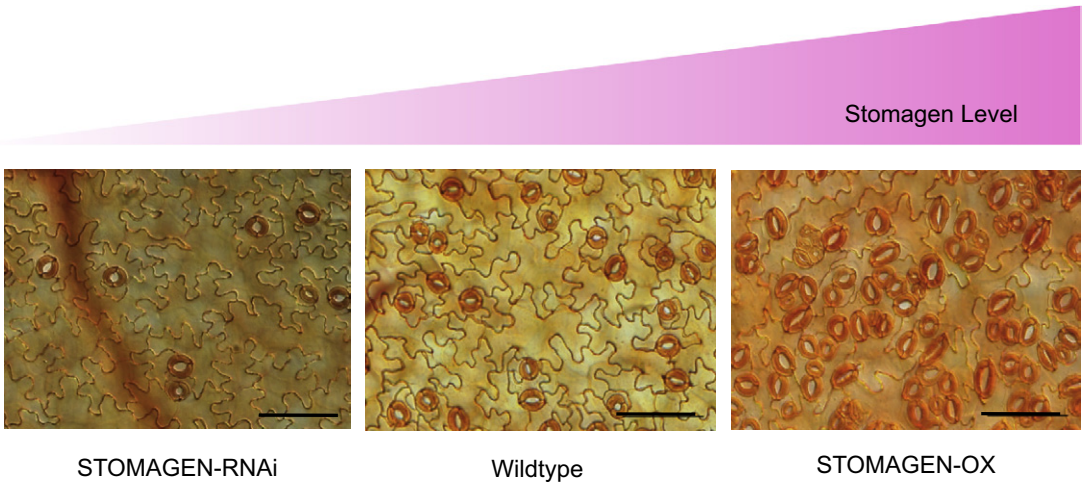

PLATE 8. Stomagen/EPFL9 positively regulates stomatal density. Differential interference contrast (DIC) images of the abaxial epidermis of mature leaves of wild-type Arabidopsis, a STOMAGEN-silenced line (STOMAGEN-RNAi), and a STOMAGEN-overexpressing line (STOMAGEN-OX). The expected accumulation level of stomagen is illustrated above panels. Stomata were stained with Safranin-O. Scale bars = 100μm. ***(Chapter 13, see page 69).***

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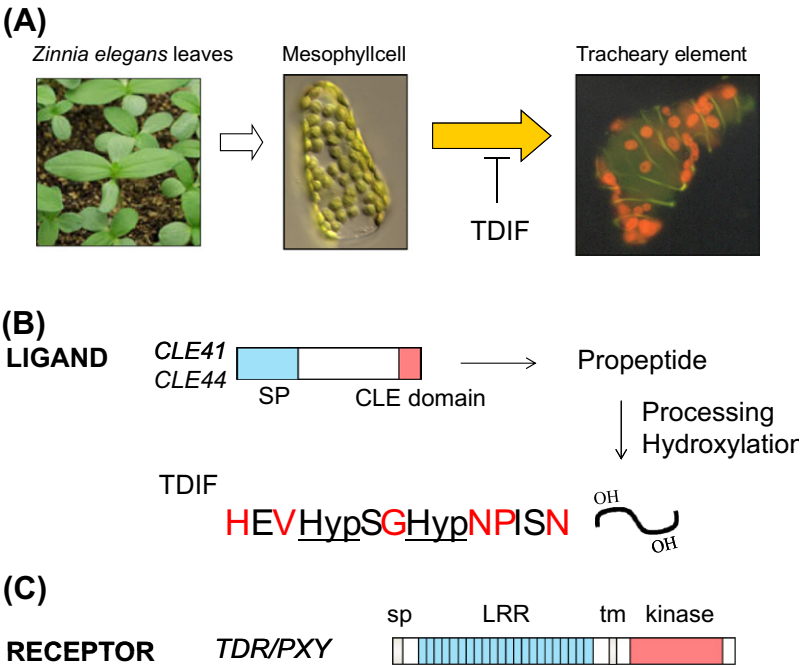

PLATE 9. Identification and characterization of TDIF. (A) TDIF was isolated as a factor inhibiting TE differentiation from the Zinnia xylogenic culture. (B) Maturation of TDIF peptide through translation as a propeptide, processing, and hydroxylation. TDIF is produced as a 12-amino acid peptide with two hydroxyproline residues. Bold characters indicate indispensable amino acids for TDIF activity. (C) Structure of TDR/PXY encoding a TDIF receptor, which consists of a signal peptide (sp), leucine-rich-repeat domains (LRR), a transmembrane domain (tm), and a kinase domain. ***(Chapter 14, see page 72).***

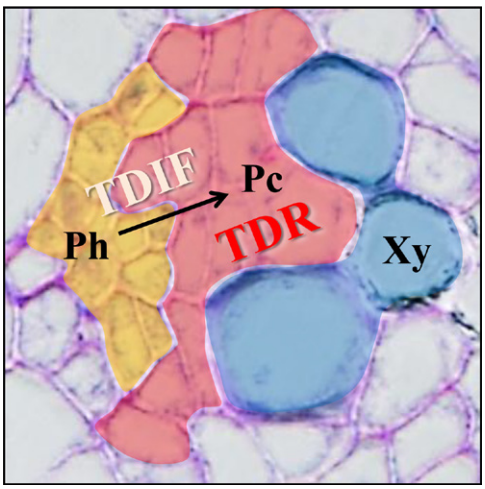

PLATE 10. Non-cell-autonomous function of the TDIF signal TDIF is secreted from phloem cells and functions in procambial cells in a non-cell-autonomous fashion. Ph, Pc, and Xy indicate phloem, procambium, and xylem, respectively. ***(Chapter 14, see page 72).***

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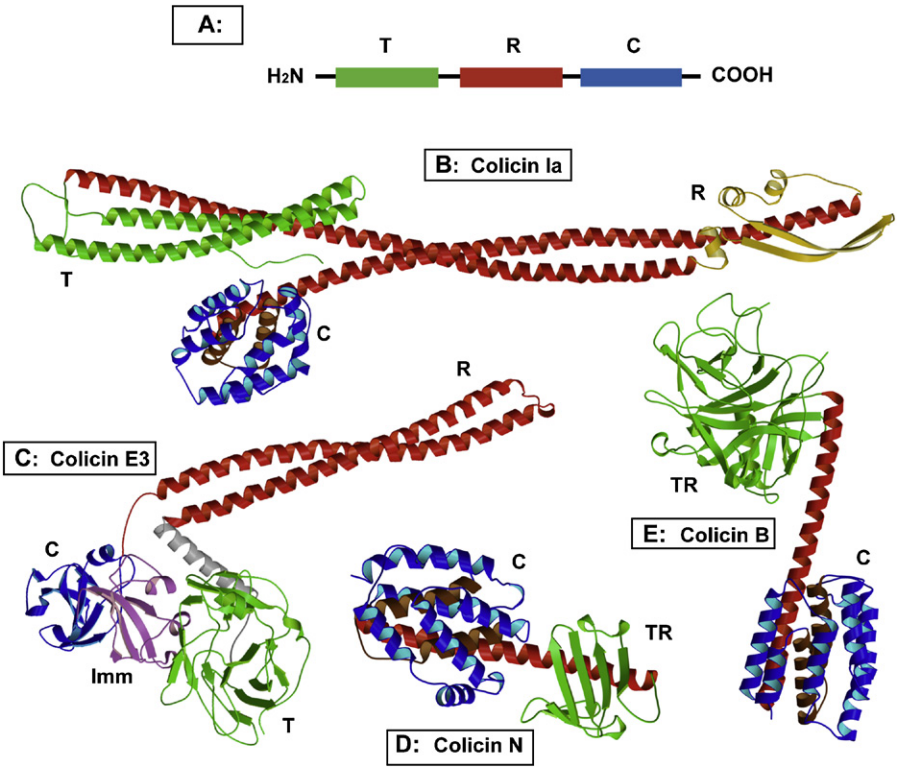

PLATE 11. (A) Generic domain structure of colicin polypeptides (top): T, translocation; R, receptor binding; C, catalytic or activity containing domain (B – E). Ribbon diagrams of structures of pore-forming colicins, Ia **(B)** , B **(E)** , and N **(D)** , and of rRNase colicin E3 **(C)** . Note the prominent long (210 and 100 Å in colicins Ia and E3) coiled-coil structure of the R-domain. Structures, predominantly a-helical, have also been obtained of the C-domains of (i) colicins A and E1 by X-ray diffraction and (ii) of E7 and E9 by solution NMR. The structures of E3 and E7 contain bound immunity protein, as is characteristic of the nuclease colicins. ***(Chapter 17, see page 94).***

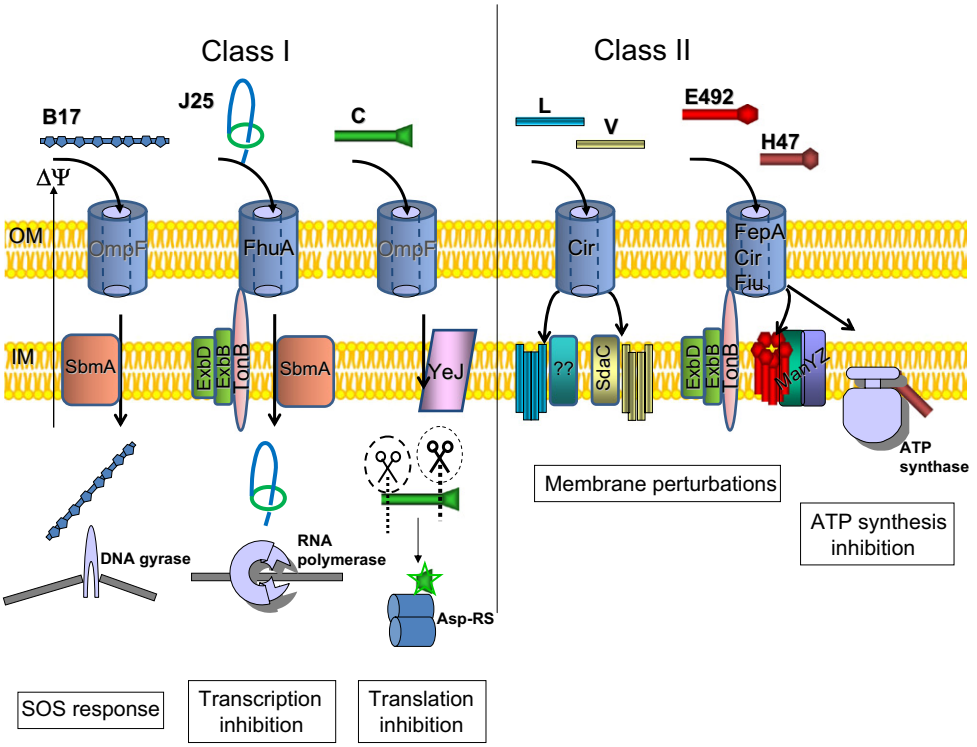

PLATE 12. Synthetic scheme of the mechanisms of action of microcins of classes I and II. ***(Chapter 20, see page 133).***

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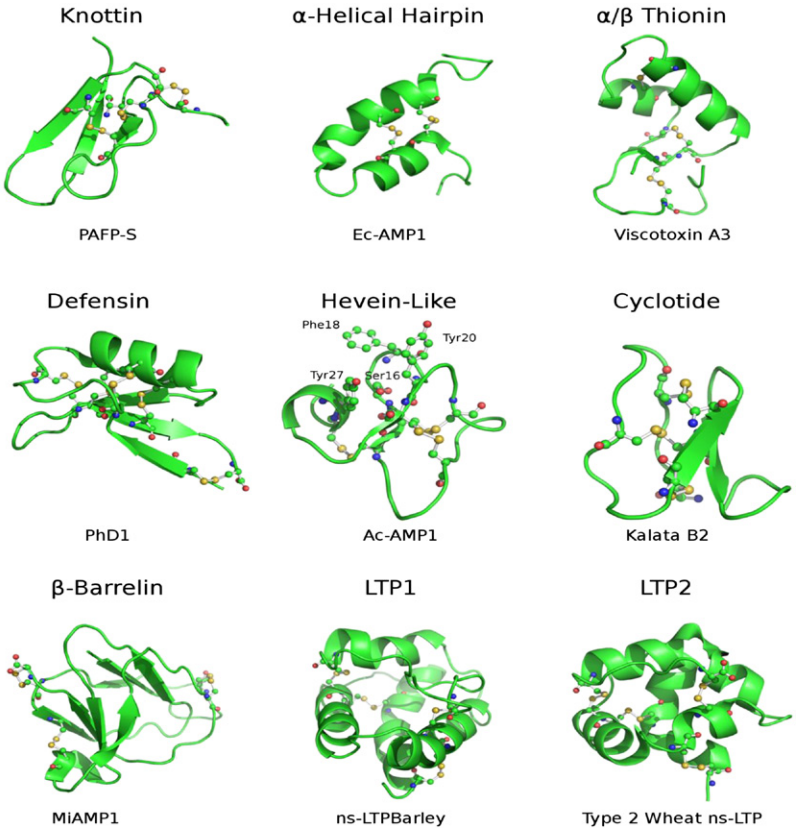

PLATE 13. Three-dimensional structures of different antifungal plant peptide families. Disulfide bridges are shown as ball and stick. In hevein-like peptides, the active residues are also shown as ball and stick and labeled. ***(Chapter 26, see page 172).***

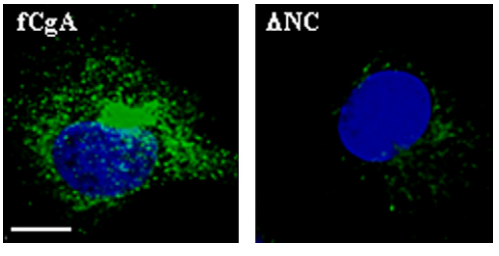

PLATE 14. Granulogenic activity of frog CgA. COS7 cells transfected with the cDNA encoding full-length CgA (fCgA) or CgA deleted in its N- and C-terminal parts (ΔNC) were incubated with a rabbit antiserum directed against frog CgA (CgA107–234) and examined for fCgA immunoreactivity using confocal microscopy. Scale bars, 10 μm. ***(Chapter 49, see page 346).***

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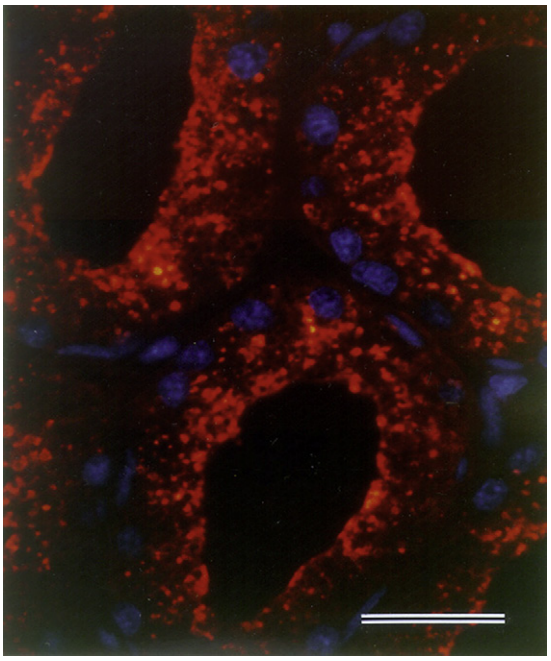

PLATE 15. Immunofluorescence micrograph showing the localization of sodefrin in the abdominal gland of a red-bellied newt. The sodefrin antibody labels the epithelial cells red with rhodamine. Nuclear DNA is stained blue with 4′ 6-diamino-2-phenylindole. The bar represents 50μm. From Ref. 16. Copyright 2000 by the Zoological Society of Japan. ***(Chapter 54, see page 385).***

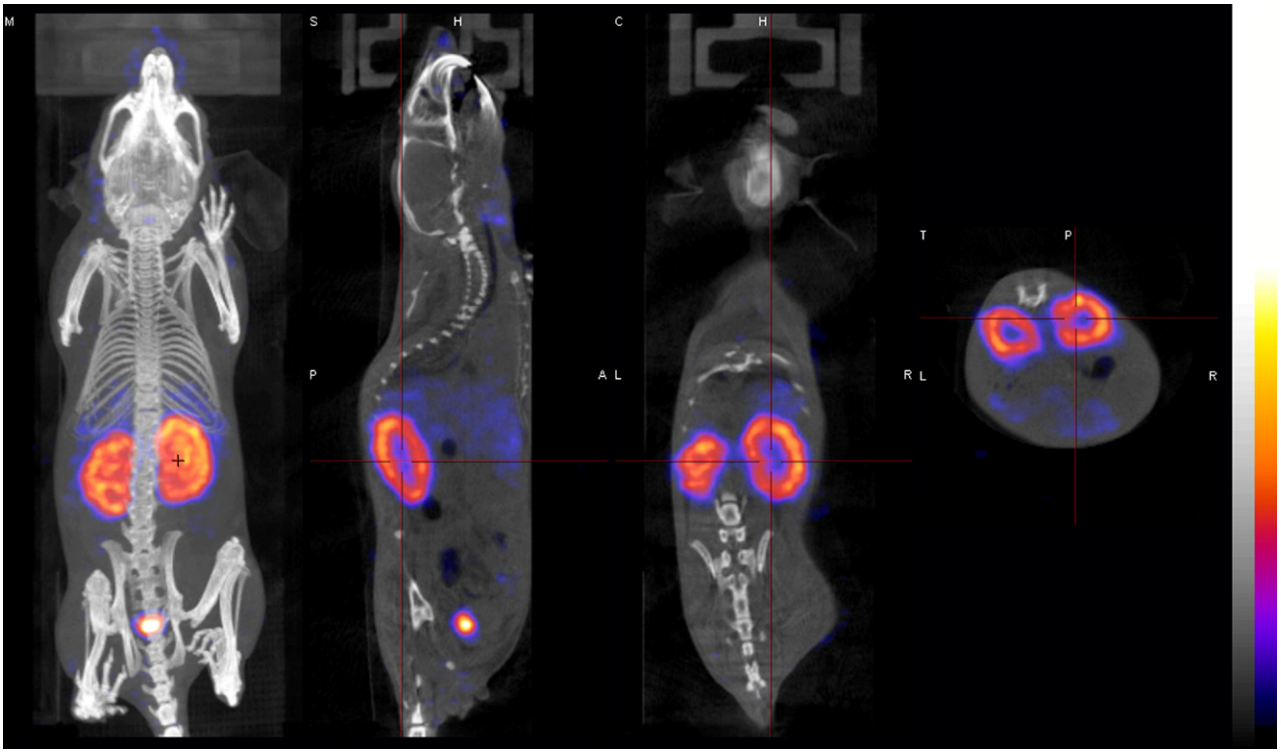

PLATE 16. *In vivo* tomographic whole-body imaging of [^{125}I]-Tyr-maurocalcine biodistribution in CD-1 mice 15 min tail i.v. post-injection of 37 MBq. From the left to the right, 3D rendering, sagittal, coronal, and transverse views of tracer activity. ***(Chapter 57, see page 414).***

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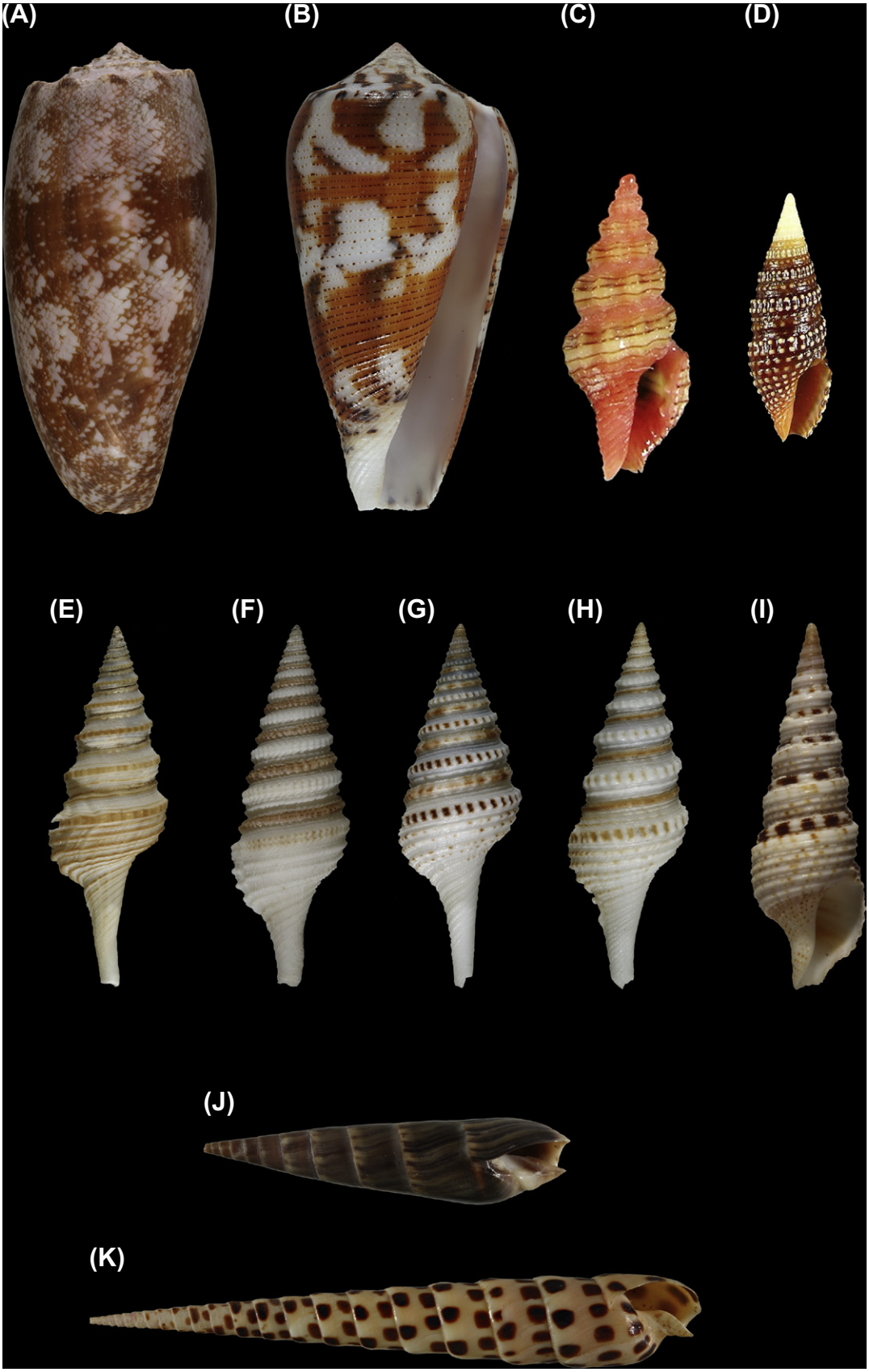

PLATE 17. Shells of venomous marine snails from which peptides have been characterized. As described in the text, four classes of venom peptides have been characterized. Best known are **conopeptides** ("conotoxins") that come from the venoms of cone snails such as the geography cone, Conus geographus (A) and the magician's cone, Conus magus (B). Other classes of venom peptides include **clathurellipeptides** from clathurellid conoideans such as the recently described Lienardia totopotens, Rosenberg, 2011(C); **crassipeptides** from crassispirid snails such as Crassispira cerithina (D); **turripeptides**, from the family Turridae such as Gemmula speciosa (E), Gemmula diomedea (F); Gemmula kieneri (G), Gemmula sogodensis (H), and Lophiotoma olangoensis (I), **augerpeptides** from species in the family Terebridae such as Hastula impages (J), and Terebra subulata (K). ***(Chapter 61, see page 438).***

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PLATE 18. The gigantic Antarctic heteronemertine Parbolasia corrugatus and several adult starfish with diameters of approximately 30cm. ***(Chapter 65, see page 485).***

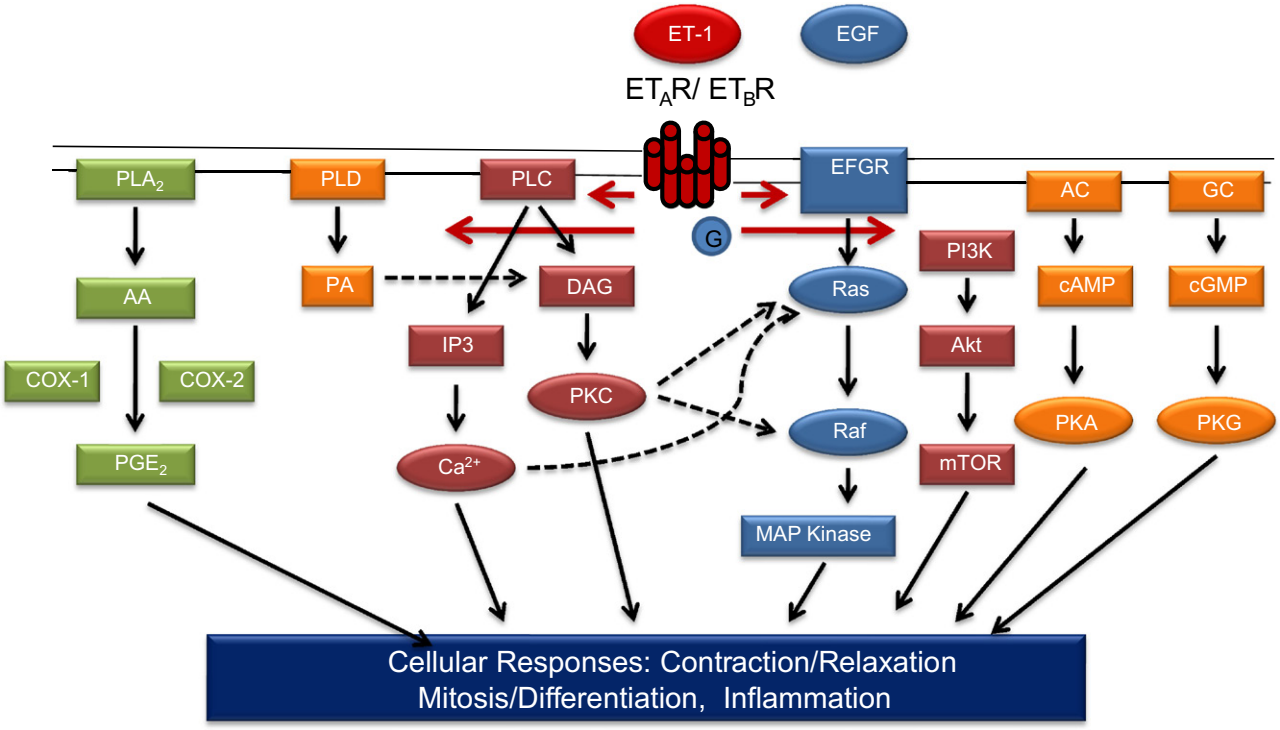

PLATE 19. Schematic representation of the signal transduction pathways associated with stimulation of the Endothelin receptors. Upstream transduction molecules activated (phosphorylated) by the G-protein are shown at the top of each pathway and include the Epidermal Growth Factor Receptor (EGFR), Phospholipase A2, C, D (PLA2, PLC, PLD), Adenylate Cyclase (AC), Guanylate Cyclase (GC), and Phosphoinositide 3-Kinase (PI3K). Downstream effectors include phosphatidyl inositol 3 kinase (IP3), diacylglycerol (DAG), phospholipase A (PA), cyclic GMP (cGMP), protein kinase A, C, G (PKA, PKC, PKG), Akt, mTOR, cyclooxygenase 1, 2 (COX-1, COX-2), and prostaglandin E2 (PGE2). ***(Chapter 70, see page 513).***

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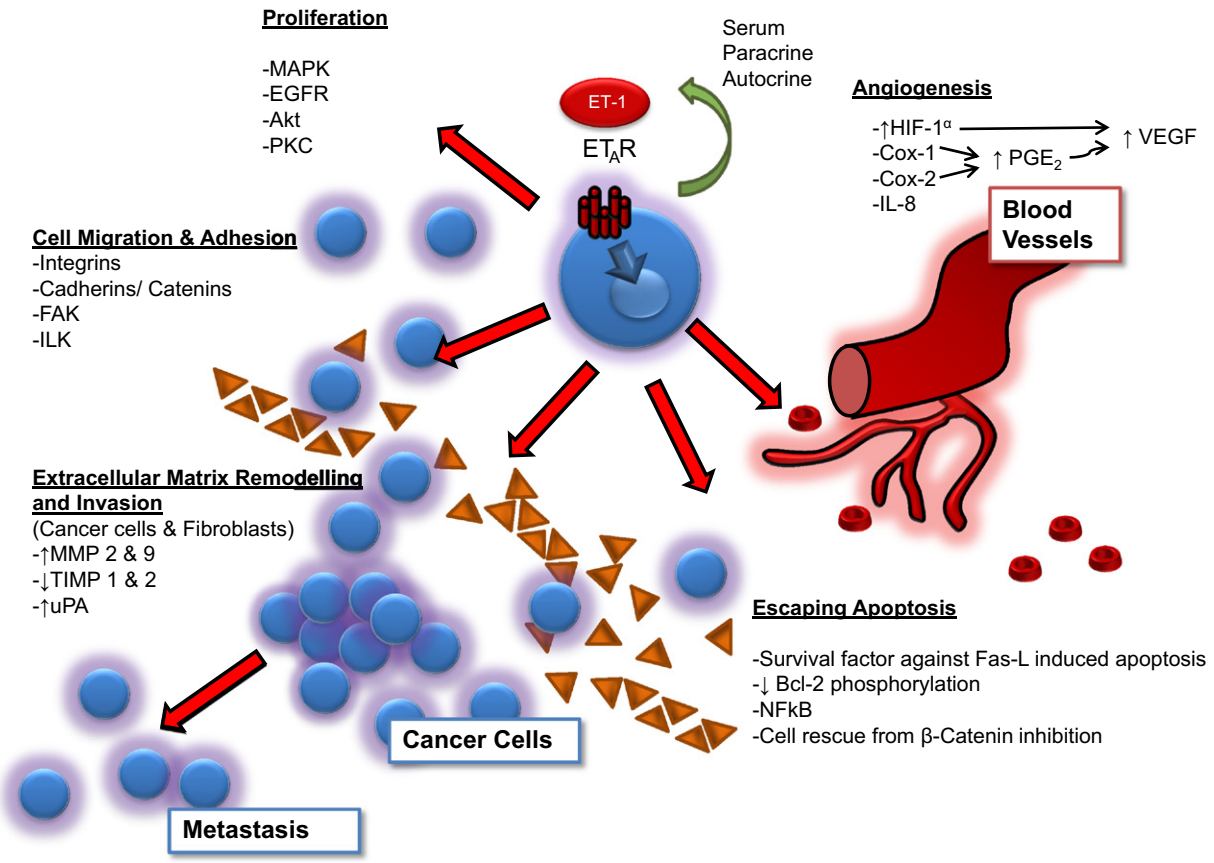

PLATE 20. Summary of the role of ET-1 in the spread of cancer. ***(Chapter 70, see page 514).***

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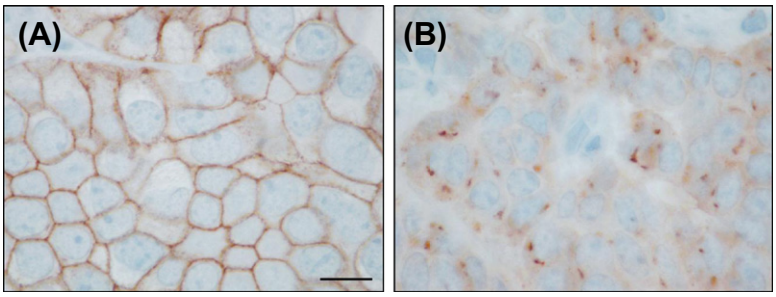

PLATE 21. Immunohistochemical detection of somatostatin receptors in human tumors using the sst2A-specific antibody UMB-1. A shows the membrane-bound receptors in an neuroendocrine tumor from an octreotide-naive patient, B shows internalized sst2A in a tumor from an octreotide-treated patient. Bar = 0.01 mm. ***(Chapter 75, see page 551).***

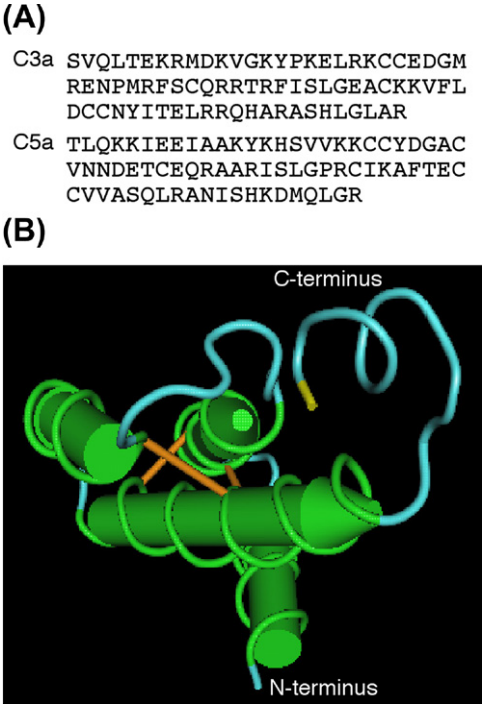

PLATE 22. Structure of anaphylatoxins. (A) Primary sequences of human C3a (NCBI accession no. = P01024) and C5a (NCBI accession no. = P01032). (B) The tertiary structure of human C5a. Green, α-helices; blue, N-terminus, the three loops connecting the α-helices, and the C-terminal tail; brown, disulfide bonds; yellow, C-terminal arginine residue. ***(Chapter 85, see page 627).***

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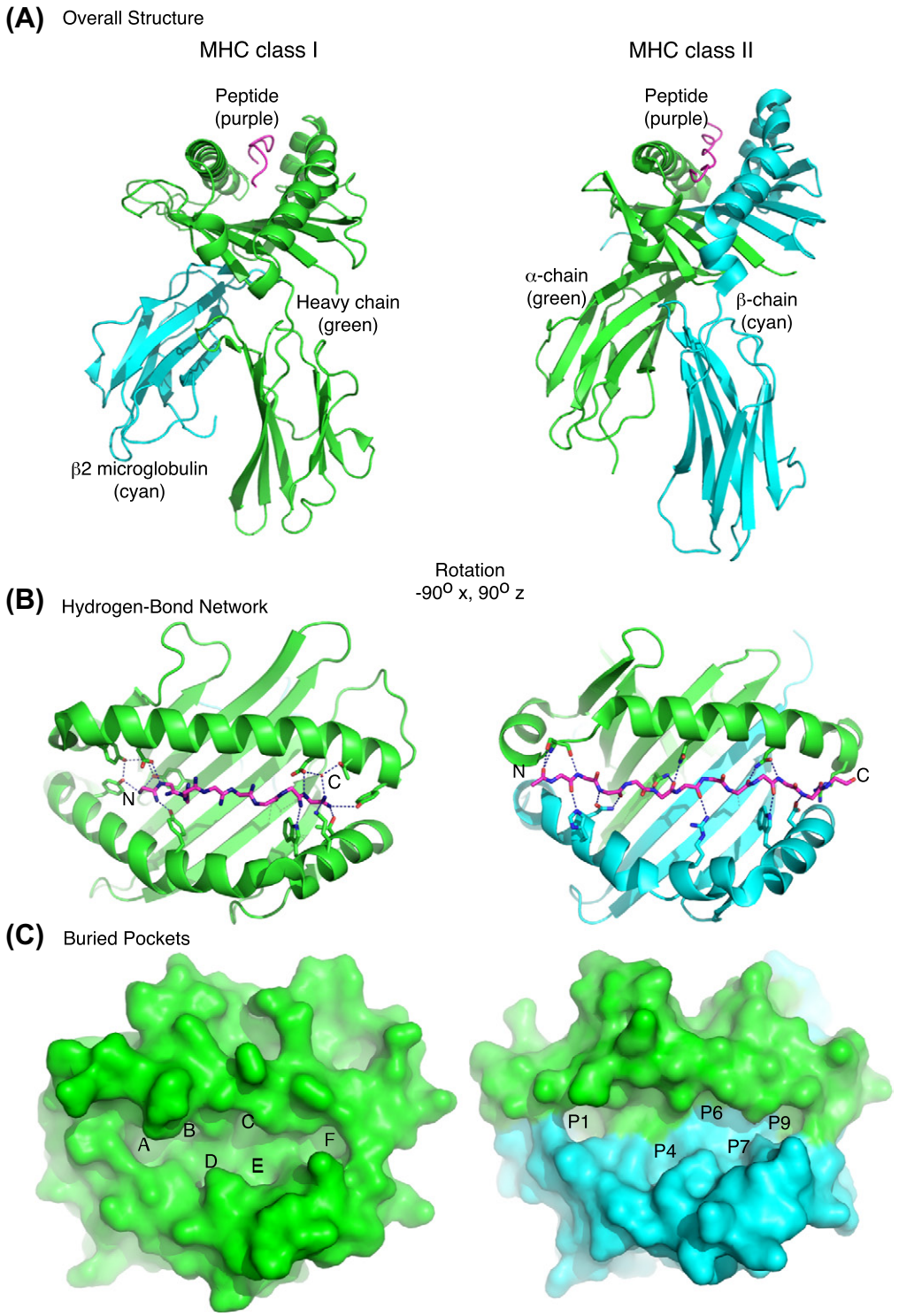

PLATE 23. (A) Side view of MHC-class I (left) and MHC-class II (right) showing the peptide-binding site at the top and the membrane proximal immunoglobulin domains at the bottom. (B) Looking down on the peptide-binding site, which is formed by α-helices that flank the peptide and supported by an eight-strand β-sheet. Conserved hydrogen bonds that stabilize the peptide backbone are indicated as dashed lines in each structure. Note that in MHC-class II, the α- and β-chains together combine to form the groove, and peptide extends out of the peptide-binding site. The peptide N- and C-termini are indicated with N and C respectively. (C) The same view as (B), but in surface rendering with the peptide removed. The pockets that serve as anchor points for peptide side chains are labeled A–F for MHC-class I and P1–P9 for MHC-class II. PBD files 1S9Y and 1DLH were used to generate this figure. ***(Chapter 93, see page 689).***

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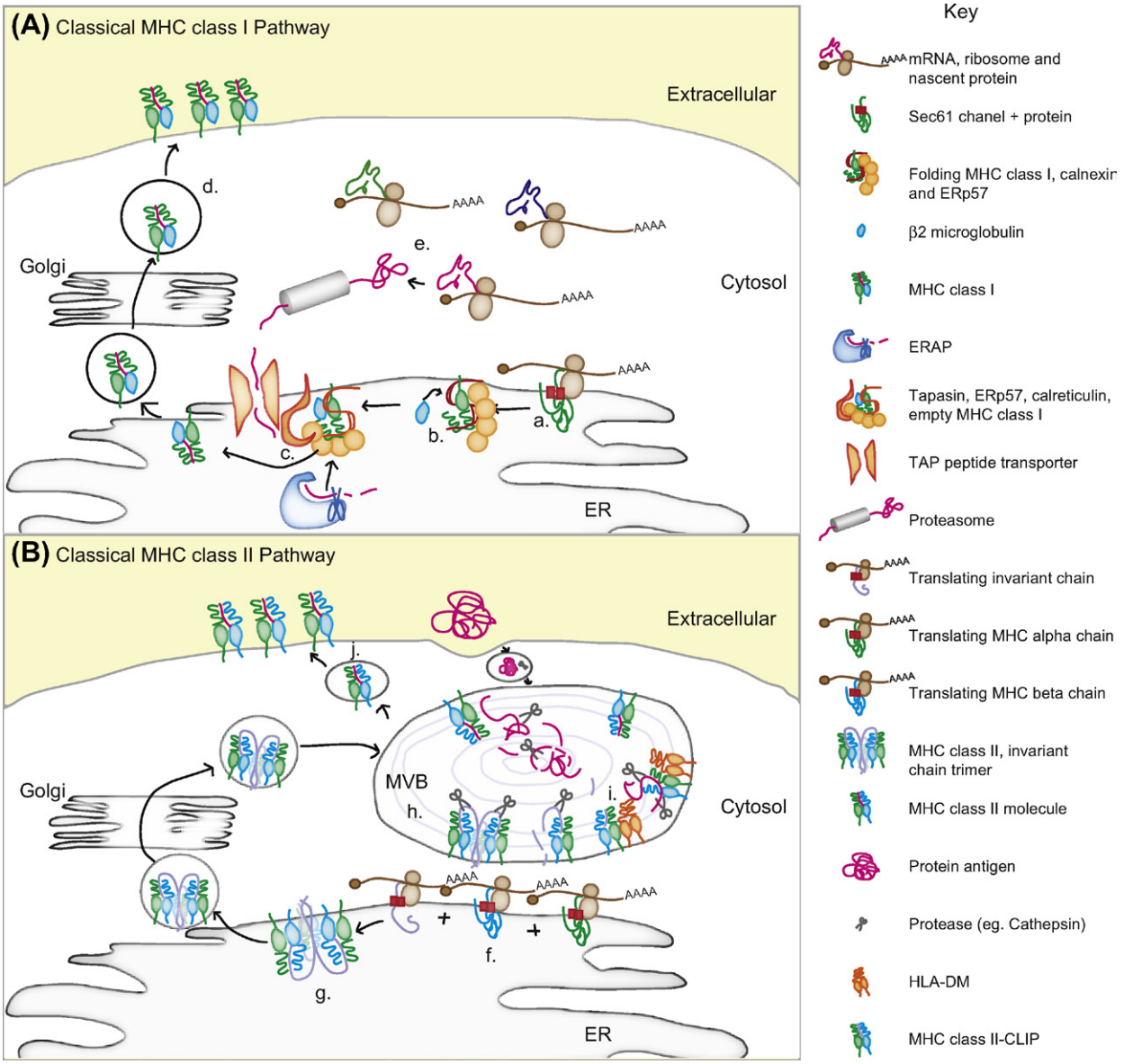

PLATE 24. (A) MHC-class I heavy chains are synthesized in the ER (a) and then partially folded with assistance from calnexin and ERp57 (b). After β2 microglobulin joins the MHC-class I heavy chain the heterodimer is incorporated into the peptide-loading complex and loaded with peptides transported from the cytoplasm to the ER lumen by TAP and trimmed to the correct size by ERAP family proteases (c). Peptide-loaded MHC-class I is then transported via the Golgi to the cell surface (d). Proteasomes cleave proteins in to peptides, which can be selected by TAP for transport into the ER (e). (B) MHC-class II chains and invariant chain are synthesized in the ER (f) and fold together into a nonameric complex with 3 invariant chains, and 3 MHC-class II molecules (g). A region of the invariant chain (CLIP) occupies the peptide-binding groove of MHC-class II. This complex is exported via the Golgi to multivesicular bodies in the endocytic compartment (h). Proteolytic cleavage of invariant chain occurs to release MHC-class II molecules and allow HLA-DM mediated release of CLIP and replacement with peptides from endocytozed proteins (i). MHC-class II molecules are transported to the cell surface for display to $CD4^+$ T-cells (j). ***(Chapter 93, see page 690).***

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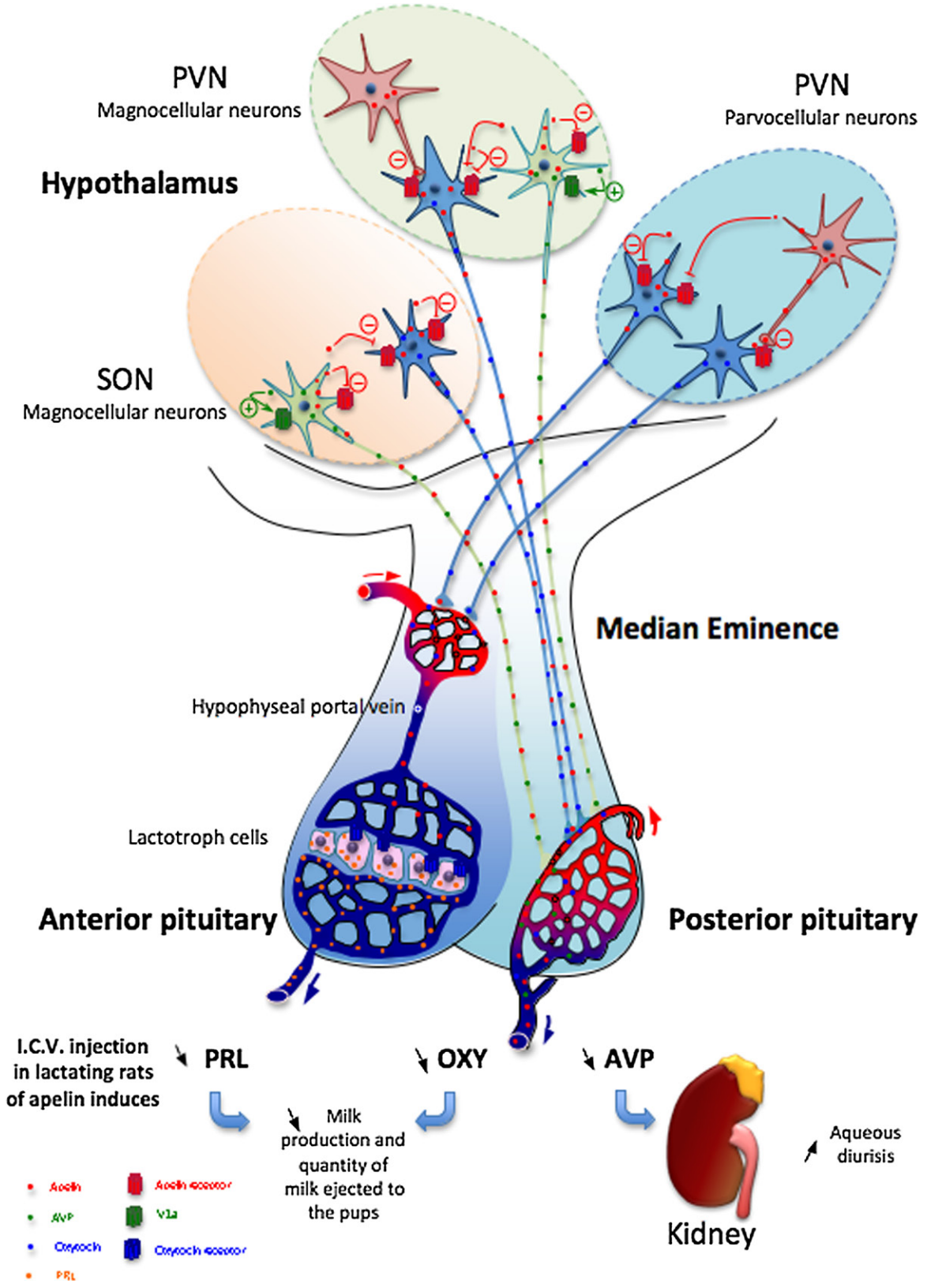

PLATE 25. Putative schema of apelin of the interactions with AVP and oxytocin. In lactating rats, apelin and its receptor are colocalized with AVP and oxytocin in the SON and PVN magnocellular neurons. Apelin and its receptor are also colocalized with oxytocin in parvocellular neurons. ICV administration of apelin inhibits the phasic electrical activity of AVP and oxytocin neurons. The decrease of AVP neuron activity results in a decrease of AVP secretion into the bloodstream and thereby the increase of aqueous diuresis at the kidney level. In contrast, the decrease of oxytocin neuron activity in magnocellular neurons and in parvocellular neurons would certainly decrease the secretion of oxytocin and PRL in the bloodstream in the anterior pituitary respectively. Adapted from De Mota et al.[7] and Bodineau et al.[2] ***(Chapter 96, see page 719).***

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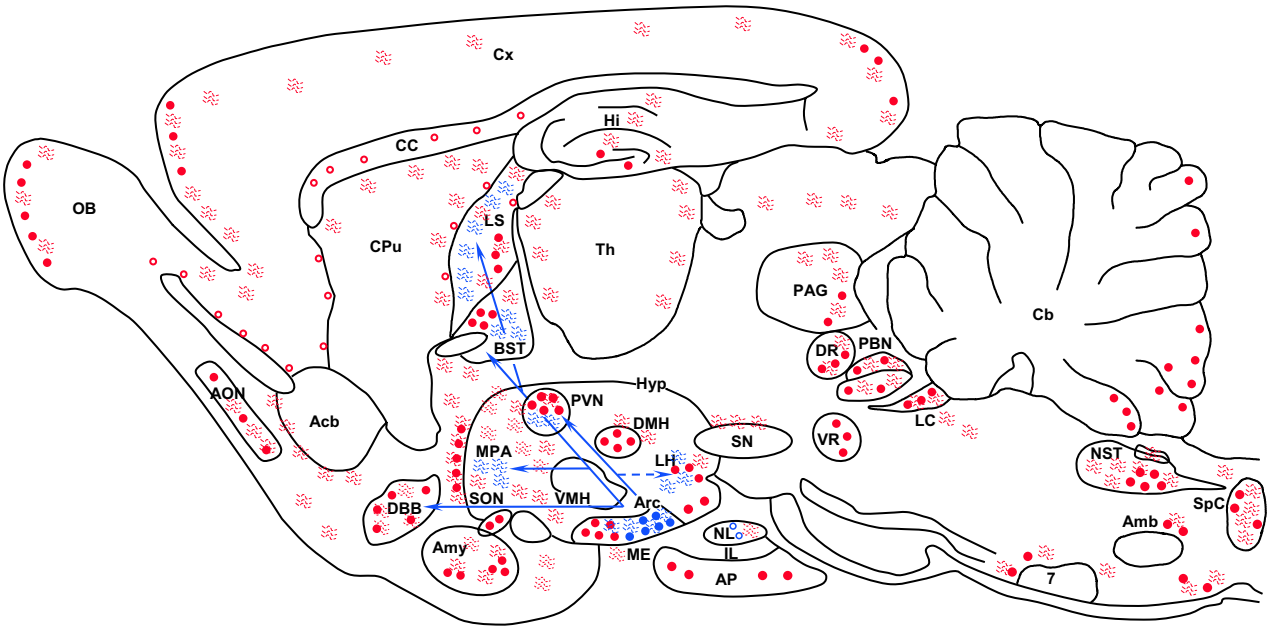

PLATE 26. Schematic illustration of the contrasting distribution of galanin and GALP positive neurons and their innervations in rat brain. Cells that express mRNA (and immunoreactivity) for galanin (black dots) and GALP (grey dots) are illustrated, along with the approximate localization of their immunoreactive nerve fibers/terminals (small black and grey lines). Non-neuronal GALP expression in astrocytes (pituicytes; grey circles) and galanin expression in oligodendrocyte and other progenitors (black circles) is also illustrated. The two major GALP pathways proposed from the arcuate nucleus to the paraventricular nucleus and to other hypothalamic and limbic regions are indicated by grey arrows. The relative topography of the many, widespread galanin pathways has been omitted for clarity. ***(Chapter 103, see page 767).***

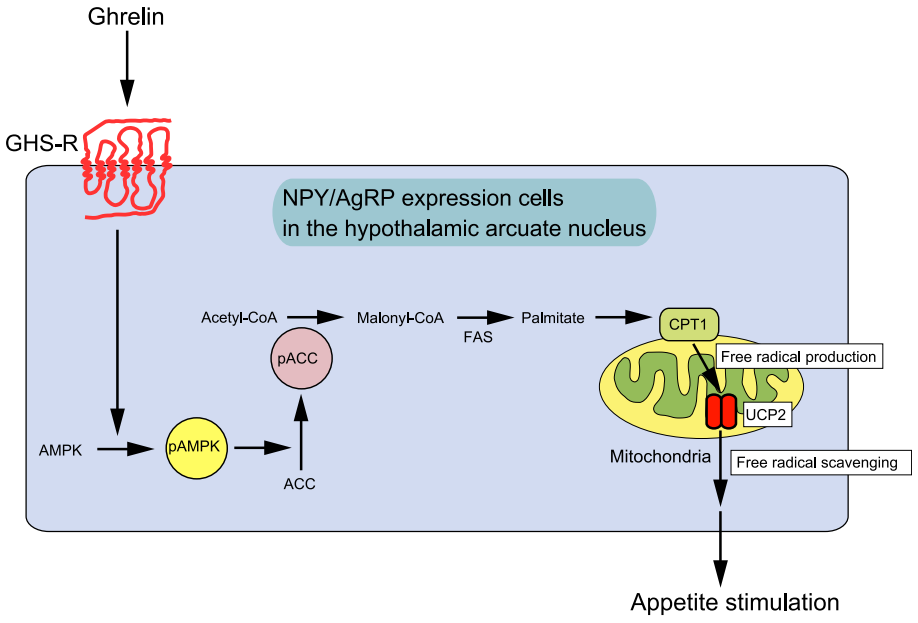

PLATE 27. Intracellular action of ghrelin in NPY/AgRP-expression cells in the hypothalamic arcuate nucleus. In NPY/AgRP-expression cells in the hypothalamic arcuate nucleus, ghrelin stimulates AMP-K and regulates fatty acid metabolism, leading to CPT1 activation. Ghrelin's effects on mitochondrial respiration are dependent on the function of UCP2 and hence result in an increase of food intake. ***(Chapter 104, see page 780).***

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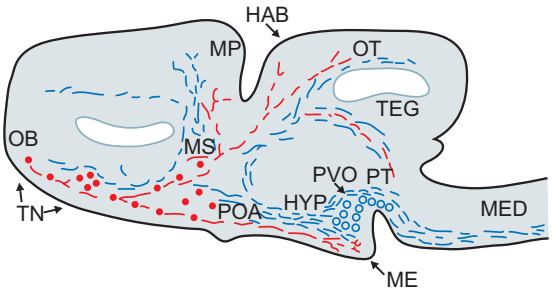

PLATE 28. GnRH-immunoreactive neuronal systems in an amphibian Taricha granulosa. Mammalian GnRH antiserum labeled cell bodies (red circles) in the anterior forebrain, including terminal nerve, medial septum, and anterior preoptic area, and a restricted set of projections to the medial pallium, habenula, and median eminence (red lines). GnRH II antiserum-labeled cell bodies (blue circles) in the paraventricular organ and posterior tubercle and a diffuse system of fibers to most brain area (blue lines). Abbreviations: HAB, habenula; HYP, hypothalamus; ME, median eminence; MED, medulla; MP, medial pallium; MS, medial septum; OB, olfactory bulb; OT, optic tectum; POA, preoptic area; PT, posterior tubercle; PVO, paraventricular organ; TEG, midbrain tegmentum; TN, terminal nerve. Adapted from Muske.[17] ***(Chapter 106, see page 795).***

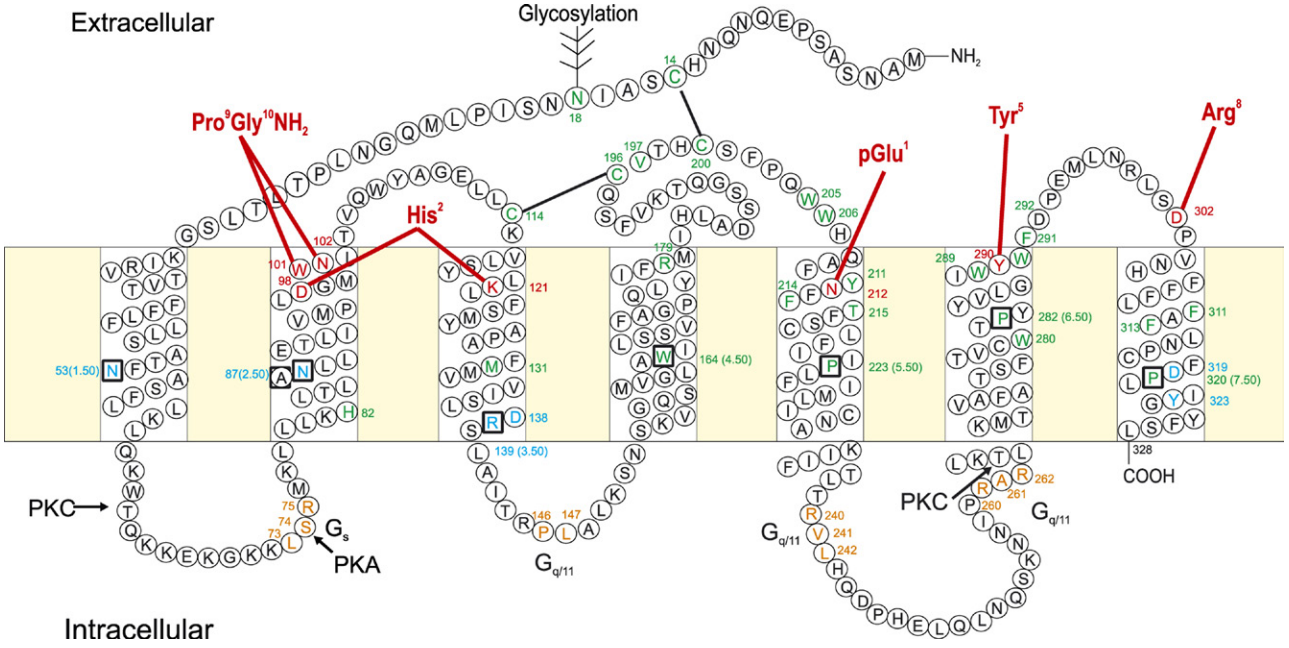

PLATE 29. Two-dimensional representation of the human GnRH receptor: the 7-(TM) α-helical domains (boxed) are connected by three extracellular loops (ECLs) and three intracellular loops (ICLs). Residues in squares are ones that are highly conserved throughout the rhodopsin family of GPCRs. Ligand-binding residues (red) and residues believed to be important in receptor structure or binding pocket configuration (green) are shown. These include residues involved in disulfide bond formation (black lines) and a glycosylation site. Residues involved in receptor activation are shown in blue. Residues involved in coupling to G proteins are shown in orange. Putative PKC and protein kinase A (PKA) phosphorylation sites are indicated. The intermolecular interactions between GnRH I residues and the receptor are indicated with red lines. Residues shown to alter selectivity for relative binding affinities of GnRH I and GnRH II are shown in bold circles. Figure modified from Ref. 12 with permission. ***(Chapter 106, see page 796).***

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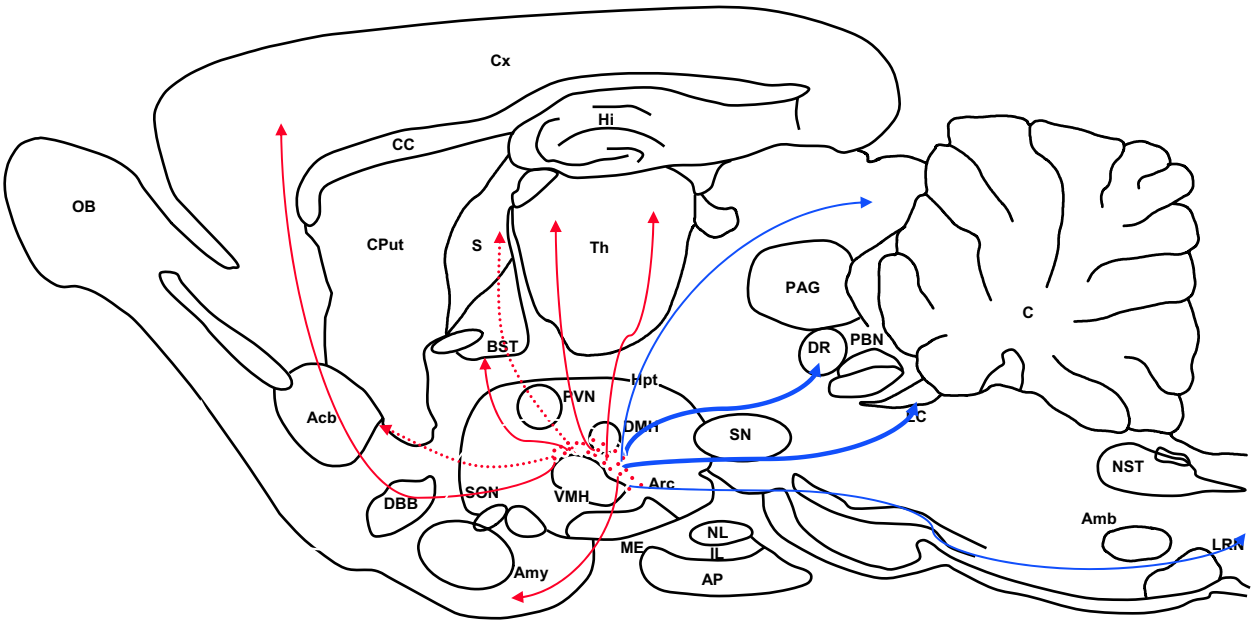

PLATE 30. Schematic of the distribution of Hcrt projections originating in the lateral hypothalamus. A very dense intrahypothalamic network of Hcrt fibers can be detected by immunohistochemistry in the DMH, VMH, Arc and PVN. Another set of fibers can be followed along the medial thalamus (Th). An ascending projection connects Hcrt with the ventral forebrain and amygdalar structures (Amy) and diffuse sparse innervation of neocortical areas (Cx). Descending efferent areas include the main monoaminergic nuclei such as the noradrenergic locus coeruleus (LC), serotonergic median and dorsal raphe (DR) and dopaminergic fibers passing through the ventral tegmental area. A direct monosynaptic connection to the spinal cord has also been described. DMH-dorsomedial hypothalamus; VMH- ventromedial hypothalamus; Arc- Arcuate nucleus; PVN- Paraventricular hypothalamic nucleus. ***(Chapter 108, see page 813).***

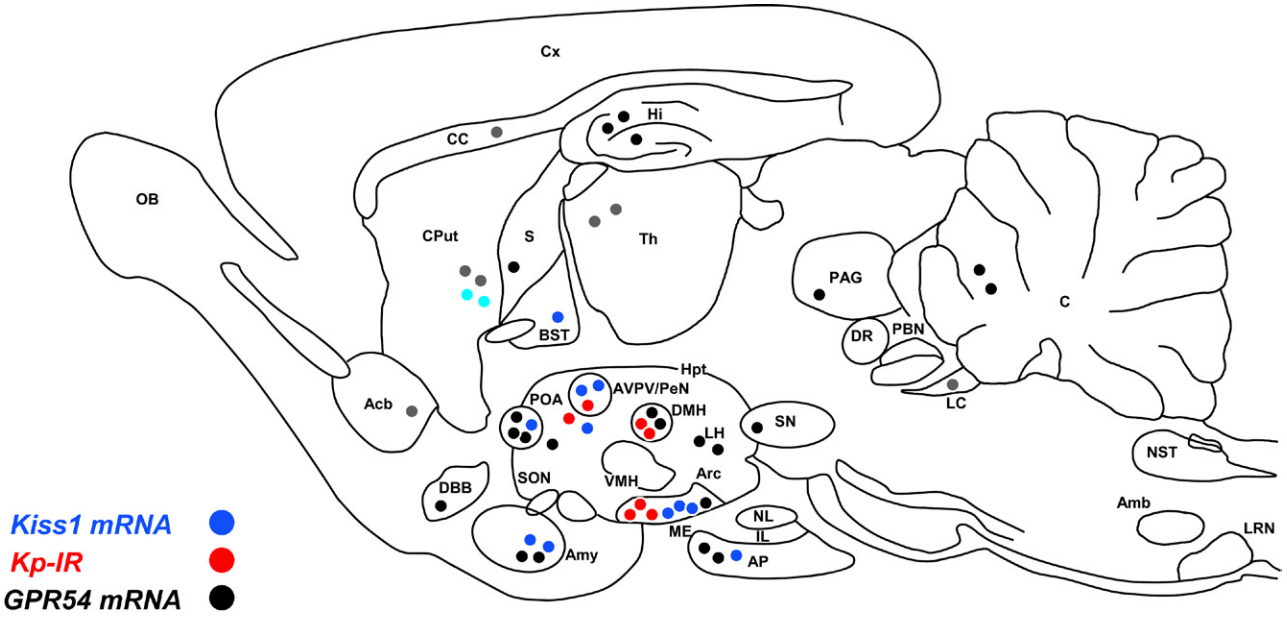

PLATE 31. Anatomical distribution of Kiss1 mRNA (blue dots), kisspeptin immunoreactivity –Kp-IR- (red dots) and GPR54 mRNA (black dots) in the different brain areas of rodent brain. In the mouse and rat, Kiss1 mRNA expression has been detected in the hypothalamus, mostly in the arcuate nucleus (Arc) as well as in the anteroventral periventricular area (AVPV) and the adjacent periventricular nucleus (PeN). In addition, the presence of Kiss1 mRNA has been reported in the preoptic area (POA), amygdala (Amy) and bed nucleus of stria terminalis (BST). In addition, the expression of KISS1 gene has been detected in the basal ganglia in humans (as detected by RT-PCR; denoted by light blue dots). In the rat brain, Kp-IR has been identified mainly in neurons in the Arc and the AVPV/PeN (the latter, especially in the female), with additional signals being detected in the dorsomedial hypothalamic nucleus (DMH), among other CNS areas. In addition, multiple Kp-IR fibers, originating from the Arc and AVPV/PeN populations and projecting to various hypothalamic and extrahypothalamic areas, have been mapped; yet, for the sake of simplicity, these are not specifically depicted in the scheme. Finally, in the rat, Gpr54 mRNA has been detected in different forebrain areas, including the diagonal band of Broca (DBB), septum, POA, anterior and lateral hypothalamus (LH), and DMH. Likewise, prominent expression of GPR54 has been observed in the hippocampus (Hpt), locus coeruleus (LC), Amy and periaqueductal gray (PAG), among others. The functional relevance of such expression sites remains to be elucidated, except for the POA, where the expression of GPR54 in GnRH neurons has been thoroughly documented. In addition, in the human brain, scattered expression of GPR54 mRNA has been reported, including basal ganglia –CPut-, Hpt, substantia nigra (SN-) and, at low levels, thalamus (Th-), cerebellum (C-) and corpus callosum. These are denoted by light gray dots. ***(Chapter 109, see page 821).***

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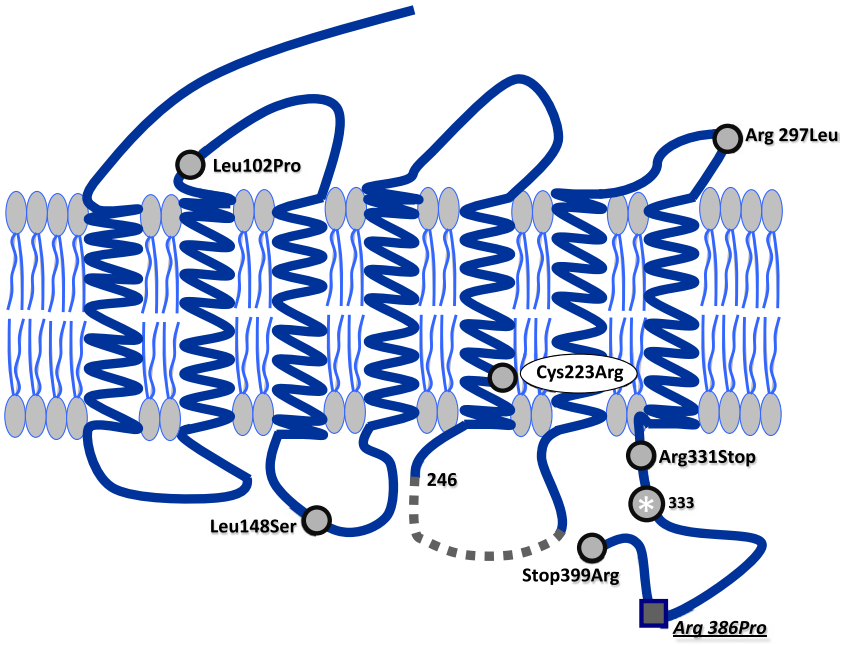

PLATE 32. Schematic representation of deletions and disease-causing mutations of GPR54 gene reported so far in humans. Inactivating mutations are indicated as black dots, with reference to the reported amino acid substitution. In addition, the location of the predicted deletion reported by de Roux et al. is marked as gray dots, while the position of the predicted insertion (1001_1002insC), which results in a shift of the open-reading frame is denoted by an asterisk. Finally, the only activating mutation of GPR54 described so far in humans, as potential cause for precocious puberty, is denoted as an open square. ***(Chapter 109, see page 825).***

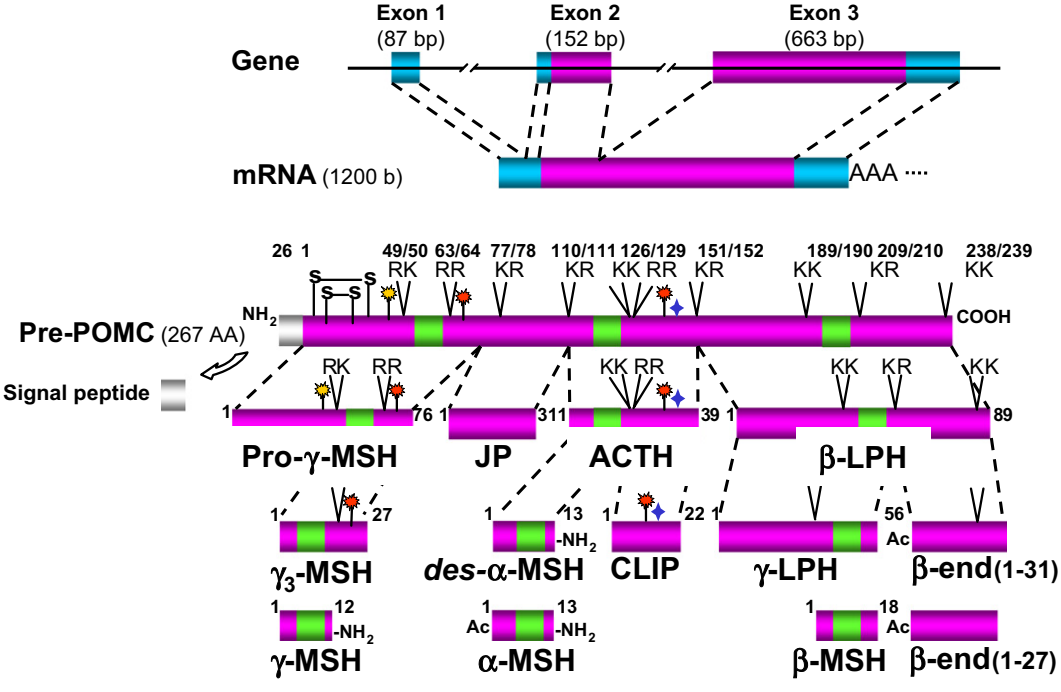

PLATE 33. Structural organization of the human POMC gene and processing of the precursor in neurons. : paired basic residue cleavage sites where processsing of POMC occurs to yield various peptide products. The numbers of the amino acid residues contributing to the cleavage site are indicated. K = Lys ; R = Arg, : MSH core sequence, : site of N-glycosylation, : site of O-glycosylation, : site of phosphorylation, CLIP : corticotropin-like intermediate lobe peptide, JP : joining peptide. ***(Chapter 111, see page 839).***

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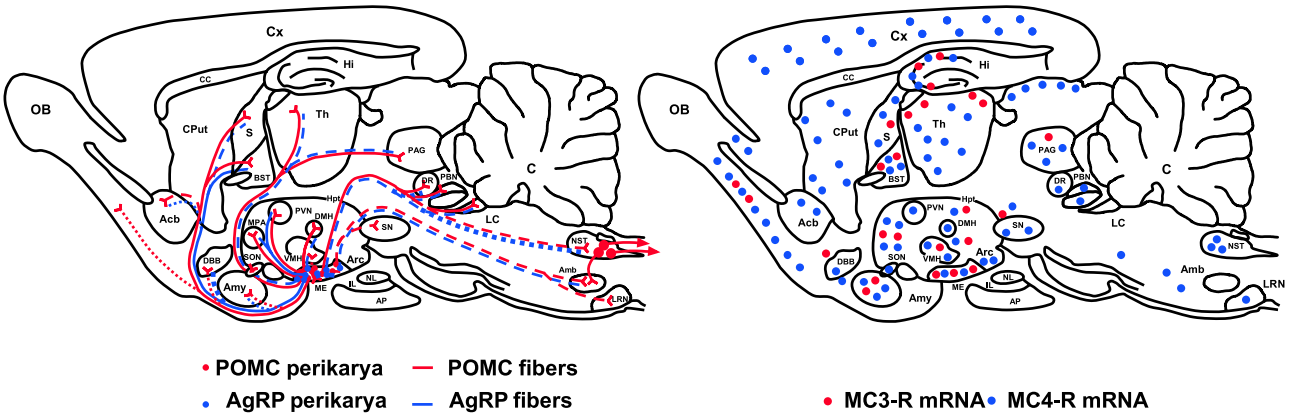

PLATE 34. Sagittal section of the rat brain depicting the distribution of POMC and AgRP neuronal systems (left). Solid lines denote high density, and dashed lines denote moderate density of fibers. Comparative distribution of MC3-R and MC4-R mRNA expression in the rat brain (right). ***(Chapter 111, see page 840).***

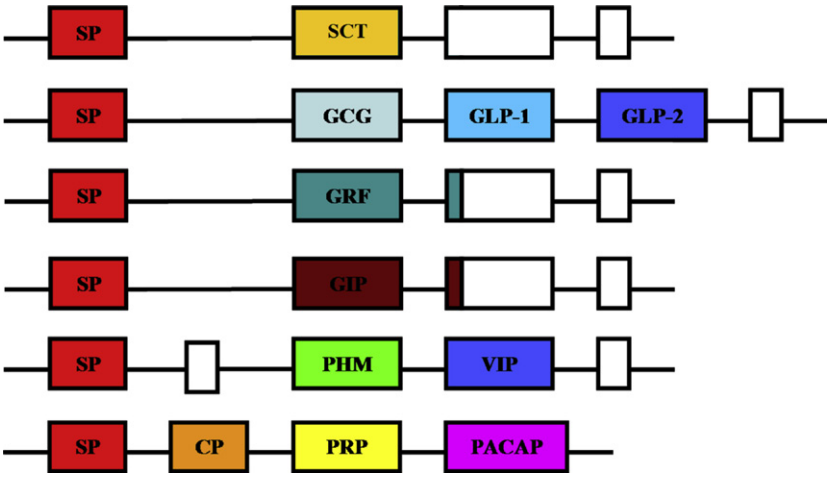

PLATE 35. The secretin superfamily genes. Boxes denote exons while introns are depicted by lines, which are both not drawn to scale or proportional for ease of gene alignment. The following abbreviations denote: signal peptide, SP; cryptic peptide, CP. ***(Chapter 123, see page 925).***

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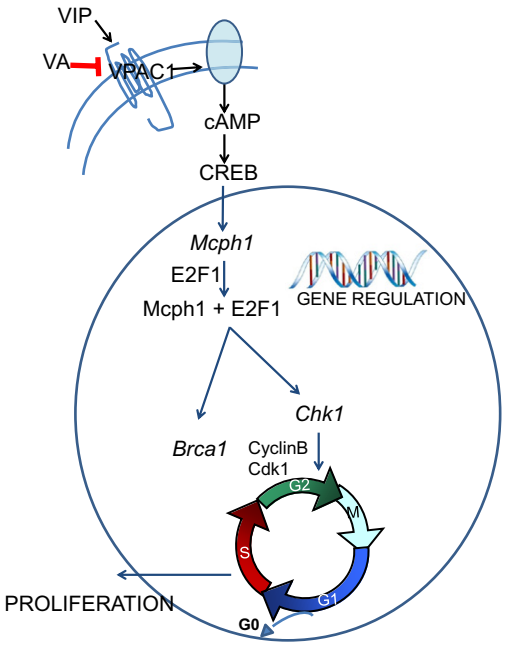

PLATE 36. Current hypothesis depicting how VIP can induce mouse neural stem cell progenitor proliferation through VPAC1–PKA–Mcph1–Chk1 signaling pathway. VIP proliferative effects are mediated by VPAC1 coupled to the cAMPc–PKA pathway, and this action is blocked by VA. PKA can phosphorylate CREB that potentially binds to one of the 3 cAMP-responsive element sequences on *Mcph1* gene. VIP blockade induces a decrease of *Mcph1* gene expression. *Mcph1* in turn regulates *Chk1* and *Brca1*, two key check points of the cell cycle, through its interaction with E2F1 on the promoter of both genes. VIP blockade then induces a decrease of neural stem cell proliferation through VPAC1–cAMP–PKA–Mcph1–Chk1 signaling pathway. ***(Chapter 128, see page 970).***

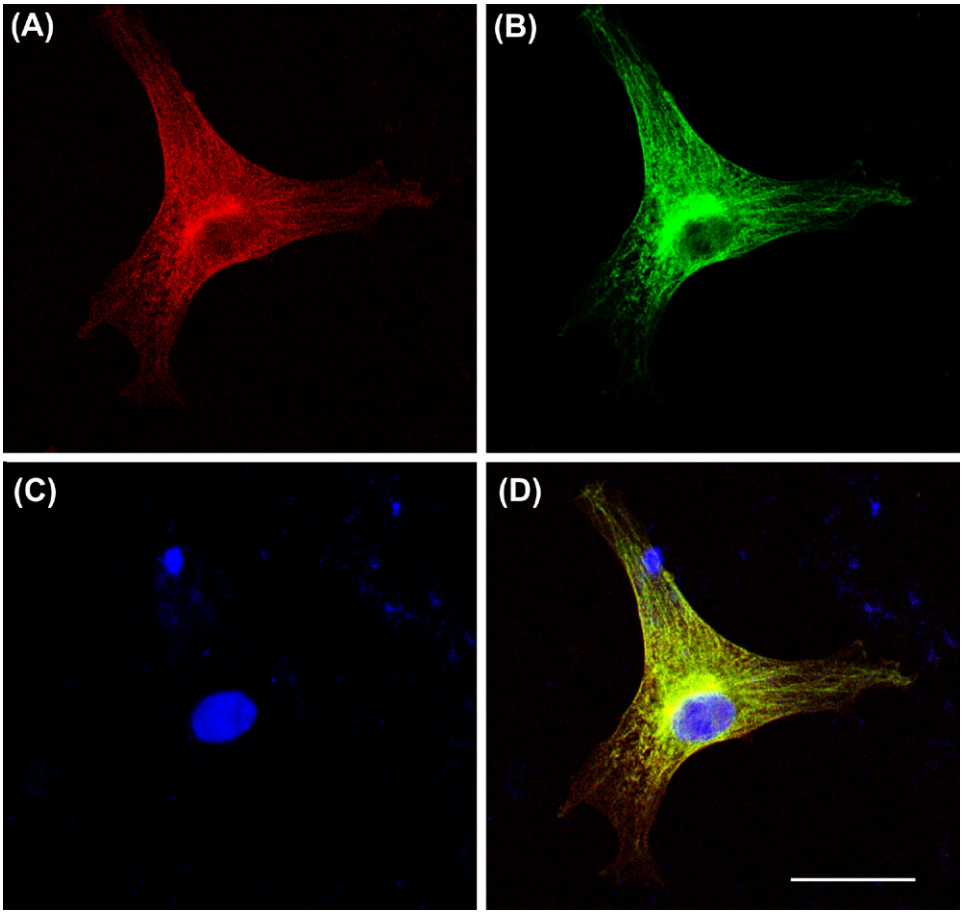

PLATE 37. Confocal images of Cos7 cells in culture stained for AM (A), tubulin (B), DAPI (C), and the combination of the 3 colors (D). Note that AM decorates all the microtubules of the cell. Bar = 20 μm. ***(Chapter 131, see page 991).***

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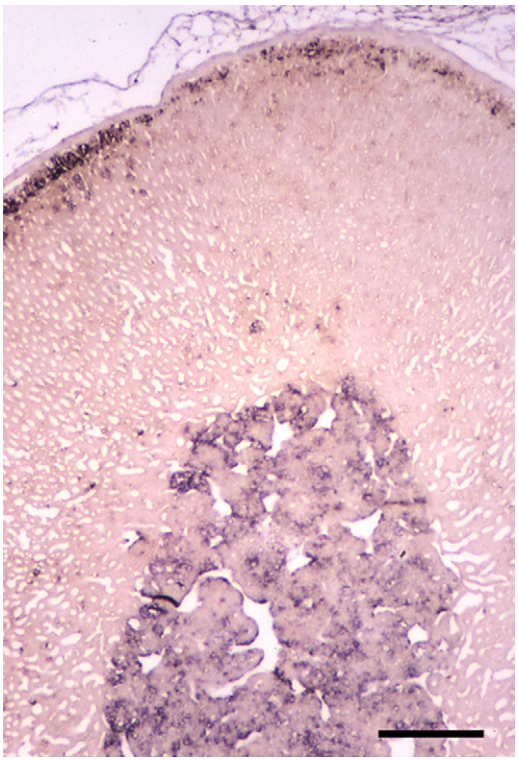

PLATE 38. Distribution of proadrenomedullin mRNA in a section of rat adrenal gland by in situ hybridization. Proadrenomedullin expression is highest in the medulla and in the zona glomerulosa of the cortex. Bar = 150 μm. ***(Chapter 131, see page 992).***

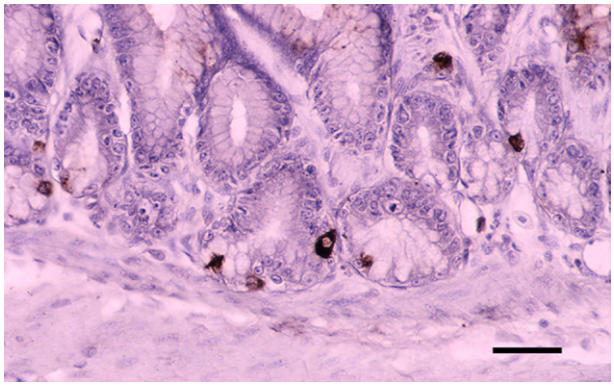

PLATE 39. Paraffin section of the stomach of the lizard Anolis sagrei, stained for PAMP. Note the numerous PAMP-positive cells belonging to the diffuse endocrine system located in the deep portion of the gastric glands. Hematoxylin was used to provide nuclear counterstaining (blue). Bar = 25 μm. ***(Chapter 131, see page 993).***

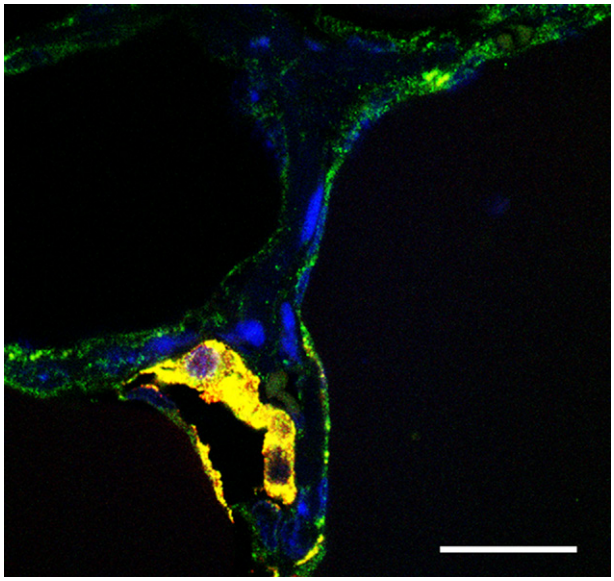

PLATE 40. Confocal microscopy image of a monkey thyroid gland stained for AM (green), calcitonin (red), and DAPI (blue). Colocalization of both immunoreactivities in the C-cells is shown by the yellow hue. Bar = 30 μm. ***(Chapter 131, see page 994).***

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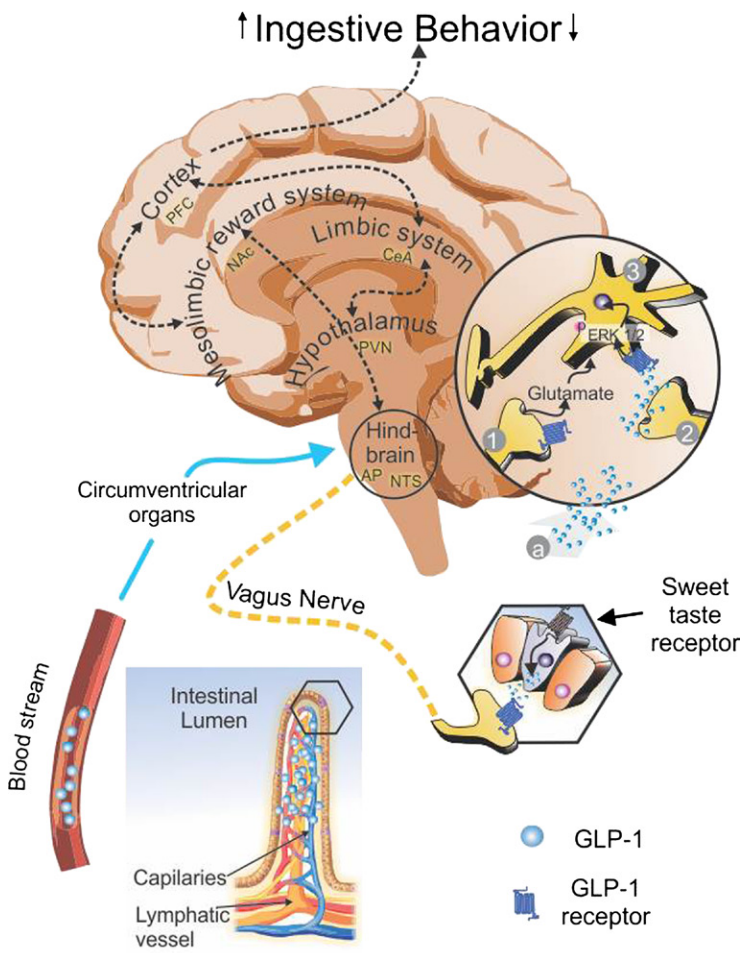

PLATE 41. Scheme of GLP-1 satiation intestinal luminal nutrients trigger GLP-1 release through taste receptor mechanisms present in enteroendocrine L-cells. GLP-1 can inhibit eating (1) through a paracrine action on GLP-1 receptors (GLP-1Rs) on intestinal vagal afferents that connect the gut to the nucleus tractus solitarii (NTS) in the hindbrain; and (2) through an endocrine action on the brain, provided that sufficient GLP-1 escapes inactivation by dipeptidyl peptidase-4 to act through circumventricular organs such as the area postrema (AP) and perhaps elsewhere. Although hindbrain processing of the GLP-1 signal seems to be sufficient to inhibit eating, rostral brain areas such as the hypothalamic paraventricular nucleus (PVN), the nucleus accumbens (NAc) and the central nucleus of the amygdala (CeA) may also be involved. See the text for further details. ***(Chapter 149, see page 1113).***

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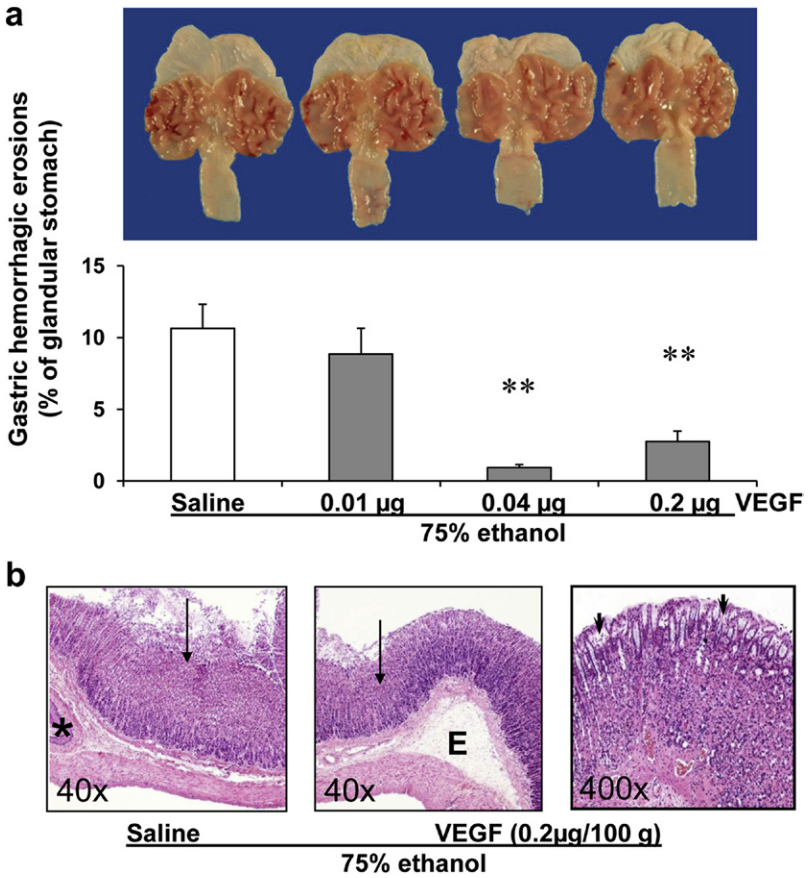

PLATE 42. Gastroprotection by VEGF in experimental gastric erosions. (a): Gross appearance and quantitative analysis of gastric hemorrhagic erosions induced by 75% ethanol in rats pretreated with recombinant rat VEGF164. (b): In control rats, hemorrhagic erosions involve about half of the gastric mucosa (arrow) in the glandular stomach (*). In VEGF-pretreated rats, only mild superficial (arrow) or no mucosal damage is seen, in addition to edema (E) in lamina propria (arrowheads) and submucosa.**$p < 0.01$. ***(Chapter 159, see page 1177).***

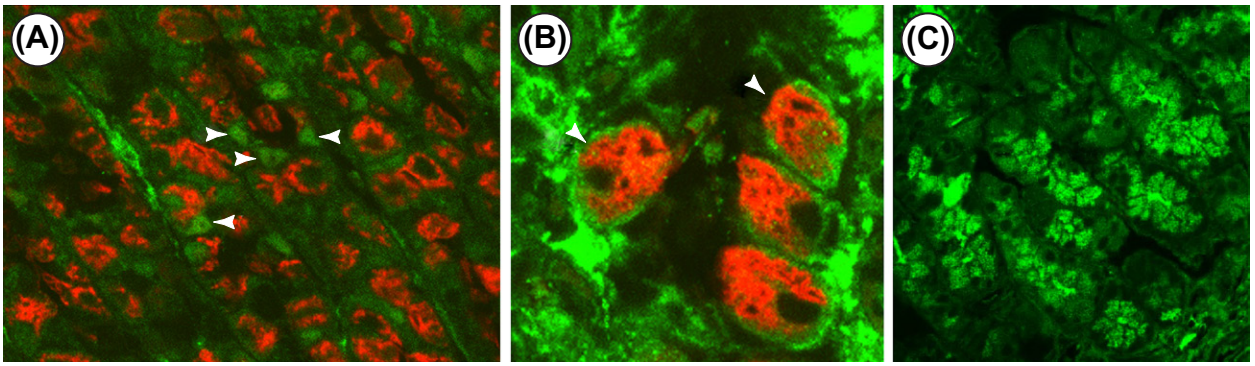

PLATE 43. Immunofluorescence detection of apelin containing cells in rat stomach. **Panel A**. In the isthmus–neck region of the rat gastric mucosa, mucous neck cells produce apelin (green fluorescence, arrowheads) whereas parietal cells do not produce apelin. Parietal cells are identified by immunostaining for the H^+/K^+ATPase/proton pump (red fluorescence). **Panel B**. In the upper portion of the gastric mucosa parietal cells stain for apelin (arrowheads). Notice that apelin staining (green fluorescence) is stronger at the cell periphery suggesting that cellular localization of the proton pump differs from that of apelin. **Panel C**. Immunofluorescence detection of apelin-containing cells in the base region of the stomach mucosa (green fluorescence). Notice the granular nature of the apelin staining. (With permission from Susaki E, Wang G, Cao G, et al. Apelin cells in the rat stomach. Regul Pept 129:37–41, 2005.) ***(Chapter 160, see page 1183).***

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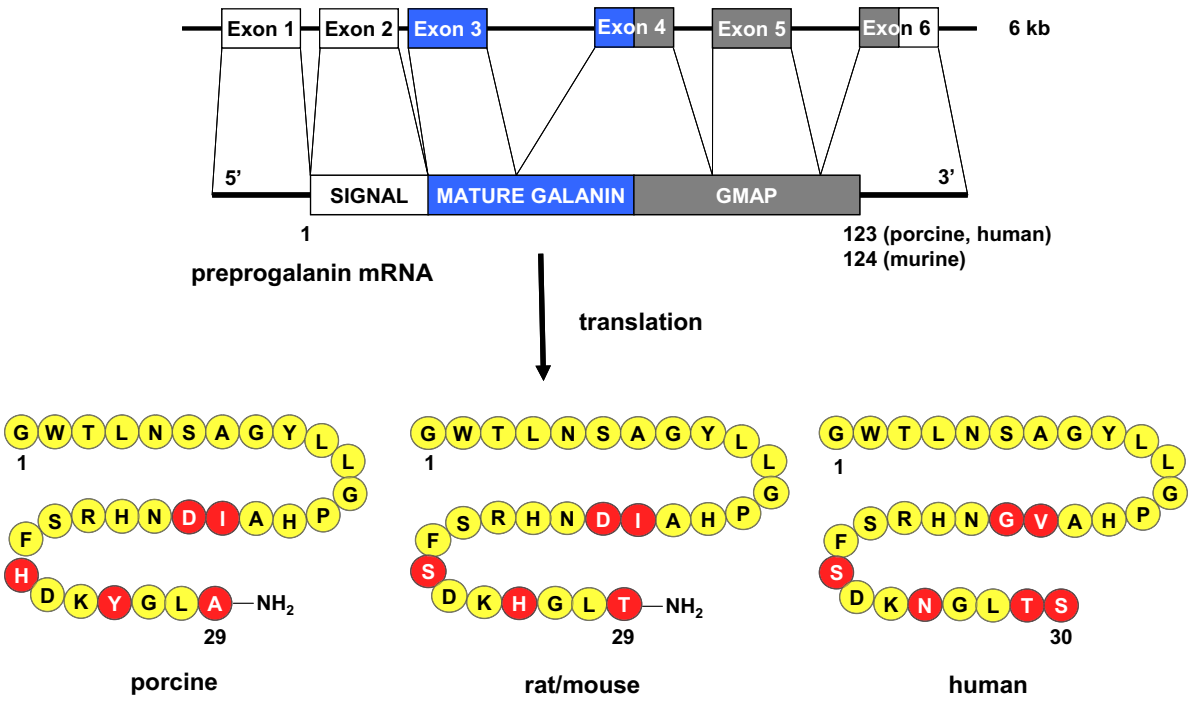

PLATE 44. Schematic diagrams showing the predicted transcription of galanin gene and the translation of preprogalanin mRNA to produce the mature peptide galanin in porcine, rat, mouse, and human. The first exon encodes the 5′-untranslated region of preprogalanin mRNA. Exon 2 starts with a translation initiation codon of the signal peptide and terminates before the proteolytic site preceding the mature galanin peptide. The first 13 amino acids of galanin are encoded by exon 3; the remaining 16 amino acids and most of galanin message associated peptide (GMAP) by exons 4 and 5. The remaining portion of GMAP and the polyadenylation site are located in exon 6. Note: 29 amino acids with C-terminal amidation in the pig, rat, and mouse, but 30 amino acids without C-terminal amidation in humans, and gray circles indicate residues that are different from human. ***(Chapter 164, see page 1211).***

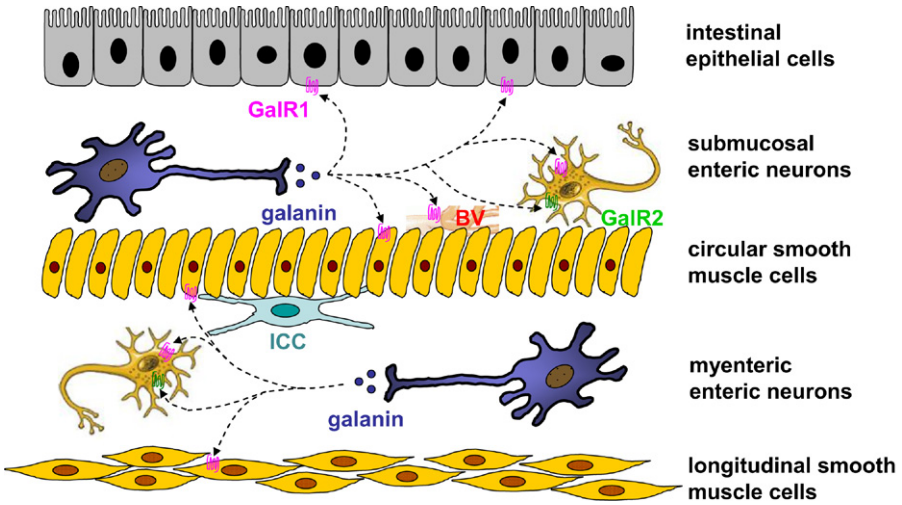

PLATE 45. Schematic diagrams showing the distributions of galanin-containing nerve cell bodies and nerve fibers, galanin receptors (GalR1, GalR2, not yet GalR3), and acting sites of galanin (indicated by dashed arrows) in the gut. BV, blood vessel; ICC, interstitial cells of Cajal. ***(Chapter 164, see page 1212).***

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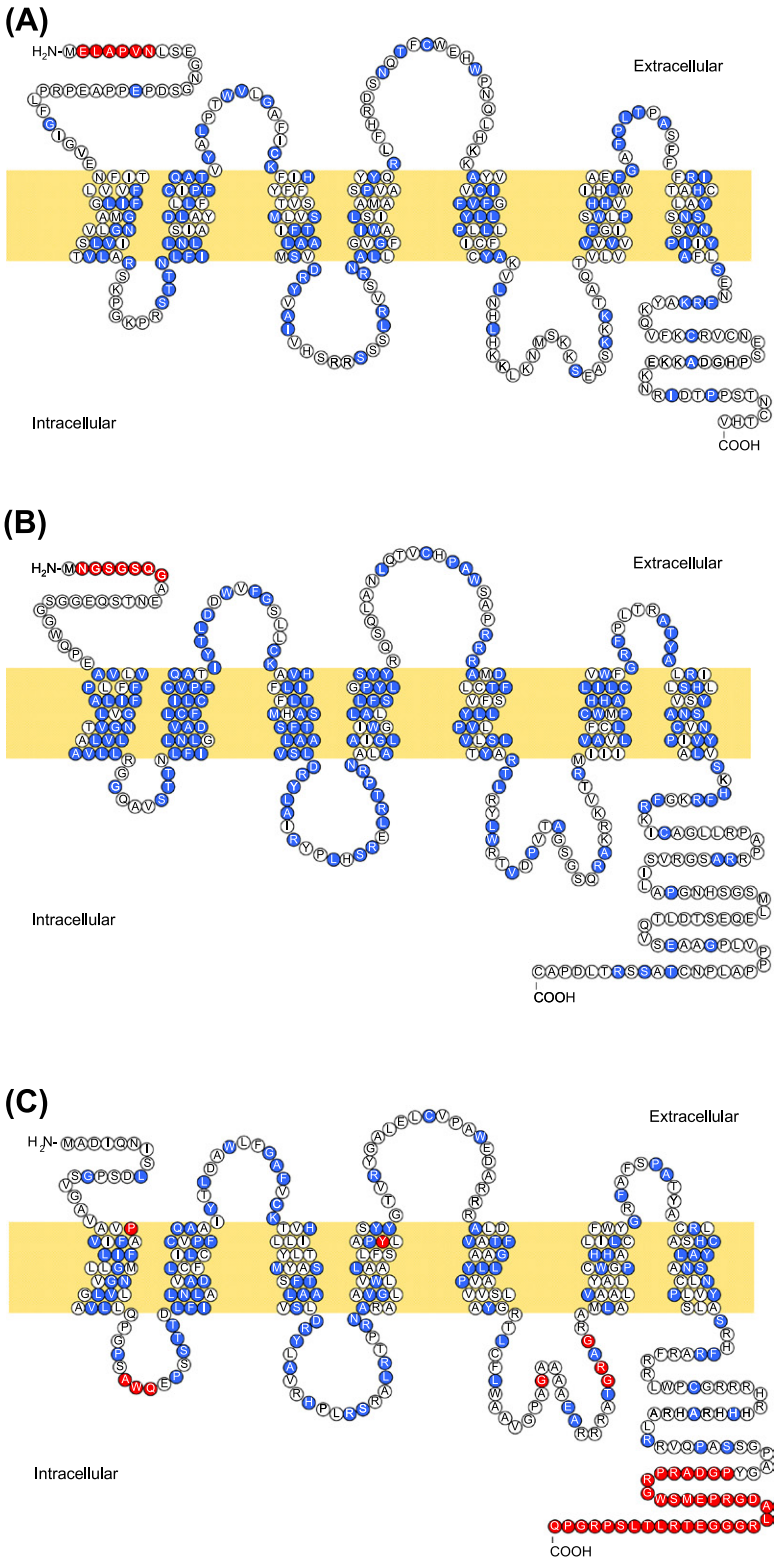

PLATE 46. Schematic diagrams showing the amino acid sequences of seven-transmembrane domain receptors of GalR1 (A), GalR2 (B) and GalR3 (C) of rat. In A, blue circles indicate residues that are identical with those in B; red circles indicate residues that are absent in B. In B, blue circles indicate residues that are identical with those in C; red circles indicate residues that are absent in C. In C, blue circles indicate residues that are identical with those in A; red circles indicate residues that are absent in A. (Reprinted from Ref. 4 with permission from Elsevier). ***(Chapter 164, see page 1213).***

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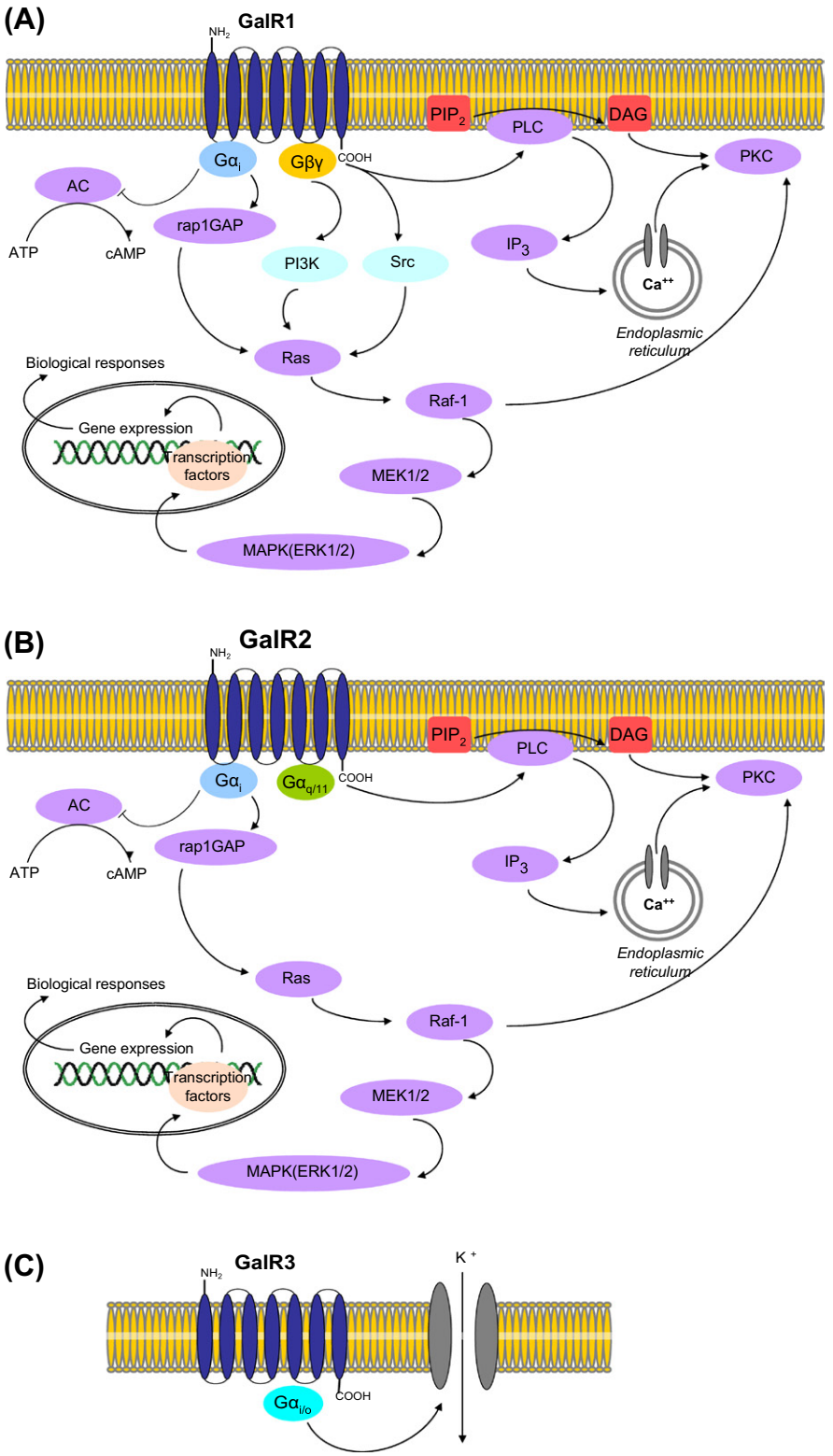

PLATE 47. Schematic diagrams showing the proposed signaling pathways of GalR1 (A), GalR2 (B), and GalR3 (C). Note: stimulations are indicated by ↓ lines, and inhibitions are indicated by lines. Abbreviations: AC, adenylate cyclase; DAG, diacylglycerol; IP3, inositol triphosphate; MAPK, mitogen-activated protein kinases; MEK, mitogen-induced extracellular kinase; PI3K, phosphatidylinositol 3-kinase; PIP2, phosphatidylinositol bisphosphate; PKC, protein kinase C; PLC, phospholipase C; rap1GAP, rap1 GTPase-activating protein 1. ***(Chapter 164, see page 1215).***

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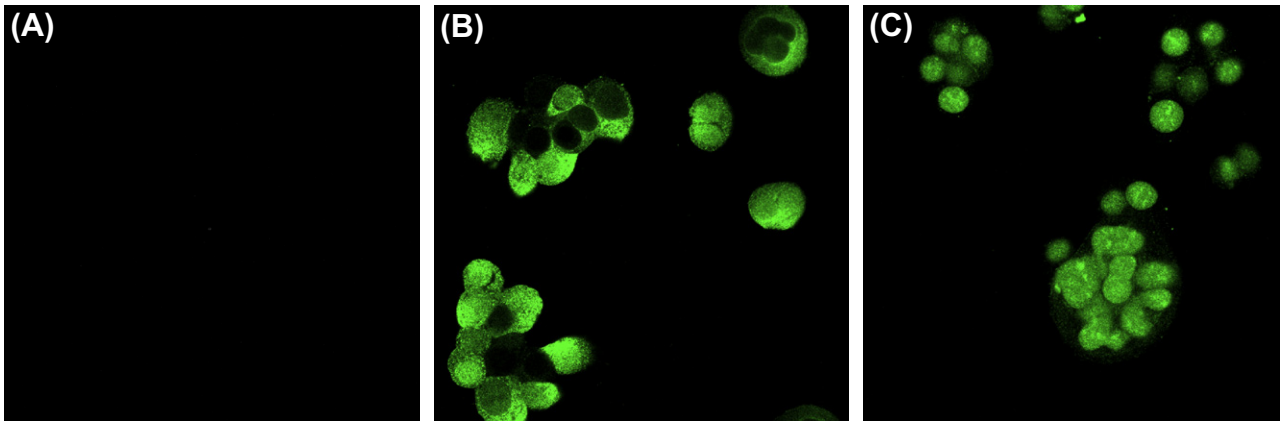

PLATE 48. Confocal MIcrograph of BON cells stained with (B) anti-PAC1 antibody or (C) anti-PACAP antibody. ***(Chapter 175, see page 1291).***

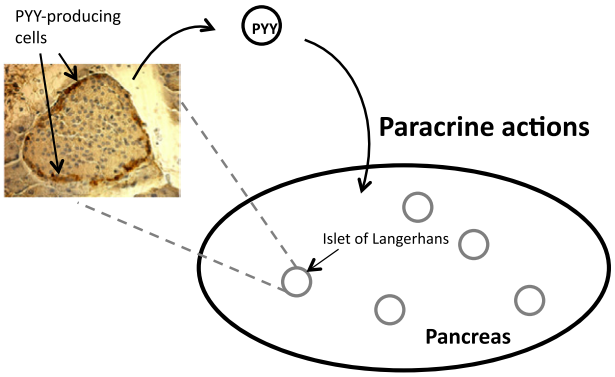

PLATE 49. Pancreatic secretion of PYY from the islets of Langerhans to exert paracrine effects. ***(Chapter 178, see page 1308).***

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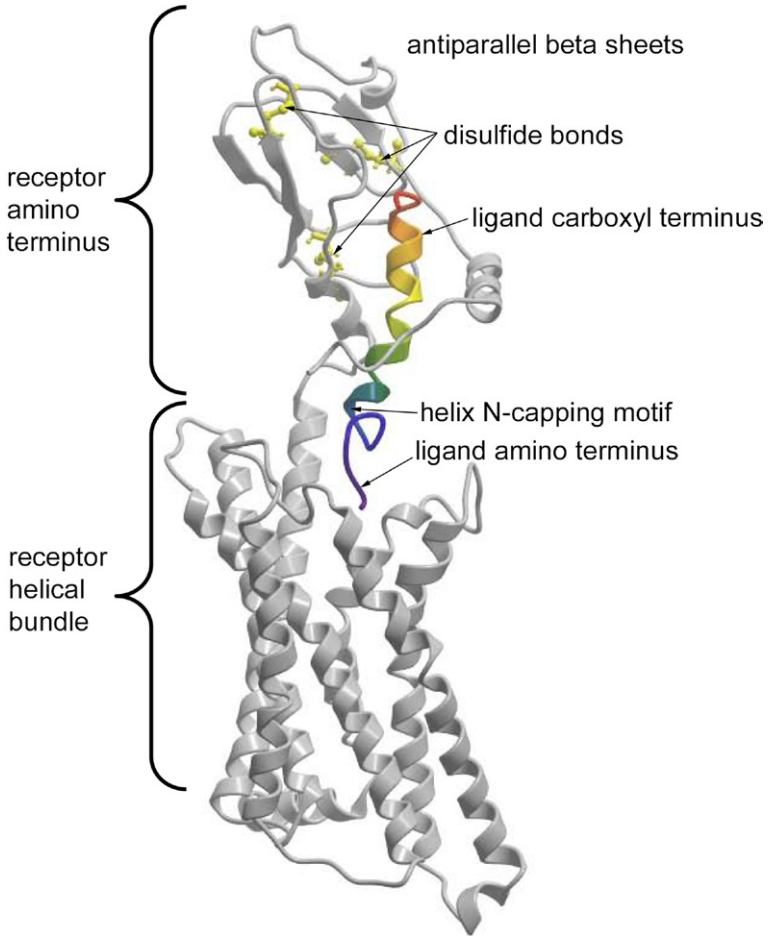

PLATE 50. Illustration of the structure of the secretin receptor. Shown is a prototypic family B GPCR in gray, with the extracellular amino-terminal domain above the intramembranous helical bundle domain. Within the amino terminus are three highly conserved disulfide bonds (yellow), two antiparallel beta sheet regions, and several loop and helical structures. The peptide ligand is shown colored from purple-to-red, representing amino-to-carboxyl-terminal regions. The carboxyl terminus is illustrated in the typical alpha helix conformation ending in the conserved helix N-capping motif and the biologically important amino terminus directed toward the helical bundle. ***(Chapter 179, see page 1317).***

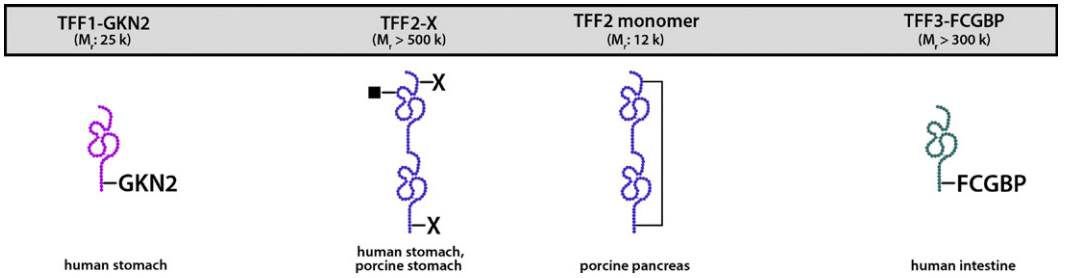

PLATE 51. Predominant TFF forms in the mammalian GI tract. Schematically represented are the TFF1–GKN2 heteromer, the monomeric (including the additional disulfide bridge), and the high molecular weight forms of TFF2 (partner X is not known thus far), and the TFF3–FCGBP heteromer. The various TFF heteromers are disulfide-linked. Also indicated is the N-glycosylation site of human gastric TFF2 (rectangular knob). ***(Chapter 182, see page 1339).***

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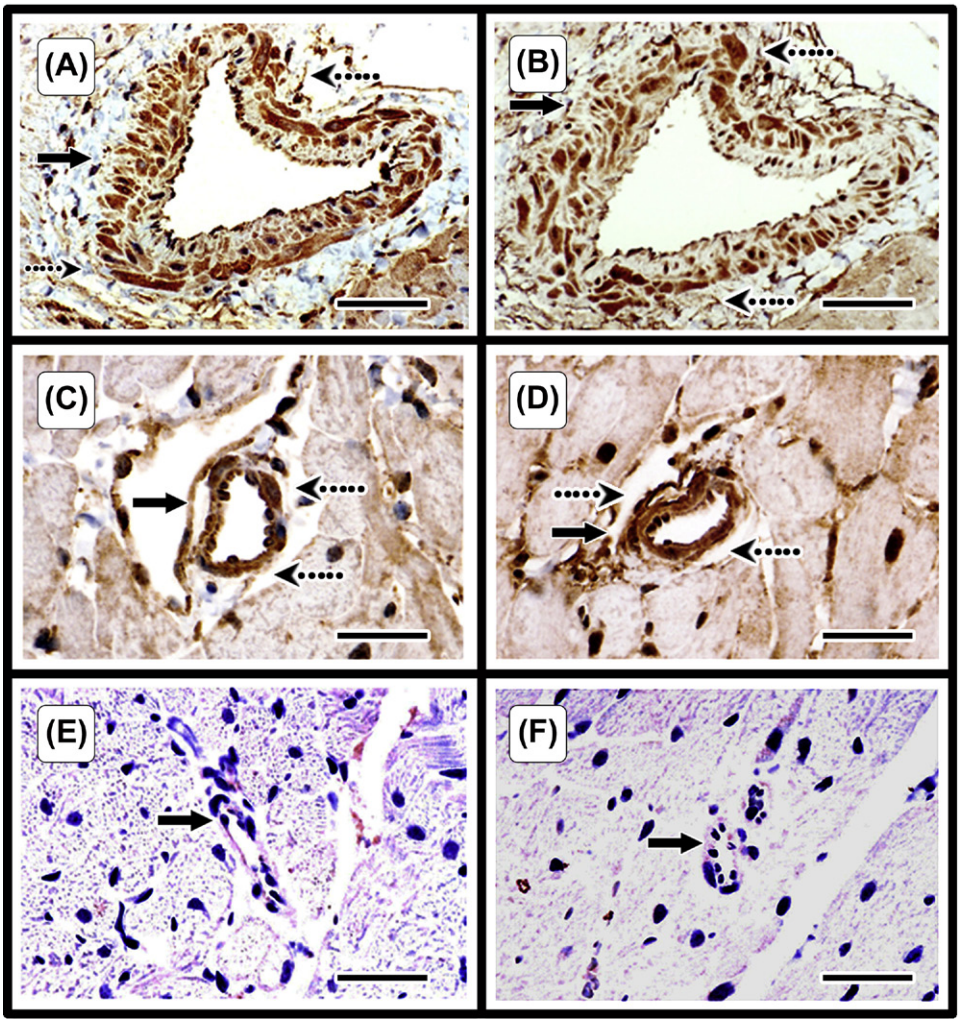

PLATE 52. Presence of VPAC1 (A, C) and VPAC2 receptors (B, D) in the left ventricular coronary artery (A, B) and coronary arterioles of the left ventricle (C, D) of the rat as determined by immunohistochemistry. Negative controls are also shown (E, F). The primary stain is Dako DAB (brown) and the counterstain is hematoxylin (blue). Scale bar represents 50 μm for panels A and B and 25 μm for panels C, D, E, and F. Large arrow denotes location of artery or arteriole. Dashed arrow denotes regions expressing high levels of VPAC1 or VPAC2. ***(Chapter 196, see page 1445).***

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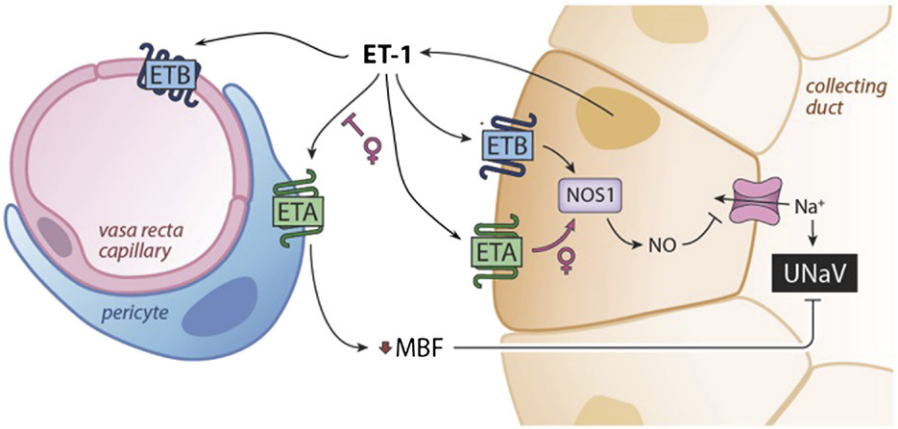

PLATE 53. Basic function of the endothelin (ET-1) system in the renal medulla. (Used with permission from Morgan & Claypool Life Sciences.[22]) ***(Chapter 200, see page 1478).***

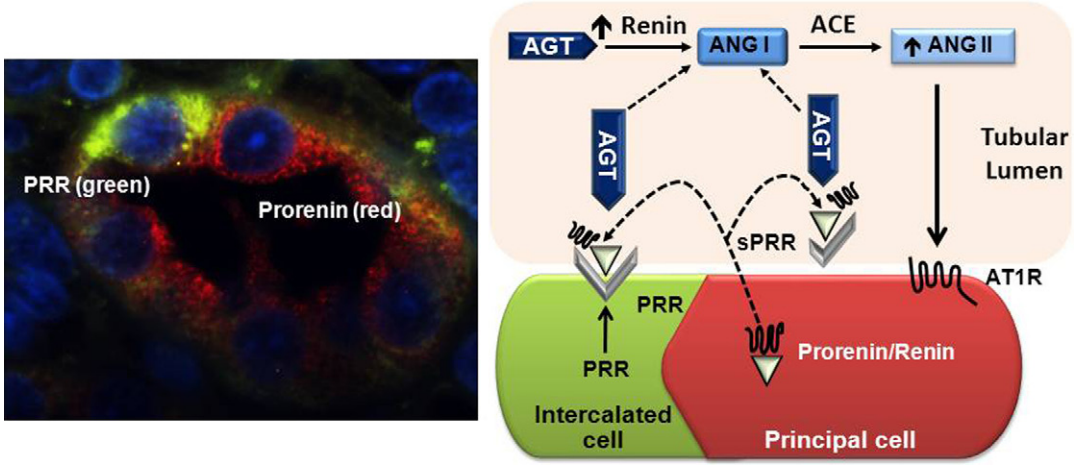

PLATE 54. Renin and (pro)renin receptor interaction in the collecting duct. Prorenin and renin stimulation in the principal cells of the collecting ducts may increase Ang I formation in the tubular lumen and increased Ang II generation. The presence of (pro)renin receptor at the surface of the intercalated cells along with its soluble form in the tubular lumen increases the catalytic efficiency for renin to form Ang I. In addition, the pro(renin) receptor bound to the membrane of the intercalated cells may anchor prorenin secreted by the principal cells to reduce the washout of prorenin or renin into the urine. ANG — angiotensin; AGT — angiotensinogen; AT1R — angiotensin II type 1 receptor; PRR — (pro)renin receptor; sPRR — soluble form of the (pro) renin receptor; ACE — angiotensin converting enzyme. ***(Chapter 204, see page 1505).***

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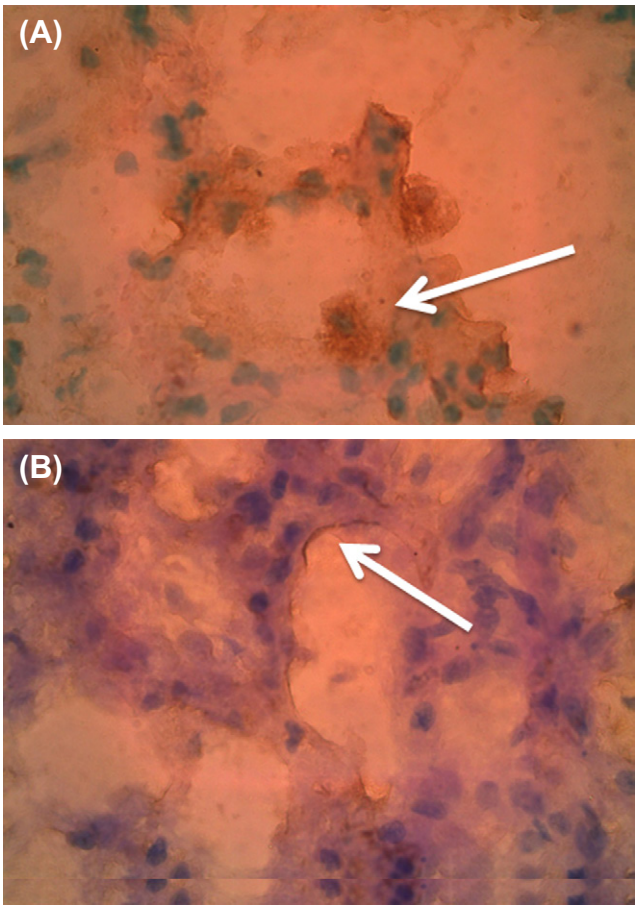

PLATE 55. Cultured rat lung slices freshly isolated were exposed for 24 h to CSC (5 μg/ml), as described elsewhere.[19] After fixation in 4% paraformaldehyde for 30 min at room temperature, lung slices (20 μm thick coronal sections) were permeabilized in PBS containing 0.2% Triton X-100 (30 min) and exposed to rabbit anti-rat B_1R antibody (1:500; 120 min),[19] washed, incubated with biotin-conjugated antirabbit secondary antibody (1:300; 60 min), washed and incubated with horseradish peroxidase (HRP)-conjugated streptavidin antibody (1:300; 60 min). After final washing, sections were incubated with PBS 0.05% 3,3'-Diaminobenzidine (DAB) + 0.015% H_2O_2 (5 min) for revelation. Nucleus (blue) was counterstained by incubating slides in Mayer's hematoxilin solution (5 min). Slides were coverslipped using DPX mountant (Fluka) and examined under an inverted microscope. Positive B1R staining (brown) is seen on (A) type II pneumocytes (great alveolar cells), according to the large/cuboidal (arrowed) morphology of stained cells, and (B) type I pneumocytes (simple squamous alveolar cells), according to the thin/stretched (arrowed) morphology of stained cells. Magnification = 400×. Pictures are representative of a minimum three slices per rat from three different rats. ***(Chapter 207, see page 1521).***

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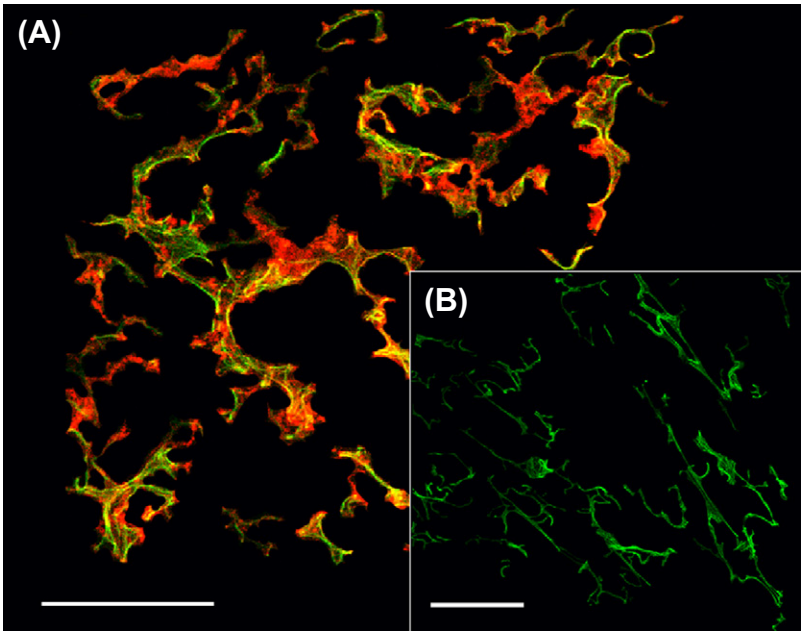

PLATE 56. Rat lung slices treated with 5 μg/ml CSC (A) or vehicle (B) for 24 h were exposed to [Nα-Bodipy]-des-Arg^9-BK, a specific fluorescent ligand for B1R[29] and to Anti-EpCAM (1:1000), a specific antibody against epithelial cells (Abcam, Cambridge, MA; stock number: AB68892). The experimental protocol for confocal microscopy is described elsewhere.[29] The B1R fluorescent dye is red (ext: 550 nm/em: 570 nm) and Anti-EpCAM dye is green (ext: 505 nm/em: 515 nm). Colocalization between B1R and epithelial cells is shown in yellow. Scale bar = 100 μm, magnification 20×. Confocal pictures are representative of a minimum four slices per rat from three different rats. ***(Chapter 207, see page 1522).***

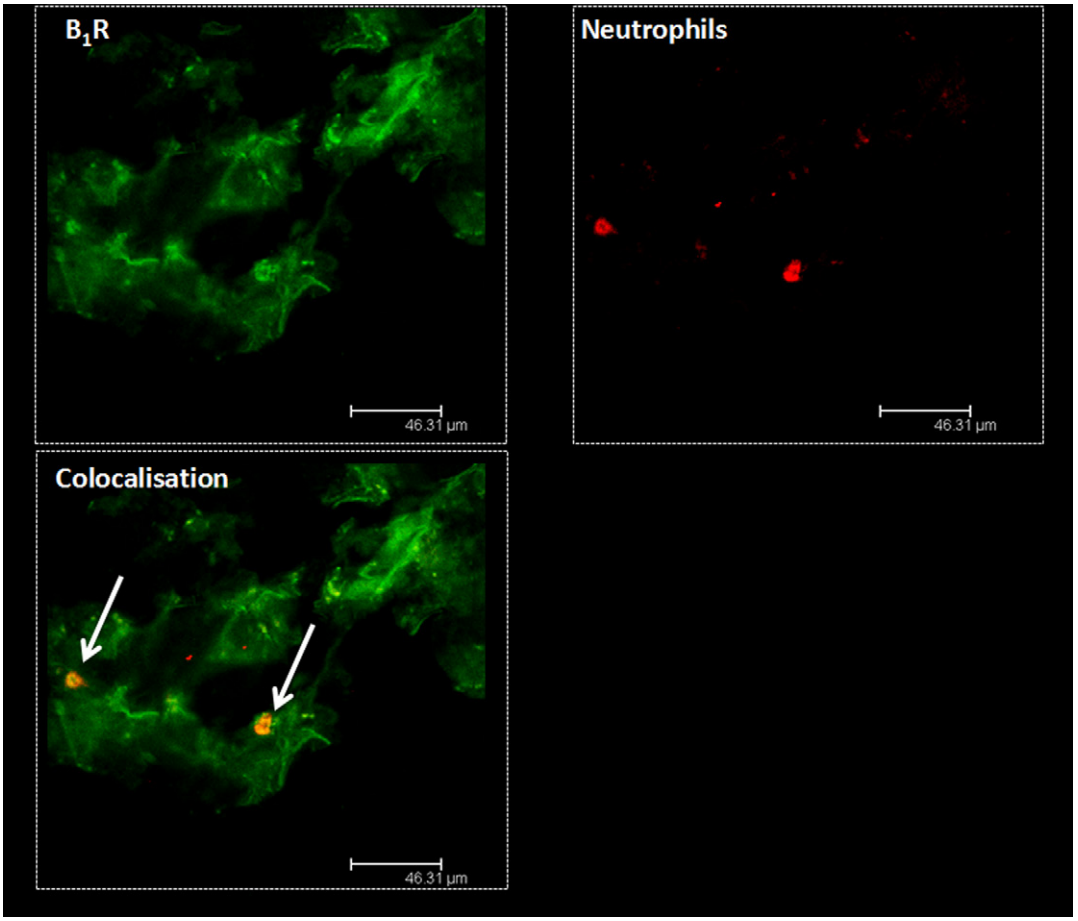

PLATE 57. Rat lung slices treated with 5 μg/ml CSC for 24 h were exposed to [N$^{\alpha}$-Bodipy]-des-Arg^9-BK, a specific fluorescent ligand for B1R[29] and to Antineutrophil elastase, a specific antibody against neutrophil (1:1000) (Abcam, Cambridge, MA; stock number: AB21595). Colocalization between B1R and infiltrating neutrophils in yellow is indicated by two arrows in the bottom panel. The fluorescent ligand for B1R is green (ext: 505 nm/em: 515 nm) and the Antineutrophil elastase dye is red (ext: 550 nm/em: 570 nm). Scale bar = 46.31 μm. Confocal pictures are representative of a minimum four slices per rat from three different rats. ***(Chapter 207, see page 1523).***

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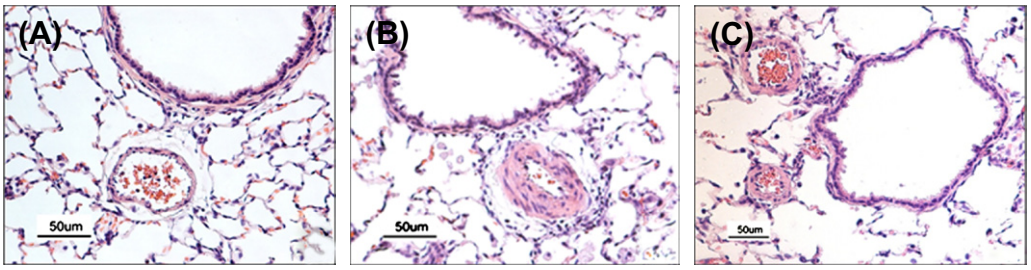

PLATE 58. VIP corrects pulmonary vascular and RV pathology in monocrotaline (MCT)-induced PAH in rats: (A) H&E section of normal control lung, showing alveoli, bronchus, and small PA. No evidence of inflammation or vascular thickening. (B) Lung section 3 weeks after injection of MCT, showing vascular thickening and inflammatory cell infiltrates. (C) 3 weeks after VIP treatment, almost complete reversal of MCT-induced pathology. ***(Chapter 209, see page 1539).***

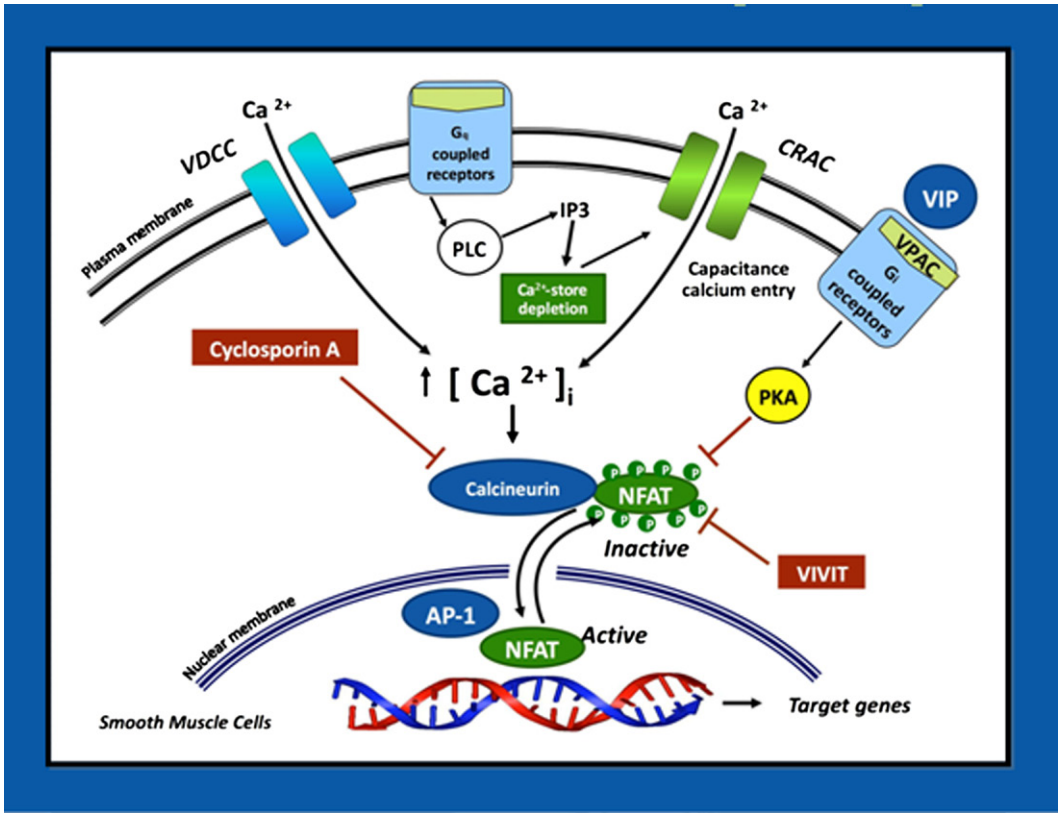

PLATE 59. Calcineurin–NFAT signaling and its inhibition by VIP. In resting cells, NFAT resides in the cytoplasm, where it is maintained in a phosphorylated, inactive state. Stimulation of cell-surface receptors coupled to phospholipase C (PLC) results in calcium mobilization, initially from intracellular stores, followed by influx of Ca^{2+} through specialized voltage-dependent Ca^{2+} channels (VDCC) and Ca^{2+} release-activated Ca^{2+} channels (CRAC). On activation by Ca^{2+}, the phosphatase calcineurin dephosphorylates NFAT, triggering its activation and nuclear translocation. In the nucleus, NFAT interacts with other transcription factors, including activator protein (AP)-1, to stimulate gene expression. When NFAT is rephosphorylated, it is exported back to the cytoplasm. The immunosuppressive drug cyclosporin A inhibits calcineurin interactions with all its substrates, whereas VIVIT selectively inhibits NFAT activation. VIP seems to inhibit this pathway, but its mechanism of action is yet to be determined. IP3: inositol-1,4,5-triphosphate. ***(Chapter 209, see page 1541).***

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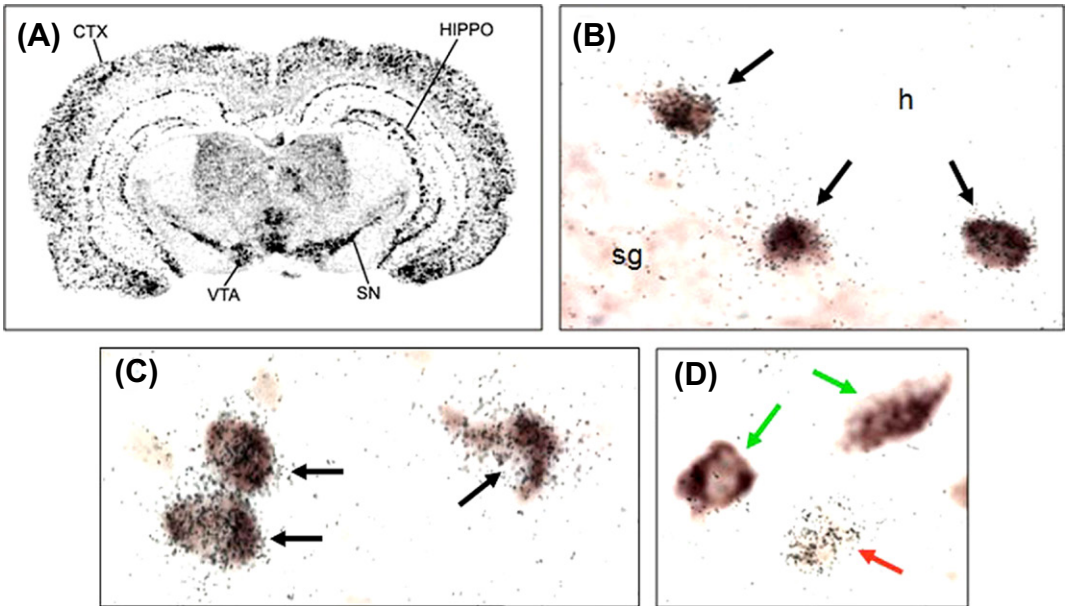

PLATE 60. (A) Distribution of ErbB4 mRNA-expressing neurons in rat cortex and midbrain as revealed by in situ hybridization. The vast majority of the scattered ErbB4 mRNA-labeled cells in the cerebral cortex (CTX) and hippocampal formation (HIPPO) represent GABAergic interneurons. In the substantia nigra (SN) and ventral tegmental area (VTA) of the midbrain, ErbB4 is expressed by virtually all of the dopaminergic mesotelencephalic neurons. (B,C) Double-labeling in situ hybridization demonstration of the extensive coexistence of ^{35}S-labeled ErbB4 mRNA and digoxygenin-labeled glutamic acid decarboxylase (GAD, the biosynthetic enzyme for GABA) mRNA in neurons of the rat hippocampal dentate gyrus (B) and primary motor cortex (C). Black arrows denote double-labeled cells. (D) Demonstration of the *non-coexistence* of ^{35}S-labeled ErbB4 mRNA (red arrow) and digoxygenin-labeled GAD mRNA (green arrows) in the rat substantia nigra. h, hilus; sg, stratum granulosum. ***(Chapter 223, see page 1635).***

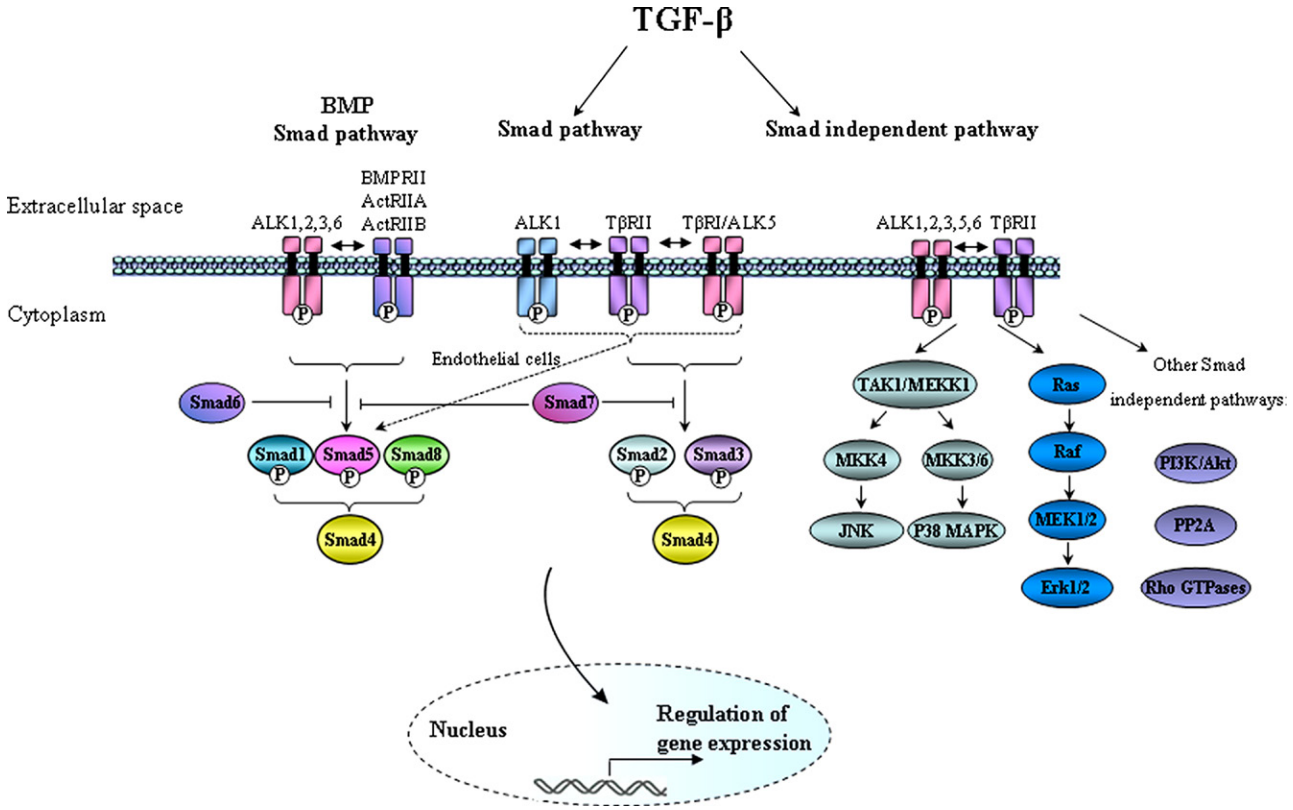

PLATE 61. TGF-β signaling through Smad-dependent and Smad-independent pathways. TGF-β binds a complex of transmembrane receptor serine/threonine kinases (TβRII and TβRI) and induces phosphorylation and activation TβRI by TβRII. In most cells, TGF-β interacts with TβRII and ALK5, but in endothelial cells, it can also signal via ALK1. BMPs signal via BMPRII, ActRIIA, and ActRIIB, and type I receptors ALK1, 2, 3, and 6. Activated type I receptors induce the phosphorylation of specific receptor regulated Smads (R-Smads: Smad1, 2, 3, 5, and 8). ALK5 induces phosphorylation of Smad2 and Smad3, and ALK1, 2, 3, and 6 mediate phosphorylation of Smad1, Smad5, and Smad8. Activated R-Smads form heteromeric complexes with common mediator Smad4, which accumulate in the nucleus, where they can act as transcription factor complexes and regulate the expression of specific target genes. Inhibitory Smads, such as Smad6 and Smad7, can antagonize TGF-β signaling by inhibiting the activation of R-Smads. TGF-β activated receptor complex (TβRI and TβRII) can activates Smad-independent signaling pathways, such as extracellular-signal regulated kinase (Erk), p38 mitogen-activated protein kinase (p38 MAPK), c-Jun N-terminal kinase (JNK), phosphatidylinositol-3-kinase (PI3 K/Akt), Src, and pathways involving Rho GTPases. ***(Chapter 225, see page 1648).***

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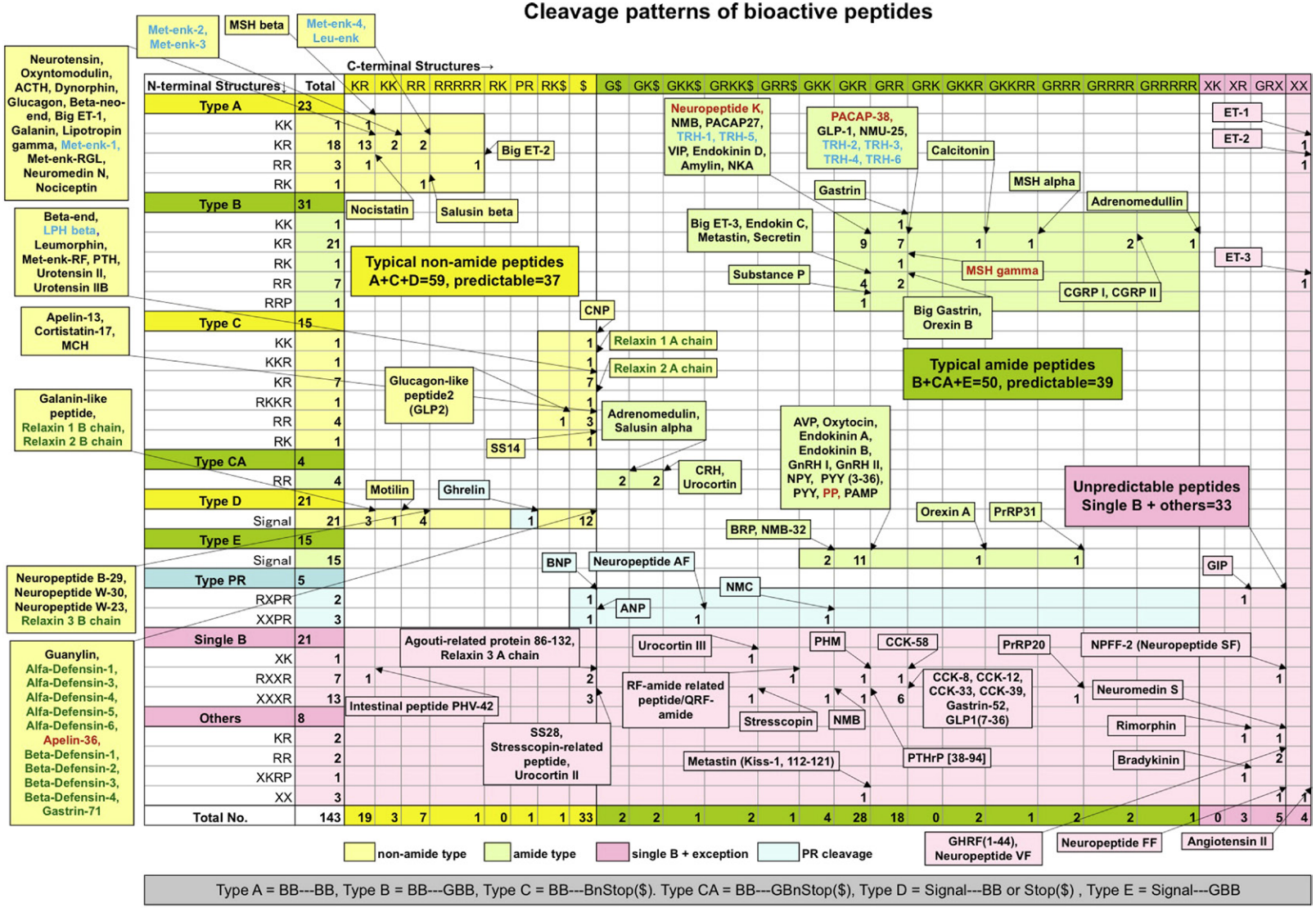
Cleavage patterns of bioactive peptides



Peptides written in red, blue, or green are difficult to predict, because those written in red contain KR and RR in the molecule, those written in blue are ≤5 or ≥61 amino acids in length, and those written in green contain more than 6 cysteine residues.

PLATE 62. Processing Patterns of Known Bioactive Peptides ***(Chapter 239, see page 1745).***

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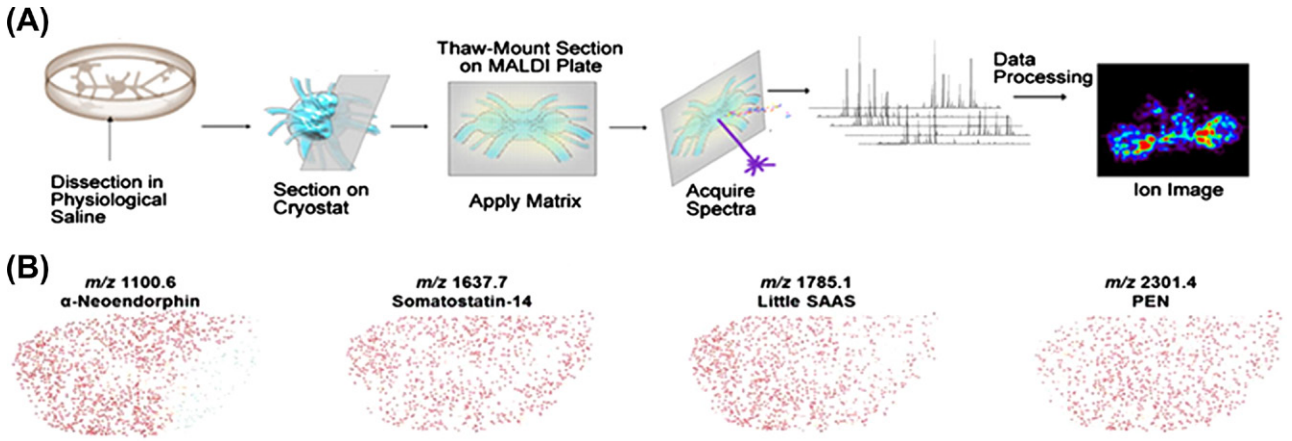

PLATE 63. Schematic of MS imaging protocols with representative ion images. (A) Typical procedures for MSI. Figure adapted and modified from Ref. 9 with permission. (B) Molecular distribution maps of four endogenous peptides detected in spinal cord using the stretched sampling approach and MS imaging. Figure adapted and modified from Ref. 34. ***(Chapter 240, see page 1752).***

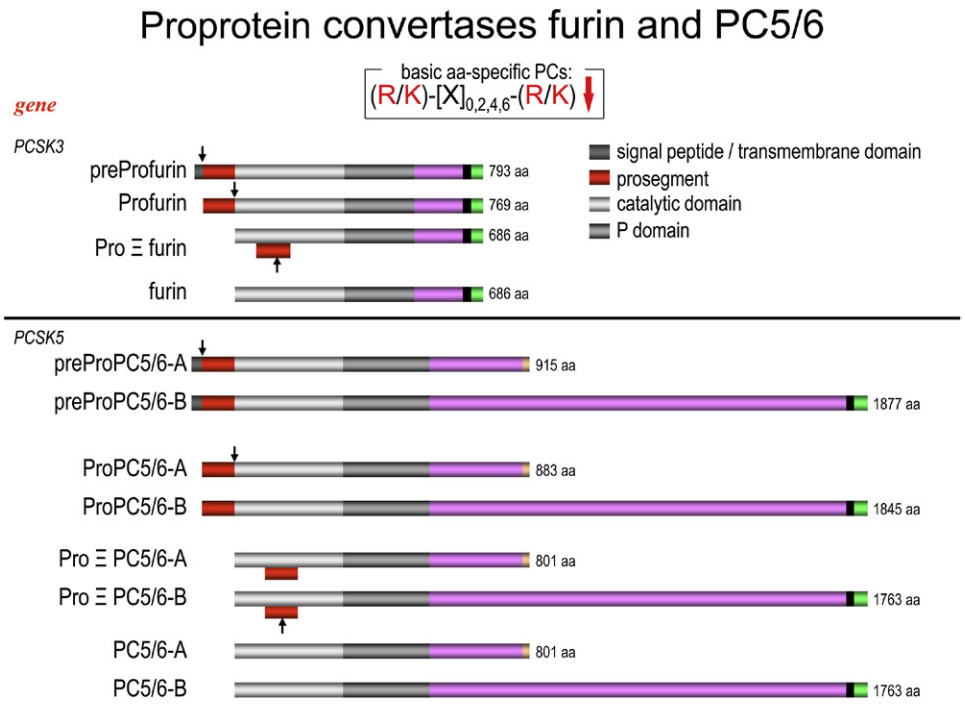

PLATE 64. Schematic representation of the processing of preProfurin and preProPC5/6. The number of aas in each form is emphasized, as well as the gene symbol, and the different domains. The arrows point to sites of either signal peptidase cleavage or autocatalytic cleavages. ***(Chapter 247, see page 1804).***

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